Table 3. Efficacy of first-line chemotherapy

	CR	PR	SD	PD	Total	RR, %	DCR, %
Cohort A	0	2	13	15	30	6.7 (0.8–22.1)	50 (31.3–68.7)
Cohort B	2	1	5	2	10	30 (6.7–65.3)	80 (44.4–97.5)
Cohort C	4	17	23	5	49	42.9 (28.8–57.8)	89.8 (77.8–96.6)

Figures in parentheses are 95% CI.

Table 4. Prognostic factors for OS

	Univariate analysis			Multivariate analysis		
	patients	MST, days	p	hazard ratio	95% CI	p
Gender						
Male	59	466	0.4212			0.791
Female	30	303		1.082	0.606–1.930	
Age, median 61						
≥61	46	455	0.5071			0.91
<61	43	446		0.969	0.559–1.680	
Histology						
Intestinal	30	455	0.5388			0.948
Diffuse	59	446		0.981	0.556–1.733	
Stage						
II	17	304	0.4825	1.115	0.444–2.800	0.744
III	50	455		1.274	0.667–2.432	
IV	22	479				
Measurable lesion						
Present	51	547	0.0243			0.004
Absent	38	285		2.181	1.279–3.720	
Metastatic sites						
1	80	455	0.0154			0.011
≥2	9	268		2.89	1.281–6.524	
DFI						
<1 year	43	351	0.365	1.349	0.786–2.315	0.277
≥1 year	46	521				
Adjuvant chemotherapy						
None	49	547	0.0061			0.008
S-1	30	287		2.635	1.346–4.747	
Oral 5-FU	10	451		0.98	0.422–2.274	

Discussion

Although adjuvant chemotherapy with S-1 has recently become the standard treatment for stage II-III gastric cancer patients after curative gastrectomy in Japan based on the result of the ACTS-GC trial [9], nearly 30% of patients still relapse, despite the adjuvant S-1 treatment. Since the total number of patients with recurrence after adjuvant S-1 is increasing, it is of great concern to discern whether adjuvant S-1 affects the subsequent clinical course of the patients after recurrence. We, therefore, retrospectively evaluated the effect of adjuvant S-1 on survival following recurrence and the efficacy of first-line chemotherapy given at the time of relapse in patients with recurrent gastric cancer.

As shown in figure 1, patients initially treated with adjuvant S-1 had shorter survival following the recurrence than those receiving no adjuvant treatment (MST 287 vs.

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Nakazuru/Mita/Tsujinaka

547 days, p = 0.0034). Similarly, adjuvant chemotherapy was reported to have a negative impact on outcome after recurrence in other types of cancer such as colon and breast [20, 21]. As for the results of subset analysis of cohort A shown in figure 2, there may be some controversies. MST of the patients who relapsed after completion of 12 months of S-1 adjuvant chemotherapy was 464 days, equivalent to that of 451 days in cohort B. Although the duration of S-1 adjuvant chemotherapy showed no effect on OS after recurrence, this lack of statistical difference between the subgroups might be due to the small sample size. However, at least, the patients who discontinued S-1 adjuvant chemotherapy within 12 months because of recurrence were very unlikely to be salvaged by the additional chemotherapy given at the time of relapse. Although there was an imbalance of initial stage of the primary tumor between cohorts A and C, as shown in table 1, MSTs at stage II-III and IV in cohort A were 237 and 479 days, respectively, while they were 588 and 290 days in cohort C, respectively, with no significant difference between stage II-III and IV. Furthermore, on multivariate analysis in table 4, S-1 adjuvant chemotherapy but not initial stage was confirmed as an independent prognostic factor for OS after recurrence. Absence of a measurable lesion and presence of multiple metastatic sites also significantly correlated with inferior survival on multivariate analysis. MSTs of patients whose metastatic lesions involved the peritoneum (n = 36), bone/skin (n = 6), lymph nodes (n = 34) and liver (n = 19) were 285, 209, 609 and 426 days, respectively. Prior receipt of S-1 adjuvant chemotherapy as well as absence of a measurable lesion and presence of multiple metastatic sites contributed to the poor prognosis following tumor recurrence. These prognostic factors identified in this study might become useful factors of stratification for future clinical trial design in patients with recurrent gastric cancer.

With respect to the efficacy of first-line chemotherapy given at the time of relapse, patients who had received S-1 adjuvant chemotherapy showed a significantly lower RR than those receiving no adjuvant treatment: 6.7 versus 42.9% (p = 0.0007) as shown in table 3. Likewise, in patients with recurrent breast cancer, adjuvant chemotherapy was demonstrated to be a significant factor in predicting a poor response to first-line chemotherapy after recurrence [21]. As for the choice of first-line regimen given at the time of relapse, about two thirds of patients in cohort A received non-S-1-based therapy after adjuvant S-1. Although 1 retrospective study reported the invalidity of S-1-based chemotherapy as first-line treatment for recurrent disease after adjuvant S-1 in terms of a significantly lower RR, DCR as well as shorter progression-free survival compared to non-S-1-based chemotherapy [22], it still remains a problem to be clarified prospectively whether patients failing S-1 adjuvant chemotherapy should subsequently be treated with non-S-1-based regimens. In fact, in cohort A, patients treated with non-S-1-based chemotherapy showed an MST of 287 days with RR of 9.5% and DCR of 52.4%, while those with S-1-based regimens demonstrated an MST of 268 days with RR of 0% and DCR of 33.3%, with no significant difference among them. These findings suggest that patients who recurred following S-1 adjuvant chemotherapy must have extremely aggressive tumors refractory to any kind of further chemotherapy.

The poor outcome following relapse in patients who had received adjuvant S-1 might be speculatively interpreted as follows. While noncurative adjuvant chemotherapy might eradicate sensitive tumor cells, adjuvant S-1 could screen and select biologically more aggressive cellular clones with intrinsic resistance to cytotoxic agents that progress more quickly once recurrence is identified, or could induce acquired cellular resistance to further chemotherapy, like anthracyclines which induce the development of multidrug resistance [23]. In either case, the tumor mass would be constituted mainly of resistant cells at the time of relapse or, as a consequence, a poor response to first-line chemotherapy and a shorter OS would be expected following recurrence. In a recent report [24], adjuvant S-1, compared to surgery alone, was shown to deteriorate recurrence-free survival as well as OS after curative gastrectomy when confined to patients with high intratumoral mRNA expression of thymidylate synthase (TS). Although high TS expression is well correlated with resistance to 5-FU [25] derived from S-1, these findings suggest that biologically more aggressive cancer cells could be induced by the S-1 administration in a tumor with high TS expression.

Irrespective of types of regimens, adjuvant chemotherapy was reported to be significantly associated with a low probability of response to first-line chemotherapy and shorter survival following recurrence in patients with recurrent breast cancer [21]. However, in the present study, adjuvant treatment with 5-FU agents other than S-1 showed modest effects on OS and RR compared to adjuvant S-1, though adjuvant S-1 adversely affected OS and RR in recurrent gastric cancer patients, as shown in figure 1 and table 3. It is not clear whether this difference in adverse effect between S-1 and other 5-FU agents depends on the ability in inducing chemoresistant cells of the respective agent.

DFI was not significantly prognostic of survival following recurrence on either univariate or multivariate analysis in this study, as shown in table 4. There have been some controversies about the effect of DFI on OS after recurrence. Patients recurred with longer DFI had superior survival to those with shorter DFI in recurrent colon cancer [20]. On the contrary, the MST of metastatic patients pretreated with adjuvant chemotherapy was independent of DFI in recurrent breast cancer [21]. In this study, the numbers of patients with a DFI less than 1 year, from 1 to 2 years, from 2 to 3 years and more than 3 years were 43, 29, 9 and 8, respectively. It remains possible for DFI to become a prognostic factor if the number of patients with a long DFI increases.

Of note, the MST of 287 days following recurrence in patients initially treated with adjuvant S-1 after curative gastrectomy was similar to that of 7 months yielded by the subsequent chemotherapy to S-1 in advanced/recurrent gastric cancer [12, 14]. No matter who received the first-line chemotherapy of S-1 as an adjuvant one or not, the OS after the usage of S-1 might be the same in patients who had recurrent tumor left after S-1 administration.

Although we believe that this is the first report demonstrating that patients initially treated with S-1 adjuvant chemotherapy had significantly inferior survival following recurrence and poorer response to first-line chemotherapy, compared with those without any adjuvant treatment after curative gastrectomy, it should be noted that the present study is a retrospective small-sized analysis performed at a single center. The results shown here warrant further study to elucidate the effect of S-1 adjuvant chemotherapy in patients with recurrent gastric cancer and to investigate an optimal regimen for patients relapsed after adjuvant S-1, though a prospective randomized study seems infeasible because adjuvant S-1 has become the standard treatment for stage II-III gastric cancer patients in Japan.

Acknowledgement

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INTERNATIONAL FORUM

Evaluation of risk factors for the development of cirrhosis in autoimmune hepatitis: Japanese NHO-AIH prospective study

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Abstract Autoimmune hepatitis (AIH) is a chronic and progressive liver disease characterized by histological interface hepatitis and circulating autoantibodies. Our aims were to evaluate risk factors that contribute to the outcome and, particularly, the development of liver cirrhosis in a prospective multicenter cohort study of AIH. One hundred and seventy-four patients were enrolled. Histologically 21 (12.1%) had cirrhosis at the initial observation and the remaining 153 showed chronic or acute hepatitis at presentation. Among the latter 153 patients, 14 developed cirrhosis during the follow-up period (mean 8.0 years). Demographic, clinical, and laboratory indices associated with the development of cirrhosis were identified. Patients who developed cirrhosis differed in mean levels of alanine aminotransferase (ALT; 158 ± 182 vs. 441 ± 423 IU/ml) and platelet counts (14.7 ± 5.5 vs. $19.4 \pm 6.9 \times 10^4/\mu l$) at presentation and received lower doses of corticosteroid (13.9 ± 15.8 vs. 31.8 ± 85.5 mg/day). In a multivariate analysis, an independent predictor for progression to cirrhosis was an older age of onset (≥ 60 years). AIH patients with cirrhosis, or those who developed cirrhosis, had a worse survival. AIH patients with an older age of onset were likely to develop cirrhosis, and careful observation and aggressive treatments are necessary for such patients.

Keywords Autoimmune hepatitis · Liver cirrhosis · Multicenter cohort study · Outcome

Introduction

Autoimmune hepatitis (AIH) is a chronic liver disease characterized by unresolving inflammation affecting the parenchymal cells of the liver [1]. The disease predominantly affects females and is characterized by elevated levels of serum transaminase enzymes, the presence of autoantibodies, and increased gamma globulins, particularly immunoglobulin G (IgG) [2]. Most patients achieve biochemical and histological remission after immunosuppressive treatments, and the outcome has been generally reported as good [3]. The natural history and outcome of AIH are largely defined by the degree of hepatic inflammatory activity and progression to liver cirrhosis [3]. But, even in patients with established liver cirrhosis, appropriate treatment ameliorates the histological fibrosis [4]. A link between the development of cirrhosis and recurrent relapses has been suggested, and one study demonstrated that the development of cirrhosis was significantly associated with relapses [5]. Therefore, it would be useful to understand what factors can determine the progression to or resolution of cirrhosis during the course of AIH. To better understand the natural history and outcome of AIH, a nationwide multicenter cohort study seemed to be needed, and here we describe the clinical presentation and course of 174 consecutive AIH patients enrolled in the Japanese National Hospital Organization (NHO) AIH register. We particularly assessed factors predictive of the development of cirrhosis.

The members of the NHO-AIH Study Group are listed in the Appendix.

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Patients and methods

Study population

There were 189 patients initially enrolled in the register of the Japanese NHO Liver-Network Study and, of these, 174 were prospectively followed between 1995 and 2008 as a multicenter cohort study. All patients satisfied the 1999 revised criteria of the International Autoimmune Hepatitis Group (AIHG) for a diagnosis of definite (91 cases) or probable (83 cases) AIH [6]. Each patient had received conventional treatment, usually corticosteroid drugs, and some (66/174, 37.9%) had received ursodeoxycholic acid (UDCA). The study protocol was approved by the Ethics Committees of all involved institutes.

Clinical and histological assessments

Standard laboratory tests of liver inflammation and function were assessed at each examination and these follow-up data were collected at 1-year intervals.

Liver tissue from percutaneous biopsy specimens was available for the majority of the patients at the study entry (131/174, 75.3%) and at subsequent follow-up examination for some (38/174, 21.8%). The histological variables examined included degree of fibrosis (0, absent; 1, expansion of fibrosis to parenchyma; 2, portal-central or portal-portal bridging fibrosis; 3, presence of numerous fibrous septa; 4, multinodular cirrhosis). The histological diagnosis of cirrhosis required loss of normal lobular architecture, reconstruction of hepatic nodules, and the presence of regenerative nodules [7]. Anti-nuclear antibodies (ANA) and smooth muscle antibodies (SMA) were measured by indirect immunofluorescence, and cut-off titers were 1:40. In patients with risk factors for nonalcoholic fatty liver disease (NAFLD), a definite diagnosis of AIH was confirmed by the liver histological findings [8]. Differential diagnosis of primary biliary cirrhosis (PBC) was performed according to the diagnostic criteria as described previously [9].

Variables at study entry

Demographic and other characteristics of the 174 patients were recorded at the baseline assessment and included gender; age at diagnosis; duration of symptoms or other evidence of liver diseases; markers of infection with hepatitis viruses (HBV, HCV); alcohol intake; coexistence of other autoimmune diseases; serum levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase, and bilirubin; platelet counts; and prothrombin time. Clinical relapse was defined as an increase of serum ALT levels to more than threefold the upper limit of the normal range (ULN) [10]. Data were collected and stored in a database.

Statistical analysis

For quantitative data, analysis was performed using the Mann–Whitney rank test for comparison of two independent groups. Differences in proportions were analyzed by Fisher's exact test when the number of subjects was <5, and the χ^2 test for 2 × 2 tables was used when the number of subjects was >5. Univariate and multivariate analyses were performed using the Cox proportional hazard model with SPSS software (Chicago, IL, USA). The p values for entering variables for the multivariate Cox proportional hazard model were <0.1.

Results

Baseline data at entry

Of the original 189 patients registered as having AIH, 15 were excluded from the analysis for the following reasons: an alternative diagnosis was made; medical records were incomplete; or they were lost to follow-up (Fig. 1). The remaining 174 patients were eligible for the study. Table 1 shows other demographic data for the cohort at entry. Data on autoantibodies were incomplete, in that data for SMA were lacking in 95 patients and data for ANA were lacking in 1 patient. Of the remaining patients, 144 of 173 (83.2%) had a positive test for ANA (>1:80) and 31 of 79 patients (39.2%) had a positive test for SMA (>1:80).

Development of cirrhosis

Among the 174 eligible patients, 21 (12.1%) had cirrhosis at the time of presentation, and among the remaining 153 patients without cirrhosis, 14 developed cirrhosis during follow-up (Fig. 1). For these 14 patients the time of progression to cirrhosis (mean ± standard deviation, SD) was 9.1 ± 4.4 years. Table 2 compares the clinical parameters of patients who did, or did not, develop cirrhosis during the follow-up period. Patients who developed cirrhosis were older at the onset of symptoms and had significantly lower levels of aminotransferases. The initial mean dose (± SD) of corticosteroid (prednisolone) was significantly lower in patients who developed cirrhosis compared with the dose in those who did not (13.9 ± 15.8 vs. 31.8 ± 8.5 mg/day). On time-dependent univariate analysis (Cox proportional hazard model), the development of cirrhosis was predictive by older age at onset (≥60 years, $p = 0.014$), being without steroid treatment ($p = 0.047$), and having lower serum ALT levels ($p = 0.007$) at presentation (Table 3).

Fig. 1 Flow diagram of patient selection and clinical outcome of autoimmune hepatitis (AIH) patients in the present cohort study. *LC* Liver cirrhosis
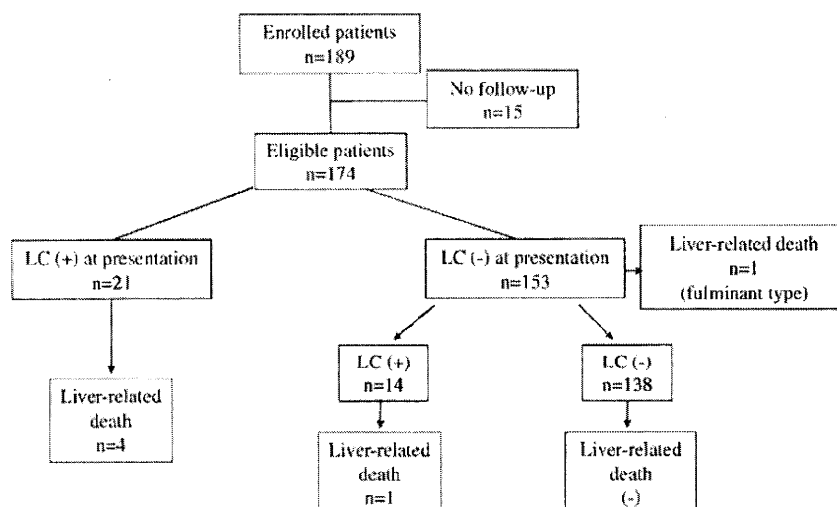

Table 1 Baseline characteristics of autoimmune hepatitis (AIH) patients

	$n = 174$
Mean age (years)	
Age ≥60	89
Age <60	85
Gender (male/female)	16/158
Mean age at presentation (years)	56.7 ± 13.9 (16–84)
Other autoimmune diseases	45 (25.9%)
Mean follow-up (years)	8.0 ± 4.5 (0.1–21)
Baseline laboratory values	
AST (<40 IU/l)	396.83 ± 460.80 (29–2718)
ALT (<40 IU/l)	413.25 ± 427.05 (18–2020)
ALP (<112 IU/l)	443.16 ± 263.47 (112–2135)
Bilirubin (mg/ml)	4.08 ± 5.80 (0.27–31.8)
Albumin (3.5–5.0 g/l)	3.77 ± 0.61 (2.00–5.10)
IgG (500–1300 mg/dl)	2511.57 ± 904.80 (210.2–5199)
Platelets (15–40 × 10^4/μl)	18.70 ± 7.4 × 10^4 (2–42 × 10^4)
ANA+ (≥1:40)	144/173 (83.2%)
SMA+ (≥1:40)	31/79 (39.2%)
Cirrhosis at presentation	21 (12.1%)
Received treatment	
PSL alone	88 (50.6%)
PSL + UDCA	35 (20.1%)
PSL + Aza	2 (1.1%)
UDCA alone	31 (17.8%)
Relapse	47 (27.0%)

AST aspartate aminotransferase, *ALT* alanine aminotransferase, *ALP* alkaline phosphatase, *ANA* anti-nuclear antibodies, *SMA* anti-smooth muscle antibodies, *PSL* prednisolone, *UDCA* ursodeoxycholic acid, *Aza* azathioprine

By multivariate Cox analysis, an older age at onset (≥60 years, hazard ratio 3.9, 95% confidence interval [CI] 1.1–14.3, $p = 0.039$) was associated independently with the risk of cirrhosis development (Table 4). Thus, the AIH patients with an older age at onset had a greater risk of developing cirrhosis. We also analyzed the association of serum ALT levels after 1 year of treatment and the progression of liver cirrhosis. However, we could not find any association between ALT levels after 1 year and the progression to liver cirrhosis (Table 5).

Finally, we assessed the clinical and histological features in all of the registered AIH patients, paying particular attention to the group of patients with AIH presenting at ≥60 years (Table 6). These 89 (51.0%) patients who developed AIH at age 60 years or more had a mean age of 67.6 years (range 60–84 years), whereas at the time of diagnosis the 85 younger patients had a mean age of 45.3 years (range 16–59 years). For the two groups, there were no significant differences in values for liver function tests, or frequencies of concomitant autoimmune diseases (24.7 vs. 27.1%). In patients who underwent liver biopsy, histological fibrosis scores (stage) were not significantly different between the older-onset and younger-onset AIH patients. Furthermore, in the whole cohort, there was no difference in the frequency of cirrhosis at presentation between older-onset and younger-onset patients.

Discussion

AIH is a chronic progressive liver disease caused by an autoimmune destruction of hepatic parenchymal cells. Essential clinical diagnostic criteria include histological interface hepatitis, hypergammaglobulinemia, and characteristic serum autoantibodies [1]. The precise pathogenic processes in AIH are uncertain. They likely depend on a genetic predisposition of the host leading to reactivity to particular self-antigens, which causes hepatic inflammation by presumed immune-cell-mediated cytotoxicity

Table 2 Baseline characteristics of AIH patients who developed cirrhosis

	Cirrhosis (+) $n = 14$	Cirrhosis (−) $n = 138$	p
Mean age (years)			
Age ≥60	11	65	0.025
Age <60	3	73	
Gender (male/female)	1/13	12/126	0.659
Mean age at presentation (years)	63.4 ± 8.9	56.0 ± 14.2 (16–84)	0.068
Other autoimmune diseases	3 (21.4%)	36 (26.1%)	0.494
Mean follow-up (years)	9.1 ± 4.4 (2–15)	7.6 ± 4.4 (0.8–21)	0.225
Baseline laboratory values			
AST (<40 IU/l)	144.00 ± 109.58 (48–459)	425.36 ± 480.24 (29–2718)	0.016
ALT (<40 IU/l)	158.50 ± 182.18 (30–613)	440.81 ± 423.49 (18–2020)	0.002
ALP (<112 IU/l)	554.50 ± 509.16 (181–2135)	441.59 ± 232.80 (112–1555)	0.927
Bilirubin (mg/ml)	2.76 ± 3.89 (0.6–15.8)	4.09 ± 5.88 (0.27–31.8)	0.964
Albumin (3.5–5.0 g/l)	3.49 ± 0.77 (2–4.6)	3.87 ± 0.54 (2.3–5.1)	0.113
IgG (500–1300 mg/dl)	2478.64 ± 696.67 (1490–3489)	2421.85 ± 874.18 (210.2–4891)	0.677
Platelets (15–40 × 10^4/μl)	14.7 ± 5.5 (2–23 × 10^4)	19.9 ± 6.9 (2–42 × 10^4)	0.013
ANA+ (≥1:40)	11/14 (78.6%)	117/138 (84.8%)	
SMA+ (≥1:40)	0/5	31/68 (45.6%)	
Received treatment			
Mean PSL mg/day (range)	13.92 ± 15.83 (0–40)	31.77 ± 85.53 (0–1000)	0.048
PSL ≥20 mg	6 (42.9%)	90 (65.7%)	
PSL alone	4 (28.6%)	74 (53.6%)	
PSL + UDCA	3 (21.4%)	25 (18.1%)	
PSL + Aza	0	2 (1.4%)	
UDCA alone	5 (35.7%)	23 (16.7%)	
Relapse	2 (16.7%)	37 (26.8%)	0.269
Liver biopsy specimen available at presentation	($n = 8$)	($n = 96$)	
Stage of fibrosis			
F0	2 (25.0%)	8 (8.3%)	
F1	2 (25.0%)	31 (32.3%)	
F2	2 (25.0%)	30 (31.3%)	
F3	2 (25.0%)	27 (28.1%)	
F4	0	0	

AST aspartate aminotransferase, *ALT* alanine aminotransferase, *ALP* alkaline phosphatase, *ANA* anti-nuclear antibodies, *SMA* anti-smooth muscle antibodies, *PSL* prednisolone, *UDCA* ursodeoxycholic acid, *Aza* azathioprine

mechanisms [11]. Cytokines and adipokines play an important role in the progression of AIH [12, 13]. In the present study we assessed, in particular, determinants of the progression to liver cirrhosis in the Japanese NHO multi-center AIH cohort study. Our data demonstrated that onset in later life (≥60 years) was one independent risk factor for the development of liver cirrhosis.

It was reported earlier that older-onset AIH patients (≥60 years) had a higher frequency of cirrhosis at the time of presentation than younger-onset AIH patients (≤30 years), 33 vs. 10% [14]. However, in our study, the older-onset AIH patients (≥60 years) did not exhibit a greater degree of hepatic fibrosis at presentation compared with younger-onset AIH patients according to liver biopsy findings. However, our data do suggest that hepatic fibrosis progresses more extensively in older-onset AIH patients. Hepatic fibrosis is a dynamic and highly regulated process that has been shown to progress and regress [15]. Intrahepatic inflammation with the release of cytokines and participation of chemokines stimulates the transition of perivascular hepatic stellate cells into fibrogenic myofibroblasts, and these activated cells proliferate and synthesize fibrillar collagens and other matrix proteins [16]. Our findings suggest that aging in itself may potentiate these processes of fibrogenesis.

Table 3 Variables associated with increased risk factors for developing liver cirrhosis (univariate Cox proportional hazard model)

Characteristics	Subgroup	LC		HR (95% CI)	p value
		Yes (n = 14)	No (n = 138)		
Gender	Male	1 (7.1%)	12 (8.7%)	1.682 (0.209–13.556)	0.625
Age (years)	<50	2 (14.3%)	37 (26.8%)	0.476 (0.105–2.154)	0.335
	50–59	1 (7.1%)	36 (26.1%)	0.159 (0.021–1.233)	0.078
	≥60	11 (78.6%)	65 (47.1%)	4.965 (1.380–17.862)	0.014
Other autoimmune disease	(+)	3 (21.4%)	36 (26.1%)	0.672 (0.186–2.420)	0.543
ALT	<100	8 (57.1%)	30 (21.7%)	4.490 (1.508–13.369)	0.007
	100–500	4 (28.6%)	62 (44.9%)	0.496 (0.155–1.585)	0.237
	≥500	2 (14.3%)	46 (33.3%)	0.361 (0.080–1.623)	0.184
PSL	(−)	7 (50.0%)	37 (26.8%)	3.004 (1.016–8.879)	0.047
	1–19 mg/day	1 (7.1%)	11 (8.0%)	0.578 (0.075–4.460)	0.599
	20–39 mg/day	4 (28.6%)	47 (34.1%)	0.832 (0.260–2.665)	0.757
	≥40 mg/day	2 (14.3%)	43 (31.2%)	0.385 (0.085–1.737)	0.214
Relapse	(+)	2 (14.3%)	36 (26.1%)	0.437 (0.098–1.956)	0.279

ALT alanine aminotransferase, *LC* liver cirrhosis, *HR* hazard ratio, CI confidence interval, *PSL* prednisolone

Table 4 Multivariate analysis of predictive factors for cirrhosis in AIH patients (Cox proportional hazards model)

Variables	p	HR (95% CI)
Age ≥60 years	0.039	3.917 (1.074–14.290)
ALT <100	0.054	2.987 (0.979–9.108)
PSL (−)	0.174	2.160 (0.711–6.559)

ALT alanine aminotransferase, *PSL* prednisolone, *HR* hazard ratio

Previous studies have demonstrated that the suppression of inflammatory activity promotes the degradation of a fibrotic liver matrix. Miyake et al. [17] reported that an inability to maintain normal transaminase levels was an independent prognostic factor for the development of cirrhosis. In the present study, we did not find any association between the frequency of clinical relapses and progression to cirrhosis. Further large-scale studies will be needed to elucidate the links between the degree of remission and fibrogenesis in AIH.

Dufour et al. [4] reported in 1997 that, in a small number of AIH patients, fibrosis and even cirrhosis were reversible

with appropriate treatments. These findings suggest that liver cirrhosis in AIH is reversible, likely due to reduced hepatic inflammatory activity in response to immunosuppressive treatments. Another interesting aspect of our study is that the initial steroid doses were lower in AIH patients who progressed to cirrhosis compared with the initial doses in those who did not. The immunological mechanism of liver injury in AIH is still not fully understood, but both cellular and humoral immune reactions appear to be involved in the liver inflammation [18]. Successful immunosuppressive treatments will arrest AIH progression to cirrhosis. Older patients have been shown to enter remission to a degree similar to that in younger patients, and treatment failure is less often during maintenance periods in the older patients. Therefore, the conventional combination regimen, prednisolone and azathioprine, can be recommended in older AIH patients with active hepatic inflammation in the presence or absence of advanced liver fibrosis.

Based on the results of the present study, we propose that one of the host-dependent factors, olderage at onset, determines the frequency of the progression of AIH to

Table 5 ALT levels 1year after the first treatment and progression to liver cirrhosis (univariate Cox proportional hazard model)

ALT levels (at 1 year)	LC		HR (95% CI)	p value
	Yes (n = 10)	No (n = 120)		
<×1 ULN	6 (60.0%)	78 (65.0%)	1.065 (0.296–3.831)	0.923
×1–×2 ULN	2 (20.0%)	28 (23.3%)	0.616 (0.130–2.915)	0.541
×2–×3 ULN	0	5 (4.2%)		
≥×3 ULN	2 (20.0%)	9 (7.5%)	2.195 (0.446–10.808)	0.334

ALT alanine aminotransferase, *LC* liver cirrhosis, *HR* hazard ratio, *ULN* upper limit of normal range

Table 6 Baseline characteristics of elderly-onset AIH patients

	Age ≥60 years n = 89	Age <60 years n = 85	p
Mean age at presentation (years)	67.63 ± 5.42 (60–84)	45.32 ± 10.67 (16–59)	
Gender (male/female)	9/80	7/78	0.668
Other autoimmune diseases	22 (24.7%)	23 (27.1%)	0.725
Mean follow-up (years)	7.1 ± 4.4 (0.1–21)	8.9 ± 4.5 (1–17)	0.010
Baseline laboratory values			
AST (<40 IU/l)	366.20 ± 431.64 (31–2718)	428.89 ± 492.57 (29–2350)	0.573
ALT (<40 IU/l)	355.29 ± 395.57 (18–1832)	473.93 ± 454.50 (22–2020)	0.046
ALP (<112 IU/l)	476.14 ± 310.91 (126–2135)	407.77 ± 198.53 (112–986)	0.205
Bilirubin (mg/ml)	3.52 ± 5.30 (0.3–28.4)	4.68 ± 6.30 (0.27–31.80)	0.580
Albumin (3.5–5.0 g/l)	3.61 ± 0.63 (2.0–4.9)	3.94 ± 0.55 (2.0–5.1)	0.000
IgG (500–1300 mg/dl)	2570.29 ± 865.76 (1051–4956)	2447.05 ± 952.43 (210–5199)	0.360
Platelets (15–40 × 10^4/µl)	16.5 ± 6.0 × 10^4 (2–29 × 10^4)	20.9 ± 8.1 × 10^4 (2–42 × 10^4)	0.000
ANA+ (≥1:40)	74/88	70/85	0.760
SMA+ (≥1:40)	11/37	20/42	0.104
Cirrhosis at presentation	12 (13.5%)	9 (10.6%)	0.558
Received treatment			
Mean PSL dose (mg/day)	33.57 ± 105.12 (0–1000)	25.54 ± 20.34 (0–60)	0.499
PSL alone	42 (47.2%)	46 (54.1%)	
PSL + UDCA	21 (23.6%)	14 (16.5%)	
PSL + Aza	1 (1.1%)	1 (1.2%)	
UDCA alone	17 (19.1%)	14 (16.5%)	
Relapse	25 (28.1%)	22 (25.9%)	
Histology			
Stage			
F0–F2	38/55	36/55	0.684
F3–F4	17/55	19/55	

AST aspartate aminotransferase, *ALT* alanine aminotransferase, *ALP* alkaline phosphatase, *ANA* anti-nuclear antibodies, *SMA* anti-smooth muscle antibodies, *PSL* prednisolone, *UDCA* ursodeoxycholic acid, *Aza* azathioprine

cirrhosis. It has been shown that aging is associated with dysregulated cytokine function and decreased capacity for accurate DNA repair, both of which factors may promote the development of liver fibrosis [19, 20]. However, in autoimmune inflammation, the precise relationships between aging and the progression of liver fibrosis are yet to be determined, and further studies are needed.

Appendix

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Original Article

Hepatitis C virus-specific CD8+ T cell frequencies are associated with the responses of pegylated interferon-α and ribavirin combination therapy in patients with chronic hepatitis C virus infection

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Aim: Hepatitis C virus (HCV)-specific cytotoxic T lymphocytes (CTLs) play critical roles in elimination of the HCV-infected hepatocytes. However, the mechanism of HCV elimination by pegylated interferon-α (peg-IFNα) plus ribavirin is not fully understood. We examined HCV-specific CTL responses during this combination therapy.

Methods: CD8+ T cells were isolated from 16 HCV infected patients treated by this combination therapy and were subjected to IFN-γ enzyme-linked immunospot (ELISPOT) assay.

Results: The numbers of IFN-γ spots against HCV Core or NS3 protein-derived peptides in HCV patients before treatment were similar to those in healthy donors, and those in HCV patients significantly increased 4 weeks after the initiation of combination therapy. All HCV Core or NS3 proteins-derived peptides specific CD8+ T cells responses in pre-treated patients were not associated with ALT levels and HCV viral loads of HCV patients before treatment. And those

in pre-treated patients were similar between sustained virologic responder (SVR) patients and non-SVR patients. Significant increase of HCV Core or NS3 proteins-derived peptides specific CD8+ T cells responses between before and 4 weeks after this combination therapy were observed in SVR patients, but not in non-SVR patients.

Conclusions: These results demonstrated that significant increase of HCV-specific CD8+ T cells at 4 weeks after the initiation of IFN treatment might be associated with the elimination of HCV. Our findings suggest that the reactivity against HCV Core and NS3 proteins-derived peptides might be useful in predicting the clinical outcome of the combination therapy of peg-IFNα and ribavirin.

Key words: chronic hepatitis C, HCV-specific CTL, IFN-γ ELISPOT, peg-IFNα, ribavirin

INTRODUCTION

CHRONIC INFECTION OF Hepatitis C virus (HCV) often leads to cirrhosis and hepatocellular carcinoma (HCC), which causes the poor prognosis of HCV-infected patients.[1,2] Combination therapy of pegylated interferon-α (Peg-IFNα) plus ribavirin is standard treat-

ment for patients with chronic hepatitis C (CH-C), and sustained virologic response (SVR) in this combination therapy occurs in about 40–60% of genotype 1 patients,[1,2] which can improve the prognosis of HCV-infected patients. HCV-specific cytotoxic T lymphocytes (CTLs) is believed to play essential roles in determining the course of chronic infection,[3] and the insufficient activation, dysfunction, suppression of CTLs may cause persistent infection of HCV.[4-6] The elimination of HCV by HCV-specific CTLs is believed to consist of second slope of decay after viral decay during the first 24–48 h of IFN therapy.[7] However, the detail immune mechanism of HCV elimination by this combination therapy is not fully understood. In addition to direct antiviral

property of Peg-IFNα and ribavirin against HCV infection, this combination therapy might have immunomodulatory activity. IFN-α enhances the maturation of antigen-presenting cells and CD4+ T cell function, but with little effect on CTLs. In contrast, ribavirin could induce a switch from Th2 to Th1 profile.[8] Although the base line immune responses of CTLs have been reported to be associated with the achievement of SVR in a few reports[7,8], even now there are relatively little reports examining the detail of HCV-specific CTL responses during this combination therapy.

IFN-γ enzyme-linked immunospot (ELISPOT) assay allows detection of finally differentiated effector CTLs, which means the ELISPOT data reflect the in vivo situation.[9–11] In the current study, we evaluated the HCV Core and NS3 proteins-derived peptides specific CD8+ T cells responses of the HCV infected patients by IFN-γ ELISPOT assay and examined the relationship between CTL activity and the clinical outcome of the combination therapy of Peg-IFNα plus ribavirin. The frequencies of HCV-specific CD8+ T cells in pre-treated HCV patients were not associated with antiviral activity of this combination therapy in SVR. However, the significant increase of HCV-specific CD8+ T cells at 4 weeks after the starting of IFN treatment could be observed in SVR patients, but not in non-SVR patients. Our findings suggest that the reactivity against HCV Core and NS3 proteins-derived peptides might be useful in predicting the clinical outcome of this combination therapy.

MATERIALS AND METHODS

Patients

SIXTEEN PATIENTS CHRONICALLY infected with HCV were examined for HCV specific CTL responses during the combination therapy of Peg-IFNα plus ribavirin. All patients enrolled in this study were infected with HCV genotype 1b with a high viral load and were HLA-A2 positive. The patients who were infected with other viruses (Hepatitis B virus, Human immunodeficiency virus) or had other forms of liver disease (alcohol liver disease, autoimmune hepatitis) were excluded from this study. Informed consent, under an Institutional Review Board-approved protocol, was obtained from each patient. All patients received Peg-IFNα-2b (PEGINTRON, Schering-Plough, Kenilworth, NJ) plus ribavirin (REBETOL, Schering-Plough) for the duration of the study of 48–72 weeks. In only one patient (Patients#11), treatment was stopped at 24 weeks because this patient remained HCV-RNA positive after

24 weeks and developed significant side effect. To evaluate the antiviral activity, serum HCV RNA levels were quantified during the combination treatment. Serum HCV RNA level was quantified using the COBAS AMPLI-CORE HCV MONITOR test (version 2.0; Roche Diagnostics, Branchburg, NJ). SVR was defined as the absence of detectable serum HCV RNA at 24 weeks after the end of the combination therapy. All treated patients were assessed the antiviral responses (SVR or non-SVR) as previously described.[12] The characteristics of patients with chronic HCV infection were summarized in Table 1.

CD8+ T cells isolation from peripheral blood mononuclear cells (PBMC)

PBMC was obtained from 16 treated HCV infected patients before IFN treatment (pre-IFN) and 4 weeks after starting of this combination therapy (IFN-4week) and six healthy donors. CD8+ T cells were isolated from PBMC by magnetic cell sorting using CD8 MicroBeads according to the manufacturer's instructions (Miltenyl Biotech, Auburn, CA). More than 95% of the cells were CD8+ lymphocytes.

IFN-γ ELISPOT assays for HCV Core and NS3 protein-derived peptide-specific CD8+ T cells responses

To evaluate the frequencies of CD8+ T cells recognizing peptide epitopes, IFN-γ ELISPOT assay were performed as previously described.[11] Briefly, 96-well multiscreen hemagglutinin antigen plates (Millipore, Billerica, MA) were coated with 10 μg/mL of anti-human IFN-γmAb (1-D1K; Mabtech, Stockholm) in phosphate-buffered saline (PBS) overnight at 4°C. Unbound antibody was removed by four successive washing with PBS. After blocking the plates with RPMI 1640/10% human serum (1 h, 37°C), 1×10^5 CD8+ T cells were co-cultured with 2×10^4 T2.DR4 cells (HLA-A2 positive peptide-presenting cells generously provided from Dr Walter J. Storkus, University of Pittsburgh, School of Medicine, Pittsburgh, PA) pulsed with HCV Core and NS3 derived peptides (a final concentration of 10 μg/mL). HLA-A2-restricted HCV Core protein derived peptides ($Core_{35–44}$, YLLPRPGPRL, $Core_{131–140}$, ADLMGYIPLV) or NS3 protein derived peptides ($NS3_{1073–1081}$, CINGVCWTV, $NS3_{1406–1415}$, KLVALGINAV) were synthesized as previously described.[13] Negative control wells contained CD8+ T cells with T2.DR4 cells pulsed with $HIV-nef_{190–198}$ peptide (AFHHVAREL). After 24 h incubation of the plates, cells were removed from the ELISPOT well by washing and captured cytokine was detected at sites of their secretion

Table 1 Characteristics of patients with chronic hepatitis C virus (HCV) infection

Subject	Age	Sex	HCV-RNA (KIU)	ALT (U/l)	Treatment duration	SVR
1	43	F	440	17	48 week	SVR
2	56	M	2000	146	48 week	non
3	49	F	1200	31	72 week	SVR
4	49	M	340	106	48 week	SVR
5	65	F	3800	24	72 week	SVR
6	58	M	320	25	48 week	SVR
7	56	M	2551	24	48 week	non
8	55	M	939	43	48 week	SVR
9	46	M	1200	64	48 week	SVR
10	46	M	1059	42	48 week	SVR
11	43	M	407	91	24 week	non
12	63	F	1621	61	48 week	non
13	63	F	1841	63	48 week	non
14	47	M	458	41	48 week	SVR
15	36	M	1024	79	48 week	non
16	61	F	677	148	48 week	non

ALT, alanine aminotransferase; F, female; M, male; non, non-SVR; SVR, sustained virologic response.

by incubation for 2 h with biotinylated mAb anti-human IFN-γ (7-6B-1, Mabtech) at 2 µg/mL. Plates were washed six times and avidin-peroxidase complex (Vectastain Elite Kit, Vector Laboratories, Burlingame, CA) were added for 1 h. Unbound complex was removed by washing and 3-Amino-9-ethylcarbazole substrate (Sigma, St Louis, MO) was added for 5 min. The data are represented as mean IFN-γ spots per 100 000 T cells analyzed.

Statistics

All values were expressed as the mean and standard deviation (SD). The statistical significance of differences between the groups was determined by applying Mann–Whitney U-test. We defined statistical significance as $P < 0.05$.

RESULTS

Analysis of HCV derived peptide-specific IFN-γ release of peripheral blood CD8+ T cells in ELISPOT assay

WE ASSESSED PERIPHERAL blood CD8+ T cell responses against HCV derived peptides (Core$_{35-44}$, Core$_{131-140}$, NS3$_{1073-1081}$, NS3$_{1406-1415}$) in 16 HLA-A2+ HCV patients and 6 healthy donors. As shown in Figure 1, the numbers of IFN-γ spots (per 100 000 CD8+ T cells) observed for T cell responses against HCV peptides in pre-IFN patients were as low as those observed in healthy HLA-A2+ donors. In contrast, significant eleva-

tions of ELISPOT reactivity to three peptides (Core$_{131-140}$, NS3$_{1073-1081}$, NS3$_{1406-1415}$) were observed in IFN-4week patients compared with healthy donors. The number of IFN-γ spots against Core$_{35-44}$ peptides in IFN-4week patients also tended to be higher than those in healthy donors. In treated HCV patients, the numbers of IFN-γ spots against all four HCV derived peptides in IFN-4week patients were significantly higher than those in pre-IFN patients (Fig. 1). We also examined whether the frequencies of HCV-specific CD8+ T cell responses were associated with sex difference. The frequencies of CTLs against all four peptides were similar between males and females before and 4 weeks after starting treatment (data not shown).

HCV-specific CD8+ T cell responses in pre-IFN patients were not associated with the antiviral activity of the combination therapy of Peg-IFNα-2b plus ribavirin

We examined the association between HCV-specific CD8+ T cell responses in pre-IFN patients and ALT levels or HCV viral load before treatment. No association was observed between the frequencies of HCV-specific CD8+ T cells in pre-IFN patients and ALT levels or HCV viral load of pre-treated patients (Fig. 2).

We next examined whether HCV-specific CD8+ T cell responses in pre-IFN patients were associated with the antiviral activity of this combination therapy. As shown in Figure 3, the frequencies of CD8+ T cell responses against all four HCV proteins-derived peptides in
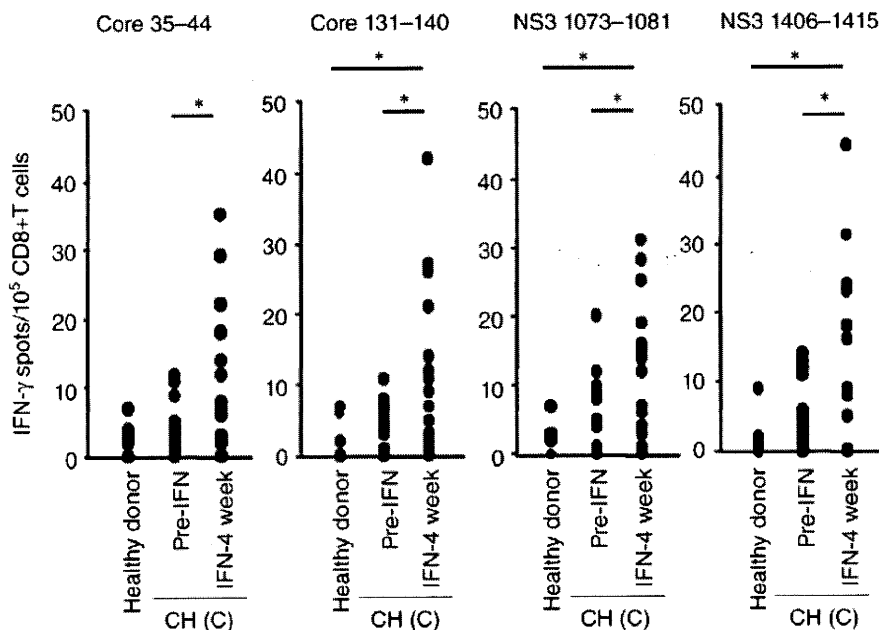
Figure 1 Interferon (IFN)-γ enzyme-linked immunospot (ELISPOT) analysis of hepatitis C virus (HCV)-specific CD8+ T cell responses in HCV patients treated with the combination therapy of peg-IFNα plus ribavirin. Peripheral blood CD8+ T cells were isolated from HLA-A2+ healthy donors and chronic hepatitis C (CH-C) patients. The CH-C patients were treated with the combination therapy of peg-IFNα plus ribavirin and PBMC were isolated from pre-treated patients (Pre-IFN) and treated patients 4 weeks after starting treatment (IFN-4week). HCV-specific CD8+ T cell responses were evaluated by IFN-γ ELISPOT as outlined in "Materials and Methods". Data are reported as IFN-γ spots/ 100 000 CD8+ T cells and represent the mean of triplicate determinations. T cell reactivity against T2.DR4 cells pulsed with HLA-A2-presenting HIV-nef$_{190-196}$ epitope served as the negative control in all cases, and this value was subtracted from all experimental determinations to determine HCV specific spots numbers. Each symbol within a panel represents the response of an individual donor to the indicated HLA-A2-presenting HCV Core- or NS3-peptides. *$P < 0.05$.

pre-IFN patients were not significantly different between SVR, the group of the patients who were observed SVR, and non-SVR, the group of the patients who were not observed SVR. These results suggested that the baseline HCV-specific CD8+ T cell responses in HCV patients were not associated with the antiviral activity of this combination therapy.

Significant early elevation of HCV-specific CD8+ T cell responses were associated with the antiviral activity of the combination therapy of Peg-IFNα plus ribavirin

We examined the association between early elevation of HCV-specific CD8+ T cell responses and the antiviral activity of this combination therapy. We evaluated the frequencies of CD8+ T cell responses against HCV proteins-derived peptides before and 4 weeks after starting treatment. As shown in Figure 4, in SVR patients, the frequencies of CD8+ T cell responses against all four HCV peptides (Core$_{35-44}$, Core$_{131-140}$, NS3$_{1073-1081}$, NS3$_{1406-1415}$) increased significantly 4 weeks

after starting treatment. In contrast, the frequencies of CD8+ T cell responses against all four HCV peptides did not increase in non-SVR patients. These results demonstrated that significant early elevation of HCV-specific CD8+ T cell responses were associated with the antiviral activity of this combination therapy.

DISCUSSION

HCV-SPECIFIC CD8+ CTLs have been reported to play a significant role in the elimination of HCV in acute hepatitis of HCV.[4,9] In contrast, in chronic infection of HCV, HCV-specific CD8+ T cell responses were weak and were directed against a limited series of epitopes compared with acute hepatitis.[9] These might cause persistent infection of HCV in the HCV infected host. However, conflicting results have been reported with respect to HCV-specific CD8+ T cell responses on the antiviral activity of IFN therapy. IFNα monotherapy may promote viral clearance by enhancing the host CTL responses.[14,15] But Rehermann et al. reported that CTL
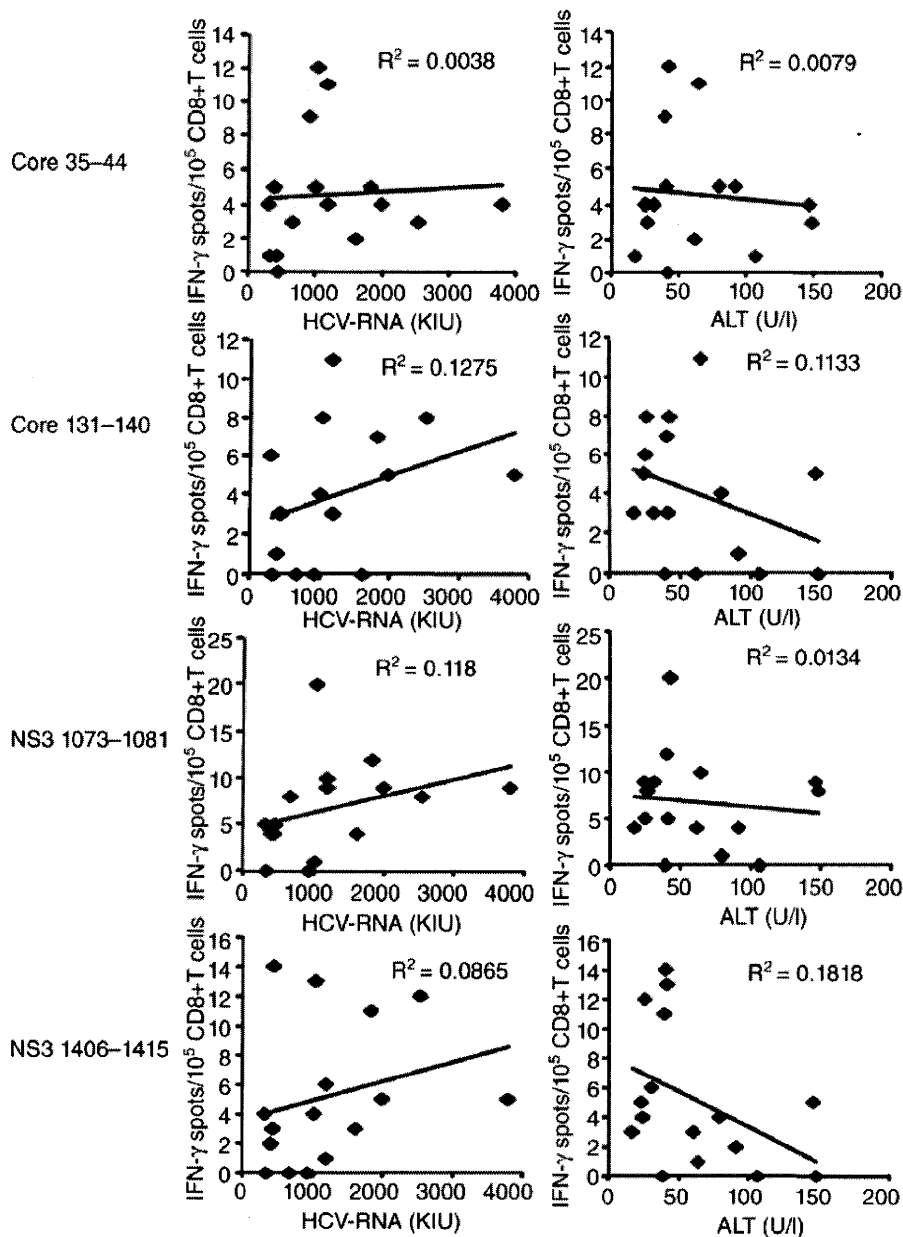
Figure 2 The association between the hepatitis C virus (HCV)-specific CD8+ T cell responses of pre-IFN patients and the serum alanine aminotransferase (ALT) levels or the HCV viral load of patients before treatment. The frequencies of HCV Core and NS3 proteins-derived peptides specific CD8+ T cell responses in pre-IFN HCV patients were evaluated by interferon (IFN)-γ enzyme-linked immunospot (ELISPOT). We examined the association between the frequencies of HCV Core and NS3 proteins-derived peptides specific CD8+ T cell responses in pre-IFN HCV patient and the serum ALT levels or HCV viral loads of patients before treatment.

precursor frequencies against a range of HCV epitopes did not change during or after the course of IFNα monotherapy.[13] Recently, the combination therapy of PegIFNα plus ribavirin is standard treatment in the treatment of HCV infected patients with the better results of viral clearance compared with IFNα monotherapy. This suggested that this combination therapy might modify the HCV specific CD8+ T cell responses. We evaluated HCV-specific CD8+ T cell responses by IFN-γ ELISPOT assay, a functional assay of T cells. Significant increase of the frequencies of HCV-specific CD8+ T cells between pre-IFN and IFN-4week could be

observed in SVR patients, but not in non SVR patients. This is consistent with the previous report of evaluating the frequencies of HCV-specific CTLs by direct ex vivo staining with HCV-specific pentamers.[16] Thus the evaluation of reactivity against HCV Core and NS3 proteins-derived peptides might be useful in predicting the clinical outcome of this combination therapy.

It has been reported that complete early virologic response (cEVR), which means HCV RNA negativity at week 12, is strongly related to SVR in the combination therapy of Peg-IFNα plus ribavirin.[12,17] cEVR itself has been reported to be an independent predictive factor of
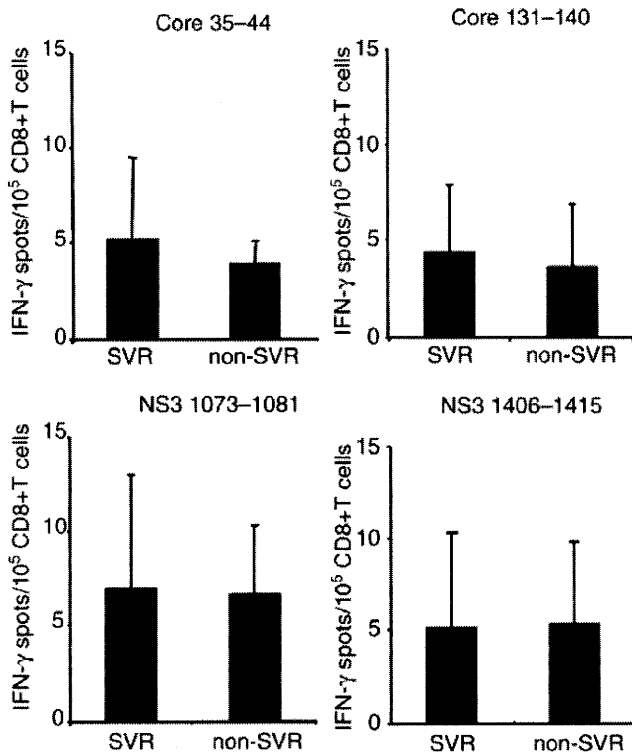
Figure 3 Comparison of the frequencies of hepatitis C virus (HCV)-specific CD8+ T cells in pre-treated HCV patients between sustained virologic response (SVR) and non-SVR. HCV Core and NS3 proteins-derived peptides specific CD8+ T cell responses in pre-treated HCV patients were evaluated by interferon (IFN)-γ enzyme-linked immunospot (ELISPOT). We analyzed the association between the HCV-specific CD8+ T cell responses and the achieving of SVR. SVR: patients who were observed SVR, non-SVR: patients who were not observed SVR.

SVR.[1,12] We also examined the association between cEVR and early elevation of HCV-specific CD8+ T cell responses. The frequencies of CD8+ T cell responses against all four HCV derived peptides in pre-IFN patients were not significantly different between cEVR and non-cEVR (Tatsumi T, unpublished data). In cEVR patients, the frequencies of CD8+ T cell responses against three HCV peptides Core$_{35-44}$, Core$_{131-140}$, NS3$_{1406-1415}$) increased significantly 4 weeks after the starting treatment and those against NS3$_{1073-1081}$ peptide tended to increase although these were not significant. In contrast, the frequencies of CD8+ T cell responses against all four HCV peptides did not increase in non-cEVR patients (Tatsumi T, unpublished data). The cEVR results were almost similar to those of the SVR results. Although we could not evaluate the HCV RNA levels at 4 week after starting treatment, the cEVR results sug-

gested that early elevation of the frequencies of HCV-specific CD8+ T cell responses might reflect the decrease of viral load of HCV.

CD8+ CTL activities in pre-treated HCV patients have been reported to be very low.[7,18,19] Consistent with the previous observations, the frequencies of HCV specific CD8+ T cell in pre-treated patients were also low in our study. The frequencies of HCV-specific CD8+ T cells in pre-treated patients were not associated with the HCV viral load and the serum ALT levels of patients before treatment. Several reports demonstrated that the baseline presence of HCV-specific CTLs prior to treatment was associated with viral clearance.[7,18] However, the frequencies of HCV-specific CD8+ T cells in pre-treated patients were not associated with the achievement of SVR in our study. In previous other reports, whole PBMC isolated from treated patients were used to evaluate the antiviral activity of HCV-specific CD8+ T cells. In our study, enriched CD8+ T cells obtained by magnetic sorting methods were used to enhance the sensitivity for the detection of HCV-specific CD8+ T cells. Both ELISPOT and staining with tetramers/pentamers could be applied for immunological monitoring for peptide-specific CTLs.[20] ELISPOT can detect activated functional CTLs, and tetramers/pentamers staining can detect peptide-specific CTLs.[20] In our study, we assessed the HCV-specific CD8+ T cell responses by IFN-γ ELISPOT, which is the most well-established methods and has already applied for immunological monitoring in cancer patients.[11] Recently perforin- or granzyme B-ELISPOT assays have also been reported. However, due to limitations in cell numbers of PBMC isolated from HCV patients, we were unable to apply another system of immunological monitoring and test other functional molecules. If we can apply these ELISPOT assays, we could directly evaluate the cytotoxic activity of HCV-specific CTLs.

In our study, the frequencies of HCV-specific CD8+ T cells in pre-treated patients were similar between SVR and non-SVR patients. In contrast, significant increase of the frequencies of HCV-specific CD8+ T cells between pre-IFN and IFN-4week could be observed in SVR patients, but not in non SVR patients. Caetano et al. evaluated the HCV-specific CD8+ T cells by HLA class I pentamers specific for the one HCV-Core epitope and one NS3 epitope which were same as we used.[16] They demonstrated that the increase of the frequencies of HCV-specific CTLs at 1 month after starting treatment was mainly due to terminally differentiated cells as well as, to a lesser extent, central memory cells in SVR patients and, in contrast, the increase of HCV-specific
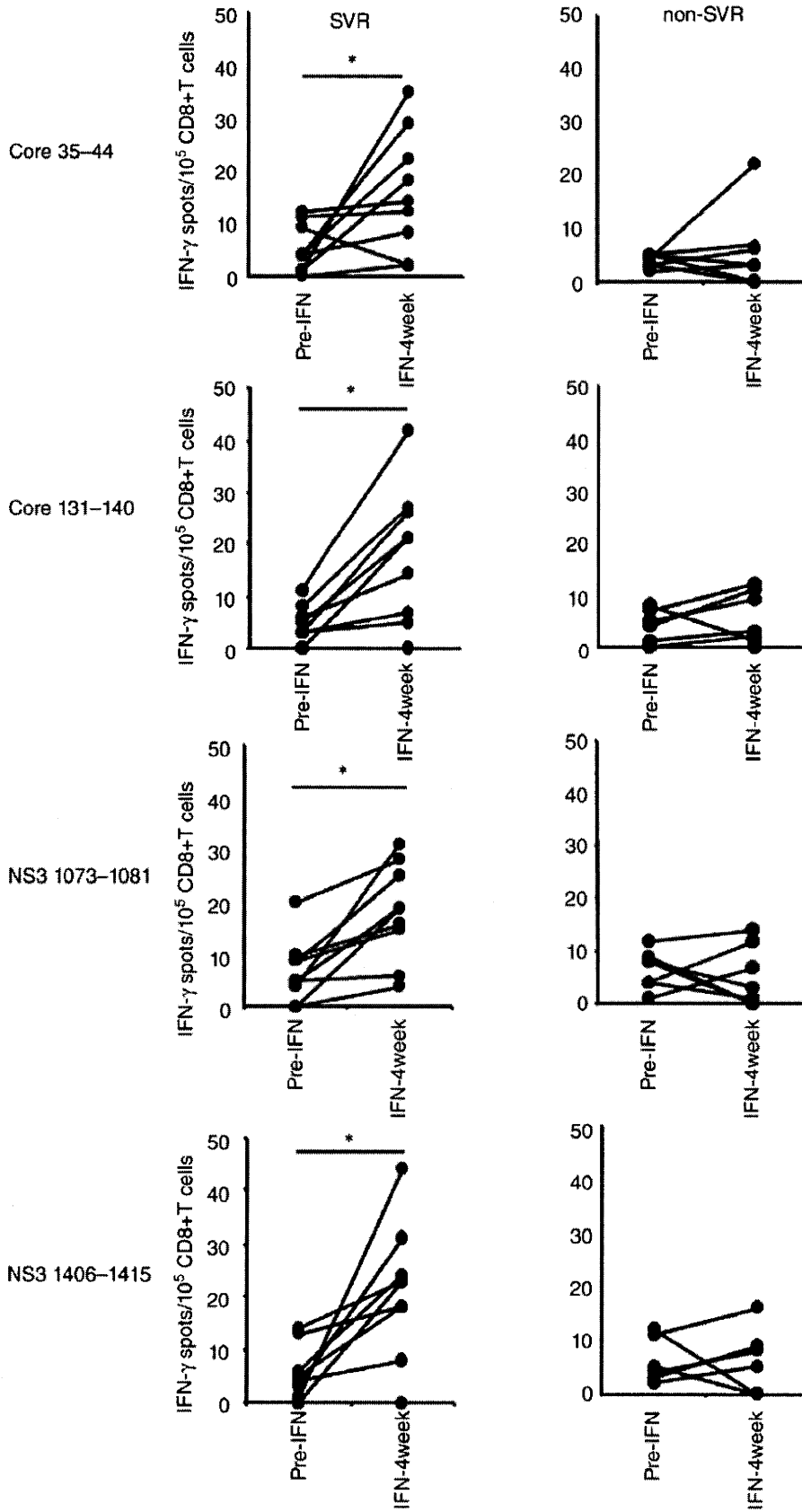
Figure 4 Analysis of the association of the change of hepatitis C virus (HCV)-specific CD8+ T cell responses between pre-IFN and IFN-4week chronic hepatitis C (CH-C) patients with the achieving sustained virologic response (SVR). Peripheral blood CD8+ T cells were isolated from pre-IFN and IFN-4week patients. HCV-specific CD8+ T cell responses were evaluated by interferon (IFN)-γ enzyme-linked immunospot (ELISPOT) assay. We analyzed the association of HCV-specific CD8+ T cell responses in treated CH-C patients with the achieving SVR. Each symbol within a panel represents the response of an individual donor to the indicated HLA-A2-presenting HCV Core or NS3 protein-derived peptides. The treated patients were divided into two groups; SVR group and non-SVR group. *P < 0.05.

pre-terminally differentiated CD8+ T cells was also observed in non-SVR patients.[16] These results suggested that CTLs maturation efficiently occurred in SVR patients. HCV or HCV-gene products have been reported to inhibit the maturation pathway of CTLs.[5,21] Thus the decrease of viral load during this combination therapy may induce CTL maturation.

We demonstrated that the achievement of SVR in this combination therapy was associated with the early elevation of HCV-specific CD8+ T cell responses, but not with the pre-treated levels of HCV-specific CD8+ T cell responses. These results suggested, at least, that the enhancement of HCV-specific CD8+ T cell responses might play critical roles in the second slope of viral clearance by this combination therapy. The increasing frequencies of HCV-specific CD8+ T cells have also been reported to be associated with SVR during the combination therapy by evaluating with pentamers of HCV-specific peptides.[16] Ribavirin has immunomodulatory effect with a switch from Th2 to Th1 cytokine profile.[22] The combined use of pegIFNα and ribavirin might have more immunomodulatory effect to generate HCV specific CTLs. However, even now, this should be elucidated to develop better treatment of chronic hepatitis C.

Although CTL responses to HCV are multi-specific,[13,23] we and others tested only small part of the known CTL epitopes of HCV, which do not comprise all potential HLA A2-restricted CTL epitopes of HCV. HCV may have mutated and escaped from the CTL responses to the corresponding epitopes in the chronically infected patients. The epitopes used in our study have been applied to the detection of HCV-specific CTLs in several other previous studies,[5,15,16] which support the usefulness of the selected epitopes. Our results demonstrated that the increases of the frequencies of CD8+ T cells against four synthesized peptides were associated with the antiviral activity of this combination therapy. Thus the selected epitopes used in our experiments were probably stable, at least, during the 4 weeks after starting treatment.

In spite of recent progress for HCV treatment, there remains significant room for improvement. To date, a variety of viral factors and host factors that correlate with SVR in the combination therapy have been noted. Recently, in addition to viral factors and host factors, response and adherence to treatment have been noted.[2] To establish the better treatment, the detail mechanism of HCV elimination should be elucidated. In the present study, we demonstrated that early enhancement of HCV-specific CD8+ T cell responses was associated with the achieving SVR in this combination therapy. These

suggest that activation of antiviral CTLs might be involved in the elimination of HCV. The early elevation of HCV-specific CTL responses in treated HCV patients may be a candidate for predicting SVR in this combination therapy.

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