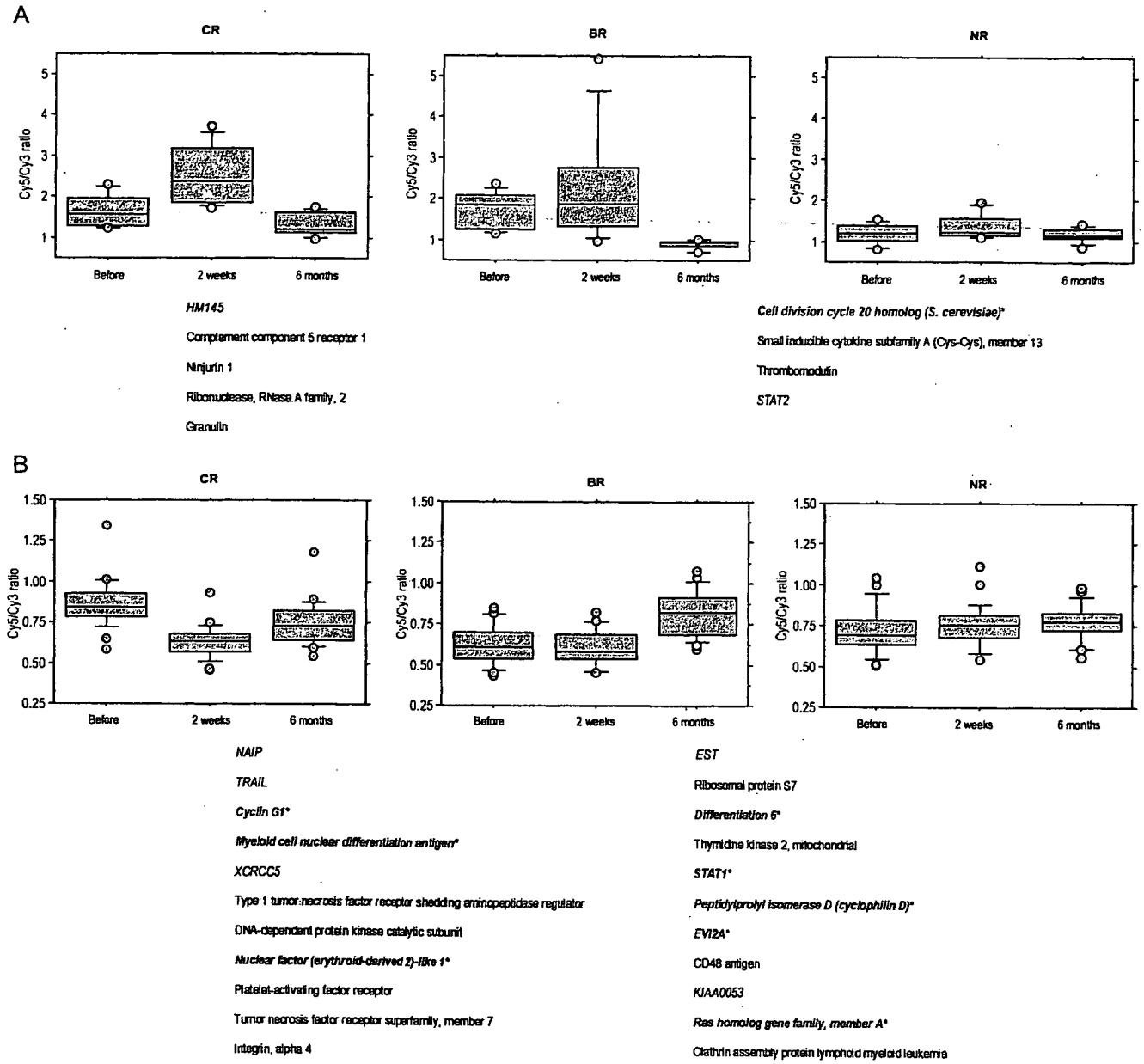
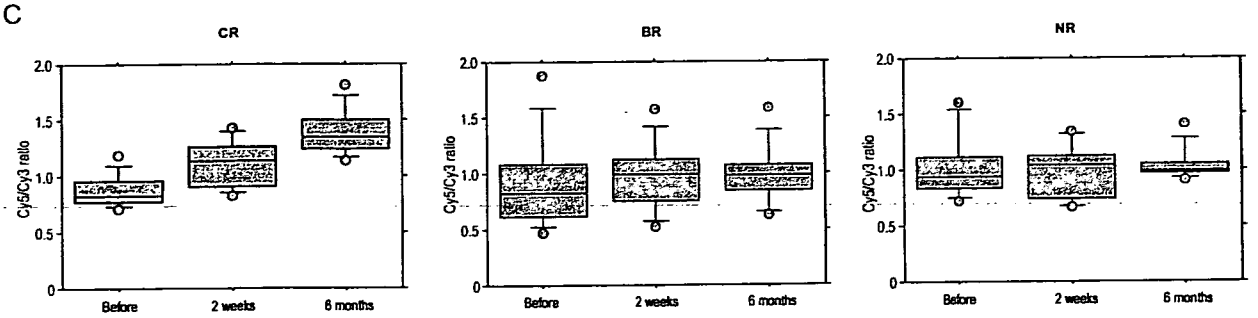


Figure 4. Gene-expression patterns. By use of projective adaptive resonance theory, 86 genes with changes in gene expression before and 2 weeks after the start of interferon (IFN) therapy were selected. For the complete responder (CR) group, changes in the expression of the 86 genes due to IFN therapy were classified into the following 5 patterns, on the basis of self-organizing maps (GeneCluster): up-regulated at 2 weeks after the start of IFN therapy and then down-regulated after the end of IFN therapy *(A)*; down-regulated at 2 weeks after the start of IFN therapy and then up-regulated after the end of IFN therapy *(B)*; up-regulated at 2 weeks after the start of IFN therapy and also up-regulated after the end of IFN therapy *(C)*; up-regulated at 2 weeks after the start of IFN therapy and then returned to normal after the end of IFN therapy *(D)*; and down-regulated at 2 weeks after the start of IFN therapy and also down-regulated after the end of IFN therapy *(E)*. Representative genes are listed under each pattern. Asterisks indicate genes that are included in table 5.

expression ratios for liver-infiltrating lymphocytes showed >1-fold increases compared with hepatocytes, thus indicating that most genes were preferentially expressed in lymphocytes. Interestingly, the genes with increased expression in liver-infiltrating lymphocytes tended to be expressed at lower levels in PBMCs (figure 1*C*).

Serial changes in the differentially expressed genes listed in table 2 during IFN treatment are shown in figure 2*A*. In the

C



*Serine (or cysteine) protainase inhibitor, clade A**

Ribosomal protein S27 (metallopanstimulin 1)

Eukaryotic translation elongation factor 2

Epidermal growth factor receptor

Hemopoietic cell kinase

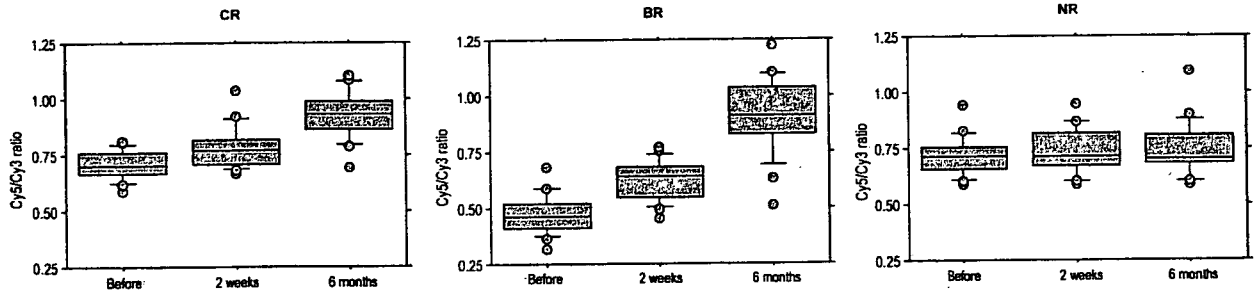
Bruton agammaglobulinemia tyrosine kinase

LITAF

Hemopoietic cell kinase

GZMA

D



Interleukin 2 receptor beta chain

ICE

Ribosomal protein S16

Glutamate dehydrogenase 1

Small nuclear ribonucleoprotein polypeptide N

Mutant lymphocyte-specific protein tyrosine kinase

*ADP-ribosyltransferase**

Interleukin enhancer binding factor 1

CD3D antigen, delta polypeptid

Fc fragment of IgE, low affinity II, receptor

Selectin L (lymphocyte adhesion molecule 1)

Ikaros/LyF-1 homolog (hlk-1)

Semaphorin (CD100)

Mitochondrial 2,4-dienoyl-CoA reductase

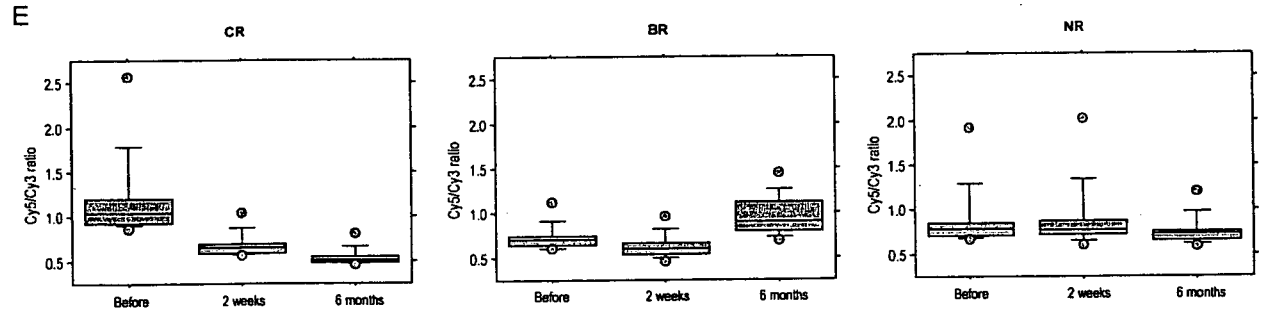
*Ras-related C3 botulinum toxin substrate 2**

E2L1

Citrate synthase

ERP72

E



Baculoviral IAP repeat–containing 2*

*MIHC**

NRAS-related gene

Reticulocalbin 2, EF-hand calcium binding domain

*ERCC5**

Integrin, beta 1

Rho-associated, coiled-coil containing protein kinase 1

*Apoptosis inhibitor 1**

Uracil-DNA glycosylase

Damage-specific DNA binding protein 1 (127kD)

CD69 antigen (p60, early T-cell activation antigen)

Figure 4. *(Continued.)*

Table 5. Ten gene combinations selected by the SWEEP operator method for the construction of chronic hepatitis C class prediction 2 weeks after the start of interferon (IFN) therapy.

Combination	Input	Gene name	GenBank accession no.	Accuracy, %	
				Training	Test
1	1	*ERCC5*	NM_000123	55.3	45.5
	2	Serine (or cysteine) proteinase inhibitor clade A member 1	NM_000295	85.6	54.5
	3	Ras homolog gene family member A	NM_001664	80.3	70.5
2	1	Baculoviral IAP repeat–containing 2	NM_001166	47.7	41.7
	2	Serine (or cysteine) proteinase inhibitor clade A member 1	NM_000295	80.3	53.8
	3	Ras homolog gene family member A	NM_001664	80.3	70.5
3	1	Cyclin G1	NM_004060	36.6	44.0
	2	Ras-related C3 botulinum toxin substrate 2	NM_002872	79.6	61.4
	3	*EST*		70.5	56.8
4	1	Ecotropic viral integration site 2A	NM_001003927	41.7	25.8
	2	Peptidylprolyl isomerase D (cyclophilin D)	NM_005038	60.6	46.2
	3	Cyclin G1	NM_004060	77.3	67.4
5	1	***Myeloid cell nuclear differentiation antigen***[a]	NM_002432	55.3	25.8
	2	Cyclin G1	NM_004060	85.6	64.4
	3	ADP-ribosyltransferase (NAD+; poly [ADP-ribose] polymerase)	NM_001618	80.3	87.1
6	1	Integrin β1	NM_033666	47.7	19.7
	2	Cyclin G1	NM_004060	80.3	62.9
	3	***STAT1***[a]	NM_139266	80.3	68.2
7	1	Differentiation 6 (septin 2)	NM_004404	28.8	25.8
	2	Cyclin G1	NM_004060	75.0	64.4
	3	Cell division cycle 20 homolog *(S. cerevisiae)*	NM_001255	90.2	87.9
8	1	*MIHC*	NM_001165	28.8	25.0
	2	Cyclin G1	NM_004060	75.0	64.4
	3	Cell division cycle 20 homolog *(S. cerevisiae)*	NM_001255	90.2	87.9
9	1	Apoptosis inhibitor 1 (baculoviral IAP repeat–containing 3)	NM_001165	28.8	25.0
	2	Cyclin G1	NM_004060	75.0	64.4
	3	Cell division cycle 20 homolog *(S. cerevisiae)*	NM_001255	90.2	87.9
10	1	Nuclear factor (erythroid-derived 2)–like 1	NM_003204	25.0	25.8
	2	Cyclin G1	NM_004060	75.0	63.6
	3	ADP-ribosyltransferase (NAD+; poly [ADP-ribose] polymerase)	NM_001618	88.6	81.8

[a] Genes that present similar expression patterns during IFN and ribavirin combination therapy.

CR group, the expression profiles of genes that were either up- or down-regulated before IFN therapy were similar to those of healthy volunteers 6 months after the end of IFN therapy (figure 2*A*, CR group). On the other hand, in the NR group, expression of genes that were either up- or down-regulated before IFN therapy tended to remain up- or down-regulated 6 months after the end of IFN therapy (figure 2*A*, NR group). This suggests that the changes in gene-expression profiles of patients with CH-C before IFN therapy reflect the state of HCV infection.

We performed real-time PCR to corroborate the microarray data. Real-time PCR revealed that *CD69* was up-regulated in patients with CH-C and that *CCR2* and *IL7R* were down-regulated in patients with CH-C (figure 2*B* and table 2).

Relationship between PBMC gene-expression profiles and IFN response. We then analyzed the relationship between PBMC gene-expression profiles before the start of IFN therapy and IFN response. Because the regimen of IFN treatment was different in group A and group B patients, we first focused on group A patients (table 1). In hierarchical clustering analysis using all genes before IFN therapy, no clustering was seen in the CR, BR, or NR groups. Conventional supervised learning methods, such as support vector machine and nearest neighbor (BRB-ArrayTools), could not discriminate between the CR, BR, and NR groups. Therefore, we applied the FNN-SWEEP method to predict the outcome of IFN therapy. Before FNN-

Table 6. Comparison of interferon (IFN)–stimulated gene (ISG) expression induced by IFN.

The table is available in its entirety in the online edition of the *Journal of Infectious Diseases*.

SWEEP analysis, nonspecific genes or genes with errors were eliminated by the PART method. The 32 genes screened by PART are shown in figure 3. Topoisomerase (DNA) I *(TOP1)* and interleukin 2 receptor β *(IL2RB)* were up-regulated in the CR group, hemoglobin γG *(HBG2)* and monocyte chemotactic protein were up-regulated in the BR group, and chemokine (C-C motif) ligand 4 and ras-related C3 botulinum toxin substrate 2 *(RAC2)* were up-regulated in the NR group. Genes selected by PART were subjected to the FNN-SWEEP method to construct a class prediction model. Consequently, we selected 10 gene combinations by the SWEEP operator method for CH-C class prediction before the start of IFN therapy (table 4). The most effective gene combination for the prediction of an IFN response was *TOP1;* catenin (cadherin-associated protein) β1 (88 kDa); and *RAC2.* The accuracy of the training and test sets were high, at 91.0% and 89.1%, respectively.

Changes in gene-expression profiles over the course of IFN therapy. We next focused on the changes in gene-expression profiles over the course of IFN therapy and their relationship with IFN response. Using PART, 86 genes with changes in expression between before and 2 weeks after the start of IFN therapy were selected. To investigate the relationship between the 86 genes with changes due to IFN therapy and the efficacy of IFN therapy, changes in the expression of the 86 genes were determined for the CR group. On the basis of self-organizing maps, changes in gene expression in the CR group were classified into the following 5 patterns (figure 4): pattern A, up-regulated 2 weeks after the start of IFN therapy and then down-regulated after the end of IFN therapy; pattern B, down-regulated 2 weeks after the start of IFN therapy and then up-regulated after the end of IFN therapy; pattern C, up-regulated 2 weeks after the start of IFN therapy and also up-regulated after the end of IFN therapy; pattern D, up-regulated at 2 weeks after the start of IFN therapy and then returned to normal after the end of IFN therapy; and pattern E, down-regulated at 2 weeks after the start of IFN therapy and also down-regulated after the end of IFN therapy. Patterns A and B represent gene groups with temporary changes during IFN therapy, whereas patterns C, D, and E represent gene groups with changes after the end of IFN therapy and are thought to be attributable to viral eradication or normalization of hepatic function. Interestingly, very little change was seen in the patterns for the NR group. Therefore, changes in gene expression are also useful in predicting therapeutic efficacy. From the 86 genes isolated by PART, the SWEEP operator method was used to identify 10 gene combinations, and therapeutic efficacy was predicted according to the FNN-SWEEP method (table 5). The results showed that the accuracy for gene combinations 7, 8, and 9 was high, at 90.2%. LOOCV confirmed the high accuracy (87.9%) of prediction using these gene combinations. These combinations included the following genes that are important for predicting therapeutic efficacy: *CDC20* was classified as belonging to pattern A; cyclin G1 and differentiation 6 were as belonging to pattern B; and *MIHC* and apoptosis inhibitor 1 were as belonging to pattern E (figure 4).

IFN and ribavirin combination therapy. We then investigated the usefulness of the above-mentioned genes in predicting the efficacy of IFN and ribavirin combination therapy. It has been shown that concurrent ribavirin administration improves the rate of CR. In addition, the changes in gene expression during combination therapy are due not only to IFN but also to ribavirin. Thus, the results for monotherapy may not be applicable to combination therapy. However, changes in the expression of several genes—CD2 antigen (p50), *IL2RB, HBG2,* myeloid cell nuclear differentiation antigen *(MNDA),* and *STAT1*—were shown to be extremely useful for distinguishing CR from NR in IFN and ribavirin combination therapy (tables 4 and 5).

DISCUSSION

HCV load, genotype, and fibrosis have been listed as factors that influence the effectiveness of IFN therapy [4, 5], but these factors are not sufficient, and other predictive factors are needed. Unlike liver-biopsy specimens, PBMCs can be easily collected, and collection can be repeated as necessary. We analyzed the gene-expression profiles of PBMCs in patients with CH-C by use of cDNA microarrays under the hypothesis that gene expression in PBMCs is indicative of IFN efficacy and CH-C disease state. In addition, changes in the gene-expression profiles of PBMCs were analyzed during the course of IFN therapy to clarify the relationship between gene-expression profiles of PBMCs and IFN response.

Interestingly, the gene-expression profiles of PBMCs from patients with CH-C and from healthy volunteers were different, and this was confirmed by hierarchical clustering analysis and supervised learning analysis using support vector machine. When patients with CH-C and healthy volunteers were compared, gene expression in the JAK-STAT cascade, humoral immune response, and G protein–coupled receptor protein signaling pathway differed markedly. In most patients with CH-C, expression of these genes is activated, and HCV infection is thought to bring about changes in the gene expression in PBMCs. Several chemokine- and cytokine-related genes, such as *CCR2* and *IL7R,* were down-regulated. Although the reason for this was not clear, expression of these genes in liver-infiltrating lymphocytes was up-regulated. Therefore, the down-regulation of immune-related genes may represent increased levels of liver-infiltrating lymphocytes accompanying hepatitis. Interestingly, when the chronological changes in PBMC gene-expression profiles were analyzed for the CR group, the profiles at 6 months after the end of therapy were similar to those of healthy volunteers. Therefore, the changes in gene-expression

profiles before IFN therapy were due to HCV infection. On the other hand, the gene-expression profiles of the NR group before IFN therapy were similar to those at 6 months after the end of IFN therapy (figure 2*A*).

Unfortunately, it was not possible to differentiate between CR, BR, and NR patients on the basis of gene-expression profiles of PBMCs by use of nonsupervised learning methods, such as hierarchical clustering, before IFN therapy. Therefore, we used FNN theory for CH-C class prediction. The most attractive feature of FNN is that causality between input and output variables can be described very accurately as explicit if-then roles obtained from the constructed model. For the purpose of analyzing numerous genes in a short time, FNN combined with the SWEEP operator method was developed (FNN-SWEEP method) and has been shown to be a precise, simple tool for predicting patient survival on the basis of microarray data [28, 29]. In addition, by first filtering genes by use of PART, the accuracy of the FNN-SWEEP method was further increased [30]. In the present study, a total of 32 genes were identified by PART on the basis of genetic changes before therapy, and, in the CR group, expression of genes such as *TOP1*, *IL2RB*, prothymosin α *(PTMA)*, and ADP-ribosyltransferase was up-regulated, thus indicating active cellular proliferation. In the NR group, the expression of genes indicating activated cytotoxic T cells—such as granzyme, CD2 antigen, *RAC2*, and natural killer cell transcript 4—was up-regulated. Because these genes were up-regulated by IFN therapy in the CR group, they were thought to be up-regulated before therapy in the NR group. Lempicki et al. reported elevated expression of endogenous IFN/innate immune response genes in PBMCs from NR patients coinfected with HCV and HIV [31]. This suggests that, in many NR patients, few immune effector cells are active or that these effector cells cannot infiltrate the liver and remain in the peripheral blood.

To further investigate the above-mentioned points, changes in the gene-expression profiles of PBMCs were determined during the course of IFN therapy. On the basis of expression profiles before and 2 weeks after the start of IFN therapy, 86 genes were selected. These genes did not include as many IFN-α–stimulated genes as were noted in liver [25–27] (table 6), but they included valuable immune regulatory genes.

On the basis of self-organizing maps, changes in gene expression in the CR group were then classified into 5 patterns (figure 4). These gene groups represent genes with temporary changes due to IFN therapy and those with changes after the end of IFN therapy. Gene groups with changes after the end of IFN therapy are thought to be involved in viral eradication or the normalization of hepatic function. Interestingly, little change was seen in any of the patterns in the NR group. In efficacy prediction by the FNN-SWEEP method, the accuracy for the gene combinations 7, 8, and 9 was high, at 90.2%, thus suggesting that changes in gene expression 2 weeks after the start of IFN therapy are also useful in predicting therapeutic efficacy.

We also investigated whether these genes are useful in predicting the efficacy of IFN and ribavirin combination therapy. Changes in gene expression during combination therapy were due not only to IFN but also to ribavirin, and the results for monotherapy could not simply be applied to combination therapy. However, changes in the expression of several genes—CD2 antigen (p50), *IL2RB*, *HBG2*, *MNDA*, and *STAT1*—were shown to be extremely useful for distinguishing CR from NR in IFN and ribavirin combination therapy.

Unfortunately, because the number of subjects in the present study was small, the genes that were identified as predictors for IFN monotherapy were not necessarily predictors for IFN and ribavirin combination therapy. However, the present study was the first to show that responses to IFN therapy could be predicted on the basis of changes in gene expression by PBMCs, and further investigations in greater numbers of patients are required.

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Analysis of hepatitis C virus-specific CD8+ T-cells with HLA-A*24 tetramers during phlebotomy and interferon therapy for chronic hepatitis C

KYOSUKE KAJI, YASUNARI NAKAMOTO and SHUICHI KANEKO

Department of Gastroenterology, Graduate School of Medicine, Kanazawa University, Kanazawa, Japan

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Abstract. Hepatitis C virus (HCV)-specific, HLA class I-restricted, CD8-positive (CD8+) T lymphocytes are thought to contribute to viral clearance as well as liver disease in chronic hepatitis C. For the patients who do not respond to interferon (IFN) therapy, phlebotomy can be used as a tool to reduce inflammation and lower transaminase levels; however, the immunological aspects have not been clearly defined. In this study, we evaluated the HCV-specific CD8+ T-cell responses during phlebotomy and IFN therapy using HLA-A*24 tetramers in 6 Japanese patients with chronic hepatitis C. During phlebotomy, 4 of the 6 cases achieved a biochemical response, but there was no clear correlation between its efficacy and HCV viral loads or changes in frequencies or activation status of tetramer-positive T-cells. In contrast, the frequencies of tetramer-positive cells and the proportions of T-cells expressing activation marker HLA-DR were higher in sustained viral responders than in transient responders to IFN therapy. Furthermore, expression of the activation marker was enhanced in the initial period of IFN therapy. The results suggest that the immunological aspects of phlebotomy obviously differ from those of IFN therapy and these differences may provide clues as to a therapeutic strategy of their combination for patients who do not respond to IFN monotherapy.

Introduction

Interferon (IFN) treatment is a radical therapy for the elimination of hepatitis C virus (HCV), but many patients do not respond to it; so called 'non-responders'. There have been recent advances in treatment, such as combination therapy with Peginterferon α-2a or α-2b and ribavirin (1-4). To date, there are no therapies for HCV elimination with a sufficiently high success rate and low rate of adverse events.

For non-responders to IFN therapy, secondary treatment is needed to lower serum transaminase levels, slow the progression of fibrosis and reduce the occurrence of hepatocellular carcinoma (5). Phlebotomy is one of the treatments used to reduce inflammation and lower serum transaminase levels (6).

Several studies have examined the correlation between HCV and iron levels. Smith *et al* reported that the progression of fibrosis is faster in patients with chronic hepatitis C with congenital hemochromatosis than in those with normal iron levels (7). Fontana *et al* reported that combination therapy with IFN and phlebotomy may be more effective than IFN monotherapy in patients with chronic hepatitis C (8). We reported the hemosiderin deposition may be a predictive parameter for the efficacy of IFN therapy (9). Mandishona *et al* reported that excess iron may promote the occurrence of hepatocellular carcinoma (10).

The mechanism by which phlebotomy decreases transaminase levels is thought to involve a decrease in the toxic effects of superoxide produced by iron excess (11). However, no studies have been reported regarding the immunological effects of phlebotomy that may be important in mitigating liver injury.

HCV-specific CD8+ cytotoxic T lymphocytes (CTLs) contribute to viral clearance in acute, self-limited hepatitis C as well as to liver cell injury in the more frequent cases with chronic hepatitis C (12-16). In a study using HLA-A*24 tetramer, we previously showed that a close correlation exists between the HCV-specific CD8+ T-cell profile and hepatic fibrosis in HCV-infected Japanese patients, most of whom are HLA-A*24 positive (17). In this study, we analyzed HCV-specific CD8+ T-cell responses during phlebotomy and IFN therapy and observed a correlation between changes in the HCV-specific CD8+ T-cell profile and the therapeutic effects of each treatment.

Materials and methods

Patients. Patients with chronic hepatitis C presented at Kanazawa University Hospital between June 2000 and June 2001 were included in this study. Their selection and diagnosis were based on the following criteria: 1) age from 20 to 70

Correspondence to: Dr Shuichi Kaneko, Department of Gastroenterology, Graduate School of Medicine, Kanazawa University, 13-1 Takara-Machi, Kanazawa 920-8641, Japan
E-mail: skaneko@m-kanazawa.jp

Key words: chronic hepatitis C, phlebotomy, interferon therapy, tetramers, HLA-A*24

Table I. Clinical characteristics of patients.

Patients	Age (years)	Sex (M/F)	HCV Serogroup	HCV-RNA (KIU/ml)	ALT (IU/l)	HAI Stage (F)	HAI Grade (A)	ALT change during phlebotomy	Response to IFN therapy
1	47	F	2	69	40	1	1	Not decreased	SVR
2	60	M	2	88	104	3	2	Decreased	TVR
3	43	M	1	>500	97	3	2	Decreased	TVR
4	65	M	1	1.9	80	1	1	Decreased	SVR
5	55	M	2	0.7	39	3	2	Decreased	SVR
6	51	M	2	>500	110	1	1	Not decreased	TVR

Serum HCV RNA was quantified with the Amplicore HCV Monitor ver.3. HAI, histological activitiy index; SVR, sustained viral responder; TVR, transient viral responder.

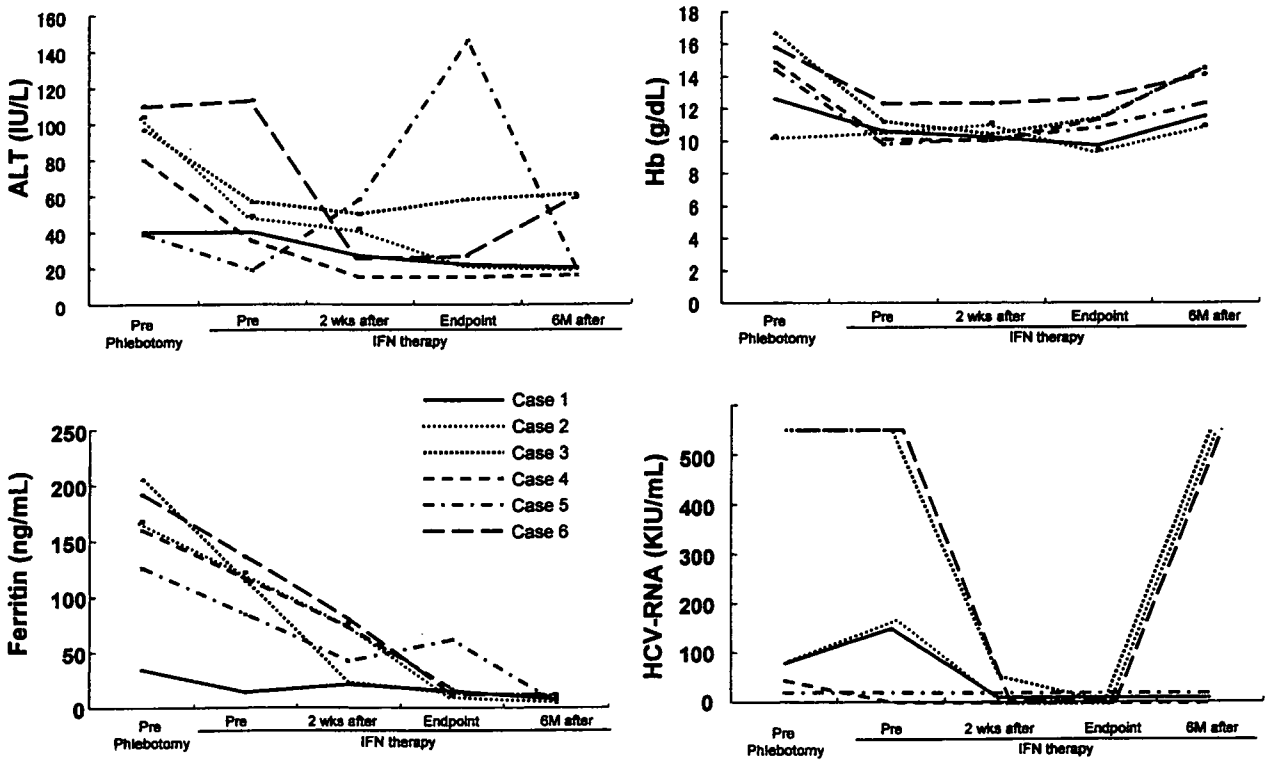

Figure 1. Trends over time for alanine aminotransferase (ALT), hemoglobin, ferritin and HCV-RNA levels during phlebotomy and interferon (IFN) therapy in patients with chronic hepatitis C. Phlebotomy was performed by removing 200 to 400 ml blood per session, once or twice weekly. The sessions were continued until achieving the target levels of hemoglobin (10 g/dl) and/or ferritin (10 ng/ml). After achieving the target levels, IFN-α-2b was administered at a dose of 6 to 10 MU per day for 2 weeks and then 3 times a week for 22 weeks. Each line indicates a single patient.

years; 2) elevated serum aminotransferase (ALT; >50 IU/l) at least once within 1 year; 3) hemoglobin greater than 13.0 g/dl in males or greater than 11.0 g/dl in females; 4) no liver cirrhosis; and 5) HLA-A*24 positive. All cases provided written informed consent.

Phlebotomy and IFN therapy. Phlebotomy was performed by removing 200 to 400 ml blood per session, once or twice weekly. The sessions were continued until achieving the target level of hemoglobin (10 g/dl) and/or ferritin (10 ng/ml). After achieving the target level, IFN-α-2b was administered at a dose of 6 to 10 MU per day for 2 weeks and then 3 times a week for 22 weeks.

Complete blood cell count, liver function tests, HCV-RNA determinations and T-cell analysis were performed pre-phlebotomy, just before IFN administration, 2 weeks after IFN therapy, immediately after IFN therapy and 6 months after IFN administration.

Patients whose transaminases decreased during phlebotomy, were recorded as biochemical responders and the others as non-responders. With respect to the HCV-RNA level, patients whose HCV-RNA levels were undetectable both at the end-points of IFN therapy and even at 6 months after the IFN therapy completion were designated as sustained viral responders (SVR) and those whose HCV-RNA were undetectable at the end point of IFN therapy but reappeared 6 months after the end of IFN therapy were designated as transient viral responders (TVR) (1).

*Synthesis of HLA-A*2402-peptide tetramers.* Five peptides were selected to synthesize HLA-A*2402-peptide tetramers (17): HCV E2 717-725 (EYVLLLFLL), NS3 1292-1300

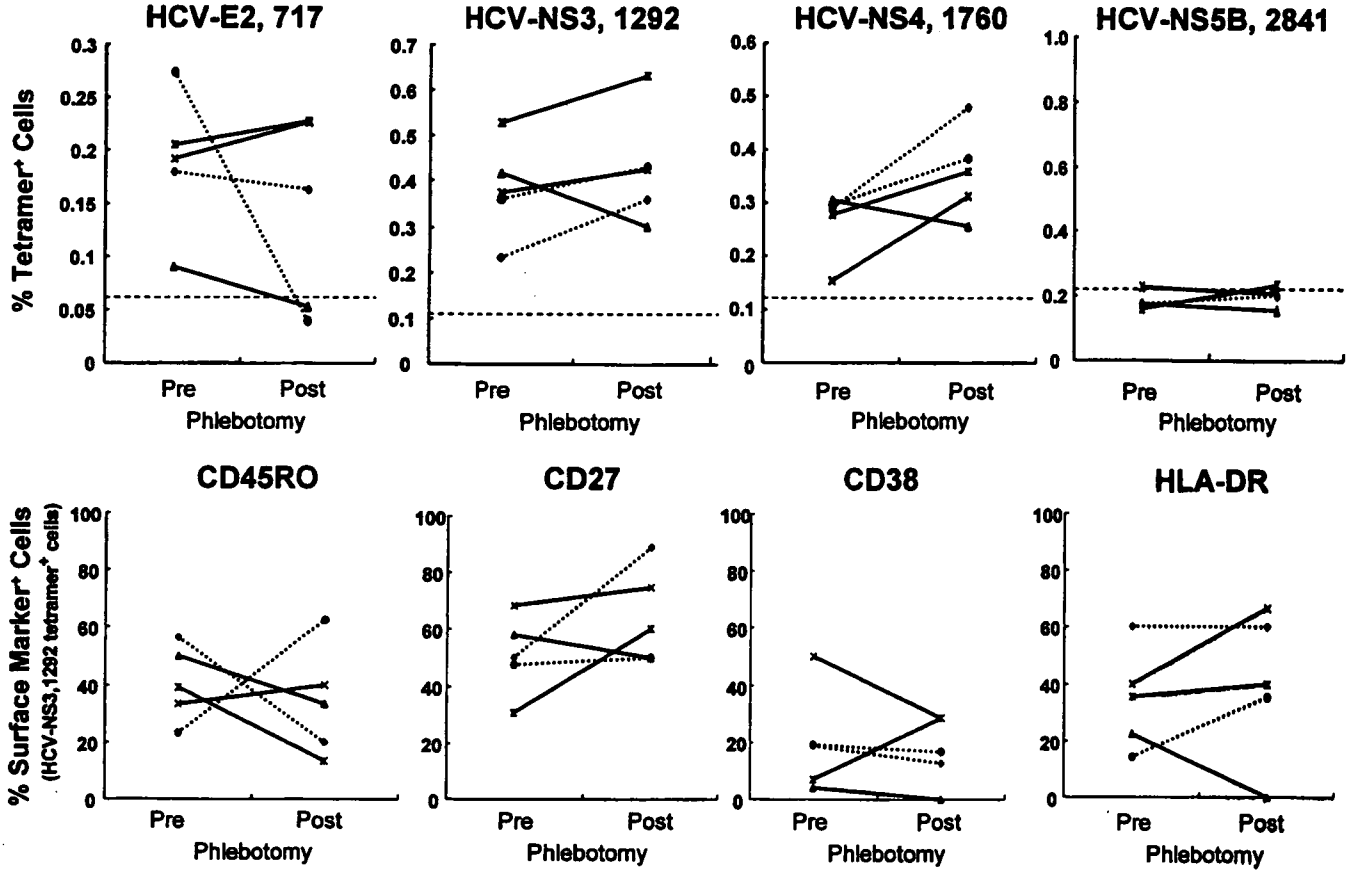

Figure 2. Changes in frequencies of four types of HLA-A*24 tetramer-positive cells and surface markers of tetramer HCV-NS3, 1292-positive cells during phlebotomy. Solid lines represent biochemical responders to phlebotomy and broken lines represent biochemical non-responders (subjects whose ALT did not decrease during phlebotomy). Horizontal broken lines indicate the cut-off value for each HLA tetramer, as mentioned in Materials and methods. There was no correlation between the biochemical effects of phlebotomy and the frequencies of tetramer-positive T-cells or the phenotypes of tetramer-positive cells.

(TYSTYGKFL), NS4 1760-1768 (FWAKHMWNF), NS5B 2841-2849 (RMILMTHFF) and NS5B 2870-2878 (CYSIEPLDL). Three of them, E2 717-725, NS3 1292-1300 and NS5B 2870-2878 were selected because they have been reported to bind to HLA-A*24 with good affinity (IC_{50} <500 nM) in a direct peptide binding assay (18). The other two peptides were chosen because they were conserved within the reported major HCV genotypes 1a and 1b sequences (19-21).

The cut-off values for positive staining with the tetramers was 2 SD above the mean for all control subjects studied previously (17): 0.064% for tetramer HCV-E2.717, 0.11% for tetramer HCV-NS3.1292, 0.12% for tetramer HCV-NS4 1760, 0.22% for tetramer HCV-NS5B.2841 and 0.10% for tetramer HCV-NS5B.2870.

Tetramer staining and flow cytometry. Peripheral blood mononuclear cells (PBMCs) were isolated from heparinized venous blood by separation using Ficoll-Hypaque (Sigma Chemical Co., St. Louis, MO) density gradient centrifugation. Freshly isolated PBMCs were stained with tetrameric complexes and antibodies and were then analyzed. The following monoclonal antibodies (mAbs) were used; anti-CD8-Cy-Chrome (CyC) (HIT8a), anti-CD-4-Allophycocyanin (APC) (SK3), anti-CD14-APC (MΦP9), anti-CD19-APC (SJ25C1), anti-CD45RA-FITC (HI100), anti-CD27-FITC (M-T271), anti-CD38-RITC (HIT2) and anti-HLA-DR-FITC (L243) (BD PharMingen, Sand Diego, CA). Freshly isolated cells ($1x10^6$) were washed, resuspended in 200 μl PBS without calcium and phosphate, and stained with 40 μg/ml of tetrameric complexes for 30 min at room temperature. Subsequently, antibodies against cell surface proteins were added and incubated for an additional 30 min at room temperature. Cells were washed, fixed with 1% formalin/PBS, and analyzed on a FACSCalibur™ flow cytometer. Data were analyzed with CELLQuest™ software (Becton Dickinson, San Jose, CA).

Results

Clinical course. The 6 cases studied included 5 males and 1 female (Table I). They ranged in age from 43 to 60 years. Of the patients, 4 had serogroup 2 HCV and 2 had serogroup 1 HCV. Serogroup 1 HCV is known to be more common than serogroup 2 in Japan (22).

Phlebotomies were performed in all cases without significant adverse events over a period of 7 to 20 days. Eventually, the total volume of blood removed was from 600 to 2800 ml (mean = 1600 ml). In 4 of the 6 cases, transaminase levels decreased during phlebotomy, but there was no effect of phlebotomy on HCV viral loads (Fig. 1).

IFN treatments were associated with lower HCV viral loads. At the endpoints of the treatments, HCV-RNA disappeared from sera of all 6 cases. Six months after IFN therapy, HCV reappeared in 3 cases (transient viral responder,

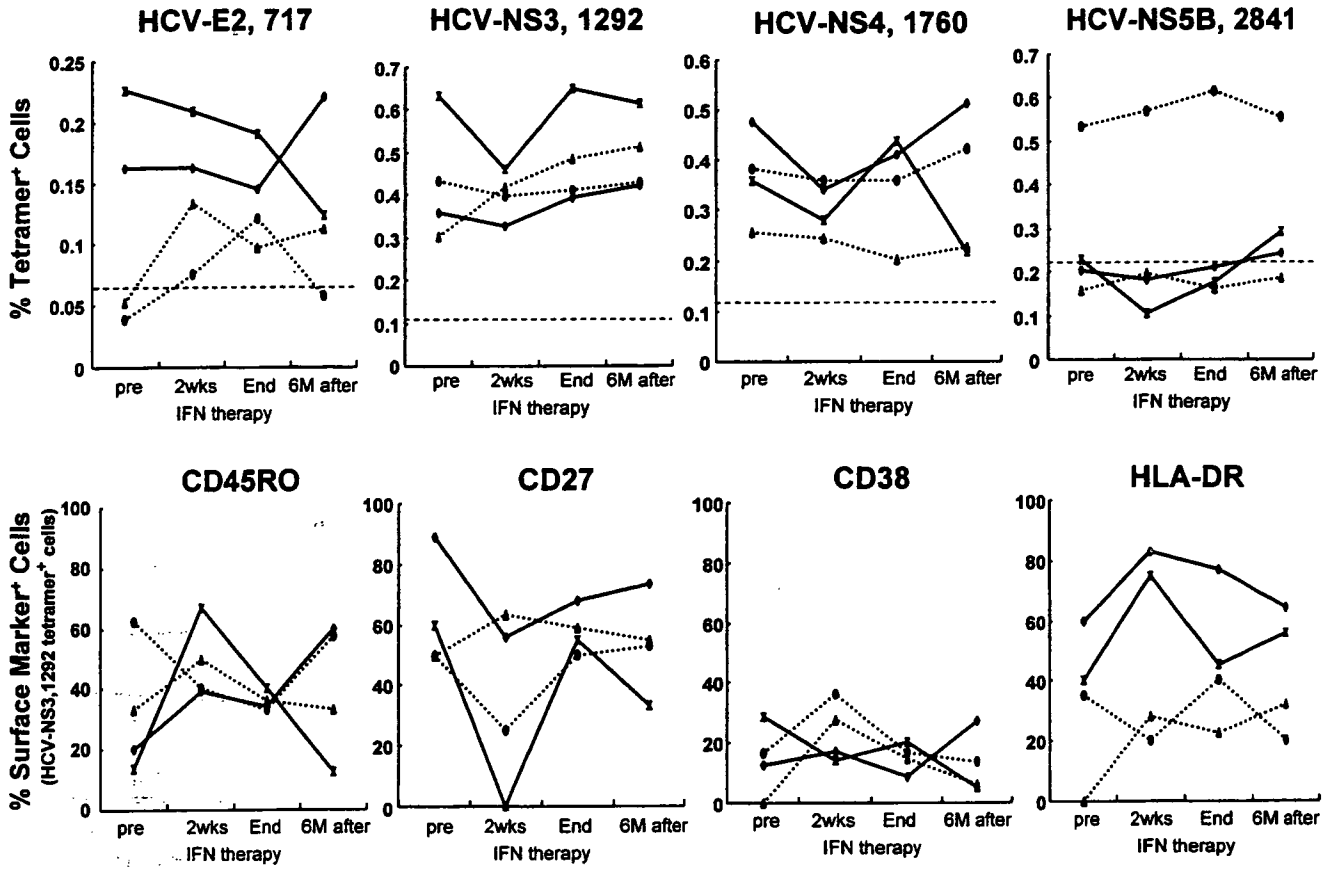

Figure 3. Changes in frequencies of four types of HLA-A*24 tetramer-positive cells and surface markers of tetramer HCV-NS3, 1292-positive cells during IFN therapy. Solid lines represent viral responders to IFN therapy (sustained viral responders, SVRs) and the broken lines represent transient viral responders (TVRs) whose HCV reappeared within 6 months after the end of IFN therapy. Horizontal broken lines in the upper four panels indicate the cut-off values for HLA tetramers, as mentioned in Materials and methods. The HLA-DR positive rates among tetramer (HCV-NS3, 1292)-positive cells were higher in SVRs than in TVRs at the start of IFN treatment and further increased after 2 weeks of IFN therapy.

TVR) and in the other 3 cases HCV remained below the detection limit (sustained viral responder, SVR) (Fig. 1).

HCV-specific CD8+ T-cell responses during phlebotomy. During phlebotomy, we analyzed HCV-specific CD8+ T-cell responses in 5 cases (patients 1, 3, 4, 5, and 6); and among them, 3 cases were biochemical responders to phlebotomy and the other 2 cases were non-responders (Table I and Fig. 2). The numbers of HLA-A*24 tetramer-positive T-cells were above the cut-off levels for all 3 of the tetramers, HCV-E2.717, HCV-NS3.1292, and HCV-NS4.1760. There was no correlation between the biochemical effects of phlebotomy and the frequencies of tetramer-positive T-cells. We also analyzed the phenotypes of tetramer-positive cells by staining CD45RO, CD27, CD38 and HLA-DR, but we did not observe a correlation between the biochemical effects and the phenotypes. The data indicate that phlebotomy displayed its therapeutic effects for the patients with chronic hepatitis C without affecting the frequencies and phenotypes of HCV-specific CD8+ T-cell responses.

HCV-specific CD8+ T-cell responses during IFN therapy. During and after IFN therapy, we analyzed HCV-specific CD8+ T-cell responses in 2 sustained responders (patients 1 and 5) and 2 transient responders (patients 3 and 6) (Table I and Fig. 3). The three different tetramer-positive T-cells, HCV-E2.717, HCV-NS3. 1292 and HCV-NS4.1760 were also detectable at levels above the cut-off during IFN therapy. The frequencies of HCV-E2.717 positive cells were higher in SVR patients than in TVR patients before, during and 6 months after IFN therapy. Interestingly, activation marker HLA-DR positive rates among tetramer-positive cells were higher in SVR patients than in TVR patients before IFN treatment and increased further after 2 weeks of IFN therapy and beyond. These results demonstrate that IFN therapy exerted its beneficial effects on the patients with high frequencies of the HCV-specific CD8+ T-cells and enhanced expression of the activation markers, suggesting that the efficacy of IFN therapy for chronic hepatitis C may be mediated by the virus-specific T-cell dependent immunity.

Discussion

For patients with chronic hepatitis C who do not respond to IFN therapy, other treatments to reduce inflammation and decrease transaminase levels are indicated to slow the progression of fibrosis and to lower the incidence of hepatocellular carcinoma. Phlebotomy is one of the therapies used to decrease the inflammation in the liver. In this study, we analyzed HCV-specific CD8+ T-cell responses in 6 patients with chronic hepatitis C treated by phlebotomy followed by IFN therapy. HLA-A*24 tetramer HCV-E2.717ÅAHCV-

NS3.1292 and HCV-NS4.1760 positive T-cells were detected at levels above the cut-off values. During phlebotomy, there was no correlation between the effectiveness of treatments and virological and immunological parameters, such as HCV viral loads, frequencies of tetramer-positive cells and their phenotypes of activation status, although 4 of the 6 cases achieved biochemical improvement. During IFN therapy, interestingly, the frequencies of HCV-E2.717 positive cells were higher in SVR patients than in TVR patients. Additionally, proportions of HLA-DR positive cells among tetramer-positive cells were higher in SVRs than in TVRs at the start of treatment; the proportions increased after 2 weeks of IFN administration and remained elevated during the follow-up periods.

Phlebotomy is thought to be effective by correcting iron excess in chronic hepatitis C. The previous studies have reported that the progression of fibrosis is faster in chronic hepatitis C patients with congenital hemochromatosis (7), that combination therapy with IFN and phlebotomy may be more effective than IFN monotherapy (8), that hemosiderin deposition may be a predictive factor for IFN efficacy (9) and that dietary iron overload may be a risk factor for hepatocellular carcinoma (10). Inhibition of the toxic effects of superoxide or excess iron has been postulated as a mechanism underlying the therapeutic effects of phlebotomy (11); however, the possible involvement of immunological mechanisms had not been addressed.

Recently developed HLA-class I peptide tetramers, consisting of fluorescently-tagged tetrameric complexes of HLA heavy chains folded around epitope peptides, allow the sensitive and precise enumeration of T lymphocytes with specific T-cell antigen receptors (TCR) (23,24). With regard to HCV infection, this technology revealed that epitope-specific $CD8^+$ T lymphocytes are not only detectable in *in vitro* expanded $CD8^+$ T lymphocytes but also in freshly isolated PBMCs at more than 10-fold higher frequencies than those previously reported (25). Furthermore, the technology has facilitated the phenotypic, functional and molecular analysis of virus-specific immune responses at the single cell level (25). Additionally, by means of tetramers, the relative frequencies of T lymphocytes specific for different epitopes were observed to change during the course of viral infection (26). We have reported the frequency, phenotype and clinical significance of HCV-specific $CD8^+$ T lymphocytes using five different HLA-A*24 tetramers in HCV-infected Japanese patients (17).

Manfras *et al* reported that increased oligoclonality of circulating $CD8^+$ T-cells in chronic HCV infection was an indicator of a poor clinical reponse to IFN-α therapy; that IFN-α therapy enhanced the differentiation of $CD8^+$ T-cells towards a late differentiation phenotype ($CD28^-$ $CD57^+$); and that in cases of virus elimination, there was disappearance of expanded, terminally-differentiated $CD8^+$ cells (27). In our study, we found that the HLA-DR positive $CD8^+$ T-cells increased after 2 weeks of IFN therapy. On the other hand, during phlebotomy, there was no correlation between the improvement of liver function parameters and the frequencies of tetramer-positive cells or changes in the levels of activation markers. These findings may indicate that the mechanisms of phlebotomy and IFN therapy differ immunologically.

This is the first study to observe the alteration of HCV-specific T-cells, not only during IFN therapy, but also during the phlebotomy and the findings suggest that there may be important differences in their immunological aspects. The use of a combination of therapies which have different but complimentary mechanisms may be more beneficial for the treatment of chronic hepatitis C.

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Intrahepatic interleukin-8 production during disease progression of chronic hepatitis C

Yoshiya Tachibana [a,b], Yasunari Nakamoto [a,b], Naofumi Mukaida [a,b], Shuichi Kaneko [a,b,*]

[a] *Department of Gastroenterology, Graduate School of Medical Science, Kanazawa University, 13-1 Takara-machi, Kanazawa 920-8641, Ishikawa, Japan*
[b] *Department of Molecular Oncology, Cancer Research Institute, Kanazawa University, 13-1 Takara-machi, Kanazawa 920-8641, Ishikawa, Japan*

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Abstract

The current study was designed to investigate the contribution of chemokines to the pathogenesis of chronic hepatitis C and hepatocellular carcinoma (HCC) by measuring the production of IL-8, monocyte chemoattractant protein-1 (MCP-1) and macrophage inflammatory protein-1 alpha (MIP-1α). A solid-phase sandwich enzyme-linked immunosorbent assay (ELISA) was established to quantitate serum concentrations of the chemokines. Expression of chemokines in liver tissues was evaluated immunohistochemically using specific monoclonal antibodies. As the severity of chronic hepatitis escalated, serum IL-8 levels increased progressively. Moreover, in the hepatocellular carcinoma (HCC) patients, IL-8 concentrations were positively correlated with the macroscopic staging of HCC, and inversely correlated with the duration of the survival periods. The results demonstrate that IL-8 production may be augmented upon the malignant transformation of hepatocytes in chronic hepatitis C.

Keywords: Interleukin-8; Monocyte chemoattractant protein-1; Macrophage inflammatory protein-1 alpha; Sandwich enzyme-linked immunosorbent assay; Chronic hepatitis C; Hepatocellular carcinoma

1. Introduction

Chemokines are known not only to mediate the recruitment of inflammatory cells such as neutrophils or lymphocytes, but also to regulate the balance of helper T cells (Th1/Th2) as well as the activation of antigen-presenting dendritic cells, and thus to be deeply involved in immune responses. Moreover, chemokine-mediated cellular responses are known to be involved in neovascularization nd fibrosis, and since chemokines have growth factor activity, their association with malignant transformation has been suggested [1,2].

Recent findings that the core and nonstructural 5A (NS5A) proteins of hepatitis C virus (HCV) induce the expression of interleukin (IL)-8 gene *in vitro* have suggested that chemokines may be

* Corresponding author. Tel.: +81 76 265 2233; fax: +81 76 234 4250.
E-mail address: skaneko@m-kanazawa.jp (S. Kaneko).

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involved in the progression of chronic hepatitis (CH) and the development of liver cirrhosis (LC) and hepatocellular carcinoma (HCC) during the course of persistent HCV infection [3]. Thus, in this study we examined a possible correlation of serum concentrations of three chemokines, IL-8, monocyte chemoattractant protein-1 (MCP-1) or macrophage inflammatory protein-1 alpha (MIP-1α), detected by a sandwich enzyme-linked immunosorbent assay (ELISA) system with the severity of chronic hepatitis C. The results suggest that IL-8 production is enhanced progressively with escalating severity of liver disease and the development of HCC.

2. Materials and methods

2.1. Patients

The patients in this study included 30 cases of CH, 29 cases of LC and 30 cases of HCC, who had been attending in Kanazawa University Hospital from April, 1999 to April, 2000. Participants eligible for the study were anti-HCV antibody negative, between 20 and 80 years of age. All the patients were positive for anti-HCV antibody and serum HCV RNA was quantitated with the Amplicore HCV Monitor, version 2. In addition, 17 patients without chronic liver disease and also negative for anti-HCV antibody were enrolled as controls. All studied patients were negative for both hepatitis B surface antigen (HBsAg), HIV and alcoholic liver disease. In order to exclude the effects of inflammation other than liver diseases in our analyses, control subjects were selected with white blood cell (WBC) counts and C-reactive protein (CRP) values within normal range. There were significant differences in age, platelet count, alanine transaminase (ALT) activity and hepaplastin test (HPT) values among the four groups, i.e., CH, LC, HCC and control. These findings were considered to reflect differences in the pathological states among the groups (Table 1). This study was approved by the local ethics committee, and patients gave consent for the use of samples in these experiments.

2.2. Sandwich ELISA for IL-8, MCP-1 and MIP-1α

Serum concentrations of IL-8, MCP-1 and MIP-1α were determined by sandwich ELISA [4,5]. Each well in 96-well plates was coated with 100 μl of either anti-IL-8, anti-MCP-1 or anti-MIP-1α monoclonal antibody overnight at 4 °C. The wells were then treated with blocking solution (1% BSA-PBS) for 1 h at 37 °C. Serum samples were diluted with Tween-PBS containing 0.5% BSA and 100 μl of the samples were added to the wells and incubated at overnight 4 °C. Then, 100 μl of rabbit polyclonal antibodies against each of the chemokines (1 μg/ml) was added to the wells and the plates were incubated for 2 h at 37 °C. Thereafter, alkaline phosphatase conjugated anti-rabbit IgG was added to the wells and the plates were incubated for 2 h at 37 °C. Finally, 1 M diethanolamine (pH 9.8) containing 1 mg/ml *p*-nitrophenyl phosphate was added and the optical density of each well at 405 nm was measured using a microplate reader.

2.3. Criteria for clinical and pathological study

Serum chemokine concentrations were compared with the severity of chronic hepatitis C, macroscopic stages of HCC and the survival periods of the patients. Pathological classification of HCC was performed using general criteria for the clinical and pathological study of primary HCC [6].

2.4. Immunohistochemistry

Paraffin embedded sections of liver tissues were immunostained with mouse monoclonal IgG antibody against IL-8 at dilutions of 1:20 as described previously [7,8]. Then, horseradish peroxidase-labeled anti-mouse IgG

Table 1
Clinical characteristics of patients studied

	CH ($n=30$)	LC ($n=29$)	HCC ($n=30$)	Control ($n=17$)	P
Age (year)	48.8 ± 11.1	58.0 ± 8.4	66.6 ± 6.0	58.6 ± 15.6	<0.01*
Sex (M/F)	23/7	18/11	18/12	12/5	NS[a]
WBC (/μl)	5050 ± 1560	4360 ± 1470	3940 ± 1630	5760 ± 1850	NS*
Plt ($\times 10^4$/μl)	17.3 ± 5.1	9.9 ± 4.7	10.3 ± 5.1	20.7 ± 6.8	<0.01*
CRP (mg/dl)	0.12 ± 0.14	0.20 ± 0.45	0.80 ± 1.60	0.30 ± 0.56	NS*
ALT (IU/l)	100.0 ± 82.8	76.6 ± 52.8	83.7 ± 104.6	25.0 ± 19.0	<0.05*
HPT (%)	79.8 ± 11.4	70.1 ± 15.6	60.3 ± 14.6	92.5 ± 32.8	<0.01*

Note. Results are expressed as means ± SD.

Abbreviations: WBC, white blood cell; Plt, platelet; CRP, C-reactive protein; ALT, alanine transaminase; HPT, hepaplastin test; CH, chronic hepatitis; LC, liver cirrhosis; HCC, hepatocellular carcinoma; NS, not significant.

[a] Fisher's exact test.

* Kruskal–Wallis test.

(Vector Laboratories, Inc., Burlingame, CA), was added and incubated. Immunocomplexes were detected with diaminobenzidine (Sigma Chemical Co, St. Louis, MO).

2.5. Statistical analysis

Differences between groups were analyzed for statistical significance using one-way ANOVA and the Mann–Whitney U test. Qualitative variables were compared by means of Fisher's exact test. Factors significantly associated with the progression of liver disease were determined by multivariate logistic regression analysis. All tests were two-tailed, and a P-value of <0.05 was considered statistically significant.

3. Results

3.1. Serum chemokine levels in patients with chronic hepatitis C

Correlation of serum chemokine levels with the severity of chronic liver disease was examined in patients with chronic hepatitis C (Fig. 1). The detection limits of our ELISA systems for IL-8, MCP-1 and MIP-1α were 10, 40 and 10 pg/ml, respectively. Serum IL-8 levels were elevated progressively as the disease severity increased escalated: control group, 17.43 ± 1.11 pg/ml; CH group, 18.75 ± 2.32 pg/ml; LC group, 32.12 ± 3.80 pg/ml; and HCC group 49.13 ± 11.03 pg/ml ($P < 0.01$). In contrast, there was no correlation between serum MCP-1 concentrations and the severity of chronic hepatitis C: control group, 209.56 ± 26.33 pg/ml; CH group, 219.22 ± 54.55 pg/ml; LC group, 192.75 ± 59.52 pg/ml; and HCC group, 302.67 ± 44.52 pg/ml ($P = 0.057$). In addition, serum MIP-1α levels did not correlate with disease severity: control group, 21.26 ± 9.26 pg/ml; CH group, 27.83 ± 14.57 pg/ml; LC group, 17.99 ± 6.63 pg/ml; and HCC group, 28.37 ± 7.95 pg/ml ($P = 0.051$). The data suggest that IL-8 production may be induced in the process of disease progression in chronic HCV infection.

3.2. Serum IL-8 levels in patients with HCC classified according to the severity liver damage

Since serum IL-8 levels were high in the HCC group, as shown in Fig. 1, we examined whether there were any differences in serum IL-8 concentrations among patients with varying stages of HCC. Namely, we evaluated the correlation between serum IL-8 levels and the degree of liver damage, which reflects the hepatic reserve in HCC patients: liver damage A, 29.98 ± 6.59 pg/ml; liver damage B, 58.80 ± 35.37 pg/ml; and liver damage C, 81.54 ± 25.11 pg/ml. There was a tendency for increased values with the progression of liver damage, although this effect was not significant.

3.3. Serum IL-8 levels in patients with HCC classified according to macroscopic staging

A correlation between the macroscopic staging of HCC and serum IL-8 levels was examined (Fig. 2): stage I, 33.10 ± 10.79 pg/ml; stage II, 46.12 ± 20.93 pg/ml; stage III, 16.27 ± 5.36 pg/ml; stage IV-A, 41.25 ± 12.86 pg/ml; and stage IV-B, 153.20 ± 47.22 pg/ml. Serum IL-8 values of patients in stage IV-B were significantly higher than those of patients in other stages. Thus, patients with advanced HCC accompanying remote metastasis (stage IV-B) were found to show elevated IL-8 levels, compared with patients without remote metastasis. In addition, we observed the elevation of IL-8 following the detection of HCC bone metastasis in two cases of stage IV-B whose serial samples were preserved (Fig. 3), suggesting that serum levels of IL-8 may directly reflect the onset of HCC remote metastasis.

3.4. Serum IL-8 levels in patients with HCC classified according to survival periods

When a correlation between serum IL-8 levels and the survival periods of patients with HCC was evaluated, patients with poor prognosis gave significantly higher val-

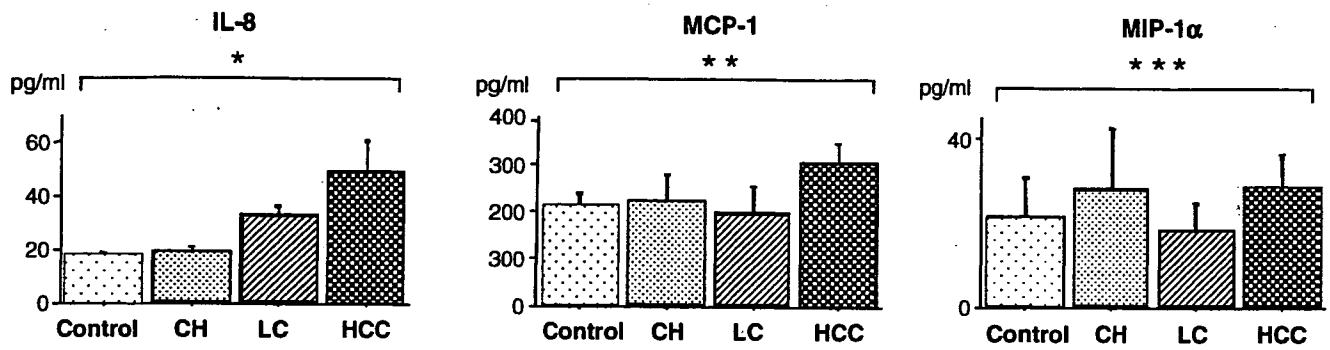

Fig. 1. Serum chemokine levels in patients with chronic hepatitis C, including 30 cases of chronic hepatitis (CH), 29 cases of liver cirrhosis (LC), 30 cases of hepatocellular carcinoma (HCC) and 17 controls. Serum IL-8 levels were elevated with the progression of disease: *F: 4.63, $P < 0.01$ when compared by analysis of ANOVA. Serum MCP-1 and MIP-1α concentrations were not correlated with disease severity: **F: 0.99, $P = 0.057$; ***F: 0.27, $P = 0.051$, respectively.

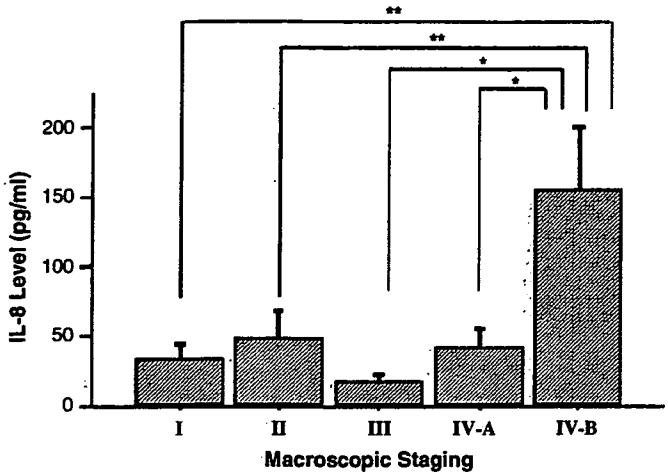

Fig. 2. Serum IL-8 levels in patients with HCC classified according to macroscopic staging. Stage I ($n = 3$) of macroscopic stage by the Liver Cancer Study Group of Japan; T1, N0, M0. Stage II ($n = 4$); T2, N0, M0. Stage III ($n = 7$); T3, N0, M0 or T1-3, N1, M0. Stage IV-A ($n = 12$); T4, N0-1, M0. Stage IV-B ($n = 4$); T1-4, N0-1, M1. In the HCC patients, IL-8 concentrations were positively correlated with macroscopic stages. F: 5.51, $P < 0.01$ when compared by analysis of ANOVA. $^{*}P < 0.01$ when compared by Mann–Whitney U test. $^{**}P < 0.05$ when compared by Mann–Whitney U test.

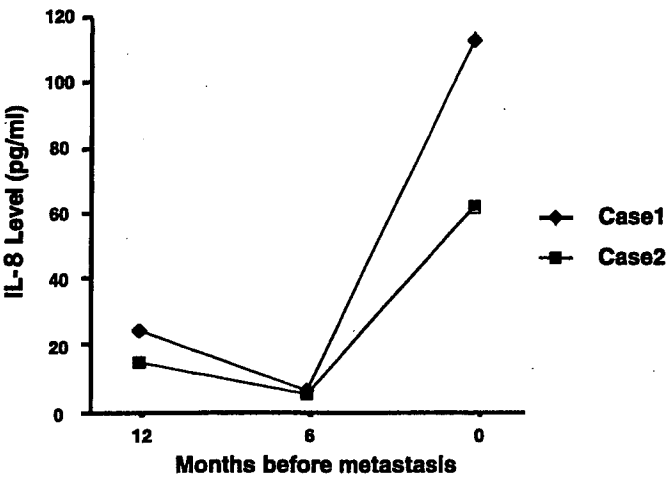

Fig. 3. Course of serum IL-8 levels in patients with HCC accompanying remote metastasis. Both cases 1 and 2 show the elevation of IL-8 following the detection of HCC bone metastasis.

ues (Fig. 4): over a 1-year survival period, 22.05 ± 5.90 pg/ml; over 3 months but less than 1 year, 64.34 ± 19.78 pg/ml; and less than 3 months, 132.72 ± 54.16 pg/ml. Furthermore, we performed multivariate logistic regression analysis between the prognosis of patients with HCC, and their ages, platelet counts, prothrombin times (PT), albumin levels, alpha-fetoprotein (AFP), IL-8 levels and the presence or absence of ascites (Table 2). The results indicated that AFP was not a factor that determined the prognosis, but IL-8 concentration was found to be an independent risk factor for a poor prognosis, as well as platelet count and serum albumin concentration elevated.

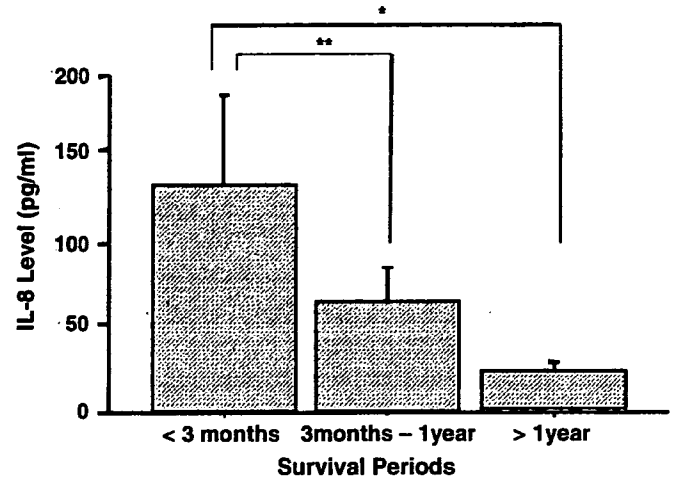

Fig. 4. Serum IL-8 levels in patients with HCC classified according to survival periods, <3 months ($n = 3$), 3 months–1 year ($n = 12$) and >1 year ($n = 16$). IL-8 concentrations were inversely correlated with the length of the survival periods. F: 6.40, $P < 0.01$ when compared by analysis of ANOVA. $^{*}P < 0.01$ when compared by Mann–Whitney U test. $^{**}P < 0.05$ when compared by Mann–Whitney U test.

3.5. IL-8 expression in liver cells

To identify IL-8 producing cells in the liver, an immunohistochemical analysis of liver tissues was performed. IL-8 was strongly stained in the cytoplasm of HCC cells, was weakly stained in the cytoplasm of some hepatocytes in LC, and was undetectable in hepatocytes from control tissue (Fig. 5). The data indicated that IL-8 is produce upon the malignant transformation of hepatocytes.

4. Discussion

The current study demonstrates that of the three chemokines, IL-8, MCP-1 and MIP-1α, determined by ELISA in patients with chronic hepatitis C, serum concentrations of IL-8 alone were increased, correlating with the progression of liver disease. Notably, the levels of IL-8 were significantly increased in patients with advanced HCC with remote metastasis and IL-8 levels were elevated in patients with poor prognoses. Interestingly, immunohistochemical analysis showed that IL-8 was detectable mainly in the cytoplasm of HCC cells. These findings suggest that the expression of IL-8 may be augmented upon the malignant transformation of hepatocytes during the course of chronic HCV infection.

IL-8 is known to be closely associated with pathological states of CH through the activation of inflammatory cells such as granulocytes or T lymphocytes. The levels of IL-8 were elevated in

Table 2
Characteristics of patients with HCC classified according to survival periods

	>3 months ($n = 3$)	3 months–1 year ($n = 12$)	<1 year($n = 16$)	Logistic regression	
				Regression coefficient	P^*
Age (year)	68.7 ± 3.8	68.3 ± 6.0	65.0 ± 6.3	3.067	0.2157
Plt ($\times 10^4/\mu l$)	9.3 ± 3.7	11.4 ± 6.1	9.6 ± 4.7	6.737	0.0344
PT (s)	14.3 ± 1.7	12.5 ± 1.5	12.2 ± 1.5	1.862	0.3942
Alb (mg/dl)	3.4 ± 1.0	3.4 ± 0.5	3.8 ± 0.9	9.013	0.0110
AFP (ng/ml)	57600 ± 57600	29300 ± 28900	252 ± 137	1.593	0.4509
IL-8 (pg/ml)	132.7 ± 54.2	64.3 ± 21.6	22.1 ± 5.9	10.196	0.0061
Ascites	2/3	2/12	2/16	0.003	0.9984

Note. Results are expressed as means ± SD.
Abbreviations: Plt, Platelet; PT, prothrombin time; Alb, albumin; AFP, alpha-fetoprotein; IL-8, interleukin-8.
* Kruskal–Wallis test.

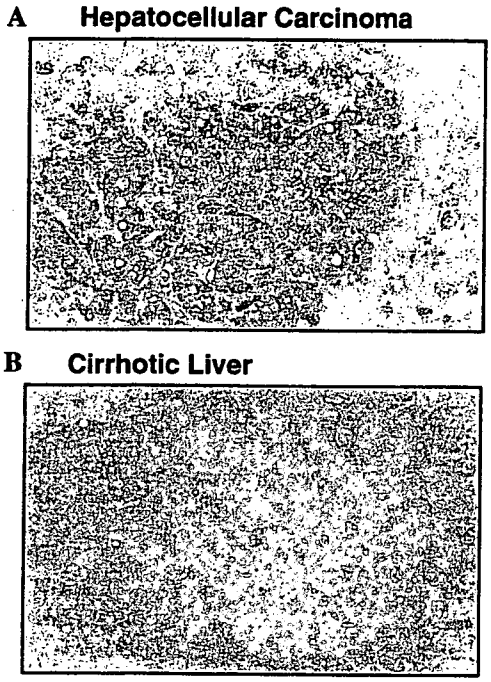

Fig. 5. Immunohistochemical analysis of liver tissues stained with the anti-IL-8 antibody. Five surgically resected HCC tissues were examined, in three cases IL-8 was strongly stained in the cytoplasm of HCC cells. A representative case of HCC stage II was shown in (A), whose serum IL-8 level was 28 pg/ml. IL-8 was weakly expressed in the cytoplasm of some hepatocytes in the cirrhotic liver (B) and was undetectable in hepatocytes of the control tissues (not shown). Original magnification 200×.

patients with acute and chronic liver damages, e.g., viral hepatitis, autoimmune hepatitis or alcohol-induced liver dysfunction [9–14]. Moreover, it has been reported that there is a correlation between IL-8 levels and the severity of liver disorders, including HCV infection [15–18]. Recently it has also been reported that the core and NS5A proteins of HCV induce the expression of the IL-8 gene [3], and that serum IL-8 levels in chronic hepatitis C patients are associated with resistance to interferon treatment [19], suggesting that IL-8 plays an important role in the maintenance of persistent infection with HCV. In the current study, serum IL-8 levels increased as the disease progressed from CH to LC and further to HCC, suggesting that the increase may be due not only to immune response against persistent HCV infection but to the development of HCC.

There has been no report, to our knowledge, investigating the possible correlation between serum MCP-1 or MIP-1α level and the pathology of chronic liver disease. The present results did not indicate a significant correlation between them. Therefore, we conclude that persistent HCV infection may not increase serum MCP-1 or MIP-1α levels.

Among the many chemokines, IL-8 level has been reported to show a tendency to increase during the progression of cancers of the stomach [20], pancreas [21], lung [22] and prostate [23]. In patients with HCC, IL-8 was shown to be expressed in the cytoplasm of hepatoma cells and in vascular endothelial cells of tumors [7,24,25], suggesting that the angiogenetic activity of IL-8 may contribute to the growth of HCC. In addition, we have observed that neither of the two IL-8 receptors, CXC chemokine receptor (CXCR) 1 or CXCR2, is detectable in HCC cell lines or tissues [5], suggesting that the growth promotion of HCC cells by IL-8 may be an indirect effect. IL-8 has the capacity to recruit various inflammatory cells that eventually produce proinflammatory cytokines including IL-1. Recently, we found that IL-1 enhances the production of CC chemokine ligand 3 (CCL3) which may interact with the CC chemokine receptor (CCR) 1 on HCC cells and contribute to tumor pro-

gression [5]. Moreover IL-8 levels were reported to be correlated with the growth of breast cancer cells having a high metastatic activity [26]. In line with these observations, our study similarly indicated that serum IL-8 levels were highly elevated in patients with HCC accompanied by remote metastasis (stage IV-B). Furthermore, we observed that serum IL-8 values rose following the detection of HCC bone metastasis in two cases. These findings suggest that IL-8 may promote the attachment and growth of cancer cells at extrahepatic sites. Moreover, IL-8 levels in cervical cancer tissues were shown to be correlated with the prognosis of patients [27]. Otherwise, IL-8 levels can simply correlate with overall tumor burden at advanced stages of HCC. Consistent with these observations, our study also showed that serum IL-8 levels increased significantly in patients with poor prognoses and whose survival periods were less than 1 year, as compared with patients with better prognoses. When we performed multivariate regression analyses of possible prognosis factors, IL-8, as well as platelet counts and albumin levels, was found to be a significant factor. These findings suggest that serum IL-8 levels can be a marker predicting the prognosis of patients with HCC.

This study indicates that IL-8 may be involved in the progression of chronic hepatitis C and the development of HCC. There is a report indicating the significant correlations of IL-8 levels with tumor size and disease stage in chronic hepatitis B as well [28], suggesting that IL-8 may be a useful biological marker of HCC invasiveness and a prognostic factor for HCC patients. The molecular biological mechanisms explaining these findings remain to be clarified in the future by using HCC cell lines or animal models.

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Prolonged, NK Cell-Mediated Antitumor Effects of Suicide Gene Therapy Combined with Monocyte Chemoattractant Protein-1 against Hepatocellular Carcinoma

Tomoya Tsuchiyama,* Yasunari Nakamoto,* Yoshio Sakai,* Yohei Marukawa,* Masaaki Kitahara,* Naofumi Mukaida,† and Shuichi Kaneko[1]*

Tumor recurrence rates remain high after curative treatments for hepatocellular carcinoma (HCC). Immunomodulatory agents, including chemokines, are believed to enhance the antitumor effects of tumor cell apoptosis induced by suicide gene therapy. We therefore evaluated the immunomodulatory effects of a bicistronic recombinant adenovirus vector (rAd) expressing both HSV thymidine kinase and MCP-1 on HCC cells. Using an athymic nude mouse model (BALB/c-*nu/nu*), primary s.c. tumors (HuH7; human HCC cells) were completely eradicated by rAd followed by treatment with ganciclovir. The same animals were subsequently rechallenged with HCC cells, tumor development was monitored, and the recruitment or activation of NK cells was analyzed immunohistochemically or by measuring IFN-γ mRNA expression. Tumor growth was markedly suppressed as compared with that in mice treated with a rAd expressing the HSV thymidine kinase gene alone ($p < 0.001$). Suppression of tumor growth was associated with the elevation of serum IL-12 and IL-18. During suppression, NK cells were recruited exclusively, and Th1 cytokine gene expression was enhanced in tumor tissues. The antitumor activity, however, was abolished either when the NK cells were inactivated with anti-asialo GM1 Ab or when anti-IL-12 and anti-IL-18 Abs were administered. These results indicate that suicide gene therapy, together with delivery of MCP-1, eradicates HCC cells and exerts prolonged NK cell-mediated antitumor effects in a model of HCC, suggesting a plausible strategy to prevent tumor recurrence. *The Journal of Immunology*, **2007, 178: 574–583.**

Despite curative treatments including surgical resection and liver transplantation for hepatocellular carcinoma (HCC),[2] tumor recurrence rates remain high, probably because of insufficient therapeutic effects and the multicentric development of HCC in cirrhotic liver (1–3). Although nonsurgical treatments of HCC such as radiofrequency ablation, transcatheter arterial embolizations, and transcatheter arterial chemotherapy induce apoptosis of HCC cells, these treatments do not enhance antitumoral immunity sufficiently. Therefore, gene therapy aimed at enhancing antitumor immune responses may be a promising approach to induce sufficient inhibitory effects for the prevention of tumor recurrence.

Although killing tumor cells with cytotoxic genes such as suicide gene/prodrug systems consisting of HSV thymidine kinase (HSV-tk) and ganciclovir (GCV) may lead to the generation of effective immunity (4, 5), cell killing alone is insufficient to increase many antitumor responses (6–8). Recently, however, coexpression of HSV-tk and chemokines was found to increase tumor immunity in animal models in which neither HSV-tk nor chemokine expression alone was sufficient (9). In addition, we previously demonstrated that, at the local treatment site, the antitumor effects of the HSV-tk/GCV system were enhanced by codelivery of MCP-1, a member of the CC chemokine family (8, 10). MCP-1 has been shown to stimulate the cytotoxic activity of monocytes, enhance the expression of adhesion molecules such as CD11b and CD11c, and induce the cytotoxic and migratory activities of NK cells (11–14). Moreover, transfection of the MCP-1 into human lung adenocarcinoma cells inhibited the formation of metastases, presumably via the activation of NK cells (15). It was recently reported that NK cells can mediate long-lived, Ag-specific adaptive recall responses independently of B cells and T cells (16). These observations suggest that MCP-1 can induce specific tumor immunity by enhancing NK cell functions even in this system.

Thus, we evaluated the long-term systemic immunomodulatory effects of a bicistronic recombinant adenovirus vector (rAd) expressing both HSV-tk and MCP-1 (Ad-tk-MCP1). After the primary s.c. HCC tumors in athymic nude mice were eradiated by using Ad-tk-MCP1, the same HCC cells were injected into an another site of the same mice to prove the presence of NK cell-mediated, long-term immunity. Moreover, we explored whether innate immune responses induced by NK cells were involved in these procedures. In this study, we provide definitive evidence to indicate that codelivery of a suicide gene and MCP-1 exerts prolonged NK cell-mediated antitumor effects in this model, suggesting a plausible strategy to prevent HCC recurrence.

*Department of Gastroenterology, Graduate School of Medical Science and †Division of Molecular Bioregulation, Cancer Research Institute, Kanazawa University, Kanazawa, Japan

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[1] Address correspondence and reprint requests to Dr. Shuichi Kaneko, Department of Gastroenterology, Graduate School of Medical Science, Kanazawa University, 13-1 Takara-machi, Kanazawa 920-8641, Japan. E-mail address: skaneko@medf.m.kanazawa-u.ac.jp

[2] Abbreviations used in this paper: HCC, hepatocellular carcinoma; AGM1, asialo GM1; BNL, BNL 1ME A.7R.1 HCC cell line; DC, dendritic cell; GCV, ganciclovir; HSV-tk, HSV thymidine kinase; MMC, mitomycin C; MOI, multiplicity of infection; rAd, recombinant adenovirus vector; Ad-tk, rAd expressing HSV-tk; Ad-tk-MCP1, rAd expressing both HSV-tk and MCP-1; Ad-MCP1, rAd expressing MCP-1; Ad-lacZ, rAd expressing lacZ; $TCID_{50}$, 50% tissue culture infectious dose.

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