TABLE II. Baseline Characteristics in Relation to HBeAg Status

Factors	ALL (n = 45)	HBeAg Positive (n = 29)	HBeAg Negative (n = 16)	P
Mean age	47 ± 10.0	46.9 ± 11.4	47.1 ± 7.3	0.943
Male	33	23	10	0.222
Baseline ALT (IU/L)	153.1 ± 116.0	146.8 ± 98.7	164.7 ± 145.1	0.856
Baseline HBV-DNA (LGE/ml)*	7.1 ± 1.3	7.5 ± 1.1	6.3 ± 1.2	0.0015
Baseline core promoter mutation	35	21	14	0.492
Baseline precore mutation	17	6	11	0.0015

*More than 8.7 LGE/ml and less than 3.7 LGE/ml were calculated as 8.8 LGE/ml and 3.6 LGE/ml.

Changes of the Precore Region During the 1-Year Lamivudine Therapy

The serial analyses of the precore region are shown in Tables I and IVa. Of the 12 patients (patients 1–12) who lost HBeAg during the 1-year treatment, 5 patients (patients 1–5) had precore wild type HBV at both baseline and 1 year. In three patients (patients 9–11), a shift from precore wild type HBV to the precore mutation G1896A was detected. A reversion from the precore mutation G1896A to precore wild type HBV was detected in three patients (patients 6–8). One patient (patient 12) had the precore mutation G1896A at both baseline and 1 year. Respectively, 8 (66.7%) of 12 patients still had precore wild type HBV at 1 year despite of the loss of HBeAg.

Of the 17 patients (patients 13–29) who were persistently HBeAg-positive, 12 patients (patients 13–24) had precore wild type HBV at both baseline and 1 year. A shift to the precore mutation G1896A was detected in three patients (patients 26–28) and a reversion to precore wild type HBV was detected in one patient (patient 25). One patient (patient 29) had the precore mutation G1896A at both baseline and 1 year.

Of the 16 patients (patients 30–45) who were positive for anti-HBe at baseline, the precore mutation G1896A was detected in 11 (patients 34–40, 42–45). Four patients (patients 30–33) had precore wild type HBV at both baseline and 1 year. A shift to the precore mutation G1896A was detected in one patient (patient 41) and a reversion to precore wild type HBV was detected in seven patients (patients 34–40). Four patients (patients 42–45) had the precore mutation G1896A at both baseline and 1 year. Among these 16 patients, the frequency of patients with precore wild type HBV significantly increased from 5 (31.3%) to 11 (68.8%) after the 1-year treatment. ($P = 0.034$) (Table V).

In a total of 45 patients, including both of those who were HBeAg- and anti-HBe-positive at baseline, a reversion in the precore region was detected in 11 (64.7%) of 17 patients having the precore mutation G1896A at baseline.

Changes of the Core Promoter Region During the 1-Year Lamivudine Therapy

The serial analyses of the core promoter region are shown in Tables I and IVb. Of the 12 patients who lost HBeAg during the 1-year treatment, 9 patients (patients 1–7, 9–11) had the dual core promoter mutations A1762T, G1764A at both baseline and 1 year except 1 patient (patient 5). One patient (patient 8), with a deletion of ten base pairs of nucleotides from 1758 to 1767 in the core promoter region at baseline, had the dual core promoter mutations A1762T, G1764A at 1 year. A shift from core promoter wild type HBV to the dual core promoter mutations A1762T, G1764A was detected in two patients (patients 11 and 12). A reversion from the dual core promoter mutations A1762T, G1764A to core promoter wild type HBV was not detected in those who lost HBeAg during the 1-year treatment.

Of the 17 patients who were persistently HBeAg-positive during the 1-year treatment, 7 patients (patients 13–19) had the dual core promoter mutations A1762T, G1764A at both baseline and 1 year. At

TABLE III. Baseline Characteristics in Relation to HBeAg and Anti-HBe Status at 1 Year in HBeAg-Positive Patients

Factors	HBeAg status at 1 year: Negative (n = 12)	HBeAg status at 1 year: Positive (n = 17)	P
Mean age	48.6 ± 10.2	45.8 ± 12.4	0.52
Male	10	13	0.51
Baseline ALT (IU/L)	121.4 ± 89.7	164.6 ± 103.4	0.25
Baseline HBeAg (S/N ratio)	85.3 ± 84.6	141.3 ± 92.4	0.11
Baseline HBV-DNA (LGE/ml)*	7.24 ± 0.99	7.38 ± 1.10	0.73
Baseline core promoter mutant HBV	10	14	0.67
Baseline precore mutant HBV	4	2	0.17

*More than 8.7 LGE/ml and less than 3.7 LGE/ml were calculated as 8.8 LGE/ml and 3.6 LGE/ml.

TABLE IVa. Changes in Nucleotide Sequence at 1896 in Precore Region

	Precore nt 1896		
	Baseline	At 1 year	No. of cases (%)
HBeAg-positive at baseline (n = 29)			
HBeAg-negative at 1 year (n = 12)	Wild	Wild	5 (41.7%)
		Mutant	3 (25.0%)
	Mutant	Wild	3 (25.0%)
		Mutant	1 (8.3%)
HBeAg-positive at 1 year (n = 17)	Wild	Wild	12 (70.6%)
		Mutant	3 (17.6%)
	Mutant	Wild	1 (5.9%)
		Mutant	1 (5.9%)
Anti-HBe-positive at baseline (n = 16)			
Anti-HBe-positive at 1 year	Wild	Wild	4 (25.0%)
		Mutant	1 (6.3%)
	Mutant	Wild	7 (43.6%)
		Mutant	4 (25.0%)

baseline, one patient (patient 21) had a deletion of 23 base pairs of nucleotides from 1757 to 1779 and one patient (patient 28) had a deletion of four base pairs of nucleotides from 1760 to 1763 in the core promoter region. Both of those two patients had the dual core promoter mutations A1762T, G1764A at 1 year. Two patients (patients 24 and 29) had core promoter wild type HBV at both baseline and 1 year. A shift to the dual core promoter mutations A1762T, G1764A was detected in one patient (patient 20). A reversion to core promoter wild type HBV was detected in four patients (patients 22, 25–27). At 1 year, a deletion of 20 base pairs of nucleotides from 1756 to 1775 in the core promoter region was detected in one patient (patient 23) having the dual core promoter mutations A1762T, G1764A at baseline.

Of the 16 patients who were positive for anti-HBe at baseline, 8 patients (patients 34–38, 41–43) had the dual core promoter mutations A1762T, G1764A at both baseline and 1 year. A shift to the dual core promoter mutations A1762T, G1764A was detected in two patients (patients 39 and 44). A reversion to core promoter wild type HBV was detected in five patients (patients 30–32, 40, 45). At 1 year, a deletion of 20 base pairs of nucleotides from 1758 to 1777 in the core promoter region was detected in one patient (patient 33) having the dual core promoter mutations A1762T, G1764A at baseline. There was no patient who had core promoter wild type HBV at both baseline and 1 year.

Accordingly, the nucleotide sequence in the core promoter region remained mutant in most patients. However, a reversion from the dual core promoter mutations A1762T, G1764A to core promoter wild type HBV was observed in some patients; 6 (35.3%) of 17 patients who were persistently positive for HBeAg and 6 (37.5%) of 16 patients who were positive for anti-HBe at baseline.

Emergence of Lamivudine-Resistant Mutations

During the 1-year treatment, lamivudine-resistant mutations in YMDD motif were detected in 11 (24.4%) of the 45 patients enrolled in this study. Lamivudine-resistant mutants were detected in only 1 (8.3%) of the 12 patients who lost HBeAg. Among the 16 patients who

TABLE IVb. Changes in Nucleotide Sequence at 1762/1764 in Core Promoter Region

	Core promoter nt 1762/1764		
	Baseline	At 1 year	No. of cases (%)
HBeAg-positive at baseline (n = 29)			
HBeAg-negative at 1 year (n = 12)	Wild	Wild	0 (0%)
		Mutant	2 (16.7%)
	Mutant	Wild	1 (8.3%)
		Mutant	9 (75%)
HBeAg-positive at 1 year (n = 17)	Wild	Wild	2 (11.8%)
		Mutant	1 (5.9%)
	Mutant	Wild	6 (35.3%)
		Mutant	9 (53.0%)
Anti-HBe-positive at baseline (n = 16)			
Anti-HBe-positive at 1 year	Wild	Wild	0 (0%)
		Mutant	2 (12.5%)
	Mutant	Wild	6 (37.5%)
		Mutant	8 (50%)

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Antibody Reactive to a Hepatitis C Virus (HCV)-Derived Peptide Capable of Inducing HLA-A2 Restricted Cytotoxic T Lymphocytes Is Detectable in a Majority of HCV-Infected Individuals without HLA-A2 Restriction

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***Abstract*: Hepatitis C virus (HCV) is a single-strand RNA virus. Approximately 170 million people around the world are persistently infected and are at risk of liver cirrhosis or cancer. There is an urgent need to develop both therapeutic and diagnostic modalities of HCV. One approach to achieve these goals would be to determine highly immunodominant HCV peptides which are recognized by both cellular and humoral immunities. This study reports one such peptide, HCV-core protein at positions 35–44, having HLA-A2 binding motifs. IgG specific to this CTL-epitope peptide is consistently detectable in a majority of the patients with HCV infection regardless of the different HLA types, different disease conditions, and different HCV-genotypes tested. The sequence LPRR at positions 37–40 is considered to be the fine epitope recognized by the IgG. These results may provide new insights for the development of both therapeutic and diagnostic modalities of HCV at lower costs.**

***Key words*: HCV, Peptide, Cytotoxic T-lymphocytes, Antibody**

Extensive studies have been carried out in the past-decade in order to find immunodominant HCV-peptides, and there are many peptides capable of inducing cellular immune responses (4, 15, 19, 23, 25). None of them, however, are clinically effective. Subsequently, there is no immunization yet available, neither prophylactic nor therapeutic (7, 12). This could be, in part, due to either the weak immunogenicity of these previously reported peptides or insufficient studies regarding them. Although interferon and other reagents are effective for eliminating HCV in certain patients, they are too expensive for the majority of HCV$^+$ patients in most countries. Subsequently, there is an urgent need to determine immunodominant peptides useful for the development of effective and low-priced vaccines. In addition, there is also a need to develop a simple and low-priced diagnostic tool for HCV since the currently available kit is also expensive for the majority of people who are not living in developed countries. We previously reported that some CTL-directed peptides derived from non-mutated self-antigens were recognized by humoral immune response in both healthy subjects and cancer patients without apparent HLA-restriction (10, 16, 17, 21). Elevated levels of IgG to those CTL-epitope peptides by peptide vaccination showed a close correlation with long-term survival in vaccinated cancer patients (16, 21), whereas the class of IgG to those peptides was either lacking or unbalanced in atopic dermatitis patients (14), suggesting their positive role in host defense. In addition, there is strong evidence that

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Abbreviations: Ab, antibody; CH, chronic hepatitis; CTL, cytotoxic T lymphocyte; EIA, enzyme immunoassay; ELISA, enzyme-linked immunosorbent assay; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; HD, healthy donor; HIV-1, human immunodeficiency virus-1; HLA, human leukocyte antigen; HTLV-I, human T-lymphotropic virus type I; LC, liver cirrhosis; OD, optical density; PBMC, peripheral blood mononuclear cells; Pt., patient; RIA, radio immunoassay.

both cellular and humoral immune responses play a major role in the control of HCV infection (9, 12, 25). We then hypothesized that HCV-peptides recognized by both cellular and humoral immunities were more immunogenic than those recognized by either immunity alone, and thus are applicable for the development of both new treatment and diagnosis modalities for HCV infection. This study reports one such peptide, an HCV core peptide at positions 35–44, to which high levels of IgG are detectable in a majority of HCV-infected individuals without apparent HLA-A restriction. These results may provide a new insight useful in the development of both therapeutic and diagnostic modalities for HCV infection.

Materials and Methods

Subjects. The Institutional Ethical Review Board of Kurume University approved this study protocol, and informed written consent was obtained from all the subjects from whom sera and peripheral blood mononuclear cells (PBMCs) were taken in this study. Twelve HCV^+ patients were seropositive for anti-HCV antibodies (Abs) as determined by second- or third-generation immunoassay test. Diagnoses of these patients determined by biochemical and histological findings, ultrasound-sonography, and computed tomography were chronic hepatitis (CH, $n=5$), liver cirrhosis (LC, $n=4$), and hepatocellular carcinoma (HCC, $n=3$) at the first time of serum samplings. As negative controls, sera from 10 HDs with no history of either viral hepatitis or vaccination of HBV, and normal liver function were tested in this study.

Peptide and measurement of peptide-reactive Ab. We used 18 different kinds of synthetic peptides having HLA-A2 binding motifs (20) from well-conserved regions of HCV-genotype 1b proteins (Table 1). An HIV derived-peptide with HLA-A2 binding motif as the negative control (11) and 15 10-mer peptides possessing overlap sequences spanning HCV core 27–50 (Fig. 3B) were also used. These peptides were purchased from BIO SYNTHESIS (Lewisville, Tex., U.S.A.) or SynPep (Dublin, Calif., U.S.A). The desalted grade of the materials was used for the screening of peptides reactive to serum IgG or >90% purified materials for further studies as assessed by reverse-phase high-pressure liquid chromatography analyses. Serum levels of peptide-specific IgG were measured by means of an enzyme-linked immunosorbent assay (ELISA) employing a method previously reported (18). In brief, each peptide was

Table 1. HCV-derived peptides used in this study and serum reactivity[a]

Peptides			Serum reactivity	
			HCV patients	HDs
Number	Region	Sequence	($n=12$)	($n=10$)
1	C 35–44	YLLPRRGPRL	11	0
2	C 132–140	DLMGYIPLV	0	0
3	C 150–158	ALAHGVRAL	0	1
4	C 168–176	NLPGCSFSI	0	1
5	C 178–187	LLALLSCLTV	1	0
6	E1 220–229	ILHTPGCV	3	0
7	E2 363–372	SMVGNWAKV	0	0
8	E2 401–411	SLLAPGAKQNV	0	3
9	NS3 1073–1081	CINGVCWTV	0	0
10	NS3 1169–1178	LLCPAGINAV	1	2
11	NS3 1287–1296	KLVALGINAV	1	0
12	NS3 1406–1505	TGAPVTYSTY	1	0
13	NS4B 1789–1797	SLMAFTAAV	0	0
14	NS4B 1807–1816	LLFNILGGWV	0	0
15	NS4B 1851–1859	ILAGYGAGV	0	1
16	NS5A 2252–2260	ILDSFDPLV	0	0
17	NS5B 2692–2701	RLIVFPDLGV	1	0
18	NS5B 2727–2785	GLQDCTMLV	2	1

[a] We used 18 peptides containing HLA-A2 binding motifs that were previously shown to represent target epitopes of HLA-A2-restricted CTL responses directed to HCV antigens. We measured the levels of peptide-specific IgG in the sera of 12 patients with HCV-1b infection and 10 healthy donors (HDs). The cut-off value for this anti-HCV core 35–44 peptide at a serum dilution of 1:100 was determined as 0.18 (the mean <0.03> plus 3SD <0.05 times 3> of 10HDs). Abbreviations: C, core; E, envelope; NS, non-structure.

dissolved in dimethylsulfoxide (DMSO) and then stored at −20 C. Peptides (20 μg/well) diluted with 0.1 M carbonate buffer containing a chemical cross-linker, disuccinimidyl suberate (DSS) (PIERCE, Rockford, Ill., U.S.A.), were bound to the ELISA plate. The wells were rinsed three times with 0.05% Tween 20-PBS (PBST). The plates were blocked overnight at 4 C with Block Ace (Yukijirushi, Tokyo). Plasma or serum samples were diluted at 1:100, 1:200, and 1:400 with 0.05% Tween 20-Block Ace, and 100 μl/well of samples was added to each well. After incubation at 37 C for 2 hr, the plates were washed with PBST three times and incubated at 37 C for 2 hr with 100 μl/well of 1:1,000-diluted rabbit anti-human IgG (γ-chain-specific) (DAKO, Glostrup, Denmark). After washing three times, 100 μl/well of 1:100-diluted anti-rabbit IgG-conjugated horseradish peroxidase-dextran polymer (EnVision, DAKO) was added to each well, and the plates were incubated at room temperature for 40 min. After washing, 100 μl/well of tetramethylbenzidine substrate solution (KPL, Guildford, U.K.) was added, and the reaction was stopped by the addition of 1.0 M of phosphoric acid. Cut-off values were calculated as the mean+3SD of optical density (OD) of sera from HDs taken as a negative control, and it was 0.18 for the screening assay and 0.2 for the fine epitope analysis with desalted peptides, or 0.093 with peptides of >90% purity.

Absorption and elution of peptide-reactive IgG. To test the specificity of anti-peptide IgG in the serum samples, 100 μl/well of serum samples (1:100 dilution with 0.05% Tween 20-Block Ace) was absorbed by immobilized peptides (20 μg/well) in wells of the plate for 2 hr at 37 C. The same procedure was repeated three times, and the levels of peptide-specific IgG in the supernatant were measured by ELISA. For an elution test, Abs bound to the peptide-immobilized plates by the first absorption were eluted by 30 μl/well of 0.5 M NaCl/50 mM citrate buffer (pH 3.0), neutralized by 1.0 M Tris-HCl (pH 7.5) with 0.05% Tween 20-Block Ace, and the levels of peptide-specific IgG in the eluted fraction were measured by ELISA.

Assay for peptide-specific CTLs. The method used for the detection of peptide-specific CTLs has been reported elsewhere (11, 13). In brief, PBMCs (1×10^5 cells/well) were cultured with 10 μM of each peptide (quadricate) in wells of a U-bottom-type 96-well microculture plate (Nunc, Roskilde, Denmark) in 200 μl of culture medium. The medium consisted of 45% RPMI-1640 (Invitrogen, Carlsbad, Calif., U.S.A.), 45% AIM-V (Invitrogen), 10% FCS, 100 U/ml of IL-2 (Shionogi, Osaka, Japan), and 0.1 mM MEM non-essential amino acid solution (Invitrogen). On days 3, 6, and 9, half of the medium was removed and replaced with new medium containing the corresponding peptide (20 μg/ml). On day 12 of the culture, cells of each of the quadricate cultures were divided and placed into four wells and tested for their ability to produce IFN-γ in response to T2 cells pre-loaded with either a corresponding peptide or an HIV-peptide as a negative control in duplicate assays. The background IFN-γ production in response to the HIV peptide (<50 ng/ml) was subtracted from the values given in the data. The $CD8^+$ cells were positively purified using the CD8 Isolation Kit (DYNAL, Oslo, Norway) in certain experiments. For the inhibition test, 20 μg/ml of anti-HLA class I (W6/32, IgG2a), anti-CD8 (Nu-Ts/c, IgG2a), anti-CD4 (Nu-Th/i, IgG1b), anti-HLA-class II (H-DR-1, IgG2a), and anti-CD14 (H14, IgG2a, as an irrelevant control) monoclonal antibodies (mAbs) were used as reported previously (11).

Statistics. Statistical analyses were performed using Student's *t*-test, Mann-Whitney *U* test, and the χ^2 test. Values of $P<0.05$ were considered statistically significant.

Results

Detection of Humoral Response to HCV-Peptides

We measured the levels of IgG reactive to each of 18 peptides in the sera of 12 patients with HCV-1b infection and 10 HDs. These subjects included both HLA-$A2^+$ and -$A2^-$. The cut-off value for this anti-HCV core 35–44 at a serum dilution of 1:100 was determined as 0.18 (the mean <0.03> plus 3SD <0.05 times 3> of 10HDs). Significant levels (>0.18 OD at a serum dilution of 1:100) of IgG reactive to HCV core 35–44 were detected in the sera from all patients except for one case, but were not detected at all from any of the HDs tested. The ELISA results are shown in Fig. 1. In contrast, significant levels of IgG reactive to the remaining 17 peptides were scarcely detected or undetectable in the patients (Fig. 1) or HDs (data not shown), respectively. Therefore, the HCV core 35–44 peptide and the other control peptides with >90% purity were used in the following experiments. The peptide specificity of the IgG reactive to HCV core 35–44 was confirmed by absorption and elution tests. As expected, anti-HCV core 35–44 Ab was absorbed by the corresponding peptide, but not by an irrelevant peptide (HCV NS5A 2252–60), and it was further eluted from the corresponding peptide, but not by an irrelevant peptide, bound fraction (Fig. 2).

A fine epitope of the HCV core 35–44 peptide recognized by the patients' sera was further analyzed using 15 decamer peptides possessing overlap sequences spanning HCV core 27–50. The reactivity of the patients' IgG to the overlapping peptides was examined and the

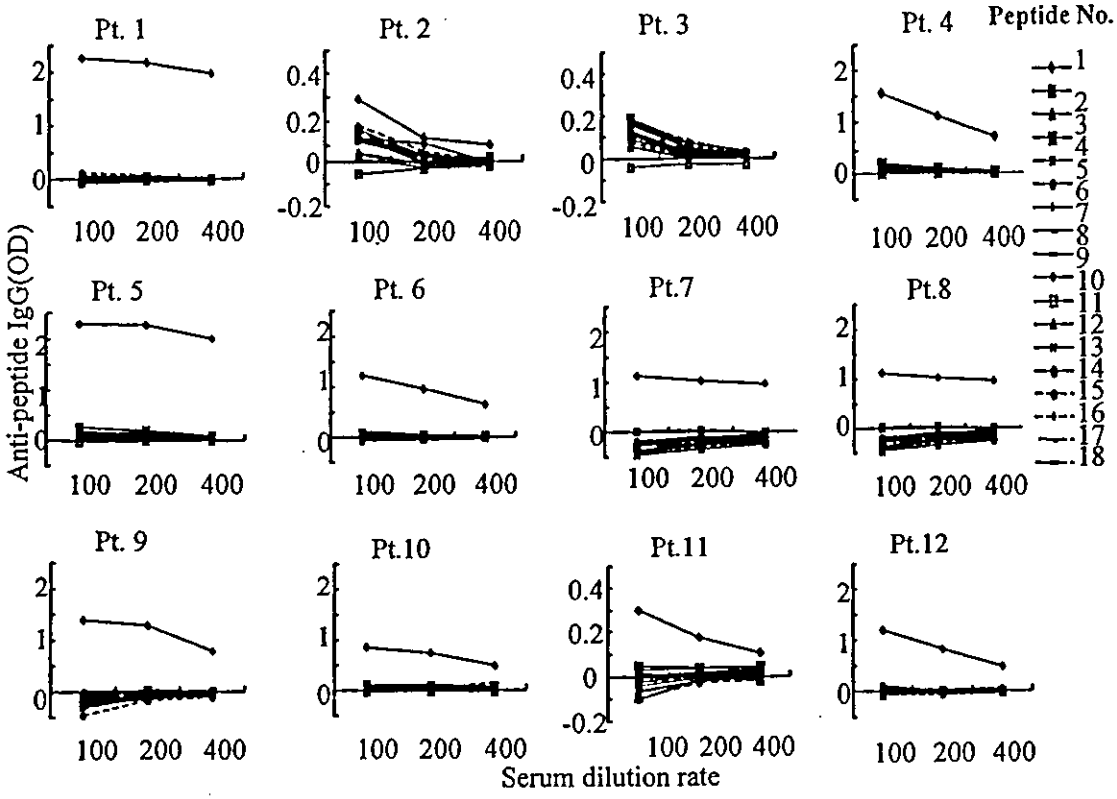

Fig. 1. Detection of anti-peptide IgG. Optical density (OD) values of each sample were assessed in serially diluted serum samples in order to estimate peptide-reactive IgG levels by ELISA. Representative results of 12 HCV$^+$ patients are shown.

representative results and summary of the data of 5 patients' sera are shown in Fig. 3A and Table 2, respectively. Seven peptides spanning HCV core 31–46 expressed positive reactivity in a majority of the cases. The common overlapping sequence of these seven peptides is LPRR at position 37–40 of the HCV core protein. Thus, this sequence, LPRR, is considered to be the fine epitope of HCV core 35–44 peptide recognized by the patients' IgG. The reactivity of the IgG to 4-mer and 7-mer peptides containing LPRR with/without N- or C-terminal GGG spacer sequences was further analyzed (Fig. 3B). The patients' IgG reacted with the native sequence decamer peptides containing LPRR. In contrast, the IgG could not bind to the 4-mer or 7-mer peptides. These results, together with the highest reactivity of the 35–44 peptide, suggest that three-dimensional conformation affected by the nested sequences might be important for the recognition of LPRR sequence by the IgG.

Induction of Peptide-Specific Cellular Response

PBMCs from HLA-A2$^+$ patients with HCV infection (n=5) and HLA-A2$^+$ HDs without HCV infection (n=9) were cultured with each of the 18 peptides (>90% purity) or a control HIV-peptide for 12 days, and were examined for their IFN-γ production in response to the corresponding peptide-pulsed T2 cells. The HCV core peptides at positions 35–44, 132–140 and 178–187, and NS3 peptide at position 1287–1296 induced significant levels (P>0.05 and net value >100 pg/ml) of IFN-γ from two, four, one, and one of five patients, respectively. In contrast, none of the peptides induced significant levels of IFN-γ in the HDs. Representative results of each of five cases whose PBMCs showed positive IFN-γ production in response to at least one peptide and a HD are given in Fig. 4A. Levels of IFN-γ produced by these PBMCs were significantly inhibited by anti-class I (W6/32) or anti-CD8 mAb, but not by any of the other mAbs tested (Fig. 4B). These results suggest that this cellular response was largely mediated by CD8$^+$, but not CD4$^+$ T cells in an HLA-class I-restricted manner.

The cytotoxicity of these peptide-stimulated PBMCs was confirmed by a 6 hr ^{51}Cr-release assay, and the representative results of the cytotoxicity to T2 cells pulsed with each of the three HCV core peptides are shown in Fig. 4C. These PBMCs showed significant levels of cytotoxicity against the T2 cells that were pre-loaded with a corresponding peptide, but not with an HIV peptide, taken as the negative control. Further, the HLA

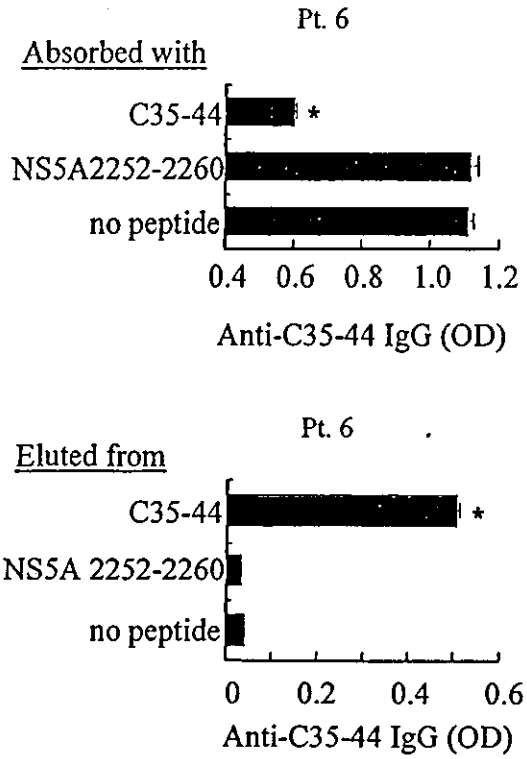

Fig. 2. Specificity of anti-peptide IgG. Each serum sample was absorbed, with either HCV core 35–44 peptide or an irrelevant (HCV NS5A 2252–2260) peptide used as the negative control, three times at 37 C followed by peptide-specific IgG ELISA. For the elution test, IgG molecules bound to the peptide-immobilized plates by the first absorption were eluted by acid and subjected for measurement of the levels of peptide-specific IgG by ELISA. The representative results from the sera of Pts. 6 and 9 are shown in this figure. Values represent the mean±SD. *Values are differed from the no peptide or irrelevant controls, $P<0.05$ by Student's t-test.

restriction and specificity of the CTL activity was confirmed by the inhibition assay. Specifically, the cytotoxicity against T2 cells pulsed with HCV core 35–44 peptide was inhibited by anti-HLA-class I (W6/32) or CD8 mAb, but not by any of the other mAbs tested, indicating that the peptide-specific cytotoxicity was largely mediated by $CD8^+$ T cells in an HLA-class I-restricted manner (Fig. 4D). These results suggest that the HCV core peptides at positions 35–44 and 132–140 are suitable candidates for use as a prophylactic as well as therapeutic vaccine for $HLA\text{-}A2^+$ patients with HCV-related diseases.

Discussion

HCV infection is a serious worldwide health problem, approximately 170 million people in the world are persistently infected with HCV and have a high risk of LC and HCC at later stages. Most of them do not live in developed countries, and thus have a rare chance of receiving either diagnosis or treatment. One solution will come, in part, from introducing an economical approach. The presently available modalities, such as IFN as a therapeutic agent and a third generation of enzyme/radio immunoassay (EIA-3/RIA-3) as a diagnostic tool, are too expensive for a majority of the infected individuals who are not living in well-developed countries. Therefore, development of both new diagnostic and therapeutic modalities with lower prices will increase the chance of these infected subjects to receive diagnosis and treatment. One way to achieve these goals would be to determine highly immunodominant HCV-peptides recognized by both cellular and humoral immunities, and provide them for the development of new therapeutic and diagnostic modalities (8, 25). Our results indicated that one peptide (HCV core at position 35–44) among the 18 kinds of peptides tested was recognized for more than 90% of the patients with HCV infection. This epitope peptide has already been reported as being capable of inducing HLA-A2-restricted CTLs (1, 6, 23–25). Therefore, our results along with those results, suggest that this core 35–44 peptide is a suitable candidate for prophylactic and therapeutic vaccines to treat HLA-A2+ patients with HCV-related diseases.

Our results show that significant levels of IgG specific to the HCV core 35–44 peptide were detectable in a majority of the HCV-infected individuals without HLA-A2 restriction. It is well known that serologically higher immunogenicity is located at the N-terminal of the HCV core protein (2, 5, 8, 22). Therefore, this region, at position 10–55, was used as one of the three key antigens for the third generation of EIA/RIA. Leucine, at position 36, and arginine or leucine, at positions 43 or 44, may be aggretopes for binding to HLA-A2 molecules (9, 20). This assumption is supported by the results from the fine epitope analysis of the HCV core 35–44 peptide using overlapping peptides. Namely, the common sequence of the overlapping peptides recognized by the patients' sera is LPRR, at position 37–40 of the HCV core protein. The results of this study, along with those of previous reports (1, 3, 6, 8, 20, 23–25), clearly indicate that the sequence at position 37–40 is one of the most immunodominant regions among the N-terminal of the HCV core protein recognized by both cellular and humoral immunities.

There is no apparent HLA-A2 restriction for the existence of anti-HCV core 35–44 peptide-reactive Ab, and the detailed mechanisms involved in this phenomenon are unclear at the present time. We also previously reported the existence of IgG reactive to certain CTL-epitope peptides derived from non-mutated self-anti-

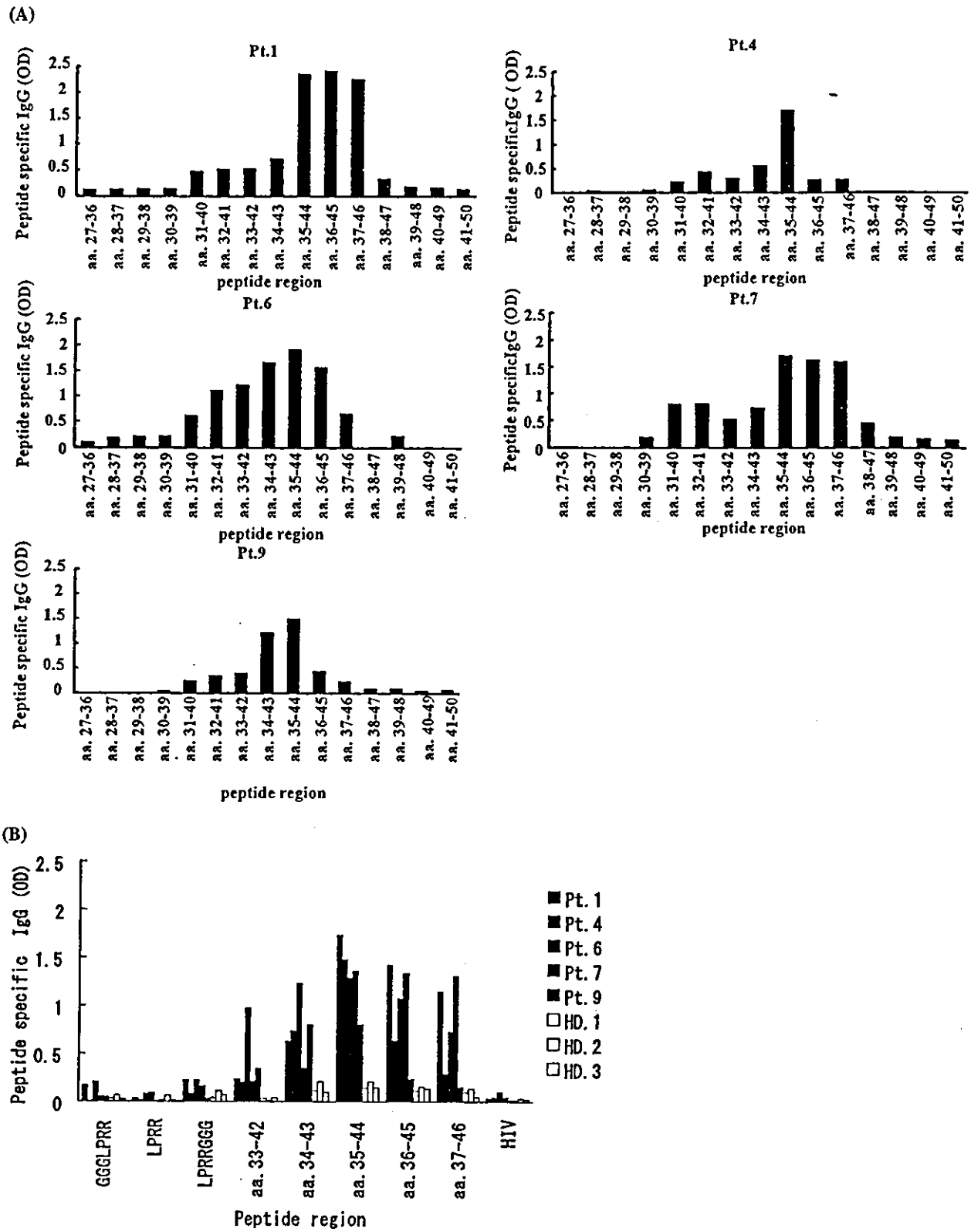

Fig. 3. Fine epitope analysis of HCV core 35–44 peptide recognized by patients' IgG. A: Representative ELISA results of the reactivity of five patients' sera (1:100 diluted) to 15 decamer peptides possessing overlapping sequences spanning HCV core 27–50. The cut-off value in these experiments was 0.15. B: Reactivity of 1:100 diluted sera from the five patients and three healthy donors (HDs) to the sequence LPRR containing peptides.

Table 2. Sequences of the overlapping peptides and a summary of the reactivity

Peptide		Ab reactivity				
Region	Sequence	Pt.1	Pt.4	Pt.6	Pt.7	Pt.9
aa.27–36	GGQIVGGVYL	–	–	–	–	–
aa.28–37	GQIVGGVYLL	–	–	–	–	–
aa.29–38	QIVGGVYLLP	–	–	–	–	–
aa.30–39	IVGGVYLLPR	–	–	–	–	–
aa.31–40	VGGVYLLPRR	+	+	+	+	+
aa.32–41	GGVYLLPRRG	+	+	+	+	+
aa.33–42	GVYLLPRRGP	+	+	+	+	+
aa.34–43	VYLLPRRGPR	+	+	+	+	+
aa.35–44	YLLPRRGPRL	+	+	+	+	+
aa.36–45	LLPRRGPRLG	+	+	+	+	+
aa.37–46	LPRRGPRLGV	+	+	+	+	+
aa.38–47	PRRGPRLGVR	+	–	–	–	–
aa.39–48	RRGPRLGVRA	–	–	–	–	–
aa.40–49	RGPRLGVRAT	–	–	–	–	–
aa.41–50	GPRLGVRATR	–	–	–	–	–

Sequences of the overlapping peptides and a summary of the reactivity of five patients' sera are summarized. Boxed sequence (LPRR) shows the fine epitope of HCV core 35–44 reactive IgG.

gens without apparent HLA-A2 restriction in both HDs and cancer patients (10, 14, 16–18, 21). One of the explanations might be that the HCV core 35–44 peptide is naturally processed by means of proteasomes, and transported into ER with a chaperone in HCV-infected cells. The peptide might be loaded to HLA-A2 or other undefined HLA-class I molecules, if any, followed by presentation on the cell surface as a HLA-peptide complex in certain subjects. In the remaining subjects, the unloaded peptides might be directly secreted from ER to outside the cells. Alternatively, naturally processed HCV core 35–44 might be directly secreted from the cytoplasm to outside the cells via an undefined pathway. Further studies are needed to resolve this issue.

The other important issue to be elucidated is the biological features of Abs to HCV core 35–44 peptide. Increases in the IgG levels of the CTL-epitope peptides of non-mutated self-peptides by peptide vaccination showed a close correlation with long-term survival in patients with advanced stages of lung cancer and gastric cancer (10, 16, 17, 21), whereas the class of IgG to these peptides was either lacking or unbalanced in atopic dermatitis patients (14, 18), suggesting its positive role in host defense. These peptide-specific Abs, however, failed to react to the whole molecules of the mother proteins. Furthermore, they did not react to tumor cells, and thus their biological features are not clarified at the present time. The reactivity of this peptide-specific Ab to HCV core protein as well as its activity to neutralize HCV remains to be answered in the near future.

This peptide-reactive Ab was undetectable in the sera of the following subjects: patients with autoimmune diseases, subjects with HBV but not HCV infection, HDs vaccinated with HBV recombinant protein, and HDs without HBV vaccination, all taken as negative controls (data not shown). Based on these results, the sensitivity and disease specificity of the anti-HCV core 35–44 IgG was calculated as 93.3 and 100%, respectively. This point could be one of the advantages as compared to the reported peptide-based immunoassay using HCV core 21–40 peptide, which has cross-reactivity with HBV infection (8). These results may provide new insight for the development of both therapeutic and diagnostic modalities for HCV infection.

The sequence of the HCV core 35–44 peptide has partial cross-reactivity to that of HIV and HTLV-I. Specifically, the sequence of HCV core 39–43 is identical to that of the HIV envelope protein and to that of HTLV-I rex protein 5–9 by database analysis. The sequence of HCV core 38–42 is also identical to that of HIV at protein 54–58. Therefore, we tested if anti-HCV core 35–44 activity was detectable in the sera of subjects with HIV infection (n=3) and HTLV-I infection (n=10), and it was detected in all three and eight of ten subjects, respectively (Takao et al., unpublished results). This cross-reactivity, however, needs to be carefully studied since the amount of samples available for analysis was too small to both test the cross-infection of HCV in these patients and the peptide specificity by means of absorption and elution tests. This study is now in progress in our laboratory.

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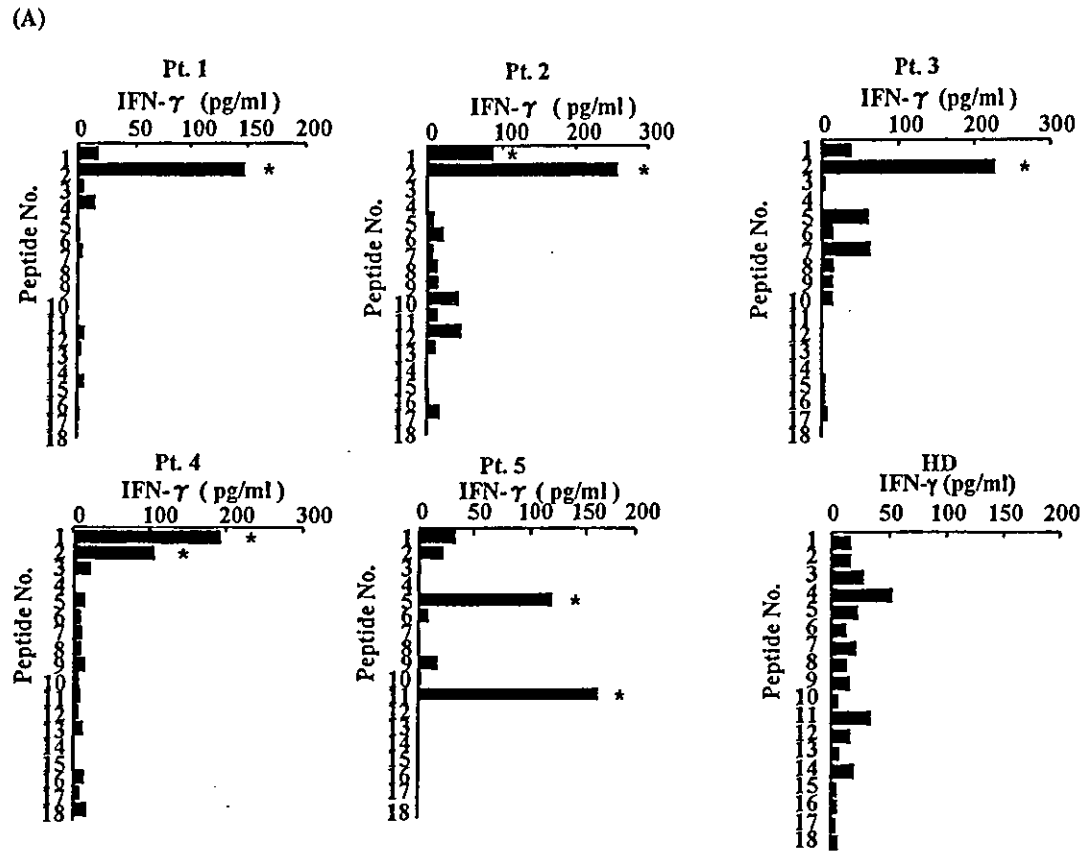

(A)
Pt. 1
IFN-γ (pg/ml)
0 50 100 150 200
Pt. 2
IFN-γ (pg/ml)
0 100 200 300
Pt. 3
IFN-γ (pg/ml)
0 100 200 300
Pt. 4
IFN-γ (pg/ml)
0 100 200 300
Pt. 5
IFN-γ (pg/ml)
0 50 100 150 200
HD
IFN-γ (pg/ml)
0 50 100 150 200
Peptide No.

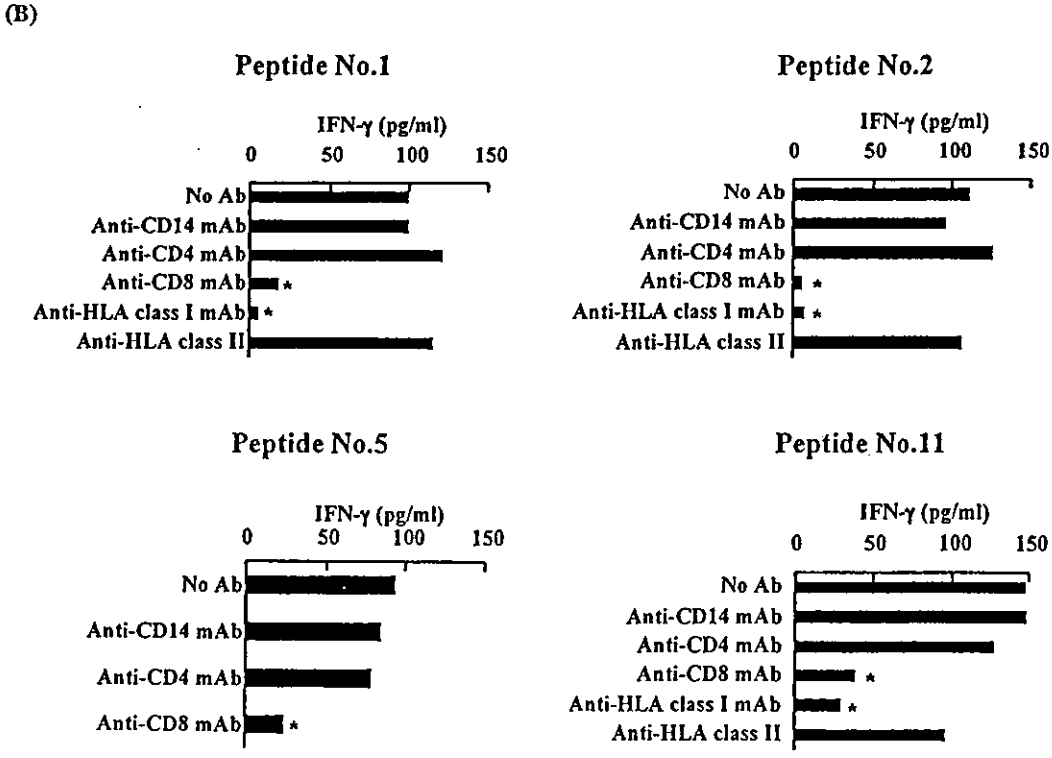
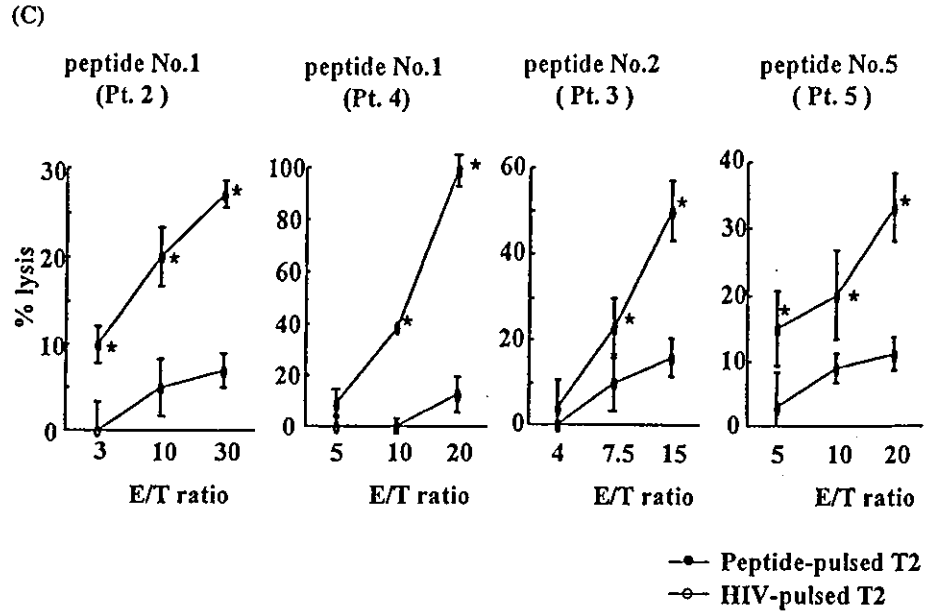

(B)
Peptide No.1
Peptide No.2
Peptide No.5
Peptide No.11
IFN-γ (pg/ml)
0 50 100 150
No Ab
Anti-CD14 mAb
Anti-CD4 mAb
Anti-CD8 mAb
Anti-HLA class I mAb
Anti-HLA class II

Fig. 4.



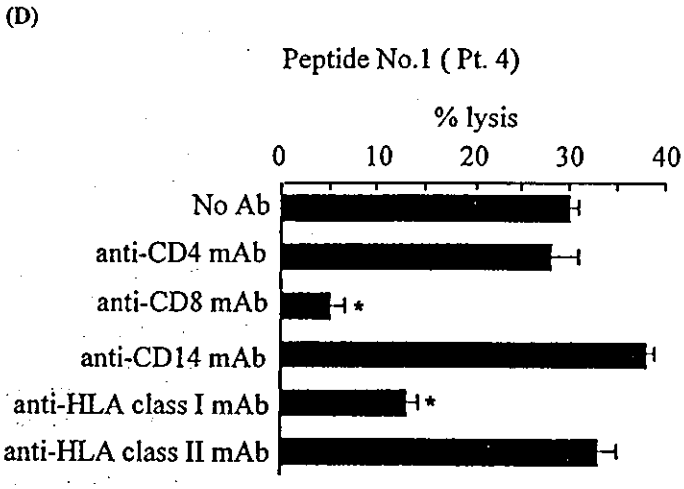


Fig. 4. Cellular responses to peptides. A: IFN-γ production. PBMCs from HLA-A2$^+$ HCV$^+$ patients (n=5) and HDs (n=5) were stimulated with each of the 18 different peptides in four different wells (15×10^4/well). On culture day 14, the peptide-stimulated PBMCs ($80–120\times10^4$/well) from each well were independently collected and divided into four equal portions. Two portions were separately tested for their ability to produce IFN-γ in response to T2 cells pulsed with a corresponding peptide, while the remaining two portions were tested using a negative control peptide (HIV). Background IFN-γ production in response to the HIV peptide (<50 pg/ml) was subtracted. The representative results of all five patients and of one HD whose PBMCs showed significant levels of IFN-γ production in response to at least one HCV-peptide are shown. *P<0.05 by Student's t-test. B: Ab-inhibition of IFN-γ production. IFN-γ production of the peptide-stimulated PBMCs in response to the peptide-pulsed T2 cells in the presence of the indicated mAbs. Anti-CD14 mAb served as negative controls. *P<0.05. C: Cytotoxicity. Peptide-stimulated PBMCs were tested for their cytotoxicity against T2 cells pulsed with each of the three HCV core peptides or an HIV-peptide as the negative control by a standard 6-hr ^{51}Cr-release assay at three different effector-to-target cell (E/T) ratios. The representative results of patiants 2–5 are shown in this figure. *P<0.05 by Student's t-test. D: Ab-inhibition of the cytotoxicity. Peptide-stimulated PBMCs were tested for their cytotoxicity against T2 cells pulsed with HCV core 35–44 peptide in the presence of anti-HLA-class I, anti-HLA-class II, anti-CD8, or anti-CD4 mAb. Anti-CD14 mAb served as the negative control. The 6-hr ^{51}Cr-release assay was performed at an E/T ratio of 10 to 1. *P<0.05 by Student's t-test.

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SOCS1 Is a Suppressor of Liver Fibrosis and Hepatitis-induced Carcinogenesis

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Abstract

Hepatocellular carcinomas (HCCs) mainly develop from liver cirrhosis and severe liver fibrosis that are established with long-lasting inflammation of the liver. Silencing of the suppressor of the cytokine signaling-1 (SOCS1) gene, a negative regulator of cytokine signaling, by DNA methylation has been implicated in development or progress of HCC. However, how SOCS1 contributes to HCC is unknown. We examined SOCS1 gene methylation in >200 patients with chronic liver disease and found that the severity of liver fibrosis is strongly correlated with SOCS1 gene methylation. In murine liver fibrosis models using dimethylnitrosamine, mice with haploinsufficiency of the SOCS1 gene ($SOCS1^{-/+}$ mice) developed more severe liver fibrosis than did wild-type littermates ($SOCS1^{+/+}$ mice). Moreover, carcinogen-induced HCC development was also enhanced by heterozygous deletion of the SOCS1 gene. These findings suggest that SOCS1 contributes to protection against hepatic injury and fibrosis, and may also protect against hepatocarcinogenesis.

Key words: cytokine • STAT • TGF-β • DNA methylation • hepatitis C virus

Introduction

Hepatocellular carcinoma (HCC) is one of the most common cancers with poor prognosis. Chronic infection with hepatitis C virus (HCV) is a major risk factor for development of HCC. In general, HCC develops only after one or more decades of HCV infection, and the increased risk is restricted largely to patients with cirrhosis or advanced fibrosis. Although HCV-infected persons with mild or nonhepatic fibrosis are not likely to get HCC, once cirrhosis is established, HCC develops at an annual rate of 1–4% per year (1), and there is a strong correlation between liver fibrosis and HCC development (2).

Suppressor of cytokine signaling-1 (SOCS1), also known as JAK binding protein, is an intracellular protein that inhibits JAK-analyzed mediated cytokine signaling by binding to JAKs. (3–5). SOCS1-deficient mice ($SOCS1^{-/-}$) die within 3 wk after birth with fulminant hepatitis accompanied with serious fatty degeneration (6, 7). The SOCS1 gene has been implicated as an antioncogene in hepatocarcinoma. Yoshikawa et al. reported aberrant methylation in the CpG island of SOCS1 that correlated with its transcription silencing in HCC cell lines (8). The incidence of aberrant methylation was 65% in the 26 human primary HCC tumor samples analyzed. However, the molecular basis for the development of HCC by SOCS1 gene silencing has not been clarified.

Because HCC development follows severe liver fibrosis, we examined SOCS1 methylation status in chronic liver injury to determine if SOCS1 genetic alteration is involved in liver fibrosis progression as well as in HCC. We found that SOCS1 gene methylation occurred at the hepatitis stage before the onset of HCC and highly correlated with liver fibrosis. Furthermore, using mouse models, we dem-

The online version of this article contains supplemental material.

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Abbreviations used in this paper: ALT, alanine aminotransferase; DEN, diethylnitrosamine; DMN, dimethylnitrosamine; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; SMA, smooth muscle actin; SOCS1, suppressor of the cytokine signaling-1; STAT, signal transducer and activator of transcription.

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onstrated that SOCS1 gene deletion augments fibrosis associated with liver damage, probably due to signal transducer and activator of transcription (STAT1) 1 hyperactivation. We propose that the SOCS1 gene is necessary for preventing hepatocarcinogenesis developed from chronic liver injury and liver cirrhosis.

Materials and Methods

Patients and Histology. A needle biopsy technique was used to obtain liver biopsy specimens from HCC patients in the Department of Internal Medicine of Kurume University Hospital and the Nagata Hospital. Tissue biopsies were stored in −70°C until analysis. Informed consent was obtained from all the patients before biopsies were conducted. Histological evaluations of the liver were based on 5–30-mm-long liver specimens obtained by percutaneous biopsy using 0.8–1.4-gauge needles. The specimens were fixed in a 10% formalin buffer and stained with hematoxylin-eosin. Biopsy specimens with signs of rejection were excluded, and histological results were classified into four categories as follows: normal, lobular hepatitis, chronic hepatitis (defined by piecemeal necrosis or fibrosis), and cirrhosis. The METAVIR score was used to classify biopsy specimens, using simplified scores for fibrosis (from F0, no fibrosis, to F4, cirrhosis; reference 9).

Methylation-specific PCR. Genomic DNA was obtained from liver biopsy samples using a standard method and bisulfite modification of genomic DNA was performed as described previously (10, 11). The bisulfite-treated DNA was amplified with either a methylation-specific or unmethylation-specific primer as described previously (8). To investigate the methylation state of SOCS1 CpG DNA, we also performed methylation assays at CpG-rich regions around the NotI landmark sites after the genomic region in question had been digested with HpaII, which is sensitive to cytosine methylation in the CCGG recognition sequence as described previously (12).

Mice and Other Animal Models. The SOCS1-deficient mouse has been described previously (13). For liver fibrosis model, dimethylnitrosamine (DMN) was administrated into 6–8-wk-old mice. Each mouse was given subsequently an intraperitoneal injection of 10 μg DMN per gram of body weight three times a week for 3 wk. The amount of DMN administered was adjusted for body weight each week. 2 d after the third series of injections,

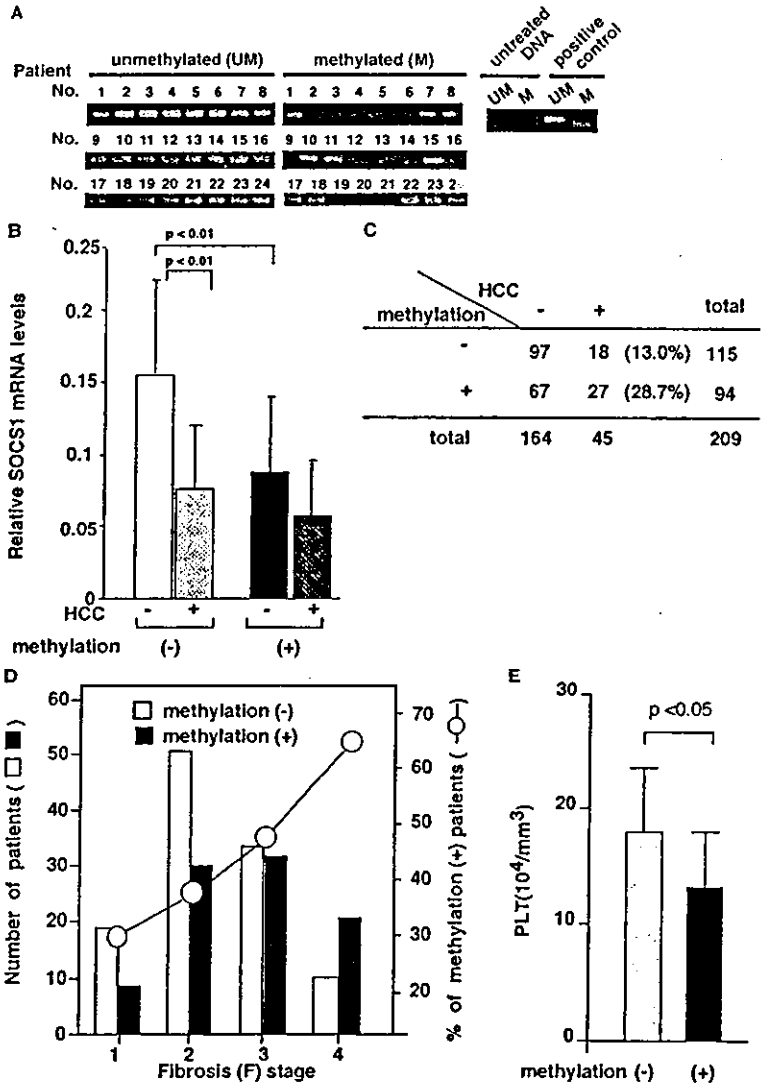

Figure 1. SOCS1 methylation in the liver of HCV positive patients and relationship to liver fibrosis and HCC. (A) Examples of methylation-specific PCR of SOCS1. Primer sets used for amplification are designated as unmethylated (UM) or methylated (M). PCR was performed using bisulfite-treated DNA from the paraffin-embedded liver tissue and PLC/PFR/5 cells (hepatoma cell line) as methylated positive control. (B) Quantification of SOCS1 mRNA levels by real-time RT-PCR in liver samples of non-HCC (−) and HCC (+) regions with SOCS1 gene methylation (n = 15) and unmethylation (n = 17). Methylation status was examined in non-HCC regions. Each group was normalized to the G3PDH values. (C) Summary of methylation status in patients and the HCC development among these patients within 12 yr. (D) The number of patients of each fibrosis score in methylation positive or negative patient groups and the percentage of methylation positive patients at each stage were plotted. (E) Serum platelet counts in patients from methylation positive or negative patient groups were scored. The bar indicates the standard deviation.

venous blood was collected and the mice were killed. Hyaluronic acid and alanine aminotransferase (ALT) were measured using standard methods. The liver was fixed with either 10% formaldehyde for histological examination or frozen immediately in liquid nitrogen for immunoblotting and the extraction of collagen as described previously (14). Liver fibrosis was quantified with Sirius red by digital image analysis as described previously (15). For carcinogenic treatment, 4-wk-old mice were given an intraperitoneal injection of 100 μg diethylnitrosamine (DEN) per gram of body weight once a week for 6 wk (16). 9 mo after the first injection of DEN, these mice were killed, and a 3-mm-thick section from each liver was prepared. An identical tumor from different sections was excluded for counts. The morphology of hepatocellular neoplasms was classified according to cell size, tinctorial properties of the cytoplasm, pattern of growth, and other morphological features as described previously (17). All experiments using these mice were approved by and performed according to the guidelines of the animal ethics committee of Kyushu University. Immunoblot analysis was performed using 20 μg of total liver protein as described previously (18).

Statistical Analysis. For statistical analysis, we used the Student's *t* test, and a 95% confidence limit (defined as $P < 0.05$) was taken to be significant.

Online Supplemental Material. The results of experiments, which complement the data presented in the paper, are shown in Figs. S1 and S2. Fig. S1 shows sequencing of 58 CpG sites in the 467-bp genomic DNA from patients with bisulfite treatment. Fig. S2 shows a gross image of the liver and sections with hematoxylin-eosin staining of hepatocarcinomas in DEN-treated SOCS1$^{+/-}$ mice. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20031675/DC1.

Results and Discussion

SOCS1 Methylation Correlates with Liver Fibrosis. SOCS1 gene deletion in mice results in severe liver inflammation, probably because of hyper IFNγ/STAT1 signaling (7). Severe liver fibrosis due to chronic liver inflammation is shown to be a major cause of HCC development after HCV infection (19). Therefore, we examined the methylation state in the CpG island of the SOCS1 gene in >200 human liver biopsy samples with HCV-associated chronic hepatitis and liver cirrhosis before the onset of HCC. Among these, 209 samples of DNA could be evaluated for methylation status. Representative methylation-specific PCR results are shown in Fig. 1 A. Sequencing analysis of 58 CpG sites in the 467-bp genomic DNA with bisulfite treatment revealed that frequent methylation occurred in the latter half (nucleotides 200–400) of the region, whereas no methylation was detected in the PCR product deter-

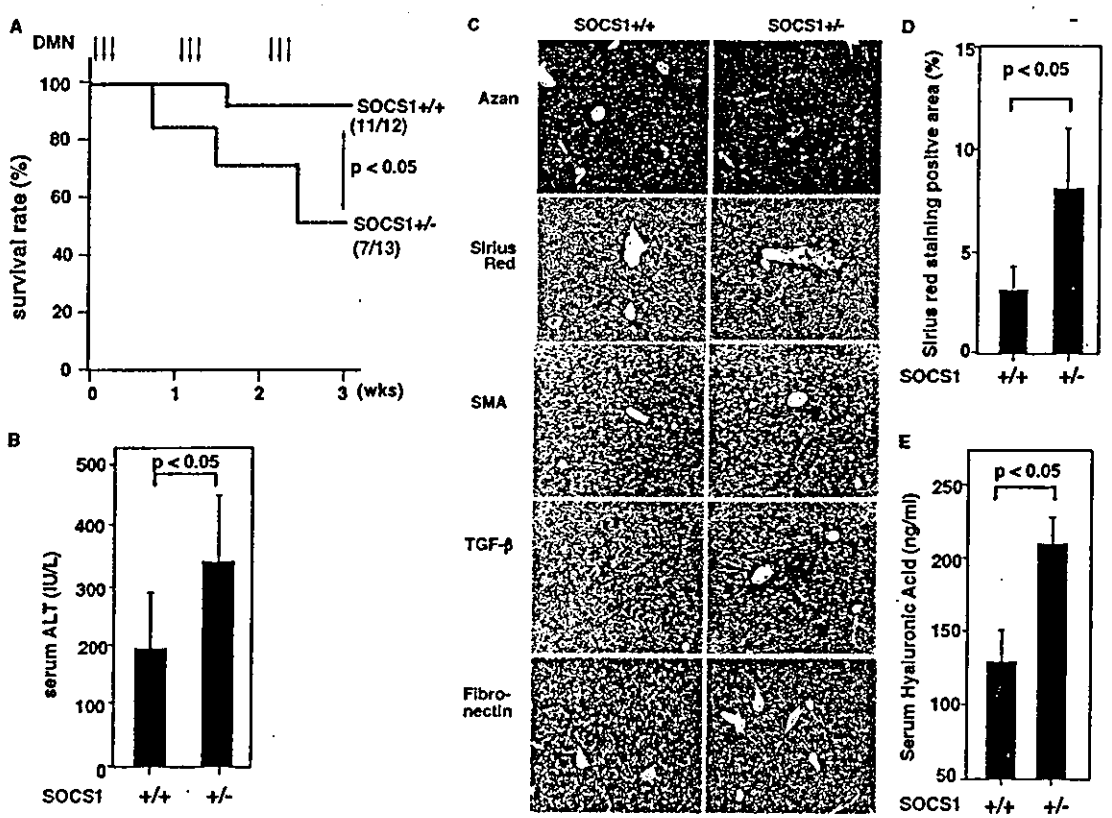

Figure 2. Liver fibrosis is enhanced in SOCS1$^{-/+}$ mice. (A) Survival rate in DMN-treated mice. DMN (10 μg/g body weight) was intraperitoneally injected on the indicated day (arrows), and mortality was examined. The numbers of mice examined and survived on day 21 are shown. (B) Serum ALT values were significantly greater in SOCS1$^{-/+}$ than in SOCS1$^{+/+}$ mice after 3 wk of DMN treatment. (C) Histological and immunohistological analysis of liver from mice treated with DMN. Sections of livers were examined using Azan and Sirius red staining and immunohistostaining with antibodies against α-smooth muscle actin (SMA), TGF-β1, and fibronectin. (D) The surface area stained with Sirius red was quantified using digital image analysis. (E) Serum hyaluronate levels in DMN-treated mice. Blood was collected 3 wk after DMN treatment. (B, D, and E) SOCS1$^{-/+}$ ($n = 6$) and SOCS1$^{+/+}$ ($n = 8$).

mined as methylation negative by methylation-specific PCR (Fig. S1, available at http://www.jem.org/cgi/content/full/jem.20031675/DC1). We confirmed the reduced expression of the SOCS1 gene in methylation positive livers using real-time PCR (Fig. 1 B). We also confirmed that SOCS1 expression in the HCC region was lower than that in non-HCC region (Fig. 1 B).

As shown in Fig. 1 C, aberrant methylation of the SOCS1 gene was present in 45% (94/209) of hepatitis patients detected by methylation-specific PCR (8). These results were confirmed by digesting genome DNA with methylation-sensitive enzymes (unpublished data and reference 12). These data indicate that DNA methylation in the liver occurred before HCC onset in a substantial percentage of hepatitis patients.

Next, we compared the grade of fibrosis and SOCS1 methylation. The progression of liver fibrosis was classified into four stages, from F1 to F4. As shown in Fig. 1 D, the frequency of SOCS1 methylation was 32% in the F1 stage, and it increased to nearly 60% in the F4 stage. The proportion of methylation significantly increased with the advancement of liver fibrosis, suggesting that the frequency of SOCS1 methylation correlated with the severity of liver fibrosis (Fig. 1 D). It has been reported that the platelet count accurately predicts significant HCV-related fibrosis (20). As shown in Fig. 1 E, the decrease in the platelet number was greater in SOCS1 methylation-positive patients ($P < 0.01$). These data reveal that SOCS1 methylation in the liver correlates with the liver fibrosis induced by hepatitis. Thus, we propose that at the earlier stages of chronic liver injury, SOCS1 methylation occurs, and the SOCS1 gene methylation ratio increases as the stage of fibrosis progresses.

SOCS1 Haploinsufficiency Enhanced Liver Fibrosis in Mice. To verify the relationship between reduced expression of SOCS1 and liver fibrosis, we used a DMN-induced liver fibrosis model and mice carrying a heterozygous deletion of the SOCS1 gene ($SOCS1^{-/+}$) as a model of SOCS1 gene silencing. An intraperitoneal injection of DMN has been shown to induce fatty degeneration of hepatocytes, activation and proliferation of hepatic stellate cells (also called Ito cells and lipocytes), infiltration by macrophages (possibly Kupffer cells), and secretion of TGF-β, which promotes fibrosis. First, we examined the mortality rate after injection of DMN (Fig. 2 A). Approximately 50% of mice with haploinsufficiency of the SOCS1 gene ($SOCS1^{-/+}$ mice) died within 3 wk, whereas >90% of $SOCS1^{+/+}$ mice survived. Serum ALT levels in $SOCS1^{-/+}$ mice were higher than those in $SOCS1^{+/+}$ mice, suggesting that more severe liver damage occurred in $SOCS1^{-/+}$ mice. As shown in Fig. 2 C, DMN treatment resulted in tissue remodeling with a matrix deposition mainly in the centrilobular area and portal tract, as assessed histologically (Azan staining and Sirius red staining) or by immunohistostaining using specific antibodies against α-smooth muscle actin (SMA), TGF-β1, and fibronectin (Fig. 2 C). These histological analyses indicate that SOCS1 heterozygous deletion cooperatively enhanced fibrosis. We also estimated fibrosis by quantitative measurement of Sirius red staining (Fig. 2 D) and by measurement of serum hyaluronate levels (Fig. 2 E), a serum marker of the progression of liver fibrosis in humans (21). These data indicate that SOCS1 gene haploinsufficiency augmented DMN-induced liver fibrosis. These findings also support the current notion that hepatic stellate cells are transformed into myofibroblasts (expressing SMA) and secrete TGF-β, thereby stimulating the production of an extracellular matrix and probably the proliferation of these cells.

Next, we used another chronic liver injury model induced by a choline-deficient, L-amino acid–defined diet. As shown in Fig. 3 (A and B), liver fibrosis, as assessed by Azan and Sirius red staining, occurred more markedly in $SOCS1^{-/+}$ mice than in the control mice ($SOCS1^{+/+}$). In addition, the amount of total collagen, a marker of fibrosis, in $SOCS1^{-/+}$ mice was higher than that in the control mice (Fig. 3 C, $SOCS1^{+/+}$). Again, SOCS1 gene haploinsufficiency enhanced diet-induced liver fibrosis. These data

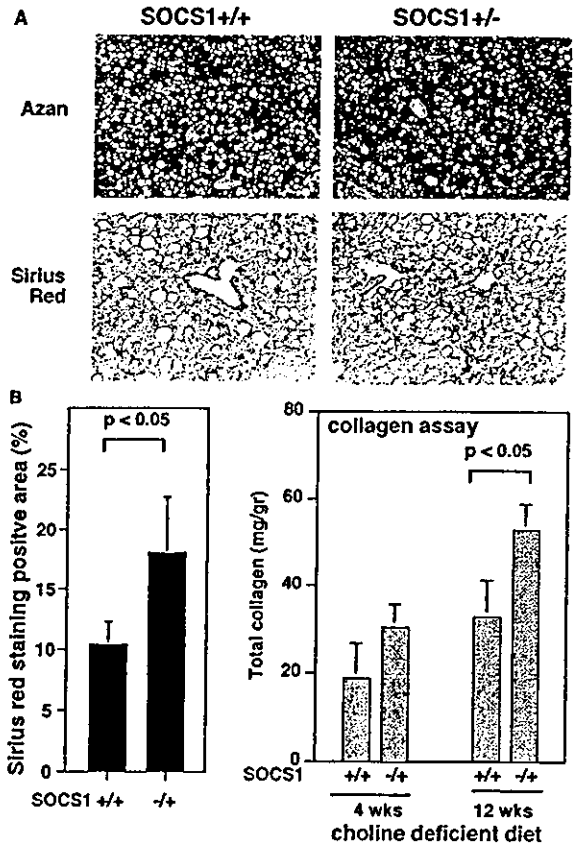

Figure 3. Fibrosis of the liver induced by a choline-deficient, L-amino acid–defined diet. (A) Histology of livers from indicated genotypes of mice fed a choline-deficient, L-amino acid-defined diet for 12 wk. Liver sections were examined histologically using Azan and Sirius red staining. (B) The surface area stained with Sirius red was quantitated using digital image analysis ($n = 4$). (C) Total collagen content of the liver of mice fed a choline-deficient diet for 4 or 12 wk. The total collagen content is shown with SD ($n = 4$).

indicate that loss of the SOCS1 gene in the liver enhances the liver fibrosis induced by liver damages.

Correlation between STAT Activation and DMN-induced Liver Fibrosis. After injection of DMN, plasma levels of a wide variety of cytokines, including IL-6 and IFNγ, were dramatically elevated (22). To elucidate the molecular basis of induction of liver fibrosis by SOCS1 gene silencing, we examined the activation status of intercellular cytokine-signaling molecules using Western blotting. As shown in Fig. 4 (A and B), RT-PCR confirmed the reduction of SOCS1 mRNA levels and elevated TGF-β production in DMN-treated SOCS1$^{-/+}$ mice. Western blotting analysis indicated that DMN injection induced phosphorylation of STAT1, STAT3, STAT5, and ERK (Fig. 4 C). The most striking correlation to fibrosis was elevated STAT1 activation and reduced STAT3 activation in SOCS1$^{-/+}$ mice, whereas STAT5 phosphorylation was almost equal between SOCS1$^{-/+}$ and SOCS1$^{+/+}$ mice. STAT1 may promote liver injury by inducing IRF1, whereas STAT3 protects cells from apoptosis by inducing Bcl-XL. Consistent with strong STAT1 activation and decreased STAT3 activation in SOCS1$^{-/+}$ mice, induction of the Bcl-XL protein was diminished, whereas the induction of the IRF-1 protein was significantly enhanced in these mice. Interestingly, SOCS3 expression was enhanced in SOCS1$^{-/+}$ mice. Because SOCS3 is a negative regulator of gp130-mediated signals (23), elevated expression of SOCS3 may explain the reduced STAT3 activation in these mice.

In the ConA-induced hepatitis model, as in the DMN-induced fibrosis model, both STAT1 and STAT3 are activated (24). Using STAT1-, IFNγ-, and IL-6–deficient mice, Hong et al. proposed that STAT1 promotes liver injury by inducing the expression of proapoptotic IRF-1 protein, whereas STAT3 activation prevents liver damage by inducing antiapoptotic signals, such as Bcl-XL (24). Our data on STAT1 and STAT3 activation induced by DMN treatment are consistent with that proposal. Increased activation of STAT1 induces IRF-1 and reduced activation of STAT3 leads decrease in Bcl-XL expression, and these, at least in part, may account for a severe liver injury in SOCS1$^{-/+}$ mice. Therefore, SOCS1 deletion may cooperatively promote liver damage and fibrosis by activation of STAT1 and repression of STAT3.

Hepatocarcinogenesis in SOCS1$^{-/+}$ Mice. Next, we examined if the SOCS1 loss of heterozygosity promotes hepatocarcinogenesis. We induced liver tumors by a well-established chemical carcinogenesis protocol using DEN. Because nitrate and nitrosamine synthesis are increased in viral hepatitis (25), DEN-induced HCC is thought to be an adequate model of hepatocarcinogenesis due to viral hepatitis. After 9 mo, liver tumors were developed. The number of tumors, including HCC in the SOCS$^{-/+}$ mice, was higher than the number of tumors in the control mice (Fig. 5). As has been found in human HCC, male mice developed more tumors than did female. Large tumor nodules were macroscopically visible in SOCS1$^{-/+}$ mice (Fig. S2, available at http://www.jem.org/cgi/content/full/jem.20031675/DC1). These tumors, whitish in color and discriminated from surrounding tissues, were consisted of hyperplastic nodules with no or mild dysplasia and typical HCCs that had a trabecular structure with or without fatty degeneration (Fig. S2). These data indicate that SOCS1 gene haploinsufficiency correlates with increased cancer risk in the liver.

Our work demonstrated that SOCS1 gene haploinsufficiency in mice and SOCS1 gene silencing by DNA meth-

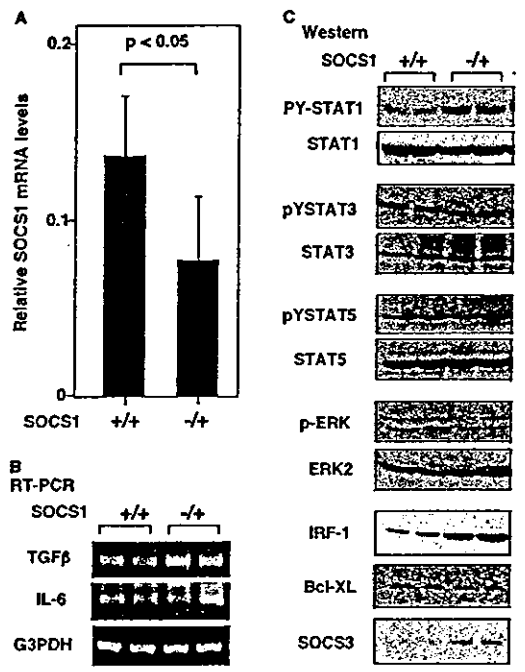

Figure 4. Activation of STATs and proapoptotic and antiapoptotic proteins in DMN-induced liver fibrosis. (A) Expression levels of SOCS1 mRNA were quantitated using real-time RT-PCR. The expression was normalized as a ratio using G3PDH mRNA as a housekeeping gene (n = 6). Each group of mice was sacrificed 3 wk after treatment with DMN. (B) RT-PCR analysis for TGF-β and IL-6 levels. Total RNA were prepared and analyzed using indicated primer sets. (C) Activation of STATs, SOCS1, and proapoptotic or antiapoptotic proteins in the liver. Total liver protein extracts were prepared and analyzed by Western blotting using indicated antibodies. Data are representative of three independent experiments with similar results. p, phosphorylated form.

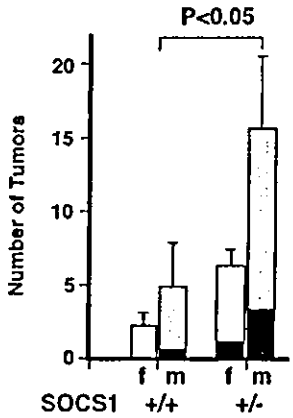

Figure 5. Hepatocarcinogenesis in SOCS1$^{-/+}$ mice. Liver tumors of SOCS1$^{-/+}$ mice 9 mo after DEN-induced tumor initiation. The total number of tumors (unshaded bars) including HCC (shaded bars) were scored from 100 series of 3-mm sections from livers of four female (f) and male (m) mice, with the indicated genotypes.

ylation in humans highly correlate with increased liver damage, liver fibrosis, and hepatocarcinogenesis, although it is unclear how liver injury contributes to fibrosis and hepatocarcinogenesis at present. Although the mechanism of aberrant gene methylation is unknown, it may be caused by increases in de novo methylation activity or by a defect of the protection mechanism against de novo methylation. However, there is no evidence of imprinting of the SOCS1 gene. Therefore, from data of SOCS1 gene methylation status of chronic hepatitis and liver cirrhosis sample, we propose that the SOCS1 gene is likely to be epigenetically methylated as liver fibrosis progresses, just as the methylation of the p16 (INK4A) tumor suppressor gene occurs during liver fibrosis (26).

It has been shown that HCC development of a person with HCV chronic infection is correlated with the severity of liver fibrosis. However, we found that SOCS1 methylation ($P = 0.0015$) is highly correlated with hepatocarcinogenesis, which is comparable to the correlation between fibrosis and HCC ($P = 0.0011$). These observations support our proposal that SOCS1 methylation can be a useful diagnostic indicator for HCC risk. SOCS1 methylation status may also partly explain the difference in individuals in the clinical course after hepatitis virus infection.

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