J Gastroenterol
DOI 10.1007/s00535-009-0155-2

ORIGINAL ARTICLE—LIVER, PANCREAS, AND BILIARY TRACT

Strong CD8$^+$ T-cell responses against tumor-associated antigens prolong the recurrence-free interval after tumor treatment in patients with hepatocellular carcinoma

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Received: 10 August 2009 / Accepted: 19 October 2009

Abstract
Aim We investigated whether tumor-specific CD8$^+$ T-cell responses affect tumor-free survival as well as the relationship between CD8$^+$ T-cell responses against tumor-associated antigens (TAAs) and the clinical course after tumor treatment in patients with hepatocellular carcinoma (HCC).
Methods Twenty patients with HCC that were treated by radiofrequency ablation or trans-catheter chemo-embolization (TACE) and in whom HCC was undetectable by ultrasonography, CT, and/or MRI 1 month after treatment were enrolled in the study. Before and after treatment for HCC, analyses of TAA (glypican-3, NY-ESO-1, and MAGE-1)-specific CD8$^+$ T-cell responses were evaluated with an interferon-γ enzyme-linked immunospot (ELISpot) assay using peripheral CD8$^+$ T-cells, monocytes, and 104 types of 20-mer synthetic peptide overlapping by 10 residues and spanning the entirety of the 3 TAAs.
Results Sixteen out of 20 patients (80%) showed a positive response ($\geq$10 TAA-specific cells/10^5 CD8$^+$ T-cells) before or after treatment. When we performed univariate analysis of prognostic factors for the tumor-free period in the 20 patients, platelet count, prothrombin time, and the number of TAA-specific CD8$^+$ T-cells after treatment were significant factors ($P = 0.027$, 0.030, and 0.004, respectively). In multivariate analysis, the magnitude of the TAA-specific CD8$^+$ T-cell response ($\geq$40 TAA-specific cells/10^5 CD8$^+$ T-cells) was the only significant prognostic factor for a prolonged tumor-free interval (hazard ratio 0.342, $P = 0.022$).
Conclusions Our results suggest that strong TAA-specific CD8$^+$ T-cell responses suppress the recurrence of HCC. Immunotherapy to induce TAA-specific cytotoxic T lymphocytes by means such as the use of peptide vaccines should be considered for clinical application in patients with HCC after local therapy.

Keywords Hepatocellular carcinoma · CD8$^+$ T-cell response · Cytotoxic T lymphocyte · ELISpot assay · Immunotherapy

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Introduction

There are about 500,000 new patients with hepatocellular carcinoma (HCC) per year worldwide. Although vaccination against hepatitis B virus (HBV) and interferon (IFN)-based therapy against hepatitis C virus (HCV) will presumably reduce the number of HCC patients in the future, the incidence of HCC is still increasing in Asia and Africa because of the previous prevalence of infection with the virus. Progress in treatments for HCC has improved the prognosis of patients with HCC. However, HCC is usually associated with cirrhosis and often recurs even after complete treatment of the tumors in the remaining part of the cirrhotic liver. Thus, there is a strong need for the development of a new intervention therapy that suppresses the occurrence or recurrence of HCC effectively and that has fewer side effects. Immunotherapy may be such a treatment and may be applicable to the clinical treatment of HCC. In fact, some clinical trials have been performed [1–3].

Cytotoxic T lymphocytes (CTLs) are thought to be potent effector cells against cancers. CTLs recognize specific antigens, and the induction of CTLs specific for tumor-associated antigen (TAA) is an attractive procedure

Published online: 20 November 2009

for tumor therapy. The MAGE-1 gene was first identified as encoding a tumor-specific antigen on MZ-2-MEL cells, a melanoma cell line, in 1991 [4]. MAGE-1 gene and protein can be detected in many cancer tissues, and three articles reported the expression of MAGE-1 in HCC as 30, 68, or 78%, respectively, in a Japanese population [5–7]. In gastrointestinal tumors, immunotherapy using both dendritic cells and MAGE peptides has been performed for patients with primary malignant melanoma of the esophagus, and this therapy was able to induce peptide-specific immune responses [8].

NY-ESO-1 antigen, a member of the cancer-testis antigen family, was initially identified by a serological analysis of recombinant cDNA expression cloning in an esophageal cancer patient [9]. NY-ESO-1 mRNA was detected in 24–37% of HCCs by reverse transcription-polymerase chain reaction [10, 11].

Glypican-3 (GPC3) consists of 580 amino acids and is a heparan sulfate proteoglycan with a potential role in the control of cell division. GPC3 mRNA was detected in 74.8% of HCC tissues, but only in 3.2% of normal liver tissues [12], and GPC3 protein was detected in 72% of HCCs, but not in normal tissue using GPC-specific antibody [13]. The GPC3 protein can also be detected in sera of 40–53% of patients with HCC [14, 15].

These three antigens are thought to be attractive targets for cancer immunotherapy because they are expressed only in tumor tissues and testis, but not in normal tissues other than testis. On the basis of previous reports, it is assumed that most HCCs would express at least one of the three TAAs. Therefore, monitoring immune responses against these TAAs might help in the development of HCC immunotherapy, such as TAA-based vaccination. In this study, we investigated how the magnitude of $CD8^+$ T-cell responses against these TAAs determined by an IFN-γ enzyme-linked immunospot (ELISpot) assay is related to other clinical data and the tumor-free interval in patients with HCC, in order to explore the clinical application of such a TAA-based immunotherapy.

Methods

Patients

Twenty patients who were diagnosed with HCC at Showa University Hospital between 2006 and 2008 were enrolled in the study. They met the following study criteria: (1) pathologically confirmed as having HCC or a lesion with characteristic imaging features of HCC based on ultrasonography, CT, and/or MRI; (2) liver function classed as Child-Pugh A or B; (3) no extrahepatic metastasis or vascular invasion; (4) no previous or simultaneous cancers other than HCC; and (5) an indication for treatment such as radiofrequency ablation (RFA) or trans-catheter chemo-embolization (TACE). RFA was performed by well-trained hepatologists using usual methods according to previous reports [16]. A 16-gauge cooled-tip ablation electrode (Covidien, Boulder, CO) was used in the procedure. TACE was performed by well-experienced hepatologists and radiologists. A microcatheter was inserted from the femoral artery to the artery feeding the HCC superselectively after conventional hepatic angiography, and then a segmental or subsegmental TACE procedure was performed using gelatin, lipiodol, and either epirubicin hydrochloride or cisplatin. All patients were followed every 1–3 months by ultrasonography, CT, and/or MRI to examine the appearance of new lesions in the liver or other organs. The recurrence-free interval was defined as the period from the month of HCC treatment to the month when a recurrent and/or metastatic HCC was first detected after treatment. Clinical data (platelet count, prothrombin time, serum AST, ALT, albumin, total bilirubin level, and AFP level) were collected 1–7 days before HCC treatment. Chronic hepatitis C was diagnosed on the basis of detectable HCV RNA in serum using the Amplicor assay (Roche Diagnostics, Tokyo, Japan). Informed consent was obtained from each patient included in this study. The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki as reflected in a priori approval by the Ethical Committee of Showa University.

Synthetic peptides of TAA

Twenty-mer peptides overlapping by 10 residues and spanning the entire MAGE-1, NY-ESO-1, and GPC3 proteins were synthesized based on the amino acid sequences reported previously as PepSets™ and purchased from Mimotopes (Clayton South, Victoria, Australia). These peptides were >80% pure. A total of 30 MAGE-1, 17 NY-ESO-1, and 57 GPC3 peptides were synthesized, as shown in Table 1. A total of 10–11 TTA peptides were pooled in a mixture (total 10 mixtures) at a concentration of 10 μg/ml each.

Preparation of $CD8^+$ T cells and monocytes from patients with HCC

PBMCs were isolated from heparinized peripheral blood by gradient centrifugation using Ficoll-Paque (Pharmacia-LKB Biotechnology, Uppsala, Sweden). As reported previously, peripheral $CD8^+$ T-cells and monocytes were separated from PBMCs using CD8 microbeads (MACS system; Miltenyi Biotec, Bergisch Gladbach, Germany) and a Monocyte Isolation Kit II (Miltenyi Biotec), respectively [17]. These cells were isolated using an autoMACS™ Pro Separator (Miltenyi Biotec). The purity of the cells was >95% on flow cytometry (data not shown).

Table 1 Synthetic peptides and peptide mixtures used in this study

Tumor-associated antigen	Peptide	Amino acid sequence
Glypican-3	GL1	1–20
	GL2	11–30
	GL3	21–40
	⋮	⋮
	GL57	561–580
MAGE-1	MG-1	1–20
	⋮	⋮
	MG-30	290–309
NY-ESO-1	NY-1	1–20
	⋮	⋮
	NY-17	161–180

Mix 1	Mix 2	Mix 3	Mix 4	Mix 5	Mix 6	Mix 7	Mix 8	Mix 9	Mix 10
GL1	GL2	GL3	GL4	GL5	GL6	GL7	GL8	GL9	GL10
GL11	GL12	GL13	GL14	GL15	GL16	GL17	GL18	GL19	GL20
GL21	GL22	GL23	GL24	GL25	GL26	GL27	GL28	GL29	GL30
GL31	GL32	GL33	GL34	GL35	GL36	GL37	GL38	GL39	GL40
GL41	GL42	GL43	GL44	GL45	GL46	GL47	GL48	GL49	GL50
GL51	GL52	GL53	GL54	GL55	GL56	GL57	MG-1	MG-2	MG-3
MG-4	MG-5	MG-6	MG-7	MG-8	MG-9	MG-10	MG-11	MG-12	MG-13
MG-14	MG-15	MG-16	MG-17	MG-18	MG-19	MG-20	MG-21	MG-22	MG-23
MG-24	MG-25	MG-26	MG-27	MG-28	MG-29	MG-30	NY-1	NY-2	NY-3
NY-4	NY-5	NY-6	NY-7	NY-8	NY-9	NY-10	NY-11	NY-12	NY-13
NY-14	NY-15	NY-16	NY-17	–	–	–	–	–	–

IFN-γ ELISpot assay

The ELISpot assay was performed using an IFN-γ ELISpot assay kit (Mabtech AB, Stockholm, Sweden) as previously described [17]. Briefly, a 96-well microtiter plate with a nitrocellulose membrane bottom (Millititer; Millipore, Bedford, MA) was coated with 100 μl anti-IFN-γ monoclonal antibody at a concentration of 15 μg/ml in phosphate-buffered saline (PBS) overnight at 4°C. Unbound antibody was removed by washing 6 times in Hanks' balanced saline solution. After blocking with AIM-V medium (Invitrogen Japan, Tokyo, Japan) containing 10% fetal bovine serum, 1×10^5 $CD8^+$ T-cells, 1×10^4 autologous monocytes, and a TAA peptide mixture at 10 μg/ml of each peptide were placed and incubated in duplicate in 100 μl AIM-V medium at 37°C in a humid atmosphere with 5% CO_2. After incubation for 18 h, the cells were removed by washing the plate 8 times with PBS. Next, 100 μl of biotin-conjugated monoclonal antibody was added to each well, and the plates were incubated further for 2 h at room temperature. Wells were washed 5 times with PBS and incubated with 100 μl streptavidin–alkaline phosphatase for 2 h. Unbound antibodies were removed by washing 6 times with PBS. Then, 100 μl of alkaline phosphatase substrate (Bio-Rad Laboratories, Richmond, CA) was added to each well and incubated until dark spots emerged. Color development was stopped by washing 3 times with water, and the plates were allowed to dry. Using an ELISpot reader (KS ELISPOT compact; Carl Zeiss, Oberkochen, Germany), the number of spot-forming cells (SFCs) per well was counted. Numbers of TAA-specific SFCs for each peptide mixture were calculated by subtracting the mean number of SFCs of 2 control wells (without stimulus) from the mean number of SFCs of 2 wells stimulated by TAA antigens. An SFC number was calculated for each patient as the sum of SFCs in each peptide mixture. ELISpot assays were performed before and 3–7 days after treatment. When TAA-specific $CD8^+$ T-cell responses were analyzed in 10 normal subjects, we were unable to detect any responses against TAA peptides in the ELISpot assay (data not shown).

Statistical analyses

The relationship between the number of TAA-specific $CD8^+$ T-cells and the recurrence-free period was analyzed

using a parametric survival model. The log-rank test was used to compare recurrence-free data for 2 groups. The effects of multiple explanatory variables on recurrence-free interval were analyzed using a Cox proportional hazards model. Statistical analyses were performed using the statistical software JMP version 5 (SAS Institute Inc., Cary, NC). Differences were considered as significant when the P value was less than 0.05.

Results

TAA-specific $CD8^+$ T-cells were detected by ELISpot assay before and after HCC treatment in most HCC patients

The characteristics of the 20 patients enrolled in this study are shown in Table 2. The 20 patients had no HCC detected by ultrasonography, enhanced CT, and/or MRI 1 month after treatment for HCC. In those patients with HCCs who had up to 3 HCCs and in whom the diameter of each lesion was 3 cm or less, the treatment was usually RFA; the remaining patients were treated by TACE. However, in a few patients (patients 2 and 5) in whom the diameter of each lesion was less than 3 cm, the physician in charge of the patient selected TACE because they could not deny the existence of more lesions that were undetectable by conventional enhanced CT. The clinical courses of the patients were followed for 3–29 months after therapy for HCC. The ELISpot assay was performed to detect $CD8^+$ T-cell responses to TAAs before and 3–7 days after treatment. The data are shown in Table 3 as SFCs (total count of TAA-specific $CD8^+$ T-cells/1 $\times$ 10^5 $CD8^+$ T-cells). Sixteen out of 20 patients (80%) showed a positive response (10 or more SFCs) for TAA peptides either before and/or after treatment. The numbers of SFCs (mean $\pm$ SD) before and after therapy were 33.8 $\pm$ 51.4 (0–161, median 16.5) and 32.9 $\pm$ 34.7 (0–130, median 23.0), respectively. Of the 20 patients, 5 (25%) and 7 (35%) showed a high TAA-specific immune response (40 or more SFCs) before and after treatment, respectively.

When we analyzed the TAA peptides recognized by $CD8^+$ T-cells, we occasionally observed that different peptide mixtures were identified as positive before and after HCC treatment (data not shown).

Change in TAA-specific $CD8^+$ T-cell response induced by HCC treatment does not correlate with recurrence-free period

The number of SFCs increased in 11 of 20 (55%) patients after treatment. In these patients, TAA-specific CTLs might have been induced by the treatment. There were no significant differences between the increase in TAA-specific $CD8^+$ T-cell response induced by the treatment and either therapeutic procedure, laboratory data, or background of the patients (data not shown). The increase in TAA-specific CTLs after treatment did not predict a better prognosis of HCC.

Table 2 Characteristics of HCC patients before HCC treatment

	$n = 20$	Median
Age (years)[a]	68.8 ± 9.4	73.0
Gender		
M	11	
F	9	
AST (IU/l)[a]	70 ± 49	52
ALT (IU/l)[a]	63 ± 43	54
PLT ($\times 10^4/\mu l$)[a]	9.8 ± 5.3	8.5
PT (%)[a]	81 ± 11	78
Alb (g/dl)[a]	3.5 ± 0.4	3.4
T-Bil (mg/dl)[a]	0.9 ± 0.4	0.9
AFP (ng/ml)[a]	86 ± 157	16
Virus		
HCV	17	
NBNC	3	
Child-Pugh class		
A	12	
B	8	
HCC size (mm)[a]	23 ± 8	23
No. HCCs		
1	9	
2	4	
3	7	
>3	0	
Treatment		
RFA	13	
TACE	5	
RFA + TACE	2	

NBNC Negative for neither HBV nor HCV infection, *RFA* radiofrequency ablation, *TACE* trans-catheter chemo-embolization

[a] Results are shown as mean ± SD

Platelet count, prothrombin time, and the magnitude of TAA-specific immune response after treatment correlate with the recurrence-free period by univariate analysis

When we analyzed the relationship between TAA-specific SFCs detected by the ELISpot assay or other clinical variates and the HCC-free interval using a parametric survival model, we found that platelet count, prothrombin time, and the TAA-specific $CD8^+$ T-cell response after treatment significantly correlated with the HCC-free interval

Table 3 Results of IFN-γ ELISpot assay in patients in whom HCCs were not detected after therapy

Patient no.	SFC before treatment (/10^5 $CD8^+$ T-cells)	SFC after treatment (/10^5 $CD8^+$ T-cells)	Recurrence-free interval (month)
1	0	0	5
2	15	31	10
3	12	15	5
4	159	130	26
5	58	4	12
6	5	99	29[a]
7	15	17	7
8	20	41	7
9	135	9	12
10	1	6	3
11	8	9	6
12	10	57	15
13	34	42	13[a]
14	6	4	12[a]
15	23	8	9
16	59	37	12
17	12	29	23
18	161	72	24
19	18	4	15
20	25	44	23[a]

SFC Spot-forming cells

[a] These patients had no recurrence detected by ultrasonography, enhanced CT, and/or MRI after treatment

($P = 0.005$, 0.007, and 0.001, respectively). When univariate analysis of prognostic factors for the HCC-free interval was performed, only platelet count ($P = 0.027$; Fig. 1a), prothrombin time ($P = 0.030$; Fig. 1b), and the number of SFCs after treatment ($P = 0.004$; Fig. 1c) were found to be significant. Child-Pugh class A tended to prolong the HCC-free interval, although this was not significant ($P = 0.066$). The other factors, including the number of SFCs before treatment ($P = 0.407$), ALT level ($P = 0.644$), albumin level ($P = 0.488$), total bilirubin level ($P = 0.340$), HCC size ($P = 0.756$), HCC number ($P = 0.486$), and the procedure used for HCC treatment (RFA or TACE, $P = 0.481$), did not affect HCC-free survival, as confirmed by the log-rank test.

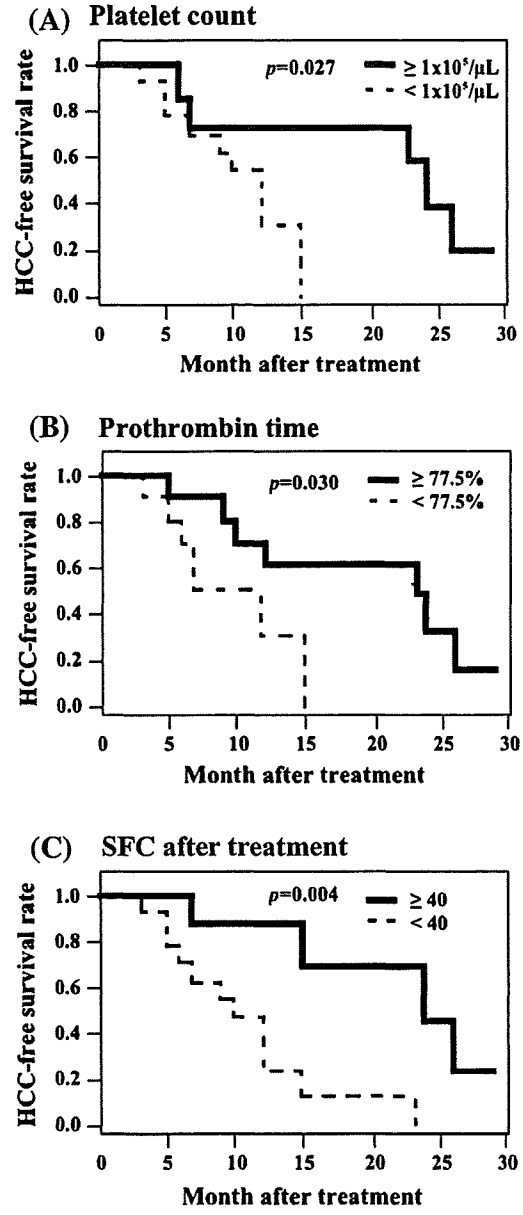

Fig. 1 Kaplan–Meier curves of HCC-free survival rate. In univariate analysis, platelet count, prothrombin time, and the tumor-associated antigen-specific $CD8^+$ T-cell response were found to be prognostic factors for the HCC-free period after treatment. Kaplan–Meier curves representing the relationship between month after treatment (HCC-free interval) and HCC-free survival rate were grouped by **a** platelet count, **b** prothrombin time, and **c** spot-forming cells (*SFCs*) specific for tumor-associated antigens after treatment

Multivariate analysis shows that the magnitude of TAA-specific $CD8^+$ T-cell responses correlates with the HCC-free interval after treatment in patients who have no detectable HCC after therapy

In a further analysis of the 20 patients with HCC who were treated by RFA or TACE and in whom no HCC was detectable 1 month after treatment, we performed multivariate analysis using a Cox proportional hazards model. On multivariate analysis, only the magnitude of TAA-specific $CD8^+$ T-cell responses (≥40 TAA-specific cells/10^5 $CD8^+$ T-cells) was the only significant prognostic factor for a prolonged tumor-free period after treatment

Table 4 Multivariate analyses of prognostic factors for tumor-free interval

Variable	Hazard ratio	95% Confidence limit	*P* value
Platelet count			
$\geq 1 \times 10^5/\mu L$	0.916	0.326–2.020	0.843
$<1 \times 10^5/\mu L$	1.000		
Prothrombin time			
≥77.5%	0.455	0.094–1.390	0.177
<77.5%	1.000		
Child-Pugh class			
A	1.464	0.539–6.813	0.493
B	1.000		
Spot-forming cells after treatment			
≥40	0.342	0.079–0.866	0.022
<40	1.000		

(hazard ratio 0.342, $P = 0.022$), as shown in Table 4. Therefore, the results suggest that TAA-specific CTLs detected after treatment are able to suppress the occurrence or recurrence of HCC in patients with no detectable HCCs after treatment.

Discussion

To determine whether TAA-specific CTLs suppress the occurrence or recurrence of HCC, we investigated the relationship between the magnitude of TAA-specific $CD8^+$ T-cell responses and the HCC-free interval in patients who had no detectable viable HCC 1 month after treatment for HCC. We found that potent TAA-specific $CD8^+$ T-cell responses, as observed 1 month after treatment for HCC, led to a prolonged HCC-free interval.

An HLA-A24-restricted MAGE-1 peptide-specific CTL line was established in a patient with metastatic melanoma [18], and an NY-ESO-1 DNA vaccine induced both antigen-specific effector $CD4^+$ and/or $CD8^+$ T-cell responses in most patients who did not show detectable pre-vaccination immune responses [19]. In addition, HLA-A2- and HLA-A24-restricted GPC3-derived peptide vaccine induced specific CTLs in mice [20]. In this study, we selected GPC3, MAGE-1, and NY-ESO-1 to monitor antigen-specific $CD8^+$ T-cell responses against HCC because they had been reported to be expressed commonly and frequently in HCC tissues [7, 11–13], and thus the combination of these TAAs would cover most HCCs. Among the 20 patients enrolled in the present study, 16 (80%) showed positive $CD8^+$ T-cell responses (10 or more SFCs) against the TAAs before and/or after the treatment. Although we did not examine the expression of TAAs in the HCC tissues, it would be expected that at least one of these three TAAs will be expressed in HCCs in patients who have a positive $CD8^+$ T-cell response against TAAs.

In patient 10, HCC recurrence was detected 3 months after treatment. Insufficient treatment or the pre-existence of intrahepatic metastases might be considered in a patient in whom HCCs are undetectable 1 month after treatment, but are detected within a few months after treatment. We expected that TAA-specific CTLs induced by treatment would suppress the development of a small HCC, which is not easily detected by conventional methods of examination. Thus, we enrolled and analyzed all patients in whom no HCC was detectable by ultrasonography, CT, and/or MRI 1 month after treatment, even if a recurrent or metastatic HCC was detected within a few months after treatment.

It is of interest whether tumor destruction by local HCC treatment would induce immune responses against HCCs. Apoptotic tumor cells are capable of inducing tumor-specific immune responses [21]. Dendritic cells, representing antigen-presenting cells, around damaged tumor cells take up tumor antigen released from the tumor cells and then migrate into draining lymph nodes [22]. There, they mature and stimulate tumor-specific helper T-cells and CTLs. In turn, the effector cells migrate into the tumor tissue and attack the tumor cells [23]. Tumor-specific immune responses were induced by a combination of direct dendritic cell injections into the HCC and radiation therapy that might induce tumor destruction [3]. When we compared TAA-specific $CD8^+$ T-cell responses before HCC treatment and those after treatment, about half of the patients (55%) showed an increased frequency of TAA-specific $CD8^+$ T-cells, which might have been induced by the treatment. However, the increase in TAA-specific CTLs did not affect the recurrence-free interval. Rather, it was the magnitude of TAA-specific $CD8^+$ T-cell responses after the treatment itself that affected the recurrence-free interval. Even if the frequency of these CTLs seemed to be decreased after treatment, they might infiltrate the liver. Furthermore, new CTLs other than pre-existing CTLs might be induced by the treatment because many TAA peptides recognized by CTLs were different between before and after the treatment. Although some patients showed a potent TAA-specific $CD8^+$ T-cell response before treatment, SFC before treatment did not correlate with the recurrence-free interval. We believe that TAA-specific CTLs are not able to control a large tumor burden by itself. As HCCs enlarge, they may secret immune suppressive factors such as TGF-β [24] and/or IL-10 [25] and modify gene expression of TAAs [26]. We speculate that TAA-specific CTLs detected after the treatment, but not detected before the treatment may be able to control HCCs. Otherwise, TAA-specific CTLs detected before the

treatment may be able to destroy a small HCC that was not detected by conventional examinations.

The ELISpot assay is a convenient means of detecting antigen-specific $CD8^+$ T-cells in a variety of diseases. We have detected HCV-specific $CD8^+$ T-cell responses in patients with acute HCV infection using this method and identified 6 new epitopes within the HCV protein [17]. In fact, we identified a novel GPC3-specific CTL epitope using this method (unpublished observation). At present, we are trying to identify more CTL epitopes among these TAAs that will be used as cancer vaccines.

In this study, we used peptide mixtures to stimulate $CD8^+$ T-cells. This procedure may mask responses to individual peptides because a peptide that interacts only weakly with HLA molecules is unable to attach to the molecule if the mixture contains 1 peptide with a high affinity for the same molecule. However, such a weak peptide would not contribute to tumor immune responses because of its weak interaction with the HLA molecules. Thus, we ignored this issue in this study.

Recurrence and multicentric carcinogenesis are major factors in determining the prognosis of HCC, and several treatments have been tried for the prevention of recurrence. IFN therapy [27, 28], treatment with acyclic retinoid therapy [29, 30], and adoptive immunotherapy [31] have been reported as effective in suppressing HCC recurrence. Preoperative hepatic function influenced early HCC recurrence in patients in whom small HCCs were resected [32]. This is consistent with our result that prothrombin time, reflecting hepatic function, affected the recurrence-free interval in the univariate analysis. In our study, higher platelet counts also contributed to a longer recurrence-free interval in the univariate analysis. In the multivariate analysis, however, only the magnitude of TAA-specific $CD8^+$ T-cell responses remained as an independent factor contributing to a longer recurrence-free interval.

Although the size and number of HCCs were reported to affect the period of HCC-free survival (recurrence) in patients with HCC treated by hepatic resection [33], they are not significant factors affecting the recurrence-free interval. Further investigation, such as the accumulation of analyses of HCC patients, is needed to clarify this issue. Sixteen out of 20 patients without detectable HCC 1 month after treatment had recurrent or metastatic HCCs during the observation period in this study. Our results suggest that the maintenance of strong TAA-specific $CD8^+$ T-cell responses for a long period may lead to a longer recurrence-free state. A long-term observation of TAA-specific immune responses should also be performed in any future study.

The results of our study suggest that strong TAA-specific $CD8^+$ T-cell responses would suppress HCC recurrence in patients with HCC who are treated by RFA or TACE and in whom any HCC is undetectable by ultrasonography, CT, and/or MRI 1 month after treatment. Since recurrence and intrahepatic metastasis are major risk factors influencing the prognosis of patients with HCC, immunotherapy to induce TAA-specific $CD8^+$ T-cells, such as a peptide vaccine, should be considered for clinical application in patients with HCC after local therapy.

Acknowledgments This study was supported in part by a grant from the Ministry of Health, Labor and Welfare of Japan (Kazumasa Hiroishi, Michio Imawari); a Grant-in-Aid for Scientific Research (C) from the Ministry of Education, Culture, Sports, Science and Technology of Japan (Kazumasa Hiroishi); and a grant for the High-Technology Research Center Project from the Ministry of Education, Culture, Sports, Science and Technology of Japan (Michio Imawari).

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Hepatitis C Virus Nonstructural Protein 5A Modulates TLR-MyD88-Dependent Signaling Pathway in the Macrophage Cell Lines

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Running title: HCV nonstructural proteins inhibit TLR-signaling pathway

Key words : HCV/TLR/MyD88/Innate immunity/Immune cells

ABSTRACT

Hepatitis C virus (HCV) infection induces a wide range of chronic liver injuries, however, the mechanism through which HCV evades the immune surveillance system remains obscure. Blood dendritic cells (DCs) play a pivotal role in the recognition of viral infection and the induction of innate and adaptive immune responses. Several reports suggest that HCV infection induces dysfunction of DCs in chronic hepatitis C patients. Toll-like receptor (TLR) has been shown to play various roles in many viral infections, however the involvement of HCV proteins in the TLR signaling pathway has not yet been precisely elucidated. In this study, we established mouse macrophage cell lines stably expressing HCV proteins and determined the effect of HCV proteins on the TLR signaling pathways. Immune cells expressing NS3, NS3/4A, NS4B or NS5A were found to inhibit the activation of the TLR2, TLR4, TLR7 and TLR9 signaling pathways. Various genotypes of NS5A bound to MyD88, a major adaptor molecule in TLR, and inhibited the recruitment of interleukin-1 receptor-associated kinase-1 to MyD88, and impaired cytokine production in response to TLR ligands. Amino acid residues from 240 to 280, previously identified as the interferon sensitivity-determining region (ISDR) in NS5A, interacted with the death domain of MyD88, and the expression of a mutant NS5A lacking the ISDR partially restored cytokine production. These results suggest that the expression of HCV proteins modulates the TLR signaling pathway in immune cells.

INTRODUCTION

Hepatitis C virus (HCV) belongs to the *Flaviviridae* family and possesses a positive, single-strand

RNA genome that encodes a single polyprotein composed of approximately 3000 amino acids. HCV polyprotein is processed by host and viral proteases, resulting in 10 viral proteins. Viral structural proteins, including the capsid protein and two envelope proteins, are located in the N-terminal third of the polyprotein, followed by nonstructural proteins. HCV infects 170 million people worldwide and frequently leads to cirrhosis and hepatocellular carcinoma (36). In over one half of patients, the acute infection evolves into a persistent carrier state, presumably due to the ability of HCV to incapacitate the activation of the host immune mechanisms. Dendritic cells (DCs) are one type of potent antigen-presenting cells *in vivo* and play a crucial role in the enhancement and regulation of cell-mediated immune reactions. Since DCs express various costimulatory and/or adhesion molecules, they can activate even naïve T cells in a primary response. The role of the response of HCV antigen-specific T cells in viral clearance or persistence has been investigated extensively in both humans and chimpanzees (6, 27, 48, 51). These studies suggest that acute HCV infections followed by viral clearance are associated with a high frequency of HCV-specific $CD4^+$ and $CD8^+$ T-cell responses that can persist (27, 51), while chronic HCV infections are characterized by weak and restricted $CD4^+$ and $CD8^+$ T-cell responses that are not sustained (51).

Toll-like receptors (TLRs) are membrane-bound receptors that can be activated by the binding of molecular structures conserved among families of microbes. More than 10 different TLRs have been identified to date (2). They are highly conserved among mammals and are expressed in a variety of cell types. TLR binding and stimulation by pathogen-associated molecules is followed by a cascade of intracellular events that culminate in the expression of multiple genes (2). TLR signaling is mediated primarily by the adaptor protein myeloid differentiation factor 88 (MyD88), which triggers the activation of transcription factors such as NF-κB essential for the expression of proinflammatory cytokine genes (2). This pathway also leads to potent production of type I interferon (IFN) through the activation of IFN regulatory factor (IRF) 7 upon stimulation of TLR7 or TLR9 (22). In contrast, toll/interleukin-1 receptor homology domain-containing adaptor inducing IFN-β (TRIF/TICAM-1) mediates the production of type I IFNs primarily through the activation of IRF3 in response to TLR3 or TLR4 stimulation (2). Type I IFN induces the maturation of DCs by increasing both the expression of costimulatory molecules such as CD80, CD86 and CD40 and antigen presentation via major histocompatibility complex class I in addition to classical endogenous antigen presentation; it also facilitates the cross-presentation of viral antigens. A cumulative report has shown that DC activation via TLR signaling is a prerequisite for the subsequent induction of vigorous T-cell responses (42). Some viral proteins have been shown to inhibit the TLR-dependent signaling pathway through interaction with the downstream adaptor molecules, suggesting that the alteration of TLR-mediated signals is one of the mechanisms of virus-induced immune modulation (49). Dysfunction of DCs has been reported in chronic hepatitis C patients due to immaturation caused by direct infection of HCV to DCs or by interaction with HCV proteins (4, 21). On the other hand, there have also been contrasting reports suggesting a lack of impairment of DC function in both chimpanzees and humans chronically infected with HCV (26, 32). Thus, at present, alterations in the TLR signaling pathway in the immune cells of chronic hepatitis C patients are not well understood.

In the present study, we examined the effect of HCV proteins on TLR function in murine macrophage cell lines stably expressing HCV proteins. The expression of NS3, NS3/4A, NS4B or NS5A was found to impair the activation of the TLR signaling pathways, and NS5A interacted with MyD88 through the interferon sensitivity-determining region (ISDR) and impaired cytokine production. To the best of our knowledge, this is the first demonstration of NS5A as an immunomodulator of TLR signaling pathways through direct interaction with an adaptor molecule in immune cells.

MATERIALS AND METHODS

Cell culture. Human embryonic kidney 293T cells, and mouse macrophage RAW264.7 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (Sigma, St. Louis, MO) containing 10% fetal calf serum (FCS). All cells were cultured at 37 °C in a humidified atmosphere with 5% CO_2.

Plasmids and viruses. DNA fragments encoding each of HCV structural and nonstructural proteins were generated from a full-length cDNA clone of genotype 1b strain J1 (1) by PCR using *Pfu* turbo DNA polymerase (Stratagene, La Jolla, CA). The fragments were cloned into pCAGGs-puro/N-Flag, in which the sequence encoding a Flag tag is inserted at the 5-terminus of the cloning site of pCAGGs-puro (37). A protease deficient NS3/4A mutant substituted Ser^{139} to Ala (S139A) was generated by the method of splicing by overlap extension and cloned into pCAGGs-puro. NS5A genes were amplified by PCR from HCV clones of strains of J1 (genotype 1b), H77c (genotype 1a, kindly provided by Dr. J. Bukh), and JFH1 (genotype 2a, kindly provided by Dr. T. Wakita), and cloned into pcDNA3.1Flag/HA (38). The NS5A deletion mutants were prepared as described previously (16). DNA fragments encoding a human MyD88, human Toll-IL-1R domain-containing adapter protein (TIRAP), and human TRIF-related adapter molecule (TRAM) were amplified by RT-PCR from total RNA of THP-1 cells and cloned into pcDNA3.1-C-myc-His (Invitrogen, Carlsbad, CA) and pcDNA3.1Flag/HA. A murine IPS-1 (mIPS-1) was amplified by RT-PCR from total RNA of RAW264.7 cells and cloned into pcDNA3.1Flag/HA. Human MyD88 deletion mutants and a mIPS-1 mutant substituted Cys^{508} to Ala (C508A) were generated by the method of splicing by overlap extension and cloned into pcDNA3.1Flag/HA. pCMVIRAK1-myc and pCMVIRAK4-myc, encoding IL-1 receptor-associated kinase (IRAK)-1 and -4, respectively, were prepared as described previously (53). pEFBossTICAM-I-HA was kindly provided by Dr. T. Seya (44). All PCR products were confirmed by sequencing by an ABI PRSM 310 genetic analyzer (Applied Biosystems, Tokyo, Japan). Vesicular stomatitis virus (VSV) (Indiana strain, NCP12.1) (19) was kindly provided by Dr. M. A. Whitt.

Establishment of stable cell lines expressing HCV proteins. pCAGGs-puro/N-Flag plasmids encoding HCV proteins were transfected into RAW264.7 cells by liposome-mediated transfection using Lipofectamine 2000 (Invitrogen) and selected with 10 μg/ml of puromycin (InvivoGen, San Diego, CA). After 2~3 weeks of selection, several clones were isolated and cell lysates of each clone were immunoblotted with each of specific mouse anti-HCV antibody (1) or anti-Flag M2 mouse monoclonal antibody (Sigma). The macrophage cell lines stably expressing HCV proteins and a control cell line obtained by transfection with an empty pCAGGs-puro vector were maintained in the presence of puromycin (10 μg/ml) throughout the experiments.

Immunoprecipitation and immunoblotting. Cells were seeded onto a 6-well tissue culture plate 24 h before transfection. The plasmids were transfected by the lipofection method, and the cells were harvested at 48 h post-transfection, washed three times with 1 ml of ice-cold phosphate-buffered saline (PBS), and suspended in 0.4 ml lysis buffer containing 20mM Tris-HCl (pH 7.4), 135 mM NaCl, and 1% Triton X-100, 10% glycerol and protease inhibitor cocktail tablets (Roche Molecular Biochemicals, Mannheim, Germany). Cell lysates were incubated for 30 min at 4°C, and centrifuged at 14,000 x g for 15 min at 4°C. The supernatant was immunoprecipitated with 1 μg of mouse monoclonal anti-Flag M2, anti-HA 16B12 (HA.11; BabCO, Richmond, CA), or anti-hexahistidine antibody (Santa Cruz Biotechnology, Santa Cruz, CA) and 10 μl of Protein G-Sepharose 4B Fast Flow beads (Amersham Pharmacia Biotech, Franklin Lakes, NJ) at 4°C for 90 min. The immunocomplex was precipitated with the beads by centrifugation at 5,000 x g for 1 min and then washed five times with 0.4 ml of 20mM Tris-HCl (pH 7.4) containing 135 mM NaCl and 0.05 % Tween20 (TBST buffer) by centrifugation. The proteins binding to the beads were boiled in 20 μl of sample buffer and then subjected to sodium dodecyl sulfate-12.5% polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to

polyvinylidene difluoride membranes (Millipore, Tokyo, Japan). The membranes were blocked with TBST containing 5% skim milk at room temperature for 1 h and incubated with mouse monoclonal anti-Flag M2, anti-HA 16B12 or anti-hexahistidine monoclonal antibody at room temperature for 1 h and then with horseradish peroxidase-conjugated anti-mouse IgG antibody at room temperature for 1 h. The cell lines (2×10^6 cells/well) were stimulated with various doses of LPS derived from *Salmonella Minnesota* (Re-595) (Sigma), PGN derived from *Staphylococcus aureus* (Sigma), R-837 (InvivoGen) or phosphorothioate-stabilized mouse CpG (mCpG) oligodeoxynucleotides (ODN1668) (TCC-ATG-ACG-TTC-CTG-ATG-CT) (InvitroGen) for the times indicated and the phosphorylation status of extracellular signal-regulated kinase (ERK) was determined by immunoblotting using antibodies specific to ERK1/2 or phosphorylated ERK1/2 (T202/Y204) (Cell Signaling Technology, Inc., Beverly, MA). Cells (1×10^6 cells/well) were treated with various doses of mouse IFN-α (PBL Biomedical Laboratories, New Brunswick, NJ) or VSV for 24 h and the phosphorylation status of double-stranded RNA-dependent protein kinase (PKR) and signal transducer and activator of transcription-1 (STAT1) was determined by immunoblotting using antibodies specific to STAT1 (Cell Signaling), phosphorylated STAT1 (Cell Signaling) or phosphorylated PKR (BioSource International, Inc., Camarillo, CA). The immune complexes were visualized with Super Signal West Femto substrate (Pierce, Rockford, IL) and detected by an LAS-3000 image analyzer system (Fujifilm, Tokyo, Japan).

Cytokine poduction and ELISA. To evaluate cytokine production in the macrophage cell lines expressing HCV proteins, cells were seeded into 96-well plates at a concentration of 1×10^5 cells/well and stimulated with various doses of LPS, PGN, R-837 or mCpG. After 24 h incubation, culture supernatants were collected and IL-6 production was determined using an OptEIA™ mouse IL-6 Set purchased from BD PharMingen (San Diego, CA).

Real-time PCR. The cell lines (3×10^6 cells/well) were stimulated with R-837, LPS, PGN, mCpG, VSV, and polyinosine-polycytidylic acid (poly I:C) (Invivogen) for the times indicated and the expression of mRNA of cytokines, chemokines, and TLR genes was determined by real-time-polymerase chain reaction (PCR). Total RNA was prepared from the macrophage cell lines using an RNeasy mini kit (Qiagen). First-strand cDNA was synthesized using a ReverTra Ace (TOYOBO, Japan) and oligo$(dT)_{20}$ primer. Each cDNA was estimated by Platinum SYBR Green qPCR SuperMix UDG (Invitrogen) following the manufacturer's protocol. Fluorescent signals were analyzed by an ABI PRISM 7000 (Applied Biosystems). The mouse Ccl2, IFN-β, IFN-α1, IFN-α4, and IL1-α genes were amplified using the primer pairs of 5'-GCATCCACGTGTTGGCTCA-3' and 5'-CTCCAGCCTACTCATTGGGATCA-3', 5'-ACACCAGCCTGGCTTCCATC-3' and 5'-TTGGAGCTGGAGCTGCTTATAGTTG-3', 5'-AGCCTTGACACTCCTGGTACAAATG-3' and 5'-TGGGTCAGCTCACTCAGGACA-3', 5'-GCTCAAGCCATCCTTGTGCTAA-3' and 5'-CATTGAGCTGATGGAGGTC-3', 5'-TTGGTTAAATGACCTGCAACAGGA-3' and 5'-AGGTCGGTCTCACTACCTGTGATG-3', respectively. The mouse TLR2, TLR3, TLR4, TLR7, TLR9 and GAPDH genes were amplified using the primer pairs of 5'-AGCTCTTTGGCTCTTCTG-3' and 5'-AGAACTGGGGGATATGC-3', 5'-AAATCCTTGCGTTGCGAAGTG-3' and 5'-TCAGTTGGGCGTTGTTCAAGAG-3', 5'-GCCTCGAATCCTGAGCAAACA-3' and 5'-CTTCTGCCCGGTAAGGTCCA-3', 5'-TCTGCAGGAGCTCTGTCCTTGA-3' and 5'-CAAGGCATGTCCTAGGTGGTGA-3', 5'-ACCAATGGCACCCTGCCTAA-3' and 5'-CGTCTTGAGAATGTTGTGGCTGA-3', 5'-ACCACAGTCCATGCCATCAC-3' and 5'-TCCACCACCCTGTTGCTGTA-3', respectively. The expression of mRNAs of each of chemokines, cytokines, and TLR were normalized with that of GAPDH mRNA.

Immunofluorescence microscopy and subcellular localization of HCV proteins in the stable macrophage cell lines. Cells were seeded on an 8-well chamber slide at 1.5×10^4 cells per well, washed twice with PBS, fixed with PBS containing 4% paraformaldehyde at 18 h of cultivation,

and permeabilized with PBS containing 0.5% Triton X-100 at 15 min. They were then incubated at 4°C for 1 h with 1 μg of mouse anti-NS5A antibody (Austral Biologicals, San Ramon, CA) or the rabbit polyclonal antibody against calregulin (Santa Cruz Biotechnology) in PBS containing 10% FCS (PBSF), and then incubated at room temperature for 1 h with 0.5 μg of Alexa Fluor 488-conjugated anti-mouse IgG (Molecular Probes) or Alexa Fluor 594-conjugated anti-rabbit IgG (Molecular Probes) after three washes with PBSF. After extensive washing with PBSF, the samples were examined with a FluoView FV1000 laser scanning confocal microscope (Olympus, Japan). To confirm the subcellular localization of the HCV proteins in the macrophage cell lines, each stable cell line was fractionated with a Subcellular Proteome Extraction Kit (Calbiochem, Darmstadt, Germany). Stepwise extraction resulted in four distinct fractions, which contained primarily cytosolic, membrane-organelle, nuclear and cytoskelton proteins, respectively. Each fraction was concentrated by Microcon (Millipore) and subjected to immunoblotting. PA28α (Biomol International, Plymouth Meeting, PA), calregulin and histone H1 (Santa Cruz Biotechnology) were used as cytoplasmic, membrane and nuclear markers, respectively.

RESULTS

Establishment of macrophage cell lines stably expressing HCV proteins. To examine the effect of HCV proteins on the TLR function of immune cells, we established murine macrophage cell lines stably expressing HCV structural or nonstructural proteins. We selected mouse macrophage RAW264.7 cells due to their high level of expression of various TLRs (3) and their high sensitivity to stimulation with TLR ligands. Processed HCV structural and nonstructural proteins were detected in each of the cell lines by immunoblot analyses using specific monoclonal antibodies (Fig. 1A). To examine the effect of HCV proteins on TLR expression in the macrophage cell lines, the mRNA of TLRs in cells expressing NS5A was determined by real-time PCR (Fig. 1B). Although slight reductions in TLR2, -3 and -4, or enhancement of TLR7 and -9 were observed, a substantial amount of mRNA of the examined TLRs was detected in the cell lines expressing NS5A and other HCV proteins (data not shown). To determine the subcellular localization of HCV proteins in the macrophage cell lines, the expression of HCV proteins was examined by confocal microscopy and cell fractionation (Fig. 1C). HCV NS5A was colocalized with the endoplasmic reticulum (ER) marker calregulin in the macrophage cell line as reported in human hepatoma cell lines (47). Other HCV proteins exhibited similar localization with NS5A (data not shown). To further confirm the subcellular localization of NS5A proteins, cytoplasmic, membrane-organelle and nuclear fractions of the cell line expressing NS5A were analyzed by Western blotting. NS5A was detected mainly in the membrane-organelle fraction.

Expression of HCV NS3, NS3/4A, NS4B or NS5A modulates TLR-dependent signaling pathway in macrophage cell lines. In order to determine the effect of the expression of HCV proteins on the TLR signaling pathway in the macrophage cell lines, we examined the ability of HCV proteins to inhibit NF-κB activation via stimulation with various TLR ligands. The macrophage cell lines were stimulated with the TLR ligands and the production of the proinflammatory cytokine IL-6 in the culture supernatants was determined by enzyme-linked immunosorbent assay (ELISA) (Fig. 2A). The expression of HCV structural proteins or NS5B had no effect on IL-6 production after stimulation with mCpG, R-837, LPS or PGN, which are ligands for TLR9, TLR7, TLR4 and TLR2, respectively. On the other hand, the expression of NS3, NS3/4A, NS4B or NS5A inhibited the production of IL-6 induced by the treatment with the ligands. These results indicate that expression of NS3, NS3/4A, NS4B and NS5A inhibits production of IL-6 through TLR dependent signaling pathway in macrophage cell lines.

In addition to proinflammatory cytokine production via NF-κB activation, stimulation of TLR also activates mitogen-activated protein kinases (MAPKs). We then examined the activation of ERK, a MAPK signaling pathway, in response to the TLR ligands in the macrophage cells expressing HCV proteins (Fig. 2B). Although the expression of the HCV structural proteins, NS3,

NS4B and NS5B did not alter the phosphorylation status of ERK1/2 in response to stimulation with the TLR7 ligand R-837, the expression of NS5A exhibited a clear inhibition of the phosphorylation of ERK1/2. To further examine the effect of NS5A expression on the MAPK cascade in response to the TLR ligands, the cells were treated with mCpG, LPS and PGN. NS5A expression was found to inhibit the phosphorylation of ERK1/2 in response to stimulation with the ligands for TLR9, TLR4 and TLR2 (Fig. 2C). In contrast, phosphorylation of c-Jun NH_2-terminal kinase (JNK) in response to stimulation with R-837 was less impaired in the macrophage cell line expressing NS5A (data not shown). These results indicate that the expression of NS3, NS3/4A, NS4B or NS5A inhibits the production of proinflammatory cytokine, and that the expression of NS5A alone induces inhibition of the MAPK cascade in response to stimulation by various TLR ligands in macrophage cells.

To further examine the effect of NS5A expression on the production of the other proinflammatory cytokine and chemokines in response to the TLR ligands, expression of mRNA of IL-1α and Ccl2 in cells expressing NS5A after stimulation with TLR ligands was determined by real-time PCR (Fig. 3A). Expression of IL-1α and Ccl2 was reduced in cells expressing NS5A by the stimulation with mCpG, R-837, LPS or PGN except for the IL-1α expression by the treatment with LPS, probably due to the TRIF-dependent activation of NF-kB. To further confirm the specific inhibition of MyD88-dependent signaling pathway by NS5A, we examined the effects of NS5A expression in the macrophage cells on the MyD88-independent/TRIF-dependent production of IFN-β (Fig. 3B). Although expression of IL-6 mRNA in cells expressing NS5A was impaired after stimulation with mCpG or LPS, expression of IFN-β was enhanced. These results suggest that expression of NS5A specifically inhibit MyD88-dependent signaling pathway.

TLR-dependent and -independent immune activation of the macrophage cells expressing NS3/4A or NS5A protein by RNA virus and dsRNA. TLR3 has been shown to sensitize cells in response to dsRNA generated by viral infection and a synthetic dsRNA analog, poly I:C, through an adaptor molecule, TRIF/TICAM-1 but not MyD88. Furthermore, RIG-I and MDA5 have been identified as cytoplasmic dsRNA detectors responding to poly I:C and viral RNAs (57, 58) sensitizing cells through an adaptor molecule, IPS-1/MAVS/VISA/CARDIF in TLR-independent manner (24, 35, 46, 55). Recently, HCV NS3/4A protease was shown to cleave not only HCV nonstructural proteins but also to inhibit viral RNAs- and dsRNA-induced IFN production through the cleavage of the adaptor molecules TRIF (28) and IPS-1 (29, 30, 33, 35). Moreover, it has been shown that NS3/4A protease inhibits dsRNA-induced immune activation in a protease-dependent manner in the human hepatoma cell lines (11).

To determine whether murine TRIF (mTRIF) is cleaved by HCV NS3/4A protease, C-terminally His-tagged mTRIF was co-expressed with N-terminally Flag-tagged NS3, NS3/4A or NS3/4A (S139A) in 293T cells. Immunoblot analyses revealed that mTRIF was not processed by HCV NS3/4A protease, probably due to differences in the amino acid sequences at the cleavage site in mTRIF (Fig. 4A). Amino acid sequences at the cleavage site of human TRIF (hTRIF) are Cys^{372} and Ser^{373}, and Pro^{372} and Ala^{373} in mTRIF (Fig. 4B). These results suggest that HCV NS3/4A protease could not inhibit the immune activation through TLR3-mTRIF-dependent signaling pathway in murine cells. We next determined the processing of IPS-1 by HCV NS3/4A protease. N-terminally Flag-tagged mouse IPS-1 (mIPS-1) or its mutant C508A replaced Cys^{508} to Ala to prevent cleavage by HCV NS3/4A protease was co-expressed with N-terminally Flag-tagged NS3, NS3/4A or NS3/4A (S139A) in 293T cells. Immunoblot analyses revealed that wild-type mIPS-1 was cleaved in cells co-expressing with the active NS3/4A protease but not in those with NS3 (Fig. 4C). mIPS-1 processing was reduced in cells co-expressing NS3/4A (S139A) as well as in those co-expressing mIPS-1 (C508A) and NS3/4A (Fig. 4C). Furthermore, we were able to detect cleavage of the endogenous mIPS-1 in the macrophage cell lines expressing NS3/4A, but not in those expressing NS3 or NS3/4A (S139A) (Fig. 4D), indicating that mIPS-1 in the murine macrophage cell lines is cleaved by HCV NS3/4A

protease, as reported in a human hepatoma cell line.

We then examined the effect of expression of NS3/4A and NS5A on the TLR-dependent and -independent immune activation induced by dsRNA. VSV and poly I:C were inoculated into the macrophage cell lines and the expression of mRNA of IFN-β and IL-1α was determined by real-time PCR (Fig. 4E). The macrophage cell lines expressing NS3/4A exhibited inhibition of IL-1α and IFN-β expression upon infection with VSV but not in response to poly I:C, whereas no inhibition was observed in those expressing NS5A. These results suggest that invasion of VSV and poly I:C is preferentially recognized in the RAW cell lines by RIG-1-IPS-1- and TLR3-TRIF-dependent signaling pathway, respectively. Inhibition of IL-1α and IFN-β expression upon infection with VSV but not in response to poly I:C, is probably due to the selective cleavage of IPS-1 but not TRIF by NS3/4A protease in the macrophage cell lines. In contrast, expression of NS5A has no effect on both TLR3-TRIF and RIG-I-IPS-1-dependent signaling pathways in the macropahge cells.

Although MyD88/IRF7-dependent production of IFN-α upon activation was reported in plasmacytoid DCs (pDCs) (17, 23), it is unclear whether the murine macrophage cells are capable of producing IFN-α in TLR/MyD88/IRF7-dependent manner. To examine the effect of NS5A expression on the IFN-α production, expression of IFN-α1 and IFN-α4 in the macrophage cell line upon infection with VSV was determined (Fig. 4E, lower). In contrast to the effect on the IFN-β production, expression of NS5A in the macrophage cells reduced the production of IFN-α1 and IFN-α4 upon infection with VSV, although inhibitory effect was weak than that of NS3/4A. These results suggest that RAW264.7 cells are capable of producing IFN-α in TLR/MyD88/IRF7-dependent manner upon infection with VSV as reported in pDCs and expression of NS5A partially counteracts this signaling pathway. However, production of type-I IFNs by the treatment with ligands for TLR7 (R-837) and TLR9 (mouse CpG) was weaker than that induced by infection with VSV in the macrophage cells (data not shown). Further study is needed to clarify the precise mechanisms of the inhibition of the TLR/MyD88/IRF7-dependent IFN-α production by the expression of HCV NS5A in human immune cells.

NS5A interacts with MyD88 in mammalian cells. The inhibition of the production of proinflammatory cytokines and chemokines, and the MAPK cascade by NS5A expression in response to stimulation by various TLR ligands without participation of TRIF- and IPS-1-dependent signaling pathways suggests that NS5A specifically inhibits the TLR-MyD88-dependent signaling pathway in macrophage cell lines. MyD88 is a critical component in the signaling pathway, and leads to the production of proinflammatory cytokines, chemokines and MAPKs. To determine the effect of the expression of HCV proteins on the TLR signaling pathway in macrophage cell lines, the interaction of the HCV proteins with the adaptor molecules in the signaling pathway of the TLR family was examined by immunoprecipitation analysis. His-tagged MyD88 was co-expressed with Flag-tagged HCV proteins in 293T cells and immunoprecipitated with the indicated antibodies. As shown in Figs. 5A and 5B, the MyD88 was coimmunoprecipitated with NS5A but not with structural and other nonstructural proteins in 293T cells.

To further confirm the specificity of the interaction of NS5A with MyD88, NS5A was co-expressed with other adaptor molecules in the TLR signaling pathway, TRAM, TIRAP or TRIF in 293T cells (Fig. 5C). NS5A interacted with MyD88 but not other adaptor molecules, suggesting that NS5A may inhibit the production of proinflammatory cytokines, chemokines and the phosphorylation of MAPKs through the counteraction of the MyD88-dependent TLR signaling pathway.

NS5A interacts with the death-domain of MyD88 through the ISDR and inhibits recruitment of IRAK to MyD88. To determine the region of NS5A responsible for interaction with MyD88, a series of deletion mutants of N-terminal Flag-tagged NS5A was constructed and

its interaction with His-tagged MyD88 was examined (Fig. 6A). The NS5A mutant covering from amino acids 1 to 280 but not that covering amino acids 1 to 200 exhibited binding to MyD88, suggesting that amino acid residues 200 to 280 of NS5A are required for interaction with MyD88. Further mutational analyses of NS5A revealed that amino acid residues 240 to 280, which overlaps the ISDR (amino acid residues 237 to 276) suggested to be involved in IFN resistance (10, 41), are required for the interaction with MyD88 (Fig. 6A). To determine the region of MyD88 responsible for interaction with NS5A, His-tagged MyD88 mutants were co-expressed with Flag-tagged NS5A in 293T cells and immunoprecipitated with anti-His antibody. A MyD88 deletion mutant lacking amino acids 1 to 50, but not one lacking amino acids 1 to 80, and a mutant possessing amino acids 1 to 70 exhibited binding to NS5A, suggesting that the amino acid residues from 50 to 70 in the death domain of MyD88 are required for interaction with NS5A (Fig. 6B).

MyD88 associates with TLRs and acts as an adapter that recruits IL-1 receptor-associated kinase, IRAK which is known as a key regulator for TLR7- and TLR9-mediated IFN-α production in pDCs (53). To determine the role of NS5A binding to MyD88 in the TLR-MyD88-dependent signaling pathway, we examined the association of IRAK with MyD88 in the presence of NS5A. Flag-tagged MyD88 was co-expressed with Myc-tagged IRAK-1 or IRAK-4 and immunoprecipitated with anti-myc antibody (Fig. 6C, left). IRAK-1, but not IRAK-4, was co-immunoprecipitated with MyD88. Although NS5A did not bind to IRAK-1, it was not possible to asses the interaction of NS5A with IRAK-4 due to degradation of NS5A in cells co-expressing IRAK-4 for unknown reasons (Fig. 6C, middle). To examine the interplay between IRAK-1 and MyD88 in the presence of NS5A, Flag-tagged MyD88 and Myc-tagged IRAK-1 were co-expressed with Flag-tagged NS5A in 293T cells. The interaction of IRAK-1 and MyD88 decreased in accord with increasing NS5A expression complex (Fig. 6C, right), suggesting that expression of NS5A may interfere with the TLR-MyD88-dependent signaling pathway through the inhibition of the recruitment of IRAK-1 to MyD88.

NS5A of other genotypes also interacts with MyD88 and inhibits the TLR signaling pathway. To determine the interaction of MyD88 with NS5A of other genotypes, Flag-tagged NS5A of genotype 1a (H77c) or 2a (JFH1) was co-expressed with His-tagged MyD88 in 293T cells. MyD88 was co-precipitated with the NS5As of genotypes 1a and 2a, although it should be noted that the interaction between the MyD88 and NS5A of genotype 2a was weaker than that of the other genotypes (Fig. 7A). To determine the region of the NS5As of genotype 1a or 2a responsible for interaction with MyD88, N-terminal Flag-tagged NS5As of 1a or 2a deletion mutants lacking amino acids 240 to 280 were constructed and their interaction with MyD88 was examined. Mutational analyses revealed that amino acid residues 240 to 280 of the NS5As of 1a and 2a were also required for the interaction with MyD88 (Fig. 7B). Amino acid alignment of the ISDR of the 1a, 1b and 2a genotypes revealed that the region of genotype 2a was less conserved than those of the other genotypes (Fig. 7C).

To determine the effect of the NS5A expression of other genotypes on the TLR signaling pathway, we established macrophage cell lines expressing NS5A of each genotype. The NS5A expression of all genotypes was found to inhibit IL-6 production after stimulation with mCpG, R-837, LPS or PGN (Fig. 7D). Although association of NS5A of the genotype 2a to MyD88 was weaker than that of other genotypes, expression of genotype 2a NS5A in macrophage cells exhibited comparable inhibition of IL-6 production in response to the stimulation by various TLR ligands with those of genotypes 1b and 1a. These results suggest that NS5A of genotypes 1a, 1b and 2a interacts with MyD88 and inhibits the TLR signaling pathway in macrophage cell lines.

ISDR participates in the inhibition of MyD88-dependent signaling pathway by NS5A. To further confirm the inhibitory effect of NS5A on the TLR signaling pathway, we established macrophage cell lines stably expressing NS5A mutant lacking the ISDR/MyD88 binding region (Δ240-280) or lacking a region dispensable for interaction with MyD88 (Δ280-300) (Fig. 8A).

The inhibitory effect of TLR signaling in response to stimulation with mCpG, R-837, LPS or PGN by NS5A was partially restored in the cell line expressing the NS5A lacking the ISDR (Δ240-280), and comparable inhibition was observed in the cell line expressing the NS5A deletion mutant retaining the ISDR (Δ280-300) (Fig. 8B). These results suggest that the interaction of NS5A with MyD88 through the ISDR is responsible for the disruption of the TLR-MyD88-dependent signaling pathway due to the expression of NS5A in macrophage cells. Partial recovery of the TLR signaling pathway by the expression of the NS5A mutant lacking the ISDR suggests the involvement of other inhibitory mechanisms by NS5A.

Previous reports suggest that the ISDR of NS5A participates in conferring IFN sensitivity (10) and in an interaction with double-stranded RNA-dependent protein kinase, PKR (13). To determine the effect of the interaction of MyD88 with NS5A through the ISDR on the IFN signaling pathway, we examined the phosphorylation of signal transducer and activator of transcription-1, STAT1 and PKR in response to treatment with murine IFN-α and infection with VSV. The expression of wild-type NS5A and Δ280-300 mutant but not Δ240-280 mutant reduced the phosphorylation of STAT1 in response to IFN-α treatment (Fig. 8C, upper), suggesting that the ISDR/MyD88 binding region in NS5A is involved in the IFN signaling pathway. Although cells expressing wild-type NS5A reduced PKR phosphorylation, those expressing the mutant NS5A (Δ240-280 or Δ280-300) did not inhibit PKR phosphorylation upon infection with VSV (Fig. 8C, bottom), which is consistent with the previous observation that the 66 ISDR-inclusive amino acid residues (aa 237-302) are required for interaction with PKR (13). These results suggest that the expression of HCV NS5A in macrophage cells counteracts the IFN signaling pathway through the repression of STAT1 and PKR due to interaction with ISDR and its adjacent region.

DISCUSSION

The majority of HCV-infected individuals become chronic carriers, however, the mechanism of progression to chronicity remains unclear. Among HCV proteins, NS3 has been shown to be immunodominant and T cells reactive to NS3 have been suggested to play a crucial role in viral clearance, while HCV core protein is immunosuppressive (8). Treatment of immature DCs with core or NS3 protein inhibited DC differentiation, and DCs transduced to express core or E1 protein exhibited poor allogeneic T-cell response (43). The immunosuppressive potential of HCV proteins has been implicated as a mechanism of the functional subversion of T cells, natural killer (NK) cells and DCs. The association of HCV core protein with the globular domain of C1q receptor on T cells down regulates T-cell proliferation and IL-2 production (25). Additionally, HCV E2 protein displays a high affinity to the tetraspanin cell surface molecule, human CD81, which is one of the candidates of HCV entry receptor (40), and E2 cross-linking with cell surface human CD81 impairs the activation of NK cells (7, 52).

In the present study, we established macrophage cell lines stably expressing HCV proteins and examined the effects of viral proteins on TLR function. The expression of NS5A protein specifically inhibits TLR-MyD88-induced signaling by associating with the death domain of MyD88 through the ISDR spanning from amino acid residues 240 to 280 in macrophage cells. HCV NS5A is a phosphoprotein that appears to possess multiple and diverse functions in viral replication, IFN resistance and pathogenesis (34). Mutation in the ISDR has been suggested to correlate with responsiveness to IFN treatment in patients chronically infected with HCV genotype 1b (10). Furthermore, NS5A has been shown to rescue virus replication in IFN-treated cell cultures (41) and to inhibit the antiviral activity of IFN by binding to the PKR through the ISDR and its adjacent region (aa 237–302) (13, 14). However, the controversial observations have been made that the ISDR sequence variation does not account for differences in IFN sensitivity in patients (9) and also in an HCV subgenomic RNA replicon system (15). Moreover, the expression of NS5A or the entire HCV polyprotein has been reported to counteract the antiviral effect of IFN

in a PKR- and ISDR-independent manner (12). Therefore, the possibility remains that a molecule other than PKR may be involved in the NS5A-mediated inhibition of IFN (50). Restoration of the phosphorylation of STAT1 in cells expressing a deletion mutant lacking ISDR in response to IFN-α and that of PKR phosphorylation upon infection with VSV in cells expressing NS5A mutants lacking amino acid residues 240-280 (ISDR) or 280-300 may support the hypothesis that the ISDR and the adjacent region are involved in IFN sensitivity. Thus, the ISDR may participate not only in conferring IFN resistance but also in disrupting TLR-MyD88 signaling pathways in the macrophage cells.

Several viral proteins have been shown to counteract TLRs and their downstream signaling cascade. The vaccinia virus A46R protein contains a toll/interleukin-1 receptor (TIR) domain that interacts with multiple TIR-containing adaptor molecules thereby inhibiting the activation of NF-κB and IRF3 (49). Measles virus and respiratory syncytial virus have been shown to inhibit the TLR7- and TLR9-dependent IFN-inducing pathways stimulated by R848 and CpG ODN in primary human pDCs (45). HCV NS3/4A protein has been shown to influence the functions of adaptor molecules mediating TLR-dependent and -independent signaling pathways, resulting in an impairment of the induction of IFN-β as well as subsequent IFN-inducible genes (11). Recently, RIG-I and MDA5 have been identified as cytoplasmic dsRNA detectors responding to viral RNAs and poly I:C in a TLR-independent manner and recruit IPS-1 as an adaptor molecule for signal transduction (24). The uncapped 5'-triphosphate RNA generated by viral polymerases was shown to be selectively recognized by RIG-I (18, 39). In this study, we could demonstrate that invasion of VSV and poly I:C into the RAW cell lines is preferentially recognized by RIG-1-IPS-1- and TLR3-TRIF-dependent signaling pathway, respectively, and that expression of HCV NS3/4A protease selectively inhibits cytokine production upon infection with VSV through the cleavage of IPS-1. Therefore, it is feasible to speculate that expression of NS5A and NS3/4A proteins in macrophage cells may disrupt TLR-dependent and -independet signaling pathway, respectively. However, the mechanism for inhibition of the TLR signaling pathway in the macrophage cells by expression of NS3 or NS4B remains unclear.

Although there have been reports suggesting a lack of DC dysfunction in both chimpanzees and humans chronically infected with HCV (26, 32), direct infection of HCV to DCs may be a plausible mechanisms of the dysfunction of DCs in chronic hepatitis C patients (4, 21). Indeed, the HCV genome has been detected in DCs by PCR (4) and HCV was detected in a monocyte/macrophage subpopulation of peripheral blood mononuclear cells from chronic hepatitis C patients (5). Further experiments are needed to exclude the possibility of contamination of viral RNA in blood samples. Pseudotype VSV-bearing chimeric HCV E1 and E2 proteins have been shown to infect immature myeloid DCs isolated from health donors through interaction with lectins in a Ca-independent manner (20). Recently, *in vitro* replication of the HCV JFH1 clone of genotype 2a isolated from an HCV-infected patient who developed fulminant hepatitis has been reported (31, 54, 59). However, *in vitro* replication was limited in the combination of HCV clones derived from the JFH1 strain and certain human hepatoma cell lines, and a robust cell culture of 1a and 1b genotypes, the most prevalent viruses in the world and resistant to IFN therapy, has not yet been successful except for a cell culture system for H77-S strain (1a genotype), in which infectivity was significantly lower than that of JFH1 clone (56). The establishment of a robust and reliable *in vitro* replication system of various HCV isolates is essential to determine the role of HCV infection in the modulation of TLR function in immunocompetent cells.

In conclusion, we have shown that the expression of the HCV non structural proteins NS3, NS3/4A, NS4B or NS5A, impairs the activation of TLR signaling pathways in immunocompetent cells. Furthermore, NS5A protein was shown to inhibit the TLR-MyD88 signaling pathway by direct interaction with the death domain of MyD88 through ISDR. These findings suggest new aspects of virus-cell interaction that may be explored to develop a greater understand of the escape mechanisms of HCV from the host immune surveillance system and the establishment of

persistent infection. However, it remains to be proven whether the results obtained in the murine macrophage cell lines are applicable to immunocompetent cells in hepatitis C patients.

ACKNOWLEDGMENTS

The authors gratefully thank H. Murase for her secretarial work. This work was supported in part by grants-in-aid from the Ministry of Health, Labor, and Welfare; the Ministry of Education, Culture, Sports, Science, and Technology; the 21st Century Center of Excellence Program; and the Foundation for Biomedical Research and Innovation.

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