Table 2. Adverse events.

Patient no.	Fever (days)	Vomiting	Abdominal pain	Encephalopathy	Others†
1	2	No	No	No	No
2	2	No	No	No	No
3	1	No	No	No	No
4	3	No	No	No	No
5	3	No	No	No	No
6	4	No	No	No	No
7	10	No	No	No	No
8	No	No	No	No	No
9	2	No	No	No	No
10	1	No	No	No	No
11	2	No	No	No	No
12	2	No	No	No	No
13	1	No	No	No	No

†Other adverse events include myalgia, ascites, gastrointestinal disorder, bleeding, hepatic abscess and autoimmune diseases.

response in any patients. Thus, concurrent treatment with OK432-stimulated DC infusions can be performed safely at the same time as TAE in patients with cirrhosis and HCC.

Recurrence-free survival following DC infusion

A further objective of this study was to determine clinical response following DC infusion. A group of historical controls treated with TAE without DC administration was reviewed for this study (Table 3). The clinical characteristics including tumour burden and hepatic reserve were comparable between patients treated with TAE and OK432-stimulated DC transfer ($n = 13$) and those historical controls with TAE but without DC administration ($n = 22$). We compared the recurrence-free survival between these patient groups. Kaplan–Meier analysis indicated that patients treated with TAE and OK432-stimulated DC transfer had prolonged recurrence-free survival compared with the historical controls that had been treated with TAE alone (recurrence rates 360 days after the treatments; two of 13 and 12 of 22, respectively; $P = 0{\cdot}046$, log-rank test) (Fig. 2). The results demonstrated that OK432-stimulated DC transfer during TAE therapy reduces tumour recurrence in HCC patients.

NK cell activity and intracellular cytokine responses in PBMCs

To assess systemic immunomodulatory effects of OK432-stimulated DC transfer, PBMCs were isolated 1 and 3 months after treatment and NK cell cytotoxicity against K562 erythroleukaemia target cells measured using the ^{51}Cr-release assay (Fig. 3). The level of NK cell was unaltered following treatment. In addition, cytokine production capacity of lymphocyte subsets was quantitated by measuring intracellular IFN-γ and IL-4 using flow cytometry. There were also no significant changes in terms of cytokine production capacity in the CD4$^+$, CD8$^+$ and CD56$^+$ subsets in the patients treated with OK432-stimulated DCs.

Immune responses to peptide epitopes derived from tumour antigens

To assess the effects on T cell responses to tumour antigens, PBMCs were obtained 4 weeks after DC infusion, pulsed with peptides derived from AFP, MRP3, SART2, SART3 and hTERT. IFN-γ production was then quantitated in an

Table 3. Clinical characteristics of patients treated with TAE + OK-DC and TAE alone.

	TAE + OK-DC	TAE	P
No. of patients	13	22	
Age (years)	68.2 ± 9.1	70.0 ± 7.6	n.s.†
Gender (M/F)	9/4	13/9	n.s.‡
White cell count (×10^2/μl)	34.4 ± 11.6	41.4 ± 18.9	n.s.†
Lymphocytes (×10^2/μl)	10.4 ± 3.6	12.4 ± 4.7	n.s.†
Platelets (×10^4/μl)	11.5 ± 10.2	10.3 ± 5.8	n.s.†
Hepaplastin test (%)	64.6 ± 11.6	75.5 ± 24.3	n.s.†
ALT (IU/l)	56.7 ± 38.9	67.9 ± 44.6	n.s.†
Total bilirubin (mg/dl)	1.3 ± 0.7	1.1 ± 0.6	n.s.†
Albumin (g/dl)	3.4 ± 0.6	3.6 ± 0.4	n.s.†
Non-cancerous liver parenchyma (no.)			
Chronic hepatitis	0	8	
Cirrhosis (Child–Pugh A/B/C)	13 (5/8/0)	14 (6/8/0)	n.s.‡
TNM stages (I/II/III/IV-A/IV-B)	0/4/9/0/0	3/8/11/0/0	n.s.‡
No. of tumours	2.5 ± 1.3	1.9 ± 1.3	n.s.†
Largest tumour (mm)	30.2 ± 9.4	32.6 ± 15.2	n.s.†
AFP	204.8 ± 404.1	201.8 ± 544.2	n.s.†

Results are expressed as means ± standard deviation. †Mann–Whitney *U*-test. ‡Fisher's exact test. TAE, transcatheter arterial embolization; OK-DC, OK432-stimulated dendritic cells; ALT, alanine transaminase; TNM, tumour–node–metastasis; AFP, alpha-fetoprotein; Child–Pugh, Child–Pugh classification; n.s., not significant.

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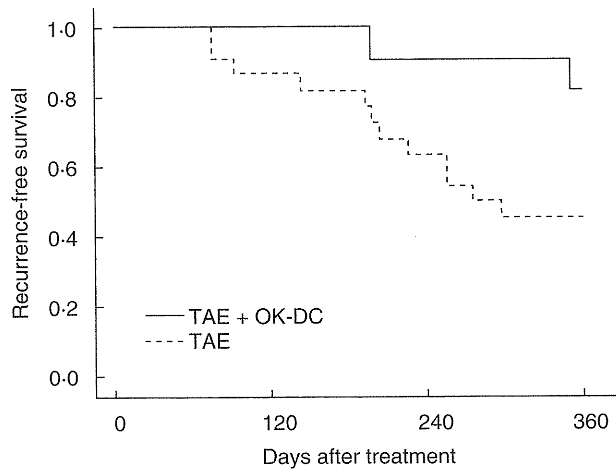

Fig. 2. Recurrence-free survival of patients treated with transcatheter hepatic arterial embolization (TAE) with [TAE + OK-stimulated dendritic cells (DC); $n = 13$] and without (TAE: historical controls; $n = 22$) OK432-stimulated DC administration. Time zero is the date of TAE. All patients underwent ultrasound, computed tomography (CT) scan or magnetic resonance imaging (MRI) of the abdomen about 1 month after treatment and at a minimum of once every 3 months thereafter. Kaplan–Meier analysis indicated that TAE + OK-DC treatment prolonged recurrence-free survival compared with the TAE-alone group (recurrence rates 360 days after the treatments; two of 13 and 12 of 22, respectively; $P = 0{\cdot}046$, log-rank test).

ELISPOT assay. Cells producing IFN-γ in response to stimulation with HLA-A24 [the most common HLA-A antigen (58·1%) in Japanese populations [35]]-restricted peptide epitopes derived from tumour antigens MRP3 and hTERT were induced in three of six HLA-A24-positive patients (numbers 2, 6 and 11) after treatment with TAE and OK432-stimulated DCs (Fig. 4). To understand the immunological and clinical significance of the T lymphocyte responses, PBMCs obtained from the historical control patients who had been treated with TAE without DC administration were also evaluated by ELISPOT. Similarly, positive reactions were observed in four (numbers t8, t19, t20 and t22) of six HLA-A24-positive patients. These data indicate that T lymphocyte responses to HLA-A24 restricted peptide epitopes of tumour antigens were induced following the TAE therapy, but no additional responses were observed as a result of OK432-stimulated DC transfer in the current study.

Serum levels of cytokines, chemokines and arginase activity

To screen for immunobiological responses induced following OK432-stimulated DC transfer, serum levels of cytokines and chemokines were measured simultaneously using the Bio-Plex multiplex suspension array system. The results were compared with the historical control patients treated with TAE without DC administration. Interestingly, serum con-

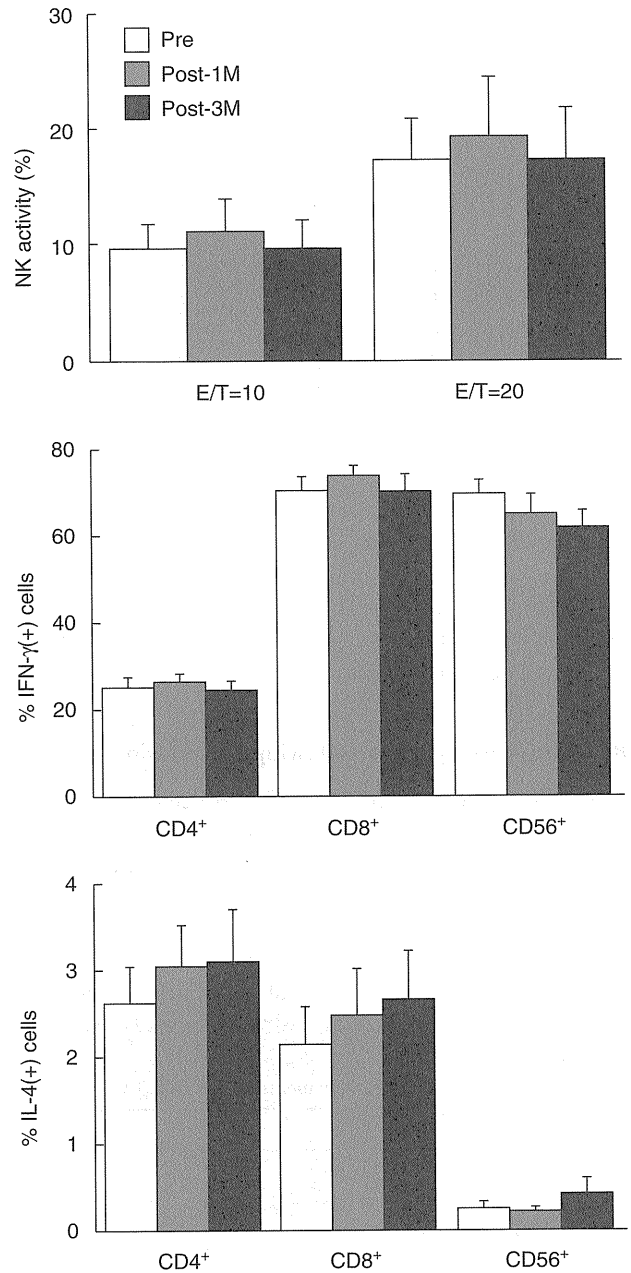

Fig. 3. Natural killer (NK) cell activity and intracellular cytokine production in peripheral blood mononuclear cells (PBMCs) of patients treated with OK432-stimulated dendritic cells (DCs) during transcatheter hepatic arterial embolization (TAE) therapy ($n = 13$). PBMCs were isolated before and 1 and 3 months after treatment and used for the analyses. Upper panel: NK cell cytotoxicity against K562 erythroleukaemia target cells was evaluated at the effector/target (E/T) cell ratios shown. NK cell activities were not changed following treatment. Middle and lower panels: PBMCs were stimulated with phorbol 12-myristate 13-acetate (PMA) and ionomycin, stained for CD4, CD8 and CD56 expression, permeabilized and stained for intracellular interferon (IFN)-γ and interleukin (IL)-4. Percentages of cytokine-positive cells were quantitated by flow cytometry. There were no significant changes in terms of cytokine production capacity in the $CD4^+$, $CD8^+$ and $CD56^+$ subsets following the treatments. The data are given as means ± standard deviation of the groups.

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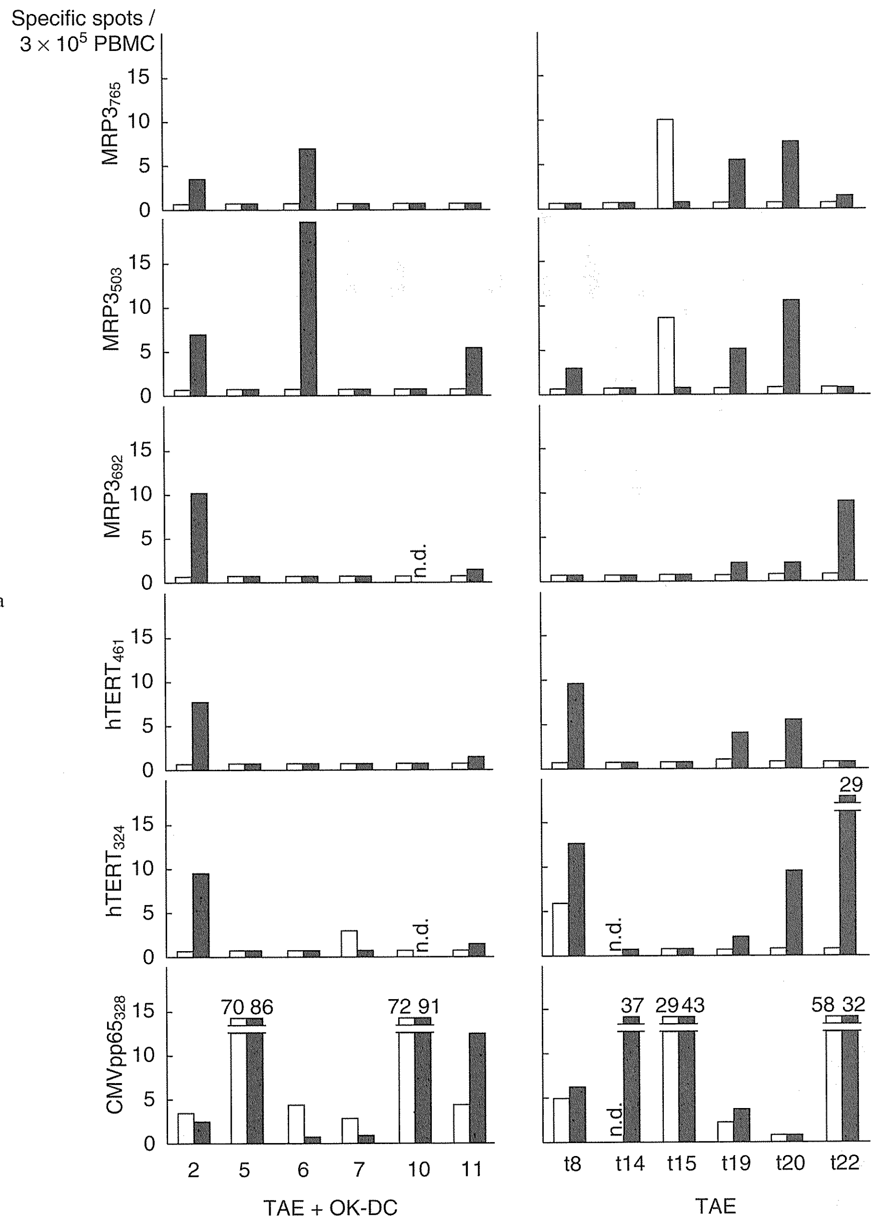

Fig. 4. Immune responses to human leucocyte antigen (HLA-DR⁻)-A24-restricted peptide epitopes derived from tumour antigens in HLA-A24-positive patients treated with OK432-stimulated DCs during transcatheter hepatic arterial embolization (TAE) therapy (numbers 2, 5, 6, 7, 10 and 11) and HLA-A24-positive historical controls treated with TAE without dendritic cell (DC) transfer (numbers t8, t14, t15, t19, t20 and t22). Peripheral blood mononuclear cells (PBMCs) were obtained before (open bars) and 1 month after the infusion (solid bars), pulsed with the peptides derived from squamous cell carcinoma antigen recognized by T cells 2 (SART2), SART3, multi-drug resistance protein 3 (MRP3), alpha-fetoprotein (AFP), human telomerase reverse transcriptase (hTERT) and interferon (IFN)-γ production was quantitated by enzyme-linked immunospot (ELISPOT). Negative controls consisted of a human immunodeficiency virus (HIV) envelope-derived peptide ($HIVenv_{584}$). Positive controls consisted of 10 ng/ml phorbol 12-myristate 13-acetate (PMA) or a cytomegalovirus (CMV) pp65-derived peptide ($CMVpp65_{328}$). The number of specific spots was determined by subtracting the number of spots in the absence of antigen from the number of spots in its presence. T lymphocyte responses to the peptide epitopes were induced following TAE therapy, but no additional responses were observed after DC transfer. Numbers denote specific spots beyond the upper limit of *y*-axis; n.d., not determined.

centrations of IL-9, IL-15 and TNF-α were greatly increased after OK432-stimulated DC infusion, in contrast to their reduction following TAE treatment alone (Fig. 5a). Furthermore, the chemokines eotaxin (CCL11) and MIP-1β (CCL4) were induced markedly after DC transfer, although they were also decreased after TAE alone. These data indicate that transfer of OK432-stimulated DC during TAE therapy induced unique immune responses that may be mediated by the cytokines IL-9, IL-15 and TNF-α and the chemokines eotaxin and MIP-1β.

In addition, serum arginase activity was reported to reflect numbers of myeloid-derived suppressor cells (MDSCs) that may inhibit T lymphocyte responses in cancer patients [36]. Therefore, serum arginase activity was measured after OK432-stimulated DC infusion, and it was found that it was increased six- or sevenfold in patients treated with TAE. However, this increase was independent of the presence or absence of OK432-stimulated DC transfer (Fig. 5b). None the less, serum arginase activity was decreased again 4 weeks after treatment with both TAE and OK432-stimulated DC transfer but tended to be maintained at a high levels in patients treated with TAE without DC transfer. However, these differences did not reach statistical significance ($P > 0{\cdot}05$). Because arginase activity is known to be relatively high in liver and HCC cells [37], the influence of tissue injury was assessed biochemically by measuring serum levels of ALT and LDH activities. We did not observe ALT or LDH elevation, indicating that the increase of arginase activity was not due to tissue damage following treatment. Collectively, these results demonstrate that infusion of OK432-stimulated

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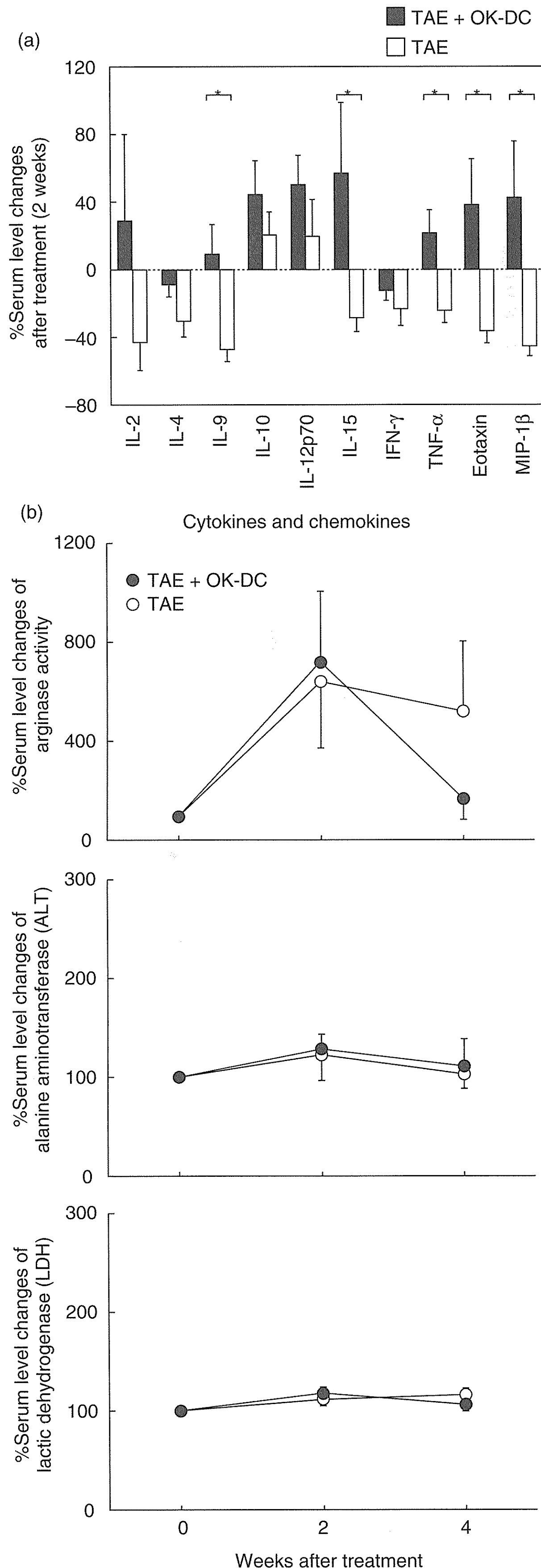

Fig. 5. Cytokine and chemokine profiling and arginase activity in sera of patients treated with OK432-stimulated dendritic cells (DCs) during transcatheter hepatic arterial embolization (TAE) therapy (TAE + OK-DC; $n = 13$) and the historical controls treated with TAE without DC transfer (TAE; $n = 22$). (a) Serum samples were examined for their content of a validated panel of cytokines and chemokines using the Bioplex assay. Percentage changes in serum levels 2 weeks after the treatments were calculated as follows: [(post-treatment level – pretreatment level)/pretreatment level] × 100. The data are means ± standard error of the mean (s.e.m.) of the groups. $^*P < 0{\cdot}05$ when compared by the Mann–Whitney U-test. (b) Serum samples were tested for arginase activity by conversion of L-arginine to L-ornithine, and for alanine aminotransferase (ALT) and lactic dehydrogenase (LDH) activities. While there was a trend for the arginase activity in the TAE + OK-DC group to decrease 4 weeks after treatment, the difference did not reach statistical significance ($P > 0{\cdot}05$). Percentage changes in serum levels 2 weeks after the treatments were calculated as follows: [(post-treatment level – pretreatment level)/pretreatment level] × 100. The data indicate means ± s.e.m. of the groups.

DCs during TAE treatment may reduce the immunosuppressive activities of MDSCs, and assist in developing a favourable environment for the induction of anti-tumour immunity.

Discussion

Although many novel strategies, including immunotherapies, have been developed in an attempt to suppress tumour recurrence after curative treatments for HCC, recurrence rates and survival times have not been improved significantly [38]. In the current study, we first established that OK432-stimulated DC administration during TAE therapy did not cause critical adverse events in patients with cirrhosis and HCC. Most importantly, DC transfer resulted in prolonged recurrence-free survival after combination therapy with TAE and OK432-stimulated DC administration. In terms of the immunomodulatory effects of DC transfer, although NK cell activity, intracellular cytokine production and T lymphocyte-mediated immune responses were not altered in PBMCs from treated patients, serum levels of IL-9, IL-15 and TNF-α and the chemokines eotaxin and MIP-1β were enhanced markedly after DC transfer. In addition, serum levels of arginase activity were decreased following DC transfer. Collectively, this study demonstrated the feasibility, safety and beneficial anti-tumour effects of OK432-stimulated DC infusion into tumour tissues for patients with cirrhosis and HCC, suggesting the ability of an active immunotherapeutic strategy to reduce tumour recurrence after locoregional treatment of HCC.

DCs were stimulated with OK432 prior to infusion into tumour tissues through an arterial catheter. OK432 was reported to activate DCs through its binding to TLR-2 and -4 [16,39] that can be used for cancer therapy [33]. The current results indicate that OK432 stimulation of immature DCs

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from HCC patients promoted their maturation processes while preserving antigen uptake capacity and enhancing tumoricidal activity, consistent with previous observations [16,19] and supporting the current strategy in which OK432-stimulated DCs were infused directly into tumour tissues. Because the tumoricidal activity of unstimulated DCs was not observed in *in vitro* experiments, OK432 stimulation obviously altered the cytotoxic properties of DCs. One of the mechanisms of DC killing was reported to be CD40/CD40 ligand interaction [19]. Further studies are needed to determine the killing mechanisms of DCs derived from HCC patients in a direct [TNF, TNF-related apoptosis inducing ligand (TRAIL), Fas ligand, nitric oxide (NO) and perforin/granzyme] and indirect (MHC-restricted) manner [40–43]. Although the main mechanism by which OK432-stimulated DCs prolonged the recurrence-free survival was not elucidated, the tumoricidal activity of mature DCs was implicated in *in vivo* enhancement of antigen presentation, co-stimulation and inflammatory cytokine production.

Very recent reports document injection of OK432-stimulated DCs into patients with cancer of the gastrointestinal tract or pancreas [44,45], but their anti-tumour effects were not defined clearly. The current study shows for the first time that OK432-stimulated DCs induce beneficial anti-tumour responses when transferred into tumour tissues during TAE therapy. The anti-tumour responses may have been enhanced as a result of optimal activation of the DCs with OK432 or combining infusion of stimulated DCs with TAE therapy. Inappropriately activated DCs may be unable to generate sufficient numbers of properly activated effector T lymphocytes [46]. As shown in Fig. 1, all these alterations could contribute to the further enhancement of anti-tumour effects compared to those in our previous study with immature DCs [20]. Furthermore, the tumour cell death-promoting therapies, e.g. chemotherapy [47] and TAE [48], can be expected to enhance the effects of therapeutic cancer vaccines by redressing the immunosuppressive tumour environment.

NK cell activity and intracellular cytokine responses in $CD4^+$ and $CD8^+$ T lymphocytes and $CD56^+$ NK cell subsets in PBMCs were not changed significantly in patients treated with OK432-stimulated DCs. Furthermore, we did not observe tumour antigen-specific T lymphocyte responses associated clearly with DC administration. The data suggest therefore that the immune responses induced by the therapy applied here were not detectable systemically. Because cytotoxic T lymphocyte responses were enhanced in patients receiving $> 3 \times 10^7$ cells [49,50], the numbers of transferred OK432-stimulated DCs were apparently not sufficient to induce responses detectable in the peripheral blood, but were enough to exert beneficial anti-tumour effects. In addition, many studies have concluded that cytotoxic T lymphocyte responses rarely predict clinical outcomes of DC-based immunotherapies [51,52] and that in many cases, also including our own studies [28,30], tumour-specific effector T lymphocytes co-exist with the tumours. Consistent with these observations, the current results suggest that cytotoxic T lymphocyte responses in PBMCs are not reliable predictors of beneficial anti-tumour effects in patients treated with the current OK432-stimulated DC strategy.

Serum levels of the cytokines IL-9, IL-15 and TNF-α and the chemokines eotaxin and MIP-1β were increased following OK432-stimulated DC transfer, but decreased after TAE therapy without DC administration. IL-9 and IL-15 belong to the cytokine receptor common gamma chain (γ_c; CD132) family, a member of the type I cytokine receptor family expressed on most lymphocyte populations [53]. IL-9 exerts pleiotropic activities on T and B lymphocytes, mast cells, monocytes and haematopoietic progenitors [54,55]. IL-15 and TNF-α are known to prime T lymphocytes and NK cells when secreted by DCs [56] and to induce anti-tumour immune responses [57]. Eotaxin is known to selectively recruit eosinophils also contributing to anti-tumour effects [58,59], and MIP-1β is a chemoattractant for NK cells, monocytes and a variety of other immune cells [60]. In addition, serum levels of arginase tended to decrease after DC transfer. Because serum arginase activity reflects the numbers of MDSCs that inhibit T lymphocyte responses in cancer patients [36], the patients treated with OK432-stimulated DCs might have developed lower levels of suppressor cells. Collectively, the results suggest that infusion of OK432-stimulated DCs may orchestrate the immune environment in the whole body that could enhance beneficial anti-tumour effects, although the precise molecular and cellular mechanisms associated with the actions of these cytokines and chemokines were not defined clearly in the current analysis.

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Disclosure

The authors have declared that no conflict of interest exists.

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Comparative Analysis of Various Tumor-Associated Antigen-Specific T-Cell Responses in Patients with Hepatocellular Carcinoma

Eishiro Mizukoshi, Yasunari Nakamoto, Kuniaki Arai, Tatsuya Yamashita, Akito Sakai, Yoshio Sakai, Takashi Kagaya, Taro Yamashita, Masao Honda, and Shuichi Kaneko

Many tumor-associated antigens (TAAs) recognized by cytotoxic T cells (CTLs) have been identified during the last two decades and some of them have been used in clinical trials. However, there are very few in the field of immunotherapy for hepatocellular carcinoma (HCC) because there have not been comparative data regarding CTL responses to various TAAs. In the present study, using 27 peptides derived from 14 different TAAs, we performed comparative analysis of various TAA-specific T-cell responses in 31 HCC patients to select useful antigens for immunotherapy and examined the factors that affect the immune responses to determine a strategy for more effective therapy. Twenty-four of 31 (77.4%) HCC patients showed positive responses to at least one TAA-derived peptide in enzyme-linked immunospot assay. The TAAs consisting of cyclophilin B, squamous cell carcinoma antigen recognized by T cells (SART) 2, SART3, p53, multidrug resistance-associated protein (MRP) 3, alpha-fetoprotein (AFP) and human telomerase reverse transcriptase (hTERT) were frequently recognized by T cells and these TAA-derived peptides were capable of generating peptide-specific CTLs in HCC patients, which suggested that these TAAs are immunogenic. HCC treatments enhanced TAA-specific immune responses with an increased number of memory T cells and induced *de novo* T-cell responses to lymphocyte-specific protein tyrosine kinase, human epidermal growth factor receptor type 2, p53, and hTERT. Blocking cytotoxic T-lymphocyte antigen-4 (CTLA-4) resulted in unmasking of TAA-specific immune responses by changing cytokine and chemokine profiles of peripheral blood mononuclear cells stimulated by TAA-derived peptides. *Conclusion:* Cyclophilin B, SART2, SART3, p53, MRP3, AFP, and hTERT were immunogenic targets for HCC immunotherapy. TAA-specific immunotherapy combined with HCC treatments and anti-CTLA-4 antibody has the possibility to produce stronger tumor-specific immune responses. (HEPATOLOGY 2011;53:1206-1216)

Hepatocellular carcinoma (HCC) is the most common primary malignancy of the liver and becoming an important public health concern.[1,2] Although many kinds of treatments have been performed for HCC, their effects are limited because the recurrence rate of HCC is very high; therefore, the development of new therapeutic options to prevent recurrence is necessary.[3,4]

To protect against recurrence, tumor antigen-specific immunotherapy is an attractive strategy. Many tumor-associated antigens (TAAs) and their epitopes recognized by cytotoxic T cells (CTLs) have been identified during the last two decades and some of them have been used in clinical trials for several cancers.[5-21] The epitopes have been under investigation for the treatment of cancer, with major clinical responses in some trials.[22,23] With regard to immunotherapy for HCC, few kinds of TAAs and their epitopes have been used and only clinical data of α-fetoprotein (AFP) have been reported.[24,25] In human trials targeting AFP, it is possible to raise an AFP-specific T-cell response using AFP-derived peptides, but this has shown little

Abbreviations: AFP, alpha-fetoprotein; CTL, cytotoxic T cell; ELISPOT, enzyme-linked immunospot; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; HLA, human leukocyte antigen; hTERT, human telomerase reverse transcriptase; IFN, interferon; Lck, lymphocyte-specific protein tyrosine kinase; MRP, multidrug resistance-associated protein; PBMC, peripheral blood mononuclear cell; TAA, tumor-associated antigen.

From the Department of Gastroenterology, Graduate School of Medicine, Kanazawa University, Kanazawa, Ishikawa, Japan.

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Address reprint requests to: Shuichi Kaneko, M.D., Department of Gastroenterology, Graduate School of Medicine, Kanazawa University, Kanazawa, Ishikawa 920-8641, Japan. E-mail: skaneko@m-kanazawa.jp; fax: 81-76-234-4250.

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antitumor effect. On the other hand, immunotherapy trials using autologous tumor lysate or dendritic cells have shown statistically significant improvements in the risk of HCC recurrence and recurrence-free survival.[26] These reports suggest that tumor antigen-specific immunotherapy is effective to reduce the recurrence rate after HCC treatment; therefore, it is necessary to find immunogenic antigens or their epitopes to develop more effective immunotherapy.

In addition, in the field of molecular targeting therapies, developments of monoclonal antibodies targeting immunomodulatory molecules to enhance antitumor immunity are progressing and some of these are under clinical trial.[27] In particular, clinical data of anti-cytotoxic T-lymphocyte antigen-4 (anti-CTLA-4) antibody have shown durable objective response and stable disease in melanoma patients.[28]

In the present study we performed comparative analysis of various TAA-specific T-cell responses in patients with HCC and examined the factors that affect the immune responses, including anti-CTLA-4 antibody. This approach offers useful information to select immunogenic TAAs and to develop a new strategy for HCC immunotherapy.

Table 1. Peptides

Peptide No.	Peptide Name	Source	Reference	Amino Acid Sequence	Number of Specific Spots in Normal Donors (Mean SD)
1	$ART1_{188}$	ART1	5	EYCLKFTKL	0.9 ± 1.1
2	$ART4_{161}$	ART4	6	AFLRHAAL	0.3 ± 0.5
3	$ART4_{899}$	ART4	6	DYPSLSATDI	0.6 ± 1.0
4	$Cyp\text{-}B_{109}$	Cyp-B	7	KFHRVIKDF	0.5 ± 0.9
5	$Cyp\text{-}B_{315}$	Cyp-B	7	DFMIQGGDF	1.2 ± 1.7
6	Lck_{208}	Lck	8	HYTNASDGL	0.3 ± 0.6
7	Lck_{486}	Lck	8	TFDYLRSVL	0.2 ± 0.8
8	Lck_{488}	Lck	8	DYLRSVLEDF	0.9 ± 1.5
9	$MAGE1_{135}$	MAGE-A1	9	NYKHCFPEI	1.0 ± 0.9
10	$MAGE3_{195}$	MAGE-A3	10	IMPKAGLLI	1.4 ± 1.7
11	$SART1_{1690}$	SART1	11	EYRGFTQDF	0.9 ± 1.3
12	$SART2_{899}$	SART2	12	SYTRLFLIL	1.0 ± 1.4
13	$SART3_{109}$	SART3	13	VYDYNCHVDL	2.1 ± 1.9
14	$Her\text{-}2/neu_{8}$	Her-2/neu	14	RWGLLLALL	1.4 ± 2.0
15	$p53_{125}$	p53	15	TYSPALNKMF	1.4 ± 1.5
16	$p53_{161}$	p53	16	AIYKQSQHM	0.4 ± 0.6
17	$p53_{204}$	p53	17	EYLDDRNTF	1.1 ± 1.5
18	$p53_{211}$	p53	17	TFRHSVVV	0.9 ± 1.9
19	$p53_{235}$	p53	17	NYMCNSSCM	2.1 ± 2.6
20	$MRP3_{503}$	MRP3	18	LYAWEPSFL	0.2 ± 0.5
21	$MRP3_{692}$	MRP3	18	AYVPQQAWI	1.5 ± 2.1
22	$MRP3_{765}$	MRP3	18	VYSDADIFL	0.9 ± 1.0
23	AFP_{357}	AFP	19	EYSRRHPQL	1.8 ± 2.0
24	AFP_{403}	AFP	19	KYIQESQAL	1.1 ± 1.5
25	AFP_{434}	AFP	19	AYTKKAPQL	0.8 ± 1.1
26	$hTERT_{167}$	hTERT	20	AYQVCGPPL	0.8 ± 1.1
27	$hTERT_{324}$	hTERT	20	VYAETKHFL	0.5 ± 0.7
28	HIV env_{584}	HIV env	32	RYLRDQQLL	1.3 ± 2.0
29	HCV $NS3_{1031}$	HCV NS3	33	AYSQQTRGL	ND
30	CMV $pp65_{328}$	CMV pp65	34	QYDPVAALF	13.3 ± 15.7

ND, not determined.

Patients and Methods

Patients and Laboratory Testing. In this study we examined 31 human leukocyte antigen (HLA)-A24-positive patients with HCC, 29 chronic hepatitis C patients without HCC, who were diagnosed by liver biopsy, and 11 healthy blood donors who did not have a history of cancer and were negative for hepatitis B surface antigen and anti-hepatitis C virus (HCV) antibody (Ab). The diagnosis of HCC was histologically confirmed in 21 patients. For the remaining 10 patients the diagnosis was based on typical hypervascular tumor staining on angiography in addition to typical findings, which showed hyperattenuated areas in the early phase and hypoattenuation in the late phase on dynamic computed tomography (CT).[29]

HLA-based typing of peripheral blood mononuclear cells (PBMCs) from patients and normal blood donors was performed as described.[19] The pathological grading of tumor cell differentiation was assessed according to the general rules for the clinical and pathological study of primary liver cancer.[30] The severity of liver disease was evaluated according to the criteria of Desmet et al.[31] using biopsy specimens of liver tissue.

All patients gave written informed consent to participate in the study in accordance with the Helsinki Declaration and this study was approved by the regional ethics committee (Medical Ethics Committee of Kanazawa University, No. 829).

Peptides, Cell Lines, and Preparation of PBMCs. Twenty-seven peptides derived from 14 different TAAs (Table 1), human immunodeficiency virus (HIV) envelope-derived peptide ($HIVenv_{584}$),[32] HCV NS3-derived peptide ($HCVNS3_{1031}$),[33] and cytomegalovirus (CMV) pp65-derived peptide ($CMVpp65_{328}$),[34] which were identified as HLA-A24 restricted CTL epitopes in previous studies, were used. Peptides were synthesized at Mimotope (Melbourne, Australia) and Sumitomo Pharmaceuticals (Osaka, Japan). They were identified using mass spectrometry and their purities were determined to be >80% by analytical high-performance liquid chromatography (HPLC). The HLA-A*2402 gene-transfected C1R cell line (C1R-A24) was cultured in RPMI 1640 medium containing 10% fetal calf serum (FCS) and 500 μg/mL hygromycin B (Sigma, St. Louis, MO), and K562 was cultured in RPMI 1640 medium containing 10% FCS.[35] PBMCs were isolated before HCC treatments as described.[20] In 12 patients their PBMCs were also obtained 4 weeks after treatments.

Table 2. Characteristics of the Patients Studied

Clinical Diagnosis	No. of Patients	Sex M/F	Age (yr) Mean ± SD	ALT (IU/L) Mean ± SD	AFP (ng/ml) Mean ± SD	Child Pugh (A/B/C)	Diff. Degree* (wel/mod/por/ND)	Tumor Size† (large/small)	Tumor Multiplicity (multiple/solitary)	Vascular Invasion (+/−)	TNM Stage (I/II/IIIA/IIIB/IIIC/IV)
Normaldonors	11	8/3	35 ± 2	ND	ND	ND	ND	ND	ND	ND	ND
Chronichepatitis	29	16/13	59 ± 10	92 ± 94	31 ± 87	27/2/0	ND	ND	ND	ND	ND
HCC	31	23/8	71 ± 4	74 ± 33	1768 ± 9103	20/10/1	11/10/0/10	22/9	20/11	9/22	10/12/3/1/2/3

*Histological degree of HCC; wel: well differentiated, mod: moderately differentiated, por: poorly differentiated, ND: not determined.
†Tumor size was divided into either "small" (≤2 cm) or "large" (>2 cm).

CTL Induction and Cytotoxicity Assay. CTL induction and cytotoxicity assays were performed as described.[20] Briefly, stimulated PBMCs were added at effector to target ratios of 100:1, 50:1, 25:1, 13:1, 6:1, and 3:1. In cases where the number of CTLs was insufficient, cytotoxicity assays were performed at effector to target ratios less than 100:1.

Interferon Gamma IFN-γ Enzyme-Linked Immunospot (ELISPOT) Assay. IFN-γ ELISPOT assays were performed as reported.[20] Responses to TAA-derived peptides were considered positive if more than 10 specific spots were detected, which is greater than the mean plus 3 standard deviations (SDs) of the baseline response detected in 11 normal blood donors (Table 1), and if the number of spots in the presence of an antigen was at least 2-fold that in its absence. Responses to HIV-, HCV-, and CMV-derived peptides were considered positive if more than 10 specific spots were detected and if the number of spots in the presence of an antigen was at least 2-fold that in its absence. In ELISPOT assay with blocking CTLA-4, anti-human CTLA-4 (eBioscience, Tokyo, Japan) was added at a final concentration of 50 μg/mL, which has been described to have maximum effect in *in vitro* cultures.[36] As a control, functional grade mouse immunoglobulin G (IgG)2a isotype control was used. The assay with blocking CTLA-4 was performed in triplicate and the results were statistically analyzed using the unpaired Student's t test.

Cytokine and Chemokine Profiling. The effect of CTLA-4 antibody on TAA-specific T-cell responses was also analyzed by cytokine and chemokine profiling. Cytokine and chemokine levels in the medium of ELISPOT assay were measured using the Bio-plex assay (Bio-Rad, Hercules, CA). These included interleukin (IL)-1β, IL-1Ra, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12p70, IL-13, IL-15, IL-17, basic fibroblast growth factor (FGF), eotaxin, G-CSF, GM-CSF, IFN-γ, IP-10, MCP-1, macrophage inflammatory protein (MIP)-1α, MIP-1β, platelet-derived growth factor (PDGF)-BB, RANTES, tumor necrosis factor alpha (TNF-α), and vascular endothelial growth factor (VEGF). Eight standards (ranging from 2 to 32,000 pg/mL) were used to generate calibration curves for each cytokine. Data acquisition and analysis were carried out using Bio-plex Manager software v. 4.1.1.

Cytokine Secretion Assay. TAA-specific IFN-γ-producing T cells were also analyzed by cytokine secretion assay. The assay was performed with the MACS cytokine secretion assay (Miltenyi Biotec K.K., Tokyo, Japan), in accordance with the manufacturer's instructions. Briefly, 5,000,000 PBMCs were pulsed with TAA-derived peptides for 16 hours and then incubated with 20 μL of IFN-γ detection antibody, 10 μL of anti-CD8-APC Ab (Becton Dickinson, Tokyo, Japan), 10 μL of anti-CCR7-FITC Ab (eBioscience, Tokyo, Japan), and 10 μL of anti-CD45RA-PerCP-Cy5.5 Ab (eBioscience, Tokyo, Japan) for 10 minutes at 4°C. After washing with a cold buffer (phosphate-buffered saline/0.5% bovine serum albumin with 2 mM EDTA), the cells were resuspended with 500 μL of cold buffer and analyzed using FACSCalibur (Becton Dickinson, Tokyo, Japan). As a positive control, $CMVpp65_{328}$-specific IFN-γ-producing T cells were also analyzed by the same methods. The number of IFN-γ-producing T cells was calculated from the results of FACS analysis and is shown as a number per 300,000 PBMCs.

Results

Patient Profile. The clinical profiles of the 11 healthy blood donors, 29 patients with chronic hepatitis C, and 31 patients with HCV-related HCC analyzed in the present study are shown in Table 2 and Fig. 1. Using TNM staging of the Union Internationale Contre Le Cancer (UICC) system (6th v.), 10, 12, 3, 1, 2, and 3 patients were classified as having stage I, II, IIIA, IIIB, IIIC, and IV tumors, respectively.

Detection of TAA-Specific T Cells in HCC Patients. First we examined the frequency of cells that specifically reacted with TAA-derived and control peptides in HCC patients. Fifty-one responses in total were observed against TAA-derived peptides. Twenty-

Patients	Response frequency per peptide (n)	(%)	TNM stage	Treatment of HCC
1	1	4	II	TAE+RFA
2	1	4	II	RFA
3	2	7	II	RFA
4	1	4	IIIc	Chemotherapy
5	0	0	I	None
6	2	7	I	RFA
7	1	4	IV	TAE+RFA
8	8	30	IIIc	Chemotherapy
9	1	4	I	RFA
10	2	7	IV	Chemotherapy
11	2	7	I	TAE+RFA
12	0	0	I	TAE+RFA
13	2	7	IV	TAE
14	3	11	IIIa	Chemotherapy
15	3	11	IIIa	TAE+RFA
16	0	0	II	RFA
17	3	11	II	TAE+RFA
18	0	0	IIIb	Chemotherapy
19	2	7	II	Operation
20	1	4	I	Operation
21	4	15	II	PEIT
22	1	4	II	TAE+RFA
23	2	7	IIIa	TAE+RFA
24	2	7	I	RFA
25	4	15	II	TAE+RFA
26	1	4	II	TAE
27	1	4	I	RFA
28	0	0	II	TAE+RFA
29	0	0	I	PEIT
30	0	0	I	TAE
31	1	4	II	RFA

Peptides	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30
Response frequency per patient (n)	1	1	1	3	2	1	0	1	1	1	2	3	6	0	0	3	3	2	1	2	2	4	2	4	2	0	3	0	5	13
(%)	3	3	3	10	6	3	0	3	3	3	6	10	19	0	0	10	10	6	3	6	6	13	6	13	6	0	10	0	16	42

Fig. 1. TAA-, HIV-, HCV-, and CMV-derived peptide-specific T-cell responses. Results of all HCC patients examined are shown. The T-cell responses were examined by IFN-γ ELISPOT assay. Responses to peptides were considered positive if more than 10 specific spots per 300,000 PBMCs were detected and if the number of spots in the presence of an antigen was at least 2-fold that in its absence. Black boxes indicate the presence of a significant IFN-γ T-cell response to peptides. Peptide sequences are described in Table 1 and characteristics of patients in Table 2.

four of 31 (77.4%) patients showed positive responses to at least one TAA-derived peptide and most of them showed responses to 1 to 4 kinds of TAA-derived peptide. Twenty-three of 27 (85.2%) TAA-derived peptides were recognized by T cells of at least one patient. Peptides 4, 12, 13, 16, 17, 22, 24, and 27 were recognized in more than two patients, suggesting that these peptides were immunogenic. Peptides 28 (HIV env_{584}), 29 (HCV $_{1031}$), and 30 (CMV $pp65_{328}$) were recognized by 0 (0%), 5 (16%), and 13 (42%) patients, respectively.

The magnitude of TAA-specific T-cell responses was assessed by the frequency of peptide-specific IFN-γ-producing T cells in the PBMC population (Fig. 2A). The range of TAA-derived peptide-specific T-cell frequency was 10-60.5 cells/300,000 PBMCs. Those specific to peptides 13 and 16 numbered more than 30 cells/300,000 PBMCs, suggesting that these peptides were immunogenic. The frequencies of T cells specific to HCV- and CMV-derived peptides were 12-22 cells and 12-92/300,000 PBMCs, respectively.

Whether these TAA-derived peptides were capable of generating peptide-specific CTLs from PBMCs was investigated in HCC patients. The seven peptides were selected according to the magnitude of TAA-specific T-cell responses determined by the frequency of T cells with a positive response. The CTLs generated with these peptides were cytotoxic to C1RA24 cells pulsed with the corresponding peptides (Fig. 2B).

Comparison of TAA-Specific T-Cell Responses Between the Patient Groups With and Without HCC. To characterize the immunogenicity and specificity of TAA-derived peptides, we compared T-cell responses to the peptides derived from TAA, HIV, HCV, and CMV among three groups consisting of normal blood donors, patients with chronic hepatitis C, and patients with HCV-related HCC. A significant TAA-specific T-cell response was not detected in normal blood donors (Fig. 3A). A response was detected in both chronic hepatitis C and HCC patient groups, but it was more frequently observed in HCC patients. HIV-specific T-cell response was not detected in any group. HCV-specific T-cell response rate was not different between the groups with chronic hepatitis C and HCC. CMV-specific T-cell response rates were similar among the three groups. Similar tendencies were observed in the analysis of individual peptides (Fig. 3B). We also examined the frequency of T cells responsive to peptides among the three groups. The mean frequency of TAA-specific T cells without *in vitro* expansion was higher in HCC patients than in

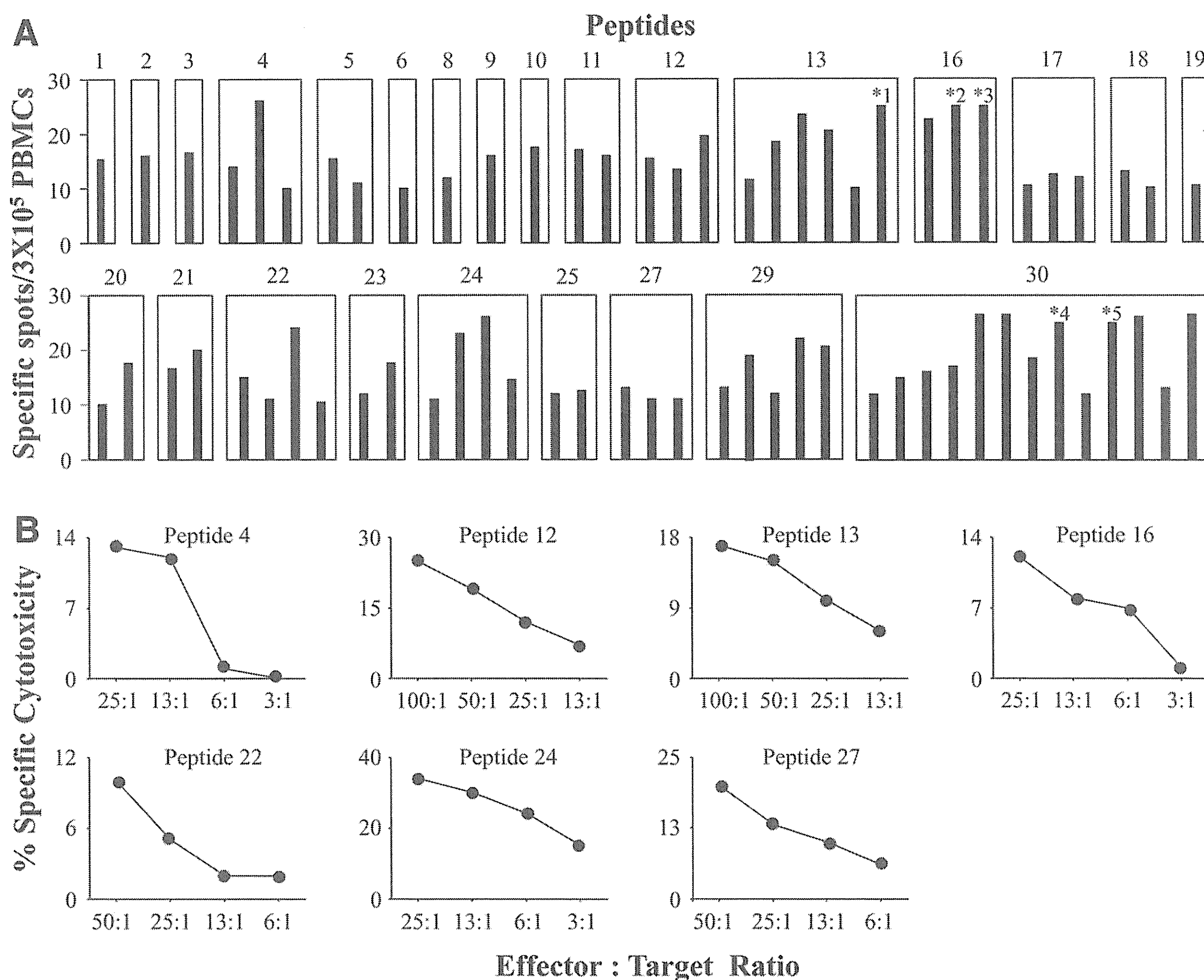
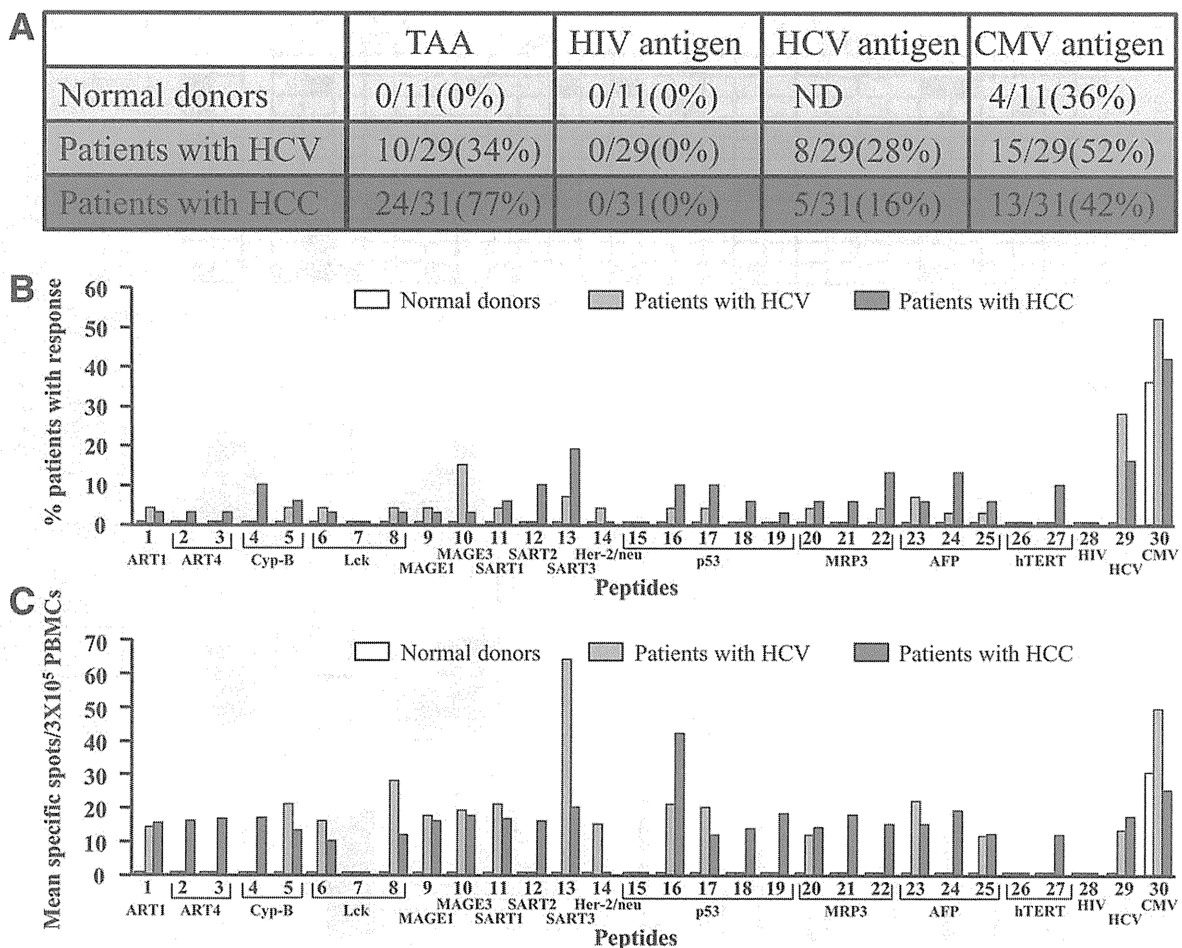


Fig. 2. Vigor of TAA-, HCV-, and CMV-derived peptide-specific T-cell responses. (A) The frequency of TAA-specific IFN-γ-producing T cells was analyzed by ELISPOT assay. Only positive responses are shown. Black bars indicate the response of one patient. *1, *2, *3, *4, and *5 denote 33, 60.5, 44, 92, and 67.5 specific spots, respectively. (B) Representative TAA-specific T-cell responses were also analyzed by CTL assay. T cell lines were generated from PBMC of the HLA-A24-positive HCC patients by stimulation with TAA-derived peptides (peptides 4, 12, 13, 16, 22, 24, and 27) (see Table 1). Expanded T cell lines were then tested for specific cytotoxicity against the corresponding peptides in a standard ^{51}Cr release assay at the indicated E:T ratios.

patients with chronic hepatitis C for 14 of 27 TAA-derived peptides (peptides 1, 2, 3, 4, 12, 16, 18, 19, 20, 21, 22, 24, 25 and 27) (Fig. 3C).

Enhancement of TAA-Specific T-Cell Responses After HCC Treatments. Several studies including our own have clarified that HCC treatments enhanced HCC-specific immune responses (19, 37, 38). In this study, we examined whether the enhancement was observed equally in all kinds of TAAs or specifically in some TAAs. For this purpose we measured the frequency of TAA-specific T cells before and after HCC treatment by ELISPOT assay in 12 cases who received transcatheter arterial embolization (TAE), radiofrequency ablation (RFA), or chemotherapy. The frequency of TAA-specific T cells increased in all patients and it was observed for 23 of 27 TAA-derived peptides (Fig. 4A). The enhancement was observed in the patients who received TAE, RFA, or chemotherapy and even in the patients without an increase in the frequency of CMV-specific T cells. Peptides 7, 14, 15, and 26, which were not recognized by T cells in all HCC patients before treatments (Fig. 1), were recognized by T cells in 1, 4, 1, and 5, respectively, of 12 patients after treatments. Representative results of enhancement of TAA-specific immune responses are shown in Fig. 4B. The frequency of TAA-specific T cells increased to 11-80 cells/300,000 PBMCs after treatments.

The enhancement of TAA-specific immune responses was also confirmed by cytokine secretion assay. Representative results are shown in Fig. 4C. In this patient (patient 25) the frequency of TAA-specific IFN-γ-producing CD8$^+$ T cells was increased from 0.4% to 1.4% of CD8$^+$ T cells after HCC treatment.



	TAA	HIV antigen	HCV antigen	CMV antigen
Normal donors	0/11(0%)	0/11(0%)	ND	4/11(36%)
Patients with HCV	10/29(34%)	0/29(0%)	8/29(28%)	15/29(52%)
Patients with HCC	24/31(77%)	0/31(0%)	5/31(16%)	13/31(42%)

Fig. 3. Comparative analysis of TAA-, HIV-, HCV-, and CMV-derived peptide-specific T-cell responses among three groups of subjects: normal donors, patients with chronic hepatitis C not complicated by HCC, and HCC patients. (A) Summary of the number of patients with a significant IFN-γ T-cell response to tumor-associated, HIV, HCV, and CMV antigens in each group. (B) Graph shows the percentage of patients in each group who showed a significant IFN-γ T-cell response to individual peptides. Peptide sequences are described in Table 1. (C) Mean frequency of peptide-specific IFN-γ-producing T cells in each group. The frequency of IFN-γ-producing T cells was analyzed by ELISPOT assay.

In this assay we also examined the naïve/effector/memory phenotype of these cells by the criterion of CD45RA/CCR7 expression.[39] Phenotypic analysis of TAA-specific, IFN-γ-producing memory $CD8^+$ T cells before and after treatment showed that the frequency of $CD45RA^-/CCR7^+$ central memory T cells was the highest, indicating that the posttherapeutic increase in these T cells is due to the increase in cells with this phenotype (Fig. 4D). In this patient the number of T cells with the $CD45RA^-/CCR7^+$ phenotype increased from 73 cells/300,000 PBMCs before treatment to 316 cells/300,000 PBMCs after treatment. Similar results were noted in five patients.

Blocking CTLA-4 Restores TAA-Specific T-Cell Responses. In previous studies including our own,[19,20,24] the CTL epitopes that correlate with the prevention of tumor progression or prognosis of HCC patients have not been identified. One of the reasons for this is considered to be that the naturally occurring T-cell responses to the epitopes are weak; therefore, recent tumor immunotherapeutic studies are moving toward modulation of T-cell responses.

CTLA-4 is recognized as a critical negative regulator of immune response; therefore, its blockade has been considered to contribute to antitumor activity.[27] In a recent study it was reported that blocking of CTLA-4 on both effector and regulatory T cell compartments contributes to the antitumor activity of CTLA-4 antibodies.[40] To examine whether similar occurs for immune response in HCC patients, we analyzed 32 separate TAA-specific T-cell responses in 15 HCC patients using 13 TAA-derived peptides. Incubation of T cells with CTLA-4 antibodies resulted in an increase of the number of TAA-specific T cells in 18 of 32 (56%) responses and in 9 of 15 (60%) patients (Fig. 5A). Fourteen and four patients showed increases of 1-10 and more than 10 TAA-specific T cells, respectively. Representative results of six patients are shown

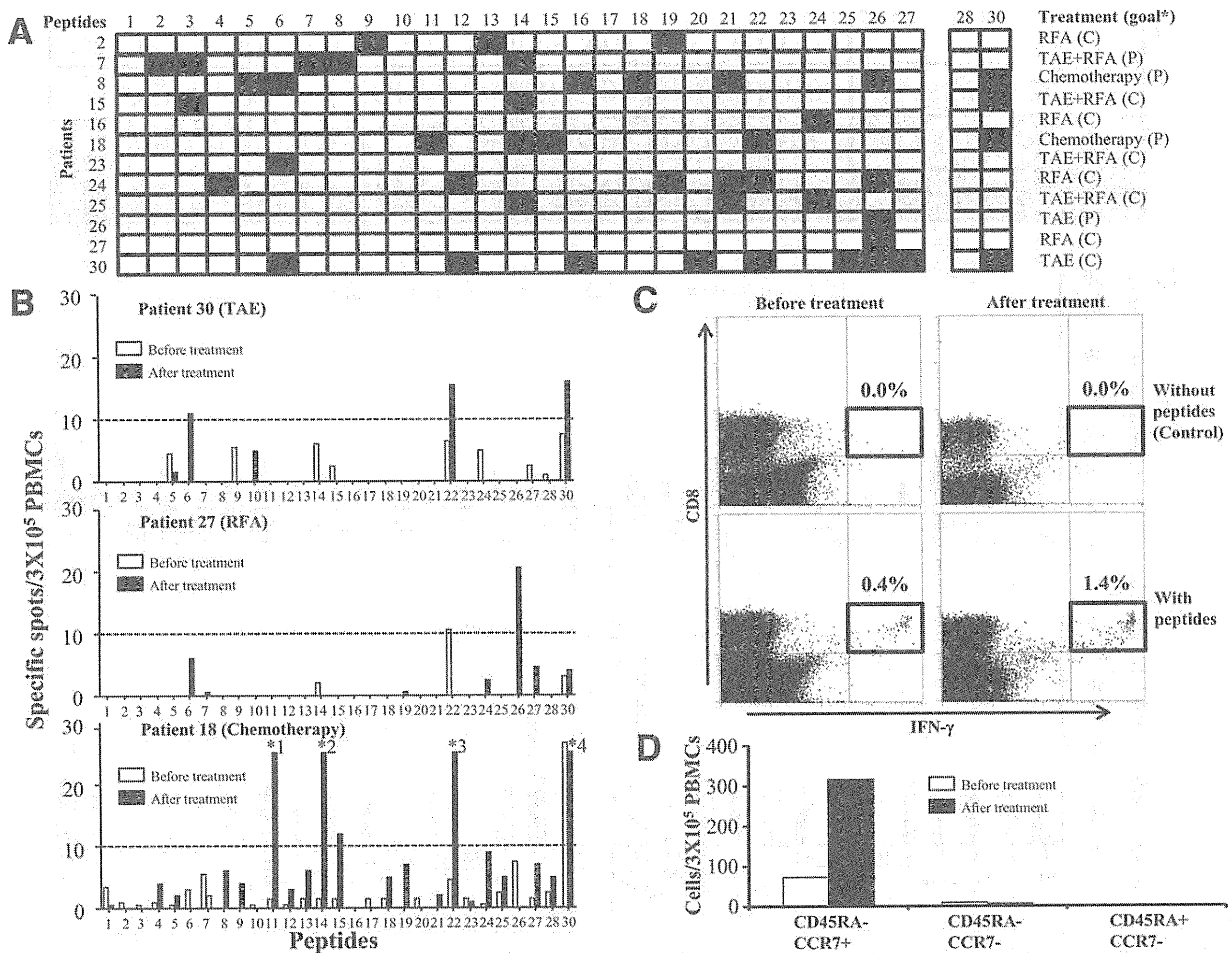


Fig. 4. Enhancement of TAA-specific T-cell responses in HCC patients after treatments. (A) Summary of patients and peptides with a significant increase of the number of IFN-γ-producing T cells (black boxes). A significant change in the IFN-γ response was defined as a more than 2-fold increase and the presence of more than 10 specific spots in ELISPOT assay after HCC treatments. The assays were performed in 12 HCC patients using 27 TAA-, HIV-, and CMV-derived peptides. Goal* shows the goal of HCC treatment. C and P denote "curative intention" and "palliative intention," respectively. (B) Representative results of ELISPOT assay are shown. White and black bars indicate the frequency of T cells before and after HCC treatments, respectively. *1, *2, *3, and *4 denote 53, 60, 80, and 121 specific spots, respectively. (C) Enhancement of TAA-specific T-cell responses was also analyzed by cytokine secretion assay. Representative results are shown (patient 25). PBMCs were pulsed with TAA-derived peptides (peptides 14, 21, and 24) for 16 hours and then analyzed for IFN-γ production. (D) IFN-γ-producing T cells were also examined for naïve/effector/memory phenotype by the criterion of CD45RA/CCR7 expression. The number of cells was calculated from the results of FACS analysis and is shown as a number per 300,000 PBMCs. White and black bars indicate the frequency of TAA-specific IFN-γ-producing T cells before and after HCC treatments, respectively. The experiments were performed in five patients and similar results were observed.

in Fig. 5B. The magnitude of TAA-specific T-cell increase was statistically significant in four patients.

To examine the effect of CTLA-4 antibodies for production of other cytokines by T cells, we measured 27 kinds of human cytokines and chemokines in the medium of ELISPOT assay. Figure 5C shows the results of cytokine production in the well with positive T-cell responses against TAA-derived peptides. The various cytokines consisting of IL-1β, IL-4, IL-6, IL-10, IL-17, eotaxin, G-CSF, GM-CSF, IFN-γ, MIP-1α, MIP-1β, RANTES, and TNF-α were increased in the medium with CTLA-4 antibodies compared with that without CTLA-4 antibodies. In contrast, increased production of these cytokines in the well without positive T-cell responses against TAA-derived peptides was not observed in medium either with or without CTLA-4 antibodies (Fig. 5D).

Discussion

In recent years, specific TAAs and their CTL epitopes have been identified in many tumors.[21] Several TAAs and their CTL epitopes, such as AFP, MAGE, and human telomerase reverse transcriptase (hTERT) have also been reported in HCC.[19,20,24,41] Although AFP-targeting immunotherapy could induce TAA-

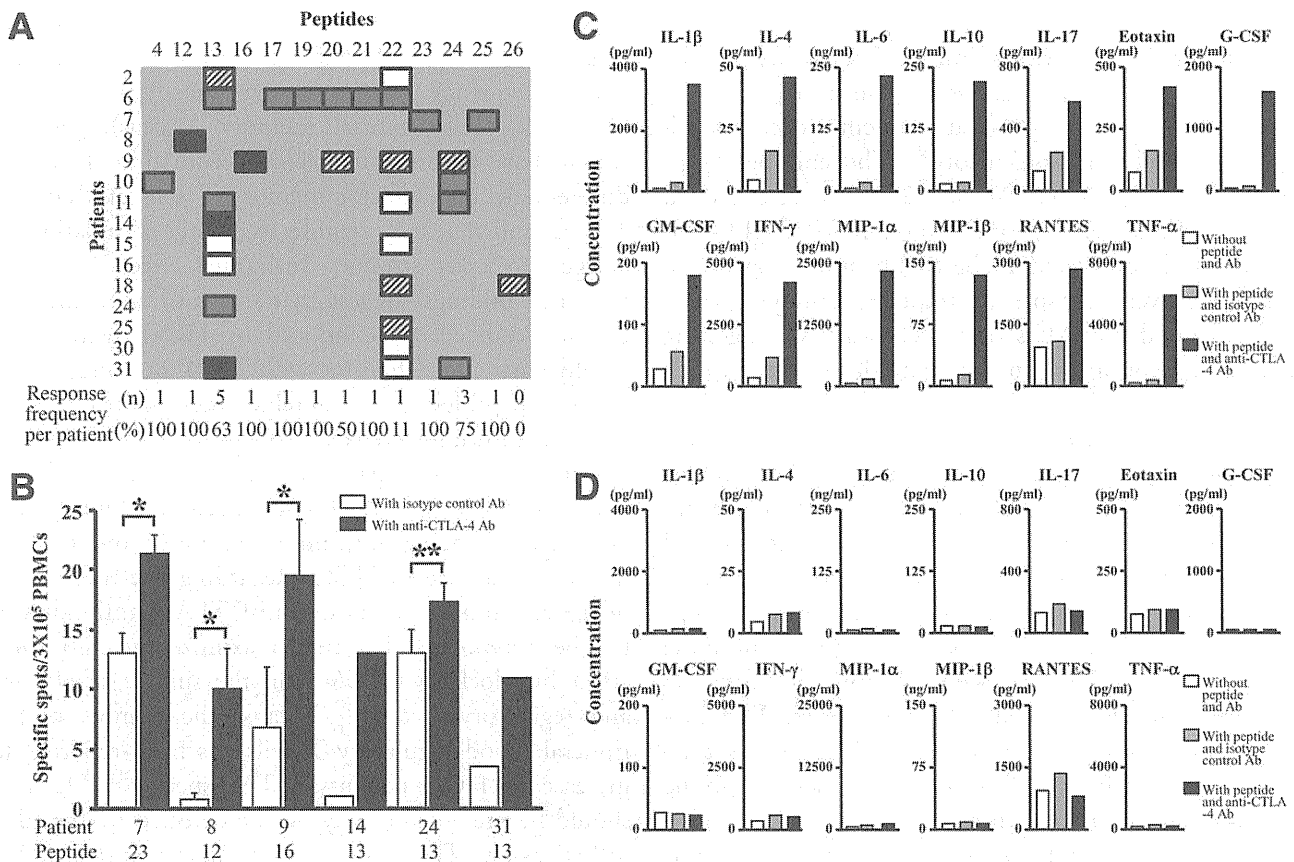


Fig. 5. Enhancement of TAA-specific T-cell responses in HCC patients by CTLA-4 antibodies. (A) Summary of patients and peptides with an increase of the number of IFN-γ-producing T cells. Black, gray, white, and hatched boxes indicate the immune responses with an increase of more than 10 specific spots, an increase of 1-10 specific spots, without change and a decrease of 1-10 specific spots, respectively. (B) Representative results of six patients are shown. Black and white bars indicate the results of assays incubated with CTLA-4 antibodies and mouse IgG2a isotype control, respectively. Data are expressed as the mean ± SD of specific spots, except for patients 14 and 31. (C) Effects of CTLA-4 antibodies on production of cytokine and chemokine. Cytokine and chemokine levels in the medium of ELISPOT assay were measured using the Bio-plex assay. The graphs indicate the concentrations of cytokine and chemokine in the medium of ELISPOT assay using PBMCs of patient 31 and peptide 13 (medium in ELISPOT assay with enhancement of T-cell response) (see A,B). The increase of cytokines and chemokines after incubation with anti-CTLA-4 antibodies was confirmed in another three experiments using PBMCs of three other patients. (D) The graphs indicate the concentrations of cytokine and chemokine in the medium of ELISPOT assay using PBMCs of patient 31 and peptide 22 (medium in ELISPOT assay without enhancement of T-cell response) (see A).

specific CTLs, no patients achieved an objective tumor response; therefore, the search for TAAs as suitable targets for HCC immunotherapy and identification of their epitopes are important issues in therapy development. However, to date, T-cell responses to previously identified TAAs or their epitopes have been measured simultaneously and comparatively in only one study involving several patients with HBV-related HCC,[42] but no T-cell responses to the many other TAAs or their epitopes have been evaluated.

In this study we performed a simultaneous, comparative analysis of immune responses to 27 different CTL epitopes derived from 14 previously reported TAAs in the peripheral blood lymphocytes of 31 HCV-related HCC patients. We noted immune responses to epitopes (peptides 4, 12, 13, 16, 17, 22, 24, and 27) derived from CypB, SART2, SART3, p53, MRP3, AFP, and hTERT in more than two patients (Fig. 1). These findings suggest the immunogenicity of these TAAs and their epitopes. In addition, the frequencies of peripheral blood CTLs specific to epitopes (peptides 4, 13, 16, 22, and 24) derived from CypB, SART3, p53, MRP3, and AFP, as detected by the ELISPOT assay, were high (≥20 specific spots/300,000 PBMCs), suggesting the high immunogenicity of these TAAs and their epitopes.

Among these immunogenic antigens the expression of p53, MRP3, AFP, and hTERT was reported in HCC.[18,19,43,44] We also previously confirmed that the expression of SART2 and SART3 was observed in 100% of human HCC tissue (data not shown). As for CypB, this protein is well known to be widely expressed in normal and malignant tissue[7]; therefore, it is considered to be expressed in HCC.

Regarding tumor immunotherapy, it has recently been reported that strong immune responses can be induced at an earlier postvaccination time using, as peptide vaccines, epitopes that frequently occur in peripheral blood CTL precursors.[23] The epitopes (peptides 4, 12, 13, 16, 22, 24, and 27) that were derived from CypB, SART2, SART3, p53, MRP3, AFP, and hTERT and considered to be highly immunogenic in this study were capable of inducing epitope-specific CTLs from the PBMCs of HCC patients, suggesting that these epitopes can be candidates for peptide vaccines.

Next, TAA-specific immune responses were compared among three groups of subjects: HCC patients, normal blood donors, and patients with chronic hepatitis C not complicated by HCC. The results showed that there were no differences in the positive rate of immune responses to CMV among the three groups and no difference in the positive rate of immune responses to HCV between chronic hepatitis C patients with and without HCC. However, TAA-specific immune responses were observed frequently only in HCC patients, indicating that these immune responses are specific to HCC.

In the present study we also analyzed factors influencing host immune responses to these TAA-derived epitopes. Previous studies have reported that treatments, such as RFA and TAE, enhance HCC-specific T-cell responses.[19,37,38] However, TAAs and their epitopes, to which these enhanced immune responses occur, have not been identified. Thus, we simultaneously measured immune responses to 27 different epitopes derived from 14 TAAs in 12 patients who were available for analysis before and after treatment. The results showed that the antigens and their epitopes to which treatment-enhanced T-cell responses occur were diverse and some of them were newly induced after HCC treatment, suggesting that HCC treatments could induce *de novo* T-cell responses and these TAAs and their epitopes can be candidates as targets for HCC immunotherapy.

Furthermore, it became clear that enhanced immune responses to TAAs were induced not only by previously reported RFA and TAE, but also by cytotoxic drug chemotherapy. The patients who received chemotherapy showed partial responses after the treatment; therefore, we considered that it induced release of TAA into the tumor environment by tumor necrosis and/or apoptosis such as the mechanism reported in RFA or TAE.[19,37,38] Thus, our findings suggest that combined cancer chemotherapy and immunotherapy is useful as a treatment for HCC.

Analysis of the memory phenotypes of the T cells thus induced showed that the phenotypes of T cells whose frequency increased were mostly $CD45RA^-$/$CCR7^+$ T cells (central memory T cells). Previous studies have reported that T cells with this phenotype differentiate into effector memory T cells and effector T cells, and that they require secondary stimulation by antigen to exert stronger antitumor effects.[39] Therefore, our findings suggest that the antitumor effect of tumor-specific T cells induced by HCC treatment is insufficient, and a booster with TAAs or epitope-containing peptides is a suitable method to further enhance antitumor effects.

Finally, we investigated the effect of anti-CTLA-4 antibodies, which have recently been in clinical trials as drugs enhancing antitumor immunity, on the host immune response to HCC. Regarding the mechanism of the antitumor activity of anti-CTLA-4 antibodies, it has been reported that they maximize the antitumor effect by blocking CTLA-4 on the surface of effector and regulatory T cells.[40] Because the number of peripheral blood regulatory T cells has been reported to increase in HCC patients,[45] TAA-specific CTLs that should be present but may not be detected by the ELISPOT assay. Therefore, in this study anti-CTLA-4 antibodies were added along with peptides to examine their effect on the ELISPOT assay.

The addition of anti-CTLA-4 antibodies resulted in an increase in the frequency of TAA-specific T cells in 60% of HCC patients. Although most patients showed an increase of only 1-10 TAA-specific T cells, the increased number of T cells was statistically significant. In addition, an increase of more than 10 TAA-specific T cells and a conversion from a negative to a positive response were observed in four patients. These results suggested that the anti-CTLA-4 antibodies unmasked IFN-γ production by CTLs. However, the function might be limited because the number of TAA-specific T cells was not changed and even decreased in some patients.

The cytokine and chemokine profiling showed that the addition of anti-CTLA-4 antibodies increased the production of not only IFN-γ but also cytokines, such as TNF-α, IL-1, and IL-6, and chemokines such as MIP-1; therefore, we speculate that the increased production of these antitumor immunity substances also plays a role in the unmasking of TAA-specific CTLs by anti-CTLA-4 antibodies. These results suggest that anti-CTLA-4 antibody is promising as a drug to enhance antitumor immunity, and that the ELISPOT assay with this antibody may serve as a more appropriate test tool to detect more HCC-specific TAAs or their epitopes.

On the other hand, recent studies have shown the important role of CD4$^+$ helper T cells in optimal function and proliferation of CD8$^+$ T cells.[46] Therefore, the lack of CD4$^+$ helper T cells or anergic CD4$^+$ T cells may explain the limited TAA-specific CD8$^+$ T-cell responses in HCC. Further studies using CD4$^+$ T-cell-depleted PBMCs or CD8$^+$ T cells expanded with TAA-derived peptide may enable identification of more immunogenic HCC-specific TAAs and their epitopes.

In conclusion, the results of this study suggest that CypB, SART2, SART3, p53, MRP3, AFP, and hTERT are promising TAAs in HCC immunotherapy, that the administration of these TAAs or peptides containing their epitopes as vaccines after HCC treatment is likely to be effective, and that the concomitant use of anti-CTLA-4 antibodies may further increase antitumor immunity. We believe that the results of this study provide useful information for the development of immunotherapy for HCC.

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Malnutrition Impairs Interferon Signaling Through mTOR and FoxO Pathways in Patients With Chronic Hepatitis C

MASAO HONDA,*,‡ KENJI TAKEHANA,§ AKITO SAKAI,* YUSUKE TAGATA,§ TAKAYOSHI SHIRASAKI,‡ SHINOBU NISHITANI,§ TAKAHIKO MURAMATSU,‖ TATSUYA YAMASHITA,* YASUNARI NAKAMOTO,* EISHIRO MIZUKOSHI,* YOSHIO SAKAI,* TARO YAMASHITA,* MIKIKO NAKAMURA,* TETSURO SHIMAKAMI,¶ MINKYUNG YI,# STANLEY M. LEMON,¶ TETSUO SUZUKI,** TAKAJI WAKITA,** SHUICHI KANEKO,* and the Hokuriku Liver Study Group

*Department of Gastroenterology, ‡Department of Advanced Medical Technology, Kanazawa University Graduate School of Medicine, Kanazawa, Japan; §Exploratory Research Laboratories, Research Center, Ajinomoto Pharmaceuticals, Co, Ltd, Kanagawa, Japan; ‖Frontier Research Labs, Institute for Innovation, Ajinomoto, Co, Inc, Kanagawa, Japan; ¶Division of Infectious Diseases, School of Medicine, The University of North Carolina at Chapel Hill, Chapel Hill, North Carolina; #Center for Hepatitis Research, Institute for Human Infections and Immunity, and Department of Microbiology and Immunology, University of Texas Medical Branch, Galveston, Texas; and **Department of Virology II, National Institute of Infectious Diseases, Tokyo, Japan

BACKGROUND & AIMS: Patients with advanced chronic hepatitis C (CH-C) often are malnourished, but the effects of malnutrition on interferon (IFN) signaling and response to treatment have not been determined. We assessed the importance of the nutritional state of the liver on IFN signaling and treatment response. **METHODS:** We studied data from 168 patients with CH-C who were treated with the combination of pegylated-IFN and ribavirin. Plasma concentrations of amino acids were measured by mass spectrometry. Liver gene expression profiles were obtained from 91 patients. Huh-7 cells were used to evaluate the IFN signaling pathway, mammalian target of rapamycin complex 1 (mTORC1), and forkhead box O (FoxO). Antiviral signaling induced by branched-chain amino acids (BCAAs) was determined using the in vitro hepatitis C virus replication system. **RESULTS:** Multivariate logistic regression analysis showed that Fischer's ratio was associated significantly with nonresponders, independent of interleukin-28B polymorphisms or the histologic stage of the liver. Fischer's ratio was correlated inversely with the expression of BCAA transaminase 1, and was affected by hepatic mTORC1 signaling. IFN stimulation was impaired substantially in Huh-7 cells grown in medium that was low in amino acid concentration, through repressed mTORC1 signaling, and increased Socs3 expression, which was regulated by Foxo3a. BCAA could restore impaired IFN signaling and inhibit hepatitis C virus replication under conditions of malnutrition. **CONCLUSIONS: Malnutrition impaired IFN signaling by inhibiting mTORC1 and activating Socs3 signaling through Foxo3a. Increasing BCAAs to up-regulate IFN signaling might be used as a new therapeutic approach for patients with advanced CH-C.**

Keywords: HCV; Liver Disease; Therapy; Diet.

Interferon (IFN) and ribavirin (RBV) combination therapy is a popular modality for treating patients with chronic hepatitis C (CH-C), but approximately 50% of patients usually relapse, particularly those with hepatitis C virus (HCV) genotype 1b and a high viral load.[1]

Recent landmark studies of genome-wide associations identified genomic loci associated with treatment responses to pegylated (Peg)-IFN and RBV combination therapy,[2,3] and a polymorphism in the interleukin (IL)-28B gene was found to predict hepatitis C treatment–induced viral clearance. Moreover, we previously showed that expression of hepatic IFN-stimulated genes (ISGs) was associated with the IL-28B polymorphism and might contribute to the treatment response.[4] In addition to the IL-28B polymorphism, host factors such as fibrosis stage and metabolic status of the liver might be associated with the treatment outcome[4,5]; however, the significance of these factors in conjunction with the IL-28B polymorphism has not been evaluated fully.

In CH-C livers, prolonged liver cell damage, fibrosis development, and microcirculation failure can lead to a state of malnutrition in hepatocytes, resulting in the impairment of multiple metabolic pathways. In patients with advanced stage CH-C, hypoalbuminemia and decreased plasma values for the Fischer's ratio of branched-amino acids (BCAA; leucine, isoleucine, and valine) to aromatic amino acids (tyrosine and phenylalanine) commonly are observed. BCAA are the essential amino acids necessary for ammonium metabolism in muscle when the liver is unable to perform this function. Recent reports have shown that BCAA activates albumin synthesis in rat

Abbreviations used in this paper: BCAA, branched-chain amino acid; BCAT1, branched chain amino-acid transaminase 1; CH-C, chronic hepatitis C; ChIP, chromatin immunoprecipitation; DMEM, Dulbecco's modified Eagle medium; FBE, Foxo binding element; FBEmut, Foxo binding element mutant; FoxO, forkhead box, subgroup O; GLuc, Gaussia luciferase; IFN, interferon; IL, interleukin; ISG, interferon-stimulated genes; mTOR, mammalian target of rapamycin; mTORC1, mammalian target of rapamycin complex 1; NR, no response; PCR, polymerase chain reaction; Peg, pegylated; p-mTOR, phosphorylated form of mammalian target of rapamycin; pS6K, phosphorylated form of p70 S6 protein kinase; pSTAT1, phosphorylated form of signal transducer and activator of transcription 1; Raptor, regulatory associated protein of mTOR; RBV, ribavirin; S6K, p70 S6 protein kinase; siRNA, small interfering RNA; SVR, sustained viral response; TR, transient response.

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primary hepatocytes[6] and cirrhotic rat liver[7] through mammalian target of rapamycin (mTOR) signaling, a central regulator of protein synthesis, by sensing nutrient conditions.[8] Thus, peripheral amino acid composition is closely related to signaling pathways in the liver.

In addition to metabolic aspects, recent reports have elucidated new functional roles for mTOR in the IFN signaling pathway. Targeted disruptions of tuberous sclerosis 2 and eukaryotic translation initiation factor 4E binding protein 1, which both inhibit mTOR complex 1 (mTORC1) signaling, substantially enhanced IFN-alfa–dependent antiviral responses.[9,10] Therefore, mTORC1 signaling might be involved in the antiviral response as well as in metabolic processes. However, these issues have not yet been addressed in terms of IFN treatment for CH-C. In the present study, therefore, we evaluated the clinical relevance of the nutritional state of the liver, as estimated by the plasma Fischer's ratio, on Peg-IFN and RBV combination therapy. We also evaluated antiviral signaling induced by BCAA using an in vitro HCV replication system.

Materials and Methods

Patients

A total of 168 patients with CH-C at the Graduate School of Medicine at Kanazawa University Hospital (Kanazawa, Japan) and its related hospitals in Japan (Table 1, Supplementary Table 1) were evaluated in the present study. The clinical characteristics of these patients have been described previously.[4] All patients were administered Peg-IFN-alfa 2b (Schering-Plough K.K., Tokyo, Japan) and RBV combination therapy for 48 weeks. The definition of the treatment response was as follows: sustained viral response (SVR), clearance of HCV viremia 24 weeks after the cessation of therapy; transient response (TR), no detectable HCV viremia at the cessation of therapy but relapse during the follow-up period; and no response (NR). Genetic variation of the IL-28B polymorphism at rs8099917 was evaluated in all patients using TaqMan Pre-Designed SNP Genotyping Assays (Applied Biosystems, Carlsbad, CA) as described previously.[4] Gene expression profiling in the liver was performed in 91 patients using the Affymetrix Human 133 Plus 2.0 microarray chip (Affymetrix, Santa Clara, CA) as described previously (Supplementary Table 1).[4]

Plasma Amino Acid Analysis

Amino acid concentrations in plasma samples were measured by high-performance liquid chromatography-electrospray ionization–mass spectrometry, followed by derivatization.[11] Detailed experimental procedures are described in the Supplementary Materials and Methods section.

Culture Medium

Huh-7 and Huh-7.5 cells (kindly provided by Professor C. M. Rice, Rockefeller University, New York, NY) were maintained in Dulbecco's modified Eagle medium (DMEM; Gibco BRL, Gaithersburg, MD) containing 10% fetal bovine serum and 1% penicillin/streptomycin (normal medium). Amino acid-free medium (ZERO medium) was prepared by mixing 5.81 g nutrition-free DMEM (Nacalai Tesque, Kyoto, Japan), 1.85 g $NaHCO_3$, 1 g glucose, and 0.5 mL 1M (mol/L) sodium pyruvate in 500 mL Milli-Q water, then sterilizing with a 0.22-μm filter (Millipore, Billerica, MA). Low amino acid media ($\times$1/5, $\times$1/10, $\times$1/30, and $\times$1/100 DMEM) were prepared by diluting $\times$1 DMEM with ZERO medium. Powdered BCAA (leucine-isoleucine-valine, 2:1:1.2) (Ajinomoto Pharma, Tokyo, Japan) was freshly dissolved with distilled water at 100 mmol/L, then applied to cultured medium at 2 mmol/L, 4 mmol/L, or 8 mmol/L.

Western Blotting and Immunofluorescence Staining

A total of 1.5 $\times$ 10^5 Huh-7 cells were seeded in normal medium 24 hours before performing the experiments. The medium was changed to low-amino-acid medium and maintained for up to 24 hours. Western blotting was performed as previously described.[12] Cells were washed in phosphate-buffered saline (PBS) and lysed in RIPA buffer containing complete Protease Inhibitor Cocktail and PhosSTOP (Roche Applied Science, Indianapolis, IN). The membranes were blocked in Blocking One-P (Nacalai Tesque). The antibodies used for Western blotting are summarized in the Supplementary Materials and Methods section.

For immunofluorescence staining, cells were fixed with 4% paraformaldehyde in PBS, then permeabilized with 0.1% Triton-X 100 in PBS. The primary anti-forkhead box O (Foxo)3a antibody (Abcam, Cambridge, MA) was used at a final concentration of 2 μg/mL in PBS containing 2% fetal bovine serum at 4°C for 16 hours. Incubation with the Alexa Fluor 488–conjugated secondary antibody (Invitrogen, Carlsbad, CA) at a 500-fold dilution in PBS containing 3% fetal bovine serum antibody was performed for 4 hours, and cells were stained with Hoechst 33258 to visualize nuclear DNA (Vector Laboratories, Burlingame, CA).

Quantitative Real-Time Detection Polymerase Chain Reaction

A total of 1.5 $\times$ 10^5 Huh-7 cells were seeded in normal medium 24 hours before performing the experiments. The medium was changed to low-amino-acid medium, to which IFN-alfa and/or BCAA was added, and maintained for 24 hours. Rapamycin treatment (100 nmol/L) was performed for 30 minutes in normal medium before a medium change. RNA was isolated using TriPure isolation reagent (Roche Applied Science), and complementary DNA (cDNA) was synthesized using the High Capacity cDNA reverse transcription kit (Applied Biosystems, Carlsbad, CA). Real-time detection polymerase chain reaction (PCR) was performed using the 7500 Real-Time PCR System (Applied Biosystems) and Power SYBR Green PCR Master Mix (Applied Biosystems) containing specific primers according to the manufacturer's