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Prolonged recurrence-free survival following OK432-stimulated dendritic cell transfer into hepatocellular carcinoma during transarterial embolization

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Summary

Despite curative locoregional treatments for hepatocellular carcinoma (HCC), tumour recurrence rates remain high. The current study was designed to assess the safety and bioactivity of infusion of dendritic cells (DCs) stimulated with OK432, a streptococcus-derived anti-cancer immunotherapeutic agent, into tumour tissues following transcatheter hepatic arterial embolization (TAE) treatment in patients with HCC. DCs were derived from peripheral blood monocytes of patients with hepatitis C virus-related cirrhosis and HCC in the presence of interleukin (IL)-4 and granulocyte-macrophage colony-stimulating factor and stimulated with 0·1 KE/ml OK432 for 2 days. Thirteen patients were administered with 5×10^6 of DCs through arterial catheter during the procedures of TAE treatment on day 7. The immunomodulatory effects and clinical responses were evaluated in comparison with a group of 22 historical controls treated with TAE but without DC transfer. OK432 stimulation of immature DCs promoted their maturation towards cells with activated phenotypes, high expression of a homing receptor, fairly well-preserved phagocytic capacity, greatly enhanced cytokine production and effective tumoricidal activity. Administration of OK432-stimulated DCs to patients was found to be feasible and safe. Kaplan–Meier analysis revealed prolonged recurrence-free survival of patients treated in this manner compared with the historical controls ($P = 0{\cdot}046$, log-rank test). The bioactivity of the transferred DCs was reflected in higher serum concentrations of the cytokines IL-9, IL-15 and tumour necrosis factor-α and the chemokines CCL4 and CCL11. Collectively, this study suggests that a DC-based, active immunotherapeutic strategy in combination with locoregional treatments exerts beneficial anti-tumour effects against liver cancer.

Keywords: dendritic cells, hepatocellular carcinoma, immunotherapy, recurrence-free survival, transcatheter hepatic arterial embolization

Introduction

Many locoregional therapeutic approaches including surgical resection, radiofrequency ablation (RFA) and transcatheter hepatic arterial embolization (TAE) have been taken in the search for curative treatments of hepatocellular carcinoma (HCC). Despite these efforts, tumour recurrence rates remain high [1,2], probably because active hepatitis and cirrhosis in the surrounding non-tumour liver tissues causes *de novo* development of HCC [3,4]. One strategy to reduce tumour recurrence is to enhance anti-tumour immune responses that may induce sufficient inhibitory effects to prevent tumour cell growth and survival [5,6]. Dendritic cells (DCs) are the most potent type of antigen-presenting cells in the human body, and are involved in the regulation of both innate and adaptive immune responses [7]. DC-based immunotherapies are believed to contribute to the eradication of residual and recurrent tumour cells.

To enhance tumour antigen presentation to T lymphocytes, DCs have been transferred with major histocompatibility complex (MHC) class I and class II genes [8] and co-stimulatory molecules, e.g. CD40, CD80 and CD86 [9,10], and loaded with tumour-associated antigens, including tumour lysates, peptides and RNA transfection [11]. To induce natural killer (NK) and natural killer T (NK T) cell activation, DCs have been stimulated and modified to

Table 1. Patient characteristics.

Patient no.	Gender	Age (years)	HLA	TNM stages	No. of tumours	Largest tumour (mm)	Child–Pugh	KPS	Post-TAE Rx
1	M	60	A11 A33	III	5	35	B	100	RFA
2	M	57	A11 A24	III	1	21	B	100	RFA
3	M	57	A11 A31	III	2	39	B	100	RFA
4	M	77	A2 A24	III	2	35	A	100	RFA
5	F	83	A11 A24	III	3	29	B	100	RFA
6	F	74	A2 A24	II	1	35	A	100	RFA
7	F	72	A24 A33	III	3	41	B	100	RFA
8	F	65	A2 A11	II	4	12	B	100	RFA
9	M	71	A2 A11	II	4	16	A	100	RFA
10	M	79	A11 A24	III	2	40	A	100	RFA
11	M	71	A2 A24	II	1	28	A	100	RFA
12	M	56	A2 A26	III	2	25	B	100	RFA
13	M	64	A2 A33	III	2	37	B	100	RFA

M, male; F, female; TNM, tumour–node–metastasis; Child–Pugh, Child–Pugh classification; KPS, Karnofsky performance scores; TAE, transcatheter arterial embolization; Rx, treatment; HCC, hepatocellular carcinoma; HLA, human leucocyte antigen; RFA, percutaneous radiofrequency ablation.

produce larger amounts of cytokines, e.g. interleukin (IL)-12, IL-18 and type I interferons (IFNs)[10,12]. Furthermore, DC migration into secondary lymphoid organs could be induced by expression of chemokine genes, e.g. C-C chemokine receptor-7 (CCR7) [13], and by maturation using inflammatory cytokines [14], matrix metalloproteinases and Toll-like receptor (TLR) ligands [15].

DCs stimulated with OK432, a penicillin-inactivated and lyophilized preparation of *Streptococcus pyrogenes*, were suggested recently to produce large amounts of T helper type 1 (Th1) cytokines, including IL-12 and IFN-γ and enhance cytotoxic T lymphocyte activity compared to a standard mixture of cytokines [tumour necrosis factor-α (TNF-α), IL-1β, IL-6 and prostaglandin E_2 (PGE_2)] [16]. Furthermore, because OK432 modulates DC maturation through TLR-4 and the β_2 integrin system [16,17] and TLR-4-stimulated DCs can abrogate the activity of regulatory T cells [18], OK432-stimulated DCs may contribute to the induction of anti-tumour immune responses partly by reducing the activity of suppressor cells. Recently, in addition to the orchestration of immune responses, OK432-activated DCs have themselves been shown to mediate strong, specific cytotoxicity towards tumour cells via CD40/CD40 ligand interactions [19].

We have reported recently that combination therapy using TAE together with immature DC infusion is safe for patients with cirrhosis and HCC [20]. DCs were infused precisely into tumour tissues and contributed to the recruitment and activation of immune cells *in situ*. However, this approach by itself yielded limited anti-tumour effects due probably to insufficient stimulation of immature DCs (the preparation of which seems closely related to therapeutic outcome [21,22]). The current study was designed to assess the safety and bioactivity of OK432-stimulated DC infusion into tumour tissues following TAE treatment in patients with cirrhosis and HCC. In addition to documenting the safety of this approach, we found that patients treated with OK432-stimulated DCs displayed unique cytokine and chemokine profiles and, most importantly, experienced prolonged recurrence-free survival.

Patients and methods

Patients

Inclusion criteria were a radiological diagnosis of primary HCC by computed tomography (CT) angiography, hepatitis C virus (HCV)-related HCC, a Karnofsky score of $\geq$ 70%, an age of $\geq$ 20 years, informed consent and the following normal baseline haematological parameters (within 1 week before DC administration): haemoglobin $\geq$ 8·5 g/dl; white cell count $\geq$ 2000/μl; platelet count $\geq$ 50 000/μl; creatinine $<$ 1·5 mg/dl and liver damage A or B [23].

Exclusion criteria included severe cardiac, renal, pulmonary, haematological or other systemic disease associated with a discontinuation risk; human immunodeficiency virus (HIV) infection; prior history of other malignancies; history of surgery, chemotherapy or radiation therapy within 4 weeks; immunological disorders including splenectomy and radiation to the spleen; corticosteroid or anti-histamine therapy; current lactation; pregnancy; history of organ transplantation; or difficulty in follow-up.

Thirteen patients (four women and nine men) presenting at Kanazawa University Hospital between March 2004 and June 2006 were enrolled into the study, with an age range from 56 to 83 years (Table 1). Patients with verified radiological diagnoses of HCC stage II or more were eligible and enrolled in this study. In addition, a group of 22 historical controls (nine women and 13 men) treated with TAE without DC administration between July 2000 and September 2007 was included in this study. All patients received RFA therapy to increase the locoregional effects 1 week later [24].

They 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, and tumour recurrences were followed for up to 360 days. The Institutional Review Board reviewed and approved the study protocol. This study complied with ethical standards outlined in the Declaration of Helsinki. Adverse events were monitored for 1 month after the DC infusion in terms of fever, vomiting, abdominal pain, encephalopathy, myalgia, ascites, gastrointestinal disorder, bleeding, hepatic abscess and autoimmune diseases.

Preparation and injection of autologous DCs

DCs were generated from blood monocyte precursors, as reported previously [25]. Briefly, peripheral blood mononuclear cells (PBMCs) were isolated by centrifugation in Lymphoprep™ Tubes (Nycomed, Roskilde, Denmark). For generating DCs, PBMCs were plated in six-well tissue culture dishes (Costar, Cambridge, MA, USA) at $1{\cdot}4 \times 10^7$ cells in 2 ml per well and allowed to adhere to plastic for 2 h. Adherent cells were cultured in serum-free media (GMP CellGro® DC Medium; CellGro, Manassas, VA, USA) with 50 ng/ml recombinant human IL-4 (GMP grade; CellGro®) and 100 ng/ml recombinant human granulocyte–macrophage colony-stimulating factor (GM-CSF) (GMP grade; CellGro®) for 5 days to generate immature DC, and matured for a further 2 days in 0·1 KE/ml OK432 (Chugai Pharmaceuticals, Tokyo, Japan) to generate OK-DC. On day 7, the cells were harvested for injection, 5×10^6 cells were suspended in 5 ml normal saline containing 1% autologous plasma, mixed with absorbable gelatin sponge (Gelfoam; Pharmacia & Upjohn, Peapack, NJ, USA) and infused through an arterial catheter following Lipiodol (iodized oil) (Lipiodol Ultrafluide, Laboratoire Guerbet, Aulnay-Sous-Bois, France) injection during selective TAE therapy. Release criteria for DCs were viability > 80%, purity > 30%, negative Gram stain and endotoxin polymerase chain reaction (PCR) and negative in process cultures from samples sent 48 h before release. All products met all release criteria, and the DCs had a typical phenotype of $CD14^-$ and human leucocyte antigen (HLA)-DR^+.

Flow cytometry analysis

The DC preparation was assessed by staining with the following monoclonal antibodies for 30 min on ice: anti-lineage cocktail 1 (lin-1; CD3, CD14, CD16, CD19, CD20 and CD56)-fluorescein isothiocyanate (FITC), anti-HLA-DR-peridinin chlorophyll protein (PerCP) (L243), anti-CCR7-phycoerythrin (PE) (3D12) (BD PharMingen, San Diego, CA, USA), anti-CD80-PE (MAB104), anti-CD83-PE (HB15a) and anti-CD86-PE (HA5.2B7) (Beckman Coulter, Fullerton, CA, USA). Cells were analysed on a fluorescence activated cell sorter (FACS0Calibur™ flow cytometer. Data analysis was performed with CELLQuest™ software (Becton Dickinson, San Jose, CA, USA).

DC phagocytosis

Immature DCs and OK432-stimulated DCs were incubated with 1 mg/ml FITC dextran (Sigma-Aldrich, St Louis, MO, USA) for 30 min at 37°C and the cells were washed three times in FACS buffer before cell acquisition using a FACS-Calibur™ cytometer. Control DCs (not incubated with FITC dextran) were acquired at the same time to allow background levels of fluorescence to be determined.

Enzyme-linked immunosorbent assay (ELISA)

DCs were seeded at 200 000 cells/ml, and supernatant collected after 48 h. IL-12p40 and IFN-γ were detected using matched paired antibodies (BD Pharmingen) following standard protocols.

Cytotoxicity assays

The ability of DCs to exert cytotoxicity was assessed in a standard ^{51}Cr release assay [19]. We used the HCC cell lines Hep3B and PLC/PRF/5 [American Type Culture Collection (ATCC), Manassas, VA, USA] and a lymphoblastoid cell line T2 that expresses HLA-A*0201 (ATCC) as target cells. Target cells were labelled with ^{51}Cr. In a 96-well plate, $2{\cdot}5 \times 10^3$ target cells per well were incubated with DCs for 8 h at different effector/target (E/T) ratios in triplicate. Percentage of specific lysis was calculated as follows: (experimental release – spontaneous release)/(maximum release – spontaneous release) × 100. Spontaneous release was always < 20% of the total.

NK cell activity

NK cell cytotoxicity against K562 erythroleukemia target cells was measured by using ^{51}Cr-release assay, according to previously published methods [26], with PBMCs obtained from the patients. All experiments were performed in triplicate. Percentage of cytotoxicity was calculated as follows: {[experimental counts per minute (cpm) – spontaneous cpm]/[total cpm – spontaneous cpm]} × 100.

Intracellular cytokine expression

Freshly isolated PBMCs were stimulated with 25 ng/ml phorbol 12-myristate 13-acetate (PMA; Sigma-Aldrich) and 1 μg/ml ionomycin (Sigma-Aldrich) at 37°C in humidified 7% CO_2 for 4 h. To block cytokine secretion, brefeldin A (Sigma) [27] was added to a final concentration of 10 μg/ml. After addition of stimuli, the surface staining was performed with anti-CD4-PC5 (13B8·2), anti-CD8-PerCP (SK1) and anti-CD56-PC5 (N901) (Beckman

Coulter). Subsequently, the cells were permeabilized, stained for intracellular IFN-γ and IL-4 using the FastImmune™ system (BD Pharmingen), resuspended in phosphate-buffered saline (PBS) containing 1% paraformaldehyde (PFA), and analysed on a flow cytometer (≈ 10 000 gated events acquired per sample).

IFN-γ enzyme-linked immunospot (ELISPOT) assay

ELISPOT assays were performed as described previously with the following modifications [28–30]. HLA-A24 restricted peptide epitopes, squamous cell carcinoma antigen recognized by T cells 2 $(SART2)_{899}$ (SYTRLFLIL), $SART3_{109}$ (VYDYNCHVDL), multi-drug resistance protein 3 $(MRP3)_{765}$ (VYSDADIFL), $MRP3_{503}$ (LYAWEPSFL), $MRP3_{692}$ (AYVPQQAWI), alpha-fetoprotein $(AFP)_{403}$ (KYIQESQAL), AFP_{434} (AYTKKAPQL), AFP_{357} (EYSRRHPQL), human telomerase reverse transcriptase $(hTERT)_{167}$ (AYQVCGPPL) (unpublished), $hTERT_{461}$ (VYGFVRACL) and $hTERT_{324}$ (VYAETKHFL) were used in this study. Negative controls consisted of an HIV envelope-derived peptide ($HIVenv_{584}$). Positive controls consisted of 10 ng/ml PMA (Sigma) or a CMV pp65-derived peptide ($CMVpp65_{328}$). The coloured spots were counted with a KS ELISPOT Reader (Zeiss, Tokyo, Japan). 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. Responses were considered positive if more than 10 specific spots were detected and if the number of spots in the presence of antigen was at least twofold greater than the number of spots in the absence of antigen.

Cytokine and chemokine profiling

Serum cytokine and chemokine levels were measured using the Bioplex assay (Bio-Rad, Hercules, CA, USA). Briefly, frozen serum samples were thawed at room temperature, diluted 1:4 in sample diluents, and 50 µl aliquots of diluted sample were added in duplicate to the wells of a 96-well microtitre plate containing the coated beads for a validated panel of 27 human cytokines and chemokines (cytokine 27-plex antibody bead kit) according to the manufacturer's instructions. These included 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, interferon gamma-induced protein (IP)-10, monocyte chemoattractant protein (MCP)-1, MIP-1α, MIP-1β, platelet-derived growth factor (PDGF)-BB, regulated upon activation normal T cell-expressed and secreted (RANTES), 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 performed using Bio-Plex Manager software version 4.1.1.

Arginase activity

Serum samples were tested for arginase activity by conversion of L-arginine to L-ornithine [31] using a kit supplied by the manufacturer (BioAssay Systems, Hayward, CA, USA). Briefly, sera were treated with a membrane filter (Millipore, Billerica, MA, USA) to remove urea, combined with the sample buffer in wells of a 96-well plate, and incubated at 37°C for 2 h. Subsequently, the urea reagent was added to stop the arginase reaction. The colour produced was read at 520 nm using a microtitre plate reader.

Statistical analysis

Results are expressed as means ± standard deviation (s.d.). Differences between groups were analysed for statistical significance by the Mann–Whitney *U*-test. Qualitative variables were compared by means of Fisher's exact test. The estimated probability of tumour recurrence-free survival was determined using the Kaplan–Meier method. The Mantel–Cox log-rank test was used to compare curves between groups. Any *P*-values less than 0·05 were considered statistically significant. All statistical tests were two-sided.

Results

Preparation of OK432-stimulated DCs

Adherent cells isolated from PBMCs of patients with cirrhosis and HCC (Table 1) were differentiated into DCs in the presence of IL-4 and GM-CSF. The cells were stimulated with 0·1 KE/ml OK432 for 3 days; 54·6 ± 9·5% (mean ± s.d.; $n = 13$) of OK432-stimulated cells showed high levels of MHC class II (HLA-DR) and the absence of lineage markers including CD3, CD14, CD16, CD19, CD20 and CD56, in which 30·9 ± 14·2% were CD11c-positive (myeloid DC subset) and 14·8 ± 11·2 were CD123-positive (plasmacytoid DC subset), consistent with our previous observations [20]. As reported [32,33], greater proportions of the cells developed high levels of expression of the co-stimulatory molecules B7-1 (CD80) and B7-2 (CD86) and an activation marker (CD83) compared to DCs prepared without OK432 stimulation (Fig. 1a). Furthermore, the chemokine receptor CCR7 which leads to homing to lymph nodes [13,34] was also induced following OK432 stimulation.

To evaluate the endocytic and phagocytic ability of the OK432-stimulated cells, uptake of FITC-dextran was quantitated by flow cytometry (Fig. 1b). The cells showed lower levels of uptake due to maturation compared to DCs prepared without OK432 stimulation, while the OK432-stimulated cells derived from HCC patients preserved a moderate uptake capacity. As expected, the OK432-stimulated cells produced large amounts of cytokines IL-12 and IFN-γ (Fig. 1c). In addition, they displayed high cyto-

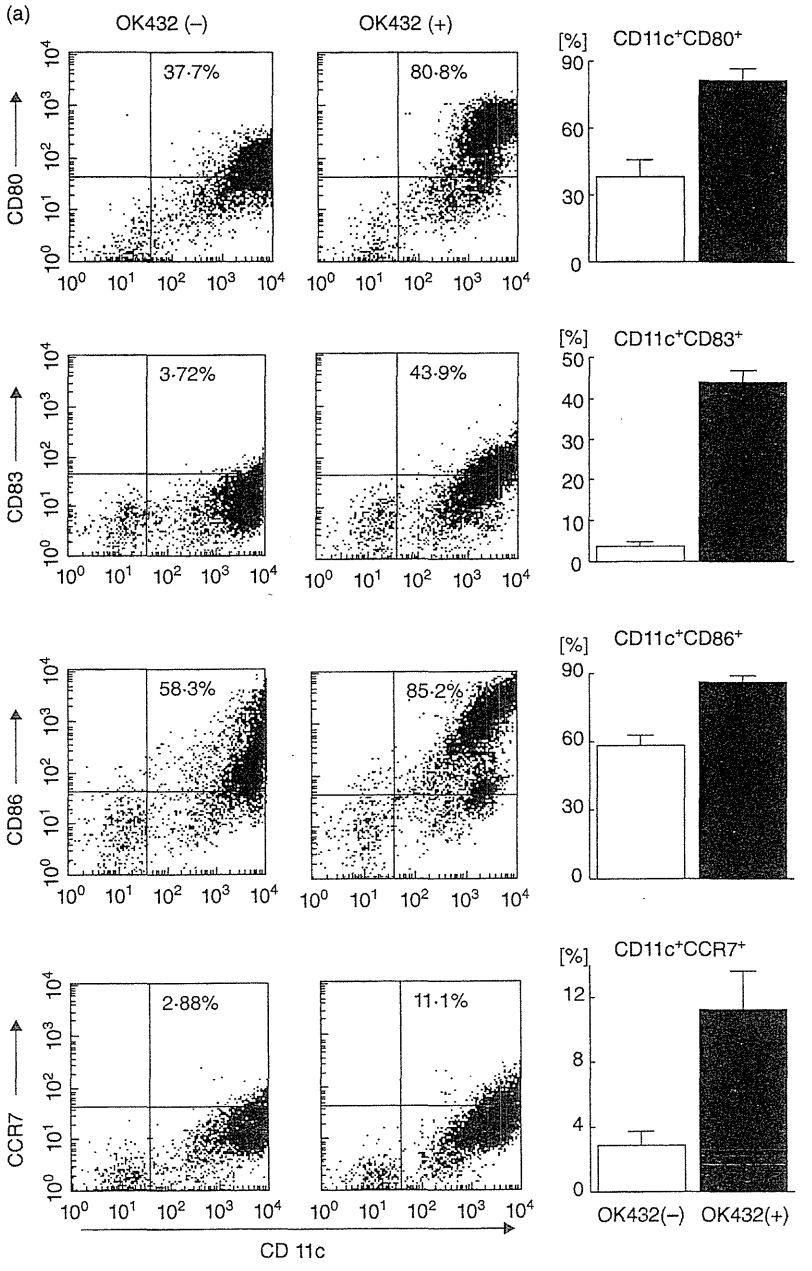

Fig. 1. Effects of OK432 stimulation on the properties of dendritic cells (DCs) generated from blood monocyte precursors in patients with cirrhosis and hepatocellular carcinoma (HCC) ($n = 13$). (a) Lineage cocktail 1 (lin 1^-) human leucocyte antigen D-related (HLA-DR^-) subsets with [OK432($^+$)] and without [OK432($^-$)] stimulation were analysed for surface expression of CD80, CD83, CD86 and CCR7. Dot plots of a representative case are shown in the left-hand panel. Mean percentages [±standard deviation (s.d.)] of positive cells are indicated in the right-hand panel. OK432 stimulation resulted in the expression of high levels of CD80, CD83, CD86 and CCR7 in the lin 1^-human leucocyte antigen D-related (HLA-DR^-) DC subset. (b) DC subsets with and without OK432 stimulation were incubated with fluorescein isothiocyanate (FITC) dextran for 30 min and the uptake was determined by flow cytometry. A representative analysis is shown in the upper panel. Mean fluorescence intensities (MFIs) (±s.d.) of the positive cells are indicated in the lower panel. OK432-stimulated cells showed lower levels of uptake due to maturation. (c) DC supernatants were harvested and the concentrations of interleukin (IL)-12 and interferon (IFN)-γ measured by enzyme-linked immunosorbent assay (ELISA). OK432-stimulated cells produced large amounts of the cytokines. The data indicate means ± s.d. of the groups with and without the stimulation. All comparisons in (a–c) [OK432(+) *versus* OK432($^-$)] were statistically significant by the Mann-Whitney *U*-test ($P < 0{\cdot}005$). (d) Tumoricidal activity of DCs assessed by incubation with ^{51}Cr-labelled Hep3B, PLC/PRF/5 and T2 targets for 8 h at the indicated effector/target (E/T) cell ratios. OK432-stimulated cells displayed high cytotoxic activity against the target cells. The results are representative of the cases studied.

toxic activity against HCC cell lines (Hep3B and PLC/PRF/5) and a lymphoblastoid cell line (T2) although DCs without OK432 stimulation lysed none of the target cells to any great degree (Fig. 1d). Taken together, these results demonstrate that OK432 stimulation of IL-4 and GM-CSF-induced immature DCs derived from HCC patients promoted their maturation towards cells with activated phenotypes, high expression of a homing receptor, fairly well-preserved phagocytic capacity, greatly enhanced cytokine production and effective tumoricidal activity, consistent with previous observations [16,19].

Safety of OK432-stimulated DC administration

Prior to the administration of OK432-stimulated DCs to patients, the cells were confirmed to be safe in athymic nude mice to which 100-fold cell numbers/weight were injected subcutaneously (data not shown). Subsequently, OK432-stimulated DC administration was performed during TAE therapy in humans, in which DCs were mixed together with absorbable gelatin sponge (Gelfoam) and infused through an arterial catheter following iodized oil (Lipiodol) injection, as reported previously [20]. Adverse events were

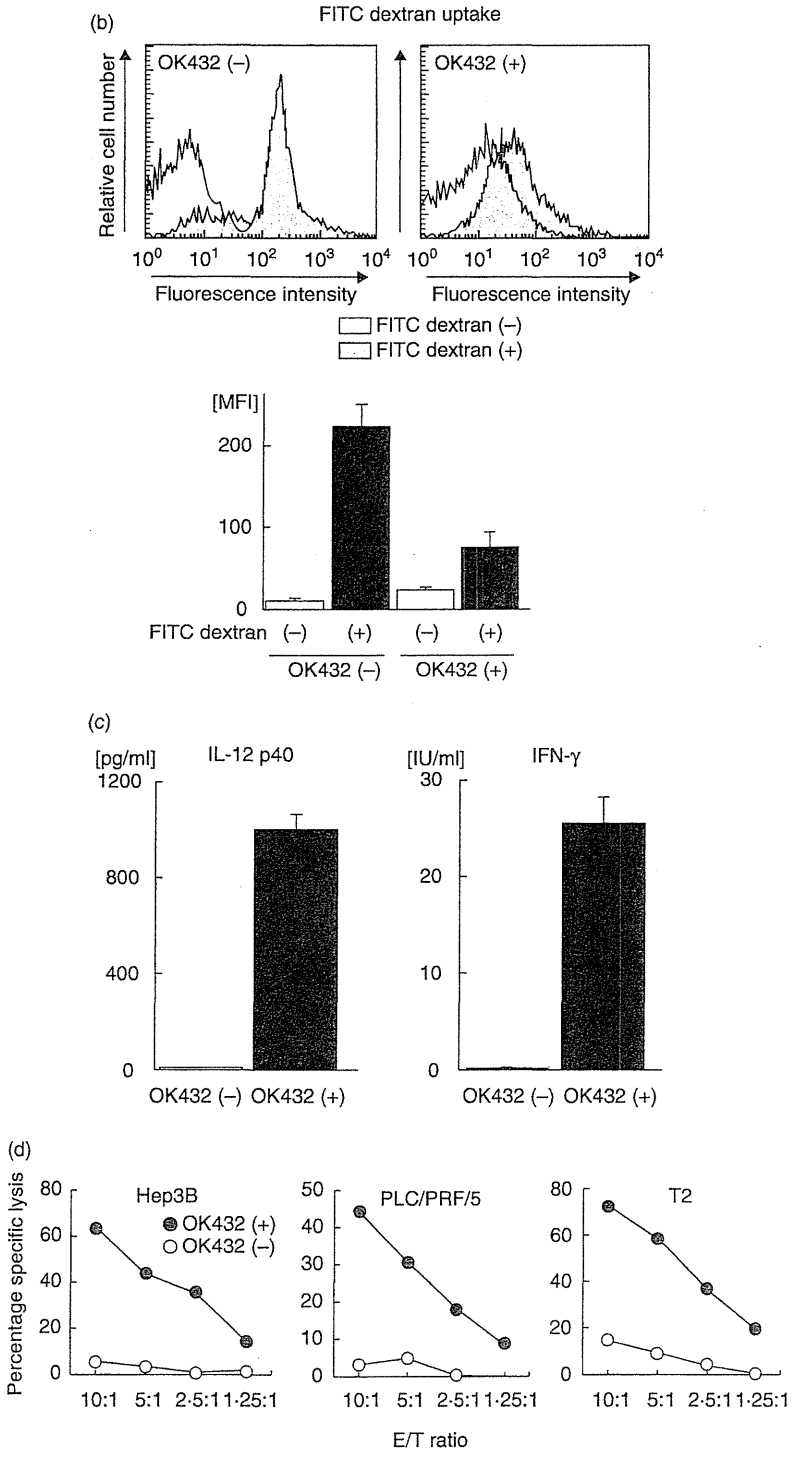

Fig. 1. *Continued*

monitored clinically and biochemically after DC infusion (Table 2). A larger proportion (12 of 13) of the patients were complicated with high fever compared to those treated previously with immature DCs (five of 10) [20], due probably to the proinflammatory responses induced by OK432-stimulated DCs. However, there were no grades III or IV National Cancer Institute Common Toxicity Criteria adverse events, including vomiting, abdominal pain, encephalopathy, myalgia, ascites, gastrointestinal disorders, bleeding, hepatic abscess or autoimmune diseases associated with DC infusion and TAE in this study. There was also no clinical or serological evidence of hepatic failure or autoimmune

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 ($\times 10^2/\mu l$)	34.4 ± 11.6	41.4 ± 18.9	n.s.†
Lymphocytes ($\times 10^2/\mu l$)	10.4 ± 3.6	12.4 ± 4.7	n.s.†
Platelets ($\times 10^4/\mu l$)	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.

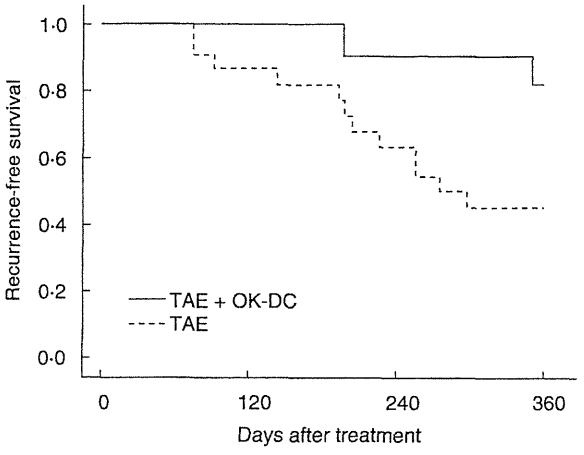

Fig. 2. Recurrence-free survival of patients treated with transcatheter hepatic arterial embolization (TAE) with [TAE + OK-stimulated dendritic cells (DC); $n = 13$] and without (TAE: historical controls; $n = 22$) OK432-stimulated DC administration. Time zero is the date of TAE. All patients underwent 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-

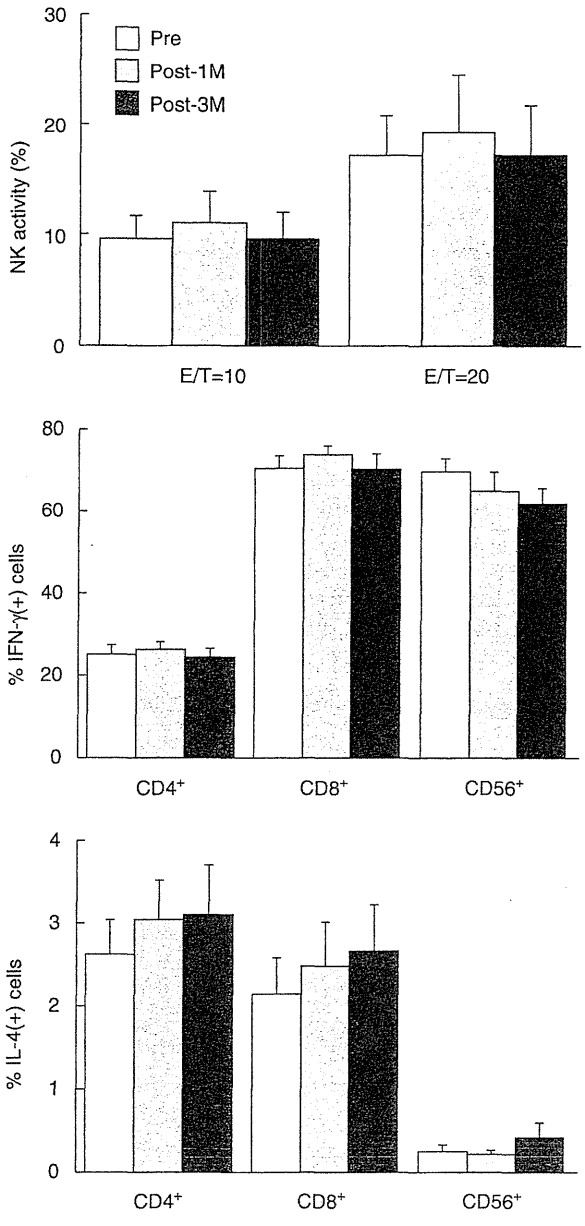

Fig. 3. Natural killer (NK) cell activity and intracellular cytokine production in peripheral blood mononuclear cells (PBMCs) of patients treated with OK432-stimulated dendritic cells (DCs) during transcatheter hepatic arterial embolization (TAE) therapy ($n = 13$). PBMCs were isolated before and 1 and 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.

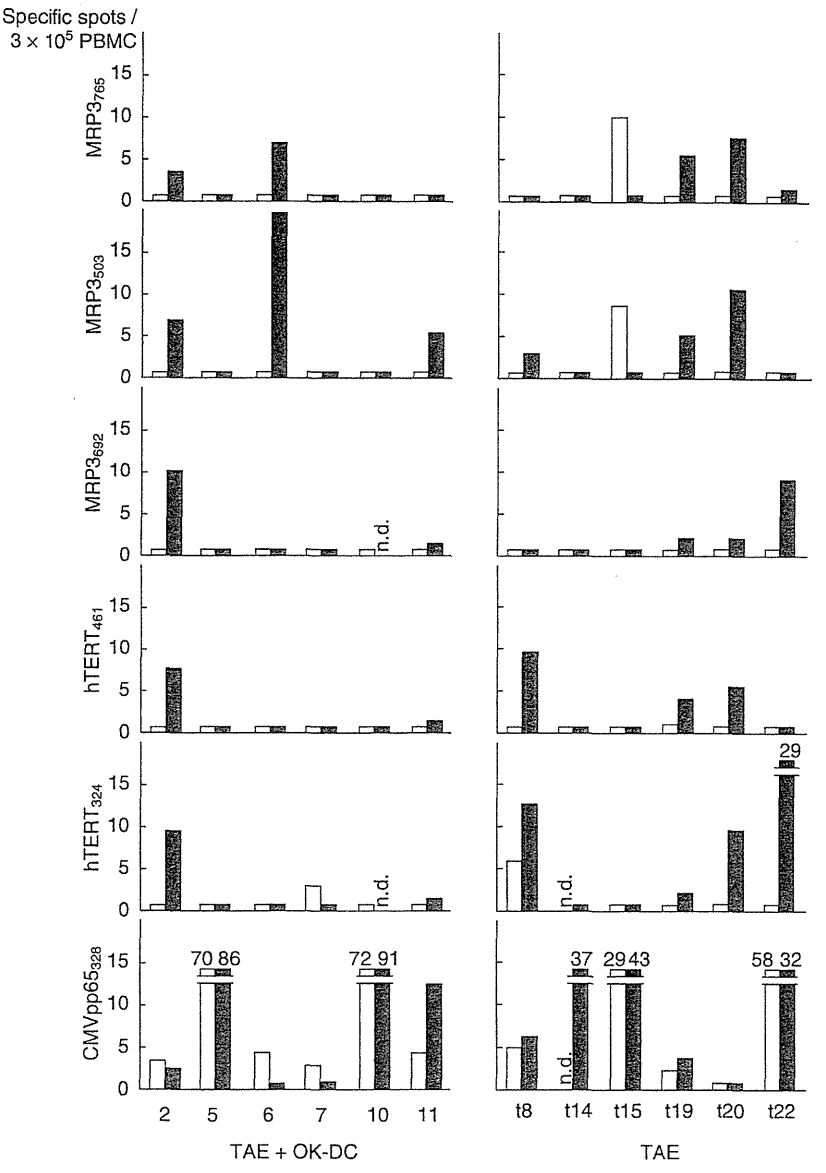

Fig. 4. Immune responses to human leucocyte antigen (HLA-DR$^-$)-A24-restricted peptide epitopes derived from tumour antigens in HLA-A24-positive patients treated with OK432-stimulated DCs during transcatheter hepatic arterial embolization (TAE) therapy (numbers 2, 5, 6, 7, 10 and 11) and HLA-A24-positive 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

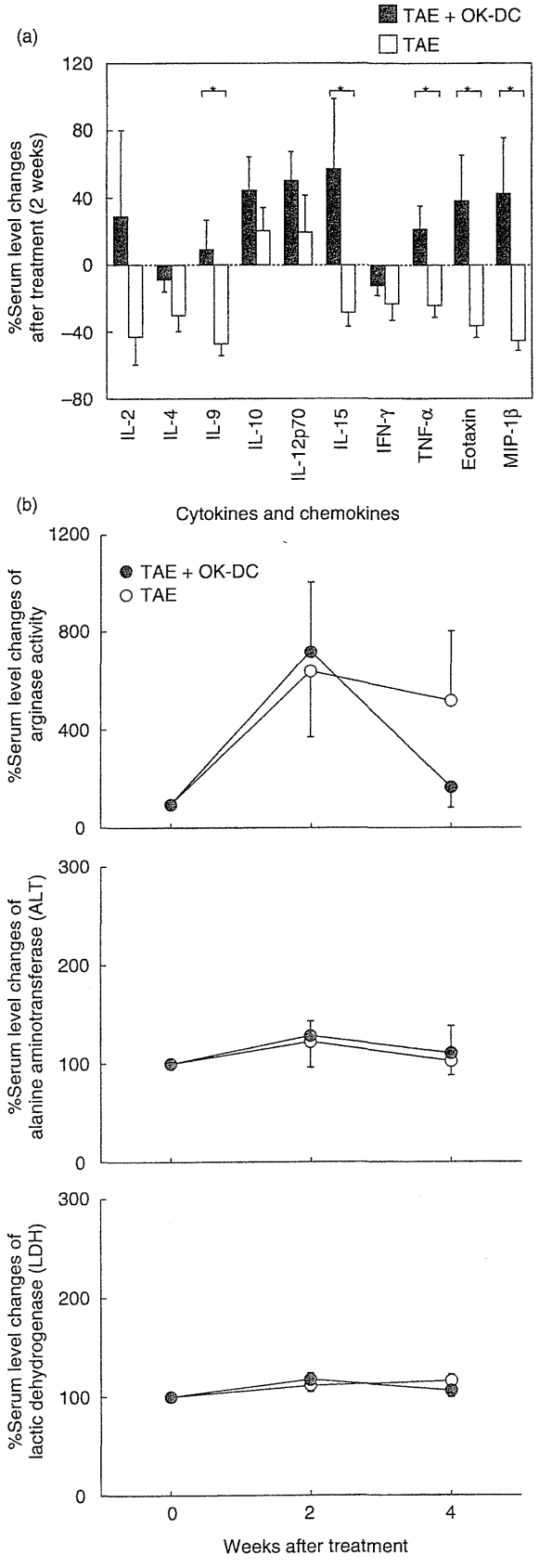

Fig. 5. Cytokine and chemokine profiling and arginase activity in sera of patients treated with OK432-stimulated dendritic cells (DCs) during transcatheter hepatic arterial embolization (TAE) therapy (TAE + OK-DC; $n = 13$) and the historical controls treated with TAE without DC transfer (TAE; $n = 22$). (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

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

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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.

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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.

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).

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.

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-

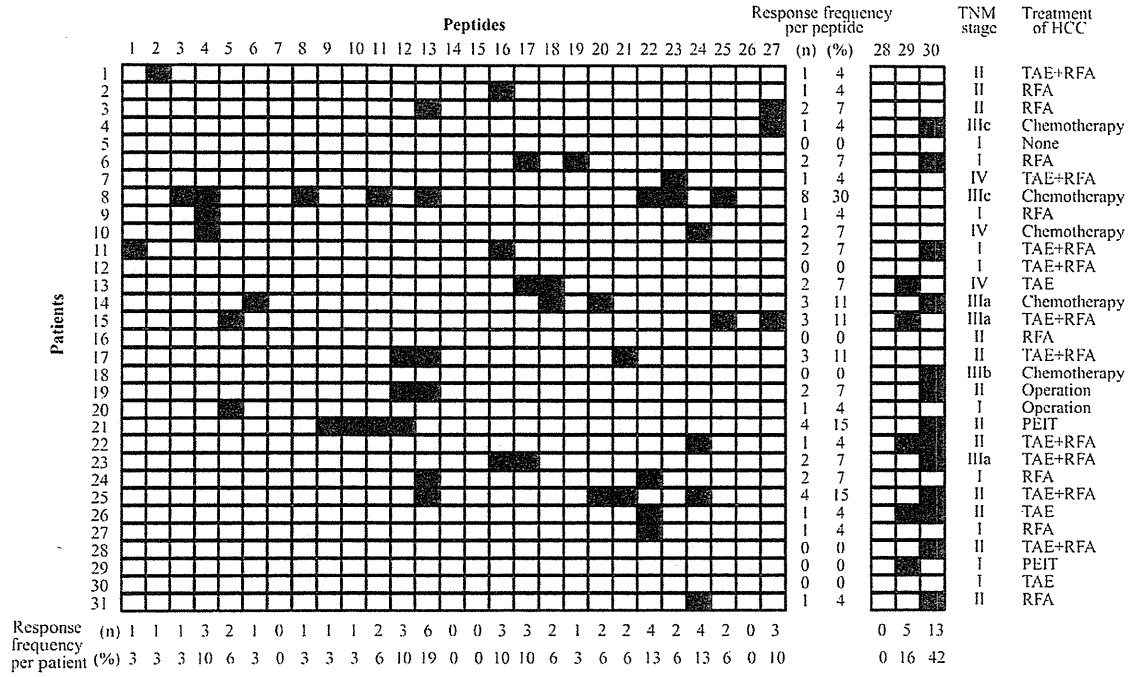

Fig. 1. TAA-, HIV-, HCV-, and CMV-derived peptide-specific T-cell responses. Results of all HCC patients examined are shown. The T-cell responses were examined by IFN-γ ELISPOT assay. Responses to peptides were considered positive if more than 10 specific spots per 300,000 PBMCs were detected and if 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

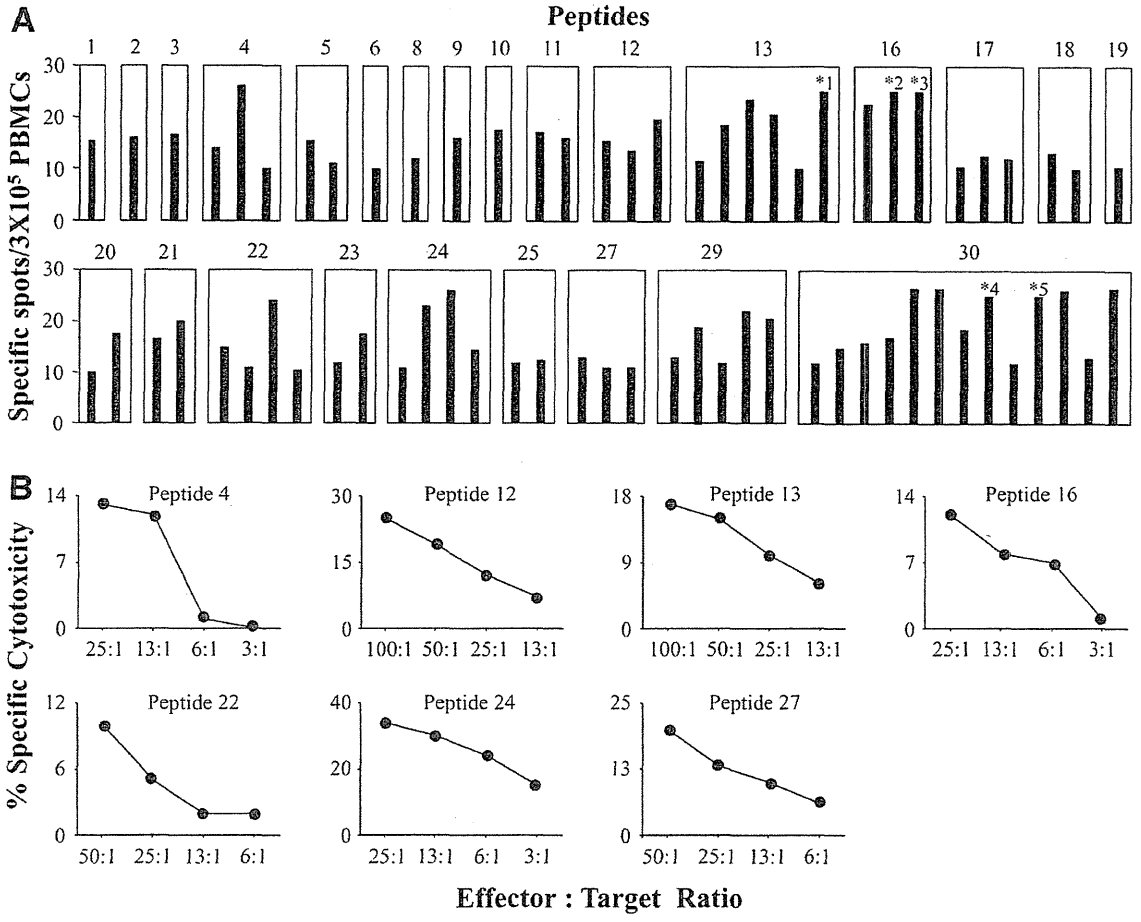

Fig. 2. Vigor of TAA-, HCV-, and CMV-derived peptide-specific T-cell responses. (A) The frequency of TAA-specific IFN-γ-producing T cells was analyzed by ELISPOT assay. Only positive responses are shown. Black bars indicate the response of one patient. *1, *2, *3, *4, and *5 denote 33, 60.5, 44, 92, 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.

A

	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%)

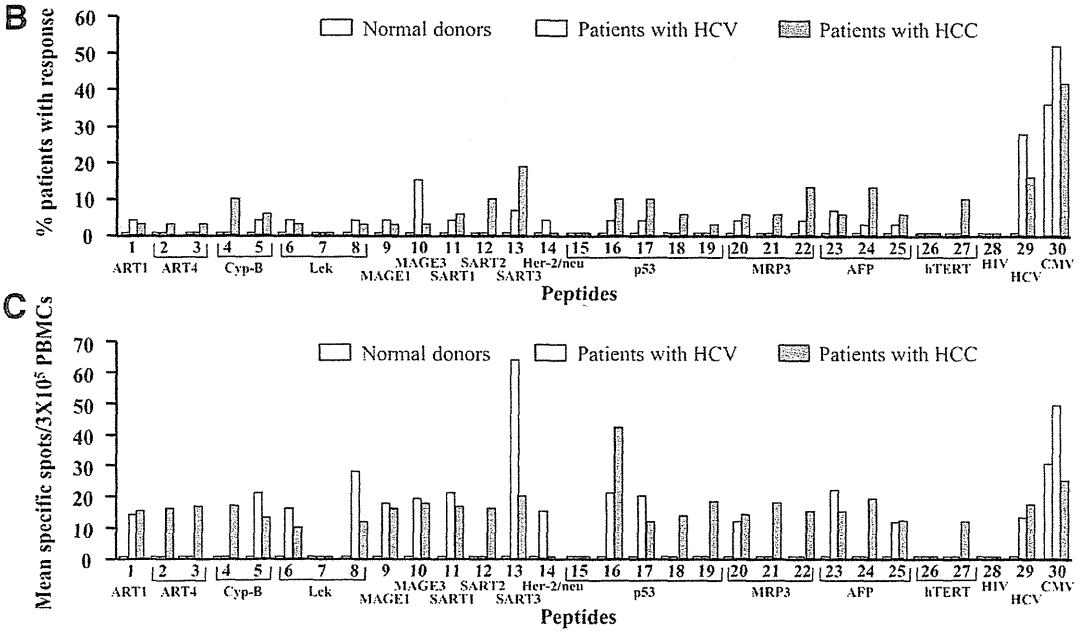

Fig. 3. Comparative analysis of TAA-, HIV-, HCV-, and CMV-derived peptide-specific T-cell responses among three groups of subjects: normal donors, patients with chronic hepatitis C not complicated by HCC, and HCC patients. (A) Summary of the number of patients with a significant IFN-γ T-cell response 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