

Figure 4. (a) Coomassie blue staining and Western blotting of culture supernatant of NIH3T3 cells transfected with pSI-*c19orf10*-FLAG. A black arrow indicates the 17-kDa *c19orf10* protein. (b) Cell proliferation assay of HLE cells supplemented with recombinant *c19orf10*-FLAG, FLAG peptides or BSA. Cell proliferation was measured in quadruplicates 72 hr after supplementation. (c) Western blotting of HLE cells supplemented with *c19orf10*-FLAG (40 ng/ml). Cells were lysed at indicated time after *c19orf10* supplementation. (d) Cell proliferation assay of HLE cells supplemented with control BSA (40 ng/ml) (white bar), *c19orf10*-FLAG (40 ng/ml) (light gray bar), *c19orf10*-FLAG (40 ng/ml) + anti-*c19orf10* antibodies (gray bar) and *c19orf10*-FLAG (40 ng/ml) + control mouse IgG (black bar).

HuH7 cells. We observed an ~50% decrease in *c19orf10* expression in Hep3B cells transfected with Si- *c19orf10* compared to the control 48 hr after transfection with statistical significance ($p < 0.0001$). In this condition, cell proliferation was suppressed to 50% compared to the control 72 hr after transfection ($p < 0.0001$) (Figs. 3c and 3d). When we performed cell cycle analysis of HuH7 cells transfected with Si-*Control* or Si-*c19orf10*, we identified an increase of G1-phase cells and a decrease of S- and G2-phase cells by *c19orf10* knockdown, suggesting that the G1 cycle arrest was caused by the knockdown of *c19orf10* (Fig. 3e).

We examined the representative MAPK/Akt pathway-associated proteins and cell cycle regulators using Western blotting 72 hr after siRNAs transfection (Fig. 3f). Interestingly, phosphorylation of c-Raf, MEK, MAPK, PI3K and pAkt was inhibited by knockdown of *c19orf10*, suggesting the involvement of *c19orf10* in the MAPK/Akt pathways. Furthermore, phosphorylation of Rb, CDK4 and CDK6 was also inhibited by knockdown of *c19orf10*, consistent with the

observation of G1 cell cycle arrest by *C19ORF10* knockdown. PTEN, p53 and phosphorylated CDC2 protein expression was not affected by knockdown of *c19orf10*.

***C19orf10* encodes the secretory protein and stimulates cell proliferation**

As the sequence of *c19orf10* suggested that it encodes a secretory protein, we transfected pSI-*c19orf10*-FLAG in NIH3T3 cells and examined the culture supernatant. Immunoprecipitation of the collected culture supernatant 48 hr after transfection using anti-FLAG antibodies indicated the existence of a 17-kDa protein (*c19orf10*), compatible with the molecular weight of the 142 amino acids protein encoded by *c19orf10* (Fig. 4a). We purified *c19orf10*-FLAG protein from the supernatant of HEK293 cells infected with Ad. *c19orf10*-FLAG using an anti-FLAG column. Supplementation of purified *c19orf10*-FLAG into the culture media for 72 hr enhanced the proliferation of HLE cells in a dose-dependent manner with statistical significance, whereas control FLAG peptides

and BSA had no effects on cell proliferation (Fig. 4b). Western blot analysis of HLE cells cultured with purified c19orf10-FLAG (40 ng/ml) or BSA control (40 ng/ml) indicated the immediate strong phosphorylation of Akt peaked 5 min after supplementation (Fig. 4c). The modest phosphorylation of GSK3 β (Ser9) and p44/42 MAPK also followed and peaked 60 min after c19orf10 supplementation. These data suggest that Akt pathway might be directly involved in the c19orf10-mediated cell proliferation signaling with the subsequent activation of MAPK pathway. Furthermore, addition of antibodies against c19orf10 to the culture media abolished the cell proliferation induced by c19orf10, whereas control IgG had no effects (Fig. 4d). Taken together, these data suggest that c19orf10 may be a growth factor overexpressed in AFP-positive HCCs and activates the Akt/MAPK pathways, potentially through the activation of an unidentified c19orf10 receptor.

Discussion

SAGE facilitates the measurement of transcripts from normal and malignant tissues in a nonbiased and highly accurate, quantitative manner. Indeed, SAGE produces a comprehensive gene expression profile without *a priori* gene sequence information, leading to the identification of novel transcripts potentially involved in the pathogenesis of human cancer.¹⁹ In our study, we identified seven SAGE tags potentially corresponding to novel genes activated in HCC. Among them, we identified the secretory protein c19orf10 activated in a subset of HCCs.

Several serum markers including AFP, DCP and Glypican 3 are currently used for the detection and/or the evaluation of the treatment for HCCs in the clinic.^{15–18,35} These markers are known as oncofetal proteins, that is, expressed in the fetus, transcriptionally suppressed in the adult organ and reactivated in the tumor. We identified that the expression of *c19orf10* positively correlated with *AFP* expression but did not correlate with the expression of *GPC3* or the biliary marker *KRT19*. As *c19orf10* was rarely detected in the normal liver, it is possible that c19orf10 is also an oncofetal protein activated in HCC. We are currently developing a system to detect serum c19orf10 in HCC patients, and the significance of the serum c19orf10 value as an HCC marker should be clarified.

Recent advancement in molecular biology has revealed the considerable diversity of transcription initiation and/or termination of genes altered in the process of carcinogenesis.

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Indeed, using 5' SAGE approach, we recently discovered the novel intronic transcripts activated in HCC.³⁶ Interestingly, when we investigated the transcription initiation of *c19orf10* using the 5' SAGE database, we identified a potential 5' splice variant initiated from the second exon of *c19orf10* (data not shown). Although we have not yet validated the presence of 5' splice variants in *c19orf10* by PCR, examination of 5' EST database also suggested the presence of the similar splice variants (GenBank Accession Number CR980295, BQ680744, BQ648461, *etc.*). Alteration of transcription initiation/termination in *c19orf10* might affect the abundance or function of c19orf10 protein, and the details of 5' splice variants in *c19orf10* should be clarified in future studies.

Molecular targeting therapy has rapidly emerged for solid tumors as well as for leukemia.^{37–39} Sorafenib is a multikinase inhibitor targeting Raf kinase in the MAPK pathway as well as VEGFR and the platelet-derived growth factor receptor.^{40,41} In our study, we identified that c19orf10 activates the MAPK and Akt/PI3K pathways and contributes to the proliferation of HCC cell lines, although we still could not discover the potential receptor of c19orf10. Development of a neutralizing c19orf10 antibody may provide novel therapeutic options for HCC patients to inhibit these signaling pathways, and its efficacy should be evaluated in the future.

Recently, c19orf10 was found to be expressed in fibroblast-like synoviocytes in the synovium using a proteomics approach.²⁹ In addition, a recent article indicated that c19orf10 was expressed in preadipocyte cells and involved in adipogenesis using two-dimensional electrophoresis mass spectrometry analysis.²⁸ Thus, c19orf10 may have pleiotropic effects on various lineages of normal organs in various developmental stages, and the clarification of its distribution and biological properties in the whole body may provide more detailed information about the function of c19orf10.

In conclusion, we have identified the protein c19orf10 that regulates the Akt/MAPK pathways and cell cycle through an unidentified mechanism in HCC. Although further studies should be conducted to detect the potential c19orf10 receptor or signaling molecules binding to c19orf10, our study suggests that c19orf10 may be a novel growth factor, a potential tumor marker and also a potential target molecule for HCC treatment.

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

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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

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 ($P = 0.046$, 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

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 pyogenes*, 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-1 β , IL-6 and prostaglandin E₂ (PGE₂)] [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 ≥ 20 years, informed consent and the following normal baseline haematological parameters (within 1 week before DC administration): haemoglobin ≥ 8.5 g/dl; white cell count $\geq 2000/\mu\text{l}$; platelet count $\geq 50\,000/\mu\text{l}$; 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 1.4×10^7 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 ⁵¹Cr 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 ⁵¹Cr. In a 96-well plate, 2.5×10^3 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 ⁵¹Cr-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% CO₂ 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 ($\approx 10\,000$ 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), SART3₁₀₉ (VYDYNCHVDL), multi-drug resistance protein 3 (MRP3)₇₆₅ (VYSDADIFL), MRP3₅₀₃ (LYAWEPSFL), MRP3₆₉₂ (AYVPQQAWI), alpha-fetoprotein (AFP)₄₀₃ (KYIQESQAL), AFP₄₃₄ (AYTKKAPQL), AFP₃₅₇ (EYSRRHPQL), human telomerase reverse transcriptase (hTERT)₁₆₇ (AYQVCGPPL) (unpublished), hTERT₄₆₁ (VYGFVRACL) and hTERT₃₂₄ (VYAETKHFLL) were used in this study. Negative controls consisted of an HIV envelope-derived peptide (HIVenv₅₈₄). Positive controls consisted of 10 ng/ml PMA (Sigma) or a CMV pp65-derived peptide (CMVpp65₃₂₈). 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 μ l 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-1 β , 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-1 α , MIP-1 β , 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 \pm 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.

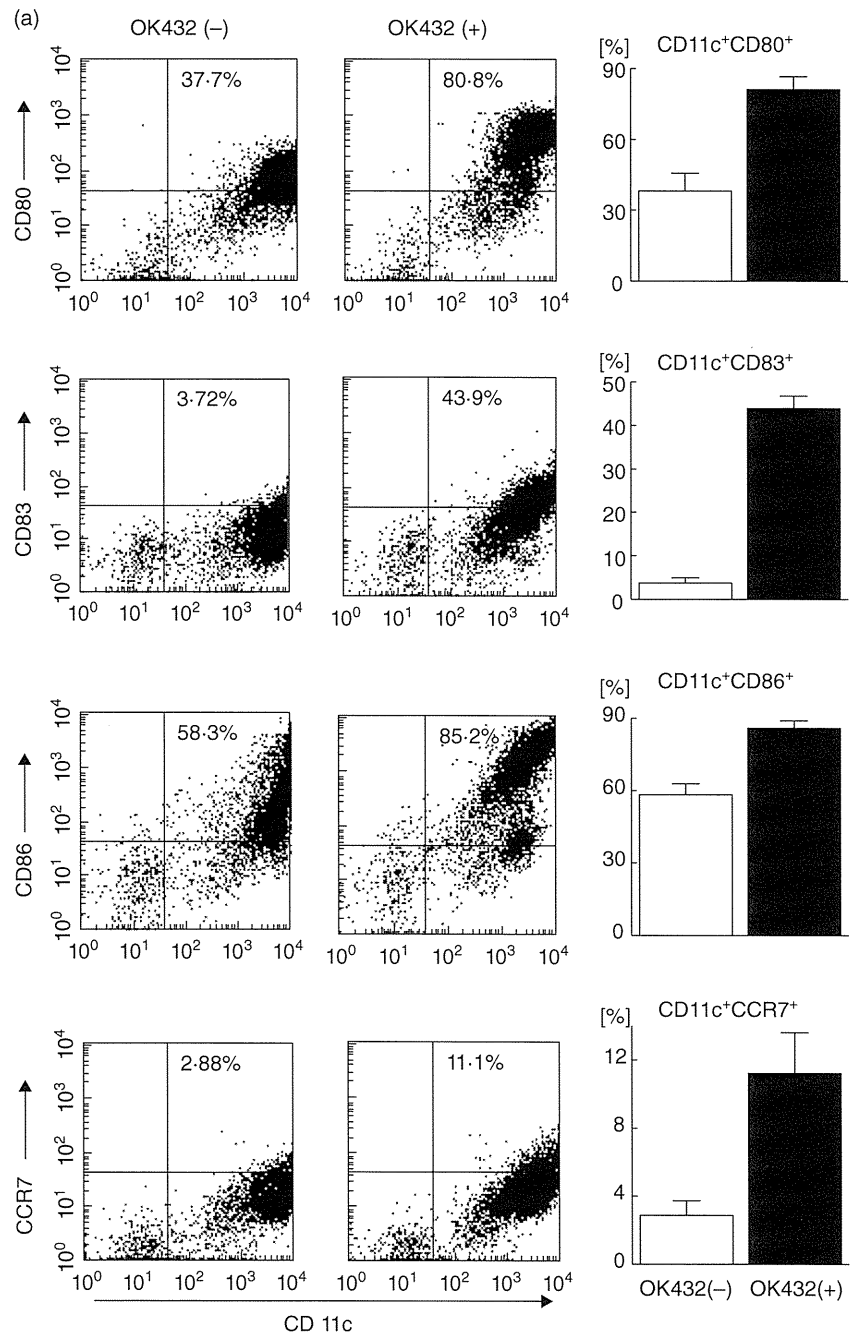
Results

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; $54.6 \pm 9.5\%$ (mean \pm 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 $30.9 \pm 14.2\%$ were CD11c-positive (myeloid DC subset) and $14.8 \pm 11.2\%$ 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 (lin¹⁻) 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 [\pm 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 lin¹⁻ 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) (\pm 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 \pm 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 ($P < 0.005$). (d) Tumoricidal activity of DCs assessed by incubation with ⁵¹Cr-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

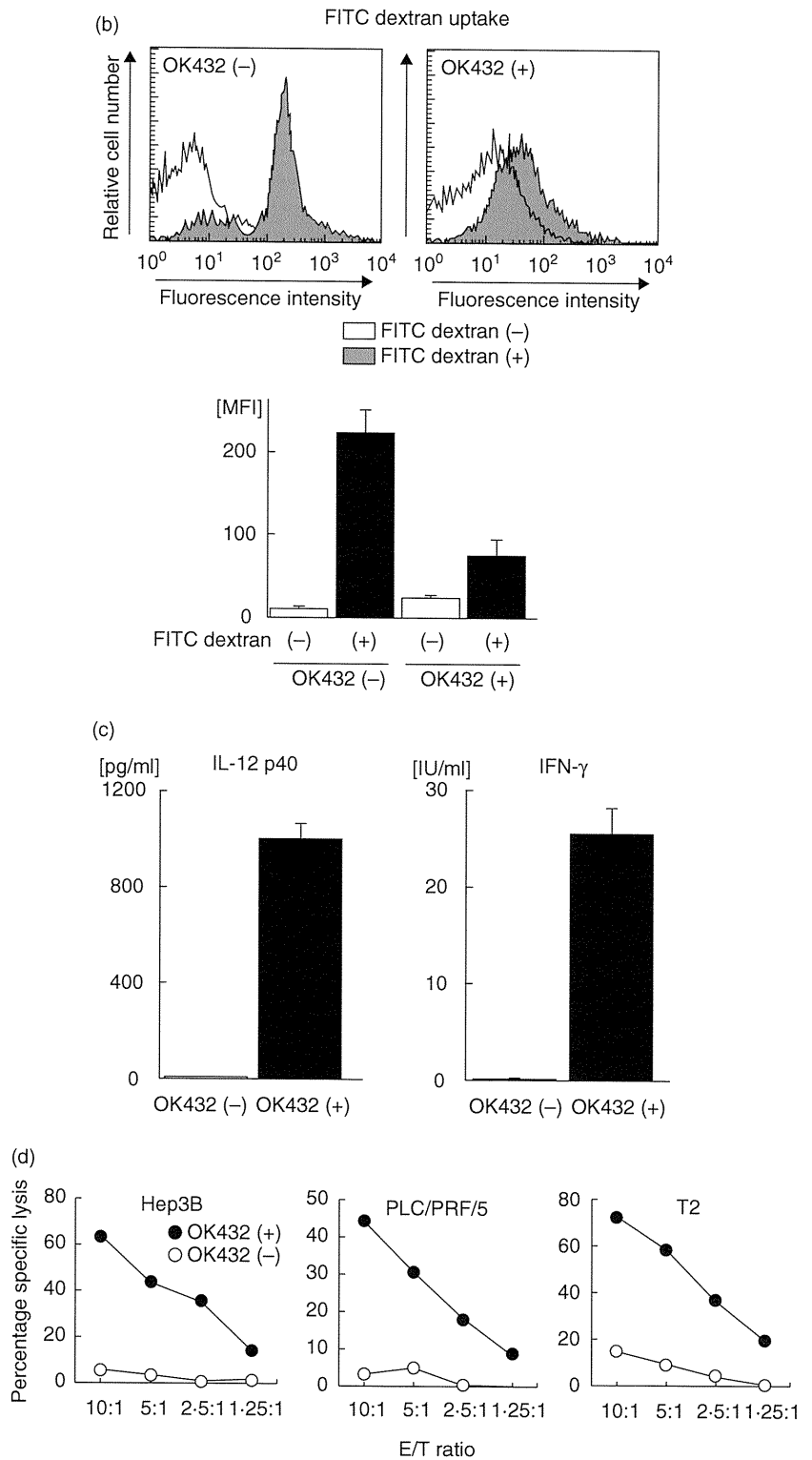


Fig. 1. *Continued*

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 com-

pared 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; $P = 0.046$, 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 ⁵¹Cr-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	<i>P</i>
No. of patients	13	22	
Age (years)	68.2 \pm 9.1	70.0 \pm 7.6	n.s. [†]
Gender (M/F)	9/4	13/9	n.s. [‡]
White cell count ($\times 10^3/\mu\text{l}$)	34.4 \pm 11.6	41.4 \pm 18.9	n.s. [†]
Lymphocytes ($\times 10^2/\mu\text{l}$)	10.4 \pm 3.6	12.4 \pm 4.7	n.s. [†]
Platelets ($\times 10^4/\mu\text{l}$)	11.5 \pm 10.2	10.3 \pm 5.8	n.s. [†]
Hepaplastin test (%)	64.6 \pm 11.6	75.5 \pm 24.3	n.s. [†]
ALT (IU/l)	56.7 \pm 38.9	67.9 \pm 44.6	n.s. [†]
Total bilirubin (mg/dl)	1.3 \pm 0.7	1.1 \pm 0.6	n.s. [†]
Albumin (g/dl)	3.4 \pm 0.6	3.6 \pm 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 \pm 1.3	1.9 \pm 1.3	n.s. [†]
Largest tumour (mm)	30.2 \pm 9.4	32.6 \pm 15.2	n.s. [†]
AFP	204.8 \pm 404.1	201.8 \pm 544.2	n.s. [†]

Results are expressed as means \pm 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.

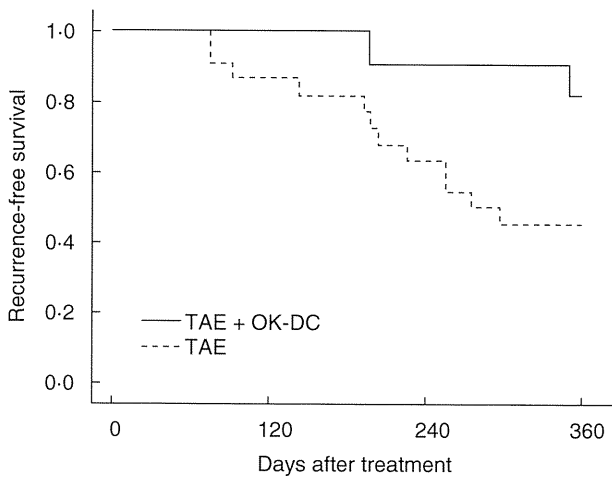


Fig. 2. Recurrence-free survival of patients treated with transcatheter hepatic arterial embolization (TAE) with [TAE + OK-stimulated dendritic cells (DC); $n = 13$] and without (TAE: historical controls; $n = 22$) OK432-stimulated DC administration. Time zero is the date of TAE. All patients underwent 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; $P = 0.046$, 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-

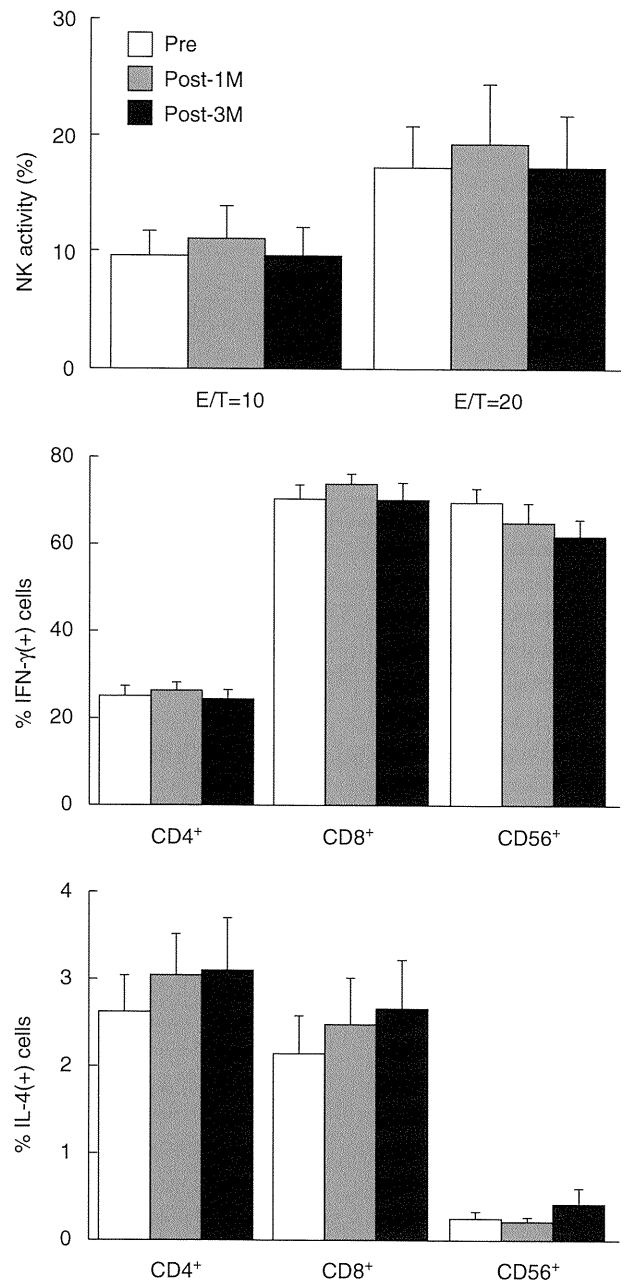
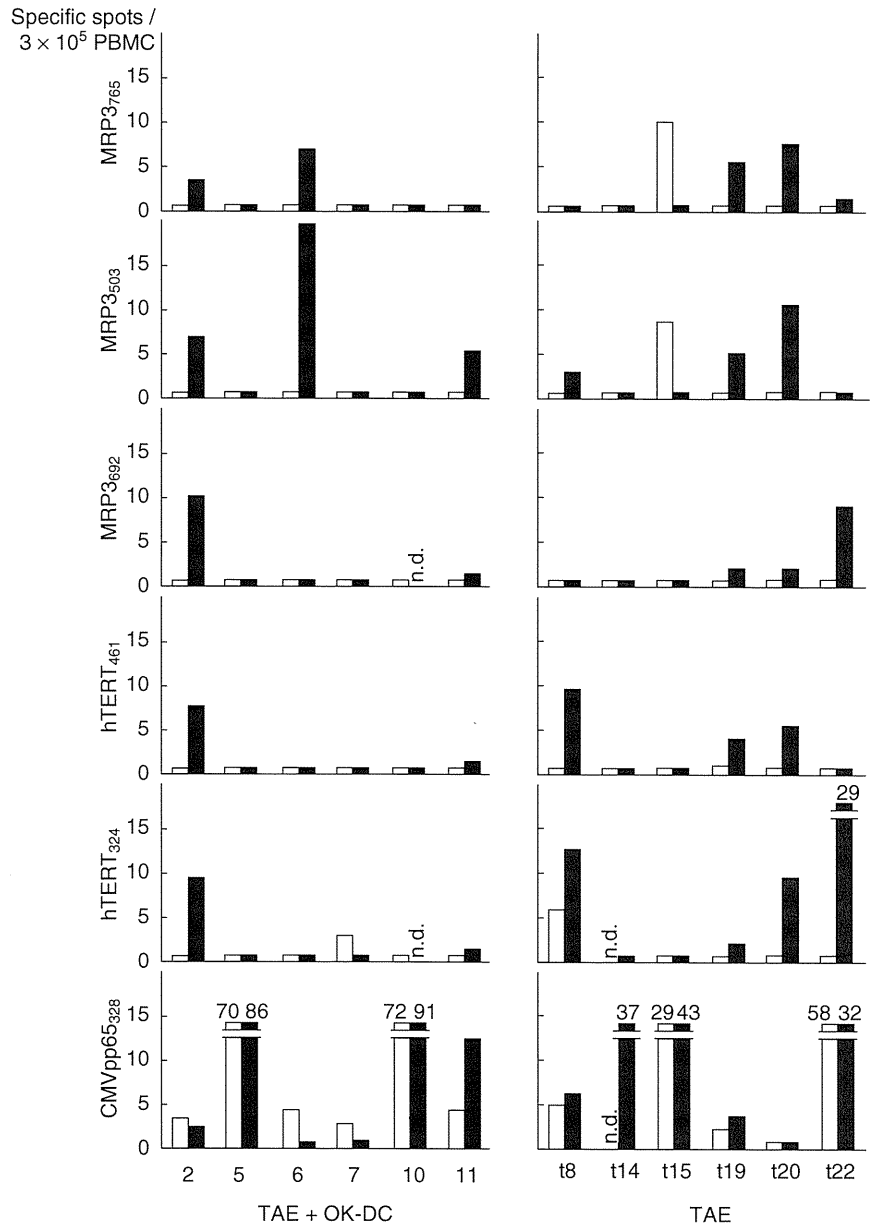


Fig. 3. Natural killer (NK) cell activity and intracellular cytokine production in peripheral blood mononuclear cells (PBMCs) of patients treated with OK432-stimulated dendritic cells (DCs) during transcatheter hepatic arterial embolization (TAE) therapy ($n = 13$). PBMCs were isolated before and 1 and 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 \pm 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 (HIVenv₅₈₄). Positive controls consisted of 10 ng/ml phorbol 12-myristate 13-acetate (PMA) or a cytomegalovirus (CMV) pp65-derived peptide (CMVpp65₃₂₈). 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.



concentrations 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-1 β (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-1 β .

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 ($P > 0.05$). 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

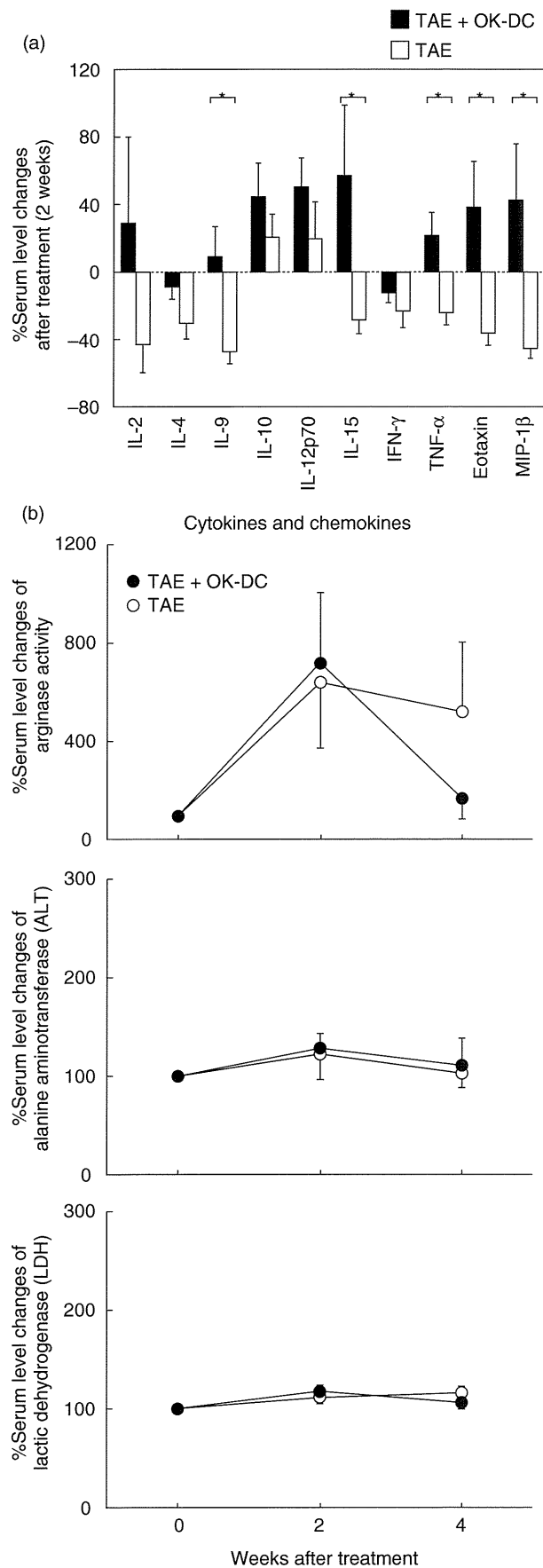


Fig. 5. Cytokine and chemokine profiling and arginase activity in sera of patients treated with OK432-stimulated dendritic cells (DCs) during transcatheter hepatic arterial embolization (TAE) therapy (TAE + OK-DC; $n = 13$) and the historical controls treated with TAE without DC transfer (TAE; $n = 22$). (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: $[(\text{post-treatment level} - \text{pretreatment level})/\text{pretreatment level}] \times 100$. The data are means \pm standard error of the mean (s.e.m.) of the groups. $*P < 0.05$ 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 ($P > 0.05$). Percentage changes in serum levels 2 weeks after the treatments were calculated as follows: $[(\text{post-treatment level} - \text{pretreatment level})/\text{pretreatment level}] \times 100$. The data indicate means \pm 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-1 β 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-1 β 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 ; 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-1 β 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

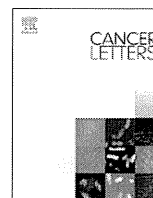
The authors have declared that no conflict of interest exists.

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Frequency of CD45RO⁺ subset in CD4⁺CD25^{high} regulatory T cells associated with progression of hepatocellular carcinoma

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ABSTRACT

The purpose of this study was to assess the properties of CD4⁺CD25^{high/low/negative} T cell subsets and analyze their relation with dendritic cells (DCs) in patients with hepatocellular carcinoma (HCC). In HCC patients, the prevalence of CD45RO⁺ cells in CD4⁺CD25^{high} T cells was increased and associated with higher frequencies of plasmacytoid DCs. Larger proportions of this T cell subset were detected in the patients with larger tumor burdens. These results suggest that increased frequencies of the CD45RO⁺ subset in CD4⁺CD25^{high} Tregs in HCC patients may establish the immunosuppressive environment cooperatively with tolerogenic plasmacytoid DCs to promote disease progression of liver cancer.

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1. Introduction

Hepatocellular carcinoma (HCC) occurs primarily in individuals with cirrhosis related to hepatitis C virus (HCV) or hepatitis B virus (HBV) infections, and alcohol abuse. HCC is the fifth most common cancer, with increasing incidence worldwide. It is characterized by high mortality, frequent postsurgical recurrence and extremely poor prognosis [1–3].

CD4⁺CD25^{high} Foxp3⁺ regulatory T cells (Tregs) have been shown to suppress immune responses by direct interaction with other immune cell types or through immune suppressive cytokines and appear crucial in maintaining immune homeostasis, mediating peripheral tolerance and preventing autoimmunity [4–6]. Increased frequencies of Tregs have been documented in the peripheral blood and in some cases the tumor microenvironment in patients

with several different tumor types [3–12]. It has been reported that, in HCC patients, increased Tregs are correlated with CD8⁺ T-cell impairment [11] and are related to poor prognosis [1].

Tregs are known to consist of heterogeneous subsets and to express various surface markers detectable by flow cytometry, including CD45RO, CTLA-4 (cytotoxic T lymphocyte associated antigen-4), GITR (glucocorticoid-induced TNF receptor-related protein), CD62L, HLA-DR, and CCR7 [8,13–15]. The role of these markers in suppressor functions mediated by human Tregs is currently under discussion [8]. It has been suggested that GITR is associated with T cell activation [16,17] and Treg subset expressing GITR are associated with disease activity in patients with Wegener's granulomatosis [17]. As for HCC, Ormandy et al. demonstrated that Tregs in HCC patients expressed high levels of HLA-DR and GITR [3]. However, there is a paucity of studies presenting the association of Treg subsets with disease progression.

In addition to Tregs, dendritic cells (DCs), a type of professional antigen-presenting cells (APCs), may be

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implicated in the regulation of immune responses. The role of human DCs in modulating Tregs is not clear [18]. It has been suggested that immature and mature myeloid DCs (mDCs) and plasmacytoid DCs (pDCs) may promote Treg cell differentiation, homeostasis and function [19]. It has been shown that lung cancer cells can convert mature DCs into TGF- β 1 producing cells, which demonstrate an increased ability to generate Tregs [20]. Conversely, Tregs can induce the generation of semimature DCs by which they can down-regulate immune responses [21]. These data suggest that there may be a mutual interaction between Tregs and DCs for the maintenance of immunosuppression.

In the present study, we evaluated the frequency and properties of CD4⁺CD25^{high}Foxp3⁺ T cells in HCC patients. Increased numbers of these cells produced more Th2 cytokine than CD4⁺CD25^{low/negative} cells. Furthermore, the proportion of CD45RO⁺ subset was increased in HCC patients. We also analyzed how the subset is related to DC frequencies, and found that some subsets were relevant to disease progression.

2. Materials and methods

2.1. Patients and healthy controls

Sixty-two HCC patients attending Kanazawa University Hospital (Ishikawa, Japan) between September 2006 and July 2008 were enrolled in this study with their informed consent. HCC was radiologically diagnosed by computed tomography (CT), magnetic resonance imaging (MRI), and CT angiography. Blood samples were taken from these HCC patients, as well as from 41 healthy controls, 17 patients with chronic hepatitis (CH) B and C and 16 patients with liver cirrhosis (LC) without a tumor. None of the patients received anticancer nor antiviral therapy at time of blood sample. Patients characteristics and disease classification are shown in Table 1.

2.2. Isolation of PBMC and CD4⁺ T cells

Peripheral blood mononuclear cells (PBMC) were isolated from freshly obtained blood by Ficoll-Hypaque (Sigma–Aldrich, St. Louis, MO). Total cell numbers were counted in the presence of a trypan blue dye to evaluate viability and immediately used for experiments. CD4⁺ T cells were isolated from freshly isolated PBMC by negative magnetic selection using the CD4⁺ T Cell Isolation Kit II (Miltenyi Biotec, Bergisch Gladbach, Germany) and QuadroMACS Separation Unit (Miltenyi Biotec) according to the manufacturer's instruction. Isolated CD4⁺ T cells were purified by >90% as measured by flow cytometric analysis using a FACSCaliber flow cytometer (BD Biosciences, San Jose, CA).

2.3. Antibodies

The following anti-human monoclonal antibodies (mAb) were used for flow cytometry: anti-CD4-PerCP, anti-CD25-APC (BD Biosciences, San Jose, CA), anti-CD45RO-FITC (PROIMMUNE, Oxford, UK), anti-CTLA-4-PE, anti-CCR7-PE,

Table 1

Clinical characteristics of hepatocellular carcinoma, liver cirrhosis, chronic hepatitis patients and healthy control.

<i>Hepatocellular carcinoma (n = 62)</i>	
Age (yrs)	68.9 ± 9.5
Gender (M/F)	37/25
Etiology of liver disease	
HBV/HCV/HBV + HCV/NBNC	19/34/2/7
TNM stages I/II/III/IV-A/IV-B	18/12/20/6/6
<i>Largest tumor (mm)</i>	
Child-Pugh A/B/C	37.6 ± 34.4
AFP (ng/mL)	41/8/3
DCP (mAU/mL)	10–35,093 (52)
<i>Liver cirrhosis (n = 16)</i>	
Age (yrs)	10–32,818 (34)
Gender (M/F)	58.3 ± 10.3
Etiology of liver disease	11/5
HBV/HCV/NBNC	4/7/5
<i>Chronic hepatitis (n = 17)</i>	
Age (yrs)	58.9 ± 10.4
Gender (M/F)	8/9
Etiology of liver disease	
HBV/HCV/NBNC	0/17/0
<i>Healthy controls (n = 41)</i>	
Age (yrs)	46.1 ± 19.1
Gender (M/F)	16/25

Note: Results except for AFP and DCP are expressed as means ± SD. AFP and DCP values are expressed as range (median). The reference range of normal values for the laboratory values: AFP < 10 ng/mL, DCP < 40 mAU/mL. M, Male; F, Female; HBV, hepatitis B virus; HCV, hepatitis C virus; NBNC, non-B non-C; TNM, tumor-node-metastasis; AFP, alpha-fetoprotein; DCP, des-gamma-carboxy prothrombin.

anti-GITR (glucocorticoid-induced TNF receptor-related protein)-PE (R&D Systems, Minneapolis, MN), anti-CD62L-FITC, anti-HLA-DR-FITC, anti-CD45RA-PE (Exalpa Biologicals, Watertown, MA), IOTest Conjugated Antibodies – (CD14 + CD16)-FITC/CD85k(ILT3)-PE/CD123-PC5 Dendritic Cells “Plasmacytoid Subset” and IOTest Conjugated Antibodies – (CD14 + CD16)-FITC/CD85k(ILT3)-PE/CD33-PC5 Dendritic Cells “Myeloid Subset” (Beckman Coulter, Miami, FL). Before use, all mAbs were titrated using normal PBMC to establish optimal staining dilutions.

2.4. Surface and intracellular staining

To determine the frequency of CD4⁺CD25^{high} T cells and the surface marker profile, CD4⁺ T cells (at least 2 × 10⁵ cells/tube) were stained with mAbs in the above described panel for 30 min on ice. Appropriate isotype antibody controls were used for each sample. Cells were washed and examined by four-color flow cytometry.

For intracellular Foxp3 and cytokine staining, 2 × 10⁵ CD4⁺ T cells/well in a 96-plate were stimulated with Leucocyte Activation Cocktail containing PMA, ionomycin, and brefeldin A, and then cultured at 37 °C in a humidified CO₂ incubator for 4 h. The activated cells were first incubated with anti-CD4-PerCP for 15 min on ice, followed by fixation and permeabilization of the activated cells for 20 min at room temperature with BD Cytofix/Cytoperm Buffer (BD Biosciences, San Diego, CA). Samples were then stained with anti-CD25-APC, anti-Foxp3-FITC (eBioscience) and PE-labeled anti-cytokine (IL-4, IL-10) antibodies (BD

Biosciences) for 15 min at room temperature. Appropriate isotype controls were included for each sample.

2.5. Flow cytometric analysis

The samples were acquired on a FACSCalibur for four-color flow cytometry. Data analysis was performed using the CellQuest software (Becton Dickinson, CA, USA).

2.6. Statistical analysis

Data are indicated as means \pm SD unless otherwise stated. The statistical significance of difference between the two groups was determined by applying the Mann–Whitney nonparametric *U* test. $P < 0.05$ was considered significant.

3. Results

3.1. Frequencies of CD4⁺CD25^{high} T cells

To evaluate the frequencies of CD4⁺CD25^{high} T cell subsets that contain Tregs, MACS-sorted CD4⁺ T cell subsets obtained from the patients with CH, LC and HCC and healthy controls were analyzed by flow cytometry following the staining with anti-CD4 and anti-CD25 monoclonal antibodies (Fig. 1A and B). Although the frequencies of CD4⁺CD25^{high} T cells were not changed in patients with CH, they were increased in patients with LC compared to the controls ($P < 0.05$). As reported, it is remarkably elevated in patients with HCC ($P < 0.0001$). The results indicated that CD4⁺CD25^{high} T cell subset containing Tregs are increased in patients complicated with liver malignancies.

3.2. Intracellular Foxp3 and cytokine production of the CD4⁺CD25^{high} T cell subset in HCC patients

The transcription factor Foxp3 is considered to be a specific marker for Tregs [22–24]. Intracellular Foxp3 levels were detected by using the specific mAb after the cell membrane permeabilization procedures (Fig. 2A). The percent of Foxp3⁺ cells in the CD4⁺CD25^{high} T cell subset in HCC patients was larger than that of CD4⁺CD25^{low/negative} subset, and it was also significantly larger than that of CD4⁺CD25^{high} T cells in healthy controls and CH patients (Fig. 2B). Thus, not only is the number of CD4⁺CD25^{high} T cells in HCC patients larger, but also the frequency of Foxp3⁺ cells in HCC patients is higher than CH patient and healthy controls. This is consistent with previous reports of Tregs in patients with other malignancies.

Intracellular production of cytokines IL-4 and IL-10 of CD4⁺CD25^{high} Foxp3⁺ T cell subset was quantitated following the stimulation with PMA/ionomycin using the specific mAbs by flow cytometry (Fig. 2C).

The levels of Th2 cytokines IL-4 and IL-10 were high in the CD4⁺CD25^{high} subsets. In addition, the levels of IL-4 and IL-10 were high in the CD4⁺CD25^{high}Foxp3⁺ T cell subset in HCC patient ($P < 0.005$) (Fig. 2D). These results suggest that the CD4⁺CD25^{high}Foxp3⁺ Treg subset in HCC patients may have a high potential to produce immunosuppressive cytokines.

3.3. Phenotypes of the CD4⁺CD25^{high} T cell subset in HCC patients

To determine the phenotypical properties of CD4⁺CD25^{high} T cell subset increased in patients with HCC, the expression levels of the seven reported surface molecules, CD45RO, CD45RO, CD62L, CCR7, CTLA-4, HLA-DR and GITR were quantitated by flow cytometry. Among the seven molecules, the proportions of CD45RO⁺, HLA-DR⁺ and GITR⁺ cells were higher in the CD4⁺CD25^{high} T cell subset in all patient groups compared to the CD4⁺CD25^{low/negative} T cell subsets, except for GITR⁺ cells in CH patients ($P < 0.05$) (Fig. 3A and B). The percentage of CD45RO⁺ cells in HCC patients were elevated compared to the patients with advanced liver diseases and healthy controls ($P < 0.01$). These data demonstrate that the CD4⁺CD25^{high} T cell subset highly expresses the surface molecule CD45RO in HCC patients, which may reflect the memory properties of T cells.

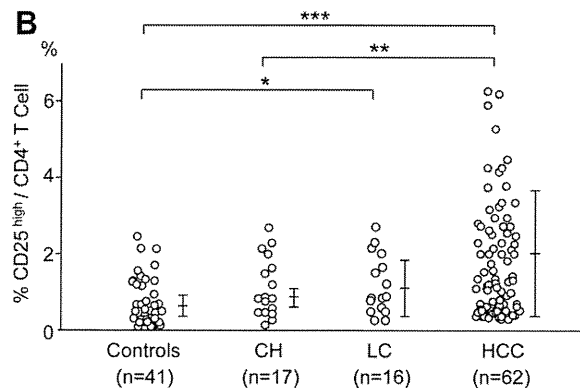
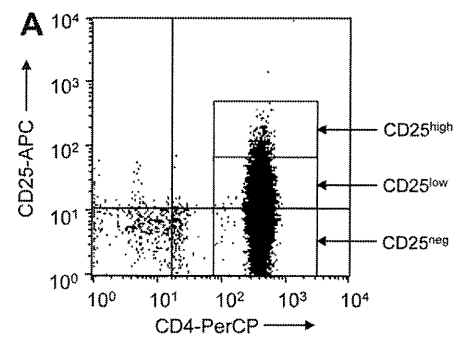


Fig. 1. Frequencies of CD4⁺CD25^{high} T cells in peripheral blood of HCC patients and controls. (A) Representative flow cytometric analysis of PBMCs (peripheral blood mononuclear cells) of an HCC patient. Freshly isolated PBMCs were labeled with anti-CD4 and anti-CD25 antibodies as described in the Materials and Methods. (B) Percentages of CD4⁺CD25^{high} T cells in the peripheral blood of HCC ($n = 62$), LC ($n = 16$), CH ($n = 17$) patient, and healthy controls ($n = 41$). Percentages for individual patient analyzed are shown. The percentages represent the proportions of CD4⁺CD25^{high} T cells in total CD4⁺ cells. The prevalence of CD4⁺CD25^{high} T cells in HCC patients was significantly higher than in healthy controls or CH patients. CH, chronic hepatitis; HCC, hepatocellular carcinoma; LC, liver cirrhosis. *Indicates $P < 0.05$, **indicates $P < 0.01$ and ***indicates $P < 0.001$.

3.4. CD4⁺CD25^{high} T Cell subset and dendritic cells of HCC patients

Several reports have suggested that the CD4⁺CD25^{high} T cell subset may interact with dendritic cells. To evaluate the frequencies of DCs in PBMC of HCC patients, whole blood cells were analyzed by flow cytometry following the staining with IOTest Conjugated Antibodies – (CD14 + CD16)-FITC/CD85k(ILT3)-PE/CD123-PC5 Dendritic Cells “Plasmacytoid Subset” and IOTest Conjugated Antibodies – (CD14 + CD16)-FITC/CD85k(ILT3)-PE/CD33-PC5 Dendritic Cells “Myeloid Subset”. HCC patients were divided into two groups according to the frequencies of CD45RO^{positive} cells in CD4⁺CD25^{high} T cell subsets (CD45RO⁺ vs. CD45RO⁺⁺). Patients with CD45RO⁺⁺ contained >83.8% positive cells in CD4⁺CD25^{high} T cells. The frequencies of CD123⁺ plasmacytoid DCs were significantly higher in CD45RO⁺⁺ group ($P < 0.05$) (Fig. 5A and B), although those of CD33⁺ myeloid DCs were not correlated with the subsets in CD4⁺CD25^{high} cells. These results showed that there are more tolerogenic plasmacytoid DCs in the PBMCs of HCC patients with higher frequencies of a memory subset of CD4⁺CD25^{high} T cells.

3.5. CD4⁺CD25^{high} T cell subset and tumor progression

To evaluate the association between CD4⁺CD25^{high} T cell phenotype and tumor progression, we compared the maximum tumor diameters, the number of tumors, tumor markers AFP (alpha-fetoprotein) and DCP (des-gamma-carboxyl prothrombin), TNM stages, Child–Pugh scores