ELISA

Target cells (1-2x10^4) were plated in flat-bottomed 96 microwell plate (Corning, Inc., Corning, NY) in DMEM medium containing 10% FBS. TIL2003 cells (5×10^4) in AIM-V medium were then added. After a 24 h-incubation period at 37°C, the amount of GM-CSF in the supernatant (100 μl) was measured using an ELISA Development Kit (TechneCorp., Minneapolis, MN) in line with the manufacturer's protocol. All experiments were performed in duplicate.

Establishment of autologous CTL clones against SP cells of MFH2003

Establishment of autologous CTL clones against SP cells of MFH2003 was performed as previously described[10]. Peripheral blood mononuclear cells (PBMCs) were obtained from an MFH2003 donor patient. CD8+ T cells were collected from PBMCs using magnetic anti-CD8 beads (Miltenyi Biotec, Gladbach, Germany). Total of 5x10^5 irradiated (100Gy) SP cells of MFH2003 and 5x10^6 CD8-T cells were distributed into five wells containing 2mL/well of AIM-V in 24-well flat-bottomed culture plates and cultured. The following day, 20 U/ml of recombinant human interleukin-2 (rhIL-2, a kind gift from Takeda Chemical Industries, Ltd., Osaka, Japan) and 10% AB human serum (HS) were added. Stimulation of the T cells was repeated at intervals of 7-10 days using SP cells. After the fourth stimulation, the CTL were plated from all five culture wells at various dilutions in round-bottomed 96 microwell plate (Corning, Inc.)

in AIM-V supplemented with rhIL-2 (200U/ml) and phytohemagglutinin (PHA; 5μg/ml, Wako Chemicals, Osaka, Japan). Irradiated LG-2 EBV cells (1×10^4 cells/ well) and allogeneic PBMCs (1×10^5 cells/ well) were added as feeder cells. Cells were incubated at 37°C. After 42 days, three resultant CTL clones were used for cytotoxic assay. One CTL clone showing specific cytotoxicity against SP cells of MFH2003 was selected and designated Tc4C-6. Cytotoxicity assay was performed as described below. Cell surface phenotypes of Tc4C-6 were assayed using an FITC-conjugated anti-CD3 antibody (BD Biosciences), PE-conjugated anti-CD4 antibody (BD Biosciences), PE-Cy5-conjugated anti-CD8 antibody (eBioscience, San Diego, California, USA), FITC-conjugated anti-CD45RA (BD Biosciences) antibody and PE-conjugated anti-CCR7 antibody (BD Biosciences). Tc4C-6 and healthy-donor PBMCs were incubated with these antibodies for 30 min on ice in the dark. After washing with PBS, the cells were fixed with 1% paraformaldehyde in PBS and analyzed by flow cytometory.

Cytotoxic assay

Specific cytotoxicity of CTL clones was measured by using the nonradioactive aCella-TOX assay (Cell Technology, Mountain View, CA) according to the manufacturer's protocol. Target cells were plated in triplicate (5000 cells per well) in round-bottomed 96-well microwells in IMDM containing 50U/well rhIL-2. Effector cells at various effector to target ratios were added as indicated. Spontaneous effector and target cell death was accomplished by including control wells of effector and target

cells at numbers corresponding to those of their various E/T ratios. To determine maximum release [total glyceralehyde-3-phosphate dehydrogenase (G3PDH) released], 10 μl of lysis reagent (0.5% NP-40/100 μl sample) was added to the target cell positive control 10 minutes after the end of assay incubation. After 12 hours of incubation at 37°C, the culture supernatant of each well was transferred into a corresponding well containing the Enzyme Assay Reagent reacting against G3PDH on a white OptiPlate-96 (PerkinElmer, Waltham, MA). Immediately the detection reagent was added to each well. Luminescence of each well was analyzed at once using an ARVO MX/Light 1420 Multilabel Luminescence Counter (PerkinElmer). All experiments were performed in triplicate. The percent cytotoxicity was calculated as follows: [(experimental G3PDH release-spontaneous G3PDH release from effector cells alone-spontaneous G3PDH release from target cells alone)/(maximum G3PDH release from target cells-spontaneous G3PDH release from target cells)] x 100. In blocking experiments, Tc4C-6 were incubated with an anti-HLA class I mAb (W6/32) or anti-HLA class Ⅱ mAb (L243) for 30 min at 37°C before cytotoxic assay.

Results

Enrichment of side population cells in MFH2003

For the isolation of side population (SP) cells as cancer stem-like cells/cancer initiating cells (CSCs/CICs), we performed SP analysis and cell sorting 95 times. As shown in Fig. 1, the proportion of SP cells of MFH2003 ranged from 0.3 to 7.2% (mean 4.0±1.7) in independent experiments. Figure 1A shows typical results of two independent SP analysis experiments. The numbers of isolated SP cells ranged from 0.1 to $6.7x10^5$ (mean $2.4±1.4\ x10^5$) from bulk MFH2003 cells. Since, for each experiment in the present study, more than $5x10^5$ SP cells were required, the variance of proportion and low number of isolated SP cells sometimes made it hard to complete experiments. Therefore, to solve this problem, we enriched SP cells by *in vitro* SP cell culture. After 7-10 days, the proportion of SP cells increased, and ranged from 9.4 to 36.2% (mean 18.6±7.4) and the resultant number of sorted SP cells was also increased more than six fold, ranging from $3.3\text{-}38.2\ x10^5$ (mean 15.3±7.7) (Fig. 1AB). The improvement of the isolation efficiency of SP cells was useful for further experiments.

Expression profiles of immune molecules on SP and MP cells of MFH2003

First, we analyzed the immune molecules on SP and MP cells of MFH2003 (Fig. 2). SP cells of MFH2003 expressed HLA class I, HLA-A24, B and C molecules on the cell surface. The expression levels of these molecules were slightly higher than on MFH2003 bulk and MP cells. Although MHC class II and CD80, which provide costimulatory signals necessary for T cell activation and survival, were not expressed on

MFH 2003 SP cells, the higher expression of MHC class I on SP cells of MFH2003 suggested that CSCs could be recognized by host cellular immunity.

Autologous TIL2003 recognized both SP and MP cells of MFH2003

Next, to evaluate whether CTLs could recognize SP cells in the tumor microenvironment, we assessed the response of autologous tumor-infiltrating lymphocyte line TIL2003 against SP and MP cells of MFH2003. TIL2003 is a CTL line we established previously from the metastatic lymph nodes of the MFH2003 patient[12]. As shown in Fig. 3, TIL2003 recognized both SP and MP cells of MFH2003. Although we could not completely deny the possibility that MP cells triggered the immune response against both SP and MP cells in the context of some antigens expressed in both SP and MP cells, these results also suggested that the CTL response against SP cells was triggered by SP cells in the tumor microenvironment

CTL clone derived from peripheral blood showed specific response against SP cells of MFH2003

To detect the peripheral specific CTL response against SP cells of MFH2003, we attempted to induce an autologous CTL clone that recognized SP cells of MFH2003, using SP cells as the stimulation antigen. We performed stimulation four times by mixed lymphocyte-tumor cell culture using purified SP cells of MFH2003 and autologous peripheral mononuclear cells. Subsequently we performed a conventional limiting dilution procedure. As a result, we obtained one CTL clone, Tc4C-6, which showed

specific cytotoxicity against SP cells of MFH2003. Tc4C-6 expressed a single V beta chain Vb5.2-3 mRNA, which was also expressed in TIL2003 (data not shown). The phenotype of Tc4C-6 was CD3+CD4-CD8+CCR7-CD45RA+, a typical effector phenotype (Fig. 4A). Moreover, Tc4C-6 showed higher cytotoxicity against SP cells than MP cells of MFH2003, purified by cell sorting (Fig. 4B and 4C). In addition, the anti-HLA class I W6/32 antibody apparently blocked the cytotoxicity of Tc4C-6 against MFH2003SP cells (Fig. 4D). These results suggested that SP cells could be killed by autologous CTL in an HLA class I-restriction manner.

Discussion

In the present study, we showed that (i) side population (SP) cells, as CSCs of MFH2003, expressed more HLA class I on the cell surface than main population (MP) cells as non-CSCs, (ii) SP cells could be recognized by autologous tumor-infiltrating lymphocytes, and (iii) an autologous CTL clone could be induced by mixed lymphocyte tumor cell culture using SP cells as antigens, and that killed SP cells rather than MP cells. These results indicated that CTL-recognizing CSCs certainly existed in the tumor microenvironment and circulating peripheral blood and that SP cells could be killed by CTLs. Thus, CTL-based immunotherapy against CSCs of bone sarcoma is a very attractive option.

Previously, Pellegatta et al. reported that dendritic cell-based vaccine therapy showed an efficient anti-tumor immune response against glioma stem cells[13]. Todaro et al. showed that gamma-delta T cells killed human colon CSC and Pietra et al. demonstrated that NK cells killed human melanoma CSCs[14, 15]. Weng et al. induced CTLs against ovarian CSCs from HLA-A2+ healthy donors using CSC-DC fusion cells and demonstrated that CTLs killed ovarian CSCs[16]. These reports suggested that CSCs could be a candidate target for immunotherapy. However, the autologous CTL response against CSCs has hither to never been investigated.

It is well documented that tumors can escape T-cell-mediated elimination by downregulation of molecules essential for immune recognition[17]. The downregulation of HLA class I molecules in tumor tissues is the major prognostic factor and has an important role in tumor immune escape[18]. We also reported that the relation of

downregulation of HLA class I to the poor prognoses of patients with osteosarcoma and Ewing's sarcoma[19, 20]. However, as shown in the present study, the expression profile of immune molecules, including HLA class I molecules on CSCs, is preserved. Therefore, CSCs might not tend to escape from cellular immune surveillance activated by CTL-based immunotherapy.

The identification of CSC-associated antigens recognized by autologous CTLs is very important, especially for the establishment of CTL-based immunotherapy in the adjuvant setting for the prevention of recurrence and metastasis. For this purpose, establishment of anti-CSC specific CTL lines is a prerequisite. Although Weng et al. assessed the CTL response against allogeneic ovarian CSCs[16], there has been no report regarding CTL lines induced by autologous CSCs. Therefore, CTL clone Tc4C-6 is the first CTL clone against CSCs induced by autologous CSCs and could serve as a good source of a probe against autologous CTL clone-defined CSC-associated antigen. Now we are trying to isolate cDNA of TCR alpha and beta chains for the establishment of a permanent probe for cDNA library expression cloning.

We observed SP cells of MFH2003 in 72 independent experiments. The proportion of SP cells in MFH2003 varied among experiments and often this was the main obstacle to completing experiments using SP cells, so enrichment of SP cells was required. The isolation of SP cells requires high technical skills, and intensive, hard laboratory work. Although we do not know why the proportion of SP cells in MFH2003 is unstable, differentiation of SP cells into MP cells in cell culture *in vitro* might contribute to the variety of the SP cell proportion. Recently, the dynamic regulation theory of cancer stem

cells was proposed[21]. A subpopulation of JARID1B-positive cells, which was expressed in SP cells, had high proliferative ability. However, not only could single JARID1B-positive cells become JARID1B-negative, but single JARID1B-negative cells could also become JARID1B-positive and acquire tumorigenicity. Such a dynamic change of the characteristics of SP and MP cells is another possible reason.

In conclusion, we showed the immunogenicity of CSCs of bone MFH using autologous TILs and a peripheral CTL clone. CTL-based immunotherapy could target CSCs of bone sarcoma for the prevention of tumor recurrence.

Acknowledgments

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Disclosure Statement

All authors have declared that no conflict of interest exists.

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Figure legends

Fig. 1. Enrichment of side population cells of MFH2003. (A) Side population (SP) cells of MFH2003 before and after enrichment of SP cells, in the presence or absence of verapamil in two independent experiments are shown. SP cells are marked by black lines. The proportion of SP cells among total living cells is indicated. (B) Summary of enrichment of SP cells. The mean proportion of SP cells in MFH2003, mean number of Hoechst33342 dye-stained bulk MFH2003 cells and mean number of resultant sorted SP cells, before and after enrichment, are shown.

Fig.2. Expression profile of immune molecules on SP and MP cells. Cell surface expression of HLA class I (HLA-A24, B and C), HLA class II, and CD80 molecules on bulk, SP and MP cells of MFH2003, LG-2, EB-B (B2003-EBV) and K562.

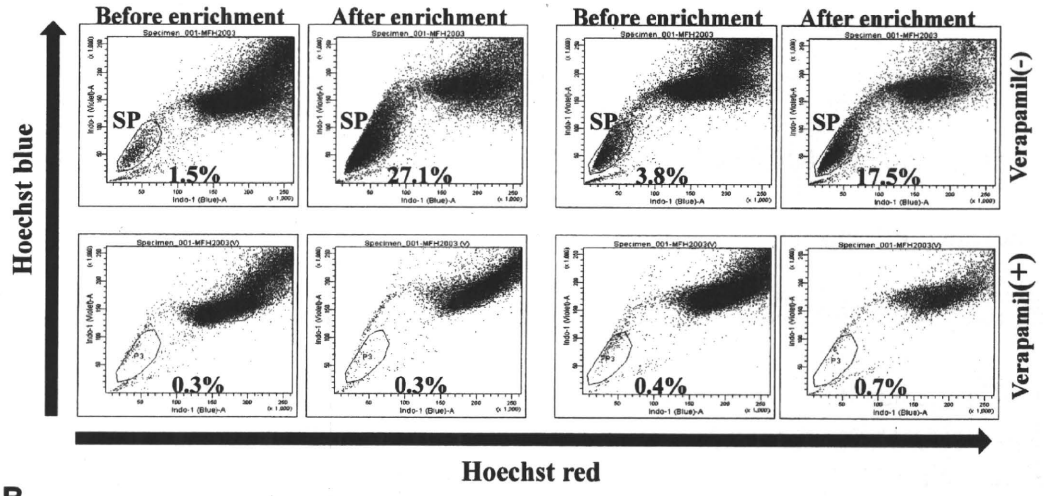
Fig. 3. Autologous TIL2003 recognized both SP and MP cells. Freshly isolated SP and MP cells of MFH2003, autologous EB-B cells (B2003-EBV) and negative control K562 cells were co-cultured with TIL2003. After 24h, culture supernatant was harvested and GM-CSF released from TIL2003 was measured by ELISA.

Fig. 4. Autologous CTL clone Tc4C-6 recognized SP cells. (A) Cell surface expression of CD4, CD8, CCR7 and CD45RA on Tc4C-6 and allogeneic PBMC of a healthy donor. (B) Re-analysis of sorted SP and MP cells. (C) Cytotoxicity of CTL clone Tc4C-6. Freshly isolated SP and MP cells, autologous EB-B cells (B2003-EBV)

and negative control K562 cells were used as target cells and co-cultured with Tc4C-6 at the indicated effector:target ratio. After 12h, CTL-mediated cytotoxicity was measured using the aCella-TOX assay as described in Materials and Methods. (D) Blocking assay of Tc4C6-mediated recognition of SP cells of MFH2003 using anti-HLA class I (W6/32), anti-HLA class II (L243) mAbs. Cytotoxicity was also measured with the aCella-TOX assay.

Kano et al. Figure 1

A

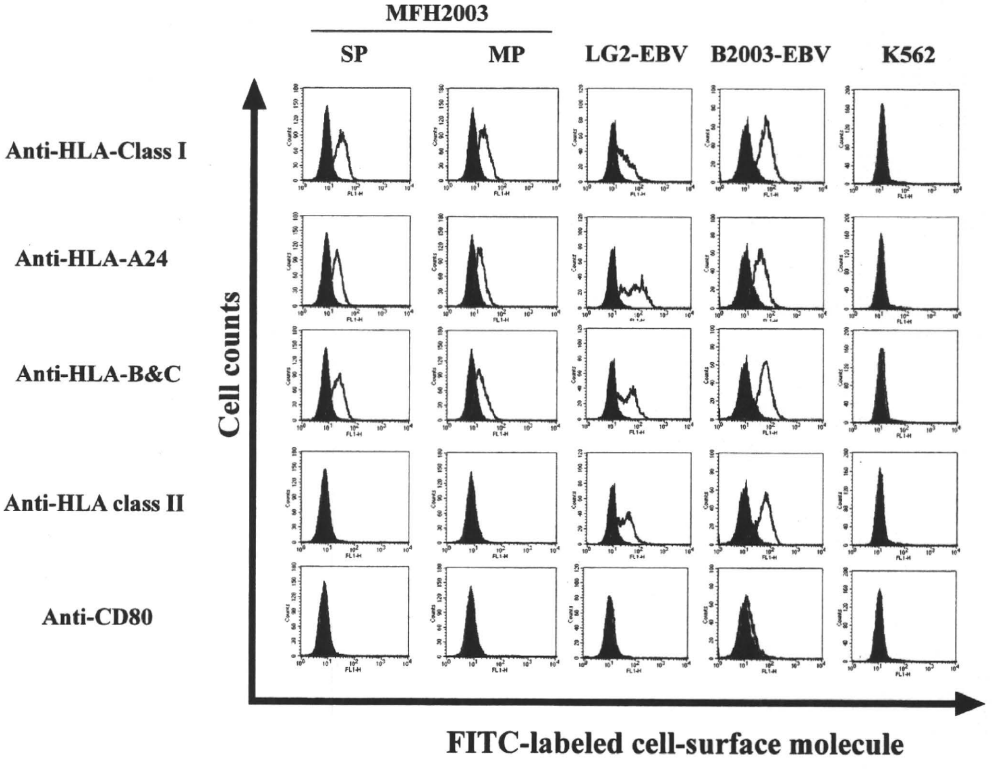


B

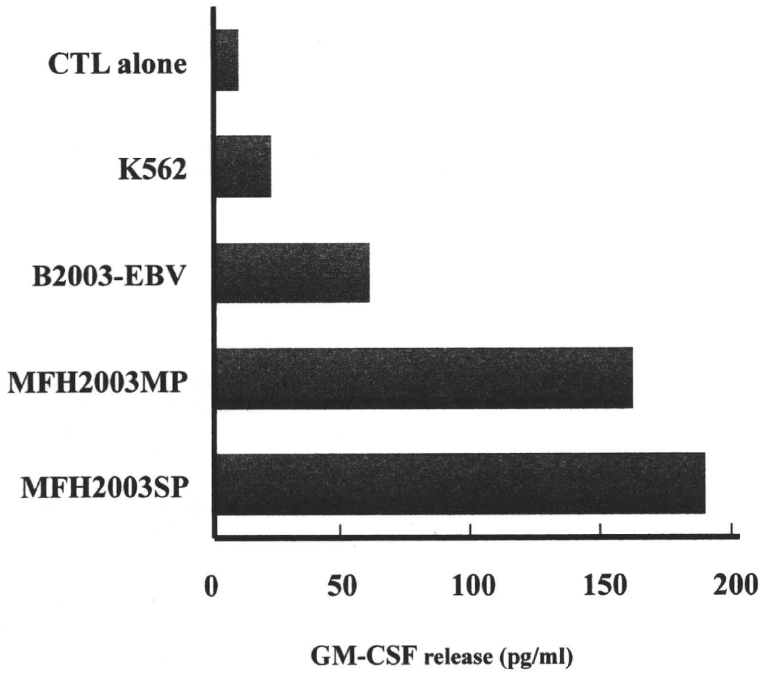
Before enrichment			
Number of experiment	Proportion of SP cells (%)	Input Bulk ($x10^7$ cells)	Output Sorted SP ($x10^5$ cells)
72	4.0±1.7(0.3-7.2)	1.7±0.8(0.6-5.7)	2.4±1.4(0.1-6.7)

After enrichment			
Number of experiment	Proportion of SP cells (%)	Input Bulk ($x10^7$ cells)	Output Sorted SP ($x10^5$ cells)
23	18.6±7.4(9.4-36.2)	2.2±1.0(0.7-4.0)	15.3±7.7(3.3-38.2)

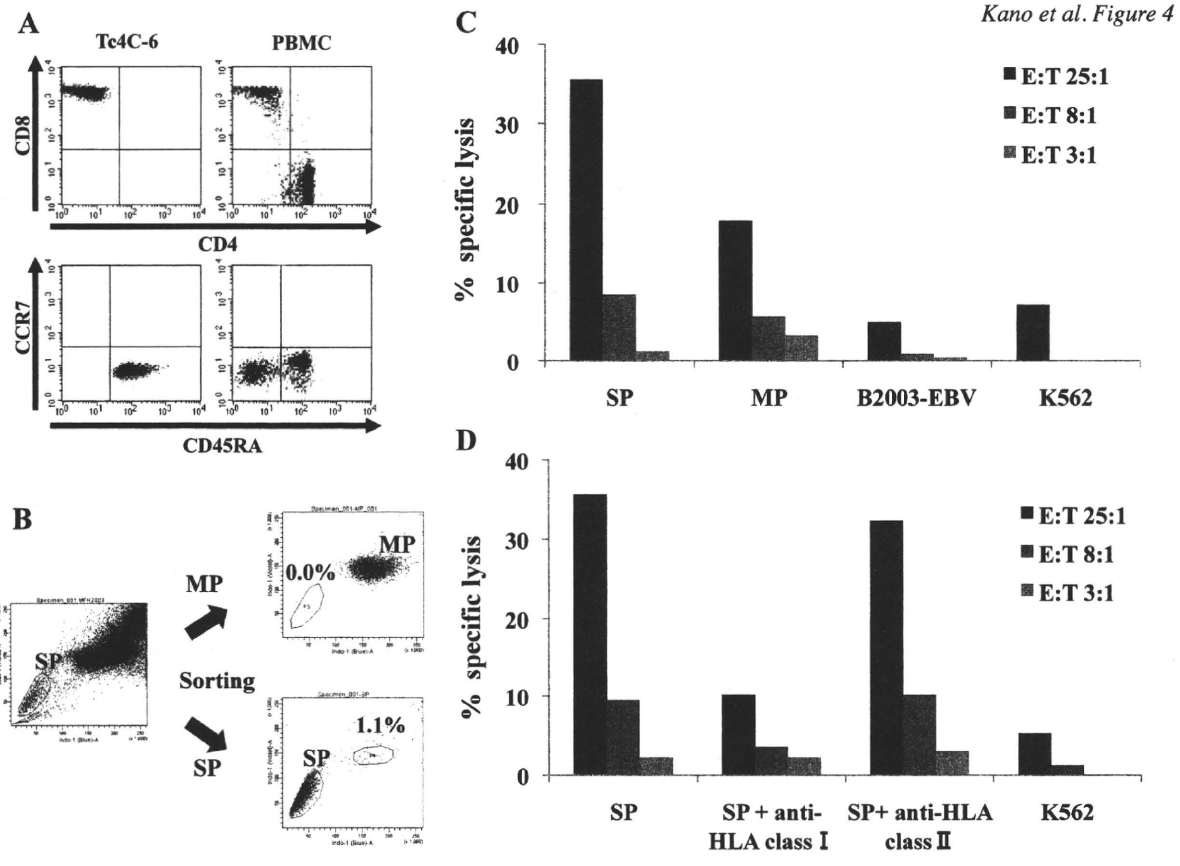
Kano et al. Figure 2



Kano et al. Figure 3



Kano et al. Figure 4



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Immunogenic enhancement and clinical effect by type-I interferon of anti-apoptotic protein, survivin-derived peptide vaccine, in advanced colorectal cancer patients

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We previously identified a human leukocyte antigen (HLA)-A24-restricted antigenic peptide, survivin-2B80-88, recognized by CD8+ cytotoxic T lymphocytes (CTL). Subsequently, we attempted clinical trials with this epitope peptide alone for some malignancies, resulting in clinical and immunological responses, although their potential was not strong enough for routine clinical use as a cancer vaccine. In the current study, to assess whether immunogenicity of the survivin-2B80-88 peptide could be enhanced with other vaccination protocols, we performed clinical trials in advanced colon cancer patients with two vaccination protocols: (i) survivin-2B80-88 plus incomplete Freund's adjuvant (IFA); and (ii) survivin-2B80-88 plus IFA and a type-I interferon (IFN), IFNα. Our data clearly indicated that, although the effect of survivin-2B80-88 plus IFA was not significantly different from that with survivin-2B80-88 alone, treatment with the vaccination protocol of survivin-2B80-88 plus IFA and IFNα resulted in clinical improvement and enhanced immunological responses of patients. Tetramer analysis of survivin-2B80-88 peptide-specific CTL demonstrated that such CTL were increased at least twofold after vaccination with this protocol in four of eight patients. In these patients, enzyme-linked immunosorbent spot (ELISPOT) results were also enhanced. Subsequent study of single-cell clone separation by cell sorting of peptide-specific CTL showed that each CTL clone was indeed not only peptide-specific but also cytotoxic against human cancer cells in the context of the expression of both HLA-A24 and survivin molecules. Taken together, these results indicate that vaccination of colon cancer patients with survivin-2B80-88 plus IFA and IFNα can be considered to be a very potent immunotherapeutic regimen, and that this protocol might work for other cancers. (*Cancer Sci*, doi: 10.1111/j.1349-7006.2011.01918.x, 2011)

Human tumor immunology research has advanced since the first human melanoma tumor antigen recognized by CD8+ cytotoxic T lymphocytes (CTL) was identified in 1992,[1] and more than 20 melanoma antigens have been reported.[2–7] Some antigens and human leukocyte antigen (HLA) class I-restricted antigenic peptides underwent clinical trials, and their adverse effects and clinical and immunological responses were studied.[8–11] Rosenberg *et al.*[4] reported on a large number of melanoma patients and found that less than 5% of patients who received peptide vaccines such as gp100 and interleukin-2 (IL-2) had a complete response.

Nevertheless, a UK-based pharmaceutical company reported that a 3-year-long observation after melanoma antigen family A, 3 (MAGE-A3) vaccine inoculation indicated a 33% reduction in the post-operative recurrence of non-small-cell lung cancers when compared with a placebo group.[12] This observation gives strong hope for future cancer immunotherapy and has prompted many different investigations for the establishment of human tumor immunotherapy.

Meanwhile, human tumor antigens of non-melanoma tumors such as colon, lung, urinary tract and soft-tissue sarcomas have been analyzed extensively in various laboratories.[13–18] In our laboratory, we have identified tumor antigens using several different experimental systems.[2,3] Using reverse immunological approaches the inhibitor of apoptosis protein (IAP) family members survivin and livin were shown to be highly immunogenic tumor antigens in addition to the fact that these two antigens were selectively expressed in tumor tissues of different tissue origins but not in normal counterparts.[19–21] The HLA-A24-restricted survivin2B80-88 nonamer peptide, which was derived from the survivin splicing variant survivin 2B from cancer patients, appears to have strong immunogenicity as assessed by CTL induction efficiency, tetramer CTL frequency and enzyme-linked immunosorbent spot (ELISPOT).[19,20]

Our group began clinical trials several years ago.[8,9,11] The HLA-A24-restricted survivin2B80-88 peptide was given subcutaneously to patients six times or more at biweekly intervals for colon, breast, lung, oral cavity, urinary bladder cancers and lymphomas. There were no severe adverse effects and, clinically, certain patients with colon, lung and urinary bladder cancers showed reductions in tumor markers and growth arrest as assessed on computed tomography (CT). However, these effects were not strong enough for the clinical requirements as decided by the Response Evaluation Criteria in Solid Tumors (RECIST) for cancer chemotherapy. When assessed with the RECIST, which requires more than 30% regression of tumors on CT, only one of 15 patients with colon cancer and three of 15 with urinary bladder cancer had a positive clinical response.

Thus, the therapeutic potential was not strong enough for routine clinical use as a cancer treatment.[2,3] In the current study, to determine if the immunogenicity of the survivin-2B80-88 peptide could be enhanced with other vaccination protocols, we performed and compared clinical trials in advanced colon cancer patients with two vaccination protocols: (i) survivin-2B80-88 plus incomplete Freund's adjuvant (IFA); and (ii) survivin-2B80-88 plus IFA and type-I interferon (IFN), IFNα. Our data clearly indicated that, although the effect with survivin-2B80-88 plus IFA was not significantly different from that with survivin-2B80-88 alone, treatment with survivin-2B80-88 plus IFA

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and IFNα resulted in clinical improvement and enhanced immunological responses of patients. We also analyzed CTL of these patients by single-cell sorting, finding that each CTL clone from the vaccinated patients was indeed not only peptide-specific but also cytotoxic against human cancer cells in the context of the expression of both HLA-A24 and survivin molecules.

Materials and Methods

Patient selection. The study protocol was approved by the Clinic Institutional Ethical Review Board of the Medical Institute of Bioregulation, Sapporo Medical University, Japan.[8–11] All patients gave informed consent before being enrolled. Patients enrolled in the present study were required to conform to the following criteria: (i) to have histologically confirmed colon cancer; (ii) to be HLA-A*2402 positive; (iii) to have survivin-positive carcinomatous lesions by immunohistochemistry; (iv) to be between 20 and 85 years old; (v) to have unresectable advanced cancer or recurrent cancer; and (vi) to have Eastern Cooperative Oncology Group (ECOG) performance status between 0 and 2. Exclusion criteria included: (i) prior cancer therapy such as chemotherapy, radiation therapy, steroid therapy or other immunotherapy within the previous 4 weeks; (ii) the presence of other cancers that might influence the prognosis; (iii) immunodeficiency or a history of splenectomy; (iv) severe cardiac insufficiency, acute infection or hematopoietic failure; (v) use of anticoagulants; and (vi) unsuitability for the trial based on clinical judgment. This study was carried out at the Department of Surgery, Sapporo Medical University Primary Hospital from December 2005 to November 2009.

Peptide preparation. The peptide, survivin-2B80-88 with the sequence AYACNTSTL, was prepared under good manufacturing practice conditions by Multiple Peptide Systems (San Diego, CA, USA).[8,9,11] The identity of the peptide was confirmed by mass spectrometry analysis and the purity was shown to be more than 98% as assessed by high-pressure liquid chromatography analysis.

The peptide was supplied as a freeze-dried, sterile white powder. It was dissolved in 1.0 mL of physiological saline (Otsuka Pharmaceutical Co., Ltd, Tokyo, Japan) and stored at −80°C until just before use.

IFA and IFNα preparation. Montanide ISA 51 (Seppic, Paris, France) was used as IFA. Human IFNα was purchased from Dainippon-Sumitomo Pharmaceutical Co. (Osaka, Japan).

Patient treatment. Two protocols were used in the current clinical study, as illustrated in Figure 1. One was a basic protocol with the survivin-2B80-88 peptide plus IFA, and the other was the survivin-2B80-88 peptide plus IFA and a type-I IFN, IFNα. In this trial, the primary end-point was safety. The second end-point was investigations about anti-tumor effects and clinical and immunological monitoring.

In the first protocol, survivin-2B80-88 at a dose of 1 mg/1 mL plus IFA at a dose of 1 mL were mixed immediately before vaccination. The patients were then vaccinated subcutaneously (s.c.) four times at 14-day intervals. This group included five patients. If patients whose disease was not far advanced hoped for continuation of this peptide vaccination therapy, we vaccinated them in the same manner after the fourth vaccination.

In the second protocol, survivin-2B80-88 plus IFA was vaccinated in a similar manner to the first protocol. In addition, in this protocol, IFNα at a dose of 3 000 000 IU was administered (s.c.) twice a week close to the site of vaccination. For this, IFNα was mixed with the peptide and IFA immediately before vaccination and administered at the time of the peptide and IFA biweekly vaccination.

Toxicity evaluation. Patients were examined closely for signs of toxicity during and after vaccination. Adverse events were recorded using the National Cancer Institute Common Toxicity Criteria (NCI-CTC).[8,9]

Clinical response evaluation. Physical examinations and hematological examinations were conducted before and after each vaccination.[8,9] A tumor marker (carcinoembryonic antigen [CEA]) was examined. Changes in tumor marker levels were evaluated by comparison of the serum level before the first vaccination and that after the fourth vaccination. Immunohistochemical study of the HLA class I expression in patients' primary colon cancer tissues was done with anti-HLA class I heavy chain monoclonal antibody EMR-8-5 (Funakoshi Co., Tokyo, Japan).

Tumor size was evaluated by CT scans or MRI by comparing the size before the first vaccination with that after the fourth vaccination. A complete response (CR) was defined as complete disappearance of all measurable and evaluable disease. A partial response (PR) was defined as a ≥30% decrease from baseline in the size of all measurable lesions (sum of maximal diameters). Progressive disease (PD) was defined as an increase in the sum of maximal diameters by at least 20% or the appearance of new lesions. Stable disease (SD) was defined as the absence of criteria matching those for CR, PR or PD.[8,9] Patients who received fewer than four vaccinations were excluded from all evaluations in this study.

***In vitro* stimulation of PBMC.** The PBMC were isolated from blood samples by Ficoll-Conray density gradient centrifugation.

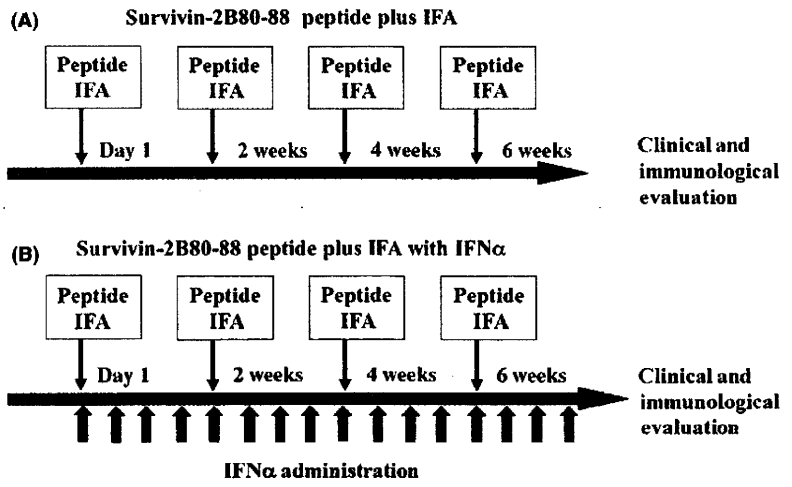

Fig. 1. Protocols of the clinical study. In the current study two protocols were used: (A) survivin-2B80-88 plus IFA and (B) survivin-2B80-88 plus IFA with IFNα. IFA, incomplete Freund's adjuvant; IFN, interferon.

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They were then frozen and stored at −80°C. As needed, frozen PBMC were thawed and incubated in the presence of 30 μg/mL survivin-2B80-88 in AIM-V medium containing 10% human serum at room temperature. Next, interleukin-2 was added at a final concentration of 50 U/mL 1 h, 2 days, 4 days and 6 days after the addition of the peptide. On day 7 of culture, the PBMC were analyzed by tetramer staining and ELISPOT assay.

Tetramer staining. FITC-labeled HLA-A*2402-human immunodeficiency virus (HIV) peptide (RYLRDQQLL) and PE-labeled HLA-A*2402-survivin-2B8-88 peptide tetramers were purchased from MBL, Inc. (Nagoya, Japan). For flow cytometric analysis, PBMC, which were stimulated *in vitro* as above, were stained with the PE-labeled tetramer at 37°C for 20 min, followed by staining with a FITC-conjugated anti-CD8 mAb (Beckton Dickinson Biosciences, San Jose, CA, USA) at 4°C for 30 min. Cells were washed twice with PBS before fixation in 1% formaldehyde. Flow cytometric analysis was performed using FACSCalibur and CellQuest software (Beckton Dickinson Biosciences, San Jose, CA, USA). The frequency of CTL precursors was calculated as the number of tetramer-positive cells divided by the number of CD8-positive cells.[8,9,11]

ELISPOT assay. ELISPOT plates were coated sterilely overnight with an IFN-γ capture antibody (Beckton Dickinson Biosciences) at 4°C. The plates were then washed once and blocked with AIM-V medium containing 10% human serum for 2 h at room temperature. CD8-positive T cells separated from patients' PBMC (5×10^3 cells/well), which were stimulated *in vitro* as above, were then added to each well along with HLA-A24-transfected CIR cells (CIR-A24) (5×10^4 cells/well), which had been preincubated with or without survivin-2B80-88 (10 mg/mL) or with a HIV peptide as a negative control. After incubation in a 5% CO_2 humidified chamber at 37°C for 24 h, the wells were washed vigorously five times with PBS and incubated with a biotinylated anti-human IFN-γ antibody and horseradish peroxidase-conjugated avidin. Spots were visualized and analyzed using KS ELISPOT (Carl Zeiss, Jena, Germany). In the present study, positive (+) ELISPOT represents a more than twofold increase of survivin-2B80-88 peptide-specific CD8 T cell IFNγ-positive spots compared with HIV peptide-specific CD8 T cell spots, whereas negative (−) represents a less than twofold increase.

Single-cell cloning and functional assessment of tetramer-positive CTL. Survivin-2B80-88 peptide tetramer-positive CTL were sorted and subsequently cloned to single cells using FACS (Aria II Special Order, BD, Houston, TX, USA). The peptide-specific cytotoxicity of each of these CTL was determined by pulsing T2A24 cells[8,9,11,20] with survivin-2B80-88 or HLA-A*2402 HIV (RYLRDQQLL) peptides. These CTL were also assessed for live tumor cell cytotoxicity against LK79 (survivin-2B positive and HLA-A24 positive), A549 (survivin-2B positive, HLA-A24 negative) and K562 (survivin-2B negative and HLA-A24 negative) target cells.

Results

Patient profiles and safety. In the first protocol with the survivin-2B80-88 peptide plus IFA, five patients were enrolled in the study (Table 1). None of the treatment interruptions was due to adverse effects of the vaccination. These five patients received the complete regimen including four vaccinations and were evaluated (Fig. 1). They consisted of three men and two women, whose age range was 50–76 years.

In the second protocol with the survivin-2B80-88 peptide plus IFA and IFNα, eight patients were enrolled in the study (Table 2). In this protocol, there were no patients who dropped out because of adverse events due to the vaccination. They consisted of four men and four women, whose age range was 33–76 years.

With respect to safety, the vaccination was well tolerated in all patients in both vaccination protocols. In patients vaccinated with the survivin-2B80-88 peptide plus IFA, no adverse events were observed during or after vaccination except for induration at the injection site. In the second protocol, the survivin-2B80-88 peptide plus IFA and IFNα, approximately half of the patients had a fever reaching almost 39°C after the vaccination, possibly due to the action of IFNα. No other severe adverse events were observed during or after vaccination.

Clinical responses. As shown in Table 1, vaccination with survivin-2B80-88 plus IFA was given to five colorectal cancer patients. The post-vaccination CEA values of four patients were increased compared with the pre-vaccination values. In the other patient (No. 5), the CEA value remained almost the same, although it was beyond the upper limit during the vaccination. As for tumor size, only one patient was considered to have SD, whereas the other four patients were considered to have PD. These outcomes suggested that vaccination with this first protocol was ineffective for clinical responses.

Table 2 summarizes the clinical outcomes for the eight patients in the second protocol with survivin-2B80-88 plus IFA and IFNα. In some patients, particularly No. 6, the post-vaccination CEA value was clearly decreased compared with the pre-vaccination value, and was within the normal limit. Other patients such as Nos 2 and 3 also had decreased post-vaccination levels of CEA, although not so large. As for tumor size evaluated by CT, four patients (Nos 1, 2, 3 and 6) were considered to have SD, but the other four patients (Nos 4, 5, 7 and 8) had PD.

Table 1. Summary of profiles of advanced colorectal cancer patients enrolled in the present study and clinical and immunological responses to vaccination with survivin-2B80-88 peptide and IFA

Patient no.	Age/sex	Adverse effects	Tumor markers pre-/post- (CEA ng/mL)	CT evaluation†	Survivin-2B80-88 peptide		
					Tetramer staining‡		ELISPOT§
					Pre-/post-	% increase	
1	76/M	No	13/20	PD	192/103	53.6	−
2	59/M	No	369/463	SD	13/16	123.1	−
3	60/F	No	685/1010	PD	60/80	133.3	−
4	72/M	No	55/64	PD	11/4	36.4	−
5	50/F	No	8/7	PD	127/97	76.4	+

†Evaluation of CT images was done by the following: PD, progressive disease; SD, stable disease. ‡CTL frequencies of pre- and post-vaccinated patients were assessed with a HLA-A24-restricted survivin-2B80-88 (AYACNTSTL) peptide tetramer, compared with a HLA-A24-restricted HIV peptide (RYLRDQQLL) tetramer used as a negative tetramer control. The number of survivin-2B80-88 peptide tetramer-positive but HIV peptide-negative CTL among 10^4 CD8 T cells is shown. §Positive (+) ELISPOT represents a more than twofold increase of survivin-2B80-88 peptide-specific CD8 T cell IFNg-positive spots compared with HIV peptide-specific CD8 T cell spots, whereas negative (−) means a less than twofold increase. CEA, carcinoembryonic antigen; CT, computed tomography; CTL, cytotoxic T lymphocyte; ELISPOT, enzyme-linked immunosorbent spot; HIV, human immunodeficiency virus; IFA, incomplete Freund's adjuvant; IFN, interferon.

Table 2. Summary of profiles of advanced colorectal cancer patients enrolled in the present study and clinical and immunological responses to vaccination with survivin-2B80-88 peptide, IFA and IFNα

Patient no.	Age/sex	Adverse effects	Tumor markers pre-/post- (CEA ng/mL)	CT evaluation†	Survivin-2B80-88 peptide		
					Tetramer staining‡		ELISPOT§
					Pre-/post-	% increase	
1	5/M	Leukopenia	Not determined	SD	53/98	184.9	–
2	62/M	No	5/3	SD	17/33	194.1	–
3	65/F	Fever	561/441	SD	11/98	890.9	+
4	72/M	No	6/8	PD	69/81	117.4	+
5	33/F	Fever	4655/8651	PD	8/24	300.0	+
6	44/F	Edema, Fever	12/2	SD	5/76	1520.0	+
7	53/F	Fever	82/117	PD	4/555	13 875.0	+
8	76/M	No	63/140	PD	32/35	109.4	–

†Evaluation of CT images was done by the following: PD, progressive disease; SD, stable disease. ‡CTL frequencies of pre- and post-vaccinated patients were assessed with a HLA-A24-restricted survivin-2B80-88 (AYACNTSTL) peptide tetramer, compared with a HLA-A24-restricted HIV peptide (RYLRDQQLL) tetramer used as a negative tetramer control. The number of survivin-2B80-88 peptide tetramer-positive but HIV peptide-negative CTL among 104 CD8 T cells is shown. §Positive (+) ELISPOT represents a more than twofold increase of survivin-2B80-88 peptide-specific CD8 T cell IFNg-positive spots compared with HIV peptide-specific CD8 T cell spots, whereas negative (–) means a less than twofold increase. CEA, carcinoembryonic antigen; CT, computed tomography; CTL, cytotoxic T lymphocyte; ELISPOT, enzyme-linked immunosorbent spot; HIV, human immunodeficiency virus; IFA, incomplete Freund's adjuvant; IFN, interferon.

Consequently, it appeared that there was a close correlation between clinical SD outcomes and a reduced CEA level.

Immune responses, single-cell cloning and subsequent functional assessment of tetramer-positive CTL. We determined if the survivin-2B80-88 peptide vaccination could actually induce specific immune responses in the patients enrolled. The peptide-specific CTL frequency was analyzed using the HLA-A24/peptide tetramer. The CTL frequency before the first vaccination (pre-vaccination) and after the last vaccination (post-vaccination) was assessed with a HLA-A24-restricted survivin-2B80-88 (AYACNTSTL) peptide tetramer, compared with a HLA-A24-restricted HIV peptide (RYLRDQQLL) tetramer for the negative tetramer control. The number of survivin-2B80-88 peptide tetramer-positive but HIV peptide-negative CD8 T cells in 10^4 CD8 T cells was determined. In the current study, ELISPOT was also carried out using these peptides.

The data are summarized in Tables 1 and 2. In the first vaccination protocol, that is, vaccination with survivin-2B80-88 plus IFA, patients did not show obvious survivin-2B80-88-specific CTL responses in the tetramer study. Only patients Nos 2 and 3 exhibited very slight enhancement in that study. Patient No. 5 was positive in the ELISPOT study, but did not show a survivin-2B80-88-specific CTL response in the tetramer study. In these patients, there was no correlation between the clinical and immune responses. However, in the second protocol with survivin-2B80-88 plus IFA and IFNα, many patients demonstrated enhanced tetramer frequency. In particular, four of the eight patients (Nos 3, 5, 6 and 7) had enhanced frequency with a more than 200% increase. It was also interesting that all four of these patients were also positive in the ELISPOT study, and two had SD by CT evaluation, suggesting that immune responses might appropriately reflect, at least in some patients, clinical responses with this second vaccination protocol.

Subsequently, we analyzed tetramer-positive CD8 T cells at the single-cell level, and determined whether these T cells had specificity for the survivin-2B80-88 peptide and cytotoxic potential against live survivin-2B-positive tumor cells in the context of HLA-A*2402. As shown in Figure 2, patient No. 6 (44 years old, female) had positive HLA class I expression in rectal tumor tissue (Fig. 2A), a reduced serum CEA level (Fig. 2B) and obvious immune responses as assessed by the survivin-2B80-88 tetramer and ELISPOT (Fig. 2C) after vaccination with the second protocol. CD8 T cells of the tetramer-positive fraction were sorted by FACS, and then cultured with 1, 3 and 10 cells/well for 7–10 days. As shown in Figure 3, almost all growing T cells were survivin-2B peptide-specific T cells, and we subsequently assessed peptide-specific cytotoxicity by using T cells of well (a) through well (h) as indicated. Consequently, as Figure 4 clearly shows, all T cells except for those in well (b) had very high peptide-specific cytotoxic potential. Furthermore, T cells in well (d) were cloned, and we obtained a T cell clone designated D-13 showing survivin-2B peptide specificity as shown in Figure 5A,B. Finally, the cytotoxic potential against survivin-2B-positive live tumor cells was assessed, confirming that the D-13 T cell clone was clearly cytotoxic against survivin-2B-positive tumor cells in a HLA-A*2402-restricted fashion. Taken together, these data clearly indicate that the second vaccination protocol was capable of inducing a strong CTL response, and in some patients might result in clinical effectiveness.

Discussion

The immunogenicity of HLA class I-restricted tumor peptides is not strong enough for routine clinical use as cancer treatment, and it is important to increase their relative and absolute immunogenicity levels by various means. Previously, our group conducted clinical trials of the survivin-2B80-88 peptide vaccine alone and with IFA in patients with various cancers.[3,8,9,11] In the current study we determined whether the immunogenicity of survivin-2B80-88 could be enhanced with other vaccination protocols. Thus, we compared clinical trials in advanced colon cancer patients with two vaccination protocols: (i) survivin-2B80-88 plus IFA; and (ii) survivin-2B80-88 plus IFA and a type-I IFN, IFNα. Our data strongly suggest that although the effect with survivin-2B80-88 plus IFA was not different from that with the survivin-2B80-88 peptide alone, treatment with survivin-2B80-88 plus IFA and IFNα resulted in clear improvement of the clinical and immunological responses of patients.

There has been less evidence concerning the extent to which peptide-specific CTL responses in patients treated with peptide vaccines could occur at the single-cell level. To assess this point, CTL of patients were sorted to the single-cell level, and we confirmed that each CTL obtained from vaccinated patients was indeed not only peptide-specific but also cytotoxic against human cancer cells in the context of the expression of both HLA-A24 and survivin molecules.

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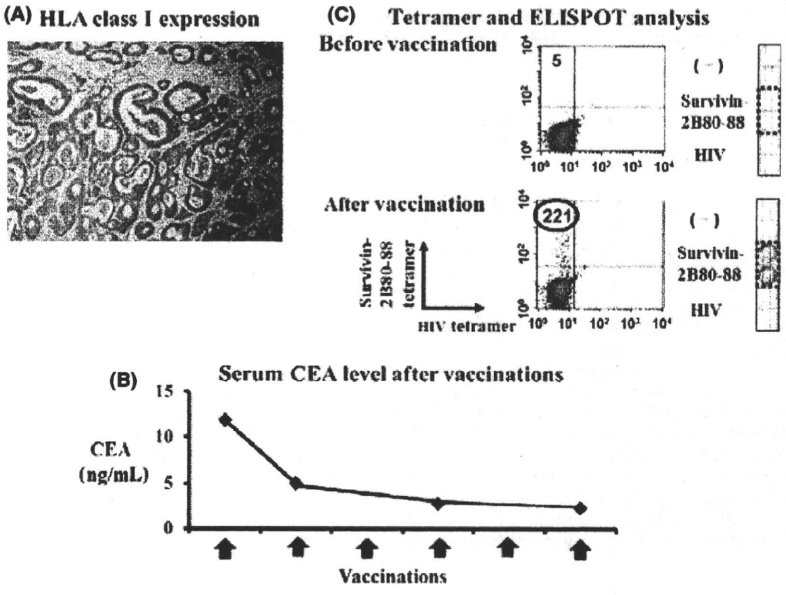

Fig. 2. Representative illustration of clinical effect and immunological analysis in patient No. 6 who was treated with survivin-2B80-88 plus IFA with IFNα. (A) Immunohistochemical analysis of HLA class I expression in primary colon cancer tissue as assessed by anti-HLA class I heavy chain monoclonal antibody EMR8-5, (B) profile of serum CEA level after vaccinations (arrows) and (C) tetramer and ELISPOT analyses before and after vaccinations. The number in the tetramer analysis indicates survivin-2B80-88 peptide-specific CD8+ T cells among 10^4 CD8+ T cells. CEA, carcinoembryonic antigen; ELISPOT, enzyme-linked immunosorbent spot; HIV, human immunodeficiency virus; HLA, human leukocyte antigen; IFA, incomplete Freund's adjuvant; IFN, interferon.

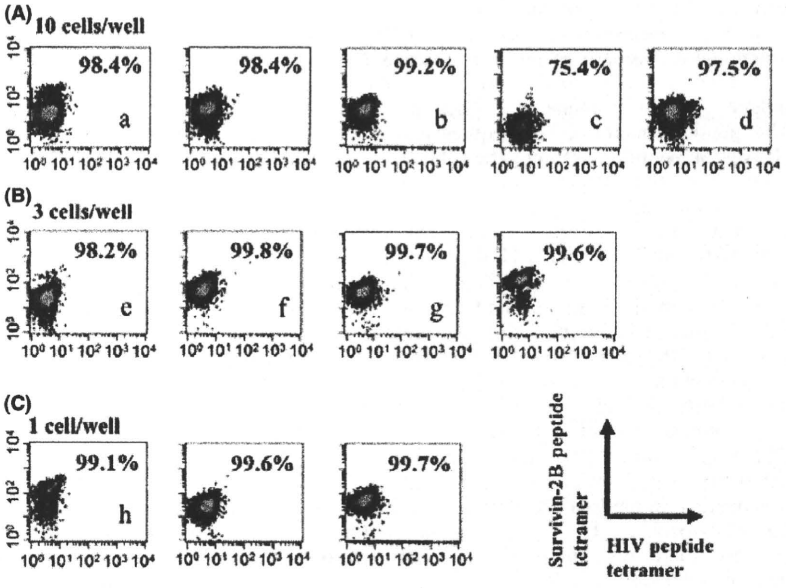

Fig. 3. Single-cell analysis of survivin-2B80-88 peptide tetramer-positive CD8 T cells. Survivin-2B80-88 peptide tetramer-positive CD8 T cells in Figure 2C (circle) were sorted and cultured at 10 (A), 3 (B) and 1 (C) cells/well for 7–10 days. Subsequently, cells were examined for their reactivity to the survivin-2B80-88 peptide tetramer.

It is well known that type-I interferons such as IFNα can work in many immunological manners to activate T cell responses.[22–25] The maturation of dendritic cells (DC) and their effect on the expression of HLA molecules seems to be the main action of this cytokine. Because of difficulties with the availability of patients' samples, we could not actually compare these features of patients' DC and primary tumor tissues before and after treatment with IFNα. However, the evidence of obvious enhancement of CTL responses and improvement of clinical responses in our study favors the two main actions described above. Indeed, a preliminary study of three colon patients who received survivin-2B80-88 plus IFA with GM-CSF did not demonstrate any clinical or immunological response, suggesting that the action of IFNα is remarkable from the aspect of being an immunogenic enhancer for human peptide vaccines. Ideally, patients should be administered IFNα alone before vaccination treatment is undertaken. However, this was practically hard to do since all patients were in the late and advanced disease stages and it was difficult to select such patients. Meanwhile, there is no evidence that administration of IFNα alone results in overt clinical benefits such as those observed in the current study. The second vaccination protocol using IFNα appeared to be highly efficient for enhancing peptide immunogenicity.

Nevertheless, we require more efficient and reasonable ways to enhance peptide immunogenicity *in vivo* with limited side-effects.[26] In our laboratory, it was observed that heat shock protein 90 (HSP90) could highly selectively target substrates such as HLA-class I restricted peptides as well as the innate immunity ligand, CpG, to the Rab5 (+) and EEA1 (+) early static endosomes of DC.[3,27–29] Consequently, HSP90 could strongly induce peptide-specific CTL responses *in vitro* and *in vivo* without obvious side-effects,[20] and it is intriguing that its action may surpass the benefits of IFNα.

Collectively, our present study may reflect one potential vaccination protocol. Since the vaccination protocol with