vivo tumorigenesis in NOD/SCID mice. In the present study, for the characterization of the immunogenicity of CSCs, we analyzed autologous CTL responses against SP cells of MFH2003 in the tumor-microenvironment and peripheral circulating blood using autologous tumor-infiltrating lymphocytes and a CTL clone, respectively.

Materials and Methods

This study was approved under the institutional guidelines for the use of human subjects in research. The patients and their families, as well as healthy donors, gave informed consent for the use of blood samples and tissue specimens in our research.

Cell lines and Culture

The cell lines used were a bone human malignant fibrous histiocytoma (MFH) cell line (MFH2003), osteosarcoma (OS2000, KIKU, NY, Huo9, HOS, U-2OS and Saos2), erythroleukemia (K562) and an Epstein-Barr virus-transformed B cell line (LG2-EBV, B2003-EBV). OS2000, KIKU MFH2003 and B2003-EBV were established in our laboratory[10-12]. The other cell lines were kindly donated or purchased from the Japanese Collection of Research Bioresources Cell Bank (Tokyo, Japan) and from American Type Culture Collection (Manassa, VA). MFH2003 and OS2000 were cultured with Iscove's modified Dulbecco's Eagle's medium (IMDM; GIBCO BRL, Grand Island, NY) containing 10% FBS and LG-2-EBV, B2003-EBV, and K562 were cultured with RPMI 1640 medium (Sigma-Aldrich, St. Louis, MO) containing 10% FBS. The others were maintained in Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich) containing 10% FBS in a 5% CO_2 incubator at 37°C.

Purification of side population cells

Cancer stem-like cells of MFH2003 were purified by side population analysis as previously described[9]. Briefly, the cell suspensions were labeled with dye Hoechst

33342 dye (Cambrex Bio Science Walkersville, Inc., Walkersville, MD). Hoechst33342 was added at a final concentration of 5.0μg/ml in the presence or absence of verapamil (75μM; Sigma-Aldrich) as an inhibitor of the ABC transporter. The cells were incubated at 37°C for 90 min with continuous shaking. At the end of the incubation, the cells were washed with ice-cold PBS with 5% FBS, centrifuged at 4°C, and resuspended in ice-cold PBS containing 5% FBS. Propidium iodide (at the final concentration of 1μg/ml; Life Technologies Corp, Carlsbad, CA) was used to gate viable cells. Flow cytometry and cell sorting were performed using a FACS Aria Ⅱ (BD Biosciences, Bedford, MA). The Hoechst 33342 dye was excited at 357 nm and its fluorescence was analyzed using dual wavelengths (blue, 402-446 nm; red, 650-670 nm).

When the proportion of SP cells was low (≤5%), the SP cells were sorted and brought into in vitro culture in 10ml of IMDM containing 10% FBS for enrichment. After more than 14 days, SP analysis and cell sorting were performed again.

Analysis of expression status of cell surface molecules

Expression of cell surface molecules was assayed as previously described[12] using an anti-HLA-A24 mAb (C7709A2.6), anti-HLA-B&C mAb (B1.23.2), anti-HLA class I mAb (W6/32), anti-HLA-class Ⅱ mAb (L243), and an anti-CD80 mAb (MAB104). Bulk, SP and main population (MP) cells of MFH2003, LG2-EBV cells, B2003-EBV cells and K562 cells were incubated with appropriate mAbs, for 40 min on ice. Then these cells were incubated with FITC-labeled second antibody and analyzed using a FACSCaliber (BD Bioscience).

ELISA

Target cells ($1\text{-}2 \times 10^4$) 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 5×10^5 irradiated (100Gy) SP cells of MFH2003 and 5×10^6 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 cytometery.

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 6.7×10^5 (mean $2.4 \pm 1.4 \times 10^5$) from bulk MFH2003 cells. Since, for each experiment in the present study, more than 5×10^5 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 3.3-38.2 $\times 10^5$ (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.

References

1 Steiner GC, Jundt G, Martignetti JA. Pathology and genetics of tumors of soft tissue and bone. In: Fletcher CDM, Unni KK, Mertens F, eds. World health organization classification of tumors. Lyon: IARC press, 2002; 294-6.

2 Capanna R, Bertoni F, Bacchini P, Bacci G, Guerra A, Campanacci M. Malignant fibrous histiocytoma of bone. The experience at the Rizzoli Institute: report of 90 cases. Cancer. 1984; 54: 177-87.

3 Bielack SS, Schroeders A, Fuchs N, et al. Malignant fibrous histiocytoma of bone: a retrospective EMSOS study of 125 cases. European Musculo-Skeletal Oncology Society. Acta Orthop Scand. 1999; 70: 353-60.

4 Bramwell VH, Steward WP, Nooij M, et al. Neoadjuvant chemotherapy with doxorubicin and cisplatin in malignant fibrous histiocytoma of bone: A European Osteosarcoma Intergroup study. J Clin Oncol. 1999; 17: 3260-9.

5 Reya T, Morrison SJ, Clarke MF, Weissman IL. Stem cells, cancer, and cancer stem cells. Nature. 2001; 414: 105-11.

6 Visvader JE, Lindeman GJ. Cancer stem cells in solid tumours: accumulating evidence and unresolved questions. Nat Rev Cancer. 2008; 8: 755-68.

7 Eyler CE, Rich JN. Survival of the fittest: cancer stem cells in therapeutic resistance and angiogenesis. J Clin Oncol. 2008; 26: 2839-45.

8 Bleau AM, Hambardzumyan D, Ozawa T, et al. PTEN/PI3K/Akt pathway regulates the side population phenotype and ABCG2 activity in glioma tumor stem-like cells. Cell Stem Cell. 2009; 4: 226-35.

9 Murase M, Kano M, Tsukahara T, et al. Side population cells have the characteristics of cancer stem-like cells/cancer-initiating cells in bone sarcomas. Br J Cancer. 2009; 101: 1425-32.

10 Nabeta Y, Kawaguchi S, Sahara H, et al. Recognition by cellular and humoral autologous immunity in a human osteosarcoma cell line. J Orthop Sci. 2003; 8: 554-9.

11 Wada T, Uede T, Ishii S, Matsuyama K, Yamawaki S, Kikuchi K. Monoclonal antibodies that detect different antigenic determinants of the same human osteosarcoma-associated antigen. Cancer Res. 1988; 48: 2273-9.

12 Tsukahara T, Kawaguchi S, Ida K, et al. HLA-restricted specific tumor cytolysis by autologous T-lymphocytes infiltrating metastatic bone malignant fibrous histiocytoma of lymph node. J Orthop Res. 2006; 24: 94-101.

13 Pellegatta S, Poliani PL, Corno D, et al. Neurospheres enriched in cancer stem-like cells are highly effective in eliciting a dendritic cell-mediated immune response against malignant gliomas. Cancer Res. 2006; 66: 10247-52.

14 Todaro M, D'Asaro M, Caccamo N, et al. Efficient killing of human colon cancer stem cells by gammadelta T lymphocytes. J Immunol. 2009; 182: 7287-96.

15 Pietra G, Manzini C, Vitale M, et al. Natural killer cells kill human melanoma cells with characteristics of cancer stem cells. Int Immunol. 2009; 21: 793-801.

16 Weng D, Song B, Durfee J, et al. Induction of cytotoxic T lymphocytes against ovarian cancer-initiating cells. International journal of cancer. 2010; [Epub ahead of print].

17 Seliger B. Strategies of tumor immune evasion. BioDrugs. 2005; 19: 347-54.

18 Marincola FM, Jaffee EM, Hicklin DJ, Ferrone S. Escape of human solid tumors from T-cell recognition: molecular mechanisms and functional significance. Adv Immunol. 2000; 74: 181-273.

19 Tsukahara T, Kawaguchi S, Torigoe T, et al. Prognostic significance of HLA class I expression in osteosarcoma defined by anti-pan HLA class I monoclonal antibody, EMR8-5. Cancer Sci. 2006; 97: 1374-80.

20 Yabe H, Tsukahara T, Kawaguchi S, et al. Prognostic significance of HLA Class I expression in ewing's sarcoma family of tumors. J Surg Oncol. 2010; [epub ahead of print].

21 Roesch A, Fukunaga-Kalabis M, Schmidt EC, et al. A temporarily distinct subpopulation of slow-cycling melanoma cells is required for continuous tumor growth. Cell. 2010; 141: 583-94.

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.

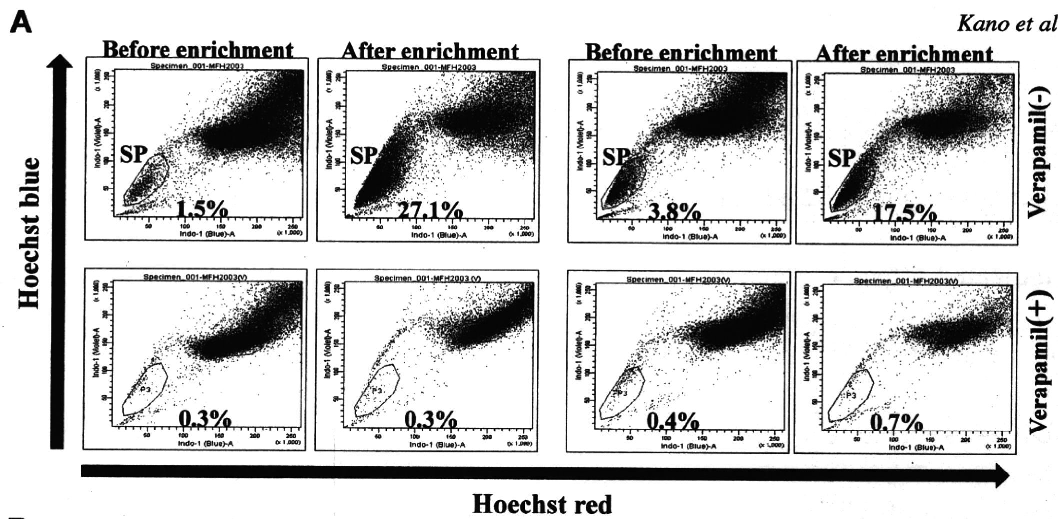
Fig. 3. Autologous TIL2003 recognized both SP and MP cells. Freshly isolated SP and MP cells of MFH2003, autologous EB-B cells (B2003-EBV) and negative control K562 cells were co-cultured with TIL2003. After 24h, culture supernatant was harvested and GM-CSF released from TIL2003 was measured by ELISA.

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

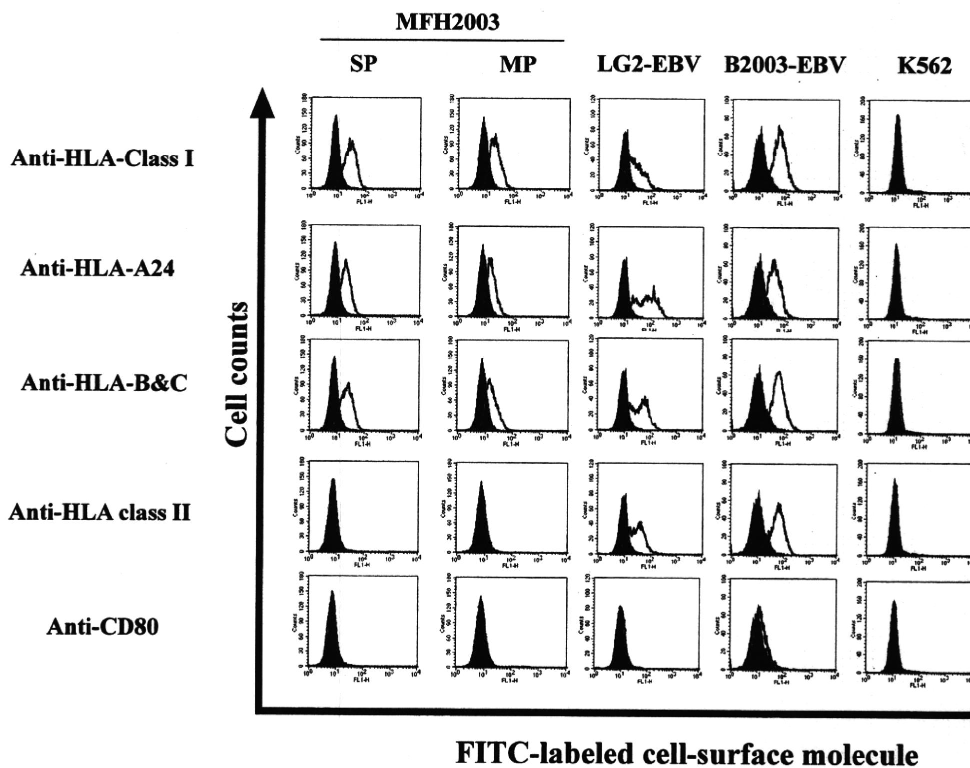


B

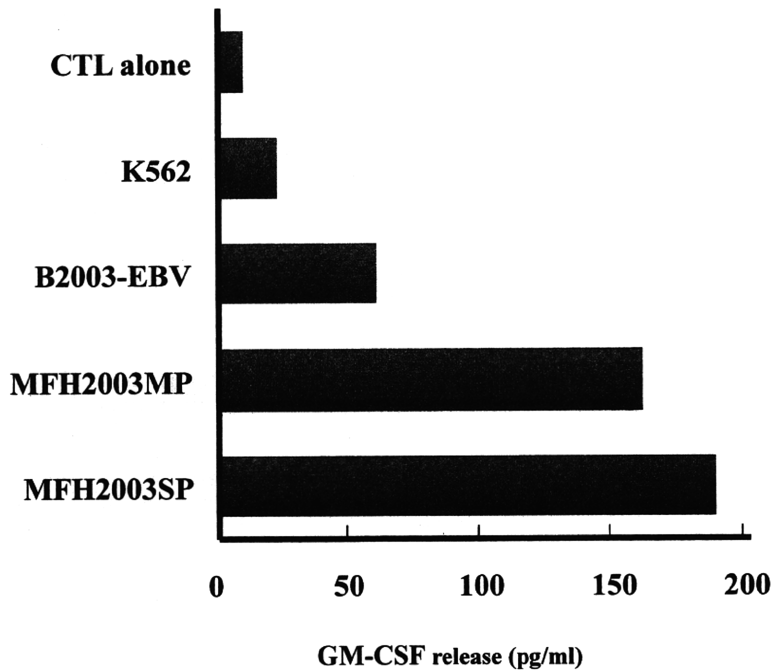
Before enrichment			
Number of experiment	Proportion of SP cells (%)	Input Bulk (x10^7 cells)	Output Sorted SP (x10^5 cells)
72	4.0±1.7(0.3-7.2)	1.7±0.8(0.6-5.7)	2.4±1.4(0.1-6.7)

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)

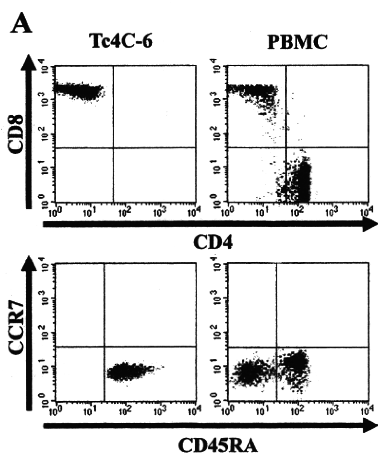
Kano et al. Figure 2

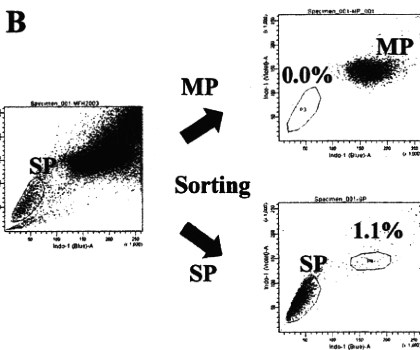


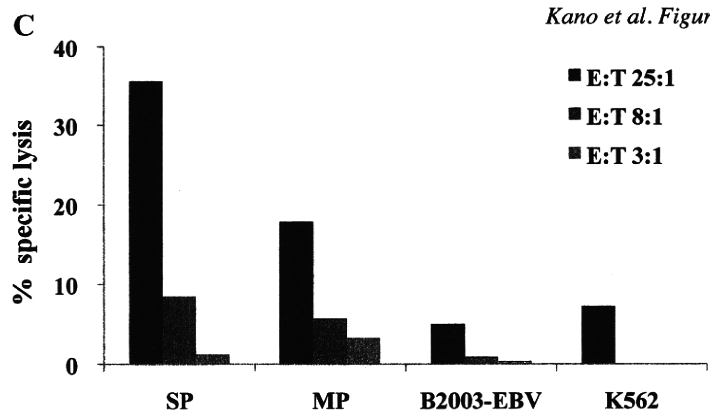
Kano et al. Figure 3

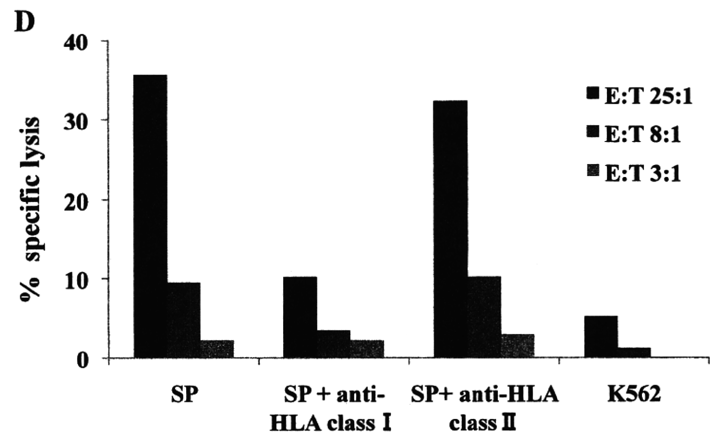


Kano et al. Figure 4









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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.

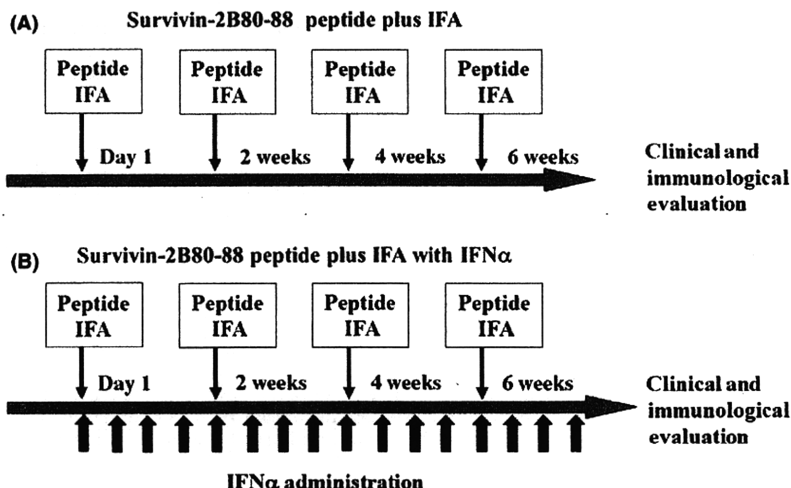

Fig. 1. Protocols of the clinical study. In the current study two protocols were used: (A) survivin-2B80-88 plus IFA and (B) survivin-2B80-88 plus IFA with IFNα. IFA, incomplete Freund's adjuvant; IFN, interferon.

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