data obtained by ELISPOT assay. $P<0.05$ was considered to be significant. Statistical analyses were made using the StatView 5.0 software package (Abacus Concepts, Calabasas, CA).

Results

IFA is an effective adjuvant for the peptide vaccine that induced strong immune responses and maintained the stability of the peptide. We attempted to verify whether emulsions of GPC3 peptide in IFA could induce a peptide-specific immune response in mouse models. The results showed that only GPC3 peptide emulsified in IFA elicited a T-cell-mediated immune response, whereas vaccination with peptide alone failed to induce any detectable immune response (Fig. 1A). In addition, we investigated the stability of GPC3 peptide alone in human serum, with the Surface Enhanced Laser Desorption/Ionization SELDI system (Bio-Rad). We applied the sample of GPC3 peptide mixed with human serum to a ProteinChip and detected the peak of GPC3 peptide. The peak value of $GPC3_{298\text{-}306}$ in buffer was about 1,000, whereas the peak of $GPC3_{298\text{-}306}$ in serum had decreased to 200, 3 min after mixing it with the serum (Fig. 1B). This finding indicated that GPC3 peptide was immediately degraded in serum. Moreover, we collected the white residue of peptide/IFA emulsions that were still present at the base of the tail of the vaccinated mice, and after applying the peptide/IFA emulsions to a ProteinChip, quantified the peak of $GPC3_{298\text{-}306}$. Mass spectrometric analysis demonstrated that the peptide was still present in a stable form in the peptide/IFA emulsions (Fig. 1C). IFA not only induced a potent immune response, but protected the peptide from various enzymes in the serum.

Dose-dependent effects of GPC3-derived peptide vaccine emulsified in IFA. Next we examined whether a more peptide-specific response was induced, when a higher dose of peptide was used. The proportion of peptide-specific CTLs among $5x10^4$ $CD8^+$ T cells was evaluated by IFN-γ Elispot assays, when mice were vaccinated with $GPC3_{298\text{-}306}$ K^d-restricted peptide doses of 5, 10, 20, or 50 μg. Peptide-specific $CD8^+$ T cell responses were observed when vaccinated with $GPC3_{298\text{-}306}$ K^d-restricted peptide doses above 10 μg (Fig. 2A). Additionally, as the peptide dose increased, peptide reactive CTLs were detected more frequently (Fig. 2A). We vaccinated A2 Tg mice with HLA-A2-restricted $GPC3_{144\text{-}152}$ peptide in the same manner. The results indicated that the higher doses of the peptide induced a greater peptide-specific immune response (Fig. 2B). We therefore, concluded that the higher the dose of peptide injected, the more peptide-specific $CD8^+$ T cells were induced. But Elispots by vaccinations of >5 μg $GPC3_{144\text{-}152}$ been seen to reach a plateau level.

Marked infiltration of subcutaneous tumor tissue by $CD8^+$ T cells in mice vaccinated with the 50 μg dose of GPC3 peptide. Immunohistochemical analysis of the tumor tissue specimens showed more intense infiltration by $CD8^+$ T cells, but not by $CD4^+$ T cells, in and/or around C26/GPC3 (Fig. 3D) or RMA-

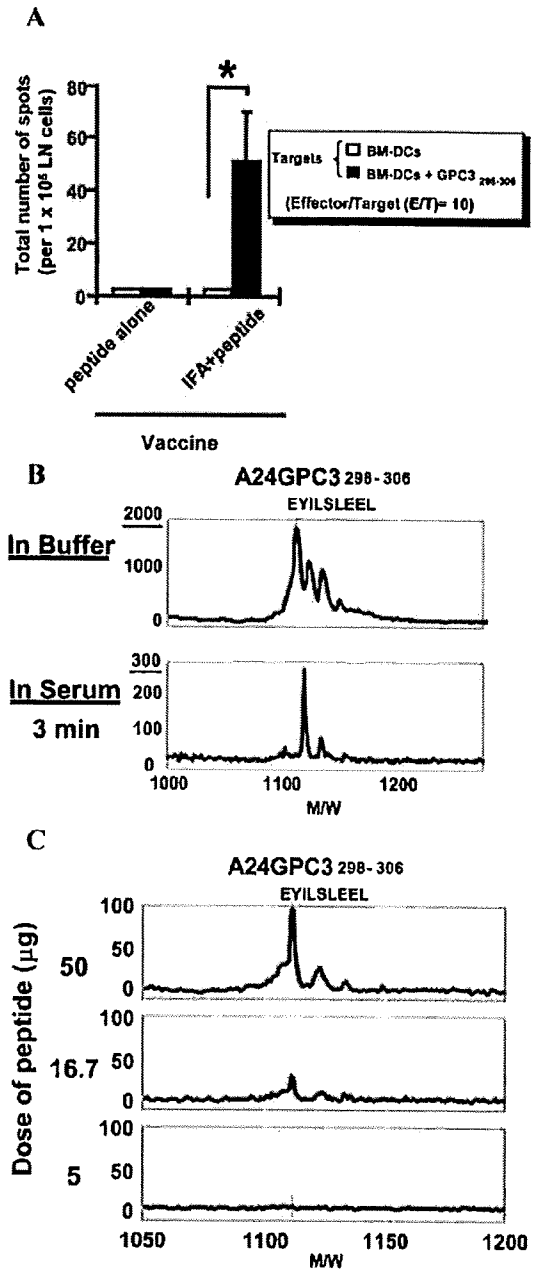

Figure 1. IFA is an appropriate adjuvant for the peptide vaccine. (A) The $GPC3_{298\text{-}306}$-specific immune response was induced by peptide emulsified in IFA. Female BALB/c mice were vaccinated twice by intradermal injection of 50 μg of $GPC3_{298\text{-}306}$ (EYILSLEEL) with IFA. Seven days after the second injection, inguinal lymph node cells were isolated and cultured with $GPC3_{298\text{-}306}$-pulsed BM-DCs for 5 days. The cells were recovered, and their Ag-specific activity was analyzed by IFN-γ Elispot assays against BM-DCs pulsed or not pulsed with $GPC3_{298\text{-}306}$. *$P<0.05$, statistically significant difference in response. (B) Stability of $GPC3_{298\text{-}306}$ (EYILSLEEL) in human serum. We measured $GPC3_{298\text{-}306}$ in the human serum 3 min after the mixture of $GPC3_{298\text{-}306}$ and human serum by SELDI-TOF mass spectrometry. The discriminating peaks of M/W 1111 represent $GPC3_{298\text{-}306}$. When GPC3 peptide was mixed with human serum, the peptide was immediately degraded by various proteases. (C) Detection of GPC3 peptide in emulsions collected from vaccinated mice. BALB/c mice were intradermally injected at the base of the tail with $GPC3_{298\text{-}306}$ emulsified in IFA. A week later we collected peptide/IFA emulsions at the base of the tail and measured GPC3 peptide contained in the emulsions. When injected with 50 μg of peptide, GPC3 peptide was detected clearly. Data are representative of 3 independent experiments with similar results in (A-C).

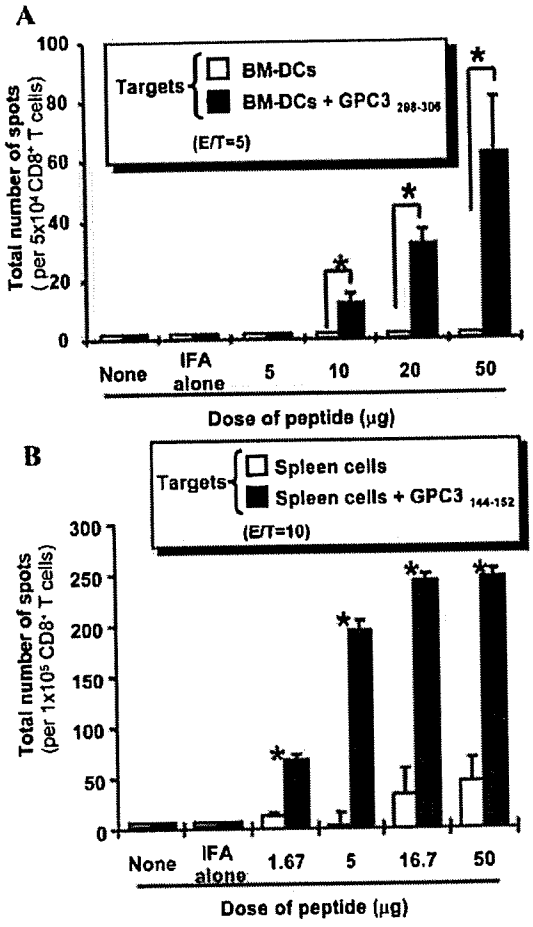

Figure 2. The higher the dose of peptide injected, the more peptide-specific CD8⁺ T cells were induced. Groups of mice were vaccinated twice at 7-day intervals with one of the dose levels of H-2K^{d}-restricted GPC3$_{298\text{-}306}$ peptide (A) or HLA-A2-restricted GPC3$_{144\text{-}152}$ peptide (B) emulsified with IFA (each group: n=3). Inguinal lymphocytes were restimulated *in vitro* with each GPC3 peptide-pulsed BM-DCs for 5 days. The recovered cells were sorted to the CD8⁺ T cells fraction by MACS and IFN-γ Elispot assays were performed. Peptide-specific CD8⁺ T cells were induced dose-dependently. Data are representative of 3 independent experiments with similar results. *P<0.05, difference in response was statistically significant.

HHD-GPC3 (Fig. 3H) tumor tissue of mice vaccinated with the 50 μg dose of GPC3 peptide than with IFA alone, and the 1.67 μg dose of GPC3 peptide (Fig. 3). This phenomenon was also observed in metastatic inguinal lymph nodes tissues (data not shown). These results also suggest that the higher the dose of peptide, the more peptide-specific CD8⁺ T cells were induced and infiltrated the GPC3-expressing tumor. However, 1.67 μg dose of GPC3$_{144\text{-}152}$ had seemed to induce a few peptide-specific CD8⁺ T cells, which corresponded to the result shown in Fig. 2B.

A second vaccination is needed to induce a peptide-specific response. Next we attempted to determine how many vaccinations were required to induce a peptide-specific immunological response. BALB/c mice were vaccinated with a 1.67, 5, 16.7, 50 μg dose of peptide once a week for 1-4 weeks, respectively. A single vaccination did not elicit a peptide-specific immune response at any of the

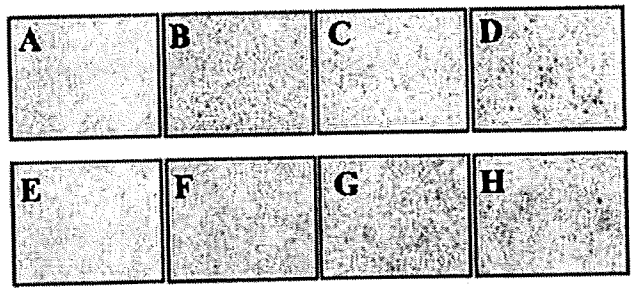

Figure 3. Infiltration by CD8⁺ T cells around and into subcutaneous C26/GPC3 (A-D) or RMA-HHD-GPC3 (E-H) tumor tissue is shown. We estimated infiltration by CD8⁺ T cells immunohistochemically; no treatment (A and E), IFA (B and F), 1.67 μg (C), 50 μg (D) of GPC3$_{298\text{-}306}$, 1.67 μg (G), 50 μg (H) of GPC3$_{144\text{-}152}$. In mice treated with 50 μg GPC3 peptide, a larger number of CD8⁺ T cells had clearly infiltrated into and/or around the tumor (D and H). But even in 1.67 μg dose of GPC3$_{144\text{-}152}$, a few peptide-specific CD8⁺ T cells were induced (original magnification, x200).

GPC3$_{298\text{-}306}$ dose levels (Fig. 4A). Induction of a peptide-specific T-cell response required at least two vaccinations and >16.7 μg dose of peptide, and no expansion of peptide-specific T cells occurred after repeated vaccinations with lower doses of peptide (Fig. 4B-D). We also compared immunological responses induced by two and five vaccinations, with 1.67 and 50 μg doses of HLA-A2 GPC3$_{144\text{-}152}$, but the same as with HLA-A24 GPC3$_{298\text{-}306}$, five vaccinations did not increase a peptide-specific response (Fig. 5).

Cytotoxity of CD8⁺ T cells primed with GPC3 peptide vaccines. We analyzed the cytotoxity of CD8⁺ T cells primed with GPC3 peptide vaccines. Their killing activity against target cells that expressed or did not express GPC3 was analyzed. The effector cells primed with the GPC3 vaccines showed a significantly higher killing activity against C26/GPC3 cells than against C26 cells, and significantly higher killing activity against RMA-HHD-GPC3 cells than against untransfected RMA-HHD cells (Fig. 6). These results suggest that the CD8⁺ T cells induced by GPC3 peptide vaccinations have cytotoxic activity against tumor cells that express GPC3 naturally.

Discussion

The stability of antigens and the immunogenicity of ISA 720 based on Western blot experiments (16) have been verified, and in the present study we showed that IFA is one of the indispensable adjuvants for peptide vaccines.

We previously reported that vaccination with GPC3$_{298\text{-}306}$ peptide-pulsed BM-DCs induced complete rejection of a C26/GPC3 tumor challenge in a mouse model (4), but in the present study, C26/GPC3 tumors in a prophylactic model were not rejected after two intradermal vaccinations with GPC3 peptide/IFA at the base of the tail even though CD8⁺ T cells by GPC3 peptide vaccine was demonstrated by immunological and immunohistological analysis (data not shown). Comparison of the capacity of peptide-pulsed BM-DCs vaccine to induce peptide-specific CTLs with the

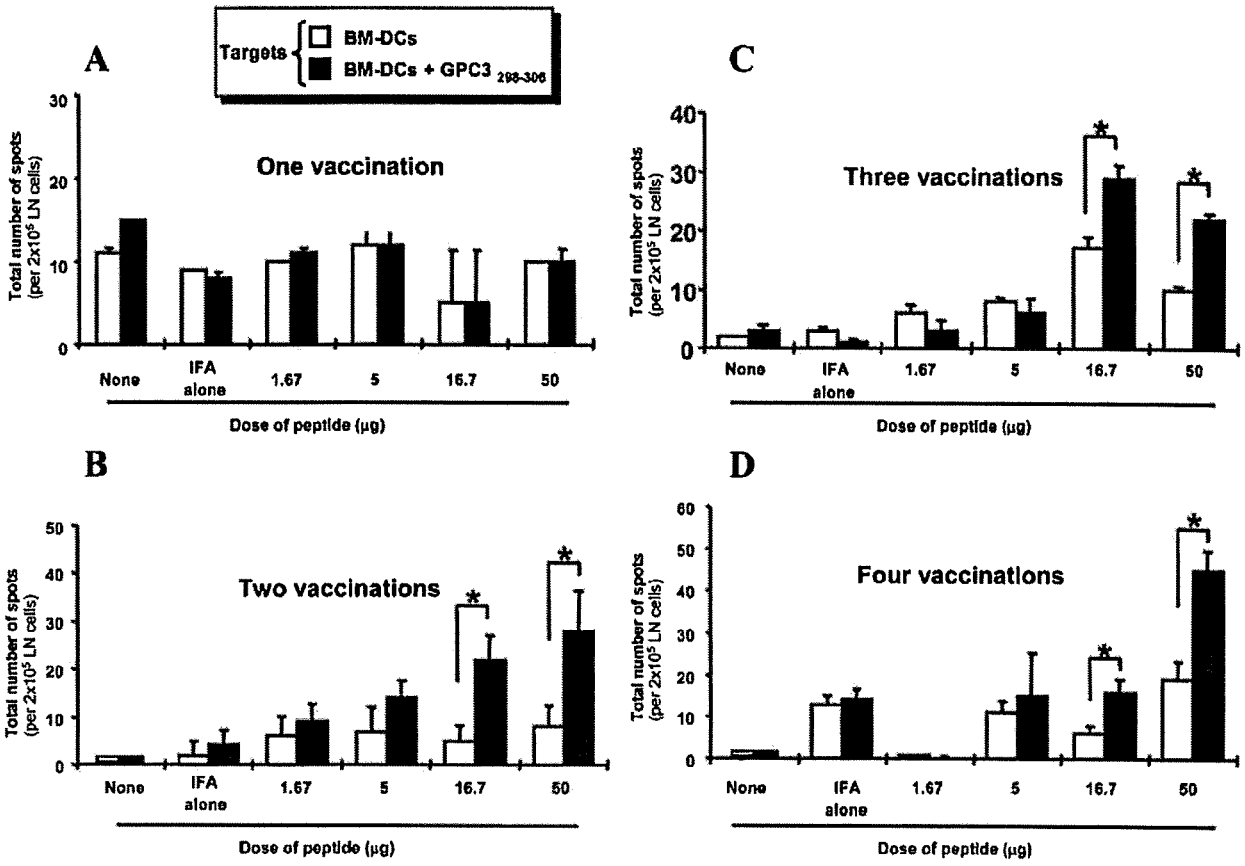

Figure 4. A second vaccination is needed to induce peptide-specific T cells. The immune responses to one (A), two (B), three (C) and four (D) vaccinations with each dose of peptide are shown. BALB/c mice (each group: n=3) were vaccinated with 1.67, 5, 16.7, or 50 μg $GPC3_{298-306}$. Seven days after the final vaccination, bilateral inguinal LNs were excised. Each lymphocyte was restimulated *in vitro* with $GPC3_{298-306}$ peptide-pulsed BM-DCs for 5 days, and IFN-γ Elispot assays were then performed against BM-DCs pulsed or not pulsed with $GPC3_{298-306}$ to count $GPC3_{298-306}$ peptide-specific CTLs. Data are representative of 3 independent experiments with similar results (A-D).

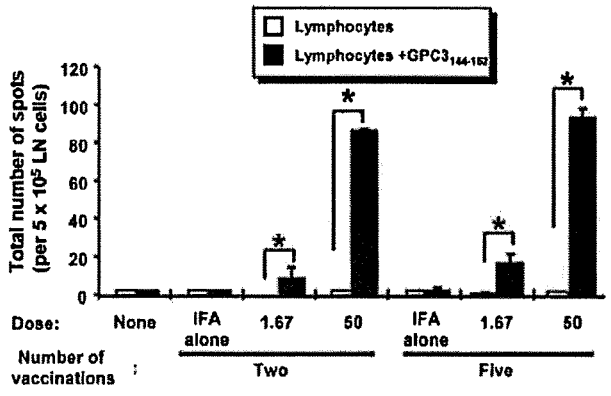

Figure 5. Comparison of immune responses after two or five vaccinations with a 1.67 μg or 50 μg dose of $GPC3_{144-152}$. A2 Tg mice (n=3) were vaccinated with 1.67 μg or 50 μg $GPC3_{144-152}$ in the same manner in Fig. 3, but the IFN-γ Elispot assay was performed using whole lymph node cells without *in vitro* culture. Data are representative of 3 independent experiments with similar results. *P<0.05, difference in response was statistically significant.

capacity of peptide/IFA vaccine by *in vitro* IFN-γ Elispot assays demonstrated that peptide-pulsed BM-DCs vaccine induced more peptide-specific CTLs (data not shown). There have been few reports of induction of tumor regression *in vivo* by peptide vaccine. Pilar *et al* recently reported finding that a combination of peptide vaccine and CpG induced stronger anticancer responses not only in a prophylactic model, but also in a therapeutic model. They reported in the same study that vaccination with peptide p66 in IFA in the absence of CpG resulted in insignificant CTL responses (17). Although other adjuvants, including CpG, were not effective in the present study (data not shown), peptide/IFA with CpG may be effective. Further study is needed.

The results of the present study showed that at least two vaccinations were necessary to elicit immunological effects. A comparison between HLA-A2-restricted $GPC3_{144-152}$/IFA and K^d-restricted $GPC3_{298-306}$ showed that $GPC3_{144-152}$ induced more peptide-specific CTLs at a lower dose. Moreover, HLA-A2-restricted $GPC3_{144-152}$ specific CTLs were induced without *in vitro* stimulation with $GPC3_{144-152}$ peptide. That may have been attributable to the difference in mouse strain. It is usually said that C57BL/6 and BALB/c mice are a prototypical Th1-type strain and a prototypical Th2-type mouse strain, respectively (18,19) and the difference in genetic background seemed to affect their susceptibility to each of the peptide vaccines.

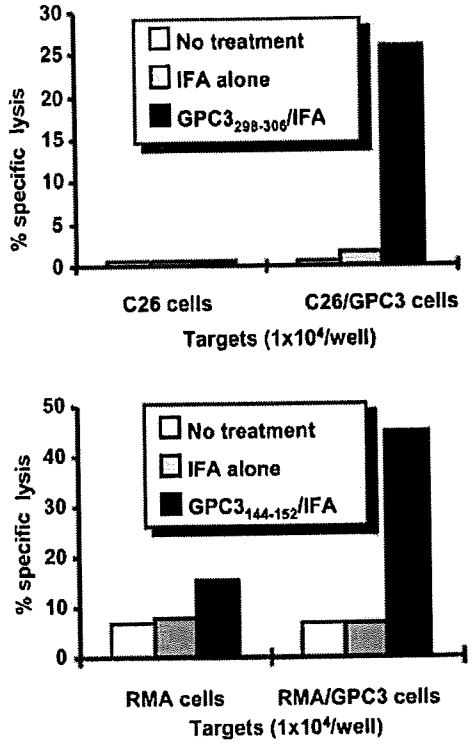

Figure 6. Cytotoxity of $CD8^+$ T cells primed with GPC3 peptide vaccine. BALB/c or C57BL/6 mice (n=2-3) were vaccinated with IFA alone or 50 μg peptide/IFA in the same manner as described above. The inguinal LN cells were cultured with $1x10^5$ peptide-pulsed BM-DCs for 5 days. The cells obtained were sorted to the $CD8^+$ T cell fraction with microbeads. Cytotoxic assays were performed with the cells to evaluate their capacity to kill $1x10^4$ C26, C26/GPC3, RMA-HHD, or RMA-HHD-GPC3 cells. Data are representative of 3 independent experiments with similar results.

In 1996, Salgaller *et al* reported that they did not detect dose dependency between 1 and 10 mg in the capacity of gp100 peptide to enhance immunogenicity in humans (9). A dose-dependent effect of peptide vaccine was shown in the present study. We are conducting a phase 1 clinical trail of GPC3-derived peptide vaccine in patients with advanced HCC at National Cancer Center Hospital East, and we are waiting for the results to determine whether a dose-dependent effect of peptide/IFA was shown in humans, the same as in the mouse model.

Acknowledgments

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Regression of intestinal adenomas by vaccination with heat shock protein 105-pulsed bone marrow-derived dendritic cells in $Apc^{Min/+}$ mice

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Heat shock protein (HSP) 105 is overexpressed in various cancers, but is expressed at low levels in many normal tissues, except for the testis. A vaccination with HSP105-pulsed bone marrow-derived dendritic cells (BM-DC) induced antitumor immunity without causing an autoimmune reaction in a mouse model. Because $Apc^{Min/+}$ mice develop multiple adenomas throughout the intestinal tract by 4 months of age, the mice provide a clinically relevant model of human intestinal tumor. In the present study, we investigated the efficacy of the HSP105-pulsed BM-DC vaccine on tumor regression in the $Apc^{Min/+}$ mouse. Western blot and immunohistochemical analyses revealed that the tumors of the $Apc^{Min/+}$ mice endogenously overexpressed HSP105. Immunization of the $Apc^{Min/+}$ mice with a HSP105-pulsed BM-DC vaccine at 6, 8, and 10 weeks of age significantly reduced the number of small-intestinal polyps accompanied by infiltration of both $CD4^+$ and $CD8^+$ T cells in the tumors. Cell depletion experiments proved that both $CD4^+$ and $CD8^+$ T cells play a critical role in the activation of antitumor immunity induced by these vaccinations. These findings indicate that the HSP105-pulsed BM-DC vaccine can provide potent immunotherapy for tumors that appear spontaneously as a result of the inactivation of a tumor suppressor gene, such as in the $Apc^{Min/+}$ mouse model. (*Cancer Sci* 2007; 98: 1930–1935)

Colorectal cancer is the third most common cancer and the fourth most frequent cause of cancer death worldwide. Every year, more than 945 000 people develop colorectal cancer worldwide, and approximately 492 000 patients die.[1] For patients with advanced stages of colorectal cancer, adjuvant systemic chemotherapy is a standard treatment. Major progress has been made by the introduction of regimens containing new cytotoxic drugs such as irinotecan and oxaliplatin; however, the new therapeutic regimens have led to only 8–9 months of progression-free survival.[2] Consequently, the development of new and effective therapeutic approaches, such as immunotherapy, is needed to expand treatment options.

The progression from normal epithelium to colorectal cancer is a multistep process involving the accumulation of multiple genetic alterations.[3] The *APC* gene, a tumor suppressor, is considered to be a gatekeeper in colon tumorigenesis,[4] and one of the earliest molecular events is the loss of function of the *APC* gene product.[5] APC forms a multimeric complex with the axis inhibition protein (AXIN)2 and glycogen synthase kinase 3β, which regulates the nuclear accumulation of β-catenin, a signal transducer of the wnt pathway.[6] When the APC–β-catenin complex is destabilized because of *APC* mutations, β-catenin binds and activates transcription factors that regulate the expression of potent oncogenes such as *c-Myc* and *c-Met*.[7] The importance of the *APC* gene product was confirmed by the demonstration that 80% of all sporadic colorectal cancers are characterized by one or more mutations in the *APC* gene, approximately 60% of which result in the expression of a truncated version of the APC protein.[8]

The $Apc^{Min/+}$ mouse has a nonsense mutation from T to A in the *Apc* gene at codon 850, homologous to the human germline and somatic *APC* mutation.[9] Although homozygous mice die before birth, all heterozygous mice develop multiple adenomas throughout their intestinal tract at an early age.[10] The $Apc^{Min/+}$ mouse model is unique in that tumors appear spontaneously in the intestinal tract, rather than as a result of induction by a carcinogen. This model is particularly advantageous for testing preventive agents targeted against early stage lesions because adenomas grow to a grossly detectable size within a few months on a defined genetic background.[10] Because $Apc^{Min/+}$ mice develop tumors due to the inactivation of the same tumor suppressor gene known to be involved in the pathogenesis of most colon cancers in humans, this model represents a clinically relevant model of human intestinal tumorigenesis.[10] Furthermore, germline mutations in the human *APC* gene cause FAP, whose symptoms resemble those of an $Apc^{Min/+}$ mouse. Therefore, this model provides useful information about not only colon cancer but also FAP.

Heat shock proteins are soluble intracellular proteins that are expressed ubiquitously, and their expression can be induced at much higher levels due to heat shock or other forms of stress. The essential functions of HSP are to bind and protect partially denatured proteins from further denaturation and aggregation.[11] A previous study reported that HSP105 (often called HSP110), identified with serological identification of antigens using the recombinant expression cloning (SEREX) method, is overexpressed in a variety of human cancers, including colorectal, pancreatic, thyroid, esophageal, and breast carcinoma, whereas HSP105 is expressed at lower levels in many normal tissues, except for the testis.[12,13] Immunotherapy targeted at HSP105 in the mouse prophylactic model, such as HSP105-pulsed BM-DC and *HSP105* DNA vaccines, induce antitumor immunity without causing an autoimmune reaction.[14,15] These findings indicate that HSP105 itself could be considered as a valuable tumor-associated antigen for immune-based treatment of various tumors.

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Abbreviations: APC, adenomatous polyposis coli; BM-DC, bone marrow-derived dendritic cell; COX, cyclooxygenase; DC, dendritic cell; ELISPOT, enzyme-linked immunospot; FAP, familial adenomatous polyposis; HSP, heat shock protein; mAb, monoclonal antibody; MBP, myelin basic protein; MHC, major histocompatibility complex; PBS, phosphate-buffered saline.

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Another study reported that HSP105 is involved in tumorigenesis by protecting cancer cells from apoptosis.[16] The constitutive overexpression of HSP105 protein was found to be essential for various cancer cells to survive and, conversely, the apoptosis-inducing effect of HSP105 small interfering RNA (siRNA) is specific for cancer. In contrast, HSP can also stimulate an adaptive immune response against antigens bound to HSP,[17] provided that the vaccine forms a complex of recombinant HSP110 and target tumor-associated antigen.[18,19]

In the present study, $Apc^{Min/+}$ mice were used as a model of a cancer immunotherapy for human colorectal cancer. Because tumors in $Apc^{Min/+}$ mice strongly express HSP105, the efficacy of immunization with HSP105-pulsed BM-DC for preventing the development of tumors in $Apc^{Min/+}$ mice was investigated.

Materials and Methods

Mice and genotyping. Frozen embryos of $Apc^{Min/+}$ mice obtained from the Jackson Laboratory were transferred to C57BL/6J mice (purchased from Charles River Japan, Yokohama, Japan) at the Center for Animal Resources and Development, Kumamoto University. Mice at 4–5 weeks of age were characterized for the *Apc* genotype by polymerase chain reaction analysis of tail DNA with the use of allele-specific primers.[20] The concentrations of these primers were 1.0 μM (5′-TGAGAAAGACAGAAGTTA-3′), 1.0 μM (5′-TTCCACTTTGGCATAAGGC-3′), and 0.2 μM (5′-GCCATCCCTTCACGTTAG-3′). The amplification conditions were 5 min at 94°C before 35 cycles at 94°C for 1 min, 50°C for 1 min, and 72°C for 1 min, followed by a final extension at 72°C for 5 min. The mice were maintained by breeding male $Apc^{Min/+}$ mice to female C57BL/6J mice. The mice were kept under specific pathogen-free conditions and these experiments were approved by the Animal Research Committee of Kumamoto University.

Production of recombinant proteins. Highly purified recombinant mouse HSP105 was produced from *Escherichia coli* strain BL21 cells transduced with the mouse *HSP105* gene expression vector, as described previously.[14,21] We also produced highly purified recombinant MBP as a negative control, which was prepared from bacterial lysate in the same way as the preparation of recombinant HSP105. Both recombinant HSP105 and MBP were estimated to be almost endotoxin free using a Limulus amebocyte lysate assay kit (BioWhittaker, Walkersville, MD, USA), and the endotoxin contents in the materials were <10 endotoxin U/mg.

Immunizations and scoring of tumors. HSP105-pulsed BM-DC were prepared as described previously.[14,22] The mice were inoculated intraperitoneally with HSP105-pulsed BM-DC (5×10^5) suspended in 200 μL PBS at 6, 8, and 10 weeks of age. The mice were treated with BM-DC alone, MBP-pulsed BM-DC, or PBS as controls. At 12 weeks of age the mice were killed and their small intestines were removed and fixed with formaldehyde. The intestines were then opened and stained with methylene blue and the number of tumors was counted.

Western blot and immunohistochemical analysis. Western blotting and the immunohistochemical detection of HSP105 were carried out as described previously.[12,16] Rabbit polyclonal antihuman HSP105 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) was used as the primary antibody in this study. The immunohistochemical staining of $CD4^+$ and $CD8^+$ T cells was carried out as described previously.[14] mAb specific to CD4 (L3T4; BD PharMingen, San Diego, CA, USA) and CD8 (Ly-2; BD PharMingen) were used for staining.

Depletion of $CD4^+$ or $CD8^+$ T cells in mice. Rat mAb GK1.5 specific to mouse CD4 and 2.43 specific to mouse CD8 were used to deplete $CD4^+$ and $CD8^+$ T cells, respectively, *in vivo*. The 6-week-old $Apc^{Min/+}$ mice were injected with ascites (500 μg/mouse) from hybridoma-bearing nude mice six times intraperitoneally with an interval of 3–4 days between injection. Normal rat IgG (Chemicon, Temecula, CA, USA) was used as a control. The depletion of T cell subsets was monitored by a flow cytometric analysis, which showed a more than 90% specific depletion in the number of splenocytes.

ELISPOT assay. The $Apc^{Min/+}$ mice were immunized with HSP105-pulsed BM-DC or BM-DC alone at 6 and 8 weeks of age. At 10 weeks of age, spleen cells were harvested and depleted of $CD4^+$ or $CD8^+$ T cells using a magnetic cell-sorting system with antimouse CD4 mAb and antimouse CD8a (Mittenyi Biotec GmbH, Bergisch Gladbach, Germany) mAb, respectively. The purity of these T-cell subsets exceeded 95% based on a flow cytometric analysis. $CD4^-$ T cells were used as a source of $CD8^+$ T cells and antigen-presenting cells, and $CD8^-$ T cells were used as a source of $CD4^+$ T cells and antigen-presenting cells. Five hundred thousand $CD4^-$ or $CD8^-$ T cells were added to each well in triplicate cultures of RPMI-1640 medium containing 10% fetal calf serum (FCS) together with 2 μg/mL HSP105, MBP, and one with medium only at 37°C for 24 h. Then ELISPOT assays were carried out as described previously.[12]

Statistical analysis. The statistical significance of differences between the experimental groups was determined using Student's *t*-test. The overall survival rate was calculated using the Kaplan–Meier method, and statistical significance was evaluated using Wilcoxon's test. A value of $P < 0.05$ was considered to be statistically significant.

Results

Overexpression of HSP105 in intestinal adenomas of the $Apc^{Min/+}$ mice. A previous study reported that mouse HSP105 is overexpressed in liver metastasis of a murine colorectal adenocarcinoma cell line (Colon26), and in lung metastasis of a murine melanoma cell line (B16-F10).[15] The expression of HSP105 in tumors of $Apc^{Min/+}$ mice were thereby analyzed. The small intestines of $Apc^{Min/+}$ mice were excised, and the expression level of HSP105 was evaluated by both western blot and immunohistochemical analyses. The $Apc^{Min/+}$ mice developed adenomatous polyps spontaneously, predominantly in and throughout the small intestine at 4 months of age (Fig. 1a). Both western blot and immunohistochemical analyses confirmed the strong expression of HSP105 in the tumors of $Apc^{Min/+}$ mice (Fig. 1b,c). Based on these observations, the $Apc^{Min/+}$ mouse was chosen as a murine model of cancer immunotherapy targeted at HSP105.

Immunization with HSP105-pulsed BM-DC vaccine reduced the number of small intestinal polyps in $Apc^{Min/+}$ mice. The preventive effects of HSP105-pulsed BM-DC vaccination on the development of adenomatous polyps in the $Apc^{Min/+}$ mice were investigated. The mice were divided into four groups consisting of 10 mice each, inoculated intraperitoneally with PBS (group 1), BM-DC (group 2), MBP-pulsed BM-DC (group 3), or HSP105-pulsed BM-DC (group 4) at 6, 8, and 10 weeks of age. Two weeks after the last immunization, the number of tumors in the small intestine was counted.

Tumors had already developed in the small intestine of $Apc^{Min/+}$ mice at the time of the first vaccination (6 weeks of age). Each mouse had a mean of 6.3 ± 3.4 tumors at that time. The mean number of tumors at 12 weeks of age was 20.9 ± 9.6 in group 4, which was significantly less ($P = 0.006$) than the numbers in group 1 (37.8 ± 11.0), group 2 (40.8 ± 11.0), and group 3 (34.8 ± 9.5) (Fig. 2a). It was therefore concluded that the HSP105-pulsed BM-DC vaccine has the potential to prevent the growth of tumors expressing HSP105. The survival time in group 4 (175.3 ± 32.6 days) tended to be longer than that in group 1 (146.7 ± 13.0 days) and in group 2 (152.7 ± 25.5 days); however, the difference between group 4 and group 2 was not statistically significant ($P = 0.081$; Fig. 2b). No apparent abnormalities, such as weight loss, hair abnormality, or paralysis, were observed in

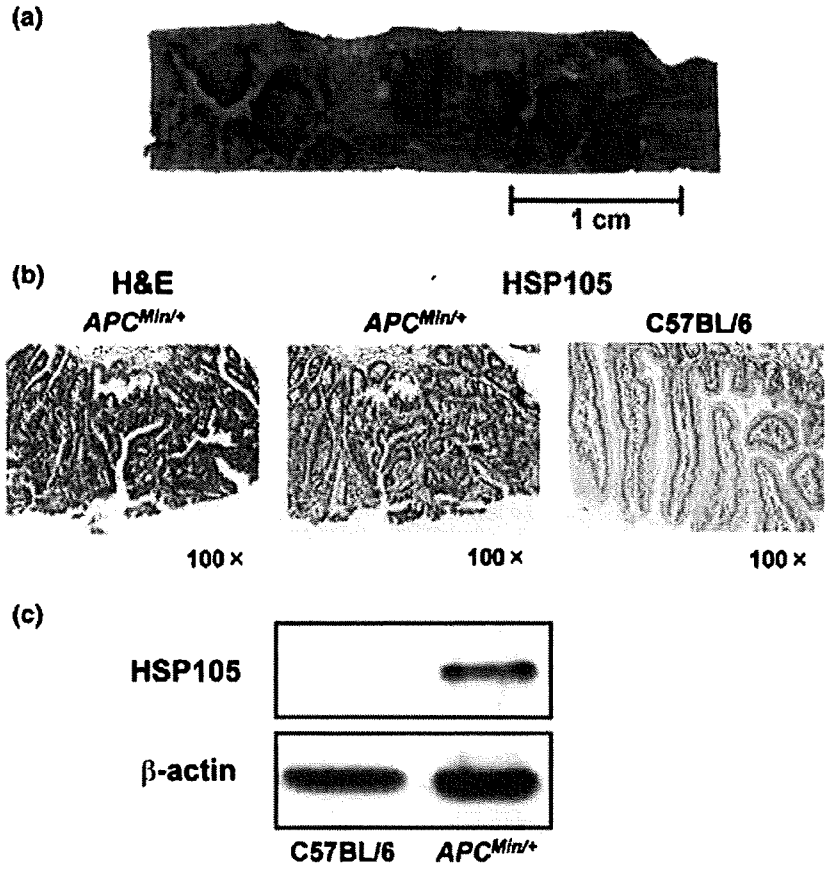

Fig. 1. Overexpression of heat shock protein (HSP) 105 in adenomatous polyps of $Apc^{Min/+}$ mice. (a) Macroscopic polyps in the small intestine of 4-month-old $Apc^{Min/+}$ mice. (b) A microscopic analysis of polyps in the small intestine of 12-week-old $Apc^{Min/+}$ mice stained with hematoxylin–eosin (left) and anti-HSP105 monoclonal antibody (middle). A normal small intestine was stained with anti-HSP105 monoclonal antibody as a negative control (right). Objective magnification was ×100. (c) Western blot analysis of HSP105 in the small intestine of 4-month-old $Apc^{Min/+}$ mice. The samples were small intestines of $Apc^{Min/+}$ and C57BL/6J mice homogenized in lysis buffer. The small intestines of three mice per group were pooled.

the mice immunized with HSP105-pulsed BM-DC, suggesting that serious autoimmunity was not observed in the mice. A histological analysis of the major organs (brain, lung, heart, liver, small intestine, kidney, and testis) of the immunized mice revealed no pathological inflammation (data not shown).

Both CD4⁺ and CD8⁺ T cells are required for antitumor immunity. To determine the role of $CD4^+$ and $CD8^+$ T cells in the reduction of tumor development in $Apc^{Min/+}$ mice immunized with HSP105-pulsed BM-DC, mice were depleted of $CD4^+$ or $CD8^+$ T cells by treatment with anti-CD4 or anti-CD8 mAb, respectively, *in vivo*. During the depletion procedure, the mice were immunized with PBS or HSP105-pulsed BM-DC vaccine (Fig. 3a). In the group of mice immunized with HSP105-pulsed BM-DC, together with inoculation of anti-CD4 mAb (35.5 ± 10.8) or anti-CD8 mAb (30.2 ± 9.6), the tumor numbers were significantly larger than those in the mice given rat IgG (18.8 ± 5.9) or left untreated (19.9 ± 7.7). The differences in the tumor numbers between the anti-CD4 mAb-treated group and the rat IgG-treated group ($P = 0.002$), and between the anti-CD8 mAb-treated group and the rat IgG-treated group ($P = 0.013$) were statistically significant. In the group of mice inoculated with PBS, the numbers of tumors in the mice given either anti-CD4 mAb (38.1 ± 5.7) or anti-CD8 mAb (38.1 ± 5.6) did not differ significantly from those in the mice given rat IgG (37.8 ± 4.8) or in the untreated mice (40.8 ± 6.1) (Fig. 3b). These results suggest that both $CD4^+$ and $CD8^+$ T cells play a crucial role in the protective antitumor immunity induced by the HSP105-pulsed BM-DC vaccine, because the HSP105-pulsed BM-DC vaccine was not effective in the mice showing a depletion of either $CD4^+$ or $CD8^+$ T cells.

Detection of HSP105-specific T cells in mice immunized with the HSP105-pulsed BM-DC vaccine. The $Apc^{Min/+}$ mice were immunized with HSP105-pulsed BM-DC or BM-DC at 6 and 8 weeks of age. At 10 weeks of age, spleen cells were harvested and depleted of $CD4^+$ or $CD8^+$ T cells using magnetic cell-sorting system, and the ELISPOT assay was carried out. The ELISPOT assay showed that the $CD8^-$ cells ($CD4^+$ T cells and antigen-presenting cells) derived from the mice immunized with HSP105-pulsed BM-DC produced a significantly larger amount of interferon-γ in response to HSP105 than did $CD8^-$ cells derived from mice immunized with BM-DC. Similar results were observed for the $CD4^-$ cells ($CD8^+$ T cells and antigen-presenting cells) (Fig. 4a). These observations clearly indicate that both HSP105-specific $CD4^+$ and $CD8^+$ T cells were induced in the mice immunized with HSP105-pulsed BM-DC vaccine.

To investigate the antitumor effect of the HSP105-pulsed BM-DC vaccination, the tumor was evaluated histopathologically. The small intestines derived from the mice used for the ELISPOT assay were stained with anti-CD4 or anti-CD8 mAb. Both $CD4^+$ and $CD8^+$ T cells infiltrated into the tumors of mice immunized with HSP105-pulsed BM-DC; however, this was not the case in tumors derived from the mice immunized with BM-DC (Fig. 4b). These results suggest that HSP105-pulsed BM-DC have the potential to sensitize many HSP105-specific $CD4^+$ and $CD8^+$ T cells to kill tumor cells.

Discussion

In the present study, the HSP105-pulsed BM-DC vaccine could sensitize HSP105-specific T cells *in vivo* and inhibited the spontaneous development of intestinal tumors overexpressing HSP105 in $Apc^{Min/+}$ mice. For diseases of germline mutations that cause malignancy throughout the body, such as FAP, novel strategies for the prevention of cancer are needed urgently because there is no satisfactory treatment for FAP. Therefore,

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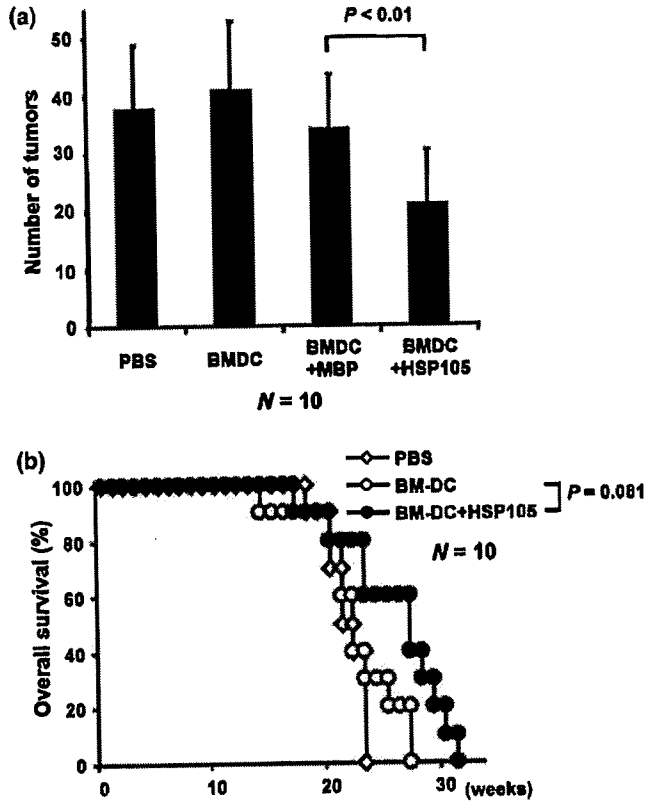

Fig. 2. Vaccination with heat shock protein (HSP) 105-pulsed bone marrow-derived dendritic cells (BM-DC) decreased the number of polyps in the small intestine of the $Apc^{Min/+}$ mice. (a) The $Apc^{Min/+}$ mice were inoculated intraperitoneally with HSP105-pulsed BM-DC (5×10^5), BM-DC alone, or myelin basic protein-pulsed BM-DC or phosphate-buffered saline (PBS) at 6, 8, and 10 weeks of age. At 12 weeks of age, the small intestines of the $Apc^{Min/+}$ mice were excised, stained with methylene blue, and the number of tumors was counted by the naked eye. Each group consisted of 10 $Apc^{Min/+}$ mice. The statistical significance of the differences in results was determined using an unpaired *t*-test. (b) The survival rate of $Apc^{Min/+}$ mice immunized with HSP105-pulsed BM-DC, BM-DC alone, or PBS as a control. The immunization protocol was the same as that of (a). The overall survival rate was calculated using the Kaplan–Meier method, and statistical significance was evaluated using Wilcoxon's test.

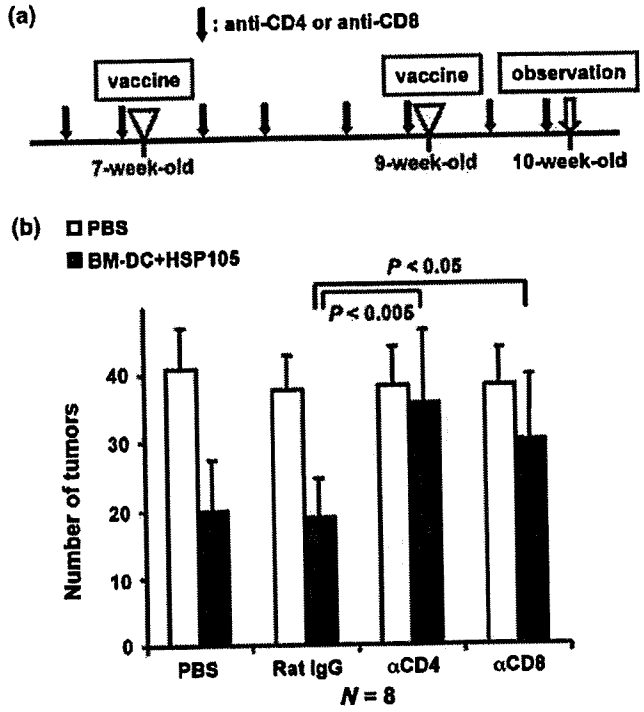

Fig. 3. Both $CD4^+$ and $CD8^+$ T cells are involved in the antitumor immunity elicited by the heat shock protein (HSP) 105-pulsed dendritic cell vaccine. (a) The protocol for the vaccination and the depletion of T cell subsets. (b) The number of polyps in the small intestine of $Apc^{Min/+}$ mice with various treatments. The number of tumors was counted as described in the legend for Fig. 2. Each group consisted of eight $Apc^{Min/+}$ mice. The statistical significance of the difference between the results was determined using the unpaired *t*-test.

the specific objective of the present study was to find out whether HSP105-pulsed DC-based immunotherapy can be used as a potent new strategy for the prevention of spontaneously arising tumors in FAP patients.

The ELISPOT assay shown in Figure 4a shows that both $CD4^+$ and $CD8^+$ HSP105-reactive T cells were primed in the mice immunized with HSP105-pulsed BM-DC. In this assay, we cannot completely rule out the possibility that responses were directed against contaminated bacteria-derived molecules in the HSP105 recombinant protein preparation. However, we consider this unlikely because practically no response was observed against BM-DC loaded with recombinant MBP protein, which was prepared from bacterial lysate in the same way as the preparation of recombinant HSP105. These recombinant proteins were purified extensively as described in a previous paper,[14] and contamination of lipopolysaccharide (LPS) or other DC-stimulants was ruled out.

Previous studies have reported that HSP105 is overexpressed specifically in a variety of human cancers and mouse tumor cells.[13,14] The present study demonstrated that HSP105 was also strongly expressed in the adenomatous polyps of $Apc^{Min/+}$ mice. In human tissue, the overexpression of HSP105 is a late event in the adenoma–carcinoma sequence, because immunohistochemical analysis revealed that HSP105 is strongly expressed in adenocarcinoma but not in adenoma.[13] Although the $Apc^{Min/+}$ mouse model has provided useful information about the pathogenesis of colorectal cancer, it is limited because it does not completely mimic the disease in humans. In humans, patients with FAP develop hundreds to thousands of adenomatous polyps, predominantly in the distal colon, and have a high risk of malignancies before the age of 40 years.[23] In contrast, $Apc^{Min/+}$ mice develop dozens to hundreds of adenomas and have a shortened life span. However, these adenomas are located mainly in the small intestine and they generally do not become malignant.[10] Furthermore, mice carrying different *Apc* mutations have been established. Tumors arising in these mice are histologically similar, but vary with respect to age of onset, number of tumors, and location.[24] Given this variation, the pattern of HSP105 expression in intestinal tumors may be different between human and $Apc^{Min/+}$ mice. Regardless of these differences, the $Apc^{Min/+}$ mice provide an appropriate model for analysis of the efficacy of the HSP105-pulsed BM-DC vaccine for inhibition of the development of human colorectal cancer, because the loss of *APC* function is the initiating event in not only FAP but also in the vast majority of sporadic colon cancers.

Recent findings regarding the cellular and molecular pathogenesis of colorectal cancer have led to the development of new targeted therapeutic options. Overexpression of COX-2 is one of the most significant observations in this respect.[25] The use of COX-2 inhibitor suppresses the development of colon cancer in

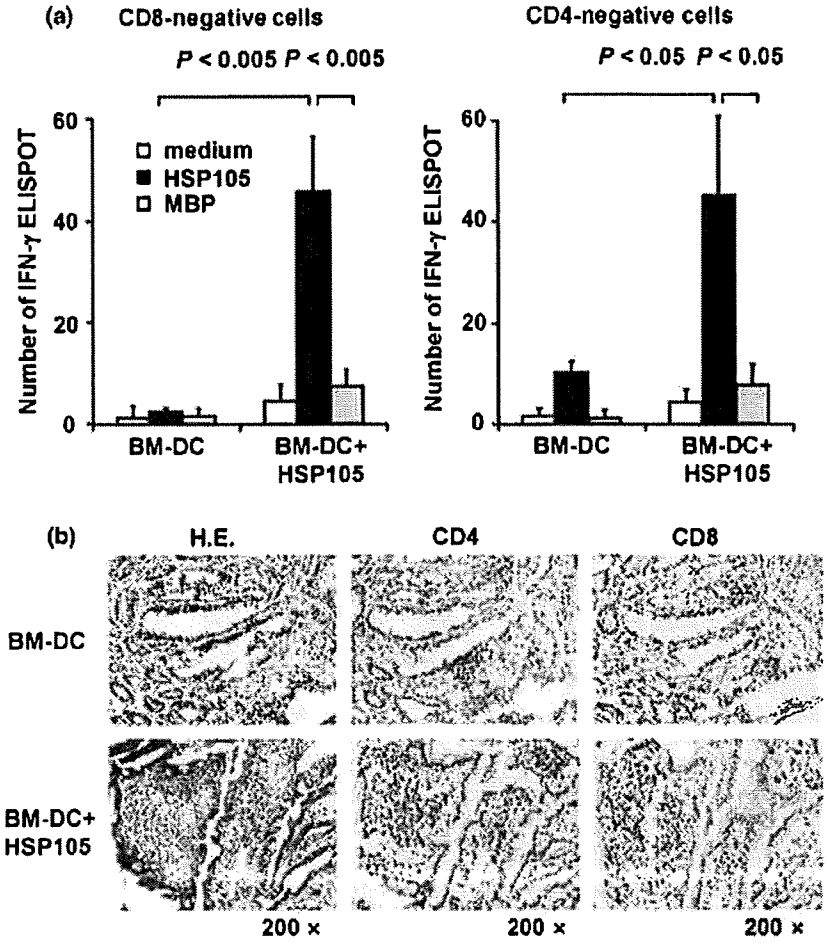

Fig. 4. Induction of heat shock protein (HSP) 105-specific T cells via immunization with HSP105-pulsed bone marrow-derived dendritic cells (BM-DC). (a) The $Apc^{Min/+}$ mice were inoculated with HSP105-pulsed BM-DC or BM-DC at 6 and 8 weeks of age. The spleen cells were harvested from 10-week-old $Apc^{Min/+}$ mice and depleted with either $CD4^+$ or $CD8^+$ cells using magnetic cell-sorting system. $CD4^-$ cells were used as a source of $CD8^+$ T cells and antigen-presenting cells, and $CD8^-$ cells were used as a source of $CD4^+$ T cells and antigen-presenting cells. Thereafter interferon-γ enzyme-linked immunospot (ELISPOT) assays were carried out. Briefly, $CD4^-$ or $CD8^-$ T cells (5×10^5) in each well were cultured together with 2 μg/mL HSP105, myelin basic protein, or medium alone for 24 h. The statistical significance of the difference in results was determined using the unpaired *t*-test. The spleens of three mice from each group were pooled. This experiment was carried out three times, with similar results. (b) The $Apc^{Min/+}$ mice were inoculated with HSP105-pulsed BM-DC or BM-DC at 6 and 8 weeks of age. The small intestines were excised from 10-week-old $Apc^{Min/+}$ mice and then were analyzed after immunohistochemical staining with anti-CD4 monoclonal antibody or anti-CD8 monoclonal antibody (magnification ×200).

sporadic cases[26] and FAP;[27] however, recent clinical trials suggest that the use of high doses of COX-2 inhibitor may have dangerous side-effects, such as increased risk of cardiovascular disease.[28] In the present study, no apparent autoimmunity was observed in the $Apc^{Min/+}$ mice immunized with HSP105-pulsed BM-DC, an observation similar to our previous findings.[14,22] In some human clinical trials of DC-based cancer immunotherapy, even in patients with advanced stages of cancer, no major toxicity nor severe side-effects were observed.[29–31] These results strongly suggest that DC-based immunotherapy is safe and feasible.

DC vaccination is now considered to be one of the most promising strategies for cancer immunotherapy.[32,33] DC are the most potent antigen-presenting cells and can present tumor antigens to stimulate a tumor-specific T-cell response. However, this does not occur in most types of cancer and in animal models of spontaneously arising tumors.[34] In the present study, immunization with HSP105-pulsed BM-DC vaccine significantly reduced the number of small-intestinal polyps in the $Apc^{Min/+}$ mice; however, the duration of survival was not prolonged as had been expected because the adenomas in $Apc^{Min/+}$ mice generally did not become malignant. Thereby, the protocol of DC-based vaccination used in the present study was not sufficient to completely prevent the occurrence of the tumors *in vivo*, and we are trying to establish a more effective immunization protocol. New strategies are now being developed to improve the clinical efficacy of DC-based vaccines, for example, the use of overexpression of Akt1 in BM-DC, suppressor of cytokine signaling 1-silenced BM-DC, and CD40-inducible DC.[35–37] The use of transfected DC in a protocol such as that used in the present study has the potential to induce a more effective antitumor response. Furthermore, it is necessary to investigate whether combinations of immunotherapy and other therapies, such as combinations of DC vaccines and chemotherapy or low-dose COX-2 inhibitors, induce a more effective antitumor response in comparison to individual therapy alone, thereby developing more effective strategies for treating colorectal cancer. Recent findings have shown the curative potential of combinations of irradiation,[38] chemotherapy,[39] and subsequent adoptive T-cell immunotherapy against established solid tumors.[40]

The abrogation of the antitumor effect of the HSP105-pulsed BM-DC vaccine, after the depletion of $CD4^+$ cells or $CD8^+$ cells via the administration of mAb, indicates that both $CD4^+$ and $CD8^+$ T cells play a critical role in the antitumor effect of HSP105-pulsed BM-DC. The report that antigen-specific $CD4^+$ T helper cells are required for the activation of $CD8^+$ effector T cells, their secondary expansion, and memory induction,[41] is consistent with the findings that $CD4^+$ T cells played an important role in tumor rejection in the present study. Peptides derived from HSP105 incorporated into BM-DC might be presented in the context of MHC class II on the surface of BM-DC to activate $CD4^+$ T cells. Subsequently, $CD4^+$ T cells produce interferon-γ and interleukin-2 to activate HSP105-specific $CD8^+$ effector T cells and facilitate the development of HSP105-specific $CD8^+$ memory T cells. Furthermore, the ELISPOT assay showed that HSP105-specific $CD8^+$ T cells were also activated by HSP105-pulsed antigen-presenting cells. These results indicate

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that HSP105-pulsed BM-DC can demonstrate peptides derived from exogenously added HSP105 not only in the context of MHC class II molecules to activate $CD4^+$ T cells but also in the context of MHC class I molecules via the mechanism of cross-presentation to activate $CD8^+$ T cells. Whole-protein-pulsed DC vaccines seem to be superior to peptide-pulsed DC because they can activate both $CD4^+$ and $CD8^+$ T cells, and it does not require a knowledge of the human leukocyte antigen (HLA) type of the cancer patients.

In conclusion, the results of the present study indicate that HSP105-pulsed BM-DC may provide a potential vaccine to combat human colorectal cancer. It is possible that immunization with HSP105-pulsed BM-DC vaccines could be useful in patients with colorectal cancer to prevent tumor recurrence after surgical resection. Although there was a noteworthy effect of this type of vaccine on the host immune response to tumors expressing HSP105, further investigation to improve the clinical efficacy of HSP105-pulsed BM-DC vaccines is called for.

Acknowledgments

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WT1 (WILMS TUMOR 1) PEPTIDE IMMUNOTHERAPY FOR CHILDHOOD RHABDOMYOSARCOMA: A Case Report

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□ *Immunotherapy using a Wilms tumor (WT1) peptide has been undergoing clinical trials for adulthood leukemia and solid cancer with promising results. In this study, the authors used WT1 peptide vaccination to treat a 6-year-old girl with metastatic alveolar rhabdomyosarcoma. She received weekly intradermal injection with HLA-A* 2404-restricted, 9-mer WT1 peptide against residual bone disease. After 3 months her bone disease disappeared, concurrent with an increase in the frequency of WT1-specific cytotoxic T lymphocytes (CTLs). A high proportion of WT1-specific CTLs with effector or effector memory phenotype were detected in peripheral blood of this patient. She is currently still on continued WT1 peptide immunotherapy in a disease-free condition for 22 months. WT1 peptide-based immunotherapy should be a promising option forhigh-risk rhabdomyosarcoma in childhood.*

Keywords childhood, rhabdomyosarcoma, WT1 peptide immunotherapy

The Wilms tumor gene WT1 was first identified as a gene responsible for Wilms tumor, a childhood renal cancer. This gene encodes a zinc finger transcription factor and plays an important role in cell proliferation, differentiation, apoptosis, and organ development by the positive or negative regulation of the expression of various kinds of genes [1]. Although the WT1 gene was first categorized as a tumor suppressor gene, recent studies showed the overexpression of WT1 mRNA in various kinds of solid tumors [2], the growth inhibition of WT1-expressing cells by WT1 antisense oligomers [2, 3], and a correlation between a high level of WT1 and a poor prognosis [15] in patients with certain kinds of tumors [4, 5], suggesting that WT1 plays an oncogenic role in human cancers. Furthermore, a sequencing study revealed the absence of mutations in the WT1 gene in tumors [2], indicating wild-type WT1 could be oncogenic.

WT1 is often overexpressed in leukemias and various types of solid tumors. Nakatsuka et al. examined overexpression of WT1 in 494 cases of human cancers and found overexpression in 30–70% of tumors of the gastrointestinal and pancreatobiliary system, urinary tract, male and female genital organs, breast, lung, brain, skin, and bone [6]. They also showed WT1 expression in 3 of 7 patients with PNET/Ewing sarcoma and in all 6 patients with rhabdomyosarcoma.

WT1 is now regarded as a molecular target for immunotherapy in various malignant tumor types. Clinical trials of WT1 peptide-based cancer immunotherapy are ongoing: WT1 peptide vaccination has been shown to be safe and clearly effective against several kinds of malignancies [7–10]. The trial for pediatric cancer is currently limited. Here, we describe the case report of a 6-year-old girl with rhabdomyosarcoma who was successfully treated with WT1 peptide-based immunotherapy.

PATIENT AND METHODS

Clinical Study

The WT1 peptide-based phase I/II clinical study was approved by the Institutional Review Board of Osaka University Hospital. Patients aged

<20 years with pediatric cancer or leukemia were eligible if they were resistant to conventional multimodal therapy. Other inclusion criteria were WT1 protein expression in solid cancer tissues or WT1 mRNA expression in leukemic cells determined by immunohistochemistry and RT-PCR, respectively; HLA-A*2402-positive; and performance status 0 to II (Eastern Cooperative Oncology Group). Patients were excluded if they had severely impaired organ function or had received chemotherapy or radiotherapy between the confirmation of residual disease and WT1 peptide vaccination.

Immunohistochemistry Determination of WT1 Expression in Solid Cancer Tissue

Formalin-fixed tissue sections (3-μm thickness) were cut from each paraffin block. After being dewaxed with xylene and rehydrated through a graded series of ethanol, the sections were microwaved for 15 min in 10 mM citrate buffer (pH 6.0) for antigen retrieval. These sections were incubated in phosphate-buffered saline containing goat serum albumin, reacted with anti-WT1 6F-H2 mouse monoclonal antibody (mAb) (Dako Cytomation. Carpinteria, CA, USA) diluted 1:50 at 4°C overnight, and then reacted with EnVision kit (Dako Cytomation) according to the manufacturer's instructions. After treatment with 3% H_2O_2 solution to reduce endogenous peroxidase activity, immunoreactive WT1 protein was visualized with diaminobenzidine tetrahydrochloride. The sections were then counterstained with hematoxylin.

WT1 Peptide Treatment Plan

The 9-mer WT1 peptide (a.a. 235–243, CYTWNQMNL) was used for immunization [11]. GMP grade WT1 peptide was purchased from Multiple Peptide Systems (San Diego, CA) as a lyophilized peptide, which was dissolved just prior to injection. After written informed consent was obtained from the patient and her parents, a skin test for an HLA-A*2402-restricted, 9-mer WT1 peptide was performed and confirmed to be negative. WT1 peptide (1 mg) was emulsified with Montanide ISA51 adjuvant (SEPPIC S.A., Paris, France) [7, 8, 12], and the emulsion was injected intradermally at several different regions, including upper arms and lower abdomen. The WT1 peptide vaccination was scheduled to be performed weekly.

Analysis of WT1-specific Cytotoxic T-lymphocytes

The procedure for cell staining was performed as described elsewhere [13]. Peripheral blood mononuclear cells were stained with phycoerythrin (PE)-conjugated HLA-A*2402-WT1 235–243 tetramer (WT1-Tet) (MBL, Tokyo, Japan). The cells were then stained with fluorescein

isothiocyanate-labeled anti-CD4, CD14, CD16, CD19, and CD5 mAbs (eBioscience, San Diego, CA), APC-Cy7-labeled anti-CD8 mAb (BD Pharmingen, San Diego, CA), ECD-labeled anti-CD45RA mAb (2H4LDH11LDB9, Beckeman Coulter, Fullerton, CA), and PE-Cy7-labeled CCR7 mAb (3D12, BD Biosciences, San Jose, CA). After this procedure, cells were analyzed with FACS Aria (BD Biosciences). CD4, CD14, CD16, CD19, and CD56-negative WT1-Tet$^+$ CD8$^+$ T cells were considered to be the WT1 peptide-specific CD8$^+$ T cells. We measured the frequency (%) of WT1-Tet$^+$ CD8$^+$ T cells among the CD8$^+$ T cells, and defined it as the WT1-specific cytotoxic T-lymphocyte (CTL) frequency. In addition, we analyzed the phenotype of WT1-Tet$^+$ CD8$^+$T cells according to their expression of CD45RA and CCR7. The WT1-Tet$^+$ CD8$^+$ T cells were phenotypically classified into four differentiation stages: naïve (CD45RA$^+$CCR7$^+$), central memory (CD45R$^-$CCR7$^+$), effector memory (CD45RA$^-$CCR7$^-$), and effector(CD45RA$^+$CCR7$^-$) [13].

CASE REPORT

A 6-year-old girl presented with a mass on her lower left leg (Figure 1A). A diagnosis of alveolar rhabdomyosarcoma was made by histopathology with presence of left inguinal PAX3-FKHR. Image studies showed a lymph node metastasis from the right axial to para-aortic legion (Figure 1B) and multiple bone metastases located on right parietal, right 4th rib, and thoracic vertebrae (Figure 1C). Bone marrow aspiration revealed aggregation of tumor cells (Figure 1D). The disease status was stage 4 and group IV.

Combination chemotherapy was started. The combination consisted of cyclophosphamide, etoposide, THP-adriamycin, cisplatin, and vincristine (course 1), followed by ifosfamide, etoposide, actinomycin-D, and vincristine (course 2). However, a new bone metastasis lesion was observed on lumbar vertebrae (Figure 2A) after two courses of chemotherapy. At this point, bone marrow aspiration showed no residual tumor cells. She then received two further courses of chemotherapy intensified with nogitecan (2 mg/m^2 × 3–5). She subsequently underwent operation on the primary site and additional two courses of chemotherapy with radiotherapy on the primary site, high-dose chemotherapy consisting of thio-TEPA and L-PAM with autologous bone marrow and peripheral blood stem cell rescue, and radiotherapy on the metastatic lymph node site. The metastatic lesions of the vertebrae and right 4th rib were not irradiated. After all these therapies were completed, no residual disease was observed at the primary site or the metastatic para-aortic site. However, the bone disease on lumbar vertebrae remained (Figure 2B). We did not perform a biopsy of the uptake region.

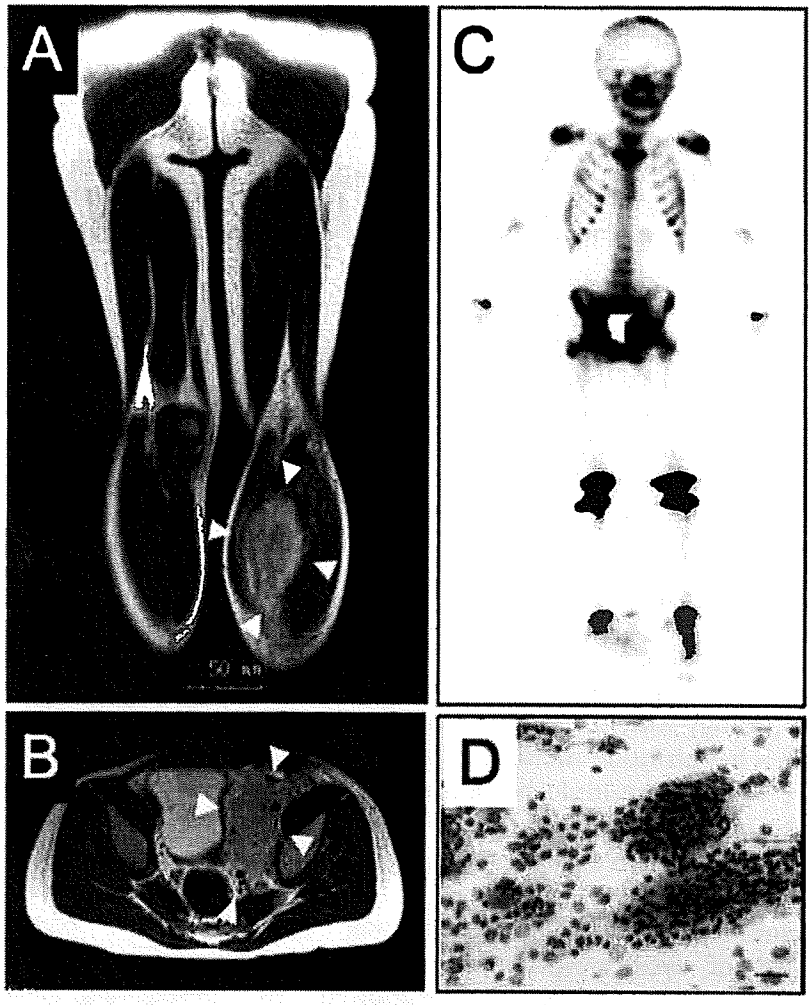

FIGURE 1 Studies at diagnosis. T2-weighted MRI images showing a mass on left leg (A, arrowheads) and metastatic swelling of para-aortic lymph nodes (B, arrowheads). (C) Scintigraphy of bone showed uptakes on right parietal, right 4th rib, and thoracic vertebrae. (D) Bone marrow aspiration showed aggregation of tumor cells.

She had HLA-A*2402 and her cancer tissue was determined by immunohistochemistry to express WT1 protein (Figure 3). She met the criteria for entry into the WT1 peptide-based clinical trial. Intradermal injection of the modified 9-mer WT1 peptide (1 mg) emulsified with Montanide ISA51 adjuvant was started from April 2005, 3 months after the last therapy (radiotherapy on the metastatic site) and continued at 1-week intervals.

The newlesions on the lumbar vertebrae remained weakly positive at the start of WT1 peptide vaccination (Figure 2B), but became negative after 3 months (12 courses) of weekly injections (Figure 2C). At 14 and 21 months after starting vaccination, scintigraphic uptake remained negative (Figure 2D, E).

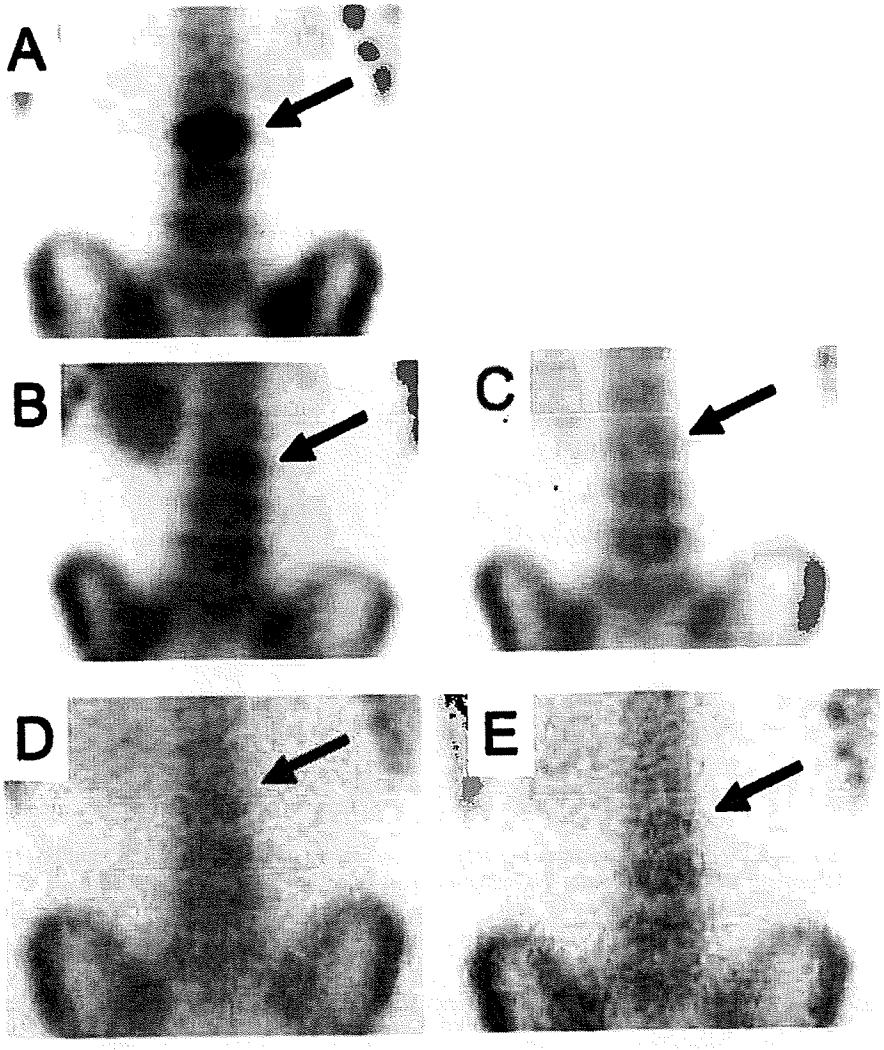

FIGURE 2 Control of new lesions of bone metastasis after the start of WT1 immunotherapy. (A) New lesions (L2, 3, 4) were observed on bone scintigraphy after two courses of combination chemotherapy. Bone scintigraphy before (B) and 3 (C), 14 (D), and 21 (E) months after WT1 vaccination. Scintigraphic uptake disappeared after vaccination. Arrow indicates L2 vertebra.

To evaluate immunological responses to WT1 peptide vaccination, WT1-specific CTL frequencies in peripheral blood and their differentiation state were analyzed by flow cytometry using WT1 tetramer. The frequency of tetramer$^+$CD8$^+$ T cells among CD8$^+$ T cells was defined as the WT1-specific CTL frequency. The frequency increased from 0.24% before vaccination to 0.37% at 1 month after the start of vaccination (1.54-fold increase). The frequency decreased to the prevaccination level at 4 months, and this was maintained at 13 months. It has recently been shown that these

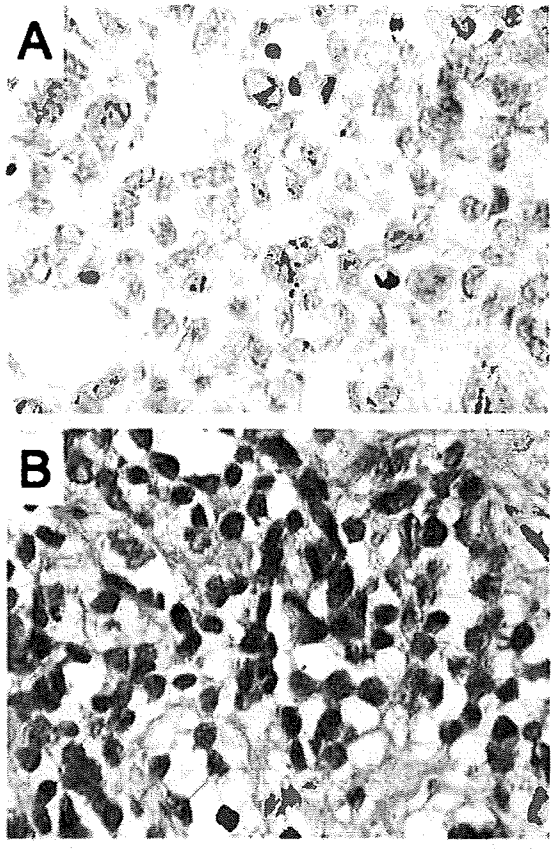

FIGURE 3 Immunohistochemical detection of WT1. Tissues were stained with anti-WT1 antibody 6F-H2 (A). WT1 protein was stained brown. The sections were then counterstained with hematoxylin (B).

CTLs can be phenotypically classified into 4 differentiation stages according to their expression of CD45RA and CCR7: naïve ($CD45RA^+CCR7^+$), central memory ($CD45R^-CCR7^+$), effector memory ($CD45RA^-CCR7^-$), and effector ($CD45RA^+CCR7^-$). Before vaccination, approximately half of $tetramer^+CD8^+$ T cells had an effector memory or effector phenotype, and these cells are considered to attack cancer cells quickly upon antigen-stimulation (Figure 4). This subset composition did not change substantially during vaccination. Compared to peripheral blood of healthy donors, in which the majority (about 80%) of $tetramer^+CD8^+$ T cells belonged to naïve phenotype [13], a high proportion of WT1-specific CTLs in peripheral blood of our patient were in an activated or differentiated stage.

No adverse effects were observed except for local erythema at the injection sites. The patient's general condition has been good without clinical relapse during WT1 peptide vaccination. The dose of WT1 peptide vaccination was increased to 2 mg from the 64th injection according to her

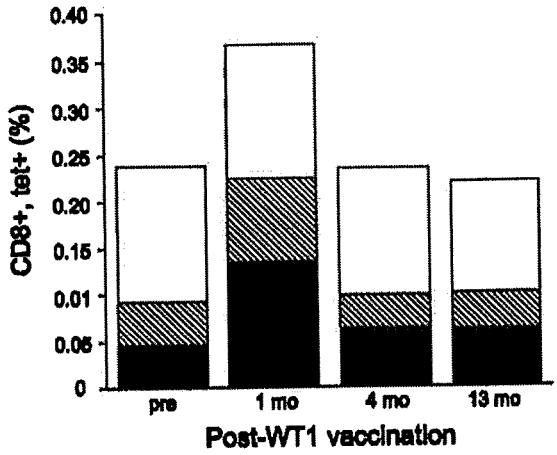

FIGURE 4 WT1-specific CTL frequencies in peripheral blood and CTLs subset composition. WT1-specific CTL frequencies are shown as percentage of WT1-tetramer$^+$CD8$^+$ T cells among CD8$^+$ T cells. CTLs were phenotypically classified into four subsets according to CD45RA and CCR7 expression: naïve (white bars), central memory (not detected), effector memory (black bars), and effector (striped bars).

weight gain. WT1 peptide vaccination has been continued to date (March 2008) without systemic adverse effects.

DISCUSSION

Rhabdomyosarcoma is the most common malignant soft tissue tumor of childhood. Patients with metastatic disease have a poor prognosis, with 5-year progression-free survival usually less than 30% [14]. Alveolar histology, confirmed by the presence of PAX3-FKHR fusion, is also associated with poor prognosis [15]. Current multidisciplinary treatment has contributed to an improvement of clinical outcomes, but control of disease is often difficult for children with metastatic alveolar rhabdomyosarcoma. Estimated 3-year event-free survival for patients with more than three metastatic sites and non-embryonal histology has been reported to be only 5% [16].

Our patient had primary disease in the lower leg with metastases on distant lymph node, bone, and bone marrow. She also developed a new metastatic bone lesion during the initial two courses of chemotherapy, indicating poor response to chemotherapy. Although she received a total of six courses of combination chemotherapy, high-dose chemotherapy, surgery on the primary site, and radiotherapy on primary and metastatic sites, bone disease remained positive. Considering her poor prognosis, we chose WT1 peptide immunotherapy. After the start of WT1 peptide immunotherapy, uptake disappeared on bone scintigraphy. Despite the resistance to initial chemotherapy, her continuing remission for more than 22 months suggests a positive effect from WT1 peptide vaccination.

The WT1 gene is physiologically expressed in some organs such as kidney, bone marrow, and pleura. Recent studies have shown that WT1-specific CTLs kill WT1-expressing tumor cells, but not normal cells. In mice immunized with MHC class I-restricted 9-mer WT1 peptides or *WT1* cDNA, WT1-specific CTLs induced killing of WT1-expressing tumor cells, but never damaged normal tissues [17, 18]. Several mechanisms have been postulated to account for WT1-specific CTLs ignoring WT1-expressing normal cells: (1) *WT1* expression levels may be different between cancer cells and normal cells; (2) mechanisms for processing of WT1 protein or presentation of WT1 peptide may be different; and (3) susceptibility of the cell membranes to CTL-producing molecules such as perforin may be different [19].

The frequency of WT1-specific CTLs is usually about 0.1% or less in healthy donors [9]. Since the frequency in our case was as high as 0.24% before WT1 peptide vaccination, this indicates that the patient had responded to the WT1 protein derived from the tumor cells and elicited WT1-specific CTLs before WT1 peptide vaccination. The frequency increased from 0.24% before vaccination to 0.37% at 1 month after starting the vaccination (1.54-fold increase). We have previously demonstrated that the emergence of clinical responses is correlated with a greater than 1.5-fold increase in tetramer$^+$ cell frequencies [9]. This finding strongly suggested that WT1 vaccination-driven induction of WT1-specific CTL responses led to a clinical effect in responders. This observation was also in line with the present case in which a greater than 1.5-fold increase in tetramer$^+$ cell frequency was observed with clinical response. Although the frequency decreased to the pre-vaccination level at 4 and 13 months, levels were maintained higher than those in healthy donors. The reason for the decrease in frequencies at later points might be explained by several mechanisms, e.g., activation-induced cell death of WT1-specific CTLs, migration of the CTLs to a tumor site, reduced stimulation of the immune system by WT1 protein owing to reduction in tumor burden (achievement of complete response). We also analyzed phenotype to evaluate the differentiation state of WT1-specific CTLs in our patient. Analysis revealed that many of the tetramer$^+$ cells had the phenotype of effector memory or effector cells, which are considered to be ready for cancer cell attack upon antigen stimulation. Taken together, the high frequencies of WT1-specific CTLs, their increase in frequency after vaccination, and the differentiated (functionally matured) state of the CTLs may contribute to the induction of clinical response.

In conclusion, WT1-peptide immunotherapy was effective with immunological response against residual disease in a child with metastatic alveolar rhabdomyosarcoma. WT1 peptide-based immunotherapy should be considered as a promising option for high-risk rhabdomyosarcoma in childhood.

Declaration of Interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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