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

Kazunori Yokomine,[1,2] Tetsuya Nakatsura,[1,3] Satoru Senju,[1] Naomi Nakagata,[4] Motozumi Minohara,[5] Jun–ichi Kira,[5] Yutaka Motomura,[1,3] Tatsuko Kubo,[6] Yutaka Sasaki[2] and Yasuharu Nishimura[1,7]

Departments of [1]Immunogenetics and [2]Gastroenterology and Hepatology, Graduate School of Medical Sciences, Kumamoto University, 1-1-1 Honjo, Kumamoto 860-8556; [3]Immunotherapy Section, Investigative Treatment Division Research Center for Innovative Oncology, National Cancer Center Hospital East, 6-5-1 Kashiwanoha, Kashiwa 277-8577; [4]Center for Animal Resources and Development, Kumamoto University, 2-2-1 Honjo, Kumamoto 860-0811; [5]Department of Neurology, Neurological Institute, Graduate School of Medical Sciences, Kyushu University, 3-1-1 Maidashi, Higashi-ku, Fukuoka 812-8582; [6]Department of Molecular Pathology, Graduate School of Medical Sciences, Kumamoto University, 1-1-1 Honjo, Kumamoto 860-8556, Japan

(Received May 12, 2007/Revised August 6, 2007/Accepted August 7, 2007/Online publication September 24, 2007)

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.

[7]To whom correspondence should be addressed.
E-mail: mxnishim@gpo.kumamoto-u.ac.jp

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.

doi: 10.1111/j.1349-7006.2007.00612.x

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

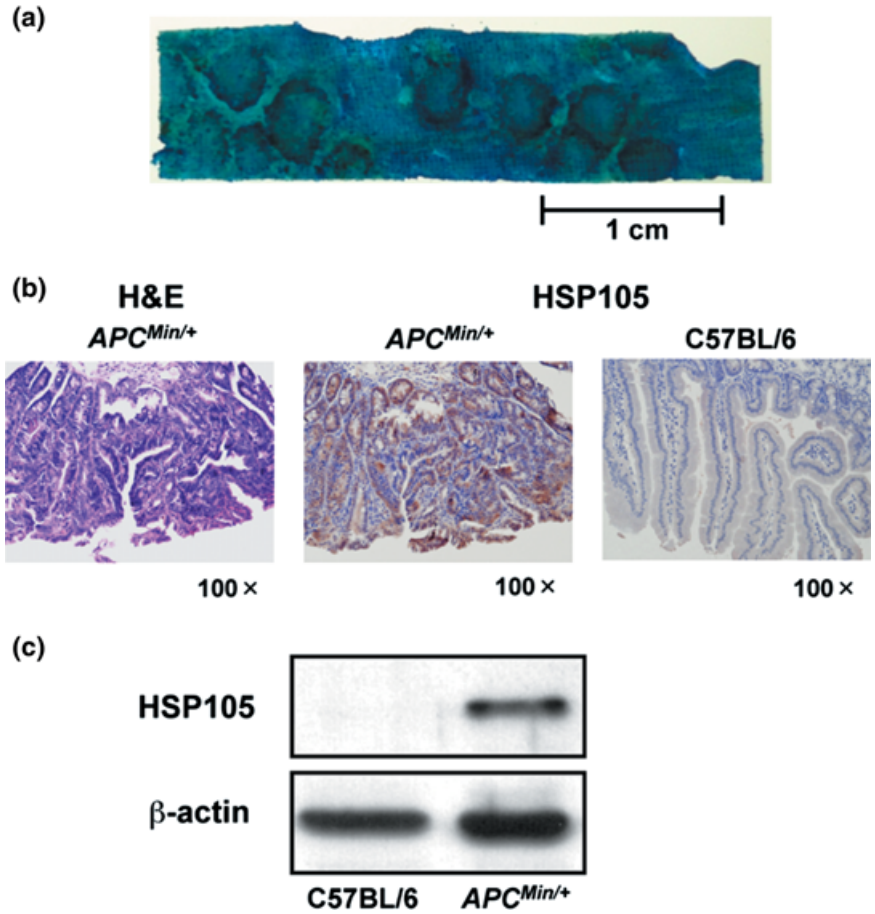

Fig. 1. Overexpression of heat shock protein (HSP) 105 in adenomatous polyps of *Apc*$^{Min/+}$ mice. (a) Macroscopic polyps in the small intestine of 4-month-old *Apc*$^{Min/+}$ mice. (b) A microscopic analysis of polyps in the small intestine of 12-week-old *Apc*$^{Min/+}$ mice stained with hematoxylin–eosin (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,

doi: 10.1111/j.1349-7006.2007.00612.x

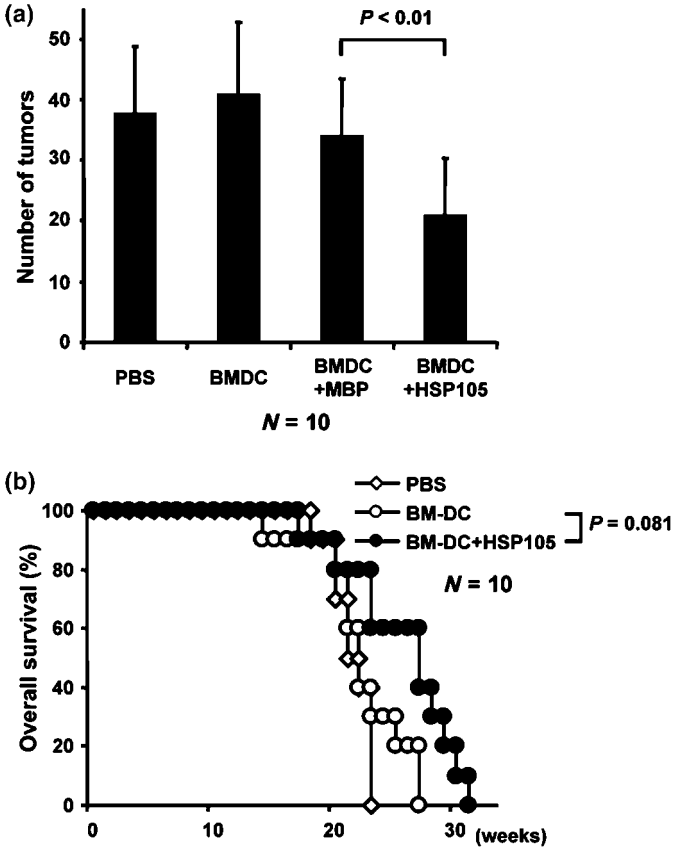

Fig. 2. Vaccination with heat shock protein (HSP) 105-pulsed bone marrow-derived dendritic cells (BM-DC) decreased the number of polyps in the small intestine of the $Apc^{Min/+}$ mice. (a) The $Apc^{Min/+}$ mice were inoculated intraperitoneally with HSP105-pulsed BM-DC (5×10^5), BM-DC alone, or myelin 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.

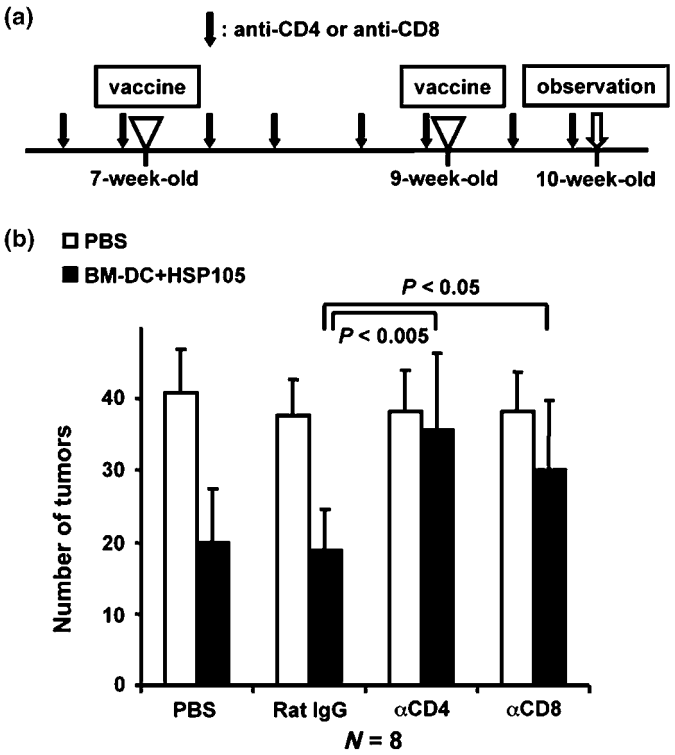

Fig. 3. Both $CD4^+$ and $CD8^+$ T cells are involved in the antitumor immunity elicited by the heat shock protein (HSP) 105-pulsed dendritic cell vaccine. (a) The protocol for the vaccination and the depletion of T cell subsets. (b) The number of polyps in the small intestine of $Apc^{Min/+}$ mice with 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

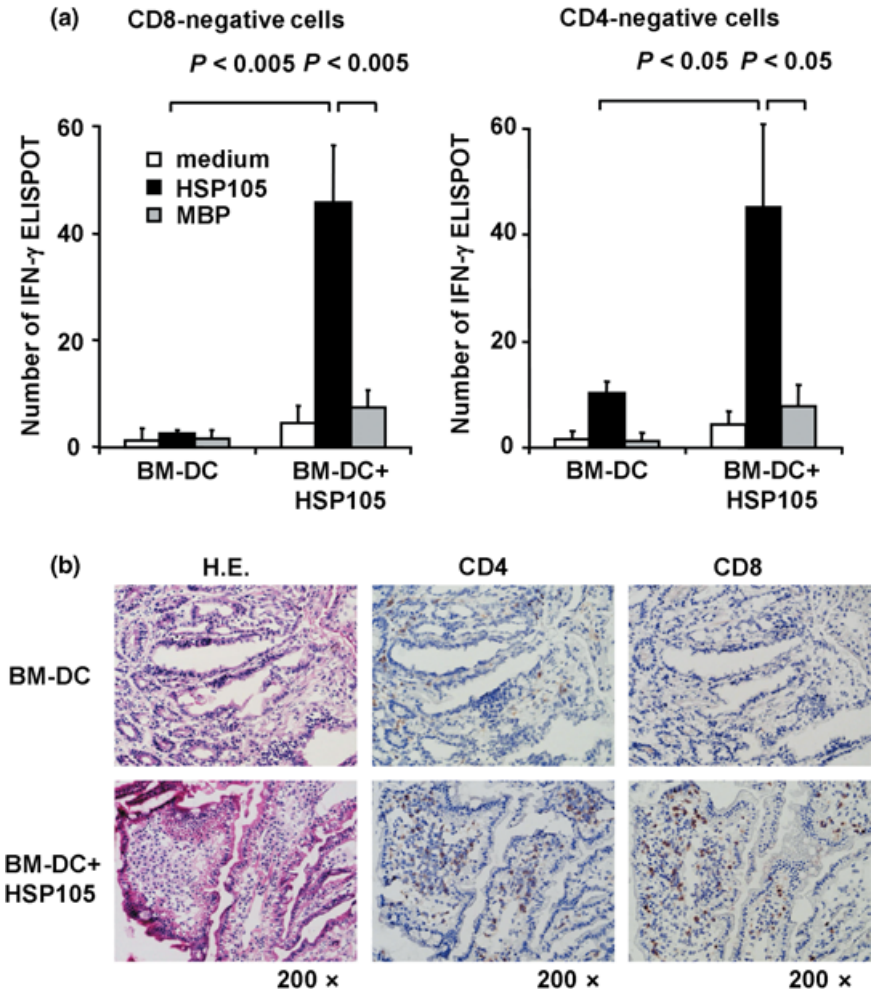

Fig. 4. Induction of heat shock protein (HSP) 105-specific T cells via immunization with HSP105-pulsed bone marrow-derived dendritic cells (BM-DC). (a) The *Apc*$^{Min/+}$ mice were inoculated with HSP105-pulsed BM-DC or BM-DC at 6 and 8 weeks of age. The spleen cells were harvested from 10-week-old *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

doi: 10.1111/j.1349-7006.2007.00612.x

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

This work was supported in part by Grants-in-Aid (no. 12213111 for Y. Nishimura, no. 14770142 for T. Nakatsura, and no. 14770142 for S. Senju) from the Ministry of Education, Science, Technology, Sports, and Culture, Japan, and The Sagawa Foundation for Promotion of Cancer Research and Meiji Institute of Health Science.

References

1 Weitz J, Koch M, Debus J, Hohler T, Galle PR, Büchler MW. Colorectal cancer. *Lancet* 2005; **365**: 153–65.
2 Tournigand C, André T, Achille E *et al*. FOLFIRI followed by FOLFOX6 or the reverse sequence in advanced colorectal cancer: a randomized GERCOR study. *J Clin Oncol* 2004; **22**: 229–37.
3 Rao CV, Cooma I, Rosa Rodriguez JG, Simi B, El-Bayoumy K, Reddy BS. Chemoprevention of familial adenomatous polyposis development in the *Apc^{min}* mouse model by 1,4-phenylene *bis*(methylene)selenocyanate. *Carcinogenesis* 2000; **21**: 617–21.
4 Kinzler KW, Vogelstein B. Cancer-susceptibility genes: gatekeepers and caretakers. *Nature* 1997; **386**: 761–3.
5 Fearon ER, Vogelstein B. A genetic model for colorectal tumorigenesis. *Cell* 1990; **61**: 759–67.
6 Kikuchi A. Modulation of Wnt signaling by Axin and Axil. *Cytokine Growth Factor Rev* 1999; **10**: 255–65.
7 Sancho E, Batlle E, Clevers H. Signaling pathways in intestinal development and cancer. *Annu Rev Cell Dev Biol* 2004; **20**: 695–723.
8 Miyoshi Y, Ando H, Nagase H *et al*. Germ-line mutations of the *APC* gene in 53 familial adenomatous polyposis patients. *Proc Natl Acad Sci USA* 1992; **89**: 4452–6.
9 Su LK, Kinzler KW, Vogelstein B *et al*. Multiple intestinal neoplasia caused by a mutation in the murine homolog of the *APC* gene. *Science* 1992; **256**: 668–70.
10 Moser AR, Pitot HC, Dove WF. A dominant mutation that predisposes to multiple intestinal neoplasia in the mouse. *Science* 1990; **247**: 322–4.
11 Feder ME, Hofmann GE. Heat-shock proteins, molecular chaperones, and the stress response: evolutionary and ecological physiology. *Annu Rev Physiol* 1999; **61**: 243–82.
12 Nakatsura T, Senju S, Yamada K, Jotsuka T, Ogawa M, Nishimura Y. Gene cloning of immunogenic antigens overexpressed in pancreatic cancer. *Biochem Biophys Res Commun* 2001; **281**: 936–44.
13 Kai M, Nakatsura T, Egami H, Senju S, Nishimura Y, Ogawa M. Heat shock protein 105 is overexpressed in a variety of human tumors. *Oncol Rep* 2003; **10**: 1777–82.
14 Yokomine K, Nakatsura T, Minohara M *et al*. Immunization with heat shock protein 105-pulsed dendritic cells leads to tumor rejection in mice. *Biochem Biophys Res Commun* 2006; **343**: 269–78.
15 Miyazaki M, Nakatsura T, Yokomine K *et al*. DNA vaccination of HSP105 leads to tumor rejection of colorectal cancer and melanoma in mice through activation of both $CD4^+$ T cells and $CD8^+$ T cells. *Cancer Sci* 2005; **96**: 695–705.
16 Hosaka S, Nakatsura T, Tsukamoto H, Hatayama T, Baba H, Nishimura Y. Synthetic small interfering RNA targeting heat shock protein 105 induces apoptosis of various cancer cells both *in vitro* and *in vivo*. *Cancer Sci* 2006; **97**: 623–32.
17 Srivastava P. Interaction of heat shock proteins with peptides and antigen presenting cells: chaperoning of the innate and adaptive immune responses. *Annu Rev Immunol* 2002; **20**: 395–425.
18 Manjili MH, Wang XY, Chen X *et al*. HSP110–HER2/neu chaperone complex vaccine induces protective immunity against spontaneous mammary tumors in HER-2/neu transgenic mice. *J Immunol* 2003; **171**: 4054–61.
19 Wang XY, Chen X, Manjili MH, Repasky E, Henderson R, Subjeck JR. Targeted immunotherapy using reconstituted chaperone complexes of heat shock protein 110 and melanoma-associated antigen gp100. *Cancer Res* 2003; **63**: 2553–60.
20 Dietrich WF, Lander ES, Smith JS *et al*. Genetic identification of *Mom*-1, a major modifier locus affecting *Min*-induced intestinal neoplasia in the mouse. *Cell* 1993; **75**: 631–9.
21 Yamagishi N, Nishihori H, Ishihara K, Ohtsuka K, Hatayama T. Modulation of the chaperone activities of Hsc70/Hsp40 by Hsp105α and Hsp105β. *Biochem Biophys Res Commun* 2000; **272**: 850–5.
22 Nakatsura T, Komori H, Kubo T *et al*. Mouse homologue of a novel human oncofetal antigen, glypican-3, evokes T-cell-mediated tumor rejection without autoimmune reaction in mice. *Clin Cancer Res* 2004; **10**: 8630–40.
23 Fearnhead NS, Wilding JL, Bodmer WF. Genetics of colorectal cancer: hereditary aspects and overview of colorectal tumorigenesis. *Br Med Bull* 2002; **64**: 27–43.
24 Boivin GP, Washington K, Yang K *et al*. Pathology of mouse models of intestinal cancer: consensus report and recommendations. *Gastroenterology* 2003; **124**: 762–77.
25 Oshima M, Dinchuk JE, Kargman SL *et al*. Suppression of intestinal polyposis in *Apc* Δ716 knockout mice by inhibition of cyclooxygenase 2 (COX-2). *Cell* 1996; **87**: 803–9.
26 Reddy BS, Hirose Y, Lubet R *et al*. Chemoprevention of colon cancer by specific cyclooxygenase-2 inhibitor, celecoxib, administered during different stages of carcinogenesis. *Cancer Res* 2000; **60**: 293–7.
27 Steinbach G, Lynch PM, Philips RKS *et al*. The effect of celecoxib, a cyclooxygenase-2 inhibitor, in familial adenomatous polyposis. *N Engl J Med* 2000; **342**: 1946–52.
28 Solomon SD, McMurray JJV, Pfeffer MA *et al*. Cardiovascular risk associated with celecoxib in a clinical trial for colorectal adenoma prevention. *N Engl J Med* 2005; **352**: 1071–80.
29 Nestle FO, Alijagic S, Gilliet M *et al*. Vaccination of melanoma patients with peptide- or tumor lysate-pulsed dendritic cells. *Nat Med* 1998; **4**: 328–32.
30 Stift A, Friedl J, Dubsky P *et al*. Dendritic cell-based vaccination in solid cancer. *J Clin Oncol* 2003; **21**: 135–42.
31 Yu JS, Liu G, Ying H, Yong WH, Black KL, Wheeler CJ. Vaccination with tumor lysate-pulsed dendritic cells elicits antigen-specific, cytotoxic T-cells in patients with malignant glioma. *Cancer Res* 2004; **64**: 4973–9.
32 Timmerman JM, Levy R. Dendritic cell vaccines for cancer immunotherapy. *Annu Rev Med* 1999; **50**: 507–29.
33 Fong L, Engleman EG. Dendritic cells in cancer immunotherapy. *Annu Rev Immunol* 2000; **18**: 245–73.
34 Gabrilovich D. Mechanisms and functional significance of tumour-induced dendritic-cell defects. *Nature Rev Immunol* 2004; **4**: 941–52.
35 Park D, Lapteva N, Seethammagari M, Slawin KM, Spencer D. An essential role for Akt1 in dendritic cell function and tumor immunotherapy. *Nat Biotechnol* 2006; **24**: 1581–90.
36 Evel-Kabler K, Song XT, Aldrich M, Huang XF, Chen SY. SOCS1 restricts dendritic cells' ability to break self tolerance and induce antitumor immunity by regulating IL-12 production and signaling. *J Clin Invest* 2006; **116**: 90–100.
37 Hanks BA, Jiang J, Singh RAK *et al*. Re-engineered CD40 receptor enables potent pharmacological activation of dendritic-cell cancer vaccines *in vivo*. *Nat Med* 2005; **11**: 130–7.
38 Reits EA, Hodge JW, Herberts CA *et al*. Radiation modulates the peptide repertoire, enhances MHC class I expression, and induces successful antitumor immunotherapy. *J Exp Med* 2006; **203**: 1259–71.
39 Casares N, Pequignot MO, Tesniere A *et al*. Caspase-dependent immnogenicity of doxorubicin-induced tumor cell death. *J Exp Med* 2005; **202**: 1691–701.
40 Zhang B, Bowerman NA, Salama JK *et al*. Induced sensitization of tumor stroma leads to eradication of established cancer by T cells. *J Exp Med* 2007; **204**: 49–55.
41 Janssen EE, Lemmens EE, Wolfe T, Christen U, von Herrath MG, Schoenberger SP. $CD4^+$ T cells are required for secondary expansion and memory in $CD8^+$ T lymphocytes. *Nature* 2003; **421**: 852–6.