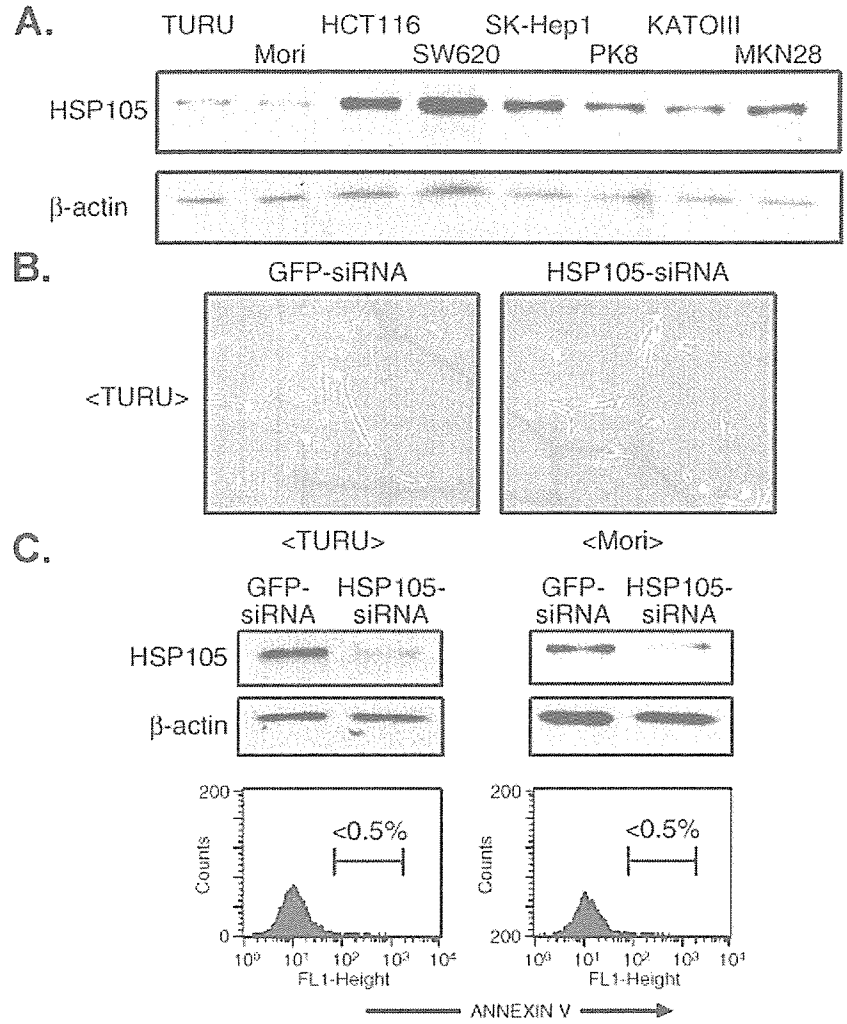

Fig. 3. No apoptosis-inducing effects of HSP105 small interfering RNA (siRNA) on human fibroblasts. (A) Western blot analysis of HSP105. The lysates of human fibroblasts Turu and Mori, and human cancer cell lines HCT116, SW620, SK-Hep1, PK8, KATO-3 and MKN28 were used and blotted with an anti-HSP105 antibody. β-Actin is shown as a quantitative control. (B) Light microscopic pictures of Turu at 72 h after transfection with 100 nM green fluorescent protein (GFP) siRNA or HSP105 siRNA. (C) Effects of siRNA on Turu and Mori. Western blot analysis of HSP105 and flow cytometric analysis of apoptotic cells detected by annexin V staining at 72 h after transfection of 100 nM GFP siRNA or HSP105 siRNA. These data are representative of at least three independent experiments. The percentages shown in the panel indicate percentage of annexin V-positive cells in HSP105 siRNA-treated cells.

independent of caspases.[33] In the present study, transfection of HSP105 siRNA caused HCT116 cells to undergo apoptosis in a caspase-dependent manner without suppressing the expression of HSP70 protein (data not shown). Our data suggest that HSP105 has a different character regarding caspase dependency in comparison to HSP70.

In the present study, HSP105 siRNA transfection induced various cancer cell lines to undergo apoptosis. These observations raise the question of how such apoptosis is induced. One possible explanation is that suppression of HSP105 activates the apoptotic pathway mediated by the p53 tumor suppressor protein. Molecular chaperones such as HSP70 and HSP90 are overexpressed in various tumor cells,[34] associating with wild-type or mutated p53 tumor suppressor proteins. Such heat shock proteins mediate stabilization, cytoplasmic sequestration and localization of p53 proteins.[35,36] In our study, HSP105 protein was bound to wild-type p53 in HCT116 cells under non-stress conditions, as shown in Fig. 6B. However, when the expression of HSP105 protein was suppressed with HSP105 siRNA, the expression of p53 protein also decreased and the p53-mediated apoptotic pathway was not activated (Fig. 6C,D). These results suggest that HSP105 stabilizes the p53 protein and protects it from degradation, but the apoptosis induced by HSP105 siRNA treatment is not mediated by the p53-dependent apoptotic pathway. We herein observed that every cancer cell line with wild-type p53 (HCT116), mutated p53 (SW620) or without p53 (KATO-3) was induced to undergo apoptosis. Further studies are needed to elucidate the biological significance of the interaction between HSP105 and p53 protein in cancer cells.

The second possible mechanism of cancer cell apoptosis induced with HSP105 siRNA treatment is the involvement of ER stress. Heat shock proteins have housekeeping functions, such as folding and degradation of various proteins. ER stress, induced by the accumulation of unfolded or malfolded proteins, induces the unfolded protein response, characterised by the induction of chaperones, the translation block and ER-associated degradation. However, if such degradation is not sufficient, then prolonged ER stress activates various apoptotic pathways, including caspase activation.[37–39]

Abnormal protein aggregation has been suspected to cause many neurodegenerative diseases, including Alzheimer's disease, Parkinson's disease and trinucleotide repeat disease. In the brain in Alzheimer's disease, HSP90 facilitates the clearance

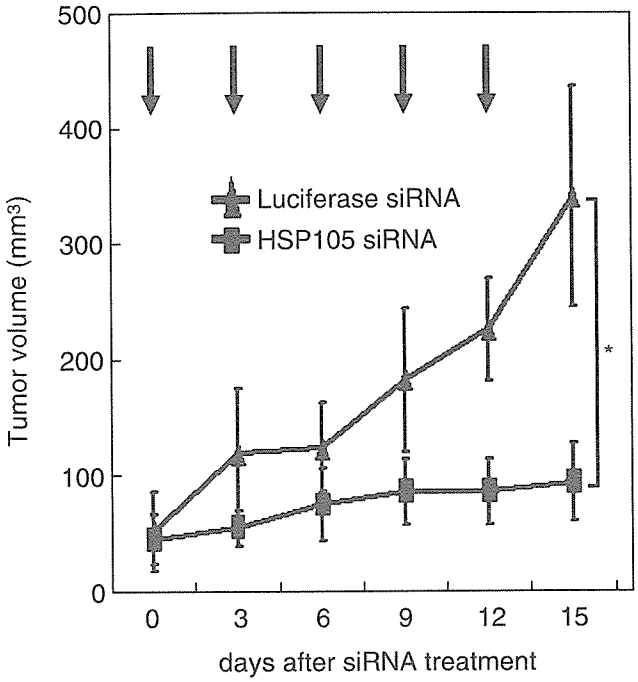

Fig. 4. The inhibitory effect of HSP105 small interfering RNA (siRNA) on the growth of established tumors in mice. (A) KATO-3 cells (2×10^6) were implanted subcutaneously into the dorsal skin of NOD SCID mice to establish growing tumors, and siRNA was injected into the tumors every 3 days (indicated by arrows). The tumor volume was measured and plotted (luciferase siRNA, ▲ HSP105-siRNA, ■). Data are mean ± SD ($n = 4$). The asterisk indicates that the difference in the tumor volume on day 15 is statistically significant between the two values as indicated by lines ($P < 0.01$).

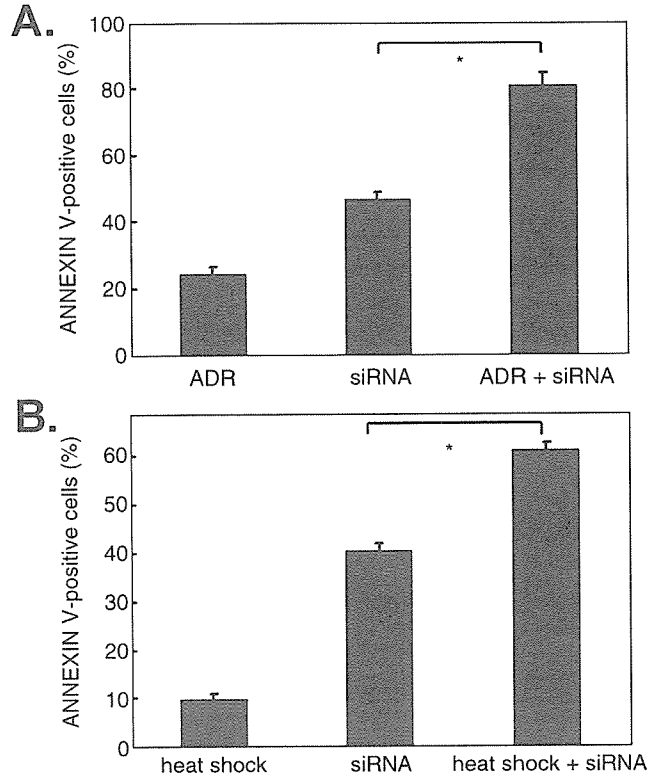

Fig. 5. The synergistic effect of HSP105 small interfering RNA (siRNA) with doxorubicin or heat shock regarding the induction of apoptotic cell death in HCT116 cells. At 12 h after transfection with 100 nM siRNA, the cells were incubated with 200 ng/mL doxorubicin (A) or treated with heat shock at 45°C for 30 min (B). Subsequently, the cells were stained with fluorescein-isothiocyanate–annexin V and analyzed by flow cytometry. Data are the mean of three independent experiments ± SD ($n = 3$). The asterisks indicate that the differences in the percentages of annexin V-positive cells are statistically significant between the two values as indicated by lines ($P < 0.001$).

of amyloid-beta.[40] In our study, HSP105 siRNA treatment induced HCT116 cells to undergo apoptosis through caspase activation. Considering these findings, we speculate that HSP105 siRNA treatment may induce aggregation of unfolded protein while also causing insufficient protein degradation, consequently leading to ER stress-mediated apoptosis, especially in cancer cells carrying mutations and aberrant expression of oncoproteins. Recent reports demonstrating that HSP105 prevents the aggregation of thermal-denatured protein *in vitro*[6] and that overexpression of HSP105 suppresses aggregation and cell toxicity in a spinal and bulbar muscular atrophy model[9] support our speculation. Regarding caspase dependency, the cleavage of PARP has been reported to be suppressed in PC12 cells overexpressing HSP105 protein and those cells were also protected from apoptosis caused by several stressors.[8] These observations are consistent with our results.

Cancer cells often have aberrantly expressed or mutated genes that lead to uncontrolled cell growth and the prevention of apoptosis, and the usage of siRNA against such targets is thus considered to be promising for cancer therapy. Several recent studies have demonstrated the effective silencing by siRNA of targets, such as β-catenin for colon cancer,[41] mutated K-ras for pancreatic carcinoma[42] and BCR/ABL fusion protein for CML.[43] In those reports, the injection of siRNA either induced target cells to undergo apoptosis or caused inhibition of their proliferation. In the present study, HSP105 siRNA induced various human cancer cell lines to undergo apoptosis both *in vitro* and *in vivo* without side effects. It is notable that the effect of HSP105 siRNA treatment was transient and not lethal in normal fibroblasts, whereas the effects of known chemical agents tend to be cytotoxic for normal cells. Indeed, human fibroblast cells treated with doxorubicin were induced to undergo apoptosis (data not shown). These data suggest that HSP105 siRNA treatment is useful for cancer therapy and it may thus be applied to various kinds of cancer patients with minimal side effects.

For patients with advanced or metastatic cancer, combination therapies using some cytotoxic agents and radiation are now often performed clinically. We expected synergistic effects of combination therapy using HSP105 siRNA and doxorubicin, which have different mechanisms of action. siRNA suppresses the expression of targeted proteins by RNA cleavage, whereas doxorubicin a DNA intercalating agent that induces apoptosis by damaging DNA. In the present study, treatment combining HSP105 siRNA with doxorubicin synergistically induced cancer cells to undergo apoptosis. We also suspected that heat shock is effective when it is combined with HSP105 siRNA because HSP105 is essential in order to protect cells from heat stress.[8]

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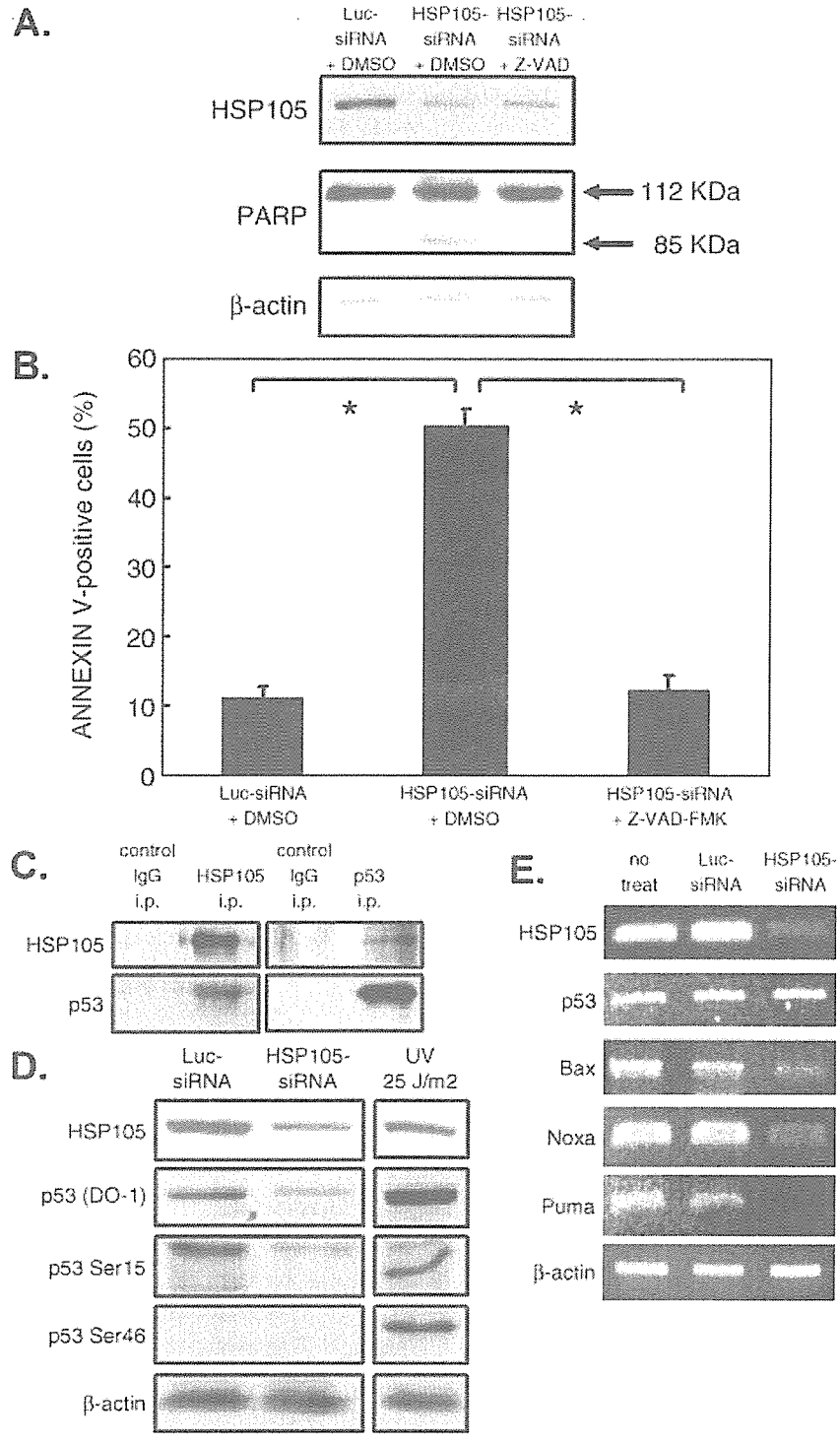

Fig. 6. Caspase-dependent and p53-independent induction of apoptosis in HCT116 cells administered HSP105 small interfering RNA (siRNA). (A) Western blot analysis of poly ADP-ribose polymerase (PARP) expression and (B) flow cytometric analysis of apoptosis induced in HCT116 cells transfected with siRNA in the presence of dimethylsulfoxide (DMSO) or Z-VAD-FMK. HCT116 cells treated with luciferase siRNA + DMSO, HSP105 siRNA + DMSO or HSP105 siRNA + 100 μM Z-VAD-FMK were cultured for 48 h and apoptotic cells were stained with annexin V. Cells were lysed and blotted with either anti-HSP105 or anti-PARP antibody. Data are the mean values of three independent experiments ± SD. The asterisks indicate that the differences in the percentages of annexin V-positive cells were statistically significant between the two values as indicated by lines ($P < 0.001$). (C,D) Western blot analysis of HSP105 and p53. HCT116 cells were lysed and immunoprecipitated with an anti-HSP105 antibody or an anti-p53 antibody (DO-1), and the proteins were blotted with either anti-HSP105 antibody or a biotin-labeled DO-1. The immunoprecipitates with rabbit polyclonal IgG and mouse monoclonal IgG2a were used as negative controls for anti-HSP105 antibody and DO-1, respectively (C). HCT116 cells transfected with luciferase siRNA or HSP105 siRNA were lysed at 48 h after transfection and blotted with the anti-HSP105 antibody DO-1, anti-phospho-p53 (Ser46) and antiphospho-p53 (Ser15). Ultraviolet light-irradiated HCT116 cell lysates were used as a positive control for p53 phosphorylation. (D) β-Actin is shown as a quantitative control. (E) Reverse transcription–polymerase chain reaction (RT-PCR) analysis of HSP105, p53, Bax, NOXA and PUMA expression in HCT116 cells transfected with siRNA. HCT116 cells transfected with luciferase siRNA or HSP105 siRNA were harvested at 24 h after transfection and the cDNAs were used for PCR analysis. cDNA extracted from the untreated HCT116 cells was used as a negative control. β-Actin is shown as a quantitative control.

As shown in Fig. 5B, the combination of HSP105 siRNA with heat shock, which is clinically applied to cancer patients as hyperthermia, exhibited a synergistic apoptotic effect in cancer cells.

In conclusion, our findings suggest that HSP105 is involved in tumorigenesis by protecting cancer cells from apoptosis, and the constitutive overexpression of HSP105 protein was found to be essential for various cancer cells to survive. We also suggest that the apoptosis-inducing effect of HSP105 siRNA is specific for cancer, therefore HSP105 siRNA may be useful as a novel therapeutic tool for patients with cancers originating from various tissues. By using effective drug delivery systems and combining this treatment with existing cytotoxic agents, an enhanced effect is thus expected.

Acknowledgments

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Immunization with heat shock protein 105-pulsed dendritic cells leads to tumor rejection in mice ☆

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Abstract

Recently, we reported that heat shock protein 105 (HSP105) DNA vaccination induced anti-tumor immunity. In this study, we set up a preclinical study to investigate the usefulness of dendritic cells (DCs) pulsed with mouse HSP105 as a whole protein for cancer immunotherapy in vivo. The recombinant HSP105 did not induce DC maturation, and the mice vaccinated with HSP105-pulsed BM-DCs were markedly prevented from the growth of subcutaneous tumors, accompanied with a massive infiltration of both $CD4^+$ T cells and $CD8^+$ T cells into the tumors. In depletion experiments, we proved that both $CD4^+$ T cells and $CD8^+$ T cells play a crucial role in anti-tumor immunity. Both $CD4^+$ T cells and $CD8^+$ T cells specific to HSP105 were induced by stimulation with HSP105-pulsed DCs. As a result, vaccination of mice with BM-DCs pulsed with HSP105 itself could elicit a stronger tumor rejection in comparison to DNA vaccination.

Keywords: Heat shock protein 105; Cancer antigen; Dendritic cells; Th; CTL

Heat shock proteins (HSPs) are soluble intracellular proteins, which are ubiquitously expressed, and their expression can be induced at much higher levels as a result of heat shock or other forms of stress. HSPs have essential functions in the regulation of protein folding, conformation, assembly, and sorting. HSPs have been shown to be molecular chaperones that function to maintain the native conformational states of proteins and prevent protein–protein aggregation [1]. HSPs can also induce the response of antigen-specific effector $CD8^+$ T cells which can protect hosts from both infection and tumor challenge [2]. Srivastava and co-workers [3,4] led to a proposal that the tumor-derived HSP–peptide complex elicits a protective immunity that is specific to a particular cancer, while HSPs derived from normal tissues did not elicit any protective immunity to the cancers tested. Immunotherapeutic clinical trials targeted at autologous tumor-derived gp96–peptide complexes are still ongoing in metastatic melanoma and colorectal carcinoma patients [5].

Dendritic cells (DCs) are powerful antigen-presenting cells (APCs) that are considered to be potent immunotherapeutic agents to promote the host immune response against tumor antigen. DCs become efficient tumor vaccines when pulsed with synthetic or natural tumor-derived peptides, transduced with tumor-derived RNA or vectors

☆ *Abbreviations:* BM-DC, bone marrow-derived DC; HSP105, heat shock protein 105; Th, helper T cell; CTL, cytotoxic T lymphocyte; MHC, major histocompatibility complex; C26 (C20), colon 26 clone 20.

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encoding for tumor-associated proteins, or directly fused to or incubated with tumor cells [6]. For effective induction of cytotoxic T lymphocytes (CTLs) by vaccination, "Cross-presentation" mediated by DCs often plays an important role. Such cross-presentation includes the antigen presentation of exogenous antigens by major histocompatibility complex (MHC) class I molecules as well as by MHC class II molecules [7,8]. HSP-chaperoned peptides were cross-presented by the MHC class I molecules of the DCs several 100-fold more efficiently than unchaperoned peptides [9]. In addition, CD91, also called α_2-macroglobulin receptor is expressed on DCs and has been shown to act as one of the receptors for HSP-chaperoned peptides to efficiently incorporate the HSP–peptide complexes [10].

We earlier reported that heat shock protein 105 (HSP105) was overexpressed in a variety of human cancers but it is not expressed in normal tissue except for the testes [11,12], thus suggesting that HSP105 itself may be a potential candidate as a target antigen for cancer immunotherapy. The amino acid sequences and expression patterns of HSP105 are very similar between humans and mice. HSP105 has been found to be immunogenic in mice and an effective anti-tumor immunity has been observed after *HSP105* DNA vaccination [13]. In the present study, we set up a preclinical study to investigate the usefulness of HSP105 as a target for cancer immunotherapy using DCs. It has been reported that HSPs can induce DC maturation and activation as determined by the upregulation of MHC class II and CD86 molecules, the secretion of IL-12 and TNFα [14,15], and migration into draining lymphoid organs [16]. On the contrary, some investigators reported that HSP-mediated maturation of DCs was caused by contaminating lipopolysaccharide (LPS) fraction because endotoxin-free HSP70 failed to induce DC maturation [17]. We herein show that the highly purified HSP105 did not induce DC maturation and that the immunization of HSP105-pulsed DC led to the tumor rejection of melanoma and colorectal cancer in mice. These findings suggested that HSP105 itself could be a valuable tumor-associated antigen applicable for DC-based immunotherapy of tumors overexpressing it.

Materials and methods

Cell lines and mice. A subline of BALB/c-derived colorectal cancer cell line Colon 26, C26 (C20), was provided by Dr. Kyoichi Shimomura (Astellas Pharmaceutical Co., Tsukuba, Japan). Other cancer cell lines were kindly provided by the Cell Resource Center for Biomedical Research Institute of Development, Aging, and Cancer, Tohoku University (Sendai, Japan). All these cell lines were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum at 37 °C in a humidified 5% CO_2 atmosphere. We used the B16-F10 melanoma cell line syngeneic to C57BL/6 mice and C26 (C20) for the tumor challenge. Female 6- to 8-week-old C57BL/6 mice (H-2^b) and BALB/c mice (H-2^d) were purchased from Charles River Japan (Yokohama, Japan). These mice were kept under specific pathogen-free conditions. These experiments were approved by the Animal Research Committee of Kumamoto University.

Production of recombinant proteins. We produced highly purified recombinant mouse HSP105 from the *Escherichia coli* strain BL21 cells transduced with the mouse *HSP105* gene expression vector, as described previously [18]. Purified proteins were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), and Coomassie brilliant blue (CBB)-stained bands were quantified by densitometry. Thereafter, by using affinity chromatography on a polymyxin B agarose gel (Sigma Chemical Co., St. Louis, MO), the endotoxin levels were decreased. We also produced highly purified recombinant myelin basic protein (MBP) as described previously [19]. Both recombinant HSP105 and MBP were estimated to be almost endotoxin free by using Limulus amebocyte lysate assay kit (BioWhittaker, Walkersville, MD), and endotoxin contents in these two materials were below 10 endotoxin U/mg.

Immunizations and tumor challenge. Bone marrow-derived DCs (BM-DC) were prepared as described previously [20]. BM-DCs were pulsed with 2 μg/ml HSP105 at 37 °C for 16 h, non-adherent and loosely adherent proliferating DCs were collected and used as HSP105-pulsed BM-DC. In tumor prevention experiments, mice were intraperitoneally inoculated with HSP105-pulsed BM-DC (5×10^5) suspended in 200 μl PBS on days −14 and −7. In parallel, groups of mice were injected with BM-DC alone or PBS as controls. Tumor challenge was initiated by subcutaneous injection with B16-F10 cells (1×10^4) or C26 (C20) cells (3×10^4) suspended in 100 μl HBSS (Gibco, Grand Island, NY) in shaved right flanks on day 0. Tumor occurrence was observed twice a week. The tumor size was evaluated by measuring two perpendicular diameters using calipers.

Flow cytometric analysis. Staining of cells and analysis on a flow cytometer (FACScan; BD Biosciences) were done as described previously [21]. Antibodies and reagents used for staining were as follows: FITC-conjugated anti-I-A^b (clone 28-16-8S; mouse IgG2a; Caltag, Burlingame, CA), R-PE-conjugated anti-mouse CD80 (clone RMMP-1; rat IgG2a; Caltag), R-PE-conjugated anti-mouse CD86 (clone RMMP-2; rat IgG2a; Caltag), FITC-conjugated anti-mouse CD4 (clone L3T4; rat IgG2a; BD PharMingen, San Diego, CA), FITC-conjugated anti-mouse CD8 (clone Ly-2; rat IgG2a; BD PharMingen), FITC-conjugated mouse IgG2a control (clone G155-178; BD PharMingen), and R-PE-conjugated rat IgG2a control (clone LO-DNP-16; Caltag).

Depletion of $CD4^+$ T cells and $CD8^+$ T cells in mice. Rat monoclonal antibodies (mAbs) GK1.5 specific to mouse CD4 and 2.43 specific to mouse CD8 were used to deplete $CD4^+$ T cells and $CD8^+$ T cells in vivo, respectively. The mice were injected with ascites (0.1 ml/mouse) from hybridoma-bearing nude mice intraperitoneally on days −18, −15, −11, −8, −4, and −1 and the tumor cells were inoculated on day 0. Normal rat IgG (Sigma, St. Louis, MO; 200 μg/mouse) was used as a control. The depletion of T cell subsets was monitored by a flow cytometric analysis, which showed more than a 90% specific depletion in the number of splenocytes.

Immunohistochemical analysis. Immunohistochemical detection of HSP105 was done as previously described [11,12]. Rabbit polyclonal anti-human HSP105 (Santa Cruz, Santa Cruz, CA) was used as the primary antibody in this study. Immunohistochemical staining of CD4 and CD8 was done as previously described [22]. For the terminal deoxynucleotidyl transferase-mediated nick end labeling (TUNEL) method, we used ApopTag Fluorescein In Situ Apoptosis Detection Kits (Serologicals Corporation, Norcross, GA).

Induction of $CD4^+$ T cells and $CD8^+$ T cells specific to HSP105. The mice were inoculated intraperitoneally with HSP105-pulsed BM-DC on days −14 and −7. Spleen cells were harvested on day 0, and $CD4^+$ T cells and $CD8^+$ T cells were purified using the magnetic cell sorting system (MACS) with anti-mouse CD4 (L3T4) mAb and anti-mouse CD8α (Ly-2) mAb, respectively. The purity of these T cell subsets exceeded 95% by a flow cytometric analysis. $CD4^+$ T cells or $CD8^+$ T cells (3×10^5/well) were separately incubated in RPMI 1640 medium supplemented with 10% horse serum, IL-2 (100 U/ml), and 2-ME (50 μM) together with the irradiated (4500 Gy) HSP105-pulsed BM-DC in 24-well culture plates. BM-DCs (3×10^4/well) pulsed with 2 μg/ml HSP105 for 16 h were irradiated (4500 Gy) and added to the culture wells for the restimulation once a week. After the third restimulation in vitro, both proliferation and cytotoxicity assays were performed as described previously [23]. For the

control of ^{51}Cr-release assay, $CD8^+$ T cells isolated from the mice immunized with BM-DCs alone were restimulated in vitro with BM-DCs alone once a week and used as effector cells.

ELISPOT assay. HSP105-specific IFN-γ production of T cells was quantified using the appropriate ELISPOT kit (BD Biosciences, San Diego, CA) according to the manufacturer's instructions. $CD4^+$ T cells or $CD8^+$ T cells were incubated with the BM-DC alone, BM-DCs pre-pulsed with HSP105, or BM-DCs pre-pulsed with myelin basic protein (MBP) as a control at 37 °C for 24 h. Each BM-DC was pre-pulsed with 2 μg/ml protein at 37 °C for 16 h. The spots were automatically counted and subsequently analyzed using the Eliphoto system (MINERVA TECH, Tokyo, Japan).

Statistical analysis. The statistical significance of the differences in the findings between the experimental groups was determined by 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

HSP 105 does not induce maturation of DCs

To analyze the direct effect of HSP 105 used in this study on BM-DCs, BM-DCs were incubated with HSP105, LPS as a positive control, and left untreated for 16 h. As shown in Fig. 1, no significant difference was observed in the levels of surface expression of CD80, CD86, and I-A^b between untreated BM-DCs and HSP105-pulsed BM-DCs. Moreover, HSP105-pulsed BM-DCs microscopically did not show any morphological changes in comparison to the untreated BM-DC. On the contrary, LPS-pulsed BM-DCs exhibited markedly increased expression of these three molecules. Although it is reported that HSPs could induce DC maturation and activation [14–16], the recombinant HSP105 used in this study including little LPS (below 10 endotoxin U/mg) did not show such activity. Thereafter, we evaluated the antigenicity of HSP105 to induce anti-tumor immunity.

The HSP105-pulsed BM-DC vaccine induced anti-tumor immunity against the lethal challenge of B16-F10 and C26 (C20)

We recently reported that mouse HSP105 was also overexpressed in the liver metastasis of C26 (C20) cells, and lung metastase of the B16-F10 cells, and that *HSP105* DNA vaccination inhibited the growth of these tumors [13]. In this study, we investigated the effects of HSP105 vaccination based on DCs on the growth of B16-F10 and C26 (C20) tumor cells in vivo. The objective was to determine whether prophylactic vaccination induced significant immunity against tumor growth and a prolonged survival. The protocol of vaccination in this study is shown in Fig. 2A. The results shown in Fig. 2B demonstrate that immunization with HSP105-pulsed BM-DC markedly inhibited the growth of B16-F10 tumors in comparison to other groups ($P < 0.01$). As shown in Fig. 2C, five of eight (62.5%) mice immunized with HSP105-pulsed BM-DC remained tumor free and survived for 100 days after the tumor challenge. In contrast, the mice vaccinated with BM-DC alone (12.5%) or PBS (0%) showed little protection against the growth of B16-F10 tumor in comparison to the observations in mice treated with HSP105-pulsed

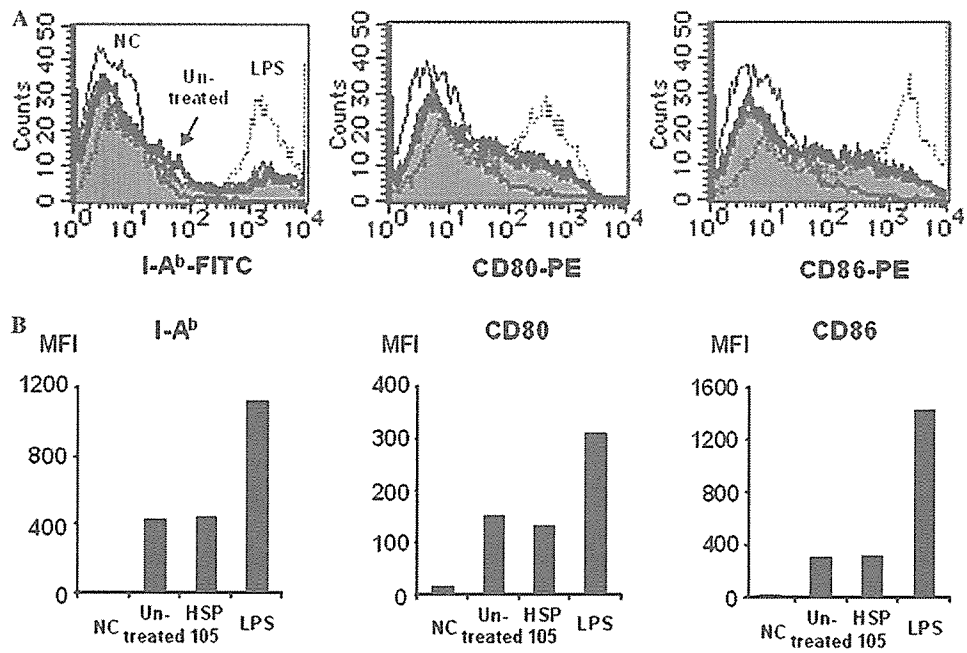

Fig. 1. Expression levels of cell surface I-A^b, CD80, and CD86 on BM-DCs, HSP105-pulsed BM-DCs, and LPS-pulsed BM-DCs were analyzed by flow cytometric analysis. BM-DCs were pulsed with 2 μg/ml HSP105, 1 μg/ml LPS or left untreated for 16 h. (A) The expression levels in HSP105-pulsed BM-DCs (filled histogram), LPS-pulsed BM-DCs (dotted line), and untreated BM-DCs (thick line), and the profiles of cells treated with isotype matched Ig as a negative control for staining (thin line). (B) The mean fluorescence intensity (MFI) of I-A^b, CD80, and CD86 staining in the cells. The results are representative of three independent experiments with similar results.

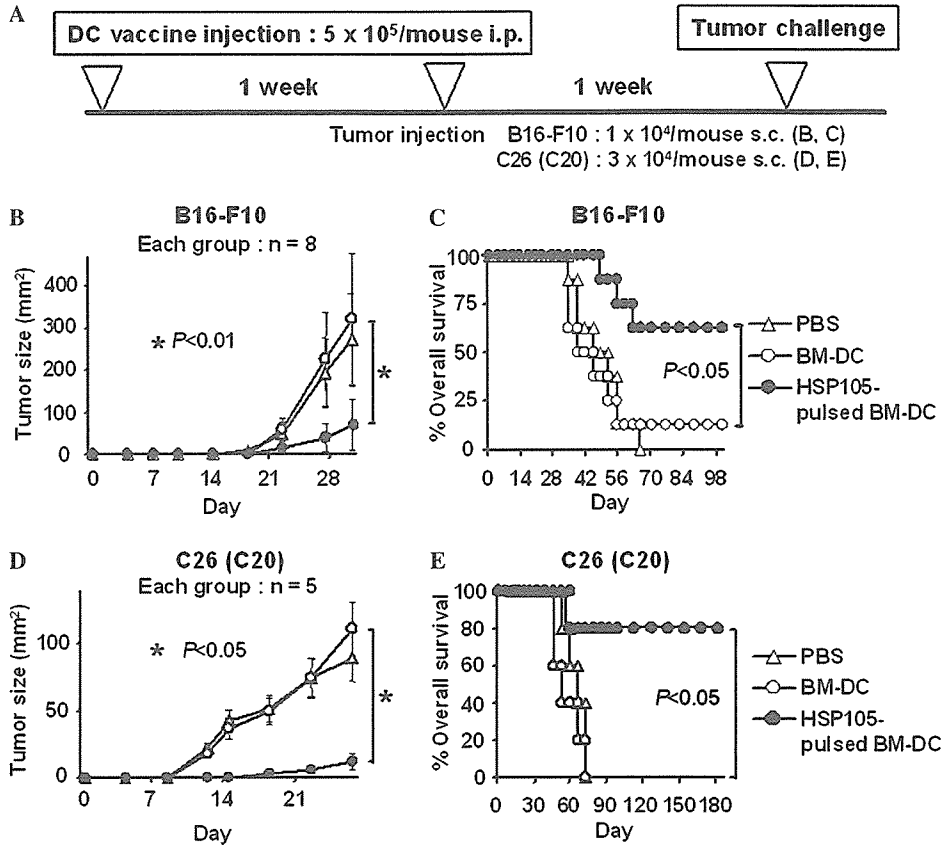

Fig. 2. Protection against tumor growth of B16-F10 and C26 (C20) cells by immunization with HSP105-pulsed BM-DC vaccine. (A) Protocol of the vaccination. The mice were immunized with PBS, BM-DC alone, and HSP105-pulsed BM-DC on 14 and 7 days before the tumor challenge. Seven days after the second immunization, the mice were challenged with B16-F10 cells s.c. (B,C), or C26 (C20) cells s.c. (D,E). (B,D) The tumor size was evaluated by measuring two perpendicular diameters. The result is presented as the mean area of tumor ± SE, and we evaluated statistical significance of the differences between each group using the unpaired Student's *t* test. (C,E) The mice in each group were observed for their survival period. The statistical significance of the differences between each group was evaluated using Wilcoxon's test.

BM-DC ($P < 0.05$). Similar results were observed in a prophylactic immunotherapy model using C26 (C20). Four of five (80%) mice immunized with HSP105-pulsed BM-DC completely rejected the C26 (C20) (3×10^4) cells (Figs. 2D and E), whereas tumors grew rapidly and all five mice died within 70 days in control mice treated with PBS or BM-DC alone. These results suggest that the HSP105-pulsed BM-DC vaccine is a potent vaccine that can efficiently induce specific anti-tumor immunity.

Both $CD4^+$ T cells and $CD8^+$ T cells are required for anti-tumor immunity

To determine the role of $CD4^+$ T cells and $CD8^+$ T cells in the protection against B16-F10 and C26 (C20) tumor cells induced by HSP105 vaccination, we depleted mice of $CD4^+$ T cells or $CD8^+$ T cells by the treatment with anti-CD4 or anti-CD8 mAb in vivo, respectively. During the depletion procedure, the mice were immunized with HSP105-pulsed BM-DC vaccine and challenged with B16-F10 or C26 (C20) cells (Fig. 3A). In both B16-F10 and C26 (C20) models, mice depleted of $CD4^+$ T cells, and $CD8^+$ T cells developed aggressively growing tumors after the challenge in comparison to the findings in control mice treated with rat IgG ($P < 0.05$) (Figs. 3B and D). The mice depleted of $CD4^+$ T cells or $CD8^+$ T cells all died by 52–65 days, whereas more than 50% of the control mice survived for 70 days ($P < 0.05$) (Figs. 3C and E). These results suggest that both $CD4^+$ T cells and $CD8^+$ T cells play crucial roles in the protective anti-tumor immunity induced by the HSP105-pulsed BM-DC vaccine.

Vaccination of HSP105-pulsed BM-DCs induced infiltrations of both $CD4^+$ T cells and $CD8^+$ T cells into tumor cells, but not into normal organs

Four of five (80%) mice immunized with the HSP105-pulsed BM-DCs completely rejected challenges of C26 (C20) cells (3×10^4) (Fig. 2E). To ascertain whether these rejections were induced by $CD4^+$ T cells or $CD8^+$ T cells, the subcutaneous inoculation of many C26 (C20) cells (1×10^6) into the right flank was done at 7 days after the second vaccination. After tumor formation, we removed the tumor and immunohistochemically stained it using anti-CD4 mAb, anti-CD8 mAb, and the TUNEL method. The infiltration of $CD4^+$ T cells and $CD8^+$ T cells into C26

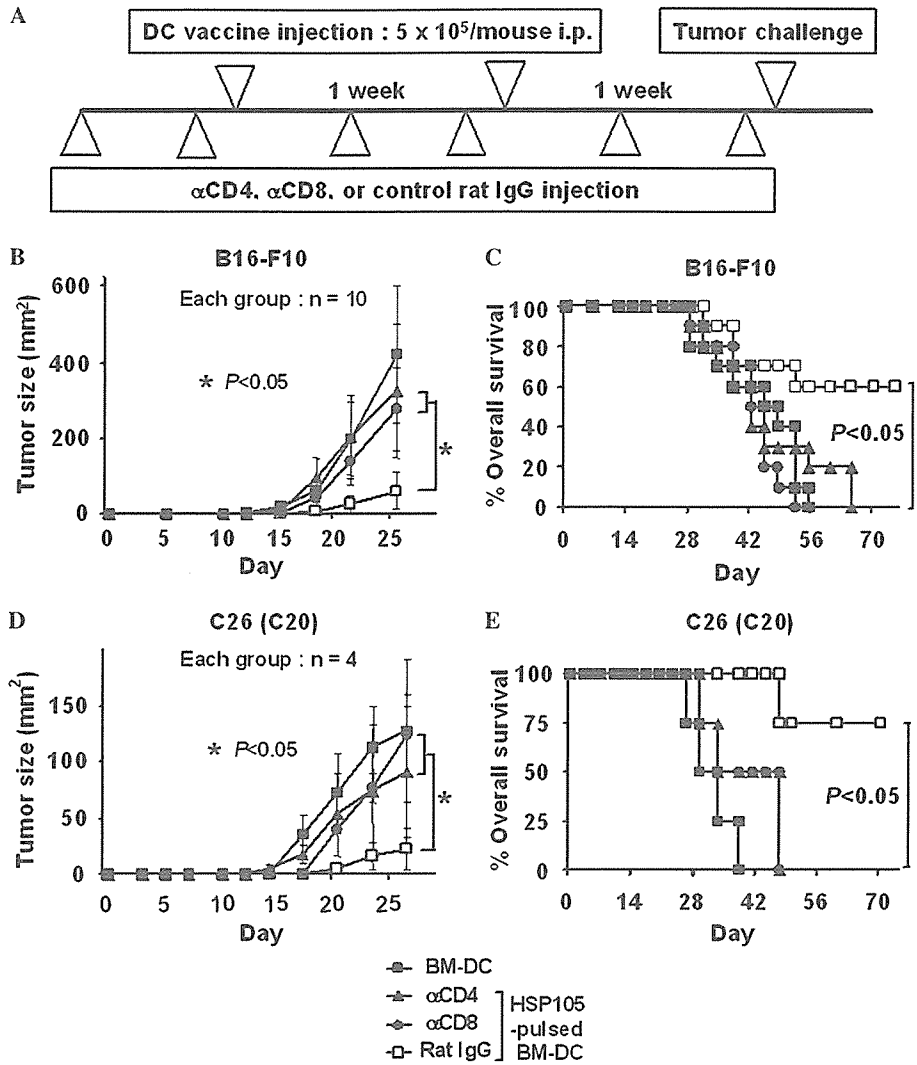

Fig. 3. Both $CD4^+$ and $CD8^+$ T cells are involved in the antitumor immunity elicited by the HSP105-pulsed DC vaccine. (A) Protocol for the vaccination and the depletion of T cells. C57BL/6 mice and BALB/c mice were challenged s.c. with B16-F10 cells and C26 (C20) cells, respectively. (B,D) The tumor size was evaluated by measuring two perpendicular diameters. The result is presented as the mean area of tumor ± SE, and we evaluated the statistical significance of the differences between each group using the unpaired Student's *t* test. (C,E) The mice in each group were observed for their survival period. The statistical significance of the differences between each group was evaluated using Wilcoxon's test.

(C20) tumors and some apoptotic C26 (C20) tumor cells were observed in the mice vaccinated with HSP105-pulsed BM-DCs, but never in the mice vaccinated with unpulsed BM-DCs (Fig. 4A). These results suggest that HSP105-pulsed BM-DCs have the potential to sensitize many HSP105-specific $CD4^+$ T cells and $CD8^+$ T cells to kill C26 (C20) tumor cells.

We evaluated the risk of autoimmunity by immunization against self-HSP105. Both BALB/c and C57BL/6 mice immunized with HSP105-pulsed BM-DC were apparently healthy without any abnormality such as dermatitis, arthritis, or neurological disorders. The tissues of the mice immunized with HSP105-pulsed BM-DC were histologically examined. The brain, liver, heart, kidneys, and spleen had normal structures and did not show any pathological changes suggestive of an immune response, such as the infiltration of $CD4^+$ T cells and $CD8^+$ T cells or tissue destruction and repair. Although we used female mice for the experiments described above, we also immunized male mice with HSP105-pulsed BM-DC to ascertain whether immunization with HSP105-pulsed BM-DC induced autoimmunity in the testis in which HSP105 is strongly expressed. However, no sign of autoimmunity was observed in the testis (Fig. 4B).

Induction of HSP105-specific $CD4^+$ T cells and $CD8^+$ T cells by immunization with HSP105-pulsed BM-DC

$CD4^+$ T cell lines specific to HSP105 were established from spleen cells derived from mice vaccinated with HSP105-pulsed BM-DC. $CD4^+$ T cells were separated from spleen cells and the purity of these cells was more than 95% by flow cytometric analysis. These cells were restimulated with irradiated and HSP105-pulsed DCs once

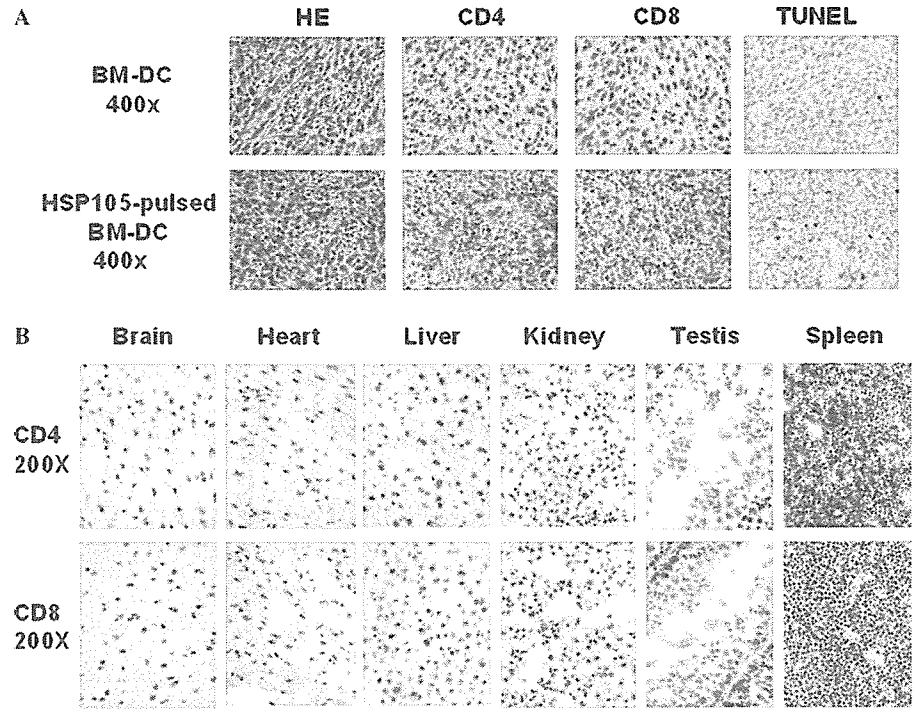

Fig. 4. Vaccination of HSP105-pulsed BM-DCs induced infiltrations of both $CD4^+$ T cells and $CD8^+$ T cells into C26 (C20) tumor and induced the apoptosis of C26 (C20) tumor cells. (A) C26 tumors removed from the mice vaccinated with BM-DCs or HSP105-pulsed BM-DCs were analyzed using immunohistochemical staining with anti-CD4 mAb, anti-CD8 mAb, and the TUNEL method on 4 days after the inoculation of tumor cells (1×10^6). (B) Normal tissue specimens of mice vaccinated with HSP105-pulsed BM-DCs were examined histologically and immunohistochemically. Objective magnification was 200×. The spleen was used as a positive control for the staining of both $CD4^+$ and $CD8^+$ cells.

a week. After three restimulations, both an ELISPOT assay and a proliferation assay were performed. The ELISPOT assay showed that HSP105-sensitized $CD4^+$ T cells produced IFN-γ in response to BM-DCs prepulsed with HSP105 but not an irrelevant MBP (Fig. 5A). As shown in Fig. 5B, HSP105-sensitized $CD4^+$ T cells proliferated in the presence of BM-DCs prepulsed with HSP105 but not MBP. These observations clearly indicated that HSP105-specific $CD4^+$ T cells were included in the T cell line.

We investigated whether HSP105-specific $CD8^+$ T cells were also induced with HSP105-pulsed DC vaccination. $CD8^+$ T cells were purified (>95%) from spleen cells of vaccinated mice and restimulated with irradiated and HSP105-pulsed DCs once a week. After three restimulations, the ELISPOT assay and 6 h ^{51}Cr-release assay were performed to detect the HSP105-specific CTL responses (Figs. 5C and D). The $CD8^+$ T cell line exhibited a HSP105-specific production of IFN-γ in an ELISPOT assay when cells were stimulated with BM-DCs prepulsed with HSP105 but not MBP ($P < 0.01$), however, the number of spots was smaller than that of $CD4^+$ T cells (Fig. 5C). $CD8^+$ T cells immunized with HSP 105-pulsed DC demonstrated a significant cytolytic activity against the B16-F10 cells pretreated with IFN-γ to induce the expression of MHC class I molecules on the cell surface, whereas $CD8^+$ T cells from mice immunized with BM-DC alone revealed little cytolytic activity ($P < 0.005$) (Fig. 5D). The induction of HSP105-specific $CD8^+$ T cells by the immunization in vivo with HSP105-pulsed BM-DC and the stimulation of the $CD8^+$ T cell line in vitro with the HSP105-pulsed BM-DC strongly suggested that these HSP105-specific $CD8^+$ T cells were induced by the cross-presentation of HSP105 by BM-DCs.

Discussion

HSPs are classified into several families based on their apparent molecular weights, such as HSP105/110, HSP90, HSP70, HSP60, HSP40, and HSP27 [24]. HSP105 consists of HSP105α and HSP105β. HSP105α is a constitutively expressed 105-kDa HSP that is induced by a variety of stresses, whereas HSP105β is a 90-kDa truncated form of HSP105α that is specifically induced by heat shock at 42 °C [24]. In this study, we used the mouse HSP105α protein. The cDNA sequence of murine HSP105 is almost the same as that of the Chinese hamster HSP110 [25,26], so HSP105 belongs to a member of the HSP105/110 family. We recently reported by the immunohistochemical analysis that HSP105 is overexpressed in a variety of human tumors [12], the liver metastasis of the C26 (C20) cells in the BALB/c mice, and lung metastasis of the B16-F10 cells in the C57BL/6 mice [13]. We examined the expression of HSP105 in the mouse cancer cell lines using a Western blotting analysis and found that HSP105 was strongly expressed in all 7 mouse cell lines tested (data not shown).

Many studies have shown that certain HSPs purified from a tumor can function as an effective vaccine against the same

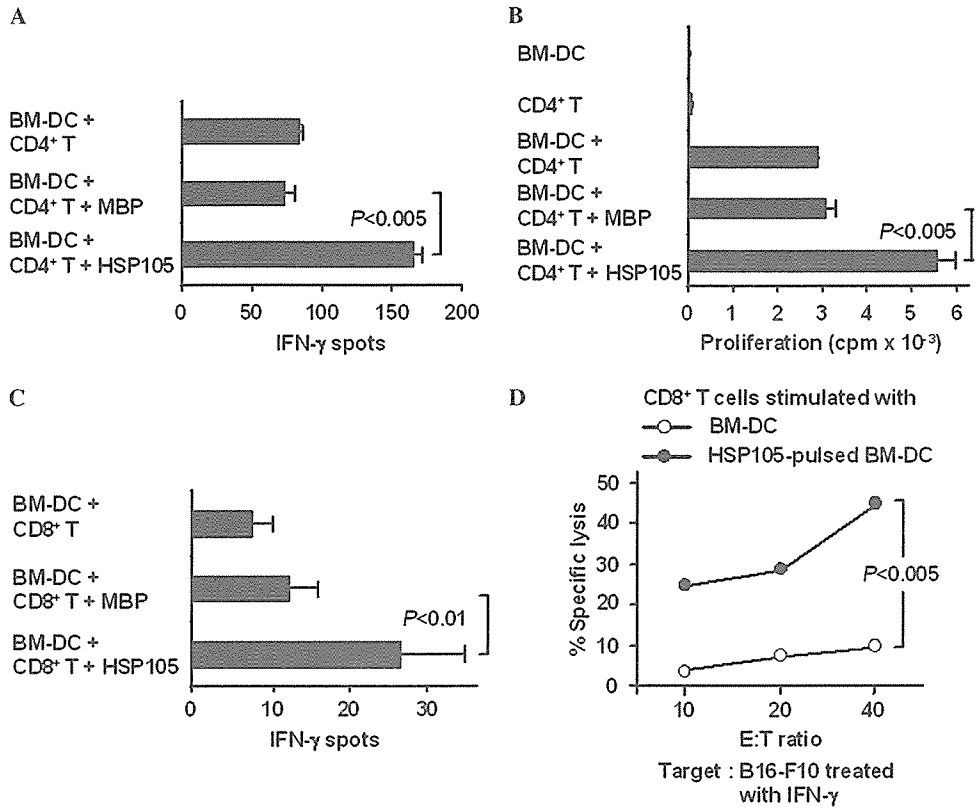

Fig. 5. Induction of HSP105-specific $CD4^+$ T cells and $CD8^+$ T cells by stimulation with HSP105-pulsed BM-DCs. (A) An ELISPOT assay for IFN-γ production by $CD4^+$ T cell lines stimulated with HSP105 protein-pulsed BM-DCs. $CD4^+$ T cells derived from the mice vaccinated with HSP105-pulsed BM-DC were stimulated in vitro with HSP105-pulsed BM-DC three times. For the ELISPOT assay, these $CD4^+$ T cells were co-cultured with BM-DC prepulsed with HSP105, MBP, or unpulsed BM-DC for 24 h. (B) Cell proliferation of $CD4^+$ T cell lines stimulated with HSP105-pulsed BM-DCs was determined by measuring [^{3}H]thymidine incorporation. $CD4^+$ T cells were co-cultured with BM-DC prepulsed with HSP105, MBP, or unpulsed BM-DC for 72 h. (C) An ELISPOT assay for IFN-γ production by $CD8^+$ T cell lines stimulated with HSP105-pulsed BM-DCs. $CD8^+$ T cells derived from mice vaccinated with HSP105-pulsed BM-DC were stimulated with HSP105-pulsed BM-DC three times in vitro. For the ELISPOT assay, these $CD8^+$ T cells were co-cultured with BM-DC prepulsed with HSP105, MBP, or unpulsed BM-DC for 24 h. (D) $CD8^+$ T cells stimulated with HSP105-pulsed BM-DC or BM-DC alone (control) were examined for their CTL activity against B16-F10 cells treated with IFN-γ (10^3 U/ml) using 6 h ^{51}Cr-release assay. The results were analyzed using the mean values of a triplicate or a quadruplicate assay. The data shown in A–D are each representative of three independent experiments with similar results.

tumor by stimulating T cells with tumor-specific peptides bound to HSPs. Subjeck and co-workers [27,28] reported that tumor-derived HSP110–peptide complexes also stimulated tumor immunity as other HSP families did in mice. Despite studies establishing a chaperoning effect of HSPs, one impediment to the full-fledged acceptance of HSPs as peptide-transporting vehicles is the lack of mass spectrometric data directly identifying HSP-associated peptides [29]. Stress-inducible proteins can be recognized by natural killer cells and CTLs as whole antigens expressed on the surface of stressed cells in humans [30]. Proteins dramatically upregulated or modified under stressful conditions should lead to increased presentation as do peptides presented by HLA class I molecules. About 25 HSP-derived peptides bound by HLA class I molecules have been identified through mass spectrometry [30]. Cancer patients have been reported to possess CTLs specific to HSP60-derived peptide [31], while HLA-A*0201-restricted HSP70-derived CTL epitopes have been identified in both an HLA-A*0201 transgenic mouse model and in humans [32]. In this study, although we did not identify HSP105-derived epitope peptides for $CD4^+$ T cells and $CD8^+$ T cells, we did prove that HSP105 itself could induce both $CD4^+$ Th-cells and $CD8^+$ CTLs specific to HSP105 as a cancer antigen. Contrary to our findings, however, Subjeck and co-workers [28] reported that HSP110 immunization did not elicit anti-tumor immunity. This discrepancy could be attributed to the difference in the methods of immunization.

It has been reported that HSPs can induce the maturation and activation of DCs as determined by upregulation of MHC class II and CD86 molecules, secretion of the IL-12 and TNFα [14,15]. However, HSP105-pulsed BM-DCs did not show any changes in comparison to the untreated BM-DC, thus suggesting that HSP105 did not induce DC maturation and activation. It is unlikely that HSP105 brought tumor-derived peptides into the culture system, because the HSP105 used in this study was the recombinant protein produced in *E. coli*. Furthermore, we recently identified HSP105-derived CTL epitopes restricted by HLA-A*0201 or -A*2402 using HLA

transgenic mouse model (unpublished data). These results also supported that HSP105 served not as a mediator for maturation of DCs, but as a cancer antigen eliciting tumor immunity.

The results of the T cell depletion study showed that the depletion of either $CD4^+$ T cells or $CD8^+$ T cells abrogated the anti-tumor immune response induced by the HSP 105-pulsed BM-DC vaccine, and that both $CD4^+$ and $CD8^+$ T cells play crucial roles in the protective anti-tumor immunity. $CD8^+$ T cells are thought to serve as the dominant effector cell mediating tumor killing, in contrast, $CD4^+$ T cells are thought to have an indirect role in providing help to CTL as well as a direct role in tumor rejection [33]. It is interesting that B16-F10 tumor cells that lack MHC class I were killed in in vivo study. We suppose that $CD4^+$ T cells may have an important role in this case. Peptides derived from HSP105 bound by MHC class II on the surface of HSP105-pulsed BM-DCs activate $CD4^+$ T cells. The activated $CD4^+$ T cells can secrete IFN-γ upon stimulation with tumor local DCs presenting tumor-derived HSP105 peptides, which contribute not only to activation of $CD8^+$ T cells but also to restoration of MHC class I expression in B16-F10 cells. The activated HSP105 specific $CD8^+$ T cells can recognize the peptides derived from HSP105 in the context of MHC class I and kill the B16-F10 cells.

In the field of cancer immunotherapy, most enthusiasm has been directed toward the use of various cancer vaccines; peptide vaccines alone, peptide plus cytokines, vaccination either with recombinant virus or with naked DNA encoding tumor antigen, and peptide pulsed on DCs [34]. DCs represent the most potent antigen presenting cells and also play an important role in the induction of specific T cell response [35]. Peptides pulsed on DCs have been reported to be the most effective vaccine in comparison to DNA vaccine or peptide–adjuvant mixture [36]. In this study, 62.5% and 80.0% of the mice immunized with HSP105-pulsed BM-DC completely rejected B16-F10 cells and C26 (C20) cells, respectively. On the other hand, only 50.0% of the mice immunized with the *HSP105*-DNA vaccine rejected these tumor cells in our previous study [13]. Although a further comparative analysis of the vaccination properties of these two strategies is required, our results suggested that protein-pulsed DCs are a more powerful vaccine than the DNA vaccine.

In this study, we used BM-DCs pulsed with HSP105 but not with HSP105-derived peptide as a cancer vaccine. We think that protein-pulsed DCs thus have an advantage over peptide-pulsed DCs. DCs are the major cell type known for its capacity to cross-present antigens [37]. In this study, HSP105-sensitized $CD8^+$ T cells responded to HSP105 in vitro by the stimulation of purified $CD8^+$ T cells with HSP105-pulsed DCs. This result strongly suggested that the HSP105-specific $CD8^+$ T cells were activated via the cross-presentation of HSP105 by BM-DCs. Although it became evident that gp96- and HSP70-chaperoned peptides can be presented to CTLs by DCs in the context of MHC class I molecules [38,39], we herein provide the first evidence that HSP itself can be cross-presented to CTLs by DCs. HSP105-pulsed DC can present peptides derived from exogenously added HSP105 in the context of not only MHC class II molecules on the surface of DCs to activate $CD4^+$ T cells, but also MHC class I molecules by cross-presentation to activate $CD8^+$ T cells. We herein showed the induction of specific $CD4^+$ T cells and $CD8^+$ T cells in vivo by stimulation with HSP105-pulsed DCs. The application of the peptide-pulsed DC as potential vaccine is limited to patients with the appropriate HLA alleles. To circumvent this limitation, we have used HSP105-pulsed DC to induce a HSP105 specific T cell response. HSP105-pulsed DCs offer the advantage of potentially presenting multiple immunogenic T cell epitopes without the need of prior knowledge of the individual patient's HLA type.

The mechanism of action of HSP105-pulsed BM-DCs injected intraperitoneally is still unclear. We think that DCs injected in the abdominal cavity might immigrate into mesenteric lymphatic vessels. Some DCs stay in mesenteric lymph nodes, others circulate in the blood via the thoracic duct and finally reach the spleen and bone marrow. Recent experimental evidence suggested that peripheral DCs migrate through the lymphatic vessels to the blood [40]. Although the present study showed that intraperitoneal injection of DCs induced an effective anti-tumor immunity in mice, comparison of effectiveness to other routes of immunization with DCs, such as intravenous, subcutaneous, and intranodal, remains to be investigated.

In conclusion, our results indicate that HSP105 itself is a tumor rejection antigen which may possibly be useful for cancer immunotherapy, and that HSP105-pulsed BM-DC vaccinations can prime HSP105-specific T cells in vivo, to prevent the subcutaneous growth of B16-F10 and C26 cancer cells expressing HSP105, without inducing autoimmune destruction. Our findings suggest that HSP105-pulsed BM-DC vaccination is a novel strategy for the prevention of cancer in patients treated surgically, who are at high risk for a recurrence of the cancer. Because of the overexpression of HSP105 in a variety of human tumors [12], clinical trial of immunotherapy targeted against HSP105 may well be applicable to various cancers.

Acknowledgments

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Proportion of De Novo Cancers Among Colorectal Cancers in Japan

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Background & Aims: Adenomatous polyps are main precursors of colorectal cancers (CRCs). In Japan, de novo cancers, which do not arise from preexisting adenomas, are considered to account for a substantial number of CRCs, but the relative importance of de novo carcinogenesis remains controversial. This study estimated the proportion of de novo cancers among CRCs in Japan. *Methods:* The subjects were persons 40–79 years of age who were relatively similar to those in the general population. The subjects underwent colonoscopy between 1997 and 2001. Early cancers among CRCs detected in this study were classified as de novo cancers or polyp cancers derived from adenomas. The age-specific incidence of the early CRCs was calculated, and the proportion of de novo cancers was estimated. The lifetime risk of early CRCs was estimated. *Results:* The study group comprised 14,817 persons. CRCs were diagnosed in 189 subjects, including 83 early cancers. There were no differences with regard to size and location between de novo cancers and polyp cancers, but morphology differed. Eighty-four percent (16/19) of de novo cancers were flat elevated or depressed. The expected lifetime risk of developing early CRCs was 5.27% for men and 3.21% for women. Among persons with early cancers, the expected probabilities of developing de novo cancer were 18.6% for men, 27.4% for women. *Conclusions:* De novo cancers account for a considerable proportion in Japan. This information suggests that the recommended interval for colonoscopic examination in Japan should be shorter than that in the United States.

The incidence of colorectal cancer (CRC) has rapidly increased in Japan. At present, CRC is the fourth leading cause of death in males and the third in females.[1] Recent studies in the United States and Europe have shown that most CRCs develop from adenomatous polyps via the adenoma-carcinoma sequence; this theory is now widely accepted.[2,3] In 1977 in Japan, Kariya et al described a case of depressed cancer that did not arise from an adenomatous polyp.[4] Subsequently, Ishii et al,[5] Kudo et al,[6] and Shamsuddin et al[7] reported depressed cancers associated with invasion or metastasis, including some lesions less than 1 cm in diameter. These findings led to the theory that some CRCs develop by de novo carcinogenesis, rather than from adenomatous polyps. In Europe, the existence of small de novo cancers less than 1 cm in diameter with invasive properties was reported.[8] Several authors showed that de novo cancers lacked K-*ras* mutations on gene analysis.[9–11] Kaneko et al suggested that some small invasive cancers less than 2 cm in diameter and characterized by a nonpolypoid growth pattern and no K-*ras* mutations are due to de novo carcinogenesis.[12] These findings led to the hypothesis that de novo cancer may develop independently of the adenoma-carcinoma sequence.

CRCs developing from adenomatous polyps can be prevented by colonoscopic surveillance and treatment. Estimation of the impact of colonoscopic screening on public health requires an accurate estimate of the proportions of CRCs developing from adenomatous polyps and CRCs developing from de novo carcinogenesis. Several studies have attempted to quantify the proportion of de novo cancers among all CRCs, but estimates have ranged from as low as 3.8% to as high as 80%.[13–15] This wide discrepancy is attributed to differences in factors such as sample size, the definition of de novo cancer, and the criteria for the selection of subjects or study design. We estimated the proportion of de novo cancers among CRCs based on well-defined criteria in a large number of subjects with characteristics relatively similar to those of the general population.

Abbreviation used in this paper: CRC, colorectal cancers.

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Materials and Methods

Subjects

The subjects were persons who attended a gastroenterology clinic in Kumamoto, Japan, from 1997 through 2001. We excluded persons who had a history of polypectomy, mucosal resection, or surgery for advanced neoplasms (adenomas more than 10 mm in diameter, severe dysplasia, or cancer) within the previous 5 years; those who were referred for treatment by other clinics; and those with symptoms of bowel stenosis suspected to be caused by CRC. The study group finally comprised 14,817 persons 40 to 79 years of age who underwent total colonoscopy. When we found the area with a different color from surrounding mucosa, or slight deformity on the folds, we used chromoendoscopy or magnifying endoscopy technique during colonoscopy procedures to make the existence of a lesion or its appearance clear. The subjects had no gastrointestinal symptoms and underwent colonoscopy for screening or had a slight transient abdominal discomfort or a positive fecal occult blood test. All patients gave oral informed consent for this study, which was approved by the Ethics Committee of Hattori GI Endoscopy and Oncology Clinic.

Procedures

It is difficult to distinguish de novo cancers among advanced cancers when they are in the advanced stage of carcinogenesis because their shape has changed completely from early stage, all adenomatous components have been replaced by cancerous components, and they have accumulated genetic changes during their progression. Even Dukes A, stage I or early cancers cannot be classified as de novo or polyp cancer with morphologic appearance only because some de novo cancers become flat elevated or polypoid as they begin to invade. De novo cancers can be distinguished among early CRCs on the basis of growth pattern, existence of adenomatous components, and genetic changes.[12] De novo cancers among early cancers were diagnosed according to both of the following histologic criteria: (1) the absence of adenomatous components in the tumor and (2) all lateral margins of the tumor covered with normal mucosa and nonpolypoid growth pattern (Figure 1). All other cancers in this study were diagnosed as polyp cancers arising via the adenoma-carcinoma sequence because there remained some possibility of including unusual polyp cancers as de novo cancers if the only diagnostic criterion used were the absence of adenomatous components.

In accordance with Japanese guidelines, we defined early CRCs as cancer with mucosal or submucosal involvement and advanced CRCs as cancer with deeper involvement regardless of the presence or absence of lymph node metastases. Early CRCs were classified as either de novo cancer or polyp cancer, and the proportions of these lesions were calculated. The age distribution, size, location, and morphologic appearance of these lesions were also examined.

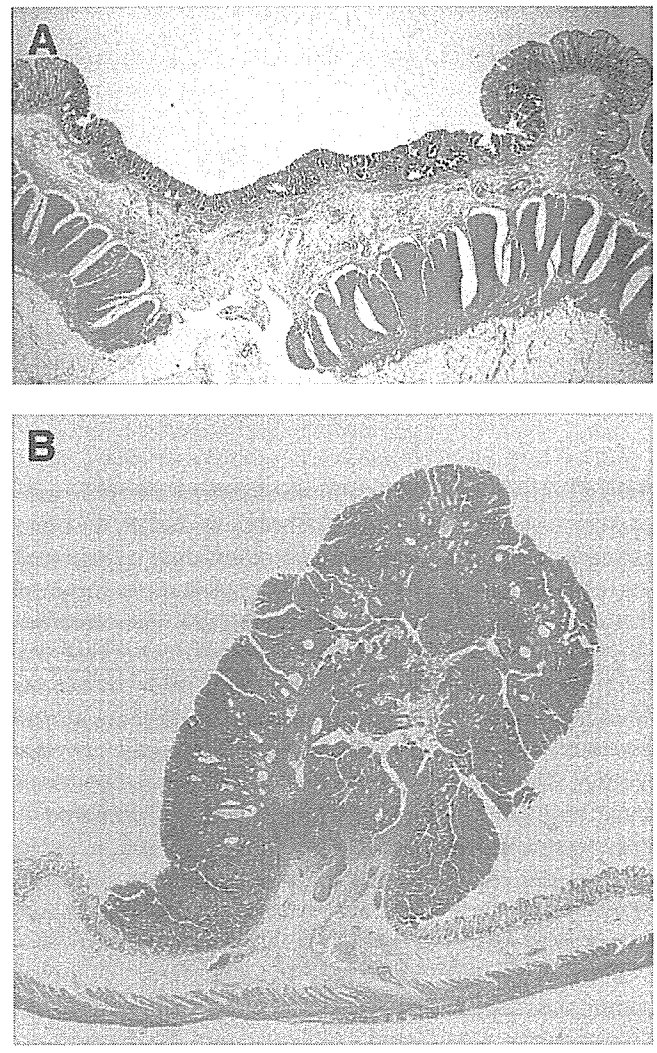

Figure 1. The histologic features of de novo and polyp cancer. (*A*) De novo cancer. The morphology is depressed type. Lateral margins are covered with normal mucosa. (*B*) Polyp cancer. Polyp includes adenomatous components.

Under the assumption that survival declines exponentially, we can convert a cumulative incidence to an incidence rate. This approximation, called the declining exponential approximation of life expectancy (DEALE) method,[16] was applied to estimate age and gender-specific incidence rates of early CRCs. The calculated incidence rates were compared with those of all stages of CRCs from the Surveillance, Epidemiology, and End Results (SEER) study in the United States.[17]

Method for Calculating Lifetime Risk

As only limited information is available for estimating the lifetime risk of early CRCs, we used a simplified approach to calculate the expected number of early CRCs and their subtypes (polyp cancer/de novo cancer) developing in persons 40 to 79 years of age.[18] The following formula was used to calculate the expected number of early CRCs in age category x.

Table 1. Chracteristics of Study Subjects

	Men (N = 6660)	Women (N = 8157)	Total (N = 14,817)
Age category, y			
40–49	1578	1636	3214
50–59	2152	2547	4699
60–69	1901	2410	4311
70–79	1029	1564	2593
Number of detected cancer	116	73	189
Early colorectal cancer	49	34	83
Advanced colorectal cancer			
≤20 mm	9	6	15
>20 mm	58	33	91
Total	67	39	106

$$a_x = (l_x - l_{x+1}) \times \frac{r_x}{m_x + r_x}$$

a_x: Expected number of early CRCs developing in age category x; l_x: number of survivors free of CRCs in age category x; r_x: incidence rate of early CRCs developing in age category x in persons free of CRCs in age category x-1; m_x: mortality rate excluding CRC-related deaths in category x.

To estimate the expected number of early CRCs developing from 40 to 79 years of age, we set l_x as 100,000 and calculated a_x for each age group (ages 40–44, 45–49, 50–54, 55–59, 60–64, 65–69, 70–74, and 75–79 years). The sum of the number for each age group was calculated to derive the total expected number of early CRCs. We also calculated the expected number of early CRCs from 40 to 79 years of age according to subtype (polyp cancer and de novo cancer). We used these data to estimate the age-specific rate of early CRCs and the proportion of de novo cancers. We also estimated the lifetime risk of all stage CRCs including early and advanced CRCs. Age-specific mortality rates excluding CRC-related deaths were derived from the Vital Statistics of Japan.[1]

Results

The characteristics of the subjects are shown in Table 1. The total number of subjects was 14,817 (men, 6660; women, 8157). There was no difference in age distribution between men and women. CRC was detected in 189 subjects (early cancer, 83; advanced cancer, 106); the incidence proportion was 1.74% (116/6660) in men, 0.89% (73/8157) in women, and 1.28% (189/14,817) overall. Of the 189 patients in whom CRCs were detected, 83 (44%) had early CRCs.

The characteristics of polyp cancers and de novo cancers detected among early CRCs are shown in Table 2. The proportion of de novo cancers was 22.9% (19/83). There were no differences with regard to size and location between them, but morphology differed. Eighty-four percent (16/19) of de novo cancers were flat elevated or depressed, whereas polyp cancers were literally polypoid.

The age-specific incidence proportions of early CRCs and their subtypes are shown in Table 3. The crude incidence proportion was 0.56% (men, 0.74%; women, 0.42%). The incidence proportions of polyp cancers and de novo cancers increased with age. In contrast to polyp cancers, de novo cancer was not found in the age 40–49-years group. The proportion of de novo cancers among all early CRCs was 22.9% (men, 18.4%; women, 29.4%).

The expected number of persons with early CRCs, ie, so-called lifetime risk, per 100,000 inhabitants 40 to 79 years of age in Japan and the estimated prevalence of de novo cancer are shown in Table 4. The expected lifetime risk of developing early CRC was 5.27% for men and 3.21% for women. Among persons with early CRC, the expected probabilities of developing de novo cancer were 18.6% (0.98/5.27) for men, 27.4% (0.88/3.21) for women, and 22.0% for the whole population.

The estimated incidence rates of early CRCs are compared with those of all stages of CRCs in the United States in Figure 2. Our data showed a higher incidence rate in the 40–55 year olds, followed by a gradual increase in the older age groups. This pattern differed from that in the United States, characterized by an exponential increase in the incidence rate of CRC with age.

Discussion

Estimation of de novo cancer is very important for setting the strategies for CRCs prevention and treatment and, simultaneously, is an issue very difficult to address correctly. To our knowledge, there have been few studies

Table 2. Characteristics of Early Colorectal Cancer

	Polyp cancer	De novo cancer	Percentage of de novo cancer
Age category, y			
40–49	10	0	0.0
50–59	18	4	18.2
60–69	20	9	31.0
70–79	16	6	27.3
Total	64	19	22.9
Size, mm			
≥20	17	5	
>10	26	7	
≤10	21	7	
Location			
Right side	22	6	
Left side	27	6	
Rectum	15	7	
Morphology			
Polypoid	64	3	
Flat elevated	0	6	
Depressed	0	10	

Table 3. Age-Specific Incidence Proportion of Early Cancer and Their Subtype

Age category, y	Number of subjects	Type of cancer		Proportion of De novo cancer in the total cases (%)	Incidence proportions of cancer (%)			
		Polyp cancer	De novo cancer		Polyp cancer	De novo cancer	All	SE
Men								
40–49	1578	5	0	0.0	0.32	0.00	0.32	0.001
50–59	2152	14	1	6.7	0.65	0.05	0.70	0.002
60–69	1901	12	5	29.4	0.63	0.26	0.89	0.002
70–79	1029	9	3	25.0	0.87	0.29	1.17	0.003
Total	6660	40	9	18.4	0.60	0.14	0.74	0.001
Women								
40–49	1636	5	0	0.0	0.31	0.00	0.31	0.001
50–59	2547	4	3	42.9	0.16	0.12	0.28	0.001
60–69	2410	8	4	33.3	0.33	0.17	0.50	0.001
70–79	1564	7	3	30.0	0.45	0.19	0.64	0.002
Total	8157	24	10	29.4	0.29	0.12	0.42	0.001
Total								
40–49	3214	10	0	0.0	0.31	0.00	0.31	0.001
50–59	4699	18	4	18.2	0.38	0.09	0.46	0.001
60–69	4311	20	9	31.0	0.46	0.21	0.67	0.001
70–79	2593	16	6	27.3	0.62	0.23	0.85	0.002
Total	14,817	64	19	22.9	0.43	0.13	0.56	0.001

SE, Standard error.

that estimated the incidence proportion of early CRC in the general population based on the results of colonoscopy in a representative number of subjects. Because the colonoscopic examination fee in Japan is considered reasonable (approximately 136 US dollars), this is often included in medical checkups and screening of the general population.

Given that repeated colonoscopic examinations at appropriate intervals allow us to detect most CRCs at a very

Table 4. Expected Number and Probability of Persons With Early Colorectal Cancer From 40 to 79 Years of Age in Japan

	Expected number of persons developing early colorectal cancer in 100,000 people			
	Total		De novo type	
	Men	Women	Men	Women
Age category, y				
40–44	316.9	305.6	0.0	0.0
45–49	313.7	303.8	0.0	0.0
50–54	679.4	270.9	45.3	116.1
55–59	661.8	267.7	44.1	114.7
60–64	816.4	477.2	240.1	159.1
65–69	771.7	466.2	227.0	155.4
70–74	919.0	576.7	229.7	173.0
75–79	790.6	541.8	197.6	162.6
Total	5269.4	3210.0	983.9	880.9
Expected probability of developing early colorectal cancer (%)	5.27	3.21	0.98	0.88

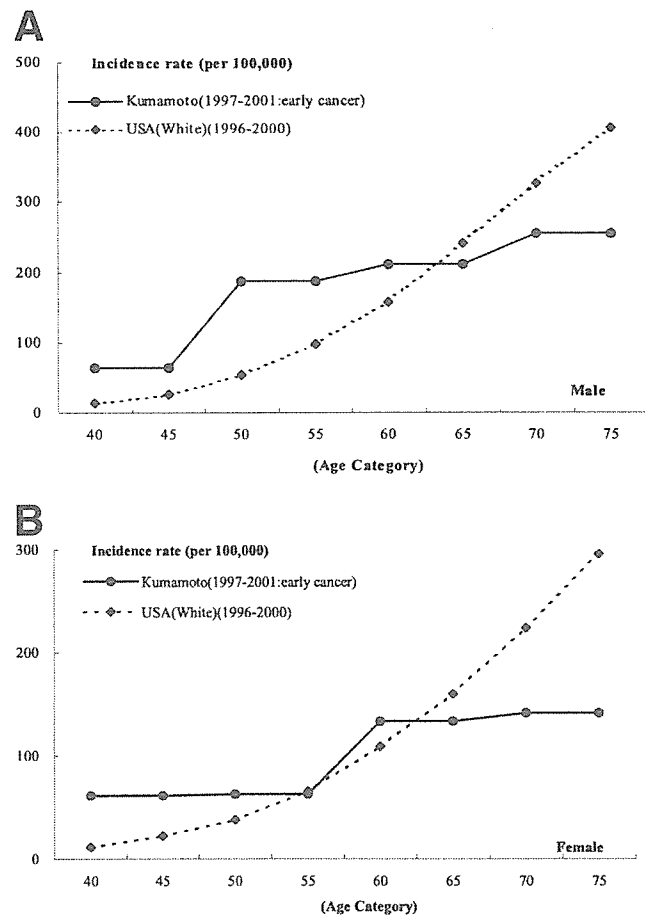

Figure 2. Age-specific incidence rates of early CRCs in Kumamoto were estimated by the DEALE method, and those rates of all stages of CRCs in the United States (*white*) were obtained from the Surveillance, Epidemiology, and End Results (SEER) Program of the National Cancer Institute.

early stage, the data can be used to predict the lifetime risk of CRC. In this study, we excluded the detection rate of advanced CRCs from the estimated lifetime risk of CRC because such cancers would probably have been detected at an earlier stage if colonoscopy had been done at appropriate intervals. In our model, such advanced cancers are included in the incidence of early CRC several years before their detection as advanced CRCs. The lifetime risk of CRC was 11.4% for men and 6.29% for women when advanced CRCs were taken into account. That was much higher than the SEER data of 6.31% for men and 5.94% for women and supports the usefulness of our model.

At present, colonoscopy is the best means to detect early CRCs. However, de novo early cancers are considered difficult to detect even by colonoscopy because the protrusion is inconspicuous.[19,20] One study has described an early cancer initially detected while it still was 6 mm in diameter that was not reconfirmed on several subsequent colonoscopic examinations, only to be detected after progression to an advanced cancer (18 mm in diameter) 3 years 4 months later.[21] On the other hand, colonoscopy has enabled the detection of some small advanced cancers 2 cm or less in diameter that were difficult to detect by barium enema because they were flat or flat elevated or depressed.

In this study, the majority of de novo cancers were flat elevated or depressed, whereas all polyp cancers were literally polypoid. At the same time, small advanced CRCs 2 cm or less in diameter accounted for 14% (15/106) of advanced CRCs. According to our criteria of de novo cancer, 80% (12/15) of small advanced CRCs were de novo. Thus, the proportion of de novo cancers might be underestimated in our study because it is difficult to detect most of them in the stage of early CRCs, even if colonoscopy is carried out at appropriate intervals.

The characteristics of our subjects were assumed to be relatively similar to those of the general population. The important risk factors for CRC such as family history were not considered because such risk factors among the general population are unknown in Japan. Instead of risk factors, the reason for a colonoscopic examination was considered. The most common reason was screening with no symptoms, slight transient abdominal discomfort, or anxiety about cancer. The medical conditions in Japan are such that people can easily undergo colonoscopic examinations at a reasonable cost. In addition, our target in this study was early asymptomatic CRC. Persons with positive fecal occult blood tests, who accounted for approximately 10% of our subjects, were included because the fecal occult blood test is a screening technique with a high sensitivity but a low specificity for CRCs, especially advanced CRCs. The inclusion of persons with positive fecal occult blood tests was therefore considered not to influence the detection of early cancers.[22] Patients who had received treatment for advanced neoplasms during the past 5 years were excluded from the study because such patients are at a decreased short-term risk and at an increased long-term risk for developing CRC.[23] The extrapolation of our data to the general population has some limitations.

The natural history of tumors cannot be followed in humans for ethical reasons. There is no way to know whether small early cancer stays small, resolves spontaneously, or goes on to become symptomatic. The origin of tumors can only be inferred on the basis of indirect methods. Most early CRCs are thought to retain their initial structural features. The origin of such early cancers can therefore be inferred on the basis of histologic appearance. Our criteria of de novo CRC might not be agreed with in Western countries. However, we believe our criteria to be most credible because the changes in the morphology, the replacement of adenomatous components by cancer components, and the genetic changes occur during the progression from an early to an advanced cancer.

Kaneko et al have demonstrated that carcinomas showing the nonpolypoid growth characteristic of small invasive CRCs do not contain K-*ras* mutations, a characteristic of adenomatous polyps. Such carcinomas therefore probably do not derive from adenomatous polyps and are most likely de novo cancers.[12] In accordance with this hypothesis, we considered carcinomas showing nonpolypoid growth pattern with none of the features of carcinomas derived from polyps to be de novo cancers. We believe that it is the most practical method to estimate the proportion of de novo cancers among patients with early CRC.

To estimate the lifetime risk of CRC and proportion of de novo cancer, we used a simplified SEER method to apply our age-specific data. CRC incidence rate, CRC mortality rate, and total mortality rate except CRC mortality rate were collected from the literature. To account for the competing risk, CRC mortality rate and total mortality rate except CRC mortality rate were treated separately in the calculation of lifetime risk of CRC. The lifetime risk of CRC was calculated by summing up age-specific incidence of CRC until the age of 80 years. Although the resulting method was not exactly the same as the SEER technique, there was no major problem in practical use.

The SEER data showed that the lifetime risk of CRC per 100,000 residents in the United States was 6.31% for

men and 5.94% for women.[17] In Japan, the incidence of CRC has increased more than 4 times during the past 20 years.[1] Our estimates of the lifetime risk for developing early CRC from 40 to 79 years of age (5.27% for men and 3.21% for women) were similar to the SEER estimates, especially in men. Similar risk levels might reflect the increasing Westernization of lifestyle in Japan or might support that some of the early cancers do not grow and do not become symptomatic or fatal. Our data showed higher incidence rates in middle age and lower incidence rates in advanced age as compared with the SEER data. The SEER data may reflect mainly the incidence of advanced CRCs. The differences in age-related incidence rates between our results and the SEER data, despite similar lifetime risks, might be associated with the time interval required for progression from early to advanced CRCs. Verification of this assumption would provide evidence that repeated colonoscopy at appropriate intervals is useful for the detection of most CRCs at an earlier stage, which can be cured by appropriate intervention.

Our results showed that de novo cancers account for approximately 22% of early CRCs. The estimation of our results is limited for the reasons described above. Estimation of de novo cancer is very important for setting the strategies to prevent and treat CRCs, and, simultaneously, it is a very difficult issue.

Our risk estimate was lower compared with that obtained in a simulation study carried out in Taiwan (30%).[24] When analyzing early CRCs and small advanced CRCs $\leq$2 cm, 30% of these cancers were de novo. This high prevalence of de novo cancer among small advanced CRCs might be attributed to the difficulty in identifying de novo cancer as early CRC because of morphologic reasons or rapid growth. In this study, we used chromoendoscopy or magnifying endoscopy technique when the existence of a lesion is suspected. Repeated colonoscopy at appropriate intervals or another new technique, such as fluoroscopy or narrow-band images, might be needed to detect most de novo cancers in the stage of early CRCs.

The National Polyp Study[25] conducted in the United States proposed that follow-up examinations after colonoscopic removal of newly diagnosed adenomas should be done every 3 years. If only 1 or 2 small (<1 cm) tubular adenomas are detected, follow-up examinations should be done every 5 years.[23] These proposals are evidence based as well as practical. However, the results of our study suggest that de novo cancers account for 22% of early CRCs, even after resection of all adenomatous polyps. Available evidence suggests that the recommended interval for colonoscopic examination in Japan should be shorter than that in the United States. The Japan Polyp Study, a large, multicenter, randomized, controlled study organized by investigators at the National Cancer Center that includes our hospital has been initiated to determine the characteristics of CRC and the most cost-effective surveillance intervals for colonoscopy in Japan.[26] That will show another aspect of the magnitude of the risk and incidence proportion of de novo cancer in Japan. Information on de novo cancer will contribute to the determination of the appropriate interval for colonoscopy and to establish the strategies for the prevention and treatment of CRCs.

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