apoptotic cells with terminal deoxynucleotidyl transferase–mediated nick end labeling methods (ApopTag fluorescein *in situ* apoptosis detection kits; Serologicals Corporation, Norcross, GA) in tumor specimens of patients with HCC were done as described previously (23, 24). In addition, immunohistochemical staining of HLA-class I in HCC tumor tissue specimens were done by using anti-HLA-class I mAb, EMR 8-5.[5]

Detection by ELISA of the serum-soluble GPC3 protein. Detection of the serum-soluble GPC3 protein was done by an indirect ELISA using the rabbit anti-GPC3 polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA) as described previously (7). We used recombinant human GPC3 protein (R&D Systems Inc., Minneapolis, MN) as a standard, and the presence of >106 ng/mL of serum GPC3 protein was considered to be positive.

Transfer of CTLs to the NOD/SCID mice implanted with a human HCC cell line. The transfer of GPC3-reactive CTLs to the immunodeficient mice implanted with a human HCC cell line was done as described previously (7). Briefly, we s.c. inoculated SK-Hep1/GPC3 cells (1×10^7) positive for both HLA-A2 and HLA-A24 at the right flank of NOD/SCID mice. When the diameter of these tumors reached 5×5 mm on day 9 after tumor inoculation into mice, we intravenously injected the mixture of GPC3 peptide-reactive CTL lines or irrelevant HIV peptides; HLA-A2-restricted SLYNTYATL peptide and HLA-A24-restricted RYLRDQQLL peptide, stimulated $CD8^+$ T cells (3×10^6) established from four HLA-A24-positive or two HLA-A2-positive HCC patients, or saline alone. T cells were i.v. injected one more times on day 14. The $CD8^+$ T cells stimulated with HLA-A24-restricted $GPC3_{298\text{-}306}$ peptide or HIV (RYLRDQQLL) peptide and derived from two independent HLA-$A24^+$ HCC patients were mixed, and injected into three NOD/SCID mice on day 9, and the mixture of peptide-stimulated $CD8^+$ T cells from two other HLA-$A24^+$ HCC patients distinct from the T cell donors at the first injection, were injected into the mice on day 14. The HLA-A2-restricted peptide-stimulated $CD8^+$ T cells from one HLA-$A2^+$ HCC patient were also injected into a NOD/SCID mouse on day 9, followed by the injection on day 14 with the peptide-stimulated $CD8^+$ T cells derived from another HLA-$A2^+$ HCC patient.

Statistical analysis. The two-tailed Student's t test was used to evaluate the statistical significance of differences in the data obtained by ELISPOT assay. The statistical significance of the differences in several factors between patients showing a successful CTL induction and other patients was assessed by a χ^2 test. $P < 0.05$ was considered to be significant. Statistical analyses were made using the StatView 5.0 software package (Abacus Concepts, Calabasas, CA).

Results

Identification of HLA-A2-restricted CTL epitopes by using HLA-A2.1 (HHD) Tgm. To identify HLA-A2-restricted epitopes by using HLA-A2.1 (HHD) Tgm, we selected nine kinds of peptides having amino acid sequences conserved between human and mouse GPC3 and having high predicted binding scores to HLA-A2 (*A*0201*; Table 1). $CD4^-$ spleen cells from HLA-A2.1 (HHD) Tgm immunized i.p. twice with BM-DCs pulsed with the mixture of these nine peptides were again stimulated *in vitro* with BM-DCs pulsed with each peptide, and we found that $CD4^-$ spleen cells stimulated *in vitro* with the $GPC3_{144\text{-}152}$ peptide produced the largest amount of IFN-γ in a peptide-specific manner in ELISPOT assays. These $CD4^-$ spleen cells (2×10^4/well), showed 36 ± 2.85 spot counts/well, in response to the BM-DCs pulsed with the $GPC3_{144\text{-}152}$ peptide, whereas they showed 23 ± 1.84 spot counts/well in the presence of BM-DCs without peptide loading ($P < 0.005$) indicating that about (36-23) / 2×10^4 = 0.065% of $CD4^-$ spleen cells were reactive to the GPC3 peptide. When we used syngeneic BM-DCs pulsed with a HLA-A2-binding HIV-derived peptide; SLYNTYATL as a control, no significant response (8.84 ± 1.73) was observed. The summation of the diameter of the IFN-γ ELISPOT observed in $CD4^-$ spleen cells stimulated with the $GPC3_{144\text{-}152}$ peptide pulsed BM-DCs was $1,878 \pm 131$ μm, that stimulated with the HIV-derived SLYNTYATL peptide pulsed BM-DCs was 437 ± 77 μm, and that observed in the presence of BM-DC without peptide loading was 762 ± 131 μm ($P < 0.001$). These assays were done thrice with similar results. As shown in Fig. 1B, the differences in the spot counts (left) or spot diameters (right) between stimulations with peptide pulsed BM-DC and BM-DC without peptide loading clearly revealed the $GPC3_{144\text{-}152}$ peptide-specific response of $CD4^-$ spleen cells. As for other peptides, no significant peptide-specific response was observed. These results suggest that the $GPC3_{144\text{-}152}$ peptide could be a CTL epitope peptide in HLA-A2.1 (HHD) Tgm, and we also expected this $GPC3_{144\text{-}152}$ peptide to be an epitope for human CTLs.

The immunization of the HLA-A2-restricted peptide, $GPC3_{144\text{-}152}$, did not induce autoimmunity in HLA-A2.1 (HHD) Tgm. It is well known that melanocyte-differentiation antigens such as MART-1 or gp100 are very useful for immunotherapy of melanoma patients, but they sometimes cause autoimmunity, such as vitiligo or uveitis, following vaccination. We previously reported that the immunization of the $GPC3_{298\text{-}306}$ peptide did not cause autoimmunity in BALB/c mouse (9). To investigate whether the immunization of mice with HLA-A2-restricted GPC3-derived peptides causes autoimmunity, the immunohistochemical staining of several organs with anti-CD4 and anti-CD8 mAb was done in HLA-A2.1 (HHD) Tgm immunized with a mixture of nine GPC3 peptides 7 days before the analysis. As shown in Fig. 2, we could not find any pathologic changes, such as lymphocyte infiltration or tissue destruction and repair in skin, lung, brain, heart, liver, and kidney of HLA-A2.1 (HHD) Tgm. The same result was also observed when mice were vaccinated with the $GPC3_{144\text{-}152}$ peptide alone ($n = 3$; data not shown). These results indicate that the $GPC3_{144\text{-}152}$ peptide-reactive $CD8^+$ CTLs do not attack the normal tissue specimens that we investigated.

Induction of GPC3-reactive CTLs from PBMCs of HLA-A2- or HLA-A24-positive HCC patients. We evaluated the cytotoxic activity of CTLs that were induced with the $GPC3_{298\text{-}306}$ or $GPC3_{144\text{-}152}$ peptide from PBMCs isolated from HCC patients. PBMCs were isolated from HCC patients positive for HLA-A24 and/or HLA-A2, and $CD8^+$ T cells sorted from the PBMCs were cocultured with autologous monocyte-derived DCs pulsed with each peptide as described in Materials and Methods. CTLs from PBMCs of HLA-$A2^+$ HCC patients stimulated with the $GPC3_{144\text{-}152}$ peptide or CTLs from PBMCs of HLA-$A24^+$ HCC patients stimulated with the $GPC3_{298\text{-}306}$ peptide exhibited cytotoxicity against peptide-pulsed target cells. The representative data of CTLs restricted by HLA-A2 or HLA-A24 were shown in Fig. 3A. The CTLs induced from PBMCs of patient A2-8 showed cytotoxic activity to T2-A0201 cells (HLA-A2+) pulsed with the $GPC3_{144\text{-}152}$ peptide, but not to T2-A0201 cells without peptide loading by ^{51}Cr release assay. The CTLs induced from PBMCs of patient A24-12 exhibited cytotoxic

[5] T. Torigoe, et al. Immunohistochemical analysis of HLA class I expression in tumor tissues revealed unusually high frequency of down-regulation in breast cancer tissues submitted.

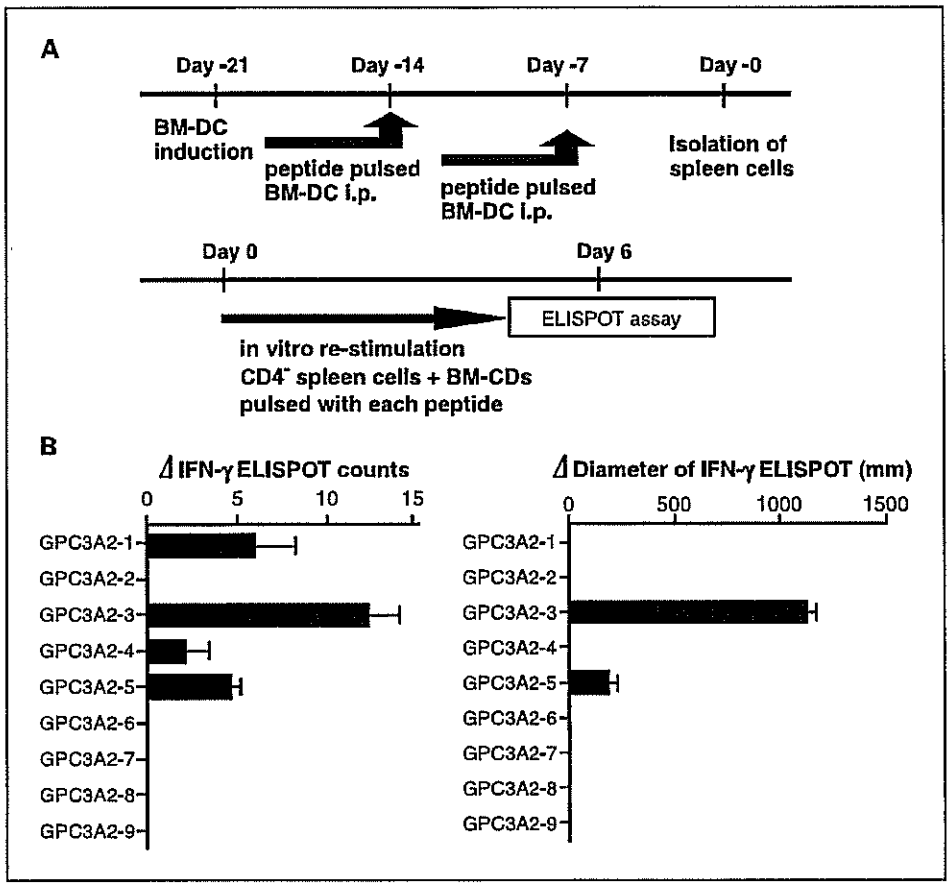

Fig. 1. Identification of HLA-A2-restricted CTL epitopes of GPC3 by using HLA-A2.1 (HHD) Tgm. *A*, protocol for identification of GPC3-derived and HLA-A2-restricted CTL epitopes. We primed the HLA-A2.1 (HHD) Tgm with BM-DCs (5×10^5) pulsed with the mixture of GPC3-derived peptides carrying HLA-A2 (*A*0201*) binding motif into the peritoneal cavity once a week for two weeks. Seven days after the last DC vaccination, spleens were collected and $CD4^-$ spleen cells (2×10^6/well) were stimulated with syngeneic BM-DCs (2×10^5/well) pulsed with each peptide *in vitro* for 6 days. We used these cultured $CD4^-$ spleen cells as responder cells in ELISPOT assay to evaluate GPC3-specific response of CTLs. *B, bar graph*, IFN-γ ELISPOT counts/2×10^4 $CD4^-$ spleen cells cocultured with peptide pulsed BM-DCs subtracted with those cocultured with BM-DCs without peptide loading (*left*). *Bar graph*, summation of IFN-γ ELISPOT diameters/2×10^4 $CD4^-$ spleen cells cocultured with peptide-pulsed BM-DCs subtracted with those cocultured with BM-DCs without peptide loading (*right*). *Columns*, mean of triplicate assays; *bars*, SE. All assays were done thrice with similar results.

activity to the C1R-A*2402 cells (HLA-A24+) pulsed with the $GPC3_{298\text{-}306}$ peptide, but not to C1R-A*2402 cells without peptide loading. These results indicate that these CTLs had peptide-specific cytotoxicity. Other CTLs induced from the nine patients A2-1, A2-2, A2-3, A2-4, A24-1, A24-3, A24-4, A24-6, and A24-7 similarly exhibited peptide-specific cytotoxicity against peptide-pulsed target cells (data not shown).

Furthermore, we used GPC3 transfectants, SK-Hep1/GPC3 (GPC3+, HLA-A2+, HLA-A24+) or SW620/GPC3 (GPC3+, HLA-A2+, HLA-A24+) as target cells and examined whether we could find GPC3-specific cytotoxic activity of CTLs. As shown in Fig. 3B, the CTLs induced from PBMCs of patient A2-3 by stimulation with the $GPC3_{144\text{-}152}$ peptide showed specific cytotoxicity against SK-Hep1/GPC3, but not against GPC3-negative SK-Hep1. Similarly, the $GPC3_{298\text{-}306}$ peptide–induced CTLs showed specific cytotoxicity against SW620/GPC3 in patient A24-7 or against SK-Hep1/GPC3 in patient A24-12, but not against SK-Hep1 or SW620, respectively, which did not endogenously express GPC3. These findings indicate that these peptides can be processed naturally in cancer cells, and the peptides in the context of HLA-A2 or HLA-A24 can be expressed on the cell surface of cancer cells to be recognized by the CTLs.

When we think about the application of GPC3 to cancer immunotherapy, the most important point is that these GPC3-reactive CTLs can exhibit specific cytotoxicity to the tumors endogenously expressing GPC3. We thus investigated whether these CTLs could kill human HCC cell lines expressing both endogenous GPC3 and the restriction HLA class I molecules. As shown in Fig. 3C, we could generate GPC3-reactive CTLs by stimulation with the $GPC3_{144\text{-}152}$ peptide and these CTLs exhibited cytotoxic activity to HepG2 (GPC3+, HLA-A2+, and

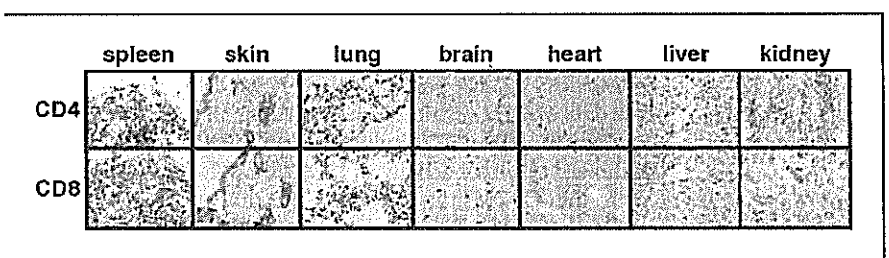

Fig. 2. Immunohistochemical staining with anti-CD4 or anti-CD8 mAb in tissue specimens of HLA-A2.1 (HHD) Tgm immunized with the $GPC3_{144\text{-}152}$ peptides. These tissue specimens were removed and analyzed 7 days after the second DC vaccination (original magnification, ×200).

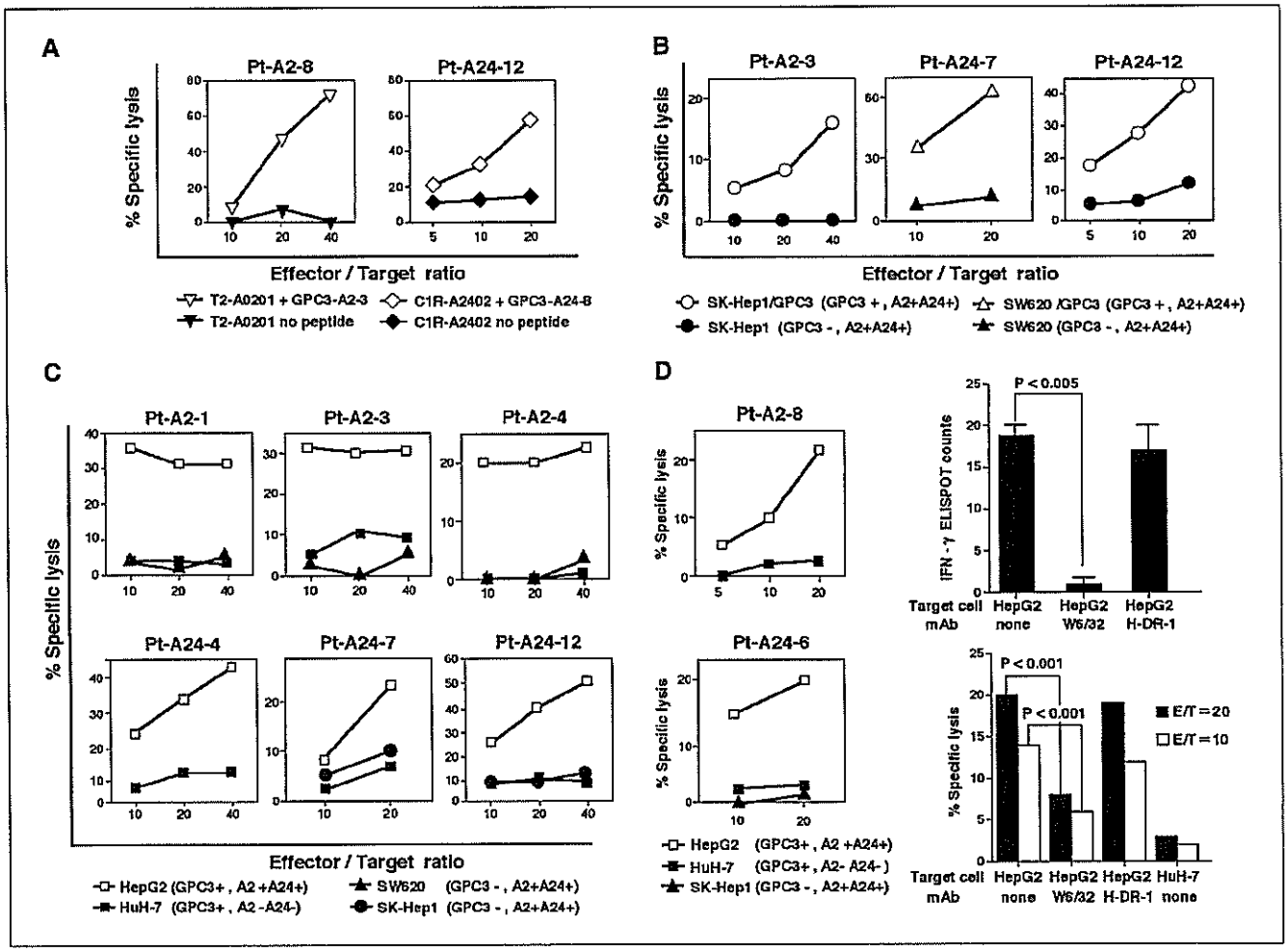

Fig. 3. CTL induction from PBMCs of HLA-A2- or HLA-A24-positive HCC patients. *A* and *B*, GPC3 peptide-reactive CTLs were generated from $CD8^+$ Tcells of $HLA\text{-}A2^+$ and/or $HLA\text{-}A24^+$ HCC patients. After three or four stimulations with autologous monocyte-derived DCs pulsed with the $GPC3_{144\text{-}152}$ or $GPC3_{298\text{-}306}$ peptide, the CTLs were subjected to a standard ^{51}Cr release assay at the indicated effector/target ratio. Their cytotoxicity against the $GPC3_{298\text{-}306}$ peptide pulsed C1R-A2402 cells orT2-A0201 cells, and each unpulsed cells (*A*), or $GPC3^-$ $HLA\text{-}A2^+$, $HLA\text{-}A24^+$ HCC cell line SK-Hep-1, $GPC3^-$ $HLA\text{-}A2^+$, $HLA\text{-}A24^+$colon cancer cell line SW620, and those cell lines transfected with the human *GPC3* gene; SK-Hep-1/GPC3 or SW620/GPC3 (*B*) were examined by a ^{51}Cr release assay. *C* and *D*, $GPC3^+$ $HLA\text{-}A2^+$, $HLA\text{-}A24^+$ HCC cell line HepG2, $GPC3^+$ $HLA\text{-}A2^-$, $HLA\text{-}A24^-$ HCC cell line HuH-7, and $GPC3^-$ tumor cell lines SW620 and SK-Hep1 were used as target cells (*left*). *Points*, percentage of specific lysis calculated based on the mean values of a triplicate assay. *D*, inhibition of cytotoxicity by anti-HLA class I mAb (*right*). After the target HepG2 cells were incubated with anti-HLA class I mAb (W6/32, IgG_{2a}) or anti-HLA DR mAb (H-DR-1, IgG_{2a}), respectively, for 1 hour, the CTLs generated from PBMCs of patient A2-8 by stimulation with $GPC3_{144\text{-}152}$ peptide (*top*) or CTLs generated from patient A24-6 using the $GPC3_{298\text{-}306}$ peptide (*bottom*) were added. IFN-γ production (*top*; IFN-γ ELISPOTassay) and cytotoxicity (*bottom*; ^{51}Cr release assay) were markedly inhibited by W6/32, but not by H-DR-1.

HLA-A24+), but not to HuH-7 (GPC3+, HLA-A2−, and HLA-A24−) or SW620 ($GPC3^-$, HLA-A2+, and HLA-A24+) in patients A2-1, A2-3, and A2-4. Similarly, we could generate GPC3-reactive CTLs by stimulation of PBMCs with the $GPC3_{298\text{-}306}$ peptide and these CTLs exhibited cytotoxic activity to HepG2, but not to HuH-7 or SK-Hep-1 ($GPC3^-$, HLA-A2+, HLA-A24+) in patients A24-4, A24-7, and A24-12.

In an HLA-class I blocking experiment, anti-HLA class I mAb W6/32 markedly inhibited the IFN-γ production stimulated with HepG2 cells in ELISPOT assay of CTLs generated from patient A2-8 by stimulation with the $GPC3_{144\text{-}152}$ peptide (Fig. 3D, top), and inhibited cytotoxic activity against HepG2 cells in ^{51}Cr release assay of CTLs generated from patient A24-6 by stimulation with the $GPC3_{298\text{-}306}$ peptide (Fig. 3D, bottom), but anti-HLA-DR mAb, H-DR-1 did not inhibit the response of CTLs. These results clearly indicate that these CTLs recognized HepG2 in a HLA-class I–restricted manner.

As shown in Table 2, we could induce GPC3-reactive CTLs from PBMCs in ~50% of either the HLA-A2− or HLA-A24-positive HCC patients. In patients A2-6, A24-5, A24-9, and A24-11 who did not express GPC3 in tumor tissues, GPC3-reactive CTLs could not be induced from their PBMCs. Among eight HLA-A2-positive HCC patients who expressed GPC3 in HCC tissue or produced soluble GPC3 in sera, patients A2-1, A2-2, A2-3, A2-4, A2-6, A2-7, A2-9, and A2-10, GPC3-reactive CTLs could be generated from the PBMCs of only four patients (50%). In patient A2-6, GPC3 was detected only in the serum but not in HCC tumor tissue. It was thought to be possible that the majority of GPC3 protein was secreted away in this type of HCC cell as described previously (7). Among six HLA-A24-positive patients who expressed GPC3 in tumor tissue, patients A24-1, A24-2, A24-3, A24-6, A24-10, and A24-12, GPC3-reactive CTLs could be generated from the PBMCs of only four patients (67%). We also examined whether it was possible to

induce GPC3-specific CTLs from PBMCs isolated from healthy donors (each HLA type, $n = 3$), but we failed to generate GPC3-specific and HLA-A2- or HLA-A24-restricted CTLs even though PBMCs were stimulated with the peptides thrice *in vitro* (data not shown). These results suggest that GPC3-reactive CTLs could only be induced in patients who expressed GPC3 in tumor tissue, thus, indicating the existence of GPC3-reactive CTL precursors in patients with GPC3$^+$ HCC. We also examined whether GPC3-reactive CTLs could be generated more frequently from PBMCs isolated from HCC patients positive for serum-soluble GPC3. As shown in Table 2, the presence of serum-soluble GPC3 did not correlate statistically with the successful induction of GPC3-reactive CTLs. As a result, we could not observe the enhancement of CTL induction efficiency via possible antigen presentation of soluble serum GPC3 through HLA-class II pathways to CD4$^+$ T cells or cross-presentation through the HLA class I pathway to CD8$^+$ T cells (25, 26) in patients positive for serum GPC3.

Inoculation of the GPC3 peptide-induced CTLs reduced growth of a GPC3$^+$ human HCC tumor cell line implanted into NOD/SCID mouse. To investigate the effects of GPC3 peptide-reactive CTL inoculation into the mice implanted with the GPC3$^+$ human HCC cell line, we s.c. inoculated SK-Hep1/GPC3 cell lines positive for both HLA-A2 and HLA-A24 into NOD/SCID mice, and i.v. injected the mixture of CTLs generated from several HCC patients positive for HLA-A2 or HLA-A24 into mice implanted with SK-Hep1/GPC3 when the diameter of these tumors reached 5 × 5 mm in size as described in Materials and Methods. The CTLs injected into mice were prepared by stimulating peripheral blood CD8$^+$ T cells with HLA-A2- or HLA-A24-restricted GPC3-epitope peptides or control-irrelevant HIV peptides as described in Materials and Methods. The tumor sizes of four individual mice in each group (Fig. 4A) and mean ± SD of tumor sizes in each group (Fig. 4B) were evaluated. After 5 days from the second inoculation of GPC3 peptide-reactive CTLs, the tumor size of SK-Hep1/GPC3 was apparently reduced in comparison to the size of tumor mass implanted into NOD/SCID mice injected with control T cells or saline alone ($P < 0.01$). These results clearly indicate the efficacy of adoptive GPC3 peptide-reactive CTL transfer therapy for GPC3$^+$ tumor in mice.

Discussion

In this article, we identified HLA-A24-restricted or HLA-A2-restricted GPC3 CTL epitope peptides, and found that

Table 2. Expression of GPC3 in HCC tissue, quantification of serum-soluble GPC3, and GPC3-specific CTL induction in HCC patients

	Age	Gender	State of tumor*	GPC3 expression†	Serum GPC3‡	HLA expression§	CTL induction‖
HLA-A2 (*A*0201*) – positive patients							
Pt-A2-1	80	F	IIIa	+	+	+	+
Pt-A2-2	72	M	II	+	+	+	+
Pt-A2-3	67	F	II	ND	+	ND	+
Pt-A2-4	54	M	I	+	–	+	+
Pt-A2-5	57	M	I	ND	–	ND	–
Pt-A2-6	66	M	I	–	+	–	–
Pt-A2-7	54	M	IIIa	+	–	+	–
Pt-A2-8	73	M	II	ND	–	ND	+
Pt-A2-9	68	F	IIIa	+	–	+	–
Pt-A2-10	54	M	II	+	+	+	–
HLA-A24 (*A*2402*)-positive patients							
Pt-A24-1	60	M	IVa	+	+	+	+
Pt-A24-2	57	M	IVa	+	+	+	–
Pt-A24-3	75	F	IIIa	+	+	+	+
Pt-A24-4	59	M	IIIa	ND	–	ND	+
Pt-A24-5	52	M	IVb	–	–	+	–
Pt-A24-6	65	M	I	ND	+	ND	+
Pt-A24-7	61	M	I	ND	–	ND	+
Pt-A24-8	74	M	II	ND	–	ND	–
Pt-A24-9	59	M	IVb	–	–	–	–
Pt-A24-10	69	M	IVa	+	+	+	–
Pt-A24-11	72	M	II	–	–	+	–
Pt-A24-12	61	M	IIIa	+	+	+	+

Abbreviations: F, female; M, male; ND, not determined.
*Tumor-node-metastasis classification.
†Positive (+) or negative (–) staining of tumor cells in contrast with peritumor normal tissue as background staining.
‡Serum levels >106 ng/mL were evaluated as positive.
§Immunohistochemical staining of the membrane of tumor cells was evaluated as positive.
‖Specific lytic activity (≧20%) at E:Tratio = 20 against HepG2 target cells was evaluated as positive by ^{51}Cr release assay.

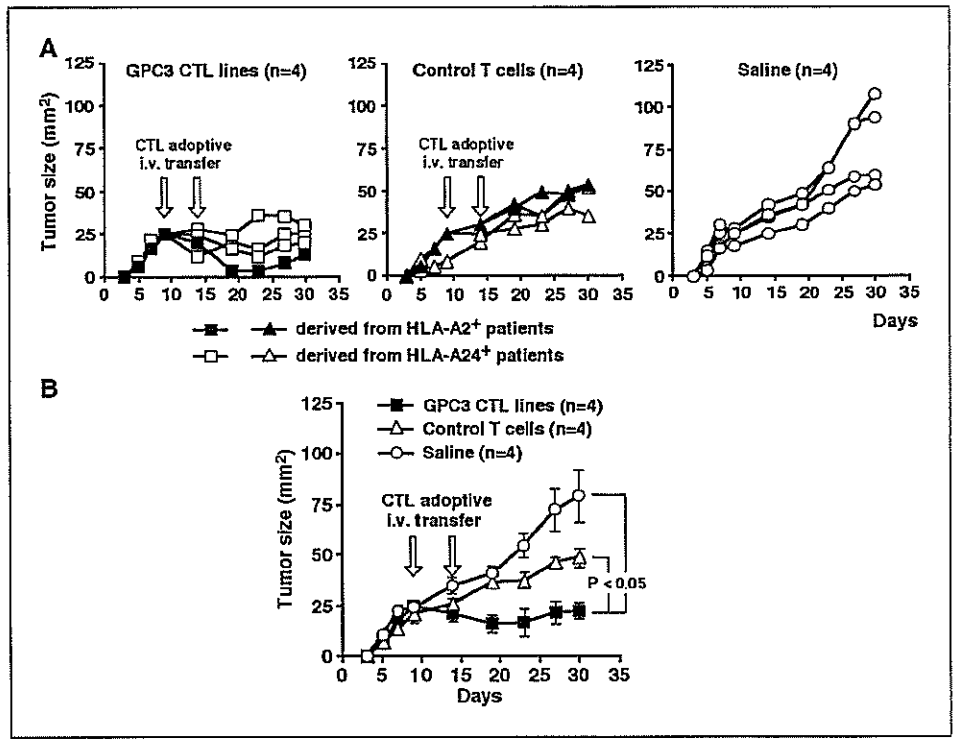

Fig. 4. Marked inhibition of growth of a GPC3-transfected human HCC cancer cell line, SK-Hep1/GPC3, engrafted into NOD/SCID mice after adoptive transfer of human CTLs induced by the GPC3 peptides. *A*, when tumor size reached 25 mm^2 on day 9 after s.c. tumor implantation, human CTLs (3×10^6) reactive to HLA-A2-restricted (■) GPC3 peptide and generated from one HLA-$A2^+$ donor, or those reactive to HLA-A24-restricted (□) GPC3 peptide and pooled from two HLA-$A24^+$ donors were i.v. inoculated. On day 14, the inoculation of CTLs generated from the donors distinct from those at the first injection was repeated. The control $CD8^+$ T cells stimulated with irrelevant HLA-A2-restricted (▲) or HLA-A24-restricted (△) HIV peptides were also injected into mice as a control. Tumor volumes in NOD/SCID mice given twice on days 9 and 14 with GPC3 epitope peptide – induced CTL lines ($n = 4$), control $CD8^+$ T cells ($n = 4$), or saline alone ($n = 4$). Tumor size was expressed in square millimeters. *B, points,* mean tumor sizes in each group of mice; *bars,* ±SD ($n = 4$). Statistical significance was evaluated using *t* test.

GPC3-reactive CTLs could be generated from PBMCs stimulated with these peptides in ~50% of HCC patients. Vaccination based on these peptides did not induce autoimmunity in HLA-A2.1 (HHD) Tgm of a B57Bl/6 background. We previously identified the $GPC3_{298\text{-}306}$ peptide to be a CTL epitope in BALB/c mouse, and we expected that this GPC3 peptide might also be present in human CTL in a HLA-A24-restricted manner. As expected, we could generate HLA-A24-restricted and the $GPC3_{298\text{-}306}$ peptide-reactive human CTLs in this study. As a result, BALB/c mice may be useful for identifying HLA-A24-restricted CTL epitopes. HLA-A2.1 (HHD) Tgm was reported to be a versatile animal model for the predinical evaluation of peptide-based immunotherapy (12, 13). We could also find its usefulness for the identification of HLA-A2-restricted antigenic epitope in this study.

In this study, we wanted to identify the most effective major CTL epitopes derived from GPC3. As a result, we used BM-DCs derived from HLA-A2.1 (HHD) Tgm and pulsed BM-DCs with the mixture of GPC3 peptides for the vaccination of mice. Some of the peptides tested stimulated the weak response of CTLs in an ELISPOT assay, and these peptides might also be useful for future analysis. It was recently reported that peptides having a weak affinity to MHC, which could not be predicted by a BIMAS system, could induce peptide-reactive CTLs with a cytotoxic activity (27). To search for more peptides that can be applicable for immunotherapy, it may be necessary to check these minor CTL epitopes in the future. In this study, the GPC3-derived peptides predicted to have high binding affinity to HLA-A2 molecules and having the amino acid sequences conserved between human and mouse GPCs were selected for the analysis. When we analyzed the amino acid sequence of human GPC3 protein, all of the top 28 human GPC3 peptides having high binding scores (>100) to HLA-A2 molecules shared the same amino acid sequences with mouse GPC3. Therefore, it is unlikely that we excluded many candidates of human GPC3-derived and HLA-A2-restricted CTL epitopes from the analysis by selecting the peptides having amino acid sequences shared between human and mouse GPC3. Furthermore, we have to consider the differences in the T cell repertoire in mice and humans. Thereby, we may miss GPC3 peptides recognized by human CTLs but not by mouse CTLs.

Considering ideal immunogenic target molecules for cancer immunotherapy, it is important to select a tumor antigen that could not be lost by tumor cells through immunoediting (28, 29). Recently, Capurro et al. reported that GPC3 is involved in the carcinogenesis and proliferation of HCC via regulation of noncanonical Wnt signals (30). Therefore, it may be possible that tumor cells cannot lose the GPC3 expression in order to continue to grow. Furthermore, according to an immunohistochemical analysis of the expression of HLA-class I molecules using newly developed specific mAb, EMR 8-5,[5] we found that almost all HCC cells expressed HLA-class I as far as we could examine (Table 2). For these reasons, we think that GPC3 is a very useful candidate as a target tumor antigen for the immunotherapy of HCC. We and others previously reported that the expression of GPC3 in HCC was detected from an early stage and the quantification of the soluble GPC3 protein in sera was useful for a diagnosis of HCC at an early stage (5, 7). As a result, GPC3-based immunotherapy might be able to prevent the appearance of HCC in patients with hepatitis B or C–based liver cirrhosis.

In this study, we found that it is possible to induce GPC3-reactive CTLs by the stimulation of PBMCs with the two major GPC3 epitopes *in vitro* in 50% of the HCC patients having an appropriate *HLA-class I* allele. However, it is necessary to investigate more patients to estimate the probability of a

successful induction of GPC3-reactive CTLs in HCC patients. We intended to know whether there was any correlation between successful induction of GPC3 peptide-reactive CTLs and prognosis or CTL infiltration into tumor tissue of these patients, therefore, we investigated the seven index cases; patients A2-10, A24-1, A24-2, A24-4, A24-9, A24-11, and A24-12, to see whether there was any correlation between successful induction of GPC3 peptide-reactive CTLs and prognosis or CTL infiltration into the tumor tissue of these patients. In three patients, A24-1, A24-4, and A24-12, who could generate GPC3 peptide-reactive CTLs, patient A24-12 recurred at 6 months after operation. In four patients, A2-10, A24-2, A24-9, and A24-11, who failed to induce GPC3-peptide-reactive CTLs, patient A24-9, whose HCC did not express GPC3, recurred at 6 months after operation, and patient A24-2 recurred at 3 months after operation and died 3 months after recurrence. These three recurred patients had extremely strong tumor invasion to the vasculature. Therefore, it was difficult to evaluate the correlation between the positive CTL response and clinical improvement at the present stage, and we have to increase the number of patients investigated and to do further statistical analyses on these relationships. In patients who could be examined for the infiltration of CD8-positive cells into their tumor specimens and for the existence of terminal deoxynucleotidyl transferase–mediated nick end labeling–positive cells in tumor tissue, patients A2-10, A24-1, A24-2, and A24-9, there was no strong correlation between the positive GPC3 peptide-reactive CTL response and for the existence of CD8-positive or terminal deoxynucleotidyl transferase–mediated nick end labeling–positive cells in the tumor tissues (data not shown). As shown in Fig. 4, we observed a regression of the tumor masses in NOD/SCID mice implanted with SK-Hep1/GPC3 and transferred i.v. with the GPC3 peptide-reactive CTLs in comparison to the mice injected with control $CD8^+$ T cells or saline alone. Although the regression of tumor growth was observed for 2 weeks after the second transfer of CTLs, the tumors began to enlarge again after that period. We thought it was important to continue the transfer of CTLs again and again to obtain continuous regression of the GPC3-expressing tumor. These data suggest that the adoptive i.v. transfer of GPC3-reactive CTLs into mice bearing $GPC3^+$ tumors was useful to inhibit tumor growth in the mouse tumor model.

In addition, it is most important to confirm the usefulness of GPC3-specific *in vivo* cancer immunotherapy in patients with HCC. Investigation of the presence of GPC3-specific CTLs in patients with melanoma are also eagerly awaited. We previously reported that DC differentiated *in vitro* from mouse embryonic stem cells transfected with the mouse *GPC3* gene (24, 31) induced protective immunity against mouse melanoma cell line B16 F10 (32). We are now preparing a translational study of GPC3-based immunotherapy to reduce the risk of recurrence in HCC patients treated surgically. We will try to use the GPC3 epitope peptides identified in this study first, whereas in the second phase, we will make a trial of the peptide-pulsed DC vaccine. We expect that GPC3-based immunotherapy may be a novel treatment strategy that could potentially help to prevent the appearance, advance, and/or recurrence of HCC and melanoma.

Acknowledgments

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Synthetic small interfering RNA targeting heat shock protein 105 induces apoptosis of various cancer cells both *in vitro* and *in vivo*

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We previously reported that heat shock protein 105 (HSP105), identified by serological analysis of a recombinant cDNA expression library (SEREX) using serum from a pancreatic cancer patient, was overexpressed in various human tumors and in the testis of adult men by immunohistochemical analysis. In the present study, to elucidate the biological function of the HSP105 protein in cancer cells, we first established NIH3T3 cells overexpressing murine HSP105 (NIH3T3-HSP105). The NIH3T3-HSP105 cells acquired resistance to apoptosis induced by heat shock or doxorubicin. The small interfering RNA (siRNA)-mediated suppression of HSP105 protein expression induced apoptosis in human cancer cells but not in fibroblasts. By a combination of siRNA introduction and doxorubicin or heat shock treatment, apoptosis was induced synergistically in a human colon cancer cell line, HCT116. *In vivo*, siRNA inoculation into the human gastric cancer cell line KATO-3 established in the flank of an NOD SCID mouse suppressed the tumor growth. This siRNA-induced apoptosis was mediated through caspases, but not the p53 tumor suppressor protein, even though the HSP105 protein was bound to wild-type p53 protein in HCT116 cells. These findings suggest that the constitutive overexpression of HSP105 in cancer cells is involved in malignant transformation by protecting tumor cells from apoptosis. HSP105 may thus be a novel target molecule for cancer therapy and a treatment regimen using synthetic siRNA to suppress the expression of HSP105 protein may provide a new strategy for cancer therapy. (*Cancer Sci* 2006; 97: 623–632)

Heat shock protein 105 (also called HSP110)[1] is a stress protein belonging to the HSP105/110 family that is expressed constitutively in most tissues at low levels, whereas HSP105 mRNA is expressed at high levels in mouse and rat brain. At the protein level, high expression levels have been reported only in the brain of mice.[1–4] Like other heat shock proteins, HSP105 plays an important role as a chaperone under physiological conditions. HSP105 is induced by various stressors and plays an important role in protecting cells from the cytotoxic effects mediated by such stressors. HSP105 is composed of an ATP-binding domain, a β-sheet, a loop and α-helical domains similar to those observed in the HSP70 family of proteins, and it binds to non-native protein substrates, thereby preventing the aggregation of denatured protein through an interaction with the β-sheet domain of HSP105.[2,5–7]

The rat neuronal cell line PC12 transfected stably with murine *HSP105* exhibited resistance to caspase-mediated apoptosis induced by stressors.[8] In a spinal and bulbar muscular atrophy model, the transient expression of tAR containing an expanded polyglutamine tract caused aggregates of polyglutamine in COS-7 and SK-N-SH cells and then induced the cells to undergo apoptosis. In contrast, in cells cotransfected with *tAR* and *HSP105*, both the aggregation of polyglutamine and the degree of it's cell toxicity decreased.[9] In contrast, HSP105 has demonstrated apoptosis-enhancing activity during murine embryogenesis.[10–13] These observations suggest that HSP105 is involved in the regulation of apoptosis.

We previously reported that HSP105 is overexpressed in various human tumors, including colon cancer cells but not colorectal adenomas, thus suggesting that the overexpression of HSP105 is a late event in the colorectal adenoma–carcinoma sequence.[14,15] Recent studies have also demonstrated that the expression level of HSP105 is elevated in highly metastatic colon cancer cell lines, and is correlated with advanced clinical stages and positive lymph node involvement.[16]

RNA interference is used widely for manipulating biological systems, and has also been utilized successfully as a therapeutic material in experimental animals.[17–19] Synthetic siRNA strongly inhibits the expression of target proteins when they are transfected with cationic liposomes, which are thought to be safer than viral vectors for human therapy. The local injection of synthetic siRNA against VEGF [20] or sphingosine 1-phosphate receptor-1[21] into established tumors has been reported recently to suppress angiogenesis and tumor growth.

The role of HSP105 in cancer cells has yet to be elucidated. In the present study, we first transfected the *HSP105* gene

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Abbreviations: CML, chronic myelocytic leukemia; DMEM, Dulbecco's modified Eagle's medium; ER, endoplasmic reticulum; FCS, fetal calf serum; FITC, fluorescein-isothiocyanate; GFP, green fluorescent protein; HBSS, Hanks' balanced salt solution; HSP70, heat shock protein 70; HSP105, heat shock protein 105; IRES, internal ribosomal entry site; NIH3T3-HSP105, NIH3T3 cells overexpressing murine HSP105; PARP, poly ADP-ribose polymerase; PBS, phosphate-buffered saline; PI, propidium iodide; RT-PCR, reverse transcription–polymerase chain reaction; SEREX, serological analysis of a recombinant cDNA expression library; siRNA, small interfering RNA; tAR, truncated androgen receptor; VEGF, vascular endothelial growth factor.

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into NIH3T3 cells to examine the function of those cells, while also treating those transfectants with several stressors. To investigate whether the suppression of HSP105 expression affects the growth of cancer cells, we then introduced synthetic siRNA specific to HSP105 into several human cancer cell lines both *in vitro* and *in vivo*. We herein report that HSP105 has an anti-apoptotic function and that an overexpression of HSP105 is essential for cancer cells to survive.

Materials and Methods

Cell lines and culture

The human pancreatic cancer cell line PK8 and the murine fibroblast cell line NIH3T3 were kindly provided by the Cell Resource Center for Biomedical Research Institute of Development, Aging and Cancer, Tohoku University (Sendai, Japan). The human hepatoma cell line SK-Hep1, human colon cancer cell line SW620, and human gastric cancer cell lines KATO-3 and MKN28 were kindly provided by Dr K. Itoh, Kurume University (Kurume, Japan). The human colon cancer cell line HCT116 was kindly provided by Dr B. Vogelstein, Johns Hopkins University (Baltimore, MD, USA). Primary normal fibroblast strains Turu and Mori were kindly provided by Dr M. Yamaizumi, Kumamoto University (Kumamoto, Japan).

NIH3T3, HCT116, SW620, Turu and Mori were all cultured *in vitro* in DMEM, and SK-Hep1, PK8, KATO-3 and MKN28 were cultured in RPMI medium supplemented with 10% FCS and 1% penicillin and streptomycin in a 5% CO_2 atmosphere at 37°C.

Mice

C57BL/6 NOD SCID mice were kindly provided by Dr S. Okada, Kumamoto University. The mice were maintained at the Center for Animal Resources and Development of Kumamoto University and they were handled in accordance with the animal care policy of Kumamoto University.

Plasmid construction and transfection

To obtain pCAGGS-IRES-neo-R, a DNA fragment containing an IRES, the neomycin-resistance gene *neo-R* was inserted into the mammalian expression vector pCAGGS. A cDNA fragment encoding the murine HSP105 protein was inserted into pCAGGS-IRES-neo-R. Cell transfection was carried out using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's recommendations in six-well multiplates. At 48 h after transfection, the cells were replated and were then cultured in the presence of an appropriate concentration of G418 for 1 week.

RT-PCR analysis

Total RNA was isolated using either TRIZOL reagent (Gibco BRL, Rockville, MD, USA) or RNeasy spin column kits (Qiagen, Valencia, CA, USA). Total RNA from normal human colon tissue was purchased from Clontech (Palo Alto, CA, USA). RT-PCR analysis was carried out as described previously.[22] Briefly, 1 μg of total RNA was converted into cDNA in 20 μL of reaction buffer. Each PCR regime involved an initial denaturation step at 94°C for 5 min followed by 24–33 cycles for each type of cDNA. All samples were then processed at 94°C for 1 min, 58°C for 1 min and 72°C for 1 min

The primer sequences were as follows: *HSP105* forward 5′-ATGAAGTGATGGAATGGATG-3′ and reverse 5′-TTTGGTTTCGGTTGTGTTAC; *NOXA* forward 5′-AGATGCCTGGGAAGAAG-3′ and reverse 5′-AGTCCCCTCATGCAAGT-3′; *PUMA* forward 5′-TGTAGAGGAGACAGGAATCCACGG-3′ and reverse, 5′-AGGCACCTAATTGGGCTCCATCTC-3′; *Bax* forward 5′-AGCGGCGGTGATGGACGGGTC-3′ and reverse 5′-TCCAAGGCAGCTGGGGCCTCA-3′; and *p53* forward 5′-CCATGGCCATCTACAAGCAG-3′ and reverse 5′-AGGGTGAAATATTCTCC-3′. PCR products were visualized by ethidium bromide staining after separation on a 2% agarose gel.

Detection of cell apoptosis

Cells in the early phase of apoptosis were detected by staining with annexin V, which binds to a phosphatidyl serine marker specific for the early phase of apoptosis, using an annexin V-FITC apoptosis detection kit (BioVision, Mountain View, CA, USA). The NIH3T3-mock cells and NIH3T3-HSP105 cells were incubated at 45°C for 90 min or with 200 ng/mL doxorubicin. The cancer cell lines were treated with either 100 nM or 200 nM siRNA, with 100 nM siRNA and 200 ng/mL doxorubicin or with 100 nM siRNA and heat shock (45°C, 30 min). The cells were harvested at the times indicated and were then stained with FITC-conjugated annexin V for flow cytometric analysis according to the manufacturer's recommendations. In the caspase inhibition assay, 100 μM Z-VAD-FMK (Sigma, St Louis, MO, USA) was added 1 h before siRNA transfection.

To detect the late phase of apoptosis, DNA fragmentation in the cells was evaluated by staining with PI and flow cytometric analysis as follows: NIH3T3-mock cells and NIH3T3-HSP105 cells treated with doxorubicin were collected by tripsinization, washed with PBS, fixed in cold 70% ethanol, and stored at −20°C until staining. After fixation the cells were washed in PBS and incubated with 100 μg/mL of RNaseA for 30 min at room temperature, before staining with 25 μg/mL of PI. Flow cytometry was carried out using a FACScan flow cytometer (BD Biosciences, San Jose, CA, USA) and the data were analyzed using CellQuest software (BD Biosciences).

Immunoprecipitation and western blot analysis

The cell samples were lysed in appropriate amounts of lysing buffer (200 mM NaCl, 20 mM Tris [pH 7.4], 1% Nonidet P-40, 1 mM sodium orthovanadate [WAKO, Osaka, Japan], 10% glycerol, plus one protease inhibitor tablet [Roche Applied Sciences, Penzberg, Germany]). Hsp105 and p53 were immunoprecipitated with rabbit polyclonal anti-HSP105 antibody and mouse monoclonal anti-p53 antibody (DO-1; Santa Cruz Biotechnology, Santa Cruz, CA, USA), respectively, together with protein A beads (Pierce, Rockford, IL, USA). The proteins were analyzed using 7% or 10% sodium dodecylsulfate–polyacrylamide gel electrophoresis and were then transferred onto a nitrocellulose membrane. HSP105 was blotted with the above-described antibody and PARP was blotted with rabbit polyclonal anti-PARP antibody (Santa Cruz Biotechnology). p53 was blotted with DO-1 or DO-1 and labeled with biotin using the Mini-biotin-XX Protein Labeling Kit (Molecular Probes, Eugene, OR, USA). Phospho-p53 (Ser46) and phospho-p53 (Ser15) were blotted with rabbit polyclonal antibody specific to phospho-p53 (Ser46) and mouse monoclonal antibody specific to phospho-p53 (Ser15) (New England Biolabs, Beverly, MA, USA), respectively, and then with horseradish peroxidase-conjugated rabbit antimouse IgG or donkey antirabbit IgG

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(Amersham Biosciences, Piscataway, NJ, USA), respectively, as secondary antibodies. The bands were visualized by enhanced chemiluminescence (Amersham Biosciences).

siRNA and *in vivo* siRNA treatment

The siRNA duplexes were purchased from Dharmacon Research (HSP105-siRNA and luciferase; Lafayette, CO, USA), Qiagen (GFP) and Invitrogen (HSP105-siRNA-2). The siRNA sequences used were as follows: HSP105, UUGGCUGCAACUCCG-AUUGTT; HSP105-siRNA-2, UGUACAUUACCUUUAUU-CCAACUCC; luciferase, CGUACGCGGAAUACUUCGATT; and GFP, GCAAGCUGACCCUGAAGUUCA. The transfection of siRNA oligonucleotides was carried out using oligofectamine (Invitrogen) according to the manufacturer's recommendations in a six-well plate.

KATO-3 cells (2×10^6) were suspended in 100 μL of HBSS solution (Gibco, Langley, OK, USA), injected subcutaneously into the dorsal skin of NOD SCID mice and were then allowed to grow. After 10 days, siRNA solutions were injected locally into tumors every third day. siRNA solutions were prepared by incubating 1 nmol of siRNA and 20 μL of oligofectamine in 5% glucose solution for 15 min at room temperature. The tumor volume was calculated using the equation

$$V = (L \times W^2) \times 0.5,$$

where V = volume, L = length and W = width.

Statistical analysis

The two-tailed Student's *t*-test was used to determine the statistical significance of differences in the percentage of the cell fraction evaluated by flow cytometric analysis, and in tumor size between the treatment groups. A value of $P < 0.05$ was considered to be significant. Statistical analyses were carried out using the StatView 5.0 software package (Abacus Concepts, Calabasas, CA, USA).

Results

NIH3T3-HSP105 cells acquired resistance to apoptosis induced by stressors

To elucidate the biological function of HSP105 in cancer cells, we first transfected the murine HSP105 expression vectors or an empty vector into murine embryonal fibroblast NIH3T3 cells, which are known to express smaller amounts of HSP105 than cancer cells. Subsequently, NIH3T3-mock cell lines and NIH3T3-HSP105 cell lines overexpressing HSP105 protein were established after selecting cells with G418 (Fig. 1A). There was no difference in either the morphology or the proliferative characteristics between these two cell lines cultured in DMEM supplemented with 1, 5 or 10% FCS (data not shown).

In a previous study, Hatayama *et al.* reported that rat neuronal cells overexpresing murine HSP105 were able to avoid undergoing apoptosis induced by several kinds of stressors.[8] We therefore examined the anti-apoptotic effects of HSP105 overexpression in NIH3T3-HSP105 cells. We exposed NIH3T3-mock cells and NIH3T3-HSP105 cells to heat shock at 45°C for 90 min and then detected annexin V-positive early apoptotic cells by flow cytometric analysis. The proportion of annexin V-positive NIH3T3-HSP105 cells was smaller than that of NIH3T3-mock cells ($P < 0.01$) (Fig. 1B).

We further treated these two cell lines with 200 ng/mL doxorubicin, which is known to be an inducer of apoptosis. After incubating NIH3T3-mock cells with doxorubicin for 12 h, annexin V-positive cells were detected by flow cytometric analysis. The number of annexin V-positive cells increased gradually thereafter, and almost all cells were stained with annexin V by 24 h after the treatment. However, even after 36 h incubation of cells with doxorubicin, only a small fraction (<18%) of NIH3T3-HSP105 cells were stained with annexin V. The difference in the number of annexin V-positive cells at 48 h after treatment was statistically significant ($P < 0.001$) between NIH3T3-mock and NIH3T3-HSP105 (Fig. 1C).

We next examined the DNA fragmentation of those two cell lines, which is observed during the late phase of apoptosis by staining with PI. The percentage of NIH3T3-HSP105 cells exhibiting DNA fragmentation was less than that of NIH3T3-mock cells, and the difference was statistically significant at both 36 h ($P < 0.001$) and 48 h ($P < 0.0001$) after doxorubicin treatment (Fig. 1D). These data suggest that HSP105 has an anti-apoptotic effect against apoptosis induced by heat shock and doxorubicin.

HSP105 siRNA induced various human cancer cell lines to undergo apoptosis

We previously reported that HSP105 protein is overexpressed in various human tumors and in the testis of normal adult men, but not in colon adenoma, by immunohistochemical analysis.[15] We thus examined the function of HSP105 protein in cancer cells by downregulating *HSP105* gene expression with siRNA. We used two human colon cancer cell lines, HCT116 and SW620, in which the expression of HSP105 mRNA was significantly elevated in comparison to that in normal human colon epithelium (Fig. 2A). At approximately 24 h after transfection, the adherent HCT116 cells treated with HSP105 siRNA started peeling off, and almost all of the cells had peeled off and were observed as dying cells at 48 h after transfection. In contrast, most of the HCT116 cells treated with luciferase siRNA proliferated normally (Fig. 2B). In a western blot analysis of HCT116 cells, the expression of HSP105 protein was markedly suppressed after treatment with two different HSP105-specific siRNA (HSP105-siRNA and HSP105-siRNA-2), and those cells were significantly stained with FITC–annexin V based on flow cytometric analysis (Fig. 2B,C).

Similarly, in SW620 cells after treatment with HSP105-siRNA, the expression of HSP105 protein was suppressed and a significant number of annexin V-positive cells were detected in proportion to the concentration of siRNA administered to the SW620 cells. The siRNA effect was more notable at 24 h than at 48 h after siRNA treatment in SW620 (Fig. 2C). This finding may be associated with the fact that the expression of HSP105 in SW620 was much higher than any of the other cancer cell lines tested (Fig. 3A). In addition, in other cancer cell lines, including the human hepatoma cell line SK-Hep1, human pancreatic cancer cell line PK8, and two human gastric cancer cell lines KATO-3 and MKN28, HSP105 protein expression was suppressed and all of these cell lines underwent apoptosis at 48 h after transfection of HSP105 siRNA (Fig. 2D). These data indicate that the suppression of HSP105 protein expression by siRNA can thus induce human cancer cells originating from various tissues to undergo apoptosis.

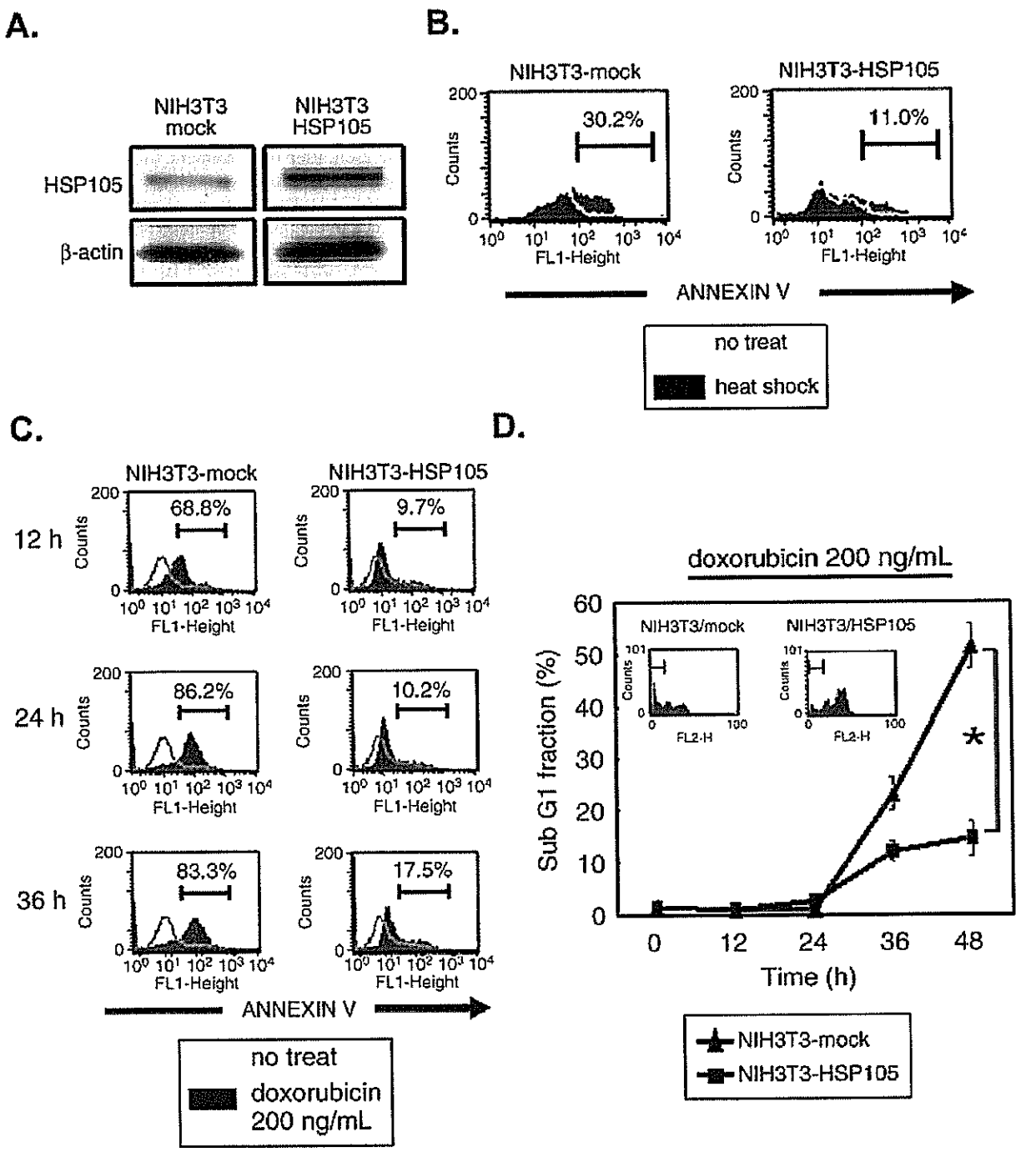

Fig. 1. Anti-apoptotic effect of HSP105 overexpression on NIH3T3 cells. (A) Western blot analysis of HSP105 expressed in NIH3T3 cells transfected with pCAGGS-IRES-neo-R or pCAGGS-IRES-neo-R-HSP105. β-Actin is shown as a control for the equal loading of protein. (B–D) Flow cytometric analyses of apoptotic cells. NIH3T3-mock cells and NIH3T3-HSP105 cells were treated at 45°C for 90 min (B) or with 200 ng/mL doxorubicin (C,D). To detect early apoptotic cells, the cells were harvested at the times indicated, stained with fluorescein-isothiocyanate–annexin V and analyzed by flow cytometry (C,D). These data are representative of at least three independent experiments. Percentages shown in the panel indicate percentage of annexin V-positive cells in heat-treated cells (B) and doxorubicin-treated cells (C). (D) Detection of DNA fragmentation by propidium iodide staining. The percentage of sub-G_1 fractions at the times indicated is shown, and the representative data of flow cytometric analysis at 48 h is shown in the panel. Data are mean ± SD ($n = 3$). The asterisk indicates that the difference in the percentages of the sub-G_1 fractions is statistically significant between the two values indicated by lines ($P < 0.001$).

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HSP105 siRNA did not induce human fibroblasts to undergo apoptosis

HSP105 is a stress-induced protein that is usually expressed ubiquitously at low levels, except in the brain and testis. To investigate the effect of HSP105 siRNA on normal cells, we applied HSP105 siRNA to human fibroblasts, Turu and Mori, generated from healthy donors. In western blot analysis, the level of HSP105 protein expressed in the Turu and Mori cells was lower than in the cancer cell lines (Fig. 3A). The expression of HSP105 protein was suppressed with HSP105 siRNA treatment, as shown in Fig. 3B,C, and the shape of fibroblast cells changed from fusiform to a round shape, but those cells were not induced to undergo apoptosis. Approximately 10 days after HSP105 siRNA transfection, both the expression of HSP105 protein and cell shape were restored (data not shown). These results indicate that the suppressive effect of HSP105 siRNA on the HSP105 expression is transient and reversible, and that the marked reduction of HSP105 protein does not have any harmful effect on normal fibroblasts under non-stressed conditions.

HSP105 siRNA treatment suppressed the growth of tumors overexpressing HSP105 *in vivo*

Small interfering RNA treatment using liposomes suppresses tumor growth *in vivo*.[21,23] To elucidate the effects of HSP105 siRNA on growing tumors *in vivo*, we injected either HSP105 siRNA or irrelevant siRNA locally into 5–7 mm KATO-3 tumors transplanted in NOD SCID mice every 3 days. As shown in Fig. 4, HSP105 siRNA suppressed tumor growth significantly in comparison to the irrelevant siRNA ($P < 0.01$). On day 15 after the first injection of HSP105 siRNA into tumors, the volumes of the tumors remained almost the same as those on day 0. During this observation period, neither abnormal behaviors nor neurological abnormalities were observed in these HSP105 siRNA-treated mice, and the expression of HSP105 protein in the brain was not suppressed by immunohistochemical analysis (data not shown).

Synergistic effects of siRNA and doxorubicin or heat shock on the *in vitro* induction of apoptosis in tumor cells

For the treatment of cancer patients with advanced, unresectable or recurrent focus, chemotherapy, radiation and other therapies are applied singly or in combination. To investigate the feasibility of combined treatment of tumor cells with HSP105 siRNA and other treatments, we treated HCT116 cells with HSP105 siRNA and either heat stress or doxorubicin. At 12 h after siRNA transfection into HCT116 cells, we added 200 ng/mL doxorubicin or treated the cells with heat shock at 45°C for 30 min, and detected the presence of apoptotic cells by staining with annexin V after 36 or 24 h incubation, respectively. As shown in Fig. 5, both combined treatments synergistically induced HCT116 cells to undergo apoptosis in comparison with the single treatment ($P < 0.001$).

Apoptosis induced by HSP105 siRNA was dependent on the caspase cascade but not on the p53 pathway

To investigate whether caspases are involved in apoptosis induced with siRNA, we examined PARP cleavage by western blot analysis using HCT116 cells with wild-type p53. PARP, a nuclear enzyme involved in DNA repair, is a well-known substrate for caspase-3, and is cleaved from a 112-kDa protein to an 85-kDa protein by the activation of caspase-3. As shown in Fig. 6A, cleavage of PARP was observed in those cells transfected with HSP105 siRNA. Moreover, this cleavage was completely inhibited by adding a pan-caspase inhibitor, Z-VAD-FMK. As a result, the apoptosis induced by HSP105 siRNA was also inhibited by Z-VAD-FMK.

We next examined whether the p53 tumor suppressor protein is involved in HSP105 siRNA-induced apoptosis. The p53 protein is located upstream of the caspase cascade and is associated with heat shock proteins such as HSP70 and HSP90.[24] As shown in Fig. 6C, the HSP105 and p53 proteins were coimmunoprecipitated with anti-HSP105 antibodies and the DO-1 in non-treated HCT116 cells. These results indicate that a proportion of HSP105 protein is bound to p53 protein in non-treated HCT116 cells. Furthermore, the expression of p53 protein decreased with HSP105 siRNA treatment at the post-transcriptional level (the mRNA expression of p53 was not suppressed), and p53 protein was not phosphorylated at serine 15 or serine 46 by this treatment (Fig. 6D,E). Furthermore, to confirm the suppression of p53 transcriptional activity, the mRNA expression of Bax, NOXA and PUMA, which are the transcriptional targets of p53-mediated apoptosis, were assessed by RT-PCR and found to be suppressed (Fig. 6E). These data suggest that HSP105 protein is thus associated with wild-type p53 protein in HCT116 cells under non-stress conditions, and HSP105 siRNA-induced apoptosis is dependent on caspases but independent of the p53-mediated apoptosis pathway.

Discussion

In the present study, we obtained the following results: (1) HSP105 protein protects tumor cells from apoptosis; (2) constitutive overexpression of HSP105 protein is essential for the survival of various kinds of cancer cells; and (3) apoptosis induced with HSP105 siRNA treatment is dependent on caspases but not p53.

Recent studies, including ours, have shown that HSP105 is overexpressed in various human tumors and that HSP105 is thus speculated to be involved in both tumorigenesis and protection of cells from apoptosis.[8,14,15] Our data obtained using NIH3T3-HSP105 cells are consistent with the findings of a recent study on neuronal PC12 cells in which the overexpression of HSP105 did not affect the growth rate of PC12 cells, but the apoptosis induced by stressors was inhibited in those cells.[8] These data indicate that HSP105 is involved in tumorigenesis through protection of cells against apoptosis.

Among the heat shock proteins, HSP70 is well characterized and HSP105 shares functional properties with HSP70. HSP70 also inhibits apoptosis induced by various stimuli.[25–27] Furthermore, HSP70 is also overexpressed in human breast cancer.[28–31] One difference in function between HSP70 and HSP105 is that HSP105 has an increased capacity to bind to denatured polypeptides in comparison to HSP70.[32] In addition, HSP105 suppresses the aggregation of denatured proteins under stress conditions in the presence of ADP, whereas HSP70 suppresses it in the presence of ATP.[7] In breast cancer cell lines, inhibition of HSP70 expression by antisense cDNA causes those cells to undergo apoptosis, and this action is

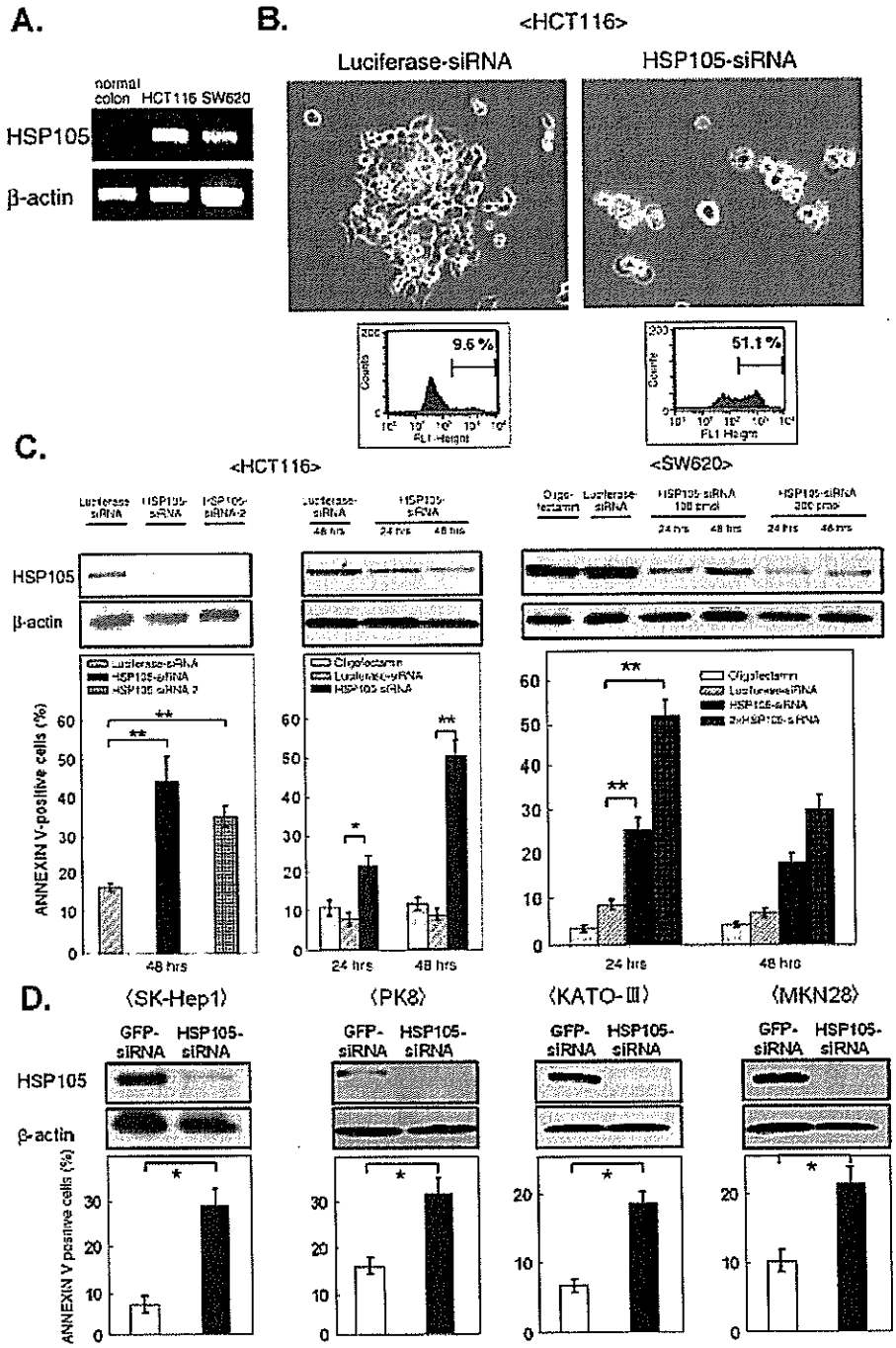

Fig. 2. Small interfering RNA (siRNA)-mediated inhibition of HSP105 expression enhanced the apoptotic cell death of human cancer cell lines. (A) Reverse transcription–polymerase chain reaction analysis of HSP105 mRNA expression in normal human colon epithelium, and in human cancer cell lines HCT116 and SW620. (B) Light microscopic pictures of HCT116 cells introduced with or without siRNA and representative flow cytometric analysis data of apoptotic cells stained with annexin V at 48 h after transfection. (C,D) Western blot analysis of HSP105 protein expression and flow cytometric analysis of apoptotic cells. HCT116 cells and SW620 cells were treated with oligofectamine, control siRNA, HSP105-siRNA (100 nM or 200 nM) or HSP105-siRNA-2 (B,C). (C) For western blot analysis, the cells were lysed at 24 or 48 h after transfection and analyzed. β-Actin is shown as a quantitative control. For flow cytometric analysis, cells were harvested at 24 or 48 h after transfection and then stained with fluorescein-isothiocyanate–annexin V and analyzed by flow cytometry. (D) Western blot analysis of HSP105 protein expression in cancer cell lines including SK-Hep1, PK8, KATO-3 and MKN28, and flow cytometric analysis of apoptotic cells at 48 h after transfection with 100 nM green fluorescent protein siRNA (□) or HSP105 siRNA (■). Data are the mean of three independent experiments ± SD. The asterisks indicate that the differences in the percentages of annexin V-positive cells are statistically significant between the two values indicated by lines (*$P < 0.01$; **$P < 0.001$).

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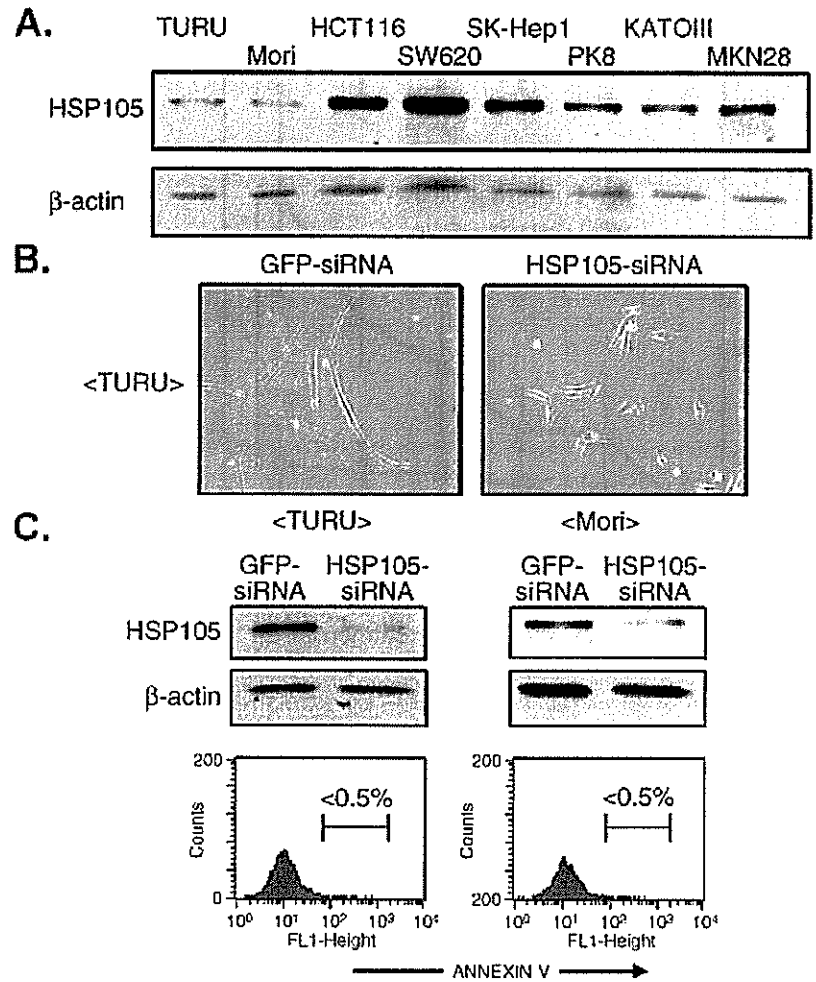

Fig. 3. No apoptosis-inducing effects of HSP105 small interfering RNA (siRNA) on human fibroblasts. (A) Western blot analysis of HSP105. The lysates of human fibroblasts Turu and Mori, and human cancer cell lines HCT116, SW620, SK-Hep1, PK8, KATO-3 and MKN28 were used and blotted with an anti-HSP105 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

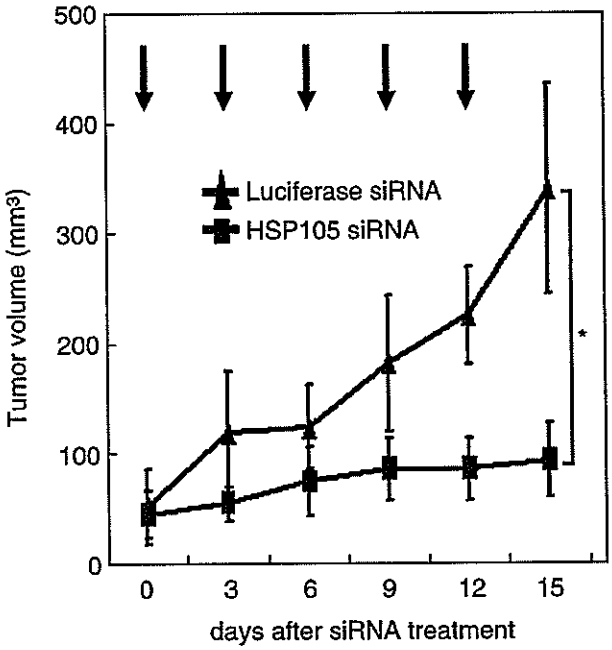

Fig. 4. The inhibitory effect of HSP105 small interfering RNA (siRNA) on the growth of established tumors in mice. (A) KATO-3 cells (2×10^6) were implanted subcutaneously into the dorsal skin of NOD SCID mice to establish growing tumors, and siRNA was injected into the tumors every 3 days (indicated 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 ($P < 0.01$).

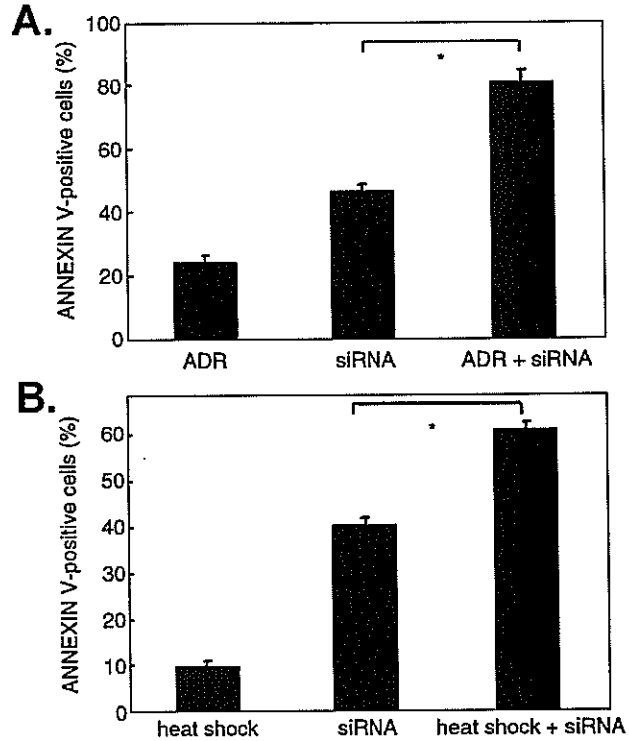

Fig. 5. The synergistic effect of HSP105 small interfering RNA (siRNA) with doxorubicin or heat shock regarding the induction of apoptotic cell death in HCT116 cells. At 12 h after transfection with 100 nM siRNA, the cells were incubated with 200 ng/mL doxorubicin (A) or treated with heat shock at 45°C 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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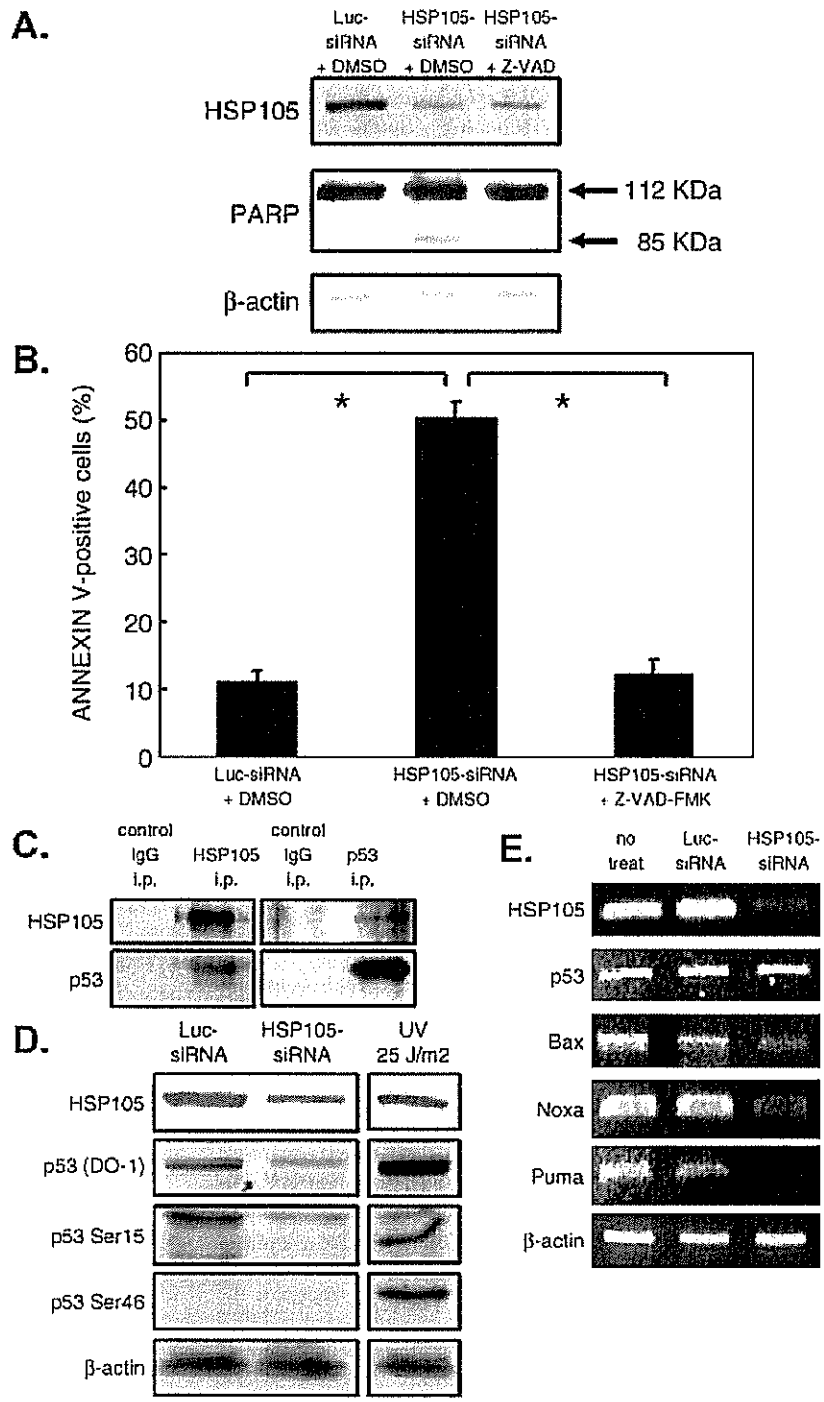

Fig. 6. Caspase-dependent and p53-independent induction of apoptosis in HCT116 cells administered HSP105 small interfering RNA (siRNA). (A) Western blot analysis of poly ADP-ribose polymerase (PARP) expression and (B) flow cytometric analysis of apoptosis induced in HCT116 cells transfected with siRNA 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 $I\text{-}A^b$ 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

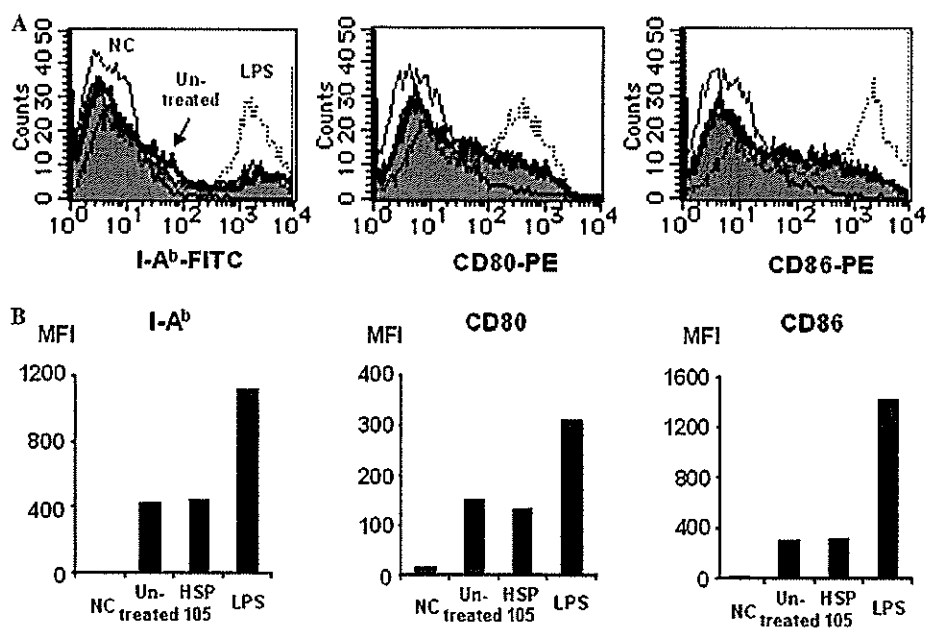

Fig. 1. Expression levels of cell surface $I\text{-}A^b$, CD80, and CD86 on BM-DCs, HSP105-pulsed BM-DCs, and LPS-pulsed BM-DCs were analyzed by flow cytometric analysis. BM-DCs were pulsed with 2 μg/ml HSP105, 1 μg/ml LPS or left untreated for 16 h. (A) The expression levels in HSP105-pulsed BM-DCs (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 $I\text{-}A^b$, CD80, and CD86 staining in the cells. The results are representative of three independent experiments with similar results.