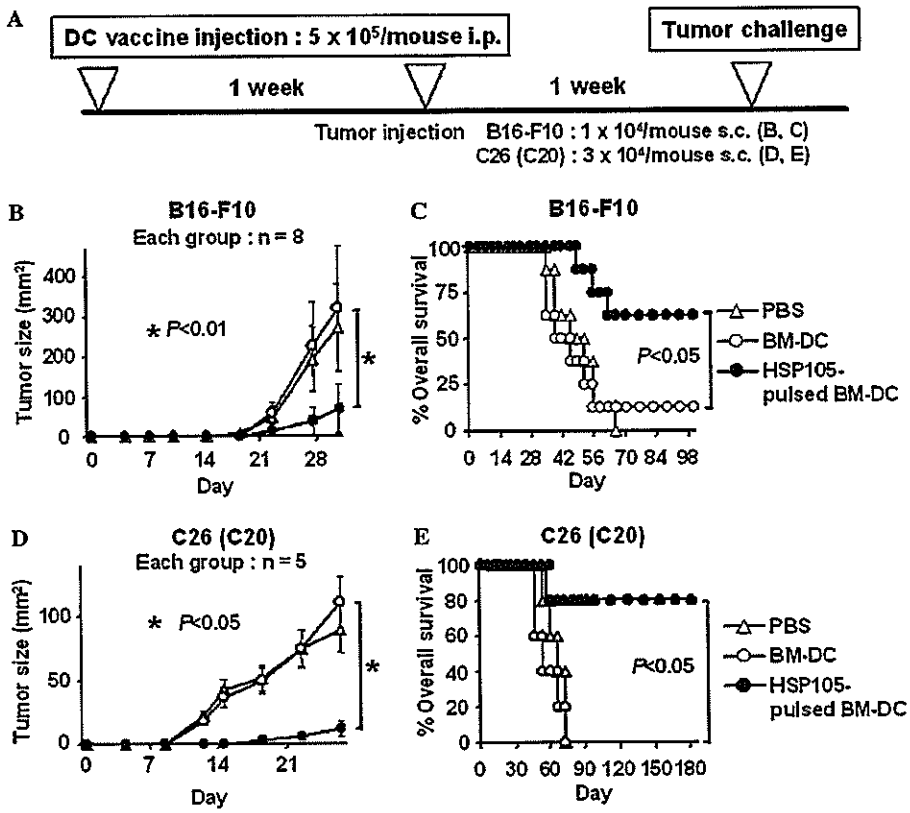

Fig. 2. Protection against tumor growth of B16-F10 and C26 (C20) cells by immunization with HSP105-pulsed BM-DC vaccine. (A) Protocol of the vaccination. The mice were immunized with PBS, BM-DC alone, and HSP105-pulsed BM-DC on 14 and 7 days before the tumor challenge. Seven days after the second immunization, 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

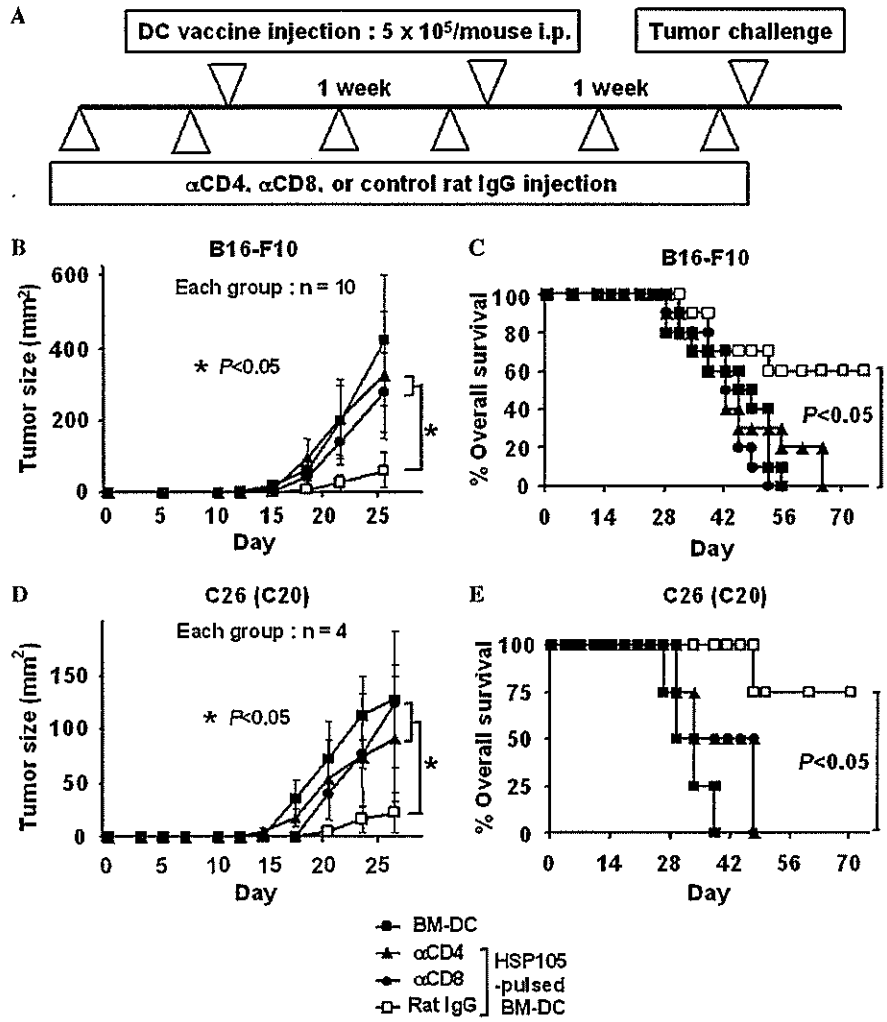

Fig. 3. Both $CD4^+$ and $CD8^+$ T cells are involved in the antitumor immunity elicited by the HSP105-pulsed DC vaccine. (A) Protocol for the vaccination and the depletion of T cells. C57BL/6 mice and BALB/c mice were challenged s.c. with B16-F10 cells and C26 (C20) cells, respectively. (B,D) The tumor 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

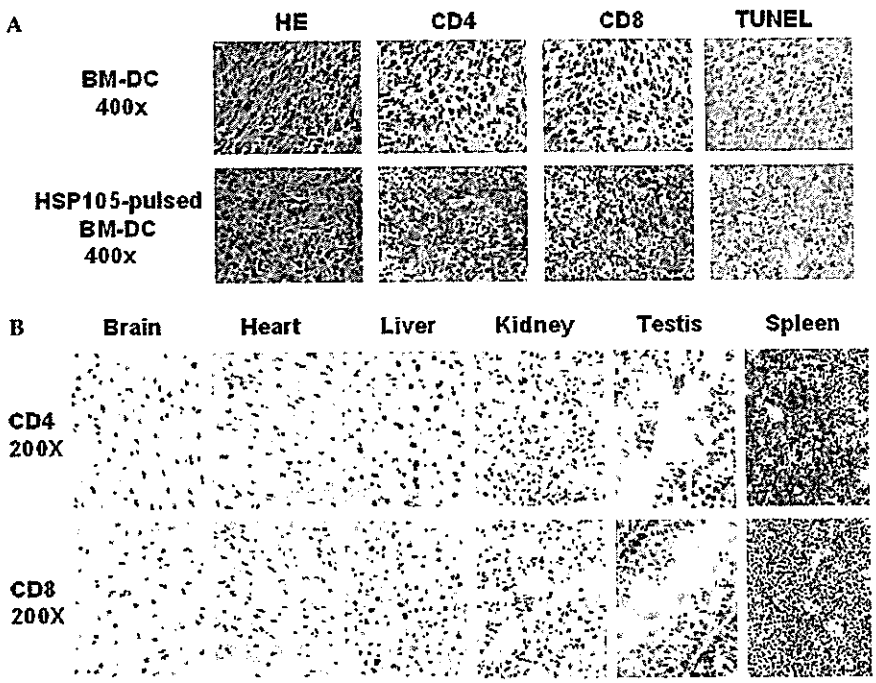

Fig. 4. Vaccination of HSP105-pulsed BM-DCs induced infiltrations of both $CD4^+$ T cells and $CD8^+$ T cells into C26 (C20) tumor and induced the apoptosis of C26 (C20) tumor cells. (A) C26 tumors removed from the mice vaccinated with BM-DCs or HSP105-pulsed BM-DCs were analyzed using immunohistochemical 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

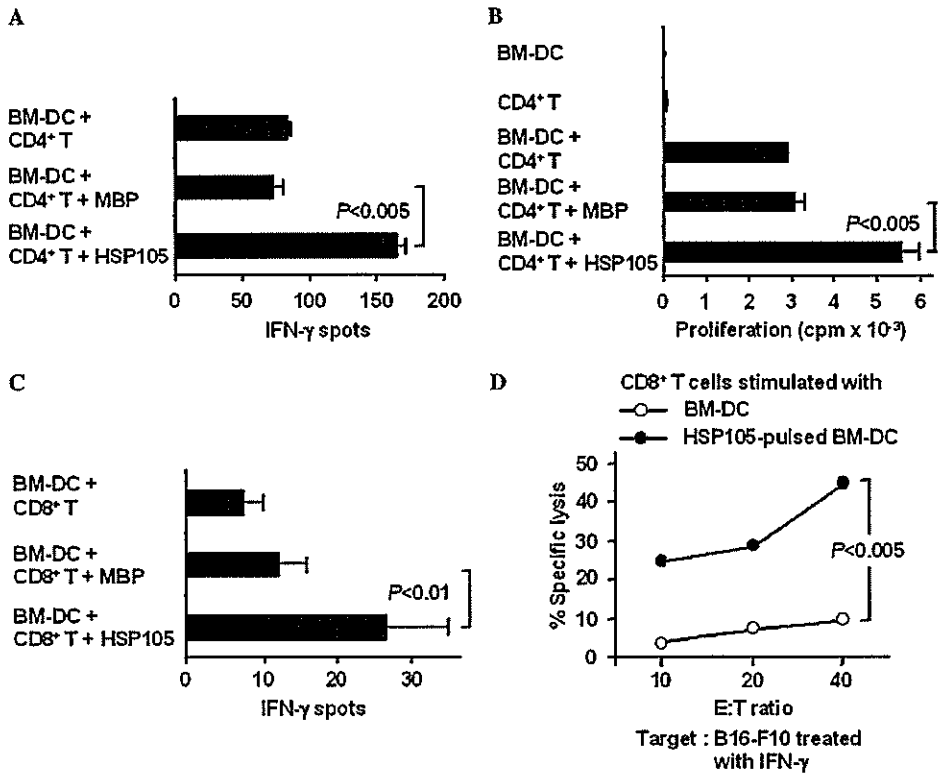

Fig. 5. Induction of HSP105-specific $CD4^+$ T cells and $CD8^+$ T cells by stimulation with HSP105-pulsed BM-DCs. (A) An ELISPOT assay for IFN-γ production by $CD4^+$ T cell lines stimulated with HSP105 protein-pulsed BM-DCs. $CD4^+$ T cells derived from the mice vaccinated with HSP105-pulsed BM-DC 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.

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Embryonic Stem Cell–Derived Dendritic Cells Expressing Glypican-3, a Recently Identified Oncofetal Antigen, Induce Protective Immunity against Highly Metastatic Mouse Melanoma, B16-F10

Yutaka Motomura,[1] Satoru Senju,[1] Tetsuya Nakatsura,[1] Hidetake Matsuyoshi,[1] Shinya Hirata,[1] Mikio Monji,[1] Hiroyuki Komori,[1] Daiki Fukuma,[1] Hideo Baba,[2] and Yasuharu Nishimura[1]

Departments of [1]Immunogenetics and [2]Gastroenterological Surgery, Graduate School of Medical Sciences, Kumamoto University, Kumamoto, Japan

Abstract

We have recently established a method to generate dendritic cells from mouse embryonic stem cells. By introducing exogenous genes into embryonic stem cells and subsequently inducing differentiation to dendritic cells (ES-DC), we can now readily generate transfectant ES-DC expressing the transgenes. A previous study revealed that the transfer of genetically modified ES-DC expressing a model antigen, ovalbumin, protected the recipient mice from a challenge with an ovalbumin-expressing tumor. In the present study, we examined the capacity of ES-DC expressing mouse homologue of human glypican-3, a recently identified oncofetal antigen expressed in human melanoma and hepatocellular carcinoma, to elicit protective immunity against glypican-3-expressing mouse tumors. CTLs specific to multiple glypican-3 epitopes were primed by the *in vivo* transfer of glypican-3-transfectant ES-DC (ES-DC-GPC3). The transfer of ES-DC-GPC3 protected the recipient mice from subsequent challenge with B16-F10 melanoma, naturally expressing glypican-3, and with glypican-3-transfectant MCA205 sarcoma. The treatment with ES-DC-GPC3 was also highly effective against i.v. injected B16-F10. No harmful side effects, such as autoimmunity, were observed for these treatments. The depletion experiments and immunohistochemical analyses suggest that both $CD8^+$ and $CD4^+$ T cells contributed to the observed antitumor effect. In conclusion, the usefulness of glypican-3 as a target antigen for antimelanoma immunotherapy was thus shown in the mouse model using the ES-DC system. Human dendritic cells expressing glypican-3 would be a promising means for therapy of melanoma and hepatocellular carcinoma. (Cancer Res 2006; 66(4): 2414-22)

Introduction

To establish effective immunotherapy for cancer, it is absolutely imperative to identify ideal tumor-specific antigens as targets of antitumor immunotherapy. In addition, the development of the methods to direct immune responses toward the antigens is essential. The manipulation of dendritic cells, specialized antigen-presenting cells, is one of the promising strategies to improve the efficacy of immunotherapy for cancer (1). Currently, numerous reports have shown that dendritic cells loaded with dead tumor cells, tumor cell lysates, tumor antigenic proteins, or peptides can induce immunity and clinical responses (2–5). However, these vaccines often induce a weak immune response that is insufficient for clinical therapy because many tumor antigens are self-antigens against which the immune system has acquired tolerance (6, 7). For loading tumor antigens to dendritic cells for anticancer immunotherapy, the gene-based antigen expression by dendritic cells is considered to be superior to loading antigen as a peptide, protein, or tumor cell lysate (8). For the efficient gene transfer to dendritic cells, the use of virus-based vectors is required because dendritic cells is relatively unsuitable for genetic modification. Clinical trials using dendritic cells genetically modified with virus vectors (e.g., monocyte-derived dendritic cells introduced with adenovirus vectors encoding for tumor antigens) are now under way (9–11). Considering the broader medical applications of this method, the drawbacks of genetic modification with virus vectors include the potential risk accompanying the use of virus vectors and legal restrictions related to it. As a result, the development of safer and more efficient means is considered to be desirable.

We recently established a novel method for the genetic modification of dendritic cells (12). In this method, we generated dendritic cells from mouse embryonic stem cells by *in vitro* differentiation. The levels of expression of MHC molecules and costimulatory molecules, CD80 and CD86, in embryonic stem cell–derived dendritic cells (ES-DC) were comparable with those of bone marrow–derived dendritic cells (BM-DC; ref. 12). The capacity of ES-DC to simulate T cells was comparable with that of dendritic cells generated *in vitro* from BM-DC. We can readily generate genetically modified ES-DC by introducing expression vectors into embryonic stem cells and the subsequent induction of their differentiation into ES-DC (13, 14). The transfection of embryonic stem cells can be done with electroporation using plasmid vectors, and the use of virus-based vectors is not necessary. Once a proper embryonic stem cell transfectant clone is established, it then serves as an infinite source for genetically modified dendritic cells. In a previous study, we showed that the *in vivo* transfer of ES-DC expressing a model tumor antigen, ovalbumin, potently primed ovalbumin-specific CTLs, thereby eliciting a protective effect against ovalbumin-expressing tumor cells (13).

Many of the genes or gene families encoding many cancer/testis antigen or oncofetal antigens have thus far been identified and regarded as ideal targets for anticancer immunotherapy (15–18). However, only a few tumor-associated antigens have been reported as the inducer of both $CD8^+$ and $CD4^+$ T-cell-mediated immune responses (19–22). Recently, we and other groups found that an oncofetal protein glypican-3, glycosylphosphatidylinositol (GPI)–anchored membrane protein, is specifically overexpressed in human

Requests for reprints: Satoru Senju and Yasuharu Nishimura, Department of Immunogenetics, Graduate School of Medical Sciences, Kumamoto University, Kumamoto 860-8556, Japan. Phone: 81-96-373-5313; Fax: 81-96-373-5314; E-mail: senjusat@gpo.kumamoto-u.ac.jp and mxnishim@gpo.kumamoto-u.ac.jp.

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hepatocellular carcinoma (23, 24). In a subsequent study, we revealed that glypican-3 is overexpressed also in human melanoma (25). An immunohistochemical analysis revealed that the tissue distribution of murine glypican-3 protein was very similar to that in humans. In a previous study, we showed that the *in vivo* transfer of glypican-3 peptide-pulsed BM-DC or glypican-3-reactive CTL line had a potent effect to protect the recipient mice from the murine glypican-3-transfected Colon 26, a colorectal cancer cell line (17).

In the current study, we found that a mouse melanoma cell line F10, which is a subline of B16, naturally expressed glypican-3. Using this cell line as a target, we elucidated the antitumor effect of therapy with ES-DC genetically modified to express murine glypican-3.

Materials and Methods

Mice. CBA and C57BL/6 mice were obtained from Clea Animal Co. (Tokyo, Japan) or Charles River (Hamamatsu, Japan) and maintained under specific pathogen-free conditions. Male CBA and female C57BL/6 mice were mated to produce (CBA × C57BL/6) F1 (CBF1) mice and all studies were done with the F1 mice syngeneic to the mouse embryonic stem cell line TT2 at 6 to 8 weeks of age. The mouse experiments met with approval by Animal Research Committee of Kumamoto University.

Cell lines. The embryonic stem cell line TT2, derived from CBF1 blastocysts (26), was maintained as described previously (12). The method for induction of differentiation *in vitro* of embryonic stem cells into dendritic cells was done as described previously (12), and ES-DC prepared from a 14-day culture in bacteriologic Petri dishes in the presence of granulocyte-macrophage colony-stimulating factor (GM-CSF) were used for *in vivo* and *in vitro* assays. C57BL/6-derived tumor cell lines, F1 and F10 sublines of B16 melanoma, a fibrosarcoma cell line MCA205 (MCA), Lewis lung cancer (3LL) and a thymoma cell line EL4, and a human hepatocellular carcinoma cell line HepG2 were provided by the Cell Resource Center for Biomedical Research Institute of Development, Aging, and Cancer, Tohoku University (Sendai, Japan). The cells were cultured in RPMI 1640 supplemented with 10% FCS. To produce glypican-3-expressing MCA (MCA-GPC3), MCA cells were transfected with pCAGGS-GPC3-internal ribosomal entry site (IRES)-puromycin-resistant (puro-R) by using LipofectAMINE 2000 reagent (Invitrogen Corp., Carlsbad, CA), selected with puromycin, and then subjected to cloning by limiting dilution in drug-free medium using 96-well culture plates (27, 28).

Generation of ES-DC expressing glypican-3. A full-length murine *glypican-3* cDNA clone was purchased from Invitrogen. A cDNA fragment encoding total glypican-3 protein was isolated from that and transferred to a mammalian expression vector pCAGGS-IRES-puro-R, containing the CAG promoter and an IRES-puro-R *N*-acetyltransferase gene cassette (29, 30), to generate an expression vector for *glypican-3*, pCAGGS-GPC3-IRES-puro-R. To generate *glypican-3*-transfected embryonic stem cell clones, TT2 embryonic stem cells were introduced with pCAGGS-GPC3-IRES-puro-R by electroporation and selected with puromycin as described previously (12). *Glypican-3*-transfectant embryonic stem cell clones were subjected to a differentiation culture to generate ES-DC as described previously (12–14). No maturation stimuli, such as lipopolysaccharide or tumor necrosis factor-α, were given to ES-DC before *in vivo* transfer. The expression of *glypican-3* in transfectant ES-DC was confirmed by reverse transcription-PCR (RT-PCR).

RT-PCR and Northern blotting. Total cellular RNA was extracted and RT-PCR was done as described previously (13, 14). Briefly, total RNA was converted into cDNA and PCR was done for 33 cycles for the quantification of *glypican-3* mRNA and for 30 cycles for the quantification of glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) mRNA. The primer sequences were as follows: *glypican-3*, sense 5′-CTGACTGACCGCGTTACTCCCACA-3′ and antisense 5′-TAGCAGCATCGCCACCAGCAAGCA-3′ and *GAPDH*, sense 5′-GGAAAGCTGTGGCGTGATG-3′ and antisense 5′-CTGTTGCTGTAGCCGTATTC-3′. The sense strand primer used for detection of transgene-derived mRNA was corresponding to the 5′ untranslated region included in the vector DNA. PCR products were visualized by ethidium bromide staining after separation over a 1% agarose gel. A Northern blot analysis was done as described previously (31). In brief, RNA samples (20 μg total RNA per lane) were subjected to electrophoresis in formalin-MOPS gels, blotted onto nylon membranes (Hybond N⁺, Amersham, Piscataway, NJ), and probed with ^{32}P-labeled DNA probe. A human *glypican-3* cDNA fragment (bp 1,639-2,139) was used as a probe. Human and murine *glypican-3* have a 90% similarity in nucleotide sequence and human cDNA probe hybridized to both human and murine *glypican-3* mRNA.

Peptides, protein, and cytokines. Eleven kinds of 9- to 10-mer glypican-3-derived peptides predicted to bind with H2-D^b or K^b were selected based on the binding score as calculated by the BIMAS software package (BioInformatics and Molecular Analysis Section, Center for Information Technology, NIH, Bethesda, MD). The peptides were synthesized by the F-MOC method on an automatic peptide synthesizer (PSSM8; Shimadzu, Kyoto, Japan) and subsequently purified by high-performance liquid chromatography. The synthetic peptides were designated as murine glypican-3-1 to -11 in ascending order of high binding score. Their amino acid sequences are as follows: murine glypican-3-1, AMFKNNYPSL; murine glypican-3-2, LGSDINVDDM; murine glypican-3-3, LTARINMEQL; murine glypican-3-4, SVLDINECL; murine glypican-3-5, TLCWNGQEL; murine glypican-3-6, YVQKNGGKL; murine glypican-3-7, GMVKVKNQL; murine glypican-3-8, RNGMKNQFNL; murine glypican-3-9, AMLLGLGCL; murine glypican-3-10, ASMELKFLI; and murine glypican-3-11, LFPVIYTQM. Murine glypican-3-11 is predicted to be restricted to H2-K^b and the others to H2-D^b. Recombinant human glypican-3 protein was purchased from R&D Systems (Minneapolis, MN). Recombinant murine GM-CSF and IFN-γ were purchased from PeproTech (London, United Kingdom).

Immunohistochemical and flow cytometric analysis. An immunofluorescence analysis to detect the expression of glypican-3 was done as described previously (16). Anti-human glypican-3 polyclonal antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). FITC-labeled goat anti-rabbit IgG (clone ALI4408; Biosource, Camarillo, CA) was used as a second antibody and propidium iodide for nuclear DNA staining. Stained samples were subjected to microscopic analysis on a confocal microscope (Fluoview FV300, Olympus, Tokyo, Japan). Immunohistochemical analysis of frozen tissue sections was done as described previously (13, 23) using monoclonal antibody (mAb) specific to CD4 (L3T4; BD PharMingen, San Diego, CA) or CD8 (Ly-2; BD PharMingen). In the flow cytometric analysis, cell samples were stained and analyzed on a flow cytometer (FACScan; BD Biosciences, Japan) as described previously (12, 14). Antibodies used for staining were as follows: FITC-conjugated mouse anti-mouse H2-D^b (clone CTDb; mouse IgG2a; Caltag, Burlingame, CA), anti-H2-K^b (clone CTKb; mouse IgG2a; Caltag) and anti-I-A^b (clone 3JP; mouse IgG2a; Caltag), R-phycoerythrin (R-PE)–conjugated anti-mouse CD11c (clone N148; hamster IgG; Chemicon, Temecula, CA), R-PE-conjugated anti-mouse CD86 (clone RMMP-2; rat IgG2a; Caltag), FITC-conjugated goat anti-mouse Ig (BD PharMingen), mouse IgG2a control (clone G155-178; BD PharMingen), FITC-conjugated mouse IgG2a control (clone G155-178; BD PharMingen), and R-PE-conjugated hamster IgG control (Immunotech, Marseille, France).

Generation of BM-DC. Generation of dendritic cells from bone marrow cells was done as described previously (17). For loading of synthetic peptides, BM-DC were incubated with a mixture of three kinds of glypican-3 peptides, murine glypican-3-2, -8, and -11 (10 μmol/L each), at 37°C for 2 hours. For loading of recombinant glypican-3 protein, BM-DC were cultured in the presence of glypican-3 protein (2 μg/mL) at 37°C for 12 hours. No maturation stimuli were given to BM-DC before *in vivo* transfer.

Induction of glypican-3-specific CTLs and cytotoxicity assay. The mice were i.p. immunized with 1 × 10^5 ES-DC twice with a 7-day interval. Seven days after the second immunization, spleen cells were isolated from the mice and cultured (2.5 × 10^6 per well) with ES-DC (1 × 10^5 per well) in 24-well culture plates in RPMI supplemented with 10% horse serum, recombinant human interleukin (IL)-2 (100 units/mL), and 2-mercaptoethanol (50 μmol/L). After the culture for 5 days, the cells were recovered and their cytotoxic activity was analyzed by ^{51}Cr release assays using MCA, MCA-GPC3, B16-F1, and B16-F10 as target cells basically by the same method as described previously (12). B16 cells were pretreated with recombinant murine IFN-γ (1,000 units/mL) before use as target cells as

reported previously (32). In some experiments, $CD8^+$ T cells and natural killer (NK) cells were isolated from effector cell preparations by using a magnetic cell sorting system (Miltenyi, Bergisch Gladbach, Germany). Positively selected cells were 95% pure as determined by flow cytometry.

ELISPOT analysis. Glypican-3-specific T cells were induced by a culture of splenocytes isolated from mice immunized with ES-DC-GPC3 by the same way as described above, except that glypican-3-derived peptides (10 μmol/L) were added to the culture instead of ES-DC-GPC3. After 5 days, the frequency of cells producing IFN-γ on stimulation with target cells (*EL4* or EL4 pulsed with each peptide, MCA or MCA-GPC3) was assessed by an ELISPOT assay as described previously (33). The spots were automatically counted and subsequently analyzed using the Eliphoto system (Minerva Tech, Tokyo, Japan).

Tumor prevention and treatment. ES-DC-GPC3 or BM-DC (1×10^5) loaded with glypican-3 peptide or protein were transferred i.p. into mice twice on days −14 and −7, and B16-F10 or MCA-GPC3 cells were challenged s.c. into the shaved back region on day 0. The tumor sizes were determined biweekly in a blinded fashion and survival rate or disease free rate was monitored. Tumor index was calculated as follows: tumor index (mm^2) = (length × width). For the i.v. challenge experiments, tumor cells (B16-F10) were injected i.v. on day 0, and 1×10^5 ES-DC-GPC3 were injected i.p. twice on days 3 and 10 as described previously (34).

***In vivo* depletion of $CD4^+$ and $CD8^+$ T cells.** The mice were transferred i.p. twice with 1×10^5 ES-DC-GPC3 on days −14 and −7 and challenged s.c.

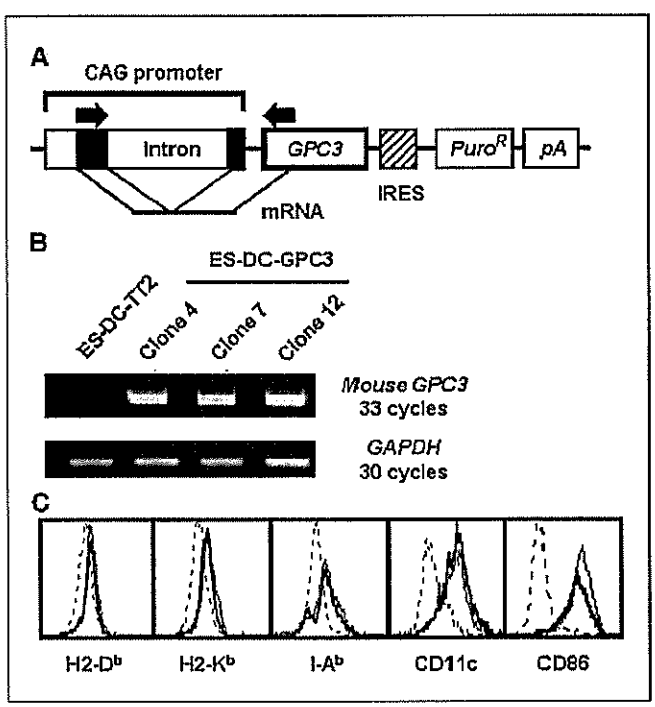

Figure 1. Establishment of ES-DC genetically modified to express murine glypican-3. *A*, structure of pCAGGS-GPC3-IRES-puro-R vector. To obtain pCAGGS-GPC3-IRES-puro-R, a cDNA fragment, including a full-length cDNA of murine *glypican-3*, was inserted into a mammalian expression vector pCAGGS-IRES-puro-R containing the CAG promoter and an IRES-puromycin *N*-acetyltransferase gene cassette. *B*, expression of *glypican-3* mRNA detected by RT-PCR analysis in transfectant ES-DC (ES-DC-GPC3). Primer sets (*arrows* in *A*) were designed to span the intron (917 bp) in the CAG promoter sequence to distinguish PCR products of mRNA origin (249 bp) from the genome-integrated vector DNA origin (1,166 bp). *Black boxes* in (*A*) indicate the 5′-untranslated region of the rabbit β-actin gene included in the CAG promoter. PCR was done at the cycles indicated for quantification of *glypican-3* mRNA and *GAPDH* mRNA. *C*, surface phenotype of genetically modified ES-DC. The expression of the cell surface H2-D^b, H2-K^b, I-A^b, CD11c, and CD86 on transfectant ES-DC was analyzed by a flow cytometric analysis. The staining patterns of ES-DC-GPC3 (*thick line*) closely coincided with those of parental ES-DC (*thin line*). *Dotted lines*, findings for isotype-matched control staining.

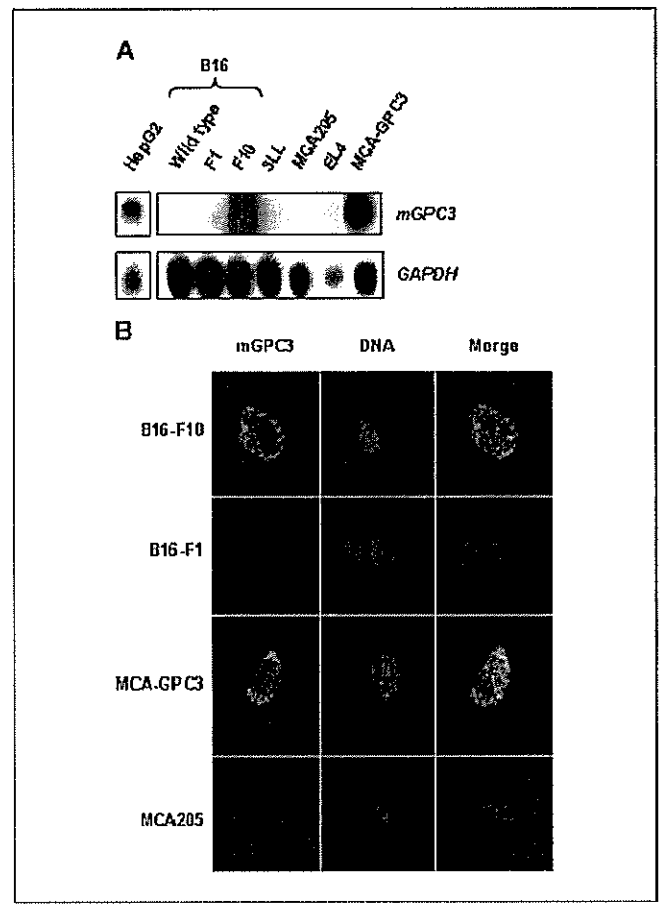

Figure 2. Expression of glypican-3 in cancer cell lines. *A*, Northern blot analysis of *glypican-3* mRNA in a human hepatocellular carcinoma cell line HepG2 (positive control) and various cancer cell lines of C57BL/6 origin. The same filters were stripped and rehybridized with *GAPDH* cDNA to assess the loading of equal amounts of RNA. *B*, immunofluorescence staining analysis of murine glypican-3 protein expressed in B16 variants F1, F10, MCA205, and MCA-GPC3. These cells were stained with rabbit anti-human glypican-3 polyclonal antibody cross-reactive to murine glypican-3 (*green*). Chromosome DNA was visualized by propidium iodide staining (*red*).

with 5×10^3 B16-F10 cells on day 0. For the depletion of T-cell subsets *in vivo*, mice were given a total of six i.p. transfers of the ascites (0.1 mL/mouse/transfer) from hybridoma-bearing nude mice or anti-asialo GM1 on days −18, −15, −11, −8, −4, and −1. Antibodies used were rat anti-mouse CD4 mAb (clone GK1.5), rat anti-mouse CD8 mAb (clone 2.43), and rabbit anti-asialo GM1 polyclonal antibody (Wako Japan; 20 μL/mouse/transfer). Normal rat IgG (Sigma-Aldrich, St. Louis, MO; 200 μg/mouse/transfer) was used as a control. The depletion of T-cell subsets by treatment with antibodies was confirmed by a flow cytometric analysis of spleen cells, which showed a >90% specific depletion.

Statistical analysis. The two-tailed Student's *t* test was used to determine the statistical significance of differences in the cytolytic activity and tumor growth between the treatment groups. $P < 0.05$ was considered to be significant. The Kaplan-Meier plot for survival was assessed for significance in the tumor challenge experiments using the Breslow-Gehan-Wilcoxon test. Statistical analyses were made using the StatView 5.0 software package (Abacus Concepts, Calabasas, CA).

Results

Generation of ES-DC expressing glypican-3. TT2 embryonic stem cells were introduced with a murine glypican-3 expression vector, pCAGGS-GPC3-IP, driven by the CAG promoter and

containing the IRES-puro-R marker gene (Fig. 1*A*), and several transfectant clones were isolated. The transfectant embryonic stem cell clones were subjected to differentiation to ES-DC, and a transfectant clone 12 expressing the highest level of glypican-3 was selected based on the RT-PCR analysis (Fig. 1*B*). ES-DC differentiated from parental embryonic stem cell line TT2 without transfection were designated as ES-DC-TT2, and ES-DC differentiated from glypican-3-transfectant embryonic stem cells were designated as ES-DC-GPC3. No significant difference was observed in the morphology and levels of the surface expression of H2-D^b, H2-K^b, I-A^b, CD11c, and CD86 between ES-DC-TT2 and ES-DC-GPC3 (Fig. 1*C*). As a result, the transfection of the *glypican-3* gene has little influence on the differentiation of ES-DC.

Expression of glypican-3 in a F10 subline of B16 melanoma. We recently revealed that the oncofetal protein glypican-3 is specifically overexpressed in human hepatocellular carcinomas and melanomas (23, 25). To establish a mouse model system to evaluate the glypican-3 as a target antigen for anticancer immunotherapy, we searched for a transplantable mouse tumor cell line naturally expressing glypican-3. We examined the expression of glypican-3 in several mouse cell lines and found that B16-F10, a subline of B16 melanoma, expressed glypican-3. In a Northern blot analysis, as shown in Fig. 2*A*, where a human hepatocellular carcinoma cell line HepG2 was used as a positive control, *glypican-3* mRNA was evidently detected in a mouse melanoma cell line B16-F10 but not in B16-W.T., B16-F1, 3LL, MCA205, or EL4. The expression of *glypican-3* mRNA was also detected in a glypican-3-transfected MCA, MCA-GPC3. Figure 2*B* shows an immunofluorescence analysis to detect expression of glypican-3 protein. In accordance with the result of the Northern blot analysis, evident expression of glypican-3 protein was detected in B16-F10 and MCA-GPC3. On the other hand, MCA205 and B16-F1 cells did not express glypican-3 protein. Glypican-3 is a GPI-anchored membrane protein, and the results shown in Fig. 2*B* indicated that glypican-3 protein localized at or around cell membrane is

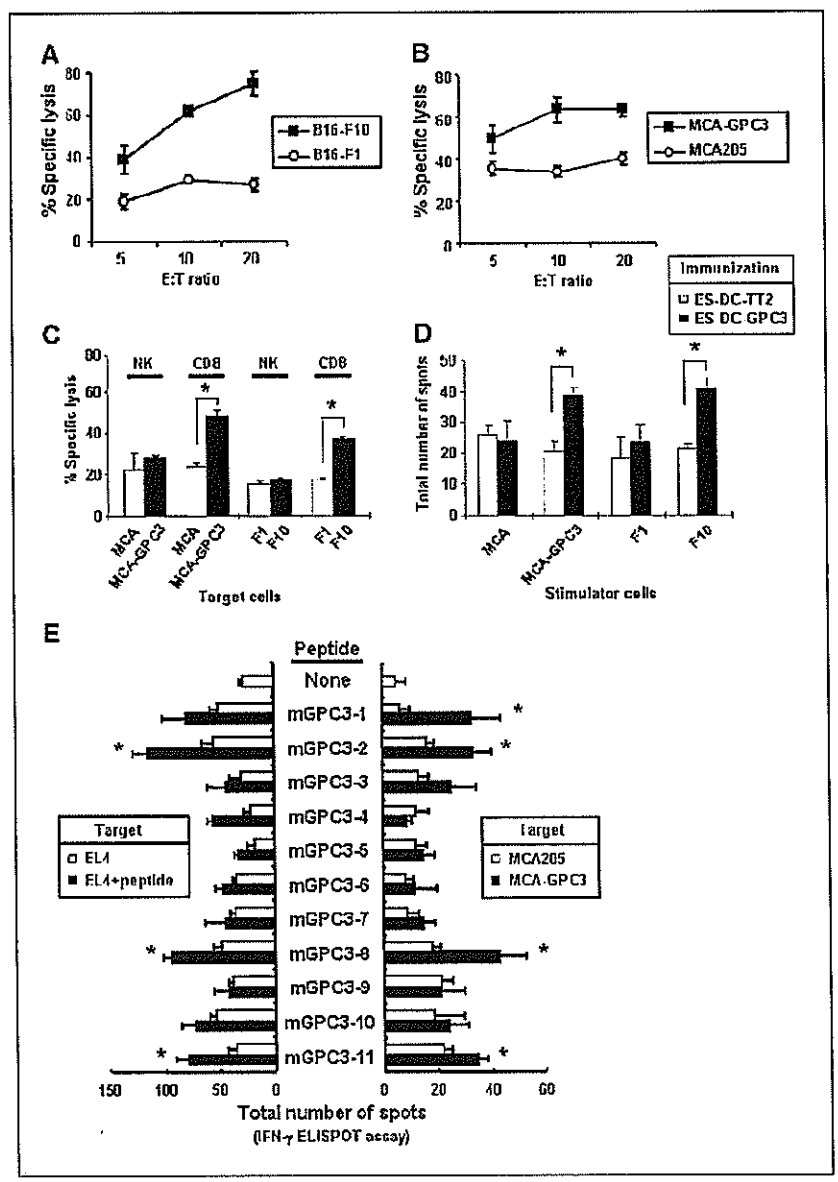

Figure 3. Priming of antigen-specific CTLs with ES-DC-GPC3. The mice were transferred i.p. twice with 1×10^5 ES-DC-GPC3 on days −14 and −7. On day 0, spleen cells from immunized mice were isolated and cultured with 1×10^5 ES-DC-GPC3 per well in the presence of recombinant human IL-2 (100 units/mL) for 5 days. ^{51}Cr release assays were done with the obtained resultant cells to evaluate the capacity to kill IFN-γ pretreated B16-F1 and B16-F10 cells (*A*) and MCA and MCA-GPC3 cells (*B*). Results are expressed as % specific lysis from triplicate assays. *C*, in addition, the resultant cells obtained in the same way were sorted to the fraction of NK cells and $CD8^+$ T cells with microbeads, and another assay was done using the targets in the same condition as in (*A* and *B*). *D*, spleen cells from mice transferred twice with 1×10^5 ES-DC-GPC3 or ES-DC-TT2, respectively, were isolated and restimulated *in vitro* with 1×10^5 ES-DC-GPC3 per well for 5 days. The resultant cells were used for IFN-γ ELISPOT assay. The assay was done in triplicate using the same targets as in (*A* and *B*). *Columns*, mean number of IFN-γ-positive spots. *E*, identification of glypican-3-derived and H2-D^b- or H2-K^b-restricted CTL epitopes by IFN-γ ELISPOT assays. The mice were immunized with 1×10^5 ES-DC-GPC3 twice with a 7-day interval. Spleen cells from mice immunized were restimulated *in vitro* with each glypican-3 peptide (10 μmol/L) and cultured for 5 days with 100 units/mL recombinant human IL-2. ELISPOT assays for 16 hours were examined against EL4 pulsed with or without each peptide and MCA or MCA-GPC3. *Columns*, mean total number of spots from quadruplicate assays. Data are representative of three independent experiments with similar results in (*A-E*). *, $P < 0.05$, differences in the responses are statistically significant between two values in (*C-E*).

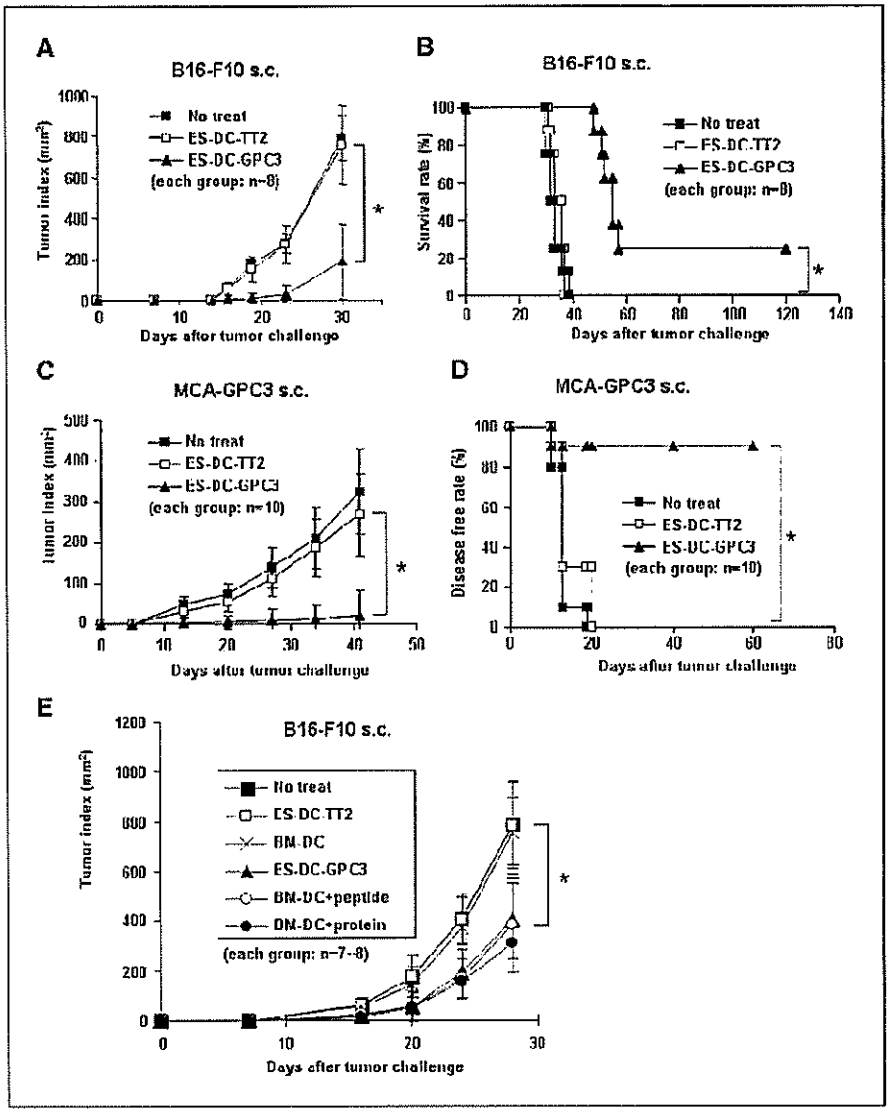

Figure 4. Suppression of tumor growth and prolongation of survival by preimmunization with ES-DC-GPC3. The mice were transferred i.p. twice with 1×10^5 ES-DC-GPC3 or ES-DC-TT2 on days −14 and −7. On day 0, the mice were challenged s.c. with 5×10^3 B16-F10 (*A* and *B*) or 1×10^5 MCA-GPC3 (*C* and *D*) expressing glypican-3. The tumor index, survival rate, or disease-free rate was monitored. *, $P < 0.05$, differences in these three indexes between the groups treated with ES-DC-GPC3 and ES-DC-TT2 were statistically significant. *E*, mice were injected i.p. with ES-DC-GPC3 or BM-DC loaded with a mixture of glypican-3 peptides, murine glypican-3-2, -8, and -11, or glypican-3 protein on the same schedule as in (*A-D*) and challenged s.c. with 5×10^3 B16-F10. Subsequently, the mice were monitored for the growth of tumor.

consistent with this, although some differences in the staining patterns among the cells were observed.

Priming of antigen-specific cytotoxic T cells with genetically modified ES-DC-GPC3. We analyzed the capacity of ES-DC-GPC3 to prime glypican-3-specific CTLs. The mice were immunized i.p. twice with ES-DC-GPC3 or ES-DC-TT2 on days −14 and −7. On day 0, the spleen cells were isolated and restimulated *in vitro* with ES-DC-GPC3 in the presence of exogenous recombinant human IL-2 (100 units/mL). After 5 days, the cells were recovered and their killing activity against target cells with or without expression of glypican-3 was analyzed. As shown in Fig. 3*A* and *B*, the effector cells primed by ES-DC-GPC3 showed a significantly higher killing activity against B16-F10 than against B16-F1, and also against MCA-GPC3 than nontransfectant MCA cells. These results suggest that the effector cells included CTLs recognizing glypican-3. In the experiments shown in Fig. 3*C*, we separated the effector cells into $CD8^+$ T cells and NK cells before the killing assay. NK cells showed activity to kill MCA and MCA-GPC3 in a similar magnitude and to kill B16-F1 and F10 in a similar magnitude, indicating that they killed target cells regardless of glypican-3 expression. On the other hand, for the $CD8^+$ fraction, the cytotoxic activity against B16-F10 was higher than that against B16-F1, and the cytotoxic activity against MCA-GPC3 was higher than that against MCA. On the contrary, spleen cells isolated from mice transferred with ES-DC-TT2 and cocultured *in vitro* with ES-DC-GPC3 exhibited the similar basal levels of killing activities directed against both B16-F1 and F10 as well as MCA and MCA-GPC3 (data not shown).

We next compared the efficiency of the induction of glypican-3-specific and IFN-γ-producing T cells primed by ES-DC-GPC3 with that primed by ES-DC-TT2. The mice were immunized twice with ES-DC-TT2 or ES-DC-GPC3 based on the above described schedule, and the splenocytes isolated from both group of mice were cocultured with ES-DC-GPC3 for 5 days. Thereafter, the frequency of glypican-3-specific T cells was analyzed by an ELISPOT analysis to detect cells producing IFN-γ. As shown in Fig. 3*D*, *in vivo* priming with ES-DC-GPC3 and ES-DC-TT2 resulted in the induction of similar frequency of cells producing IFN-γ on stimulation with cells with no expression of glypican-3, MCA or B16-F1. On the other hand, *in vivo* priming with ES-DC-GPC3 led to the induction of significantly larger number of T cells producing IFN-γ

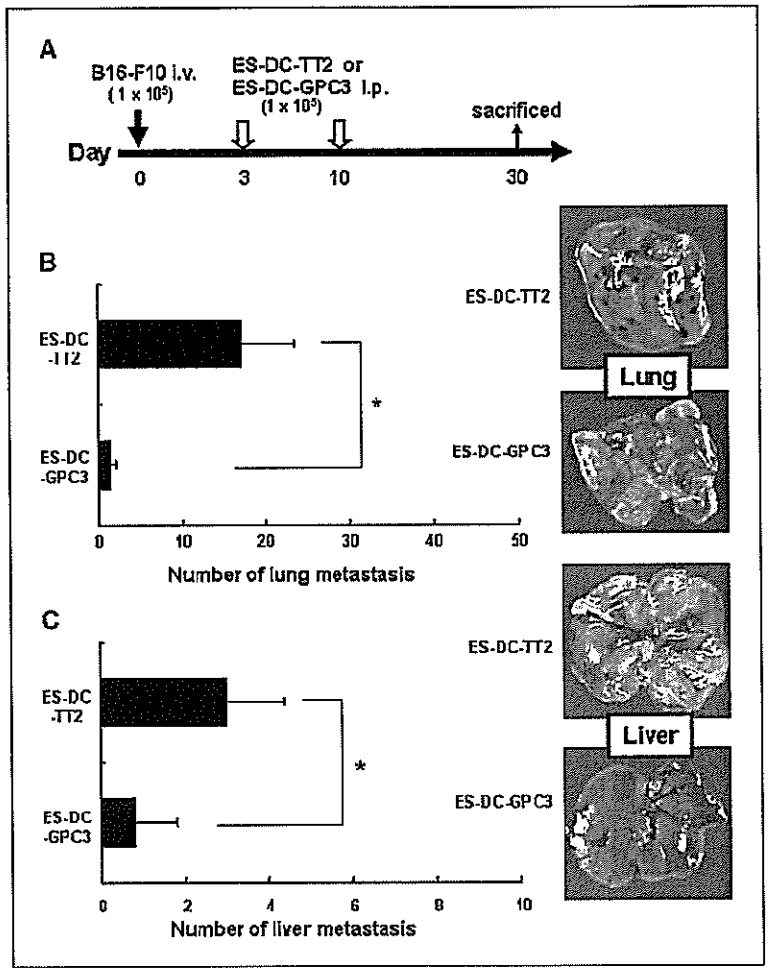

Figure 5. Suppression of tumor growth in the metastatic tumor model of B16-F10. The protocol for therapeutic immunotherapy model was indicated in (*A*). All mice were injected into tail vein with 1×10^5 F10 cells on day 0. On days 3 and 10, mice were injected i.p. with 1×10^5 ES-DC-TT2 or ES-DC-GPC3. On day 30, the mice were sacrificed and the numbers of pulmonary and liver metastases were macroscopically calculated. *Columns*, mean number of total metastases in the lung (*B*) and liver (*C*) using five mice per group. *, $P < 0.05$, differences in the number of metastases are statistically significant between the two values.

on stimulation with cells expressing glypican-3, MCA-GPC3 or B16-F10, compared with priming with ES-DC-TT2. Collectively, these results showed that glypican-3-specific CTLs were primed *in vivo* only when mice were transferred with ES-DC-GPC3, further confirming that ES-DC-GPC3 have the capacity to prime the glypican-3-specific CTLs *in vivo*.

Identification of glypican-3-derived and H2-D^b- or K^b-restricted CTL epitopes. To identify the H-2^b-restricted CTL epitopes of glypican-3, we synthesized 11 glypican-3-derived peptides carrying the binding peptide motifs for H2-D^b or K^b and designated as murine glypican-3-1 to -11 in turn. Spleen cells of the mice immunized with ES-DC-GPC3 by the same procedure as described above were stimulated *in vitro* with each of the peptides instead of ES-DC-GPC3 for 5 days. Subsequently, the frequency of glypican-3-specific CTLs was analyzed by IFN-γ ELISPOT assays. As shown in Fig. 3*E*, cells stimulated *in vitro* with murine glypican-3-2, -8, or -11 showed specific IFN-γ production on restimulation with EL4 cells prepulsed with the same peptide or MCA-GPC3. These results indicate that glypican-3-specific CTLs primed *in vivo* with ES-DC-GPC3 included those recognizing multiple glypican-3 epitopes.

Tumor preventive effects of immunization with ES-DC-GPC3. We next asked whether ES-DC-GPC3 could induce a protective immunity against tumor cells expressing glypican-3 *in vivo*. We immunized mice by the i.p. transfer of ES-DC on days −14 and −7, and the mice were challenged s.c. with 5×10^3 B16-F10 cells or 1×10^5 MCA-GPC3 on day 0. We then monitored the growth of tumors and survival of the mice. As shown in Fig. 4, immunizations with ES-DC-GPC3 provided a significant degree of protection against both B16-F10 and MCA-GPC3. On the other hand, the transfer of ES-DC-TT2 gave no significant protection compared with mice without dendritic cell transfer. Immunization with ES-DC-GPC3 did not show a protective effect against MCA or B16-F1 with no glypican-3 expression (data not shown). Collectively, the *in vivo* administration of ES-DC-GPC3 induced antitumor immunity against glypican-3-expressing tumor cells, thus resulting in a significant inhibition of the growth of tumor and prolongation of the survival time of the treated mice.

Next, we compared ES-DC-GPC3 with BM-DC preloaded with glypican-3 peptide or protein in their capacity to induce antitumor effect. We generated BM-DC from bone marrow cells of CBF1 mice and loaded them with mixture of the three major H2-D^b-restricted epitopes, murine glypican-3-2, -8, and -11 (Fig. 3*E*), or recombinant glypican-3 protein. As shown in Fig. 4*E*, ES-DC-GPC3 and peptide or protein antigen-loaded BM-DC elicited similar magnitude of protective effect against challenge with B16-F10.

Protective effect of ES-DC-GPC3 against i.v. challenge with tumor cells. We next examined the antitumor effect of ES-DC-GPC3 against i.v. challenge with B16-F10. As shown in Fig. 5*A*, the mice were i.v. inoculated with B16-F10 cells on day 0, and the mice were treated with ES-DC-TT2 or ES-DC-GPC3

twice on days 3 and 10. On day 30, mice were sacrificed and macroscopically analyzed. As shown in Fig. 5*B* and *C*, treatment with ES-DC-GPC3 significantly reduced the pulmonary and liver metastases in comparison with the treatment with ES-DC-TT2 ($P < 0.05$). Some of the mice treated with ES-DC-TT2, but not those treated with ES-DC-GPC3, died before they were scheduled to be sacrificed. Thus, the survival time of the mice treated with ES-DC-GPC3 was prolonged in comparison with those treated with ES-DC-TT2.

Identification of effector cells involved in the protection against F10 and MCA-GPC3 induced by ES-DC-GPC3. To determine the subsets of the effector cells involved in the observed protective effect against tumor cells induced by ES-DC-GPC3, we depleted $CD4^+$ or $CD8^+$ T cells in the mice by treatments with either anti-CD4 or anti-CD8 mAb. By this treatment, >90% of $CD4^+$ or $CD8^+$ T cells were depleted (data not shown). The NK cells were depleted by the treatment with anti–asialo GM1 antibody. During this procedure, the mice were immunized with ES-DC-GPC3 and challenged s.c. with B16-F10 cells. As shown in Fig. 6, depletion of $CD4^+$ T cells, $CD8^+$ T cells, or NK cells almost totally abrogated the protective immunity induced by ES-DC-GPC3, suggesting that all of three effector cell subsets were essential for the protective effect.

In a histologic analysis of the tumor tissue specimens, we observed more intense infiltration of inflammatory cells into and/or around tumor tissues of mice immunized with ES-DC-GPC3 than those of mice immunized with ES-DC-TT2. In the metastatic B16-F10 tumor tissue specimens, the infiltrating cells were found to consist of both $CD4^+$ and $CD8^+$ T cells (Fig. 6). These results also suggest that both $CD4^+$ and $CD8^+$ T cells were involved in the antitumor effect against the B16-F10 induced by ES-DC-GPC3.

Discussion

We investigated the antitumor effect of immunization with ES-DC genetically engineered to express a mouse oncofetal antigen glypican-3 against mouse tumor cells naturally expressing GPC3-F10, a subline of B16 melanoma. *In vivo* transfer of ES-DC-GPC3 primed CTL reactive to multiple glypican-3-derived epitopes. The treatment of mice with ES-DC-GPC3 elicited potent protective effect against B16-F10 in both preventive and therapeutic conditions with no evidence of any side effects, such as autoimmunity. The antitumor effect induced by ES-DC-GPC3 was specific to the tumor cells expressing glypican-3, because this treatment was not effective against B16-F1, another subline of B16 with no glypican-3 expression. The glypican-3 specificity of the antitumor effect induced by ES-DC-GPC3 was further confirmed by the observation that the treatment was effective against glypican-3-transfectant MCA205 sarcoma but not against parental MCA 205 cells. The depletion experiments and immunohistochemical analyses showed that $CD8^+$ T cells, $CD4^+$ T cells, and NK cells contributed to the observed antitumor effect.

The tumor cell lines used in this study, MCA205 and B16-F10, were derived from C57BL/6 mice and may be recognized by some fraction of NK cells of CBF1 mice. Thus, the tumor cells must be

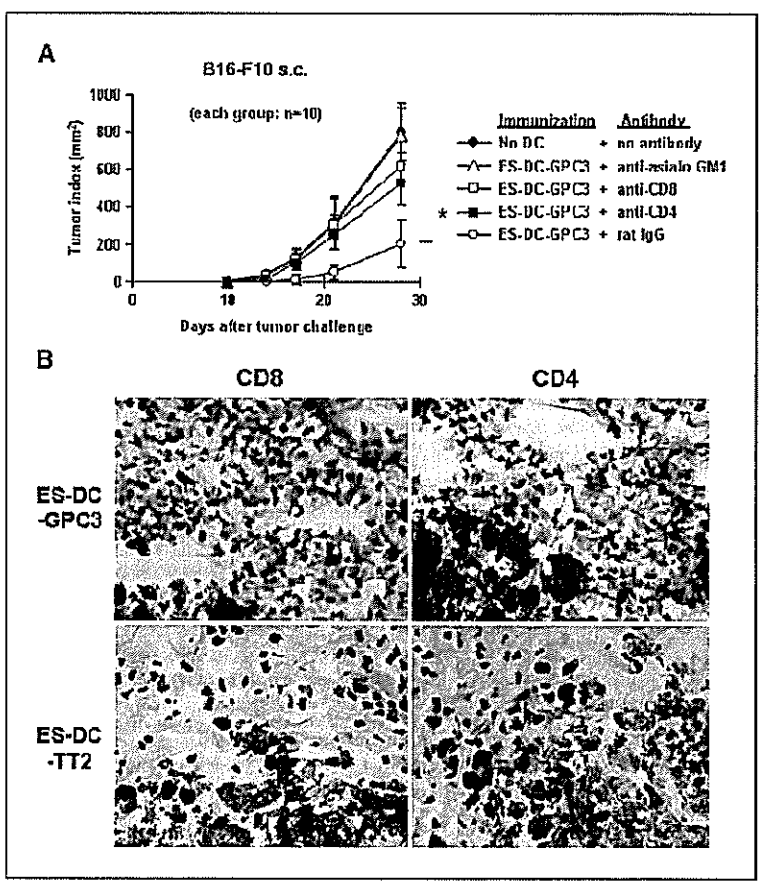

Figure 6. Involvement of both $CD4^+$ and $CD8^+$ T cells in antitumor immunity induced by ES-DC-GPC3. *A*, $CD4^+$ or $CD8^+$ T cells were depleted *in vivo* by the inoculation of anti-CD4 or anti-CD8 mAbs during immunization with ES-DC-GPC3. The mice were challenged s.c. with 5×10^5 F10 tumor cells, and the tumor size was measured and the tumor volume was represented as the tumor index. In immunization with ES-DC-GPC3, the differences in the tumor index between the mice inoculated with rat IgG and those with anti-CD4 mAb or those with anti-CD8 mAb are statistically significant (*, $P < 0.05$). The mice inoculated with anti-CD4 mAb or anti-CD8 mAb showed tumors that were the same size as those in the mice with no transfer with dendritic cells. *Points*, mean tumor index ($n = 10$ per group); *bars*, SD. *B*, infiltration of both $CD4^+$ and $CD8^+$ T cells into pulmonary metastatic tumor tissues. After the challenge with 1×10^5 F10 tumor cells as well as the pulmonary metastatic model in Fig. 5, the mice were treated twice with 1×10^5 ES-DC-TT2 or ES-DC-GPC3. Twenty days after the second treatment, frozen sections of tumor tissue were made and stained with the Giemsa method or immunostained with anti-CD4 or anti-CD8 mAb. In mice treated with ES-DC-GPC3, both $CD8^+$ and $CD4^+$ T cells apparently infiltrated into and/or around the pulmonary metastatic tumor. However, in the mice treated with ES-DC-TT2, neither $CD8^+$ nor $CD4^+$ T cells were detected in the tissue specimens. Magnification, ×400.

more immunogenic to CBF1 mice, used as the recipient mice in the present experiments, than to C57BL/6 mice. However, under the current experimental condition, all of the CBF1 mice challenged with B16-F10 or MCA-GPC3 died unless the recipient mice were treated with ES-DC-GPC3 (Fig. 4*B* and *D*), indicating that these tumor cells are invasive enough also to CBF1 mice.

In the ^{51}Cr release assay shown in Fig. 3*A* to *C*, CTL primed with ES-DC-GPC3 or ES-DC-TT2 (data not shown) exhibited weak killing activity against MCA or B16-F1 cells. Similar weak responses of spleen cells primed with ES-DC-GPC3 or ES-DC-TT2 to MCA or B16-F1 cells were also observed in the ELISPOT assay shown in Fig. 3*D*. At present, we have not yet clarified the effector cells and the target antigens that cause these "background" responses. However, such responses observed *in vitro* did not contribute to the *in vivo* antitumor effect, because the treatment with ES-DC-TT2 had no antitumor effects as shown in Fig. 4.

There was a considerable difference in the effect of treatment with ES-DC-GPC3 between the challenge with B16-F10 and that with MCA-GPC3 cells. This may be partly due to the lower expression of MHC class I on B16 compared with MCA205. B16 does not express MHC class I unless they are stimulated with IFN-γ. The indispensable role of NK cells in the antitumor effect (Fig. 6*A*) suggests that NK cells recognized B16 cells expressing a very low level of MHC class I; subsequently, NK cells produced IFN-γ to up-regulate MHC class I molecules on B16-F10 cells and to make B16-F10 cells sensitive to an attack by glypican-3-specific CTLs (35, 36).

As shown in Fig. 4, the protection against B16-F10 elicited by ES-DC-GPC3 was not complete. Treatment of the ES-DC with some maturation stimuli or loading of α-galactosylceramide, a ligand for NKT cells, to ES-DC before *in vivo* administration may have some effect to enhance the antitumor effect (37). As a future project, we are planning to generate ES-DC genetically engineered to produce cytokines, such as IL-15 or IL-21, along with glypican-3 to improve the antitumor effect.

We reported previously that the induction of immune response to glypican-3 protected the mice from a challenge with Colon 26 colon tumor cells genetically modified to overexpress glypican-3 (17). In the present study, we found the natural overexpression of glypican-3 in B16-F10 and showed that the immunization of mice with glypican-3 protected the mice from the challenge with B16-F10. Glypican-3 is one of the oncofetal proteins and the expression in normal human tissues is limited to the placenta and fetal liver (17). In addition, the tissue distribution of glypican-3 expression is very similar in mice and humans (17). As a result, our results strongly suggest that anti-melanoma and anti–hepatocellular carcinoma immunotherapy with glypican-3 seems to be an effective and safe method, and it should therefore be tested clinically.

To enable to the future clinical application of ES-DC, we recently established a method for generating ES-DC from embryonic stem cells of nonhuman primate, cynomolgus monkey, and also for genetic modification of them.[3] Considering the future clinical application of ES-DC technology, allogenicity (i.e., differences in the genetic background between the patients to be treated and the embryonic stem cells as the source for dendritic cells), we expected to cause problems. However, it is expected that human embryonic stem cells sharing some of HLA alleles with patients are available in most cases. We recently found that antigen-expressing ES-DC could potently prime antigen-specific CTL on the adoptive transfer to semiallogeneic mice, thus sharing some MHC alleles with the ES-DC and also protecting the recipient mice from subsequent challenge with tumor cells bearing the antigen (38). Immunotherapy with human ES-DC expressing glypican-3 may therefore be clinically useful as an immunotherapy of melanoma and hepatocellular carcinoma.

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[3] In preparation.

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●連載● HLA 3

新規がん胎児性抗原 Glypican-3 を標的とした肝細胞がんの免疫療法

小森宏之*, ** 中面哲也*** 本村 裕**, *** 石河隆敏** 別府 透** 馬場秀夫** 西村泰治*

Point

①肝細胞がん(HCC)は治療後も高頻度に再発をくり返すため予後不良なガンであるが，治療後の再発予防に対する有効な補助療法は確立されていない．

②がん部と非がん部の cDNA マイクロアレイ解析を用いて，さまざまながんで特異的に高発現する遺伝子を網羅的に解析できる．この方法を用いて HCC に特異的に高発現する新規がん胎児性抗原 Glypican-3(GPC3)を同定した．

③GPC3 は，ヘパラン硫酸プロテオグリカンファミリーに属する糖鎖修飾が強い GPI アンカー型膜蛋白質であり，HCC 患者の 80 %のがん組織に発現している．最近，肝細胞がんの増殖を促進することが報告されている．

④GPC3 は，HCC 患者の約 40 %の血清中に認められ，HCC の腫瘍マーカーとして有用である．

⑤マウスにおいて GPC3 を標的とするがん免疫療法が，自己免疫現象を伴うことなく奏効することを証明した．

⑥日本人の HCC 患者の約 80 %に適用できる，GPC3 由来の HLA-A2 あるいは A24 拘束性ヒト・キラー T 細胞エピトープペプチドを同定し，これを用いた HCC 免疫療法の臨床試験を開始した．

Key Words /Glypican-3(GPC3)，肝細胞がん(HCC)，がん免疫療法，ペプチドワクチン

● はじめに

肝細胞がん(HCC)の罹患者数は欧米および，アジア諸国においていぜんとして増加している．HCC は治療後も高頻度に再発をくり返すため予後不良なガンであり，ウイルス性肝炎，肝硬変から発生した，ごく初期のがんに対する早期治療法や，治療後の再発予防のために有効な補助療法の確立が望まれている．Glypican-3 (GPC3)は HCC に高発現し，腫瘍免疫の標的として理想的な胎児性抗原である．われわれは HCC に対する免疫療法の新たなターゲットとして GPC3 に着目し，その有用性に関して前臨床試験を完了し，臨床試験を開始した．

1 HCC に対する免疫療法

慢性肝炎，肝硬変患者における HCC の発症予防や，HCC 術後における術後化学療法は，いまだ開発途上にある．HCC に対する免疫療法についても，1990 年代よ

* KOMORI Hiroyuki, NISHIMURA Yasuharu/熊本大学大学院医学薬学研究部免疫識別学分野 ** KOMORI Hiroyuki, MOTOMURA Yutaka, ISHIKO Takatoshi, BEPPU Toru, BABA Hideo/熊本大学大学院医学薬学研究部消化器外科学分野 *** NAKATSURA Tetsuya, MOTOMURA Yutaka/国立がんセンター東病院臨床開発センター・がん治療開発部機能再生室

りリンフォカイン活性化キラー細胞(lymphokine-activated killer cells：LAK)細胞，腫瘍浸潤リンパ球(tumor-infiltrating lymphocyte：TIL)，末梢血単核細胞(peripheral blood mononuclear cell：PBMC)を用いた養子免疫療法，樹状細胞(dendritic cell：DC)ワクチン療法，α-フェトプロテイン(AFP)由来ペプチドワクチン療法などが試みられている．またHCCにおいて高発現するがん特異的抗原も多数報告されており，各施設でその有用性が検討されている[5]．

2 新規がん胎児性抗原 Glypican-3(GPC3)

われわれは，東京大学医科学研究所ヒトゲノムセンターの中村祐輔博士らとの共同研究により，がん部と非がん部におけるcDNAマイクロアレイ解析データ[6]を用いて，肝細胞がん(HCC)特異的に高発現する遺伝子として*Glypican-3*(*GPC3*)を同定した[1]（図①A）．

1) GPC3の機能

膜結合型のGlypicanファミリーは，現在までのところ6種類が報告されている[7]．GPC3は，580アミノ酸からなる60 kDaのコア蛋白質に糖鎖修飾が加わった膜蛋白質で，C末端がglycosyl phosphatidyl inositol(GPI)アンカーにより形質膜に結合している．Piliaらは，X染色体(Xq26)連鎖疾患である巨人症の一つである，Simpson-Golabi-Behmel症候群(SGBs)において，*GPC3*の遺伝子変異を報告している．また，*GPC3*ノックアウトマウスでも，SGBsと同様に巨大化などの表現型を示すことが報告されている．GPC3は，ある種の腫瘍細胞では増殖を抑制したり，あるいはアポトーシスの誘導に関連があると報告されている[8]．近年，GPC3コア蛋白質が直接Wntと結合することにより，Wntシグナルを活性化し，肝細胞がんの増殖を促進することが報告されている[9]．

2) HCCがん組織におけるGPC3の発現と腫瘍マーカーとしての有用性

われわれは，*GPC3*遺伝子の発現量の差が，その遺伝子産物である蛋白質量の差として反映されているか否かをRT-PCR法，ならびに組織切片における免疫組織化学的解析を用いて確認した(図①B，C)．肝臓組織は，胎児期においてGPC3を発現するが出生後発現しなくなり，HCCにおいて再び発現するため，GPC3はがん胎児性蛋白質としての性格を有している．HCC患者の約40％の血液中に可溶性GPC3が検出されるが，健常人，慢性肝炎，その他の肝疾患ではまったく検出されず，HCCの腫瘍マーカーとして有用である[1]．またHCC切除後は，血清GPC3が消失することから，治療効果の判定などの臨床への応用が期待される．

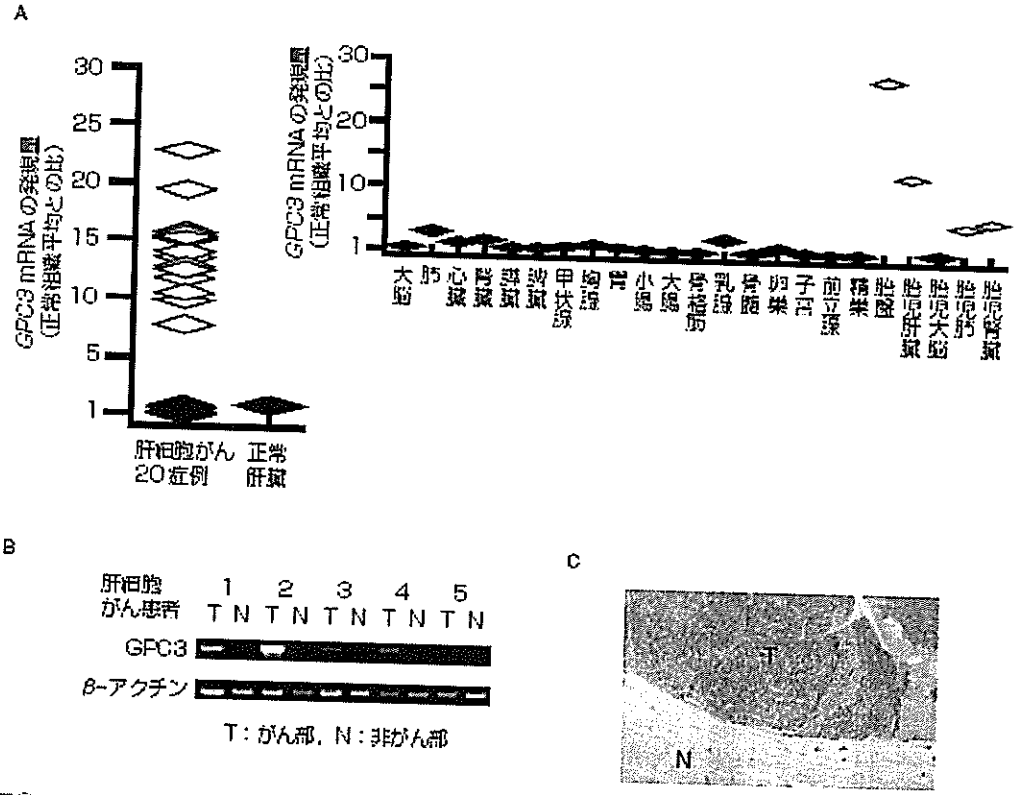
3 抗腫瘍免疫療法のターゲットとしてのGPC3の有用性

1) マウスにおける腫瘍免疫の解析

発現の組織特異性がすぐれていることから，中面らは，この新規がん胎児性抗原GPC3が，理想的な腫瘍拒絶抗原になり得るかどうかをマウスを用いて検討した．日本人の約60％が所有するヒト白血球抗原(human histocompatibility leukocyte antigen：*HLA*)遺伝子の産物の一つであるHLA-A24と，BALB/cマウスのクラスI分子のK^dに結合するペプチドの構造モチーフは，非常に類似していることがわかっている．さらに，ヒトとマウスのGPC3では，アミノ酸配列のレベルで95％以上のホモロジーを認めることから，ヒトとマウスのGPC3でアミノ酸配列が完全に一致し，HLA-A24，K^dのいずれにも結合すると予測されるGPC3由来のペプチドを合成した．このペプチドを骨髄由来樹状細胞(bone marrow-derived dendritic cell：BM-DC)に負荷してBALB/cマウスに免疫して解析することにより，K^d分子に結合して細胞傷害性T細胞(CTL)に提示される(K^d拘束性)CTLエピトープペプチドを同定した[2]．

このエピトープペプチドを負荷したBM-DCワクチンを腹腔内に予防的に投与したBALB/cマウスでは，コントロール群にくらべGPC3発現マウス大腸がん細胞株の増殖が著明に抑制された[2]．このエピトープペプチドはHLA-A24によっても提示され，ヒトでも同様にCTLエピトープとなる可能性があると思われた．

また，本村らは，マウス*GPC3*を遺伝子導入したマウスES細胞より分化誘導した樹状細胞(ES-DC-GPC3)を樹立した．ES-DC-GPC3をマウスに免疫することに



図① HCC20例のがん部，非がん部および多様な正常臓器における*GPC3*遺伝子発現のcDNAマイクロアレイ解析データ[6]とHCC組織における*GPC3* mRNAおよび蛋白質の発現(Nakatsura T *et al*, 2003[9]より改変引用)

A：HCC患者20例のがん部と非がん部における23,040種類の遺伝子の発現を比較検討し，さらに胎生期の4臓器を含む23臓器の正常組織において，各遺伝子の発現プロフィールを解析した．GPC3は，肝臓がん患者20例中16例でがん部／非がん部の発現の比が5以上(平均396.2)で，胎盤や胎生期の肝臓および腎臓に発現する以外は，ほとんどの成人の正常臓器に発現を認めない，がん胎児性抗原をコードする遺伝子であった．

B：HCC組織のがん部(T)と非がん部(N)における*GPC3* mRNAの発現の有無をRT-PCR法により検討したところ，がん部においてのみ*GPC3*遺伝子の発現を認めた．

C：HCC組織切片におけるGPC3蛋白質の発現を，抗GPC3抗体を用いた免疫組織化学的解析により確認した．

より，*in vivo*においてGPC3特異的なCTLが誘導され，移植されたGPC3発現がん細胞株の増殖と転移が抑制されることを報告した[8]．

2) HCC患者における腫瘍免疫の解析

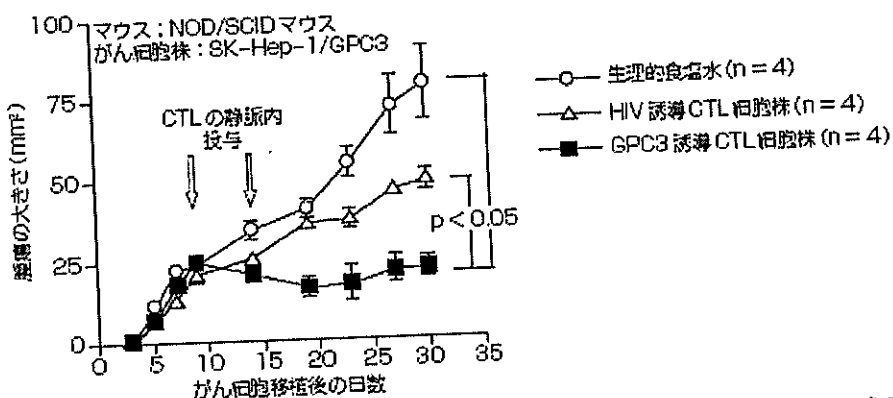
日本人のHLAークラスI対立遺伝子のうち，*HLA-A24*(*A*＊*2402*)は日本人の約60％が所有し，*HLA-A2*(*A*＊*0201*)は約20％が所有する，ありふれた対立遺伝子である．そこでヒトとマウスのGPC3に保存されたアミノ酸配列をもつペプチドで，HLA-A2(A＊0201)に結合すると推定されるGPC3由来の9～10個のアミノ酸からなるペプチドを9種類選択した．このうち，*HLA-A2*トランスジェニックマウス(HLA-A2Tgm)に最も強く，GPC3特異的CTLを誘導できるエピトープペプチドをELISPOTアッセイにて検討することにより，ペプチドA2-3：$GPC3_{144-152}$を同定した．さらに，このGPC3 A2-3ペプチドを負荷したBM-DCにて2回免疫したHLA-A2Tgmでは，重要臓器(脳，皮膚，心，肺，肝，腎)において自己免疫反応は生じておらず，その安全性が示唆された．

表① *HLA-A2* あるいは *HLA-A24* 陽性HCC患者(それぞれPt-A2，Pt-A24)の約50%において，GPC3特異的なCTLが誘導された．(Komori H *et al*. 2006[4]より改変引用)

患者	年齢	性別	がんの進行度※1	GPC3の発現※2	HLAの発現※3	CTLの誘導※4
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	+	+	−

患者	年齢	性別	がんの進行度※1	GPC3の発現※2	HLAの発現※3	CTLの誘導※4
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	+	+	+

※1 TNM分類を用いた．
※2 免疫染色を用いて，腫瘍周辺の正常組織と比較して発現を確認した．
※3 免疫染色により膜が染色された場合に，発現ありと判断した．
※4 GPC3発現HCC細胞株HepG2に対する細胞傷害活性が，E/T比20で20%以上観察された場合に，CTLを誘導できたと判断した．



図② 免疫不全マウスに移植したGPC3発現ヒトHCC細胞株に対するヒトCTL養子免疫療法の有効性(Komori H *et al*. 2006[4]より改変引用)
NOD/SCIDマウスの背部の皮下に，ヒトHCC細胞株SK-Hep-1に*GPC3*遺伝子を強制発現させたSK-Hep-1/GPC3を1×10^7個移植し，移植後9日目に5×5mmの大きさになった時点と，その5日後(移植後14日目)にCTLを8×10^7個，計2回i.v.投与した．HCC患者のPBMCをGPC3エピトープペプチドで刺激して誘導したCTL投与群(■)と，コントロールとしてHIVエピトープペプチドで誘導したCTL投与群(△)，生理食塩水のみを投与した群(○)のあいだで比較すると，GPC3特異的CTL投与群ではコントロール群にくらべ，有意に腫瘍の増殖が抑制されていた．