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

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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

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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. 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.

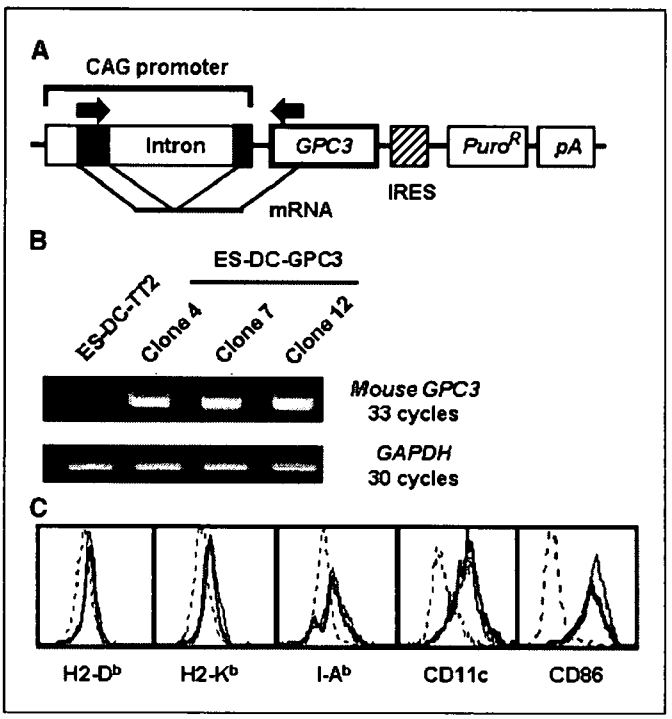

Figure 1. Establishment of ES-DC genetically modified to express murine glypican-3. *A*, structure of pCAGGS-GPC3-IRES-puro-R vector. To obtain pCAGGS-GPC3-IRES-puro-R, a cDNA fragment, including a full-length cDNA of murine *glypican-3*, was inserted into a mammalian expression vector pCAGGS-IRES-puro-R 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.

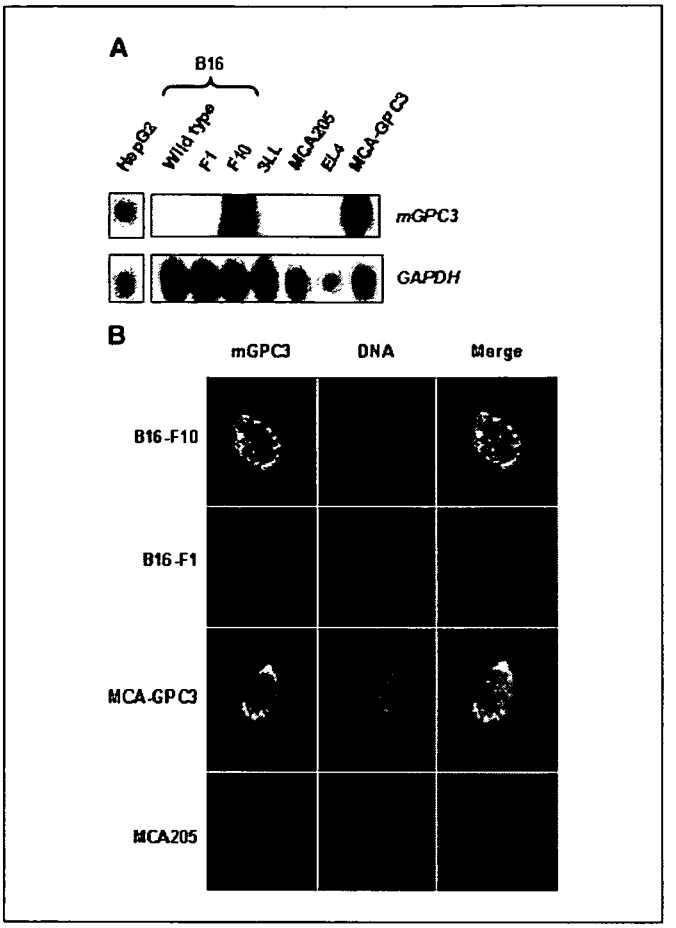

Figure 2. Expression of glypican-3 in cancer cell lines. *A*, Northern blot analysis of *glypican-3* mRNA in a human hepatocellular carcinoma cell line HepG2 (positive control) and various cancer cell lines of C57BL/6 origin. The same filters were stripped and rehybridized with *GAPDH* cDNA to assess 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*).

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

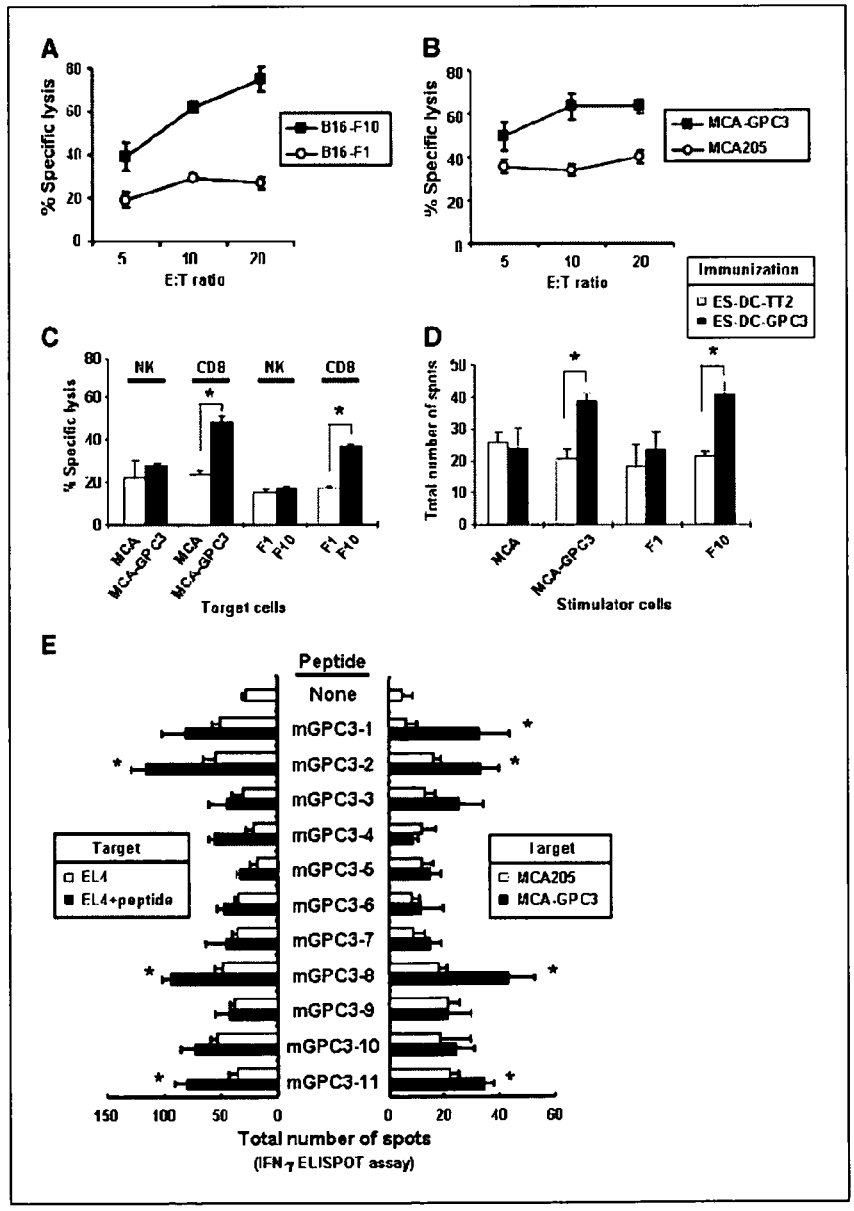

Figure 3. Priming of antigen-specific CTLs with ES-DC-GPC3. The mice were transferred i.p. twice with 1×10^5 ES-DC-GPC3 on days −14 and −7. On day 0, spleen cells from immunized mice were isolated and cultured with 1×10^5 ES-DC-GPC3 per well in the presence of recombinant human IL-2 (100 units/mL) for 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*).

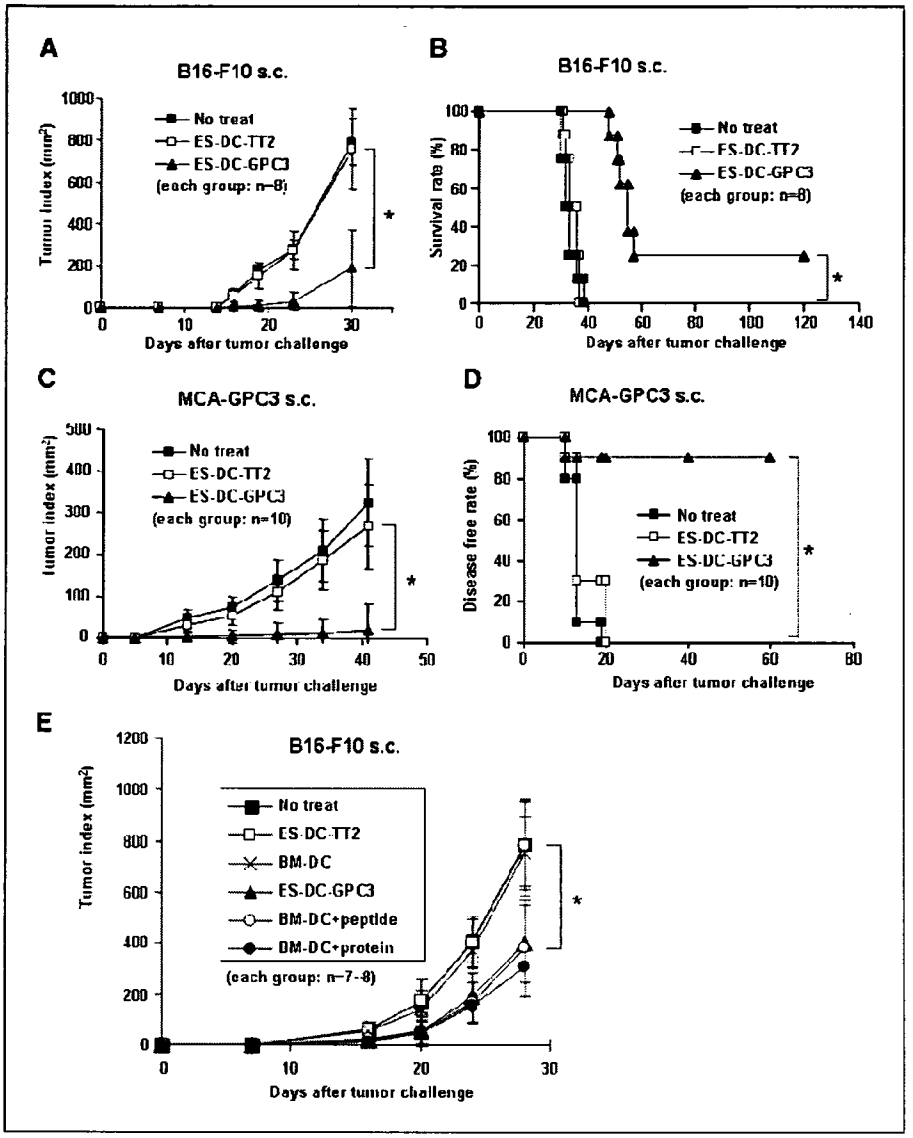

Figure 4. Suppression of tumor growth and prolongation of survival by preimmunization with ES-DC-GPC3. The mice were transferred i.p. twice with 1×10^5 ES-DC-GPC3 or ES-DC-TT2 on days −14 and −7. On day 0, the mice were challenged s.c. with 5×10^3 B16-F10 (*A* and *B*) or 1×10^5 MCA-GPC3 (*C* and *D*) 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

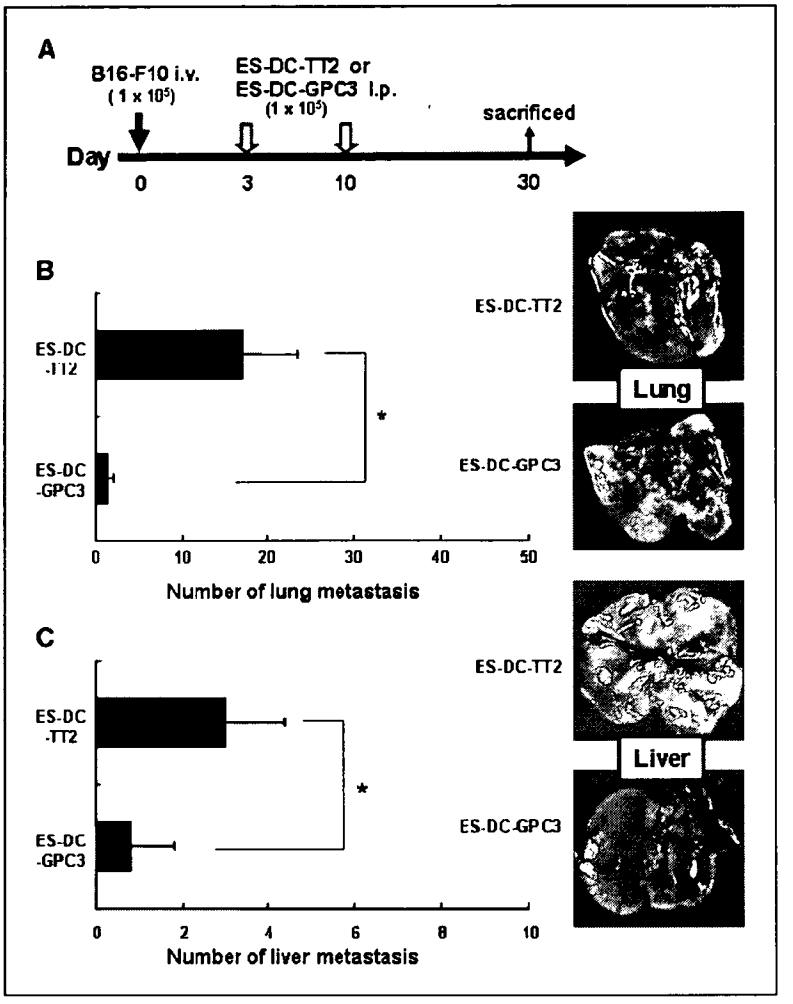

Figure 5. Suppression of tumor growth in the metastatic tumor model of B16-F10. The protocol for therapeutic immunotherapy model was indicated in (*A*). All mice were injected into tail vein with 1 × 10^5 F10 cells on day 0. On days 3 and 10, mice were injected i.p. with 1 × 10^5 ES-DC-TT2 or ES-DC-GPC3. 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

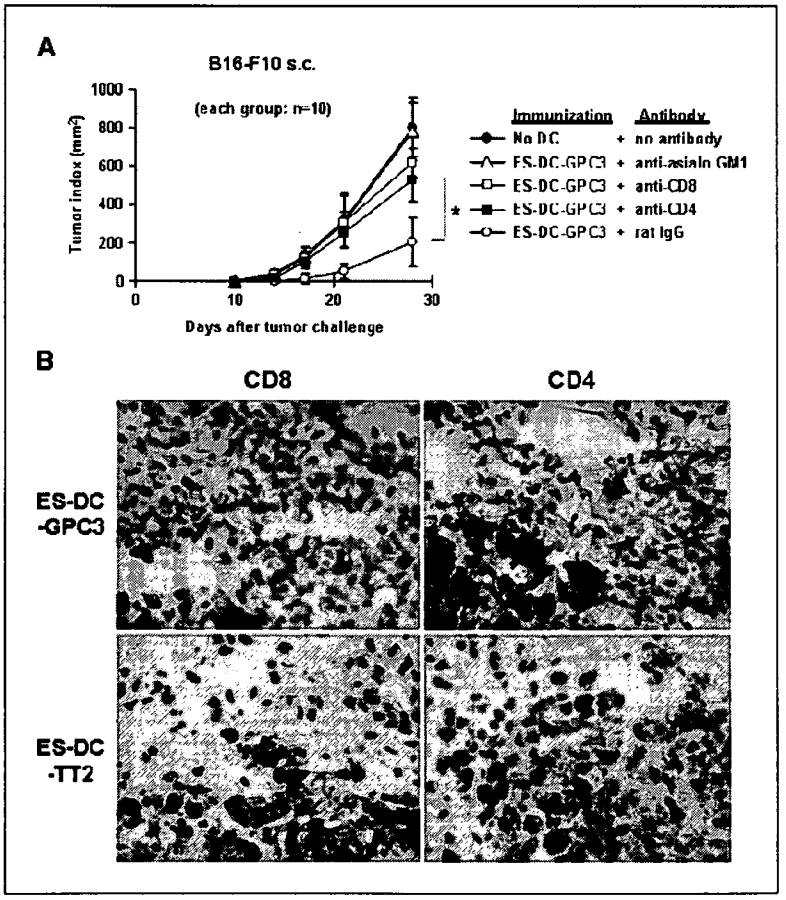

Figure 6. Involvement of both $CD4^+$ and $CD8^+$ T cells in antitumor immunity induced by ES-DC-GPC3. *A*, $CD4^+$ or $CD8^+$ T cells were depleted *in vivo* by the inoculation of anti-CD4 or anti-CD8 mAbs during immunization with ES-DC-GPC3. The mice were challenged s.c. with 5×10^3 F10 tumor cells, 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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特集

5. 皮膚科医のための臨床トピックス

悪性黒色腫の新しい血清マーカー

glypican-3とSPARC

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悪性黒色腫の新しい血清マーカー
glypican-3 と SPARC

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要約　悪性黒色腫(メラノーマ)の新しい血清マーカーとして glypican-3(GPC 3)と SPARC を同定した．GPC 3 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 アンカー蛋白であるが，肝細胞癌とメラノーマに特異的に発現している．血清 GPC 3 はメラノーマの約 40%で検出されるが，健常人では検出されない．一方，SPARC はシステインに富む酸性分泌蛋白で，メラノーマに高頻度に発現している．血清 SPARC はメラノーマの約 30%で検出されるが，健常人ではほとんど検出されない．両者は早期メラノーマにおいても検出され，いずれかが陽性を示す症例は 0 期 88%，I 期 48%，II期 71%である．両者を併用することで早期メラノーマが血清学的に診断可能になる．

キーワード　メラノーマ，腫瘍マーカー，glypican-3(GPC 3)，SPARC

影下登志郎，他：臨皮 60(5 増)：169-172，2006

はじめに

腫瘍マーカーは癌細胞または癌細胞と免疫応答をしている細胞から産生される物質である．腫瘍マーカーは悪性腫瘍の診断や転移巣の検出などに広く応用されている．

現在，悪性黒色腫(メラノーマ)の血清腫瘍マーカーとしては感度や特異性の点から 5-S-cysteinyldopa (5-S-CD)，melanoma inhibitory activity(MIA)，S 100 β 蛋白などが用いられている[1~4]．しかしながら，これらのマーカーは主として進行期で検出されるものが多い．そのため，診断よりも転移巣の検出や経過観察のモニターとして用いられる．悪性黒色腫は再発・転移を生じやすいので，それらを早期発見できる腫瘍マーカーの意義は大きく，新たなマーカーの発見が望まれる．

最近，筆者らは早期メラノーマにおいても検出できる新たな血清マーカーを同定したので紹介する．

GPC 3

GPC 3(glypican-3)はヘパラン硫酸プロテオグリカンファミリーに属する 60 kDa の糖鎖修飾が強い GPI アンカー蛋白である．その機能は不明

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* New melanoma serum markers：Glypican-3 and SPARC

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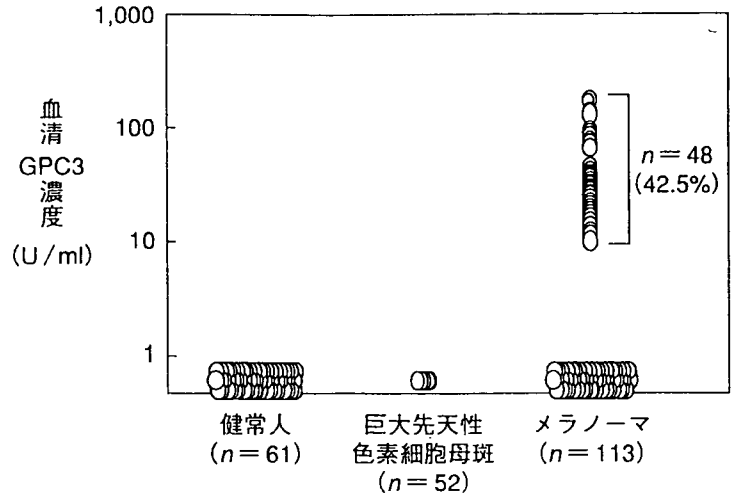

図1　健常人，巨大先天性色素細胞母斑，メラノーマでのGPC 3の比較

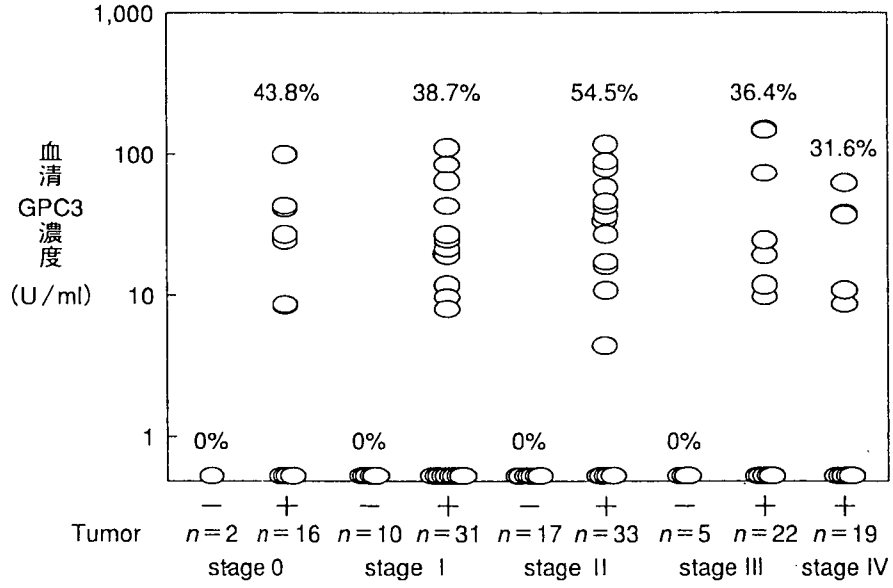

図2　メラノーマ患者血清中の各ステージごとのGPC 3の陽性率

であるが，ヒトの*GPC3*遺伝子変異がX染色体関連疾患で巨人症，嚢胞腎などを呈するSimpson-Golabi-Behmel症候群患者で見いだされている．また，*GPC3*遺伝子破壊マウスは胎生期に巨大化し，種々の奇形を発現，周産期に死亡する．さらに，ある種の腫瘍細胞では増殖の抑制やアポトーシスの誘導に関連する．

*GPC 3*は最近DNAマイクロアレイで肝細胞癌に特異的に発現している遺伝子として同定された[5]．*GPC 3*は肝細胞癌の80%に発現，胎生期では肝臓，成人では胎盤のみに発現する，いわゆる癌胎児性抗原の一種である．

*GPC 3*は肝細胞癌以外の癌では発現していないが，例外的にメラノサイト系細胞に発現している．すなわち，培養メラノーマ細胞や色素細胞母斑・メラノーマ組織の約80%にmRNAおよび蛋白レベルで発現がみられる[6]．

血清マーカーとしてのGPC 3

GPC 3は培養肝細胞癌細胞や培養メラノーマ細胞から分泌され，モノクローナル抗体を用いたELISA法で検出することができる[5,6]．さらに，肝細胞癌やメラノーマ患者の血清中でもGPC 3蛋白を検出することができる．術前メラノーマ患者血清では113人中48人(42.5%)にGPC 3蛋白が検出されたが，術後はすべて陰性化した(図

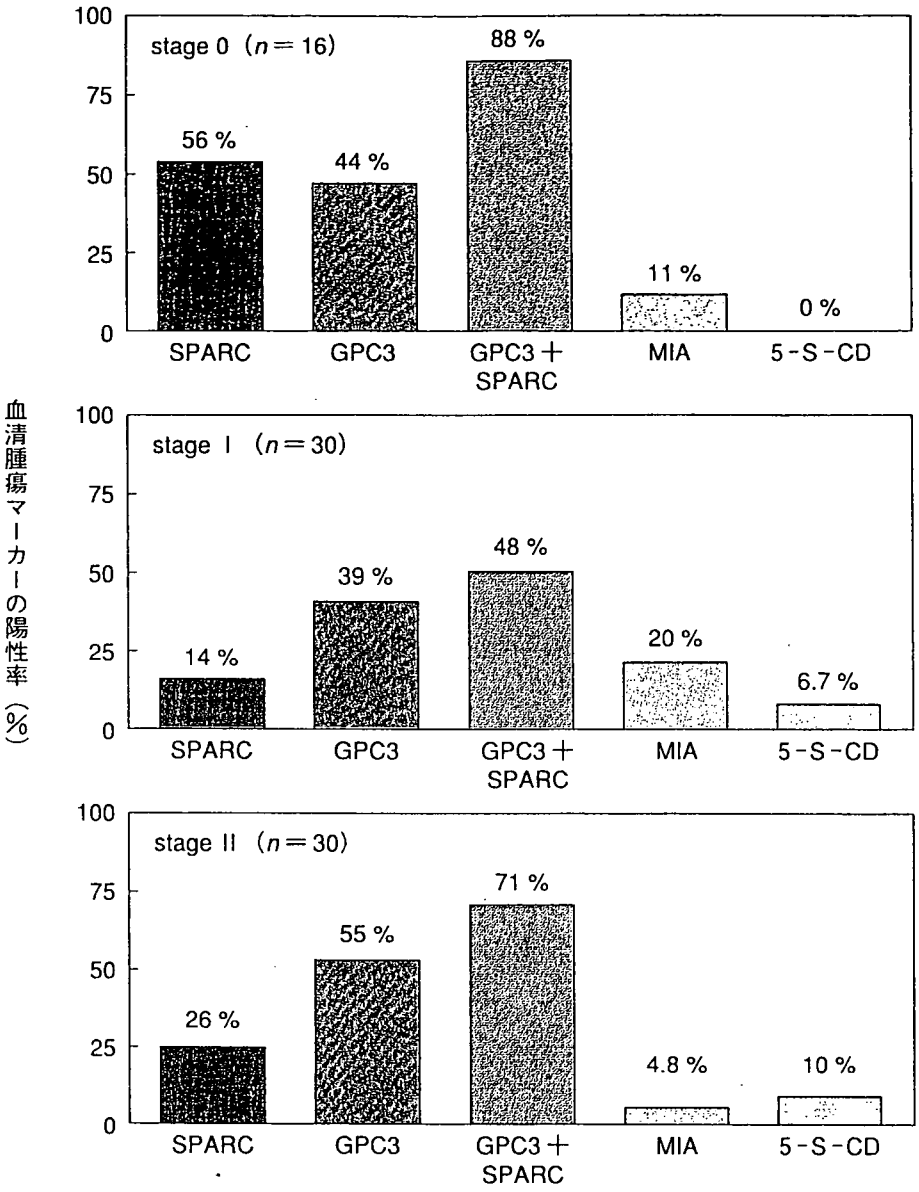

図3　SPARC，GPC 3，MIA，5-S-CD のメラノーマ各ステージでの陽性率の比較

1)[6]．陽性率は 0，I，II，III，IV期でそれぞれ 43.8，38.7，54.5，36.4，31.6%であった(図 2)．病期別での陽性率に差は認められなかったが，これまでの腫瘍マーカーと異なり，表皮内メラノーマである 0 期でも約 50%の症例で陽性を示した点が特異である．一方，mRNA および蛋白レベルで発現がみられる巨大色素細胞母斑ではまったく検出されず，その他の癌患者や健常人でも検出されず，メラノーマおよび肝細胞癌に特異性が極めて高い(図 1)．

SPARC

SPARC(secreted protein, acid and rich in cystein)は osteonectin や BM-40 とも呼ばれ，280 アミノ酸からなる 43 kDa のシステインに富む酸性分泌蛋白である．発現は骨のオステオブラストや血小板，創傷部位，さらには腫瘍内の間質細胞にみられる．メラノーマでは腫瘍細胞自体に高発現し，発現は腫瘍の進行に相関している[7]．また，色素細胞母斑でも mRNA および蛋白レベルで発現している．SPARC の機能は創傷治癒，細胞接着・増殖，分化，血管増生など細胞外基質との調節作用が示唆されている．

血清マーカーとしての SPARC

血清中の SPARC はモノクローナル抗体を用

いたELISA法で検出できる．SPARCは血小板にも存在するため，健常人においても血清に少量ながら検出される．そのため血漿での測定が望ましい．健常人の平均＋2SDを正常上限とすると，術前メラノーマ患者117人中38人(33%)，巨大色素細胞母斑6人中2人(33%)，健常人61人中3人(5%)で陽性となった[8]．病期別では0，I，II，III，IV期の陽性率はそれぞれ56，14，26，36，47%であった．GPC3同様，表皮内メラノーマでも検出できるが，病勢による上昇は認められない．術後は速やかに大部分の症例で低下する．

GPC3とSPARCの最大の特徴は，従来のメラノーマ腫瘍マーカーであるMIA，5-S-CDに比べ早期でも陽性を示すことである．GPC3とSPARCのいずれかが陽性になれば，第0，I，II期での検出率は88，48，71%となる(図3)[8]．一方，MIAの陽性率は11，20，5%，5-S-CDは0，7，10%である．

血清GPC3・SPARCはメラノーマの進行度とは相関せず，腫瘍量とも無関係である．これは両者がメラノーマの進展よりも発生に重要な役割を演じていることを示唆する．メラノーマ細胞におけるGPC3・SPARCの機能や分泌のメカニズムの解明は今後の課題である．

おわりに

メラノーマの新しい血清マーカーとしてGPC3とSPARCが有用であることが示唆された．両者を組み合わせて用いることで，血清学的に早期メラノーマの80%以上が診断可能となる．今後は多施設での追試や，臨床的にメラノーマとの鑑別が重要であるSpitz母斑や異型母斑を含めた検討が必要である．

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陪席医の一日

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やはり教授診を間近でみることのできる陪席医を経験することは有意義だと思います．具体的には，臨床，病理診断だけでなく，問診に始まり皮疹を観察するやり方，処方，必要な検査を的確にオーダーする等々です．しかしながら，診察時間内に教授診の難しい臨床や病理を理解するのは不可能です．教授診前日までには，処方内容や病理組織をチェックする，さらに難しい症例，貴重な症例があればそれらに関する文献を読み，また患者検体の解析結果もそろえておかなければなりません．

いよいよ診察が始まれば，教授自ら処置，注射をし，時間が許せば生検，切除までもされる．また，皮膚症状に合わせた微妙な処方内容の変更も勉強になります．そういう診察医としての姿勢や手技，知識を大いに学んでいます．診察中，教授から「アトピーに合併した○○は何例くらいあった？」などと聞かれることもあるし，答えられるように準備しておかなければなりません．診察が終われば，夜は患者リンパ球の表面マーカー解析や培養が待っています．また，貴重な症例があれば論文も書かなければなりません．こうして陪席医の一日が終わるのですが，夜の研究は半分楽しみながらやっているのも事実です．

陪席医を通じて得たことは，臨床症例1例1例を大切にして詳細に観察すること，日夜研究を続けその成果を臨床に還元していく姿勢です．そういうことをたたき込まれながら日々成長しているような気がいたします．

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Glypican-3（GPC3）を標的とした免疫療法の有用性の検討

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Possibilities of Glypican-3-Specific Immunotherapy for Hepatocellular Carcinoma: Hiroyuki Komori[*1,2], Tetsuya Nakatsura[*3], Toru Beppu[*1], Yasuharu Nishimura[*2] and Hideo Baba[*1] (*[1]*Dept. of Gastroenterological Surgery, and* *[2]*Immunogenetics, Graduate School of Medical Sciences, Kumamoto University,* *[3]*The Immunotherapy Section, Investigative Treatment Division, Center for Innovative Medicine, National Cancer Center East*)

Summary

The patients with hepatitis B or C based liver cirrhosis are at high risk for developing Hepatocellular carcinoma (HCC), and HCC patients treated surgically or by other therapies are also at high risk for recurrence. As a result, the prognosis of HCC remains poor, and new therapies for the prevention of cancer development and recurrence are urgently needed. We previously reported that glypican-3 (GPC3) was over expressed specifically in HCC. In this report, we found the HLA-A2 or HLA-A24 restricted GPC3 epitope peptide, and investigated whether these peptides could induce GPC3 reactive CTLs from the peripheral blood mononuclear cells (PBMCs) of HLA-A2$^+$ or HLA-A24$^+$ HCC patients. We used HLA-A2.1 (HHD) transgenic mice (Tgm) to identify the HLA-A2-restricted GPC3 epitopes. We found that these epitope peptides could induce peptide-reactive CTLs in about 50% of HLA-A2$^+$ or HLA-A24$^+$, and GPC3$^+$ HCC patients. Our study raises the possibility that these GPC3 peptides may therefore be applicable to cancer immunotherapy for prevention of cancer development and recurrence of HCC.

Key words: GPC3, HCC, Immunotherapy

要旨 肝細胞癌は治療後も高頻度に再発を繰り返すため予後不良な癌であり，発癌予防や治療後の再発予防のために有効な治療法の確立が望まれる。GPC3は肝細胞癌に高発現し，正常組織にはほとんど発現しないため，腫瘍免疫のターゲットとして理想的な癌特異的抗原である。今回最も抗腫瘍活性が高く，自己免疫現象を誘導しないHLA-A2もしくはHLA-A24拘束性CTLエピトープペプチドを同定し，GPC3をターゲットとした肝癌に対する免疫療法の有用性を検討した。HLA-A2エピトープペプチドに関しては，HLA-A2トランスジェニックマウスを用いて最も効果的にCTLを誘導できるものを決定し，さらにHLA-A2$^+$もしくはHLA-A24$^+$患者におけるCTL誘導の有無を検討した。それぞれの肝癌患者末梢血単核細胞からGPC3特異的ヒトCTLが半数以上の症例で誘導可能であった。GPC3はウイルス性肝硬変患者の発癌予防や術後再発予防における免疫療法のターゲットとして期待される。

はじめに

glypican-3 (GPC3) は肝細胞癌特異的に高発現し，近年において腫瘍マーカーとしての有用性を報告されている[1]。今回，GPC3がHCC患者に対する免疫療法のターゲットとして有用か否かを検討した。

I. 方 法

エピトープ候補ペプチドの決定: HLA-A24拘束性のエピトープは以前同定したペプチド $GPC3_{298-306}$ (EYILS-LEEL) を使用した[2]。HLA-A2拘束性エピトープは，マウスとヒトとで共通の結合モチーフをもち，HLA-A2分子への結合力の高いペプチド9種類をHLA-A2トランスジェニックマウスを免疫することで決定した。

免疫したマウスの自己免疫現象の検討: 免疫したマウスの重要臓器に対するリンパ球浸潤の有無を免疫染色し検討した。

HCC患者におけるCTLの誘導の検討: 熊本大学医学部消化器外科にて治療中のHLA-A2$^+$もしくはHLA-A24$^+$のHCC患者からインフォームド・コンセントを得た後，

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0385-0684/06/¥500/論文/JCLS

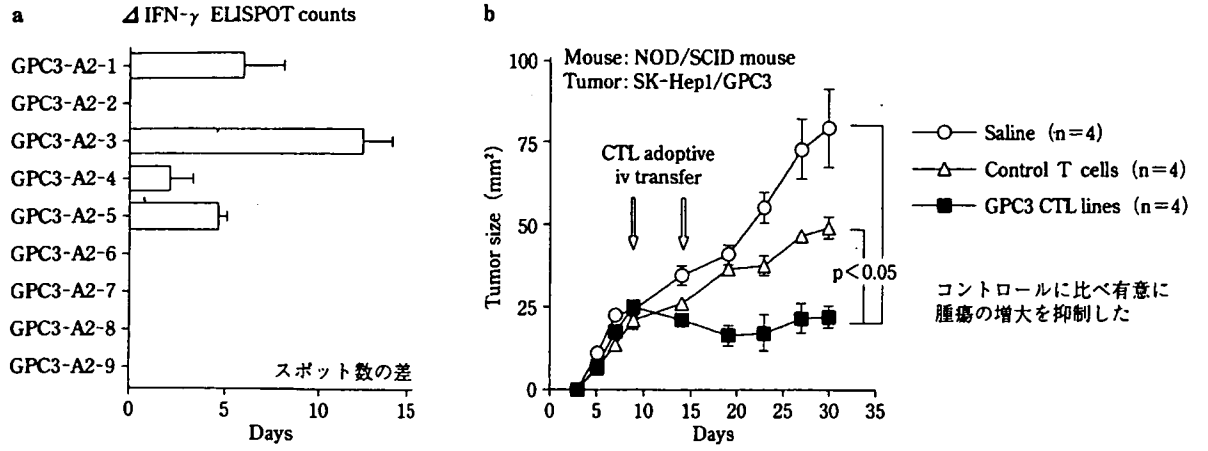

図1 GPC3-A2エピトープペプチドの決定とその抗腫瘍効果
a: ELISPOT assayによるエピトープペプチドの検討。GPC3-A2-3ペプチドを提示させたBM-DCをターゲットとした場合においてコントロールに比べ有意にIFN-γを産生した。
b: 免疫不全マウスであるNOD/SCIDマウスに移植したGPC3発現腫瘍に対する増殖抑制効果の検討。GPC3エピトープペプチドで誘導したCTLを投与すると有意に腫瘍の増大を抑制した。

HLA-A2⁺患者10人，HLA-A24⁺患者12人から血液サンプル30〜50 m*l*を採取した。そのうちCD14⁺細胞から樹状細胞を誘導しエピトープを提示させ，CD8⁺細胞を1週間ごと3回刺激した後特異性をCr release assay，ELISPOT assayにて検討した。

養子免疫療法に関する検討: 免疫不全マウス（NOD/SCIDマウス）にGPC3を移入したヒト肝癌細胞株SK-Hep1/GPC3を移植し5×5 mmの大きさとなった後，GPC3エピトープで誘導したHCC患者由来のCTLを2回iv投与し，腫瘍縮小効果を検討した。コントロールとしてHIV由来ペプチド誘導CTLもしくは生理食塩水のみを投与し比較した。

II. 結　果

エピトープ候補ペプチドの決定: ELISPOT assayを用いて検討した結果HLA-A2拘束性のCTLエピトープ候補として，$GPC3_{144-152}$(FVGEFFTDV)を同定した。図1aはこのペプチドを提示させた同種骨髄由来樹状細胞に対するスポットがコントロールと比較し，最も差があることを示す。

免疫したマウスの自己免疫現象の検討: GPC3由来ペプチドを用いた樹状細胞ワクチンにて免疫されたトランスジェニックマウスの重要臓器には，CD8⁺細胞やCD4⁺細胞の浸潤は認められず，自己免疫反応は生じていないと判断した。

HCC患者におけるCTL誘導の検討: 実際のHCC患者からのCTL誘導に関しては，HLA-A2拘束性エピトープペプチドの$GPC3_{144-152}$(FVGEFFTDV)を用いて10人中5人，HLA-A24拘束性エピトープペプチドの$GPC3_{298-306}$(EYILSLEEL)を用いて12人中6人からGPC3特異的CTLが誘導できた（表1）。

養子免疫療法に関する検討(図1b): 各群4匹ずつ検討したところ，GPC3エピトープで誘導したヒトCTL投与群がコントロールのHIV由来ペプチド誘導CTL投与群や生理食塩水投与群に比較し有意に増殖を抑制した。

III. 考　察

GPC3はWntシグナルを介してHCCの癌化と増殖に重要であると近年報告されている[3]。肝癌細胞の悪性形質転換に重要な役割を担っている分子で癌抗原として免疫系からの逃避が起こりにくい抗原であると考えられ，ゆえにGPC3はHCCの癌抗原として非常に有用であると考えられる。われわれは今回HLA-A2もしくはHLA-A24拘束性のGPC3由来CTLエピトープを同定し，これらのペプチドを用いてHCC患者の末梢血から約50%の頻度でGPC3に反応するCTLを誘導できた。これらのペプチドによるワクチンはHLA-A2 Tgmに自己免疫反応を引き起こさなかった。

今回の実験では，最も効果的にCTLを誘導し得るメジャーエピトープを決定することが目的であったため，マウスの免疫では，9種類のHLA-A2拘束性ペプチドの混合物を提示させたBM-DCを投与し，最も強い反応を示すペプチド$GPC3_{144-152}$(FVGEFFTDV)について検討した。このペプチド以外にもELISPOTにて差がみられるペプチドが数個存在したが，これらのペプチドについてはエピトープとなり得るか否かを今後検討したい。また，HLA-A2拘束性のペプチドを選択する際マウスGPC3とヒトGPC3とで共通のアミノ酸配列をもつペプチドを

表 1 HLA-A2⁺ もしくは HLA-A24⁺ 肝癌患者の約 50%において GPC3 特異的な CTL が誘導された

Patients	Age	Gender	State of tumor†	GPC3 expression*	CTL induction**
Pt-A2-1	80	F	IIIa	+	+
Pt-A2-2	72	M	II	+	+
Pt-A2-3	67	F	II	ND	+
Pt-A2-4	54	M	I	+	+
Pt-A2-5	57	M	I	ND	−
Pt-A2-6	66	M	I	−	−
Pt-A2-7	54	M	IIIa	+	−
Pt-A2-8	73	M	II	ND	+
Pt-A2-9	68	F	IIIa	+	−
Pt-A2-10	54	M	II	+	−
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	+
Pt-A24-5	52	M	IVb	−	−
Pt-A24-6	65	M	I	ND	+
Pt-A24-7	61	M	I	ND	+
Pt-A24-8	74	M	II	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	+	+

†: TNM 分類を用いた。
*: 免疫染色を用いて腫瘍周囲組織と比較した。
**: GPC3 発現肝癌細胞株 HepG2 に対する細胞傷害活性が，E/T 比 20 で 20%以上を CTL が誘導できたと判断した。

選んだが，ヒトのみにプロセスされるペプチドを見落としている可能性はあると思われる。

われわれを含めた複数の施設より GPC3 は早期の HCC でも発現し，早期の HCC の診断にも有用であると報告されている。早期から発現することで B，C 型肝炎や肝硬変から発生した肝癌の早期治療においても期待できると考えられる。

今回の検討では，約 5 割の患者から GPC3 に反応し得る CTL が誘導できた。腫瘍組織における CTL の浸潤や GPC3 反応性の CTL の誘導とその患者の予後との相関について，初発の HCC 患者 7 名に関して検討したが，相関は認められなかった。現段階では CTL の誘導と予後，脈管浸潤との相関は認めなかったが症例を増やし検討する予定にしている。

図 1b に示すように，NOD/SCID マウスに SK-Hep1/GPC3 を移植し，GPC3 反応性 CTL の養子免疫すると，コントロールに比べ有意に腫瘍の増殖を抑制した。しかしながら，二度目の養子免疫の 2 週間後から再び増殖が認められたことから，養子免疫を繰り返すことが必要と考えられた。

ま と め

われわれは HCC の新たな治療として，GPC3 をターゲットとした免疫療法の可能性を検討した。HCC の再発，進行などを予防できる有効な治療法として期待できる。

本論文の要旨は第 27 回癌免疫外科研究会において発表された。

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Highly Sensitive Detection of Melanoma at an Early Stage Based on the Increased Serum Secreted Protein Acidic and Rich in Cysteine and Glypican-3 Levels

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Abstract **Purpose:** There are no available tumor markers detecting primary melanoma at an early stage. The identification of such serum markers would be of significant benefit for an early diagnosis of melanoma. We recently identified glypican-3 (GPC3) as a novel tumor marker but could diagnose only 40% of melanomas. Thereby, we focused out attention on secreted protein acidic and rich in cysteine (SPARC) overexpressed in melanoma as another candidate for tumor marker.

Experimental Design: Secreted SPARC protein was quantified using ELISA in the sera from 109 melanoma patients, five patients with large congenital melanocytic nevus, 61 age-matched healthy donors, and 13 disease-free patients after undergoing a surgical removal. We also quantified GPC3 and 5-*S*-cysteinyldopa in the same serum samples and compared these markers for their diagnostic value.

Results: The serum SPARC concentrations in melanoma patients were greater than those in healthy donors ($P = 0.001$). When we fixed a cutoff value at the mean concentration plus 2 SD of the healthy donors, the serum SPARC was found to have increased in the sera of 36 of the 109 (33%) melanoma patients, whereas there were three (4.9%) false-positive cases of 61 healthy donors. Surprisingly, 19 of 36 patients showing increased SPARC levels were in stages 0 to II. The serum SPARC level decreased under the cutoff level in 10 of 13 patients after surgical removal. Using SPARC and GPC3 in combination thus enabled us to diagnose 47 of 75 (66.2%) melanoma patients at an early stage (0-II).

Conclusions: SPARC or its combination with GPC3 is thus considered a potentially useful tumor marker, especially for melanoma at an early stage.

The incidence rates for melanoma have steadily increased worldwide, and the mortality rates have increased as well. Several molecules have been evaluated as tumor markers to detect melanoma (1-3). Recently, several investigators reported the 5-*S*-cysteinyldopa and melanoma-inhibitory activity to be useful as a marker for melanoma progression or for monitoring metastatic melanoma (4-9). However, current methods are not sensitive enough to detect melanoma in its early stages. Thus, there is a need for new tumor markers that can detect primary melanoma in the early stages. We recently reported that glypican-3 (GPC3), which is overexpressed in melanoma, is a novel tumor marker for melanoma (10).

Secreted protein acidic and rich in cysteine (SPARC), also called osteonectin or BM-40, is a matricellular glycoprotein that modulates cellular interaction with the extracellular matrix during tissue remodeling (11). SPARC was overexpressed in primary and metastatic melanomas, and an overexpression of SPARC by melanoma cell was associated with an invasive phenotype *in vivo* (12, 13). In this study, we detected SPARC in the sera of melanoma patients at higher concentrations than in healthy donors. Indeed, SPARC was detected in the sera of 33% of all melanoma patients, irrespective of the clinical stages and even in the sera of patients with stage 0 *in situ* melanoma. Moreover, the combined use of SPARC and GPC3 will thus make it possible to diagnose melanoma, especially in the early stages (0-II).

Materials and Methods

Tissues, blood samples, and cell lines. After receiving their informed written consent, we obtained tissue, serum, and plasma samples from the patients with melanoma and large congenital melanocytic nevus treated at the Department of Dermatology, Graduate School of Medical Sciences, Kumamoto University from 1997 to 2004. We also obtained 61 serum samples and 21 plasma samples from age-matched and

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sex-matched healthy donors from Hiraki hospital (Kumamoto, Japan) after receiving their informed written consents. All samples were anonymized, numbered at random, and stored at −80°C until use. We collected the patient profiles from medical records to determine the clinical stages, according to the Unio Internationale Contra Cancrum/American Joint Committee on Cancer tumor-node-metastasis classification (14). The subjects consisted of 113 consecutive and preoperative patients with melanoma comprising 52 male and 61 female patients with an average age of 67 years (range, 22-91 years): 15 had stage 0 melanoma (*in situ*); 30 had stage I melanoma; 30 had stage II melanoma; 19 had stage III melanoma; and 19 had stage IV melanoma. Five patients with large congenital melanocytic nevus consisted of four male and one female patients with an average age of 21 years (range, 4-38 years). All patients were of Japanese nationality.

Melanoma cell lines CRL1579, G361, HMV-I, and SK-MEL-28 were kindly provided by the Cell Resource Center for Biomedical Research Institute of Development, Aging, and Cancer, Tohoku University (Sendai, Japan), and 888mel and 526mel were provided by Dr. Yutaka Kawakami (Keio University, Tokyo, Japan). The cell lines were cultured in DMEM or RPMI 1640 supplemented with 10% FCS. Human epidermal melanocytes, neonatal (HEMn), in culture medium 154S supplemented with human melanocyte growth supplements, were purchased from KURABO (Osaka, Japan).

Quantitative reverse transcription-PCR. The *SPARC* mRNA levels were analyzed using real-time reverse transcription-PCR, as described previously (15). We designed *SPARC* gene-specific PCR primers to amplify the fragments of 374 bp; *SPARC* PCR primer sequences were sense, 5′-CGAAGAGGAGGTGGTGGCGGAAAA-3′ and antisense, 5′-GGTTGTTGTCCTCATCCCTCTCATAC-3′. Reaction mixtures contained 2 μL of DNA Master SYBR green I, 1 mmol/L $MgCl_2$, 0.4 μmol/L of each primer, and 1 μL of cDNA in a total volume of 20 μL. The PCR cycles were 95°C for 10 minutes followed by 35 cycles of 95°C for 1 second, 68°C for 1 second, and 72°C for 16 seconds. For *β-actin*, we used LightCycler-Primer Set Human *β-actin* (Search LC, Heidelberg, Germany). The quantification was achieved by comparisons with an internal standard curve containing 10-fold dilutions of HEMn cDNA probe. The relative expressions of *SPARC* mRNA were calculated as the ratio of the *SPARC/β-actin* expressions from three replicate reverse transcription-PCR experiments.

Western blot analysis and immunohistochemical examination. SDS-PAGE and Western blotting were done as described previously (16). The membranes were incubated with anti-SPARC monoclonal antibody AON-5031 (Haematological Technologies, Inc., Essex Junction, VT). Immunohistochemical examination was done using the DakoCytomation EnVision+ System according to the manufacturer's instructions with minor modifications, as described previously (17). Briefly, 4-μm-thick paraffin sections were cut and stained with AON-5031 at a dilution of 1:2,000 (0.216 μg/mL). After washing, the sections were incubated for 60 minutes with polymer/horseradish peroxidase-labeled goat anti-mouse IgG at room temperature. 3,3′-Diaminobenzidine tetrahydrochloride was used as the chromogen. The intensity of staining was classified as weak; weaker than the adjacent epidermis, moderate; same as the adjacent epidermis and strong; and stronger than the adjacent epidermis. These samples were estimated independently by two observers in a blinded manner (T.K. and S.F.).

Double-determinant (sandwich) ELISA. The SPARC concentrations in the culture supernatants of melanoma cell lines, sera, and plasma were measured by ELISA in duplicated wells in each plate assay. ELISA was done as described previously (16). All samples were tested in a blinded manner. We used mouse anti-human SPARC monoclonal antibody ON1-1 (Zymed Lab, South San Francisco, CA) with 0.05 μg/well and biotinylated polyclonal goat anti-human SPARC antibody EYR01 (R&D Systems, Minneapolis, MN) with 0.01 μg/well. To obtain a serum-free culture supernatant, cells were grown to near confluence, washed twice with PBS, and kept in a serum-free medium. After 24 hours, the medium was collected and centrifuged for 10 minutes at 375 × *g* to remove debris. The samples were divided and diluted at 1:4 with 10% Block Ace (Dainippon Pharmaceutical, Osaka, Japan) to serve as samples for ELISA. The serum and plasma samples were diluted at 1:200 with 10% Block Ace as described above. In this ELISA system, human SPARC HON-3030 (Haematological Technologies) was used to estimate the standard curve to quantify the SPARC protein based on absorbance data.

Statistical analysis. We analyzed all of the data using the StatView statistical program for Macintosh (SAS, Cary, NC) and then evaluated the statistical significance using Student's *t* test, χ^2, and Fisher's exact test. Because the SPARC concentration values exhibited a normal distribution in each group, the values were analyzed using Student's *t* test. We considered $P < 0.05$ to be statistically significant.

Results

Expression of SPARC mRNA and protein in human melanoma. The expression levels of SPARC in various melanoma cell lines were determined by quantitative reverse transcription-PCR (Fig. 1A) and Western blot (Fig. 1B). SPARC was expressed in all cell lines tested, except for HMV-1, in both mRNA and protein levels. SPARC proteins in the human tissue specimens were examined by Western blotting (Fig. 1C) and an immunohistochemical analysis (Fig. 1D). The vertical growth phase of primary melanomas and lymph node metastasis expressed a large amount of SPARC, whereas large congenital melanocytic nevi and radial growth phase of primary melanomas showed a moderate expression. Normal skin, including a few melanocytes, showed a weakly positive expression (Fig. 1C). Hence, all the examined tissue samples of melanomas and large congenital melanocytic nevi were positive for SPARC protein. An immunohistochemical analysis of SPARC was made on primary melanomas (33 cases), metastatic melanomas (seven cases), and melanocytic nevus (14 cases) tissue specimens. The results obtained from primary melanoma are summarized in Table 1, and the representative strong staining of primary melanoma patient is shown in Fig. 1D. SPARC was detected immunohistochemically in all 33 independent primary melanoma lesions (weak, 7; moderate, 14; strong, 12) and in all seven metastatic lesions tested (weak, 0; moderate, 2; strong, 5). SPARC was predominantly located in the cytoplasm of malignant cells. All of the 14 melanocytic nevi lesions also showed a positive expression (weak, 4; moderate, 6; strong, 4).

Presence of soluble SPARC protein in the culture supernatants of melanoma cell lines and sera from melanoma patients. We detected soluble SPARC protein using ELISA. Soluble SPARC protein could be detected in the culture supernatants of all human melanoma cell lines tested, with the exception of HMV-1, and cultured melanocyte HEMn (Fig. 2A). The concentration of soluble SPARC secreted from each cell line into the culture supernatant did not always correlate with the expression levels of SPARC mRNA and protein (Fig. 1A and B and Fig. 2A).

Serum SPARC concentrations for 109 preoperative melanoma patients, five patients with large congenital melanocytic nevus, and 61 healthy donors are shown in Fig. 2B and Table 1. Figure 2C shows the standard curve for ELISA detection of SPARC that confirmed the linearity of the ELISA determination of SPARC concentration. According to these data, we were convinced that the range for accurate detection of serum SPARC was between 0 and 16 μg/mL by using 200-fold diluted serum samples. We could obtain reproducible results in three independent ELISA assays, and the representative results were shown. The mean ±

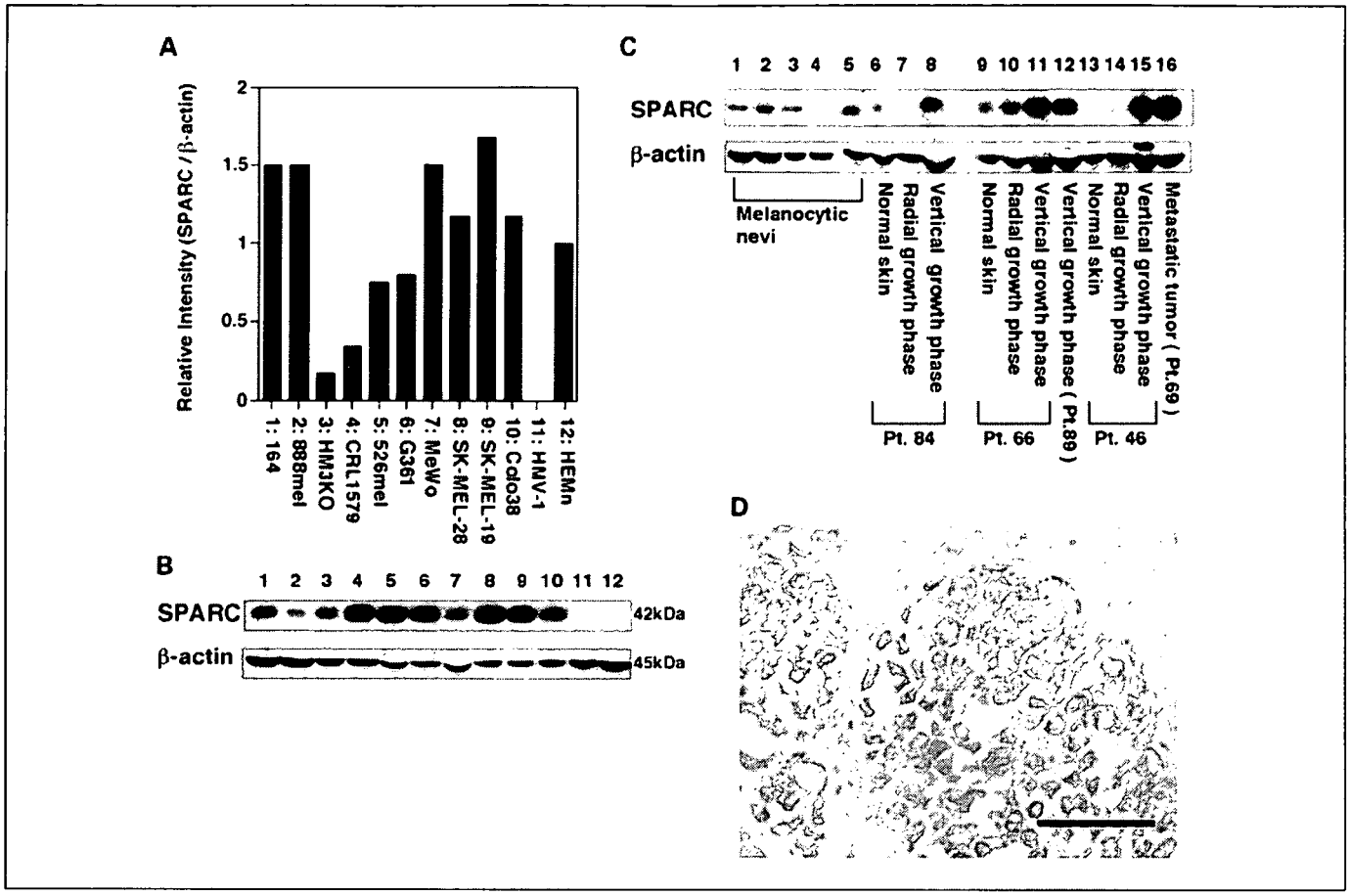

Fig. 1. Expression of SPARC mRNA and protein in human melanoma and melanocytes. *A*, differential expressions of *SPARC* mRNA in various human melanoma cell lines (*lanes 1-11*) and HEMn (*lane 12*) were analyzed using real-time reverse transcription-PCR. The relative expressions of *SPARC* mRNA were calculated as the ratio of the *SPARC*/*β-actin* expressions from three replicate reverse transcription-PCR experiments. 164; lane 2, 888mel; lane 3, HM3KO; lane 4, CRL1579; lane 5, 526mel; lane 6, G361; lane 7, MeWo; lane 8, SK-MEL-28; lane 9, SK-MEL-19; lane 10, Colo38; lane 11, HMV-I; lane 12, HEMn. *B*, Western blot analysis of human melanoma cell lines (*lanes 1-11*) and HEMn (*lane 12*) were done using anti-SPARC monoclonal antibody. The lanes were the same as those observed in (*A*). *C*, Western blot analyses of human tissues from melanocytic nevi (*lanes 1-5*) and melanoma patients (*lanes 6-16*) were done. Tissues originated from the following patients. Patient 84: lane 6, normal skin; lane 7, radial growth phase; lane 8, vertical growth phase. Patient 66: lane 9, normal skin; lane 10, radial growth phase; lane 11, vertical growth phase. Patient 89: lane 12, radial growth phase. Patient 46: lane 13, normal skin; lane 14, radial growth phase; lane 15, vertical growth phase. Tissue of metastasis to lymph node of patient 69: lane 16. The patient ID numbers are the same as those shown in Table 1. *D*, expression pattern of SPARC protein in primary melanoma. SPARC immunoreactivity in melanoma cells localized predominantly in the cytoplasm. Bar, 50 μm.

SD serum SPARC concentration in 109 preoperative melanoma patients (2.02 ± 1.02 μg/mL) was significantly greater than that in the 61 healthy donors (1.62 ± 0.36 μg/mL; $P = 0.001$, Student's t test). When the cutoff value was fixed at 2.34 μg/mL, which was the mean SPARC concentration plus 2 SD in the healthy donors, 36 of 109 (33.0%; 3.21 ± 0.70 μg/mL) melanoma patients were positive for increased serum SPARC. Thereby, the sensitivity of this assay was 33.0%. On the other hand, three (4.9%; 2.46 ± 0.08 μg/mL) and two (40%) positive cases were found in 61 healthy donors and five melanocytic nevi patients, respectively. Thus, the specificity of this assay was 92.4%. The prevalence of increased SPARC protein in the sera of melanoma patients was higher than that in healthy donors. The presence of a significant amount of SPARC in the sera from melanoma patients suggested that melanoma cells secrete SPARC in melanoma patients.

Among the 30 cases of melanoma patients in which both immunohistochemical staining and ELISA detection of serum SPARC were done, increased serum SPARC was detected in nine patients (30%; Table 1). In six of nine patients (patients 39, 50, 42, 76, 90, and 112), strong SPARC protein expression was immunohistochemically detected in their melanoma cells, and moderate SPARC protein expression in melanoma cells was observed in two patients (patients 44 ad 49). Patient 96 expressed weak SPARC protein in melanoma cells. However, 7 of 13 cases expressing strong SPARC protein did not secrete SPARC in the sera. Because we were not able to prepare serum and tissue samples from the same patients with congenital melanocytic nevus, we could not evaluate the correlation between the serum SPARC levels and the expression levels of SPARC in melanocytic nevi tissue in these patients.

Human platelets contain and secrete SPARC protein in the sera of healthy donors (18). Thus, we measured SPARC in plasma to eliminate the influence of the SPARC secreted from the platelets. The plasma concentrations of SPARC in 11 preoperative patients with melanoma and 21 healthy donors were shown in Fig. 2D. The mean SPARC value in 11 preoperative melanoma patients (0.61 ± 0.65 μg/mL) was significantly greater than that in the 21 healthy donors (0.14 ± 0.14 μg/mL; $P = 0.003$). When the cutoff value was fixed at