tumor antigenic peptides or the vaccination strategy may be sufficient to induce CTL responses but not to elicit $CD4^+$ T cells.

DNA-based immunization is potentially a powerful method for immunizing against microbial, viral, and tumor antigens through both humoral and cell-mediated immune responses.[28] The generation of T-cell immunity involves local target cell transfection and protein antigen production, which is taken up by host APC, leading to cross-presentation in draining lymph nodes; in addition, direct DNA transfection into APC in peripheral tissue has also been demonstrated.[29] Compared with orthodox vaccines consisting of tumor proteins or viral components, DNA vaccination stimulates host immunity against transgene-encoding proteins without the processes related to protein purification. In the present study, a DNA vaccine was used to activate HSP105-specific tumor immunity.

Although the SEREX method facilitated the identification of tumor antigens that could be recognized by antibodies and $CD4^+$ Th cells, few of their T cell epitopes have been determined.[2,30] We previously reported that HSP105, identified by SEREX of pancreatic adenocarcinoma, was overexpressed specifically in a variety of human cancers, including pancreatic and colon adenocarcinoma.[1,5] Other investigators identified HSP105 by SEREX using other cDNA libraries derived from tissues including colorectal cancer, melanoma, and normal testis. HSP105 are complexes associated with HSP70/HSC70,[31,32] which negatively regulate HSP70/HSC70 chaperone activity.[33] In addition, HSP105 protects neuronal cells against the apoptosis induced by various stresses.[34] 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 HSP that is specifically induced by heat shock at 42°C. HSP105β is a truncated form of HSP105α.[12] We used in this study the mouse HSP105α DNA and protein. Recently, Subjeck and colleagues reported that recombinant HSP110 and cancer antigens such as Her2/*neu* or gp100 complexes are powerful cancer vaccines.[8,9,35] Their HSP110[11] and our HSP105α are in fact the same protein.

Although they noted that HSP110 did not have immunogenic properties, we emphasize in this study that HSP105 does have a strong immunogenic action. Although we did not identify the HSP105-derived epitope peptides of $CD8^+$ T-cells or $CD4^+$ T-cells in this study, we did prove that HSP105 itself could induce both $CD4^+$ T-cells and $CD8^+$ T-cells to become reactive to tumor cells expressing HSP105. As shown in Figure 5, in a homeostatic lymphocyte proliferation model, we demonstrated that adoptive transfer of either $CD4^+$ T cells or $CD8^+$ T cells alone into sublethally irradiated mice was sufficient to reject C26 cells that do not express MHC class II molecules. To ascertain whether this is also true for B16.F10 that express both MHC class I and II molecules in the presence of interferon (IFN)-γ, further investigation is needed. As shown in Figure 6, we demonstrated that both $CD4^+$ T cells and $CD8^+$ T cells were required for rejection of B16.F10 in the induction phase. In terms of the mechanism for the rejection of C26 tumors, we have other data relating to vaccination with HSP105 protein-pulsed BM-DC instead of *HSP105* DNA vaccination. In those experiments, we also demonstrated that both $CD4^+$ T cells and $CD8^+$ T cells were required for rejection of not only B16.F10 but also C26 in the induction phase by depleting $CD4^+$ T cells and $CD8^+$ T cells using the *in vivo* administration of antibodies (unpublished data). Therefore, both HSP105-specific $CD4^+$ T cells and $CD8^+$ T cells seem to be important for the rejection of HSP105-expressing tumors in the induction phase, and either $CD4^+$ T cells or $CD8^+$ T cells can independently exert anti-C26 tumor effects in the effector phase in a homeostatic lymphocyte proliferation model.

It has been reported that antigen-specific $CD4^+$ T-cell help is required to activate memory $CD8^+$ T cells to fully functional effector killer cells.[36] The peptides derived from exogenous antigens acquired by endocytosis are typically presented on MHC class II molecules on the surface of APC, and activate $CD4^+$ T cells. We observed in this study that $CD4^+$ T cells specific to HSP105, in fact, have an important role in tumor rejection, even when tumors do not express MHC class II molecules, such as the C26 tumors used in this study. It was recently reported that tumor-specific $CD4^+$ T cells may have a pivotal role in preventing early tumorigenesis by secreting IFN-γ and stimulating the classical macrophage-activation pathway. This results in the inhibition of tumor cell growth, even when tumor cells themselves do not express MHC class II molecules.[37] To better understand the mechanism of C26 tumor rejection by HSP105-specific $CD4^+$ T cells, further studies are needed. Furthermore, peptides derived from exogenous self-antigen, HSP105, acquired by endocytosis are possibly presented by MHC class I molecules on the surface of APC by cross-presentation to activate $CD8^+$ T cells.

Because HSP are present in all organisms, low levels of human HSP-derived peptides serve as harbingers of autoimmune responses after CTL have been primed to respond to bacterial HSP-derived peptides.[38] However, because many cancers overexpress HSP, CTL-based vaccines that elicit an anti-HSP response might be effective against many different tumors.[39] Indeed, in this study, HSP105 itself evoked T-cell-mediated tumor rejection without autoimmune reactions. In the present paper, all results shown in the figures were obtained using female mice, but we have carried out the same experiment using male mice. *HSP105* DNA vaccination did not induce T-cell infiltration or damage in testis tissue (in which HSP105 is highly expressed). Furthermore, *HSP105* DNA vaccination was also able to induce antitumor immunity in male mice (data not shown), indicating that male mice did not acquire immunological tolerance to HSP105 expressed in testis tissue.

To substantiate the specificity for HSP105, we searched for mouse cancer cell lines derived from BALB/c mice and C57BL/6 mice that do not express HSP105. However, all cancer cell lines we examined strongly expressed HSP105. BALB/3T3 fibroblasts expressed HSP105 relatively weakly, but these cells unfortunately did not form tumors in mice. Further investigations are needed to clarify whether *HSP105* DNA vaccination affects the growth of some tumors that do not express HSP105.

We showed in this study that *HSP105* DNA vaccination can prime T cells to be reactive to tumor cells expressing HSP105 *in vivo*, and that growth of C26 and B16.F10 cells expressing HSP105 was prevented without inducing autoimmune destruction in murine subcutaneous CRC and melanoma models. We believe that *HSP105* DNA vaccination is a novel strategy for the prevention of CRC and melanoma in patients treated surgically who are at high risk of recurrence

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of CRC or melanoma. Whether or not HSP105 is an ideal target for immunotherapy in human cancers will continue to be investigated in our laboratory.

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Therapeutic effect of α-galactosylceramide-loaded dendritic cells genetically engineered to express SLC/CCL21 along with tumor antigen against peritoneally disseminated tumor cells

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The close cooperation of both innate and acquired immunity is essential for the induction of truly effective antitumor immunity. We tested a strategy to enhance the cross-talk between NKT cells and conventional antigen-specific T cells with the use of αGalCer-loaded dendritic cells genetically engineered to express antigen plus chemokine, attracting both conventional T cells and NKT cells. DC genetically engineered to express a model antigen, OVA, along with SLC/CCL21 or monokine induced by IFN-γ/CXCL9, had been generated using a method based on *in vitro* differentiation of DC from mouse ES cells. The ES-DC were loaded with α-GalCer and transferred to mice bearing MO4, an OVA-expressing melanoma, and their capacity to evoke antitumor immunity was evaluated. *In vivo* transfer of either OVA-expressing ES-DC, stimulating OVA-reactive T cells, or α-GalCer-loaded non-transfectant ES-DC, stimulating NKT cells, elicited a significant but limited degree of protection against the i.p. disseminated MO4. A more potent antitumor effect was observed when α-GalCer was loaded to ES-DC expressing OVA before *in vivo* transfer, and the effect was abrogated by the administration of anti-CD8, anti-NK1.1 or anti-asialo GM1 antibody. α-GalCer-loaded double transfectant ES-DC expressing SLC along with OVA induced the most potent antitumor immunity. Thus, α-GalCer-loaded ES-DC expressing tumor-associated antigen along with SLC can stimulate multiple subsets of effector cells to induce a potent therapeutic effect against peritoneally disseminated tumor cells. The present study suggests a novel way to use α-GalCer in immunotherapy for peritoneally disseminated cancer. (*Cancer Sci* 2005; 96: 889–896)

A means to induce the close cooperation of both innate and acquired immunity would be necessary for the induction of efficient antitumor therapy. Recent studies have shown DC to be potent stimulators of both innate and acquired immunity. The *in vivo* transfer of DC presenting tumor-associated antigens has proven to be efficient in the priming of CTL specific to the antigens. α-GalCer presented by DC efficiently stimulates NKT cells,[1–4] a subset of T cells implicated in the innate immunity against infection and cancer.[5–7] In addition, NKT cells stimulated by the *in vivo* administration of α-GalCer secondarily stimulate conventional T cells.[8,9] It is thus presumed that the *in vivo* transfer of DC simultaneously loaded with tumor-associated antigens and α-GalCer may stimulate both tumor-reactive T cells and NKT cells, thus resulting in a potent antitumor immunity.

Chemokines mediate leukocyte adhesion and homing, and the concordant migration of specific leukocyte subsets induced by chemokines is pivotal for the development of proper immune responses. SLC/CCL21 attracts both T cells and DC to lymphoid tissues through its receptor CCR7, and the effect of SLC is essential for the priming of naive T cells in the initiation phase of the immune response. CXCR3 and its ligands, Mig/CXCL9 and IP-10/CXCL10, mediate the migration of effector/memory T cells and NK cells to the site of inflammation. In addition, a recent study revealed that these chemokines and their receptors also mediate the migration of some subpopulations of NKT cells.[10–12]

As a means for loading the tumor-associated antigens to DC, genetic modification to express antigenic proteins has several advantages. The expression of tumor antigens by DC circumvents the need for identifying specific CTL epitopes within the protein, and by that the antigens are continuously supplied for presentation as opposed to a single pulse of peptides or tumor cell lysates.[13] For the efficient gene transfer to DC, the use of virus-based vectors is required because DC are not easy to genetically modify. Considering the clinical application, however, there are several problems related to the use of virus vectors. These include the inefficiency of gene transfer, the instability of gene expression, and the potential risk accompanying the use of virus vectors. In addition, in many countries, legal restrictions have been placed on the use of virus vectors outside of carefully isolated laboratories.

We and others have established methods to generate dendritic cells *in vitro* from mouse ES cells.[14,15] ES-DC have the

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Abbreviations: Ab, antibody; BM-DC, bone marrow cell-derived dendritic cell; CBF1, (CBA × C57BL/6) F1; CTL, cytotoxic T lymphocyte; DC, dendritic cell; ELISA, enzyme-linked immunosorbent assay; ES cell, embryonic stem cell; ES-DC, ES cell-derived dendritic cell; α-GalCer, α-galactosylceramide; GM-CSF, granulocyte macrophage colony-stimulating factor; HLA, human histocompatibility leukocyte antigen; IFN-γ, interferon-gamma; IL, interleukin; i.p., intraperitoneally; IP-10, IFN-γ-inducible 10 kDa protein; mAb, monoclonal antibody; Mig, monokine induced by IFN-γ; NK, natural killer; OVA, ovalbumin; s.c., subcutaneously; SLC, secondary lymphoid tissue chemokine.

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capacity to stimulate T cells, present antigen and migrate to lymphoid tissues upon *in vivo* administration, and these capacities of ES-DC are comparable to those of BM-DC. The genetic modification of ES-DC can be carried out without the use of virus vectors by introducing exogenous genes by electroporation into undifferentiated ES cells and the subsequent induction of their differentiation into ES-DC. We can generate multiple gene-transfectant ES-DC by the sequential transfection of ES cells with vectors bearing different selection markers.[16,17] In a previous study, we generated double-transfectant ES-DC expressing SLC or Mig along with a model tumor antigen.[16] Using these double-transfectant ES-DC, we demonstrated that the coexpression of SLC or Mig enhanced the capacity of *in vivo*-transferred ES-DC to activate antigen-specific CTL and to protect the recipient mice from a tumor cell challenge.

In the present study, we evaluated the capacity of α-GalCer-loaded ES-DC to stimulate NKT cells both *in vitro* and *in vivo*, in comparison to that of BM-DC. We next evaluated the antitumor effect of simultaneous stimulation of NKT cells and antigen-specific conventional T cells by the *in vivo* administration of α-GalCer-loaded ES-DC expressing a model tumor antigen, namely OVA. Furthermore, we addressed whether coexpression of SLC or Mig with the antigen by ES-DC could enhance the synergic antitumor effect of NKT cells and conventional T cells.

Materials and Methods

Mice

CBA and C57BL/6 mice were obtained from Clea Animal Co. (Tokyo, Japan) or Charles River (Hamamatsu, Japan) and kept under specific pathogen-free conditions. Male CBA and female C57BL/6 mice were mated to produce CBF1 mice and all *in vivo* experiments were carried out using the CBF1 mice. The animal experiments in this study were approved by the animal experiment committee of Kumamoto University (permission number A16-074).

Reagents

Recombinant mouse GM-CSF was purchased from PeproTech EC (London, UK) and α-GalCer was kindly provided by the Kirin Brewery Co. (Tokyo, Japan). Mouse IL-4 and IFN-γ ELISA kits were purchased from eBioscience (San Diego, CA, USA). Polyclonal rabbit anti-asialo GM1 Ab was purchased from Wako Chemicals (Tokyo, Japan).

Cell lines and preparation of DC

The ES cell line TT2, derived from CBF1 blastocysts,[18] was maintained as described previously.[19] MO4[20] was generated by the transfection of C57BL/6-derived melanoma B16 with the pAc-neo-OVA plasmid. The procedure for inducing the differentiation of ES cells into DC has been reproted previously.[15] ES-DC expressing OVA (ES-DC-OVA) and ES-DC expressing chemokine, SLC or Mig, along with OVA (ES-DC-OVA/SLC or ES-DC-OVA/Mig) were generated as reported previously.[16] ES-DC recovered after a 14-day culture in bacteriological Petri dishes were used for both *in vivo* and *in vitro* assays. To generate BM-DC, bone marrow cells were isolated from CBF1 mice and cultured in RPMI + 10% fetal calf serum + GM-CSF (5 ng/mL) for 7 days, according to the method reported by Lutz *et al.*[21]

Analysis of the activation of NKT cells by DC loaded with α-GalCer

Embryonic stem cell-derived dendritic cells or BM-DC were cultured in the presence of α-GalCer (100 ng/mL) or vehicle (0.00025% Polysorbate-20) alone for 22 h, washed twice, and cocultured with splenic T cells of syngeneic CBF1 mice (5×10^4 DC $+2.5 \times 10^6$ T cells/well in 24-well culture plates). Splenic T cells were isolated using nylon-wool columns, as described previously.[16] After 24 h or 5 days, the cells were recovered and analyzed on their cytotoxic activity by a 4-h ^{51}Cr-release assay using YAC-1 cells (1×10^4 cells/well) as targets in 96-well round-bottomed culture plates at the effector : target ratio indicated. The amount of IL-4 and IFN-γ in the culture supernatant was measured by ELISA. In the analysis of the stimulation of NKT cells *in vivo*, ES-DC or BM-DC loaded with either α-GalCer (100 ng/mL) or vehicle alone, as described above, were injected i.p. into syngeneic CBF1 mice (1×10^6 cells/mouse). After 24 h, the mice were killed and the cytotoxic activity of whole spleen cells against YAC-1 cells was analyzed, as described above.

Tumor challenge experiments

Indicated numbers of MO4 cells were injected s.c. into the shaved left flank region, or i.p. on day 0. On day 3, 1×10^5 genetically modified ES-DC preloaded with either α-GalCer or vehicle alone were transferred i.p. into the mice. The survival rate of the mice was monitored and, in s.c. challenge experiments, the tumor sizes were also determined biweekly in a blinded fashion. The tumor index was calculated as:

Tumor index (mm) = square root of (length × width).

In vivo depletion experiments

The mice were challenged i.p. with 1×10^5 MO4 cells on day 0 and they were injected i.p. with 1×10^5 ES-DC-OVA preloaded with α-GalCer on day 3. To deplete the specific types of cells, the mice were given a total of 14 i.p. injections (days −4, −1, 2, 5, 10, 13, 15, 19, 26, 33, 40, 47, 54 and 61) of mAb, ascites (0.1 mL/mouse/injection) from hybridoma-bearing nude mice, or polyclonal rabbit anti-asialo GM1 Ab (50 μg/mouse/injection). The mAbs used were rat antimouse CD4 (clone GK1.5), rat antimouse CD8 (clone 2.43) and mouse anti-NK1.1 (clone PK-136). Normal rat IgG (Sigma, St Louis, MO, USA) was used as a control (200 μg/mouse/injection).

Statistical analysis

Two-tailed Student's *t*-test was used to determine the statistical significance of differences in cytolytic activity and the tumor growth between the treatment groups. A value of $P < 0.05$ was considered to be significant. The Kaplan–Meier plot for survival was used to determine any significant differences in tumor challenge experiments, using the Breslow–Gehan–Wilcoxon test. In some experiments, the difference in the survival rate between treatment groups was assessed for significance using the χ^2-test. Statistical analyses were made using the StatView 5.0 software (Abacus Concepts, Calabasas, CA, USA).

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Results

Activation of NKT cells by ES-DC pulsed with α-GalCer

Mouse splenic DC and BM-DC loaded with α-GalCer have been reported to efficiently stimulate NKT cells, resulting in the rapid induction of NK cell-like cytolytic activity and the production of cytokines such as IL-4 and IFN-γ.[2,3] We examined whether ES-DC loaded with α-GalCer had the capacity to activate NKT cells, as naturally occurring DC do.

TT2 ES cell-derived non-transfectant ES-DC (ES-DC-TT2) or BM-DC were preincubated with α-GalCer, and then cocultured with splenic T cells isolated from syngeneic CBF1 mice. After 24 h, the cultured cells were recovered and their cytolytic activity against YAC-1 target cells was analyzed by a ^{51}Cr-release assay. The results shown in Fig. 1a indicate that a significant cytotoxity against YAC-1 cells was induced in the splenic T cell preparations by coculture with ES-DC loaded with α-GalCer, in comparison to the coculture with ES-DC loaded with vehicle alone. The cytotoxic activity induced by α-GalCer-loaded ES-DC-TT2 was comparable to that induced by α-GalCer-loaded BM-DC (Fig. 1a). As shown in Fig. 1b, IL-4 and IFN-γ were produced by splenic T cells cocultured with α-GalCer-loaded BM-DC or ES-DC, and a similar amount of the cytokines was produced in the culture with BM-DC and ES-DC preloaded with α-GalCer. If the coculture of T cells with α-GalCer-loaded ES-DC was extended to 5 days, the induced killing activity (Fig. 1c) and the amount of IL-4 and IFN-γ produced was increased in parallel (data not shown).

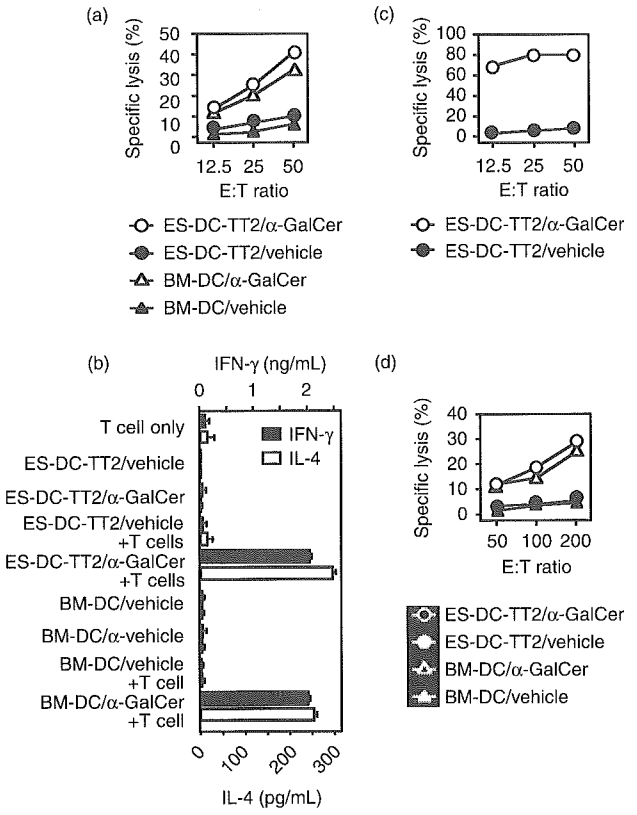

Fig. 1. Activation of NKT cells by the α-GalCer-loaded ES-DC. (a) ES-DC-TT2 or BM-DC were loaded with either α-GalCer (100 ng/mL) or vehicle (Polysorbate-20) alone for 22 h, washed extensively, and cocultured with splenic T cells of syngeneic CBF1 mice (5×10^4 DC + 2.5×10^6 T cells/well in 24-well culture plates). After 24 h of culture, the cells were recovered and the cytotoxic activity of the harvested cells against YAC-1 cells (1×10^4 cells) was analyzed using a 4-h Cr-release assay at the effector : target (E : T) ratios indicated. (b) Amounts of IL-4 and IFN-γ in the supernatant collected at the end of the 24-h coculture were quantified by ELISA. The results are expressed as the mean cytokine production of triplicate assays + SD. (c) The coculture was extended to 5 days and the killing activity of resultant cells was analyzed as in (a). (d) ES-DC or BM-DC were cultured in the presence of either α-GalCer (100 ng/mL) or vehicle alone for 18 h, washed, and injected i.p. into syngeneic CBF1 mice (1×10^6 cells/mouse). After 24 h, spleen cells were isolated from the mice and their cytotoxic activity against YAC-1 cells was analyzed as in (a). The results are expressed as the mean specific lysis of triplicate assays. The SD of triplicates were less than 2%.

We next analyzed the capacity of α-GalCer-loaded ES-DC to activate NKT cells *in vivo*. ES-DC-TT2 or BM-DC were preloaded with α-GalCer or vehicle alone in the same way as described above and i.p. injected into the syngeneic CBF1 mice. After 24 h, the mice were killed and the cytotoxic activity of whole spleen cells against YAC-1 cells was analyzed. As shown in Fig. 1d, a significant degree of cytotoxic activity was induced in the spleen cells by transfer of ES-DC loaded with α-GalCer, but it was not induced by the transfer of those loaded with vehicle alone. The capacity to evoke YAC-1 cell-killing activity of ES-DC and that of BM-DC was similar also *in vivo*. The activated NKT cells are known to activate the cytotoxic activity of NK cells.[22] It is therefore possible that the cytotoxic activity observed in these assays were mostly mediated by NK cells secondarily stimulated by NKT cells. Even so, these data collectively demonstrate that ES-DC had the capacity to present α-GalCer to activate NKT cells, and the capacity was similar to that of BM-DC both *in vitro* and *in vivo*.

Anti-tumor effect of α-GalCer-loaded ES-DC

We assessed whether the activation of NKT cells *in vivo* by α-GalCer-loaded ES-DC had any therapeutic effect against the tumor cells growing *in vivo*. MO4, originating from NK-sensitive B16 melanoma cells, were injected s.c. into the left flank region of mice and, 3 days later, the mice were treated with an i.p. injection of ES-DC-TT2 loaded with α-GalCer or vehicle alone. As shown in Fig. 2a, ES-DC loaded with α-GalCer did not show any therapeutic effect in this s.c. tumor model.

We next investigated the effect of α-GalCer-loaded ES-DC in the peritoneally disseminated tumor model. MO4 cells were injected i.p. into mice and, 3 days later, the mice were treated with an i.p. injection of ES-DC loaded with either α-GalCer or vehicle alone. As shown in Fig. 2b, which indicated the survival rate of the treated mice, the injection of ES-DC-TT2 loaded with α-GalCer elicited a significant ($P < 0.05$) but limited protective effect against the i.p. disseminated tumor cells.

Synergic therapeutic effect of α-GalCer-activated NKT cells and antigen-specific T cells against peritoneally disseminated tumor cells

In a previous study, we demonstrated that the *in vivo* transfer of ES-DC-OVA effectively primed OVA-specific CTL and induced protection against a subsequent challenge with s.c. injected MO4 cells expressing OVA.[16] We investigated

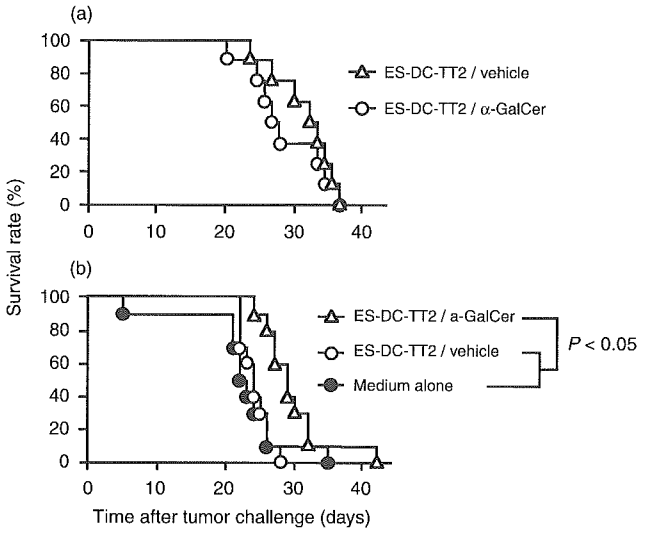

Fig. 2. Anti-tumor effect of *in vivo*-transferred α-GalCer-loaded ES-DC. The mice were (a) inoculated s.c. with MO4 cells (3×10^5 cells/mouse) to the left flank region or (b) inoculated i.p. with MO4 cells (1×10^5 cells/mouse). After 3 days, the mice were treated with i.p. injection of ES-DC-TT2 (1×10^5 cells/mouse) loaded with α-GalCer, vehicle alone or medium alone, and the mouse survival rate was monitored ($n = 10$ per group). In (b), the survival rate of the α-GalCer-loaded ES-DC-TT2-treated group was higher than that of the other two groups and the difference was statistically significant. Data are representative of four independent and reproducible experiments.

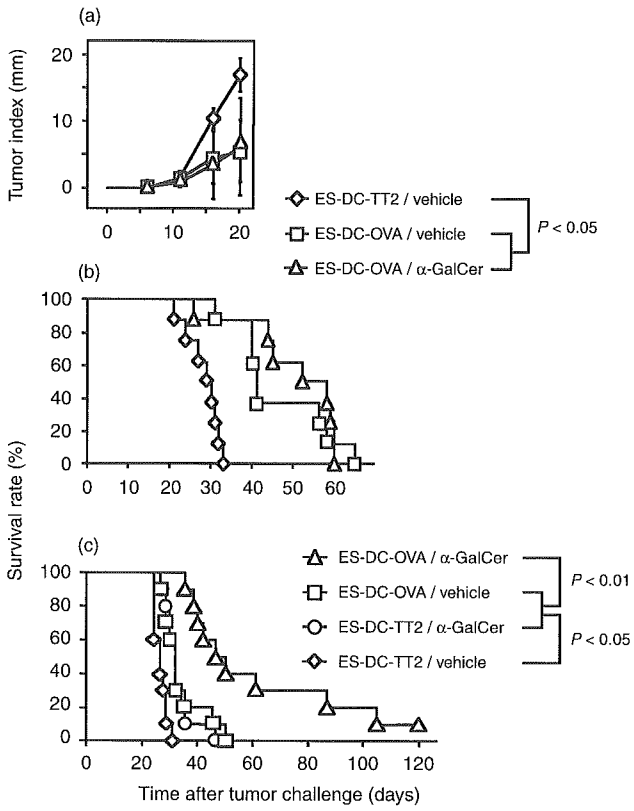

Fig. 3. Synergic effect of α-GalCer loading and the expression of tumor antigen on the protection against tumors induced by ES-DC. MO4 cells (3×10^5 cells/mouse) were injected s.c. into the left flank region of the mice and, 3 days later, the mice were treated with an i.p. injection of ES-DC-TT2 (1×10^5 cells/mouse) loaded with vehicle alone, ES-DC-TT2 loaded with α-GalCer, or ES-DC-OVA loaded with α-GalCer. After that, the tumor sizes were determined (a) and survival rate was monitored (b). Both the differences in tumor index (a) and in mouse survival rate (b) between the vehicle-loaded ES-DC-TT2-treated group and other two groups were statistically significant. (c) MO4 cells (1×10^5 cells/mouse) were injected i.p. into the mice and, 3 days later, the mice were treated with an i.p. injection of ES-DC-OVA or ES-DC-TT2 (1×10^5 cells/mouse) loaded with α-GalCer or vehicle alone. Thereafter, the mouse survival rate was monitored. The survival rate of the α-GalCer-loaded ES-DC-OVA-treated group was higher than that of the other groups and the difference was statistically significant. The survival rates of the vehicle-loaded ES-DC-OVA-treated group and α-GalCer-loaded ES-DC-TT2-treated group were higher than that of vehicle-loaded ES-DC-TT2-treated group and the difference was statistically significant. Each group included 10 mice. Data are representative of three independent and reproducible experiments.

whether the loading of α-GalCer to ES-DC-OVA before *in vivo* transfer would enhance the therapeutic effect against pre-established MO4 tumor. The mice were challenged s.c. with MO4 cells, and then 3 days later they were treated by i.p. injection of ES-DC-OVA preloaded with α-GalCer or vehicle alone. As shown in Fig. 3a,b, compared to the transfer of ES-DC-TT2, the transfer of ES-DC-OVA, loaded with either α-GalCer or vehicle alone, elicited a significant antitumor effect in this therapeutic model, as observed in the previously reported prevention (prophylactic) model.[16] However, the loading of α-GalCer to ES-DC-OVA did not improve the effect, based on either the tumor growth or the mouse survival time (Fig. 3a,b). These results suggest that the activation of NKT cells by α-GalCer loaded to ES-DC does not enhance the therapeutic effect of antigen-specific T cells against s.c. growing tumor cells.

We next investigated the effect of transfer of α-GalCer-loaded ES-DC-OVA against peritoneally disseminated tumor cells. Mice were i.p. inoculated with MO4 cells and 3 days later they were treated by an i.p. injection of ES-DC-OVA loaded with α-GalCer or vehicle alone, or ES-DC-TT2 loaded with vehicle alone. As shown in Fig. 3c, the therapeutic effect of the transfer of ES-DC-OVA loaded with vehicle alone was significant ($P < 0.05$) in comparison to the treatment with ES-DC-TT2 loaded with vehicle alone, but the effect was less marked than the effect observed in the s.c. growing tumor model (Fig. 3b). In contrast, the treatment with ES-DC-OVA loaded with α-GalCer elicited a potent effect to prolong the survival time of the mice. Given that the antitumor effect elicited by α-GalCer-loaded non-transfectant ES-DC was also limited (Figs 2b,3c), these results indicate that the NKT cells activated by α-GalCer presented by ES-DC and OVA-specific CTL primed by OVA antigen presented by the same ES-DC acted synergistically to protect the mice.

Subsets of effector cells contributing to the antitumor effect induced by α-GalCer-loaded ES-DC that expressed a model tumor antigen

To determine the effector cells exhibiting the observed antitumor effect induced by adoptive transfer of α-GalCer-loaded ES-DC expressing OVA, we carried out depletion experiments by injecting the mice with Abs specific to several subsets of effector cells during the tumor cell challenge and treatment with ES-DC. Figure 4a shows the

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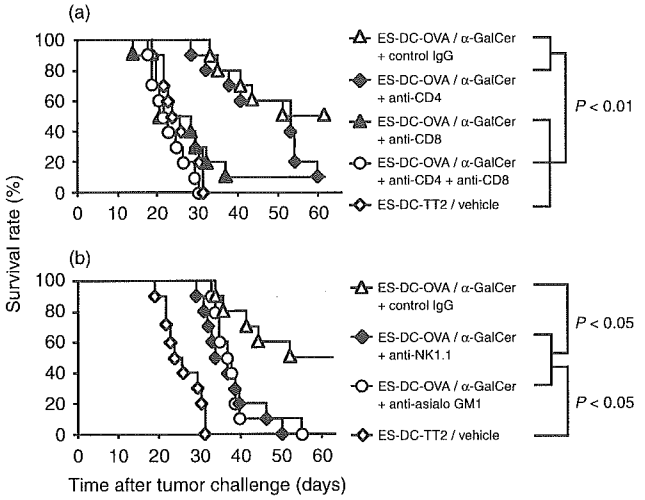

Fig. 4. Effector cells involved in the antitumor effect exerted by adoptive transfer of α-GalCer-loaded, antigen-expressing ES-DC. The mice were challenged i.p. with 1×10^5 MO4 cells on day 0 and injected i.p. with 1×10^5 ES-DC-OVA preloaded with α-GalCer on day 3. To deplete the specific types of cells, the mice were given i.p. injections of mAb or polyclonal rabbit anti-asialo GM1 Ab. The effect of the injection of anti-CD4, anti-CD8, or a combination of these two Abs is shown in (a). The effect of anti-NK1.1 or rabbit anti-asialo GM1 Ab is shown in (b). As a control, the survival of the mice treated with normal rat IgG is shown in both (a) and (b). Each group included 10 mice. In (a), the survival rates of α-GalCer-loaded ES-DC-OVA plus control IgG-treated group and α-GalCer-loaded ES-DC-OVA plus anti-CD4-treated group were higher than those of the other three groups and the difference was statistically significant. In (b), the survival rates of α-GalCer-loaded ES-DC-OVA plus anti-NK1.1 or anti-asialo GM1-treated groups and those of the other two groups were statistically different. The experiment was carried out once.

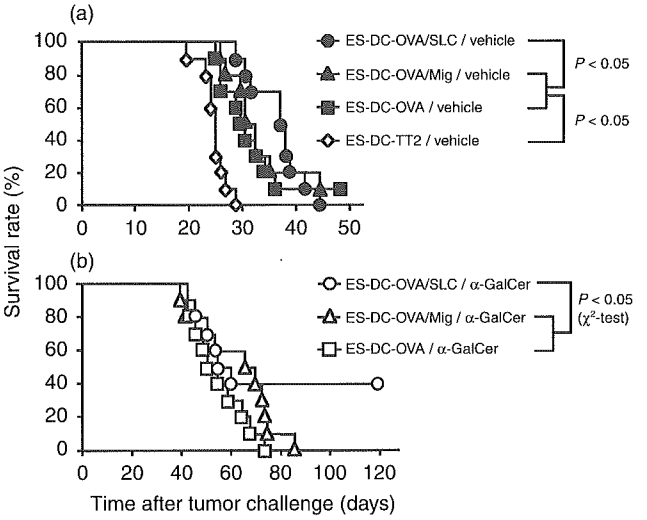

Fig. 5. Enhanced antitumor effect elicited by α-GalCer-loaded ES-DC expressing SLC along with OVA. (a) MO4 cells (1×10^5 cells/mouse) were injected i.p. into the mice and, 3 days later, the mice were treated with i.p. injection of ES-DC-TT2, ES-DC-OVA, ES-DC-OVA/Mig, or ES-DC-OVA/SLC (1×10^5 cells/mouse), all loaded with vehicle only. Thereafter, the survival rate of the mice was monitored. The differences in the survival rate between ES-DC-OVA/Mig-treated or ES-DC-OVA-treated group and the other two groups were statistically significant. (b) The mice were challenged with MO4 cells as in (a) and treated with either ES-DC-OVA, ES-DC-OVA/SLC or ES-DC-OVA/Mig, all loaded with α-GalCer. The frequency of mice from the ES-DC-OVA/SLC-treated group surviving for more than 100 days (four out of 10 mice) was significantly higher than that of the other two groups (0 out of 10 mice in each group), according to the χ^2-test. The experiment was carried out once.

effect of the injection of anti-CD4 or anti-CD8 mAbs or a combination of these two mAbs. The injection of anti-CD8 mAb almost totally abrogated the effect of the treatment with the ES-DC, thus suggesting that $CD8^+$ OVA-specific CTL played an important role in protecting the mice from the tumor. Compared to the effect of anti-CD8 mAb, the injection of anti-CD4 mAb had far less influence on the protective effect against the tumor, thus indicating the function of $CD4^+$ helper T cells to be not essential.

Figure 4b shows the effect of the injection of rabbit anti-asialo GM1 Ab, depleting NK cells, and also that of anti-NK1.1 mAb, depleting both NK and NKT cells. Treatment with either of these two kinds of Ab decreased the effect of α-GalCer-loaded ES-DC-OVA to a level similar to that elicited by vehicle-loaded ES-DC-OVA. These results indicate that NK cells played an essential role in the enhanced antitumor effect caused by the activation of NKT cells by α-GalCer.

Enhanced antitumor effect elicited by α-GalCer-loaded ES-DC expressing SLC along with OVA

We previously found that the coexpression of SLC or Mig, T cell-attracting chemokines that natural DC do not produce, along with OVA by ES-DC significantly enhanced their capacity to prime OVA-specific CTL and also to induce a protective immunity against s.c. injected MO4 cells.[16] A recent study revealed that these two chemokines induced chemotaxis not only of conventional T cells but also of some subpopulations of the NK cells and NKT cells.[10–12,23] We therefore examined whether the coexpression of such chemokine by ES-DC expressing OVA would also have an enhancing effect in protection against the i.p. growing MO4 cells.

We first assessed the effect of the expression of such chemokines by ES-DC without preloading with α-GalCer. We analyzed the capacity of ES-DC-OVA/SLC or ES-DC-OVA/Mig, ES-DC expressing OVA simultaneously with SLC or Mig, to induce protection against i.p. disseminated MO4 cells, comparing the capacity with that of ES-DC-OVA. The effect elicited by ES-DC-OVA/Mig was not higher than that elicited by ES-DC-OVA. Thus, the expression of Mig did not enhance the antitumor effect (Fig. 5a). However, expression of SLC by ES-DC did enhance the protective effect, although the effect of SLC in this i.p. tumor model was less evident than that observed in the s.c. tumor model reported previously.[16]

We next evaluated the effect of the expression of either SLC or Mig on the antitumor effect elicited by α-GalCer-loaded ES-DC-OVA. α-GalCer ES-DC-OVA/Mig and α-GalCer ES-DC-OVA exhibited a similar degree of protection, thus indicating that the coexpression of Mig by α-GalCer-loaded ES-DC-expressing OVA did not have any additive effect (Fig. 5b). In contrast, α-GalCer-loaded ES-DC-OVA/SLC exhibited a far more potent protective effect than α-GalCer ES-DC-OVA/Mig or α-GalCer ES-DC-OVA did. We observed that 40% of the mice treated with α-GalCer-loaded ES-DC-OVA/SLC completely rejected the tumor cells (Fig. 5b). These results suggest that the SLC produced by

ES-DC augmented the synergic effect of antigen-reactive CTL, α-GalCer-activated NKT cells, and probably NK cells.

As we reported previously,[16] coexpression of SLC along with OVA in ES-DC enhanced the capacity of ES-DC to prime OVA-specific CTL upon *in vivo* transfer. The data shown in the Fig. 5a also indicate that coexpression of SLC enhanced the capacity of ES-DC to induce antitumor immunity mediated by OVA-specific CTL in the absence of α-GalCer. To assess the effect of SLC produced by ES-DC on the activation of NKT or NK cells, we compared the capacity of α-GalCer-loaded ES-DC-OVA and α-GalCer-loaded ES-DC-OVA/SLC to stimulate NKT cells by an experiment similar to that shown in Fig. 1d. As a result, we observed that the capacity of α-GalCer-loaded ES-DC to induce YAC-1 cell-killing activity was not enhanced by expression of SLC (data not shown). Thus, effect of SLC produced by ES-DC to enhance the activation of NKT and NK cells was not detected at least by this short-term (24 h) assay. Based on these observations, it may be considered that the expression of SLC by ES-DC dominantly enhanced the activation of antigen-specific CTL rather than NKT or NK cells.

Discussion

In the present study, we evaluated the effect of loading α-GalCer to ES-DC expressing a model tumor antigen on their capacity to induce antitumor immunity. Upon loading with α-GalCer, ES-DC had a capacity comparable to that of BM-DC to stimulate NKT cells (Fig. 1). The *in vivo* administration α-GalCer-loaded non-transfectant ES-DC had some antitumor effect in an i.p. disseminated tumor model but not in an s.c. growing tumor model (Fig. 2). The difference in the effect of loading of α-GalCer to ES-DC in between the two models may be accounted for by the tissue distribution of NKT cells. NKT cells localize mainly in the liver, lung, spleen, bone marrow and peritoneal cavity.[6,11,24,25] In parallel with these observations, the loading of α-GalCer to ES-DC-OVA enhanced their antitumor effect against i.p. disseminated but not s.c. growing MO4 tumor cells (Fig. 3).

In a previous study, we observed that the protective effect against s.c. growing MO4 cells by transfer of ES-DC-OVA was almost totally abrogated by the depletion of either of CD4 or CD8 T cells.[16] In contrast, in the present study, the depletion of $CD8^+$ T cells but not $CD4^+$ T cells diminished the antitumor effect against i.p. MO4 cells elicited by α-GalCer-loaded ES-DC-OVA (Fig. 4a). These results indicate that CTL play a pivotal role in both conditions, and that $CD4^+$ T helper cells were not essential in the protective immunity against i.p. tumor cells on the occasion of simultaneous activation of NKT cells. The reason for the dispensability of $CD4^+$ T helper cells may be that NKT cells and NK cells, secondarily activated by NKT cells, provide help to OVA-specific CTL.[26] The data shown in Fig. 4b revealed that the depletion of NK cells decreased the effect of α-GalCer-loaded ES-DC-OVA to a degree similar to that elicited by vehicle-loaded ES-DC-OVA, indicating that NK cells played an essential role in the enhancement of the antitumor effect obtained by loading α-GalCer to ES-DC-OVA. Collectively, $CD8^+$ CTL, NKT cells and NK cells played essential roles in the antitumor effect obtained by α-GalCer to ES-DC expressing

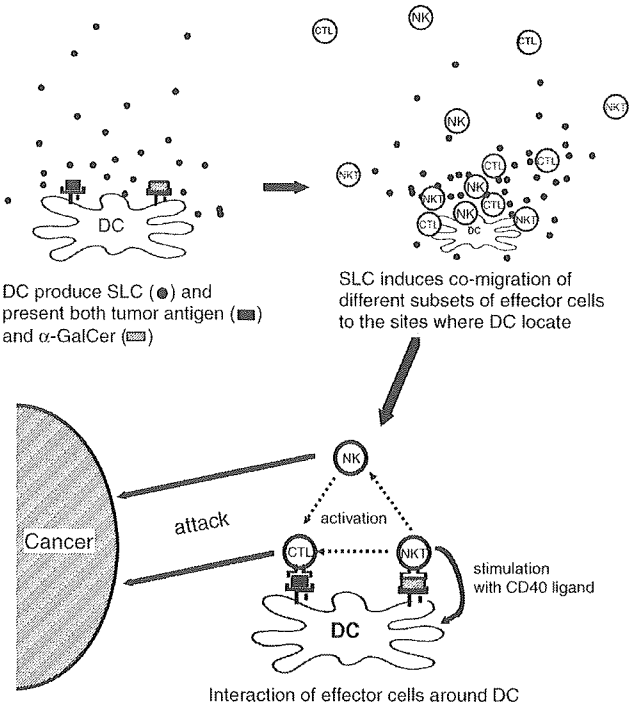

Fig. 6. A schematic depiction of the enhanced cross-talk of different subsets of effector cells induced by α-GalCer-loaded ES-DC expressing OVA plus SLC. SLC secreted by ES-DC induces the comigration of different subsets of effector cells, including NKT cells, NK cells and antigen-specific T cells, to the sites where the injected ES-DC are located. The effector cells of both innate and acquired immunity gathered around ES-DC, which present both α-GalCer and tumor antigen, thus closely interacting to develop a potent antitumor immunity.

the antigen (Fig. 6). Presumably, the sequential stimulation of NKT cells and NK cells augmented the antitumor effect of OVA-specific CTL[9] and probably the interactions of effector cells were mediated by IFN-γ and IL-2.[22,27–31]

The data shown in Fig. 5b indicate that the expression of SLC by ES-DC enhanced the antitumor effect induced by the transfer of α-GalCer-loaded ES-DC expressing OVA. SLC has been reported to attract not only conventional T cells and DC but also NKT cells.[10,23] SLC also induces chemotaxis of $CD56^{bright}$ $CD16^-$ NK cells and has a costimulatory effect on the proliferation of NK cells.[32] Thus, SLC probably induced the comigration of conventional T cells, NKT cells and NK cells to the sites where ES-DC were located, and, as a result, the close interaction of such multiple subsets of effector cells may have occurred (Fig. 6).

In the past decade, α-GalCer has been attracting attention as a novel immunostimulatory reagent for antitumor therapy. Based on the promising results of preclinical studies demonstrating antitumor effects of α-GalCer,[2,25,33] several phase I clinical studies on anticancer immunotherapy by the direct intravenous administration of α-GalCer or the administration of α-GalCer-loaded DC have been carried out.[34–37] Although the activation and expansion of NKT cells by the administration of α-GalCer has been observed, the results seemed to be unsatisfactory from the viewpoint of the clinical effect. The present study demonstrated that α-GalCer is useful for induction

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of immunity against peritoneally disseminated tumor cells, especially when it is loaded to DC genetically engineered to express tumor antigen. Although metastasis of melanoma to visceral organ sites is observed frequently in patients with advanced (stage IV) malignant melanoma, peritoneal dissemination of melanoma is very rare. Thus, we are planning another study with more clinical relevance, using models of cancer with a high tendency to peritoneal dissemination.

In recent years, a number of tumor-associated antigens have been identified. These antigens are potentially good targets for immunotherapy. To establish truly effective anticancer immunotherapy, a method for potently polarizing the immune system toward these antigens is essential. Antitumor immunotherapy with DC loaded with HLA-binding peptides derived from tumor antigens has been tested clinically in many institutions. In most cases, the DC are generated by culture of monocytes obtained from peripheral blood of the patients. To generate a sufficient number of DC for treatment, apheresis, a procedure that is sometimes invasive for patients with advanced stages of cancer, is necessary to obtain a sufficient number of monocytes as a source for DC. In addition, the culture to generate DC should be done separately for each patient and for each treatment, and thus the procedure used at present may be too labor-intensive and expensive to be applied broadly in a practical setting. Alternately, the source of ES-DC, ES cells, have the capacity to propagate infinitely. We would thus be able to use human ES cells as an infinite source of DC. In addition, we will be able to generate genetically engineered DC without the need to use virus vectors, as mentioned above. We may thus be able to generate multiple gene-transfectant ES-DC expressing tumor antigen plus immunostimulating molecules, which could be more potent in stimulating antitumor immunity than monocyte-derived DC are.

Regarding the future clinical application of ES-DC, we recently established a method for generating ES-DC from ES cells of a non-human primate, namely the cynomolgus monkey, and also for their genetic modification (unpublished data). We believe that this method would be applicable to human ES cells, although some modifications may be necessary. Considering the future clinical application of ES-DC technology, allogenicity (i.e. differences in the genetic background) between patients to be treated and ES cells as a source for DC may cause problems. However, it is expected that human ES cells sharing some HLA alleles with patients will be available for most cases. We recently found that antigen-expressing ES-DC potently primed antigen-specific CTL after the transfer to semiallogeneic mice sharing some MHC alleles with the ES-DC, and protected the recipient mice from subsequent challenge with tumor cells bearing the antigen.[38] CD1d is monomorphic and thus a CD1d-α-GalCer-complex on ES-DC can stimulate the NKT cells of any recipients. α-GalCer would thus be an ideal adjuvant to enhance the immune response toward the tumor antigens presented by ES-DC.

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Brief report

Direct recognition and lysis of leukemia cells by WT1-specific CD4+ T lymphocytes in an HLA class II–restricted manner

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Wilms tumor gene 1 product (WT1) has been recognized as an attractive target antigen of immunotherapy for various malignancies including leukemia. Because tumor-associated antigen-specific CD4+ T lymphocytes undoubtedly play an important role in the induction of an antitumor immune response, we attempted to generate WT1-specific CD4+ T lymphocytes in vitro and examined their antileukemia functions. A CD4+ T-cell line, designated NIK-1, which proliferated and produced Th1 cytokines specifically in response to stimulation with the WT1-derived peptide, $WT1_{337-347}$ LSHLQMHSRKH, in an HLA-DP5–restriced manner was established. NIK-1 exhibited cytotoxicity against HLA-DP5–positive, WT1-expressing leukemia cells but did not lyse HLA-DP5–negative, WT1-expressing leukemia cells or HLA-DP5–positive, WT1–negative cells. NIK-1 did not inhibit colony formation by normal bone marrow cells of HLA-DP5–positive individuals. This is the first report to describe WT1-specific and HLA class II–restricted CD4+ T lymphocytes possessing direct cytotoxic activity against leukemia cells. (Blood. 2005;106: 1415-1418)

Introduction

Because Wilms tumor gene 1 product (WT1) is expressed in most cases of acute leukemia but not in normal tissues, it would be an attractive target antigen for immunotherapy against various malignancies including leukemia.[1-4] Recently, we and other investigators have succeeded in generating $CD8^+$ cytotoxic T lymphocytes (CTLs) that recognize WT1-derived peptides in vitro.[5-9] These WT1-specific CTLs efficiently lysed leukemia cells and solid tumor cells, but not normal cells, in an HLA class I–restricted manner. On the basis of these findings, clinical trials of cancer vaccine using WT1 peptides have been initiated.[10,11]

Increasing evidence from both murine and human studies indicates that tumor-associated antigen-specific $CD4^+$ T lymphocytes play a central role in orchestrating the host immune response against malignancies and infectious diseases.[12] Because identification of epitopes on WT1 recognized by $CD4^+$ T lymphocytes is essential for development of effective cellular immunotherapy for malignaicies targeting WT1, we attempted to generate WT1-derived peptide-specific $CD4^+$ T lymphocytes and examined their antileukemia functions.

Study design

Approval for the present study was obtained from the Institutional Review Board of Ehime University School of Medicine. Informed consent was obtained according to the Declaration of Helsinki.

On the basis of the amino acid sequence of WT1, a comprehensive panel of 43 20-mer peptides with 10 overlapping amino acids were synthesized. The WT1 peptide–specific $CD4^+$ T-cell lines were generated as reported previously.[13] Briefly, peripheral blood mononuclear cells (PBMCs) were stimulated 3 times with synthetic peptides at a concentration of 10 μg/mL. Cells showing a positive response to a WT1 peptide were cultured continuously in interleukin-2 (IL-2)–containing culture medium, and mitomycin C–treated autologous PBMCs and WT1 peptide were added to the wells every 1 to 2 weeks.

Chromium-51 release cytotoxicity assays were performed as described previously.[14] In some experiments, the target cells were incubated with an anti–HLA-DR monoclonal antibody (mAb) (L243), an anti–HLA-DQ mAb (HU-11), or anti–HLA-DP mAb (B7/21) at an optimal concentration for 30 minutes before adding the effector cells. Cold-target inhibition assays were performed as described previously.[15]

WT1 mRNA expression levels in cells were determined by quantitative reverse-transcription polymerase chain reaction (PCR) and calculated relative to that in the human leukemia cell line K562 as described previously.[16]

The effect of WT1-specific T lymphocytes on the growth of normal bone marrow cells was examined by performing the colony-forming assays as described previously.[5]

Results and discussion

A $CD4^+$ T-cell line, designated NIK-1, which proliferated specifically in response to stimulation with one of the 20-mer WT1

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Table 1. WT1-specific and HLA-DP5–restricted cytotoxicity by NIK-1: experiment 1

Target cells	% cytotoxicity, at E/T ratio		
	10:1 ratio	5:1 ratio	2.5:1 ratio
Auto LCL (HLA-DP5–positive)			
Without WT1 peptide	10.3	6.3	3.1
With WT1 peptide	70.2	63.2	49.1
Allo LCL no. 1 (HLA-DP5–positive)			
Without WT1 peptide	8.9	7.3	3.4
With WT1 peptide	73.9	62.1	50.1
Allo LCL no. 2 (HLA-DP5–positive)			
Without WT1 peptide	5.3	5.1	4.1
With WT1 peptide	63.2	56.1	47.3
Allo LCL no. 3 (HLA-DP5–negative)			
Without WT1 peptide	1.0	0.5	0.0
With WT1 peptide	2.1	0.8	0.1
Allo LCL no. 4 (HLA-DP5–negative)			
Without WT1 peptide	2.1	0.0	0.1
With WT1 peptide	4.1	0.8	0.0

Cytotoxicity of NIK-1 against autologous (auto) and various allogeneic (allo) LCL loaded or unloaded with WT1 peptide. E/T ratio indicates effector-target ratio.

peptides ($WT1_{336\text{-}355}$ KLSHLQMHSRKHTGEKPYQC) was established. More than 99% of NIK-1 cells were $CD3^+CD4^+CD8^-$. NIK-1 appeared to produce large amounts of T helper-1 (Th1) cytokines, such as interferon-γ, IL-2, and IL-12, upon stimulation with WT1 peptide in the presence of autologous PBMCs (data not shown). In addition to the proliferative response, NIK-1 showed strong cytotoxicity against a WT1 peptide–loaded autologous B-lymphoblastoid cell line (LCL) (Table 1). The restriction element of NIK-1 appeared to be HLA-DP, because the cytotoxicity and proliferative response of NIK-1 against WT1 peptide were significantly inhibited by adding anti–HLA-DP mAb. Because the donor was an HLA-DP5 homozygote, the cytotoxicity of NIK-1 against WT1 peptide–loaded *HLA-DP5* gene–transfected L cells was examined. As expected, NIK-1 exerted cytotoxicity against WT1 peptide–loaded HLA-DP5–positive L cells but not against $HLA\text{-}DP9^+$ L cells (Table 2). In addition, NIK-1 showed cytotoxicity against WT1 peptide–loaded HLA-DP5–positive but not HLA-DP5–negative allogeneic cells (Table 1), demonstrating that NIK-1 is the HLA-DP5–restricted $CD4^+$ T-cell line.

Table 2. WT1-specific and HLA-DP5–restricted cytotoxicity by NIK-1: experiment 2

Target cells	% cytotoxicity, at 10:1 E/T ratio
Auto LCL (HLA-DP5–positive)	
Without WT1 peptide	10.5
With WT1 peptide	63.6
With WT1 peptide and anti–HLA-DR mAb	59.9
With WT1 peptide and anti–HLA-DQ mAb	62.5
With WT1 peptide and anti–HLA-DP mAb	11.6
L-DP5 (HLA-DP5–positive)	
Without WT1 peptide	5.7
With WT1 peptide	68.3
With WT1 peptide and anti–HLA-DR mAb	62.6
With WT1 peptide and anti–HLA-DQ mAb	64.7
With WT1 peptide and anti–HLA-DP mAb	8.9
L-DP9 (HLA-DP5–positive)	
Without WT1 peptide	2.5
With WT1 peptide	4.2

Cytotoxicity of NIK-1 against autologous LCL, *HLA-DP5* gene–transduced L cells, and *HLA-DP9* gene–transduced L cells loaded or unloaded with WT1 peptide in the presence or absence of anti-HLA-DR, anti-HLA-DQ, or anti-HLA-DP mAb. E/T ratio indicates effector-target ratio.

Table 3. WT1-specific and HLA-DP5–restricted cytotoxicity by NIK-1: experiment 3

WT1 peptide loaded	% cytotoxicity, at 10:1 E/T ratio
None	3.5
KLSHLQMHSRKHTGEKPYQC	73.5
KLSHLQMHSRKHTGEKPYQ	68.5
KLSHLQMHSRKHTGEKPY	73.1
KLSHLQMHSRKHTGEKP	58.6
KLSHLQMHSRKHTGEK	70.2
KLSHLQMHSRKHTGE	71.5
KLSHLQMHSRKHTG	71.2
KLSHLQMHSRKHT	73.1
KLSHLQMHSRKH	72.7
KLSHLQMHSRK	32.5
KLSHLQMHSR	7.3
KLSHLQMHS	7.5
LSHLQMHSRKHTGEKPYQC	63.9
SHLQMHSRKHTGEKPYQC	32.4
HLQMHSRKHTGEKPYQC	6.6
LQMHSRKHTGEKPYQC	6.9
QMHSRKHTGEKPYQC	4.9
MHSRKHTGEKPYQC	6.4
HSRKHTGEKPYQC	5.9
SRKHTGEKPYQC	7.2
RKHTGEKPYQC	3.5
KHTGEKPYQC	7.8
HTGEKPYQC	7.7
LSHLQMHSRKH	72.1

Cytotoxicity of NIK-1 against autologous LCL loaded and unloaded with various WT1 peptides. E/T ratio indicates effector-target ratio.

We next examined the fine epitope on WT1 recognized by NIK-1. Experiments using deletion peptides clearly demonstrated that the minimal amino acid sequence recognized by NIK-1 is $WT1_{337\text{-}347}$ LSHLQMHSRKH (Table 3).

Because NIK-1 showed WT1 peptide–specific cytotoxicity, we addressed the question of whether NIK-1 can lyse leukemia cells. Because most leukemia cell lines are HLA class II negative, only one HLA-DP5–positive leukemia cell line expressing WT1 was available. As shown in Table 4, NIK-1 exerted strong cytotoxicity against HLA-DP5–positive WT1-expressing leukemia cell lines but not against HLA-DP5–negative leukemia cell lines or HLA-DP5–positive or HLA-DP5–negative lymphoma cell lines that are negative for WT1 expression. Similarly to the cytotoxicity against cell lines, HLA-DP5–positive but not HLA-DP5–negative freshly isolated leukemia cells were lysed efficiently by NIK-1. Cytotoxicity of leukemia cells mediated by NIK-1 appeared to be restricted by HLA-DP5, because only HLA-DP5–positive leukemia cells were lysed by NIK-1 and the cytotoxicity of leukemia cells mediated by NIK-1 was inhibited by adding anti–HLA-DP mAb (Table 5).

To further confirm that the cytotoxicity of NIK-1 against leukemia cells was mediated by the specific recognition of endogenously processed WT1, we performed cold-target inhibition experiments. As shown in Table 6, the addition of WT1 peptide–loaded autologous LCL decreased the cytotoxicity of NIK-1 against leukemia cells, whereas the addition of peptide-unloaded autologous LCL had no effect on the cytotoxicity. These data strongly suggest that WT1 is naturally processed in leukemia cells and recognized by WT1-specific $CD4^+$ CTLs in the context of HLA-DP5.

We then addressed the issue of whether NIK-1 recognizes WT1 peptide expressed on normal bone marrow progenitor cells and

Table 4. Direct recognition and lysis of leukemia cells by NIK-1: experiment 1

Target cells	WT1 expression level	HLA-DP5 expression	% cytotoxicity, at E/T ratio		
			20:1 ratio	10:1 ratio	5:1 ratio
Cell lines					
C2F8; leukemia	2.3×10^{0}	+	38.8	34.4	31.2
MEG01; leukemia	8.4×10^{-1}	−	3.6	1.8	1.2
KAZZ; leukemia	1.1×10^{0}	−	2.1	2.7	0.9
IZU; lymphoma	1.1×10^{-5}	+	1.5	0.5	1.0
IKE; lymphoma	8.6×10^{-5}	+	0.7	1.8	0.5
Daudi; lymphoma	3.8×10^{-5}	−	2.0	0.3	0.4
Freshly isolated leukemia cells					
From donor no. 1 (AML M1)	3.5×10^{-1}	+	45.1	34.2	21.0
From donor no. 2 (AML M2)	2.7×10^{-1}	+	33.6	24.3	18.9
From donor no. 3 (AML M2)	8.6×10^{-2}	+	31.2	18.9	13.1
From donor no. 4 (AML M1)	6.5×10^{-1}	−	2.7	2.1	0.6
From donor no. 5 (AML M2)	7.2×10^{-1}	−	1.4	1.8	1.0
From donor no. 6 (ALL L2)	5.8×10^{-1}	−	0.0	0.3	0.0

Cytotoxicity of NIK-1 against various cell lines and freshly isolated leukemia cells. E/T ratio indicates effector-target ratio.

suppresses their growth. As shown in Table 7, after coculture with NIK-1 in the absence of WT1 peptide, the numbers of granulocyte-macrophage colony-forming units (CFU-GMs) and erythroid burst-forming units (BFU-Es) generated from bone marrow cells of 2 HLA-DP5–positive individuals were almost the same as those generated from bone marrow cells cultured alone. However, the numbers of CFU-GMs and BFU-Es decreased significantly when HLA-DP5–positive bone marrow cells were pretreated with WT1 peptide and then cocultured with NIK-1. As expected, NIK-1 had no effect on colony formation by HLA-DP5–negative bone marrow cells that had been pretreated with WT1 peptide or left untreated.

In the present study, we demonstrated for the first time the generation of WT1-specific CD4+ T lymphocytes that can recognize directly leukemia cells in an HLA class II–restricted manner. It is well known that induction of the CD8+ CTL response requires cognate CD4+ T-lymphocyte help.[17] CD4+ T lymphocytes recognize major histocompatibility complex (MHC) class II–binding peptides on antigen-presenting cells, such as dendritic cells (DCs), and their interaction may result not only in activation and priming of CD4+ T lymphocytes but also in activation of the DCs themselves.[18] Consequent to this mutual activation, DCs prime and activate CD8+ CTLs specific for tumor-associated antigens. On the basis of this scenario, it is expected that WT1-specific CD4+ T lymphocytes may be effective for efficient induction of WT1-specific CD8+ CTLs in vivo.

The other interesting finding of this study is that WT1 peptide–specific CD4+ T lymphocytes exerted strong cytotoxicity against WT1-expressing leukemia cells in an HLA class II–restricted manner. In general, endogenous antigens are degraded in the cytoplasm to oligopeptides and bind to newly synthesized MHC class I molecules. On the other hand, exogenous antigens are processed into peptides capable of binding to MHC class II molecules. However, it has recently been shown that the MHC class II pathway can process and present endogenous antigens as well as exogenous antigens. For example, virus-infected cells are recognized by CD4+ T lymphocytes in a viral antigen–specific and MHC class II–restricted manner in vitro and in vivo.[19,20] It has also been reported that tumor cells transfected with syngeneic MHC class II genes could present endogenously synthesized tumor-associated protein-derived peptides in the context of MHC class II molecules to CD4+ T lymphocytes.[21] Taken together with previous data, our present findings strongly suggest that leukemia cells can process and present endogenously synthesized WT1 protein to CD4+ T lymphocytes in the context of HLA class II molecules.

Table 5. Direct recognition and lysis of leukemia cells by NIK-1: experiment 2

Target cells	% cytotoxicity, at 10:1 E/T ratio
C2F8 cells	
No anti-HLA mAb	27.6
With anti-HLA-DR mAb	28.0
With anti-HLA-DQ mAb	30.3
With anti-HLA-DP mAb	3.1
Freshly isolated leukemia cells	
From donor no. 1 (AML M1)	
No anti-HLA mAb	45.1
With anti-HLA-DR mAb	43.2
With anti-HLA-DQ mAb	41.6
With anti-HLA-DP mAb	5.6
From donor no. 2 (AML M2)	
No anti-HLA mAb	33.6
With anti-HLA-DR mAb	31.7
With anti-HLA-DQ mAb	35.1
With anti-HLA-DP mAb	5.6

Cytotoxicity of NIK-1 against leukemia cell line and freshly isolated leukemia cells in the presence or absence of anti-HLA- DR, anti-HLA-DQ, or anti-HLA-DP mAb.
E/T ratio indicates effector-target ratio.

Table 6. Direct recognition and lysis of leukemia cells by NIK-1: experiment 3

Hot target cells and cold target cells	% cytotoxicity, at cold-hot target cell ratio			
	0	5:1	10:1	20:1
C2F8 cells				
WT1 peptide–loaded autologous LCL	31.3	22.5	14.3	9.6
Peptide-unloaded autologous LCL	31.3	30.5	29.7	28.1
Freshly isolated leukemia cells from donor no. 1 (AML M1)				
WT1 peptide–loaded autologous LCL	42.1	26.4	15.2	8.8
Peptide-unloaded autologous LCL	42.1	41.3	39.3	40.2

Cytotoxicity of NIK-1 against leukemia cells in the presence or absence of WT1 peptide–loaded autologous LCL or peptide-unloaded autologous LCL at an effector–hot target cell ratio of 10:1.

Table 7. Direct recognition and lysis of leukemia cells by NIK-1: experiment 4

Donor	Colony formation	
	CFU-GM	BFU-E
HLA-DP5–positive donor no. 1		
Without NIK-1	56 ± 5	86 ± 8
With NIK-1	59 ± 7	93 ± 10
With NIK-1 and WT1 peptide	29 ± 3	37 ± 7
HLA-DP5–positive donor no. 2		
Without NIK-1	136 ± 14	167 ± 24
With NIK-1	138 ± 8	170 ± 23
With NIK-1 and WT1 peptide	66 ± 14	62 ± 13
HLA-DP5–negative donor no. 3		
Without NIK-1	86 ± 15	96 ± 18
With NIK-1	88 ± 7	100 ± 10
With NIK-1 and WT1 peptide	85 ± 15	103 ± 8

The colony formation by normal bone marrow cells cocultured with or without NIK-1 cells in the presence or absence of WT1 peptide.

In summary, we have demonstrated WT1-specific $CD4^+$ T lymphocytes capable of producing Th1 cytokines and exerting direct cytotoxicity against leukemia cells in an HLA class II–restricted manner. Because most types of leukemic cells are positive for HLA class II expression,[22] WT1-specific $CD4^+$ CTLs may play an important role in the antileukemia response through cytotoxic activity as well as helper function for $CD8^+$ CTL induction. On the basis of this concept, we are planning a clinical trial of WT1 peptide vaccination using a combination of peptides derived from epitopes recognized by $CD4^+$ T lymphocytes as well as $CD8^+$ T lymphocytes.

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Usefulness of the Novel Oncofetal Antigen Glypican-3 for Diagnosis of Hepatocellular Carcinoma and Melanoma

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Contents

Abstract

Glypican-3 (GPC3) mRNA and protein are expressed in >80% of human hepatocellular carcinomas (HCC) but not in normal tissues except for placenta and fetal liver. The oncofetal antigen GPC3 is a glycosylphosphatidyl inositol-anchored membrane protein and may be secreted. It is a novel tumor marker for human HCC: GPC3 protein was present in sera from 40–50% of HCC patients, but was not detected in sera from patients with liver cirrhosis or chronic hepatitis, or in sera from healthy individuals. α-Fetoprotein (AFP) and PIVKA-II (protein induced by vitamin K absence or antagonist-II), are well known major tumor markers for HCC. Generally, AFP shows high positivity for HCC but also high false-positivity in detection assays. *Lens culinaris* agglutinin-reactive fraction of α-fetoprotein (AFP-L3) is a recently described marker of HCC. Detection of AFP-L3 shows a much higher specificity than AFP, but a lower sensitivity. On the other hand, detection of PIVKA-II shows a lower false-positivity, but is not always sensitive enough to detect low levels secreted by small HCCs. There was no correlation between the three tumor markers, AFP, PIVKA-II, and GPC3 in terms of their presence in HCC cells. All three tumor markers showed similar positivity in patients with HCC, detecting 80% of patients with the disease.

GPC3 is also a novel tumor marker for the diagnosis of human melanoma, especially in the early stages of the disease. Expression of GPC3 mRNA and protein was evident in tumor cells from >80% of patients with melanoma and melanocytic nevus, which is a common benign lesion. GPC3 protein was detected in sera from

40% (36/91) of melanoma patients, but not in sera from those with large congenital melanocytic nevus, or from healthy donors. Surprisingly, we detected serum GPC3 even in patients with stage 0, *in situ* melanoma. The positive detection rate of serum GPC3 at stage 0, I, and II (44.4%, 40.0%, 47.6%, respectively) was significantly higher than that of 5-S-cysteinyldopa, a well known tumor marker for melanoma (0.0%, 8.0%, and 10.0%, respectively).

Interestingly, GPC3 was highly immunogenic in mice and elicited effective anti-tumor immunity with no evidence of autoimmunity. Thus, GPC3 is useful for diagnosis of HCC and melanoma and may also have a role in immunotherapy or tumor prevention. However, studies in humans are warranted.

Primary hepatocellular carcinoma (HCC) is one of the most common solid malignancies in the world and accounts for about 1 million deaths each year. Numerically, most of these cases occur in the Far East and are related to chronic infection with the hepatitis B virus (HBV) although, proportionally, chronic hepatitis C (HCV) is more important in developed Western countries.[1] Because of the global pandemic of hepatitis B and C viral infections, the incidence of HCC is increasing rapidly in Asian and Western countries,[2] and this trend is expected to continue for the next 50 years because of the long latency between infection and the development of HCC. The prognosis of patients with advanced HCC remains poor, and novel treatment and diagnostic strategies are needed urgently. There are several tumor markers, including carcinoembryonic antigen (CEA),[3,4] carbohydrate antigens (CA) 19-9,[5] and α-fetoprotein (AFP),[6] which may be used in different settings in cancer patients, including screening measures, differentiating between malignant and benign lesions, monitoring the response to treatment, and detecting recurrences. AFP and PIVKA-II (protein induced by vitamin K absence or antagonist-II)[7] are well known tumor markers for HCC. Generally, detection of AFP shows a high sensitivity but also shows a high false-positivity. Serum AFP levels are often increased in patients with benign liver diseases such as chronic hepatitis (CH) and liver cirrhosis (LC). *Lens culinaris* agglutinin-reactive fraction of α-fetoprotein (AFP-L3) is a recently described marker of HCC. Detection of AFP-L3 shows a much higher specificity than AFP, but a lower sensitivity. On the other hand, PIVKA-II shows a lower false-positivity, but is not always sensitive enough to detect low levels of PIVKA-II in small HCCs.

Age-adjusted incidence rates for melanoma have been increasing in most fair-skinned populations in recent decades. The annual increase in incidence rate varies between populations but generally has been in the order of 37% for fair-skinned Caucasian populations. Annual incidence rates vary from >40 per 100 000 persons in Australia to <5 per 100 000 in countries of low insolation in Northern Europe. The increase in incidence rates among non-European people with darker skin has not been as consistent, although the incidence rates are generally very low and trends are difficult to determine. The incidence in these populations varies from 0.1 to 3 per 100 000 persons per year depending on the skin type and latitude.[8] In the last decade, several molecules have been evaluated as tumor markers to detect melanoma, including melanin metabolites, adhesion molecules, cytokines, and melanoma-associated antigens.[9-11] In melanoma, several tumor markers have been evaluated for use as prognostic variables, to monitor response to therapy, and to detect recurrence.[12-14] Several investigators have reported[15-18] that 5-S-cysteinyldopa (5-S-CD) is useful as a marker for melanoma progression or for monitoring metastatic melanoma. 5-S-CD is often used as a tumor marker for melanoma in Japan and the usefulness of melanoma-inhibitory activity (MIA) as a tumor marker has also been reported.[19,20] However, detection of 5-S-CD often gives a false-positive result. Serum 5-S-CD levels are often increased in patients with a large congenital melanocytic nevus, which is a benign tumor.[21] There is no available tumor marker that can detect primary melanomas at early stages, that are small, and without metastasis. In addition, current methods are not sensitive enough to detect organ metastasis at early stages. A simple, inexpensive, and non-invasive method with high sensitivity to detect a serum tumor marker would aid the management of high-risk patients who have already had the disease but are at high risk of recurrence.

1. Novel Strategies for Identification of Tumor-Associated Antigens

Cloning of the human melanoma antigen (*MAGE*) gene with cDNA expression cloning methods, has indicated that the human immune system can recognize cancer as a foreign body and can respond to it.[22] This 'genetic approach' to T-cell epitope cloning led to the identification of a large number of genes encoding tumor antigens and antigenic peptides that are recognized by tumor-reactive cytotoxic T lymphocytes (CTLs), thereby enhancing the possibility of antigen-specific cancer immunotherapy.[23-26] In

1995, tumor-derived cDNA expression libraries were screened for identification of tumor-associated antigens. The antigens were recognized by high-titered IgG antibodies present in sera of cancer patients, so called serologic identification of antigens by recombinant expression cloning (SEREX). This method allows for the systematic identification of many human cancer-associated antigens, indeed, over 1500 types of tumor antigen have been identified using the SEREX method. [27-33] cDNA microarray technology, by which investigators can obtain comprehensive data regarding gene-expression profiles, is progressing rapidly. Studies have demonstrated the usefulness of this technique for identification of novel cancer-associated genes and for classification of human cancers at the molecular level.[34-39]

2. Identification and Expression of Glypican (GPC)-3 in Hepatocellular Carcinoma (HCC) and Melanoma

2.1 Identification by cDNA Microarray Analysis of the GPC3 Gene Over-Expressed in HCC

Antigens ideal for HCC tumor immunotherapy are those that are strongly expressed in almost all HCCs, but not in normal adult tissues (except for immune-privileged tissues such as testis and placenta or fetal organs). To identify such HCC antigens, two kinds of data were used from cDNA microarrays containing 23 040 genes. One was a comparison of expression profiles between 20 HCCs (10 cases were HBV-positive and 10 were HCV-positive) and corresponding adjacent non-cancerous liver tissues.[40] The other data was from various normal human tissues.[41]

GPC3 was identified as a gene over-expressed specifically in HCC.[42] In 16 of 20 HCCs, the expression of *GPC3* mRNA in the cancer tissue was ≥5-fold higher than that in non-cancerous tissues. The *GPC3* gene was found to be over-expressed in most HCCs and the expression was not related to HBV or HCV infection. *GPC3* mRNA is highly expressed in the placenta, fetal liver, fetal lung, and fetal kidney and expression is low in most normal adult tissues. Similar observations on GPC3 have been published by other investigators, based on northern blotting studies.[43,44] Thus, like AFP, GPC3 is a novel oncofetal antigen present in HCC.

2.2 Limited Expression of GPC3 Protein in Human HCC, Melanoma, and Fetal Tissues

GPC3 has been found to be overexpressed in HCC [32,42-48] and melanoma;[49] immunohistochemical analysis of GPC3 has been conducted using various human tissues (table I).[50] Immunohistochemical staining of GPC3 in HCC and melanoma tumor cells usually had a coarsely granular pattern located near the cell membrane. Strong membrane staining was also observed in several cases. Occasionally, there was diffuse nongranular staining of the cytoplasm. In >80% of HCC, melanoma, and melanocytic nevus tumor samples there was evident expression of GPC3 mRNA and protein.[42,49,50] GPC3 protein was expressed in placenta and fetal liver, but little or no expression was observed in all normal adult human tissues tested, including brain, lung, heart, liver, kidney, mammary gland, spleen, and thymus (table I).[50]

3. Detection of GPC3 in Patients

3.1 Detection of Soluble GPC3 in Sera from Patients with HCC

GPC3 is a glycosylphosphatidyl inositol (GPI)-anchored membrane protein and may be secreted. Using an enzyme-linked immunosorbent assay (ELISA), soluble GPC3 protein could be detected in culture supernatants from four of five HCC cell lines and in sera from 40% of patients with HCC.[42] The quantification by ELISA of GPC3 protein in sera from 40 HCC (27 HCV, 8 HBV, 6 non HBV or HCV), 13 LC (8 HCV, 4 HBV, 1 non HBV or HCV), 34 CH (31 HCV, 3 HBV) patients, from other patients, and from 60 healthy donors (HDs) is indicated in table II. We detected and quantified GPC3 protein in the sera from 16 of 40 HCC patients, but not in sera from patients with LC, CH, autoimmune hepatitis (AIH), primary biliary cirrhosis (PBC), HD, and other kinds of cancers (colon, gastric, pancreatic, biliary, esophageal, lung, and breast).

Table I. The expression levels of glypican-3 protein in various human tissues as determined by immunohistochemical analysis

Expression level	Tissue
Very strong	HCC, melanoma
Strong	Placenta, fetal liver
Weak	Lung, mammary gland
Very weak or none	Liver, brain, heart, kidney, pancreas, spleen, thymus, stomach, small intestine, colon, ovary, uterus, prostate, testis

HCC = hepatocellular carcinoma.

Table II. Clinical profiles of serum donors and detection of glypican (GPC)-3 using ELISA

Disease	Mean age (y)	Sex (m/f)	UICC stage[a]					GPC3 positive rate (% of patients)
			0	I	II	III	IV	
HCC	66	36/4		1	15	14	10	16/40 (40)
Liver cirrhosis	65	6/7						0/13 (0)
Chronic hepatitis	60	15/19						0/13 (0)
Autoimmune hepatitis	65	0/2						0/2 (0)
Primary biliary cirrhosis	79	0/1						0/1 (0)
Melanoma	66	43/48	9	25	21	18	18	36/91 (40)
Large congenital melanocytic nevus	21	3/2						0/5 (0)
Healthy donors	40	25/35						0/60 (0)
Cancers								
colon	66	16/5		1	6	5	9	0/21 (0)
gastric	71	9/5		7	3	4	0	0/14 (0)
pancreatic	58	6/5		0	0	0	11	0/11 (0)
biliary	70	2/4		0	3	1	2	0/6 (0)
esophageal	59	6/0		1	0	2	3	0/6 (0)
lung	64	7/0		3	0	0	4	0/7 (0)
breast	50	0/10		4	2	2	2	0/10 (0)

a UICC classification; TNM classification of malignant tumors.

f = female; **HCC** = hepatocellular carcinoma; **m** = male; **TNM** = tumor, nodes, metastasis; **UICC** = International Union Against Cancer; **y** = year.

3.2 Comparison of Serum Concentrations of GPC3, α-Fetoprotein (AFP), and PIVKA-II in Patients with HCC

There was no correlation between the three tumor markers AFP, PIVKA-II, and GPC3 in terms of their presence in HCC cells.[42] In our study, the sensitivity of AFP, AFP-L3, PIVKA-II, and GPC3 was 20/40 (50%), 10/36 (27.7%), 20/40 (50%), and 16/40 (40%), respectively. We could not diagnose 28 of 40 (70%) HCC patients using AFP and PIVKA-II without using GPC3. However, with GPC3 we could identify an additional four patients with HCC among 12 patients; three were classified as being in a relatively early disease stage (International Union Against Cancer [UICC] stage II), hence GPC3 may be useful for diagnosis of early-stage HCC. We could diagnose 80% of our patients with HCC using AFP, PIVKA-II, and GPC3.[42]

3.3 Detection of Soluble GPC3 in Sera from Patients with Melanoma

Soluble GPC3 protein could be detected in culture supernatants of 5 of 11 melanoma cell lines and in sera from 40% of patients with melanoma.[49] The quantification by ELISA of GPC3 protein in sera of 91 preoperative patients with melanoma, five patients with large congenital melanocytic nevus, and 60 HDs who had many small melanocytic nevi was performed.[49] We detected and quantified GPC3 protein in the sera of 36 of 91 melanoma patients (39.6%), but more importantly, not in sera of patients with large congenital melanocytic nevus and HDs, whereas GPC3 mRNA and protein were expressed in melanocytic nevus tissues.[49]

There is no convincing correlation between levels of secreted GPC3 as measured by ELISA and levels of *GPC3* mRNA and protein expression determined by RT-PCR and immunohistochemical analysis in HCC and melanoma cell lines, or in HCC and melanoma tissues. About 40% of HCC and melanoma patients showed characteristics of GPC3 secretion, irrespective of GPC3 expression levels. The mechanisms of secretion of GPC3 from HCC and melanoma cells remain to be elucidated.

3.4 Comparison of Serum Concentrations of GPC3, 5-S-CD, and Melanoma Inhibitory Activity in Patients with Melanoma Classified by Stage

We compared the serum concentrations of GPC3, 5-S-CD, and MIA in patients with melanoma classified by stage.[49] Although serum concentrations of 5-S-CD and MIA increased markedly in patients with stage IV disease, percentages of serum GPC3-positive patients were almost equal among the five clinical stages. To

our surprise, we detected GPC3 in the sera of patients with very small melanomas, such as stage 0 or I. There was no correlation between the positive state of three tumor markers, GPC3, 5-S-CD, and MIA.[49] More importantly, 27 of 36 GPC3-positive patients were negative for both 5-S-CD and MIA, and many were classified as having relatively early UICC stage 0, I, and II disease. Thus, GPC3 is very useful for the diagnosis of melanoma at early stages. Finally, we could diagnose 59 of 91 (64.8%) cases of melanoma using 5-S-CD, MIA, and GPC3.

3.5 GPC3 Protein in the Sera of HCC and Melanoma Patients Disappeared After Surgical Treatments

Changes in serum levels of GPC3 before and after surgical treatments were seen in 15 pre-operative GPC3-positive patients (three HCC patients and 12 melanoma patients). For example, GPC3 protein was detectable in three patients with HCC prior to surgery, but GPC3 was not detectable after the surgery. GPC3 protein was detected in sera of 12 melanoma patients prior to surgery, but not so after the surgery, except for one patient, who could not be followed after postoperative day 27. Thus, GPC3 is useful for monitoring the response to treatment. Taken together, these results indicate that GPC3 may prove appropriate for diagnosing patients with HCC and melanoma and determining the outcome of therapy.

4. Known Biologic Properties of GPC3

In 1996, Pilia et al.[51] reported that *GPC3*, which encodes one member of the glypican family, is mutated in patients with Simpson-Golabi-Behmel syndrome. This syndrome is an X-linked disorder characterized by pre- and post-natal overgrowth, and a broad spectrum of clinical manifestations that vary from a very mild phenotype in carrier females, to infantile lethal forms in some males.[52] The list of clinical manifestations of this syndrome includes a distinct facial appearance, cleft palate, syndactyly, polydactyly, supernumerary nipples, cystic and dysplastic kidneys, and congenital heart defects.[53,54] Most *GPC3* mutations are point mutations or small deletions encompassing a varying number of exons.[55,56] Given the lack of correlation between patient phenotype and location of the mutations, it has been proposed that Simpson-Golabi-Behmel syndrome is caused by the lack of a functional GPC3 protein, with additional genetic factors being responsible for the intra- and inter-familial phenotypic variation. The development of *GPC3*-deficient mice added strong support for this hypothesis.[57] These mice have several abnormalities found in Simpson-Golabi-Behmel syndrome patients, including overgrowth, and cystic and dysplastic kidneys.

Furthermore, it was reported that GPC3 could induce apoptosis in certain types of tumor cells.[58] Some reports indicated that GPC3 expression is downregulated in tumors of different origin. They showed that, although GPC3 is expressed in normal ovary, mammary gland, and mesothelial cells, the expressions are undetectable in a significant proportion of ovarian and breast cancer, and mesothelioma cell lines.[59] In all cases where GPC3 expression was lost, the *GPC3* promoter was hypermethylated, and mutations were nil in the coding region. GPC3 expression was restored by treatment with a demethylating agent. In addition, the authors demonstrated that ectopic expression of GPC3 inhibits colony-forming activity in several of these cancer cell lines. Collectively, these data suggest that GPC3 can act as a negative regulator of growth in these cancers. As the expression of GPC3 is reduced during tumor progression in cancers originating from tissues that are GPC3-positive in adults, this reduction seems to play a role in generation of the malignant phenotype.

However, in the case of HCC tumors originating from tissues that express GPC3 only in the embryo, GPC3 expression tends to reappear with malignant transformation. Whether or not re-expression of GPC3 plays a role in progression of these tumors is unknown, i.e. why is GPC3 upregulated only in HCC and melanoma? Whether GPC3 is involved in oncogenesis in melanoma and HCC is under investigation in our laboratory.

4.1 Mouse Homolog of a Human GPC3 Evokes T Cell-Mediated Tumor Rejection Without Autoimmune Reactions in Mice

GPC3, expressed in almost all HCCs and melanomas, but not in normal tissues except for placenta or fetal liver, is an ideal tumor antigen for immunotherapy. Very recently, we reported that GPC3 could be highly immunogenic in mice, eliciting effective antitumor immunity with no evidence of autoimmunity in mice.[50] In this study, we identified a mouse GPC3-derived and K^d-restricted CTL epitope peptide in BALB/c mice. Inoculation of these GPC3 peptide-specific CTLs into subcutaneous Colon26 cancer cell tumors transfected with the mouse *GPC3* gene (C26/GPC3) led to rejection of the tumor *in vivo*. In addition, intravenous inoculation of these CTLs into sublethaly irradiated mice markedly inhibited growth of an established subcutaneous tumor. Inoculation of bone-marrow-derived dendritic cells pulsed with this peptide prevented the growth of subcutaneous and splenic C26/GPC3 tumors accompanied with massive infiltration of $CD8^+$ T cells into tumors.

Evidence of autoimmune reactions was not observed in surviving mice that had rejected tumor cell challenges.

Thus, GPC3 is useful, not only for the diagnosis of HCC and melanoma, but also for possible immunotherapy or prevention of these tumors.

5. Conclusion

The novel oncofetal protein GPC3 appears to be a novel tumor marker useful for the diagnosis of HCC and melanoma, especially in early stages of the disorder. Furthermore, we found GPC3 to be highly immunogenic in mice and elicited effective anti-tumor immunity with no evidence of autoimmunity. Thus, GPC3 may be useful not only for diagnosis of HCC and melanoma, but also for possible immunotherapy or tumor prevention. The next step is to introduce GPC3 into the clinic as a tumor marker and as a cancer antigen for immunotherapy. We are making a GPC3 ELISA kit for the diagnosis of HCC and melanoma, and are planning to conduct a clinical trial of immunotherapy for HCC using GPC3 as a cancer antigen.

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