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Immunology

Evaluation of pH-sensitive fusogenic polymer-modified liposomes co-loaded with antigen and α-galactosylceramide as an anti-tumor vaccine

Seiji OKAZAKI[1)], Tadashi IWASAKI[1)], Eiji YUBA[2)] and Shinobu WATARAI[1)]*

[1)]Division of Veterinary Science, Graduate School of Life and Environmental Sciences, Osaka Prefecture University, Izumisano, Osaka 598-8531, Japan

[2)]Department of Applied Chemistry, Graduate School of Engineering, Osaka Prefecture University, Sakai, Osaka 599-8531, Japan

ABSTRACT. pH-Sensitive fusogenic polymer-modified (pH-sensitive) liposomes co-loaded with tumor model antigen, ovalbumin (OVA), and adjuvant, α-galactosylceramide (α-GalCer) were fabricated and administered subcutaneously into mice. The ability of pH-sensitive liposomes containing OVA and α-GalCer to stimulate cellular and humoral immune responses *in vivo* was compared with OVA-encapsulating pH-sensitive liposomes as well as with OVA alone. After immunization, significant OVA-specific antibodies were detected in the serum. When sera were analyzed for isotype distribution, antigen-specific IgG1 antibody responses were noted in mice immunized with OVA alone, whereas immunization with OVA-containing pH-sensitive liposomes and with pH-sensitive liposomes containing OVA and α-GalCer resulted in the induction of OVA-specific IgG1 and IgG2b antibody responses. Moreover, more substantial production of IFN-γ and IL-4 was demonstrated in spleen cells from mice immunized with pH-sensitive liposomes having OVA and α-GalCer than OVA-containing pH-sensitive liposomes *in vitro*. Spleen cells from the immunized mice showed strong cytotoxic activity against E.G7-OVA tumor cells. In addition, prophylactic vaccination efficacy against tumor formation was evaluated. In all mice immunized with pH-sensitive liposomes having OVA and α-GalCer, immunization provided substantial protection from tumor formation. The therapeutic efficacy of pH-sensitive liposomes containing OVA and α-GalCer against already established E.G7-OVA tumors was also investigated. Tumor growth was reduced significantly in all mice treated with pH-sensitive liposomes having OVA and α-GalCer. The provided evidence on the advantage of antigen and α-GalCer co-encapsulation into pH-sensitive liposomes should be considered in the design of future cancer vaccines for prophylactic and therapeutic purposes.

KEY WORDS: cancer immunotherapy, α-GalCer, liposome, pH-sensitive liposome, tumor vaccine

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Tumor immunotherapy is expected to become an effective approach in the treatment of cancer [17]. Tumor specific peptides or proteins are used as antigen to induce an immune system and to kill cancer cells. A tumor-specific immune response starts by recognizing the cancer antigen by antigen presenting dendritic cells (DCs). The captured antigen is present on MHC class I and II molecules and the stimulated DCs then elicit the formation of anticancer effector T cells with the help of co-stimulatory molecules. Antigen educated T cells, cytotoxic T lymphocytes (CTLs), then kill the cancer cells [3]. In addition, antigen nonspecific tumor immunotherapy in which an adjuvant is involved, is also of current interest, because stimulation of the innate immune system aids in this type of therapy. Many efforts have been made in this area using tumor vaccines and the results show that the stimulation of innate immune system with a suitable adjuvant is also important for achieving tumor immunotherapy [10]. An adjuvant can reduce the amount of antigen produced and induce the desired immune responses. Because of this, the use of efficient adjuvants is also an important issue in the field of tumor immunotherapy.

Alpha-galactosylceramide (α-GalCer) is a unique adjuvant that enables the activation of both specific and non-specific immune responses. In general, adjuvants such as the toll-like receptor ligand activate innate immunity *via* the production of inflammatory cytokines and type I interferon (IFN). On the other hand, α-GalCer activates innate and adaptive immunity *via* the production of large amounts of INF-γ, which would be expected to show anti-tumor activity independent of the specific class of tumor [12].

*Correspondence to: Watarai, S.: swatarai@vet.osakafu-u.ac.jp

α-GalCer, a synthetic glycolipid, is presented by CD1d molecules, antigen-presenting molecules, on DCs to invariant T cell receptor-bearing natural killer T (iNKT) cells [24]. Recognition of the resulting α-GalCer/CD1d complex on DCs by iNKT cells stimulate both iNKT cells and DCs which in turn, induce the secretion of various pro-inflammatory cytokines and activate a broad spectrum of immune cells against the tumor [22]. Among these, IFN-γ and IL-12 are the most abundant, which can switch the immune response in favor of a Th1 driven response. Additionally, the CD40-CD154 ligation during the presentation of glycolipids to iNKT cells evokes DC maturation up-regulating other co-stimulatory ligands [4]. Based on these characteristics, α-GalCer has been regarded as a potent vaccine adjuvant [5]. However, it has been reported that soluble α-GalCer is not taken up efficiently by DCs [21]. Hence, to induce a sufficient immune response by the administration of α-GalCer, it will be necessary to control the disposition and cellular uptake of α-GalCer with the currently available delivery systems. Thus far, very few studies have been devoted to examining adjuvant effects of α-GalCer by incorporating α-GalCer into delivery systems [18].

Liposomes are lipid multilayer vesicles that have been successfully used as delivery systems for antigens, drugs and genomic material [6]. Use of liposome-associated antigens is known to induce protective immunity against microbial infections [2, 7, 9, 11, 15]. To establish more effective vaccine, in a previous study, we developed pH-sensitive liposomes, which generate fusion ability under weakly acidic conditions, by surface modification of liposomes with pH-sensitive fusogenic polymer having carboxyl groups, such as succinylated poly (glycidol) (SucPG) and reported on the potential of SucPG-modified (pH-sensitive) liposomes as a vaccine delivery system [31]. pH-Sensitive liposomes in which ovalbumin (OVA) is encapsulated as a protein antigen, were efficiently taken up by dendritic cells (DCs) and induced strong antigen presentation *via* MHC class I molecules *in vitro* [31]. In addition to protein antigen, we have also reported the utility of liposomes as a delivery system of lipid antigens, such as glycolipids [26, 27, 29, 30]. These observations suggest that tumor antigen-loaded pH-sensitive liposomes incorporating α-GalCer should exhibit the ability for the induction of an anti-tumor effect *via* activation of an immune response. To date, however, the therapeutic potential of such liposomes has not been clarified.

In this study, we investigated the ability of pH-sensitive liposomes co-loaded with tumor antigen and α-GalCer to induce tumor antigen-specific CTLs and their anti-tumor effect *in vivo*. The findings reported herein indicate that tumor antigen and α-GalCer modified pH-sensitive liposomes not only stimulate tumor antigen-specific CTLs effectively, but also induce prophylactic and therapeutic effects in the mouse tumor model.

MATERIALS AND METHODS

Materials

Dipalmitoylphosphatidylcholine (DPPC), dioleoylphosphatidylethanolamine (DOPE), OVA, bovine serum albumin (BSA) (Sigma-Aldrich Co., St. Louis, MO, U.S.A.), and α-GalCer (Enzo Life Sciences, Inc., Farmingdale, NY, U.S.A.) were commercial products. SucPG polymer was prepared as previously reported [31].

Mice

Female C57BL/6 mice (H-2^b, 6 weeks old) were purchased from Japan SLC, Inc., Hamamatsu, Shizuoka, Japan, and were maintained at the experimental facility, Education and Research Center for Experimental Animal Science, of Osaka Prefecture University. Animal experiments were conducted in accordance with the guidelines for animal experimentation in Osaka Prefecture University.

Cell lines

E.G7-OVA cells (OVA cDNA-transfectant of EL4 (C57BL/6 mouse-derived T lymphoma, H-2^b) cells) were cultured in RPMI-1640 medium (Nissui Phamaceutical Co., Ltd., Tokyo, Japan) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (SAFC Biosciences, St. Louis, MO, U.S.A.), 100 U/m*l* penicillin, 100 μg/m*l* streptomycin, 2.5 μg/m*l* fungizone, and 200 μg/m*l* G418 (Life Technologies Co., Carlsbad, CA, U.S.A.).

Preparation of antigen and α-GalCer-containing pH-sensitive liposomes

pH-Sensitive liposomes that entrap antigen and α-GalCer were prepared by the following method. DPPC (2.5 μmol), DOPE (2.5 μmol), α-GalCer (25 μg), and SucPG polymer (lipids/polymer=7/3, w/w), each dissolved in an organic solvent (DPPC, DOPE and α-GalCer, chloroform/methanol=2/1, v/v; SucPG polymer, methanol), were mixed in a conical flask. The lipids were dried on a rotary evaporator, and left to stand for 60 min in a high vacuum in a desiccator. After addition of 1 m*l* of PBS containing OVA (5 mg/m*l*), the lipid film was dispersed by vigorous vortexing. Any un-encapsulated OVA was removed by repeated centrifuging at 19,000 × *g* for 30 min at 4°C in PBS, and the resulting OVA encapsulated in α-GalCer-modified pH-sensitive liposome suspension was used for immunization. Furthermore, α-GalCer-un-modified pH-sensitive liposomes containing OVA were also prepared according to the above procedure using lipid mixture solution without α-GalCer.

The amount of OVA entrapped in liposomes was determined by the following method. Fifty μ*l* of isopropyl alcohol was added to a 50 μ*l* suspension of liposome-entrapped OVA (at 2-fold dilution in PBS), followed by vortex mixing. The protein concentration of the resulting solutions was determined using a Bio-Rad protein assay kit (Bio-Rad Laboratories, Richmond, CA, U.S.A.), with bovine plasma gamma globulin used as a standard.

Immunization of mice

Mice were divided into 3 groups (4 mice per a group). Each group was subcutaneously immunized as follows: group I, OVA solution; group II, α-GalCer-un-modified pH-sensitive liposomes that entrap OVA; group III, pH-sensitive liposomes co-loaded with OVA and α-GalCer (5 μg per a mouse). Immunization was repeated two times at 2-week intervals. One hundred μl of each formulation containing 100 μg of OVA were given per a mouse. One week after final immunization, the mice were euthanized, and sera and spleens were harvested. Sera were used for antibody assay. Mononuclear cells used for cytokine measurements and cytotoxic T lymphocytes (CTLs) assay were isolated by HISTOPAQUE 1077 (Sigma-Aldrich) density centrifugation method from spleen cell suspension.

Antibody assay

OVA was diluted with PBS (20 μg protein/m*l*) and dispensed in 50 μl/well into a 96-well microtiter plate (ASAHI TECHNO GLASS Co., Ltd., Shizuoka, Japan), followed by incubation at 37°C for an hr. The plates were washed 5 times with PBS containing 0.1% Tween 20 (washing solution). The wells were treated with 100 μl of PBS containing 1% BSA (solution A), and left at 4°C overnight to block nonspecific binding, and then washed 2 times with the washing solution. After that, 50 μl of sera diluted with solution A was added to each well. The plates were incubated at 37°C for an hr, and washed 7 times with the washing solution, and then 50 μl of horseradish peroxidase-labeled anti-mouse IgG (1:10,000 dilution in solution A; American Qualex), IgG1 (at 1:8,000 dilution in solution A; Zymed Laboratories) or IgG2b (at 1:20,000 dilution in solution A; Zymed Laboratories) solution was added as the second antibody. Following incubation at 37°C for an hr, the plates were washed 10 times with the washing solution, and 100 μl of *o*-phenylenediamine dihydrochloride substrate solution (Sumitomo ELISA Color Reagent Kit; Sumitomo Bakelite Co., Ltd., Tokyo, Japan) was reacted for 10 min at room temperature. The enzyme reaction was stopped by adding of stopping solution (Sumitomo ELISA Color Reagent Kit), and absorbance at 490 nm was measured with a microplate reader (MTP-810Lab, CORONA ELECTRIC Co., Ltd., Hitachinaka, Japan). Antibody titers are represented as the reciprocal of endpoint dilution exhibiting an optical density more than 2.5 times that of background.

Cytokine measurements and CTL assay

Spleen cells from mice in groups II and III were suspended in RPMI-1640 medium supplemented with 10% heat-inactivated FBS, 100 U/m*l* penicillin, 100 μg/m*l* streptomycin and at a density of 1×10^7 cells/m*l*, dispensed in a 24 well plate (Asahi Techno Glass), and cultured in 1 m*l* of RPMI-1640 medium containing 10% heat-inactivated FBS, 100 U/m*l* penicillin, 100 μg/m*l* streptomycin and in the presence of OVA (5 μg/m*l*). To detect antigen-specific T cell-derived cytokine production, culture supernatants were collected 3 days after incubation and kept at −80°C until assayed for cytokines. Additionally, spleen cells (effector cells) thus prepared were co-cultured with target cells (E.G7-OVA cells) at a effector/target ratio of 40:1 for 6 hr at 37°C and cytotoxicity was measured with a lactate dehydrogenase (LDH) cytotoxicity detection kit (Takara Biomedicals, Tokyo, Japan). On the other hand, the levels of Th1 and Th2 cytokines (IFN-γ and IL-4) were determined with murine cytokine assay kits (Mouse ELISA kit, Thermo Fisher Scientific K.K., Yokohama, Japan).

Evaluation of anti-tumor efficacy of subcutaneous immunization with pH-sensitive liposomes containing OVA and α-GalCer

To evaluate prophylactic vaccination efficacy of pH-sensitive liposomes co-loaded with OVA and α-GalCer against tumor formation, five C57BL/6 mice were subcutaneously immunized two times at 2-week intervals with OVA solution (group I), α-GalCer-un-modified pH-sensitive liposomes entrapping OVA (group II), or pH-sensitive liposomes containing OVA and α-GalCer (group III). One week after the final immunization, the mice were inoculated subcutaneously with 1×10^6 E.G7-OVA cells into their left backs under anesthesia. Tumor growth was monitored by measuring the major and minor axes of the tumors using digital caliper. Tumor volume was calculated using the following formula: tumor volume (mm^3)=0.4 × (major axis; mm) × (minor axis; $mm)^2$. The mice were euthanized when tumor volumes were greater than 2,000 mm^3.

Evaluation of therapeutic efficacy of pH-sensitive liposomes containing OVA and α-GalCer on tumor-bearing mice

The therapeutic efficacy of OVA encapsulated in α-GalCer-modified pH-sensitive liposomes against established E.G7-OVA tumors was investigated. E.G7-OVA tumor cells (1×10^6) were injected subcutaneously into their left backs of C57BL/6 mice on day 0. When the tumor mass become palpable (4–5 mm in length, around day 7), and day 14, the mice were immunized subcutaneously in their right backs with OVA solution (group I), α-GalCer-un-modified pH-sensitive liposomes entrapping OVA (group II), or pH-sensitive liposomes containing OVA and α-GalCer (group III). The anti-tumor activity was determined by measuring tumor growth. The mice were euthanized when tumor volumes were greater than 2,000 mm^3.

Statistical analysis

All data are presented as the means ± standard errors (SE). The statistical significance was evaluated by *t*-test. Mouse survival was analyzed by Kaplan-Mayer curves using log-rank tests. A value of $P<0.05$ was considered to be statistically significant.

RESULTS

Serum antibody responses in mice immunized with pH-sensitive liposomes co-loaded with OVA and α-GalCer

It has been reported that the SucPG-modified (pH-sensitive) liposomes, which have diameters of around 400–500 nm and

negative zeta potentials of around −11 mV, can activate immune response effectively [31]. In addition, our preliminary experiments revealed that the pH-sensitive liposomes incorporating α-GalCer at a dose of 5 μg per a mouse have high ability to activate immune response (data not shown). In this study, thus, we used pH-sensitive liposomes co-loaded with OVA and 5 μg per a mouse of α-GalCer. Antibodies against OVA were evaluated on day 0, 14 and 21 after primary immunization. The antigen-specific IgG antibody titers in group III on day 14 and 21 after primary immunization were 12,800 ± 4,525 and 153,600 ± 29,560, respectively, and were significantly higher when compared to group I (day 14, P<0.001; day 21, P<0.001). On the other hand, the antigen-specific IgG antibody titers in group II on day 14 and 21 after the primary immunization were 8,000 ± 1,600 and 153,600 ± 29,560, respectively, and were also significantly higher when compared to group I) (day14, P<0.0001; day 21, P<0.0001) (data not shown).

Furthermore, serum antibody responses were characterized by analyzing the pattern of IgG subclasses present in sera from mice in the groups I to III (Fig. 1). As shown in Fig. 1a, OVA-specific serum IgG1 antibody responses were demonstrated in the serum from 3 groups. In particular, production of anti-OVA IgG1 antibody was significantly enhanced by the administration of α-GalCer-un-modified pH-sensitive liposomes that entrap OVA (group II) and by pH-sensitive liposomes co-loaded with OVA and α-GalCer (group III) than by OVA solution (group I) (P<0.0001). On the other hand, the induction of OVA-specific serum IgG2b antibody responses was demonstrated in sera from mice in the groups II and III, although the serum IgG2b antibody activities against OVA antigens were not detected in any mice in group I (Fig. 1b). Between groups II and III, IgG2b antibody responses against OVA were higher in the group III than in the group II (Fig. 1b).

Th1 and Th2 cytokine production by spleen cells from mice immunized with OVA-containing α-GalCer-modified pH-sensitive liposomes

The induction of OVA-specific serum IgG1 and IgG2b antibody responses by the immunization with OVA-containing α-GalCer-un-modified pH-sensitive liposomes (group II) and with α-GalCer-modified pH-sensitive liposomes containing OVA (group III) suggests efficient major histocompatibility complex presentation of the antigen leading to both a humoral (IgG1) (Th2) and a cell-mediated (IgG2b) (Th1) response (Fig. 1). To characterize antigen-specific Th1 and Th2 responses, spleen cells were isolated from mice in the groups II and III and re-stimulated with OVA *in vitro*. Culture supernatants from OVA-stimulated spleen cells were then examined for the presence of Th1 and Th2 cytokines by ELISA. As shown in Fig. 2, substantial production of Th1 (IFN-γ) and Th2 (IL-4) cytokines was demonstrated in spleen cells from mice in the groups II and III. However, higher levels of both IFN-γ and IL-4 were detected in the culture supernatant harvested from *in vitro* OVA-stimulated spleen cells from mice in the group III than did spleen cells from mice in the group II (IFN-γ, P<0.08; IL-4, P<0.09) (Fig. 2a and 2b), suggesting that immunization with OVA-containing α-GalCer-modified pH-sensitive liposomes induces an antigen-specific Th1-type immune response (cellular immunity) effectively.

Induction of CTL responses by OVA and α-GalCer-co-encapsulated pH-sensitive liposomes

In systemic tumor immunity, the antigen-specific cellular immunity plays an important role in the elimination of cancerous cells. Therefore, we next estimated the induction of the antigen-specific CTL upon administration of pH-sensitive liposomes co-loaded with OVA and α-GalCer (group III) and OVA-loaded α-GalCer-un-modified pH-sensitive liposomes (group II), and compared their CTL-activation with the administration of free OVA (group I). As shown in Fig. 3, cytotoxic effects were observed in the effector cells prepared from mice in the groups II and III. Especially, splenocytes from mice in the group III induced significantly higher CTL response against OVA-expressing tumor (E.G7-OVA) cells when compared to the groups I and II (P<0.01). Higher cytotoxicity of splenocytes from mice in the group III against E.G7-OVA cells indicates that OVA-specific CTLs were induced more efficiently by immunization of α-GalCer-modified pH-sensitive liposomes than α-GalCer-un-modified pH-sensitive liposomes.

Preventive antitumor effect of immunization with pH-sensitive liposomes co-loaded with OVA and α-GalCer

Considering the CTL responses in α-GalCer-un-modified and α-GalCer-modified pH-sensitive liposomes containing OVA-immunized mice (Fig. 3), it was expected that immune responses would lead to anti-tumor activity in a transplantable E.G7-OVA tumor model. To evaluate if the CTL responses induced by the immunization were potent enough to provide an *in vivo* anti-tumor effect, the mice (five per group) were immunized two times with OVA solution (group I), α-GalCer-un-modified pH-sensitive liposomes entrapping OVA (group II) or pH-sensitive liposomes containing OVA and α-GalCer (group III). The immunized mice were challenged subcutaneously with live E.G7-OVA cells and the size of tumor was measured for 17 days. As shown in Fig. 4, mice in the group I exhibited a rapid increase in tumor volume after day 11 from the inoculation of tumor cells. On the other hand, all mice that received α-GalCer-un-modified pH-sensitive liposomes entrapping OVA (group II) or pH-sensitive liposomes containing OVA and α-GalCer (group III) completely rejected the E.G7-OVA tumor. These results indicate that the encapsulation of OVA by pH-sensitive liposomes improved anti-tumor immune responses. For OVA-loaded pH-sensitive liposomes, OVA-specific CTLs induced by these liposomes might kill the tumor cells efficiently.

Therapeutic effect of pH-sensitive liposomes co-loaded with OVA and α-GalCer on tumor-bearing mice

We finally examined therapeutic effect against an established E.G7-OVA tumor. Mice of groups I to III were inoculated with E.G7-OVA tumor cells (1×10^6) on day 0. On day 7, when the tumor mass become palpable (4–5 mm in length), tumor-bearing mice were immunized with OVA solution (group I), α-GalCer-un-modified pH-sensitive liposomes entrapping OVA (group II), or pH-sensitive liposomes containing OVA and α-GalCer (group III). The immunization procedures were repeated two times at one-

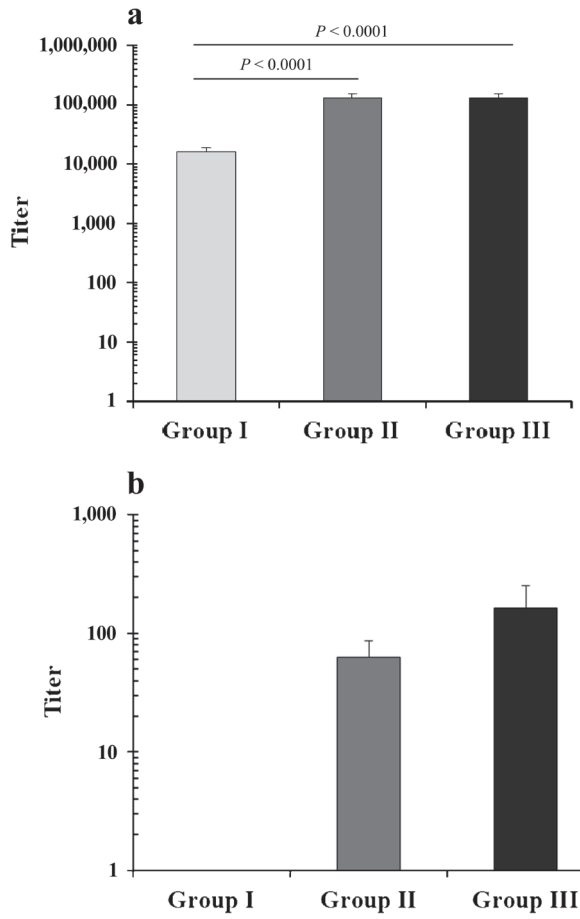

Fig. 1. Profiles of OVA-specific IgG antibody subclasses, IgG1 (a) and IgG2b (b), in mice subcutaneously immunized with pH-sensitive liposomes co-loaded with OVA and α-GalCer. Mice were immunized subcutaneously with OVA solution (group I), α-GalCer-un-modified pH-sensitive liposomes containing OVA (group II) and α-GalCer-modified pH-sensitive liposomes entrapping OVA (group III), and antibody titers were determined by ELISA on day 7 following final immunization. Results are expressed as mean ± SE in 4 different mice.

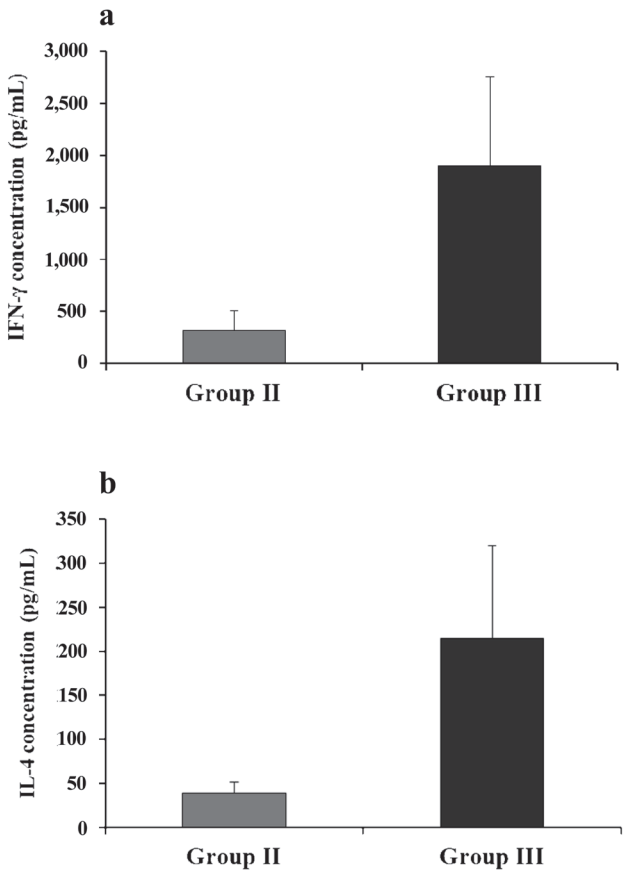

Fig. 2. Th1 (IFN-γ) (a) and Th2 (IL-4) (b) cytokine secretion by spleen cells from mice immunized subcutaneously with OVA-containing α-GalCer-modified pH-sensitive liposomes. Spleen cells were harvested on day 7 after final immunization and cultured with OVA for 3 days. Then, culture supernatants were collected for the analysis of cytokine production by ELISA. Values represent the mean ± SE of cytokine production by spleen cells of mice in the groups II and III.

week intervals. Then tumor growth and the survival of mice were monitored (Fig. 5). As shown in Fig. 5a, immunization with OVA solution (group I) exhibited no tumor suppressive effect. However, when mice were immunized with OVA-loaded α-GalCer-un-modified pH-sensitive liposomes (group II) and OVA-loaded α-GalCer-modified pH-sensitive liposomes (group III), tumor volume decreased remarkably after 6–7 days from their first administration, indicating that the OVA-specific immunity was effectively induced in mice. Furthermore, comparison of tumor suppressive effects between α-GalCer-un-modified and -modified pH-sensitive liposomes having OVA revealed that α-GalCer-modified OVA-loaded pH-sensitive liposomes (group III) showed higher anti-tumor effect than that of α-GalCer-un-modified pH-sensitive liposomes that entrap OVA (group II) (day 17, $P<0.05$; day 18, $P<0.001$; day 19, $P<0.01$; day 20, $P<0.01$) (Fig. 5a). Twenty% of mice in the group III showed complete elimination of the E.G7-OVA tumor (Fig. 5a). In addition, the survival rates of mice in the groups I to III showed in Fig. 5b. The survival rate for each group correlates with the result of tumor growth suppression showed in Fig. 5a. All mice that received OVA solution (group I) were euthanized on day 20. On the other hand, all mice treated with OVA-loaded α-GalCer-modified pH-sensitive liposomes (group III) survived for more than 25 days, by which time 80% of mice that received OVA-loaded α-GalCer-un-modified pH-sensitive liposomes (group II) had been euthanized (Fig. 5b). This result might have derived from the induction of strong Th1 responses by the α-GalCer-modified pH-sensitive liposomes (Figs. 2 and 3).

DISCUSSION

As noted in the introduction, the purpose of the present study was to evaluate the potential for the use of pH-sensitive liposomes

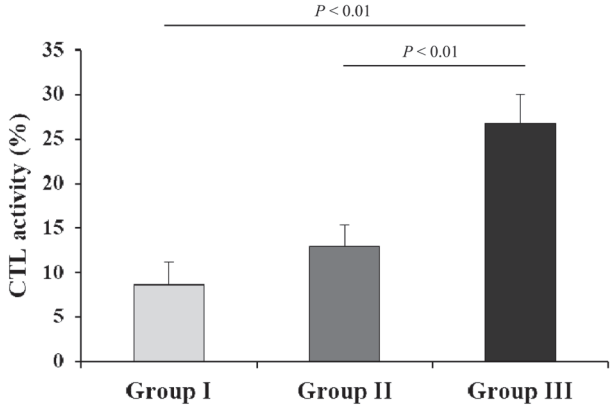

Fig. 3. CTL activity of spleen cells from mice immunized subcutaneously with OVA solution (group I), α-GalCer-un-modified pH-sensitive liposomes containing OVA (group II) and α-GalCer-modified pH-sensitive liposomes entrapping OVA (group III). Cytotoxic activity was measured by a LDH assay at an effector/target ratio of 40:1. E.G7-OVA cells were used as target cells. Data represent mean ± SE.

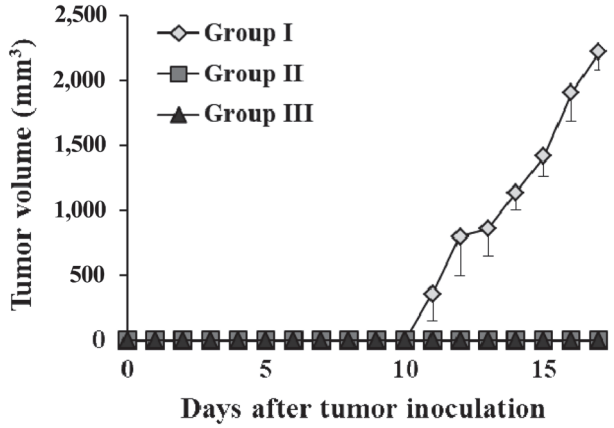

Fig. 4. Prophylactic effect of immunization with pH-sensitive liposomes co-loaded with OVA and α-GalCer against an E.G7-OVA *in vivo* tumor mice model. C57BL/6 mice were subcutaneously immunized with OVA solution (group I), α-GalCer-un-modified pH-sensitive liposomes entrapping OVA (group II), and pH-sensitive liposomes containing OVA and α-GalCer (group III) 14 and 7 days before E.G7-OVA tumor cell inoculation. Tumor volume was measured during a 17-day period post tumor inoculation. Values are means of tumor volume ± SE.

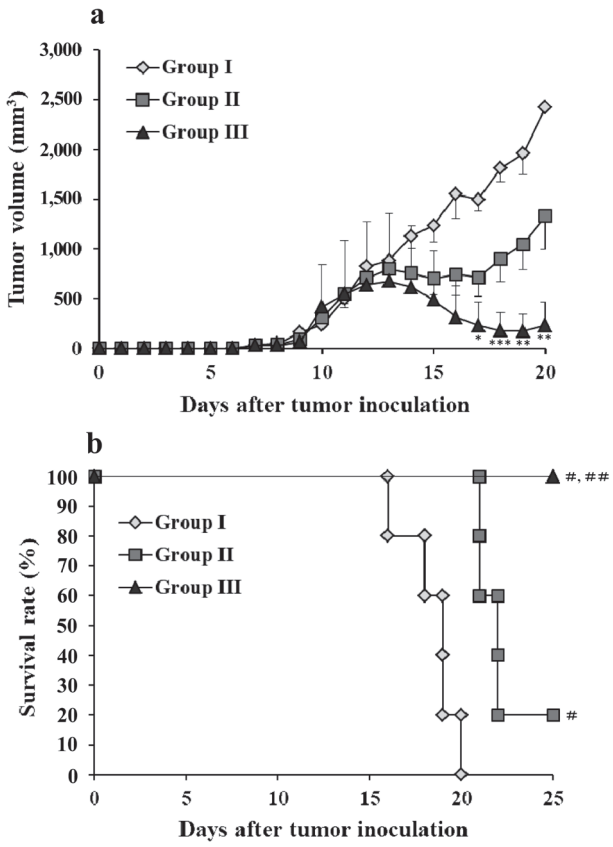

Fig. 5. Therapeutic effect of pH-sensitive liposomes co-loaded with OVA and α-GalCer against established E.G7-OVA tumor. Mice were inoculated by subcutaneous injection of E.G7-OVA tumor cells in the left dorsal area on day 0. When the tumor mass became palpable (around day 7), OVA solution (group I), α-GalCer-un-modified pH-sensitive liposomes entrapping OVA (group II), or pH-sensitive liposomes containing OVA and α-GalCer (group III) was injected into the side where the tumor had not grown. (a) Change in tumor volume, with tumor size examined every day. Each point represents mean ± SE. **P*<0.05 (day 17), ***P*<0.01 (day 19 and 20), ****P*<0.001 (day 18) when group III is compared with group II. (b) Surviving mice, expressed as a percentage of the total number of mice in each group. $^{\#}P$<0.001 when groups II and III are compared with group I; $^{\#\#}P$<0.05 when group III is compared with group II.

as a delivery system for α-GalCer to induce an anti-tumor effect *via* activation of an immune response. Although very few reports have been devoted to investigating the adjuvant effects of α-GalCer by using delivery system, particularly liposomes [19], the enhancement of adjuvant effects of α-GalCer by using pH-sensitive liposomes is clearly demonstrated in this study. This is the first report to show the anti-tumor effect of α-GalCer incorporated pH-sensitive liposomes against E.G7-OVA tumor mouse model.

It has been established that liposomes have the applicability as an adjuvant for use in vaccines [6, 8]. In this study, the subcutaneous administration of OVA-containing pH-sensitive liposomes with (group III) or without (group II) α-GalCer induced good serum IgG antibody responses directed against OVA. On the other hand, subcutaneous immunization with OVA solution (group I) was also able to induce serum IgG antibody responses against OVA. However, α-GalCer-un-modified pH-sensitive liposomes that entrap OVA (group II) and pH-sensitive liposomes co-loaded with OVA and α-GalCer (group III) induced OVA-specific IgG antibody responses in mice greater than those induced by immunizing OVA solution (group I) (data not shown). This indicates that pH-sensitive liposomes act as effective adjuvant for potentiating antibody responses in the serum when administered

by subcutaneous route, resulting in the enhancement of OVA-specific immune responses.

It has been reported that intranasal immunization with antigen-containing SucPG-modified pH-sensitive liposomes induces not only humoral immunity, but also cell-mediated immunity [28]. In the present study, it was shown that subcutaneous immunization with α-GalCer-un-modified pH-sensitive liposomes having OVA (group II) and with pH-sensitive liposomes co-loaded with OVA and α-GalCer (group III) induced not only antigen-specific IgG1 response, but also IgG2b responses (Fig. 1). Furthermore, the production of Th1 (IFN-γ) and Th2 (IL-4) cytokines was demonstrated in spleen cells from mice in the groups II (immunized with OVA-containing α-GalCer-un-modified pH-sensitive liposomes) and III (immunized with α-GalCer-modified pH-sensitive liposomes containing OVA) (Fig. 2). IgG1 antibody is regulated by Th2-type cytokines, while IgG2b antibody is regulated by Th1-cytokines [23]. Thus, these results suggest that both humoral (Th2-type) and cell-mediated (Th1-type) immunity are also induced by subcutaneous immunization with OVA-having pH-sensitive liposomes regardless of the presence of α-GalCer.

As shown in Fig. 1b, mice treated with pH-sensitive liposomes co-loaded with OVA and α-GalCer (group III) exhibited higher IgG2b antibody response against OVA than α-GalCer-un-modified pH-sensitive liposomes that entrap OVA (group II). In addition, higher levels of both IFN-γ was detected in the culture supernatant harvested from *in vitro* OVA-stimulated spleen cells from mice in the group III than did spleen cells from mice in the group II (Fig. 2a). These results suggest that α-GalCer inclusion in the pH-sensitive liposomes induced Th1-dominant immune responses. Considering that the SucPG-modified pH-sensitive liposomes can deliver antigenic proteins into cytosol of dendritic cells and activate cellular immune response [31], and also that α-GalCer induces the production of large amounts of IFN-γ [5, 16, 25], such properties of the α-GalCer-incorporated pH-sensitive liposomes might engender the Th1-derived immune response rather than the Th2-derived immune response.

The induction of the tumor-specific CTLs generally regarded as important to achieve efficient tumor immunotherapy because these CTLs attack the target cells directly and eliminate them effectively [1, 3, 20]. Therefore, antigen delivery system that can generate antigen-specific CTLs is important for the induction of effective cellular immunity with high tumor-specificity. In this study, strong OVA-specific CTL responses against E.G7-OVA cells were obtained by correcting splenocytes from the mice immunized with pH-sensitive liposomes co-loaded with OVA and α-GalCer (group III) when compared to the mice immunized with OVA-loaded α-GalCer-un-modified pH-sensitive liposomes (group II) (Fig. 3). This result indicates that the modification of α-GalCer to pH-sensitive liposomes is essential for effective induction of OVA-specific CTLs *in vivo*, and also suggests that the very high level of CTL induction ability of pH-sensitive liposomes co-loaded with OVA and α-GalCer is expected tumor-specific prophylactic and therapeutic effects against E.G7-OVA tumor-bearing mice.

To investigate the effectiveness of the immunization protocol *in vivo*, at first, we performed tumor protection experiments in a prophylactic vaccination model using a transplantable E.G7-OVA tumor cells. The mice immunized with OVA solution (group I) exhibited a significant increase in tumor volume after 11 days from tumor cell inoculation, whereas a marked tumor growth was not observed in the mice immunized with α-GalCer-un-modified pH-sensitive liposomes entrapping OVA (group II) and with pH-sensitive liposomes containing OVA and α-GalCer (group III) (Fig. 4). This observation was congruous with the results of CTL assay (Fig. 3). As inferred from the induction of CTL responses (Fig. 3), however, it might be considered that α-GalCer-modified pH-sensitive liposomes have high ability for induction of potent anti-tumor immunity *in vivo*. Thus, we further evaluated whether liposome-mediated immunization was able to eradicate an established tumor to assess the clinical usefulness (Fig. 5). α-GalCer-modified pH-sensitive liposome-based vaccination resulted in sufficient ant-tumor immunity to suppress tumor growth and results tumor rejection: twice injections of α-GalCer-modified pH-sensitive liposomes containing OVA (group III) induced anti-tumor activity in model E.G7-OVA tumor-bearing mice and 20% of the mice showed elimination of an established E.G7-OVA tumor. On the other hand, this suppressive effect was not observed in mice treated with OVA solution (group I) or OVA-loaded α-GalCer-un-modified pH-sensitive liposomes (group II). Furthermore, complete survival was observed for mice treated with the OVA-loaded α-GalCer-modified pH-sensitive liposomes, which survived for more than 25 days. These results suggest that α-GalCer-modification in pH-sensitive liposomes is important to induce systemic immune responses effectively. Indeed, it has been reported that iNKT cells directly activated by α-GalCer rapidly produce both Th1- and Th2-type cytokines, including IFN-γ and IL-4, and can contribute to the up-regulation of both cellular and humoral immune responses [14]. In addition, it was recently reported that α-GalCer can serve as an effective adjuvant for vaccine that induces substantial protective immune responses against tumor growth [13]. Thus, it is likely that subcutaneous immunization with an α-GalCer-modified pH-sensitive liposome-based vaccine with an encased tumor antigen is potentially useful clinically for ant-tumor immunotherapy.

In conclusion, successful development of new tumor vaccines requires an effective tumor-antigen delivery system and an adjuvant that induces an effective ant-tumor immune response. Our results obtained here demonstrate the effectiveness of subcutaneous administration of α-GalCer-modified pH-sensitive liposome-based tumor vaccine three perspectives. First, α-GalCer-modified pH-sensitive liposomes have potent anti-tumor adjuvant activity by subcutaneous administration, since subcutaneous administration of α-GalCer-modified pH-sensitive liposomes also induced an antigen-specific cellular immune response, as shown by production of IFN-γ and cytotoxic activity from *in vitro* stimulated spleen cells obtained from immunized mice (Figs. 2 and 3). Second, the cellular immune responses induced by subcutaneous administration of α-GalCer-modified pH-sensitive liposomes caused complete rejection of tumors challenged after immunization. Third, subcutaneous administration of α-GalCer-modified pH-sensitive liposomes led to suppression of growth and elimination of an established tumor. Based on the current results, we conclude that α-GalCer-modified pH-sensitive liposomes have excellent properties as novel antigen-delivery vehicles and have a strong adjuvant effect even *via* subcutaneous administration, suggesting that they have potential for clinical use in anti-tumor vaccination.

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