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ABSTRACT:

Cancer immunotherapy has gained much attention because of the recent success of immune checkpoint inhibitors. Nevertheless, clinical therapeutic effects of immune checkpoint inhibitors remain limited, probably because most patients have other immune checkpoint molecules or because they lack cancer-specific cytotoxic T lymphocytes. Induction of cancer-specific cytotoxic T lymphocytes requires efficient antigen delivery systems that can convey cancer antigens specifically to antigen presenting cells, can promote the endosomal escape of antigen into cytosol, and which can activate immune cells. Earlier, we reported cytoplasmic delivery systems of antigen using pH-sensitive polymer-modified liposomes. Adjuvant molecules were further incorporated into these liposomes to provide activation properties of cellular immune responses. This study further introduced cell specificity to these liposomal systems using hyaluronic acid-based pH-sensitive polymers, which are recognized by CD44 expressing on antigen presenting cells. pH-Sensitive hyaluronic acid derivative-modified liposomes showed much higher cellular association to antigen presenting cells than to fibroblasts with less CD44 expression. These liposomes achieved the delivery of model antigenic proteins into cytosol of dendritic cells and promoted Th1 cytokine production from the cells. Subcutaneous administration of these liposomes to mice induced antigen-specific cellular

immune response in the spleen, leading to tumor regression in tumor-bearing mice. Results show that pH-sensitive hyaluronic acid derivative-modified liposomes are promising as multifunctional antigen carriers having cell-specificity, cytoplasmic antigen delivery performance, and adjuvant property to induce antigen-specific cellular immunity.

KEYWORDS: *pH-sensitive liposome / hyaluronic acid / cancer immunotherapy / dendritic cell / CD44 / cellular immunity*

INTRODUCTION

Recent advancements in cancer immunology have led to novel cancer therapies using the body's own immune systems to defeat cancer. Especially, the success of immune checkpoint inhibitors in clinical cancer therapy has clearly revealed that immune systems have strong potential to kill cancer cells if immune systems are activated to suitable states or if immunosuppression in tumor microenvironments is cancelled properly.[1,2] Nevertheless, therapeutic effects of immune checkpoint inhibitors remain limited because most patients have other immunosuppression mechanisms in tumor microenvironments or lack cancer neoantigen-specific cytotoxic T lymphocytes (CTLs).[3] Therefore, an induction system for antigen-specific CTLs is being sought to establish efficient cancer immunotherapy for a broader range of cancer patients.

Antigen-specific CTLs are differentiated from CD8-positive naïve T lymphocytes via antigen presentation by antigen presenting cells (APCs). Exogenous antigens taken up by APCs, such as macrophages or dendritic cells, are generally degraded in endo/lysosomes and are presented via MHC class II molecule, which activates helper T (Th) cells. However, exogenous antigens taken up via certain specific receptor-mediated endocytosis or antigens delivered into cytosol are presented onto MHC class I molecule to induce CTLs, which is known as "cross-presentation".[4]

Earlier, we reported the use of pH-sensitive polymer-modified liposomes for promotion of cross-presentation by cytoplasmic delivery of antigen.[5–9] Carboxylated polyglycidol- or polysaccharide derivative-modified liposomes exhibit membrane fusion behaviors in acidic endo/lysosomes and achieve cytoplasmic release of model antigenic protein (ovalbumin, OVA), leading to cross-presentation.[5–9] Subcutaneous administration of these liposomes to mice induced OVA-specific CTLs; OVA-expressing tumors were regressed.[6–8,10] Immunity-inducing effects of these liposomes were improved further by the inclusion of adjuvants such as ligands for Toll-like receptors (TLRs), cationic lipids and α-galactosylceramides or by using bioactive polysaccharides as a backbone of pH-sensitive polymers.[8,11–13] However, those earlier liposomal systems were not designed to possess specificity to APCs after subcutaneous administration. Therefore, introduction of APC-targeting properties can be expected to improve the immunity-inducing functions of these systems further.

We recently developed a pH-sensitive hyaluronic acid (HA) derivative-based intracellular drug delivery system.[14] In fact, HA has specific binding ability to CD44 in cancerous cells.[15–17] After HA derivative-modified liposomes were taken up selectively by CD44-expressing tumor cells, they released anticancer drugs responding to acidic pH in endosomes.[14] CD44 is known to express not only cancer cells but also APCs.[18–20] In

addition, low molecular weight HA acts as an inflammatory mediator via recognition by CD44 and/or TLR2/4.[21–26] Therefore, we assumed that HA derivatives can be used to provide not only intracellular antigen delivery performance but also APC-targeting via CD44 and adjuvant functions (Figure 1). We examined HA derivative-modified liposomes applied to antigen delivery to APCs and their specificity to APCs, cytoplasmic antigen delivery performance, adjuvant activity, and *in vivo* immunity-inducing functions.

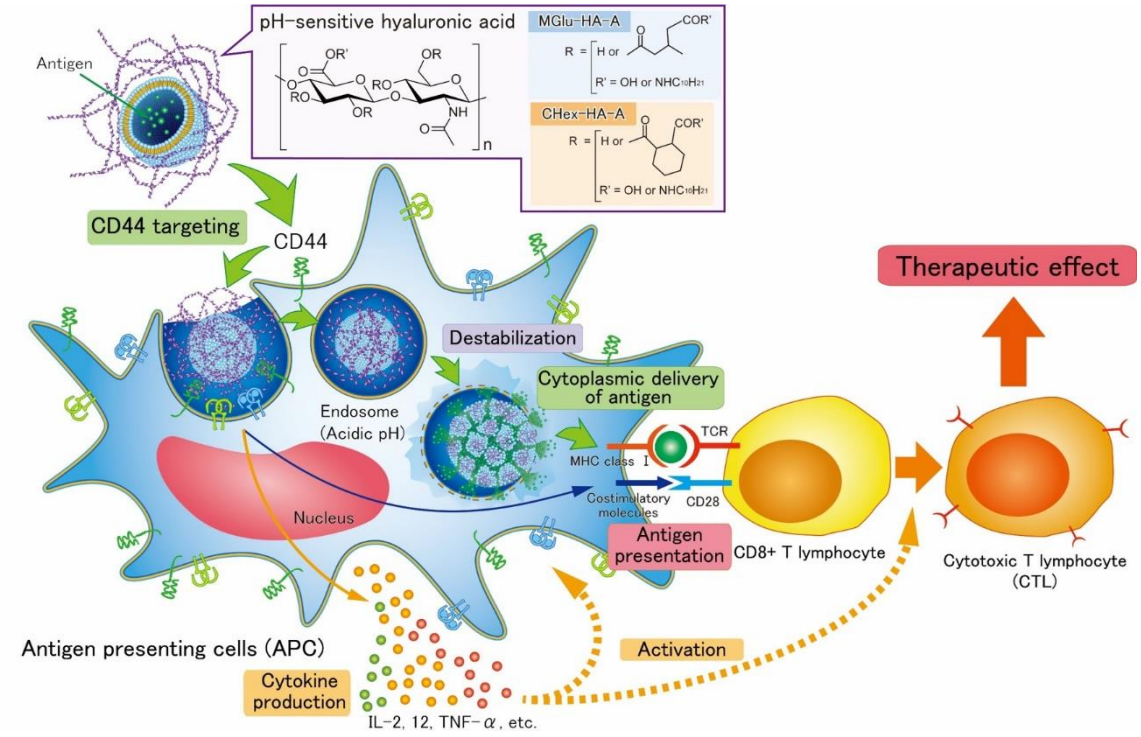

Figure 1. Cellular-immunity-inducing system using liposomes modified with pH-sensitive hyaluronic acid derivatives for CD44-specific delivery, cytoplasmic delivery of antigen, and activation of antigen presenting cells.

EXPERIMENTRAL SECTION

Materials. Egg yolk phosphatidylcholine (EYPC) was kindly donated from NOF Co. (Tokyo, Japan). OVA, bovine serum albumin (BSA), fetal bovine serum (FBS) and monophosphoryl lipid A (MPLA) were purchased from Sigma (St. Louis, MO, USA). 1,1′-Dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI) was from Life Technologies. HA derivatives, 3-methylglutarylated hyaluronic acid (MGlu-HA), 2-carboxycyclohexane-1-carboxylated hyaluronic acid (CHex-HA) and these derivatives having anchor moiety (MGlu-HA-A and CHex-HA-A) were synthesized and characterized as shown in Table S1 and S2 according to the previous literature.[14]

Preparation of Liposomes. A dry, thin membrane composed of a mixture of EYPC and HA derivatives (lipid/polymer = 7/3, w/w) was suspended in phosphate-buffered saline (PBS) containing OVA (4 mg/mL) by a brief sonication, and the liposome suspension was further hydrated by freezing and thawing, and was extruded through a polycarbonate membrane with a pore size of 100 nm. The liposome suspension was purified with ultracentrifugation for 2 h at 4 °C twice. Liposomes containing DiI were also prepared according to the procedure described above using a dry membrane composed of EYPC, HA derivatives and DiI (0.1 mol%). The concentrations of lipid and OVA in

liposome suspension were measured from using phospholipid C test-Wako and Coomassie Protein Assay Reagent, respectively.

Dynamic Light Scattering and Zeta Potential. Diameters in PBS (pH 7.4) and zeta potentials in 0.1 mM of Na_2HPO_4 (pH 7.4) of the liposomes (0.1 mM of lipid concentration) were measured using a Zetasizer Nano ZS ZEN3600 (Malvern Instruments Ltd., Worcestershire, UK). Data was obtained as an average of more than three measurements on different samples.

Cell Culture. Murine embryo fibroblast-derived NIH3T3 cell were obtained from RIKEN BRC (Ibaraki, Japan) and were grown in DMEM supplemented with 10% FBS and antibiotics at 37 °C. Immature murine dendritic cell line DC2.4 cell was provided from Kenneth L. Rock (University of Massachusetts Medical School, Worcester, MA, USA) and was grown in RPMI-1640 supplemented with 10% FBS, 2 mM L-glutamine, 100 mM nonessential amino acid, 50 μM 2-mercaptoethanol and antibiotics at 37 °C.[27] Murine monocyte macrophage cell line RAW264.7 cell was purchased from DS Pharm Biomedical (Osaka, Japan) and were grown in DMEM supplemented with 10% FBS and antibiotics at 37 °C. E.G7–OVA, which is a chicken egg OVA gene–transfected murine T lymphoma and which presents OVA with MHC class I molecules, was obtained from the American Type Culture Collection (Manassas, VA) and was grown in RPMI-1640

supplemented with 10% FBS, 2 mM L-glutamine, 10 mM Hepes, 1 mM sodium pyruvate, 50 μM 2-mercaptoethanol, 0.5 mg/mL G418 and antibiotics at 37 °C.[28]

Immunostaining of CD44 on Cells. Cells (1×10^6 cells) were suspended in PBS containing 2% FBS (100 μL). Subsequently anti-mouse CD44 antibody labeled with phycoerythrin (PE) (0.2 μg/μL, 1 μL, BD Biosciences) was added gently to the cells and incubated for 30 min at 4 °C in the dark. The cells were washed with PBS containing 0.1% BSA three times. Fluorescence intensity of these cells was determined by a flow cytometric analysis (CytoFlex, Beckman Coulter, Inc.).

Cellular Association of Liposomes. DC2.4 cells (7.5×10^4 cells) cultured for 2 days in a 24–well plate, NIH3T3 cells (5.0×10^4 cells) cultured for a day in a 24–well plate and RAW264.7 cells (7.5×10^4 cells) cultured for 2 days in a 24–well plate were washed with HBSS/PBS, and then incubated in serum–free RPMI–1640/DMEM (0.25 mL). The DiI-labeled OVA-loaded liposomes (1.0 mM lipid concentration, 0.25 mL) were added gently to the cells and incubated for 4 h at 37 °C. After incubation, the cells were washed with HBSS/PBS three times. Fluorescence intensity of these cells per cellular autofluorescence was determined by a flow cytometric analysis (CytoFlex, Beckman Coulter, Inc.). DiI fluorescence per lipid in each liposome was measured and cellular fluorescence obtained in a flow cytometric analysis was corrected using fluorescence intensity for each liposome.

Cytokine Production from Dendritic Cells. DC2.4 cells cultured for 2 days in a multi-well plate were washed with HBSS, and then incubated in serum–free RPMI–1640 medium. HA derivatives in PBS (final concentration: 0.5 mg/mL) or OVA-loaded liposomes (final concentration: 0.5 mM) were added gently to the cells, followed by incubation for 24 h at 37 ℃. After incubation, supernatants were collected and cytokine (TNF–α, IL-2 and IL–12) production was measured using an enzyme–linked immunosorbent assay kit (ELISA Development Kit, PeproTech EC Ltd.) according to the manufacture's instruction.

Animal Experiments. Female C57BL/6 mice (H–2^b, 7 weeks old) were purchased from Oriental Yeast Co., Ltd. (Tokyo, Japan). The experiments were carried out in accordance with the guidelines for animal experimentation in Osaka Prefecture University.

Treatment of Tumor-Bearing Mice with Liposomes. E.G7-OVA cells (1×10^6 cells/mouse) were subcutaneously inoculated into the left back of C57BL/6 mice under anesthesia with isoflurane. On days 7 and 14, 100 μg of OVA-loaded liposomes with or without MPLA were subcutaneously injected into the right backs of the mice under anesthesia with isoflurane. Tumor sizes were monitored from the day of tumor inoculation. Mice immunized with PBS were used as a control to confirm the development of tumor following the first inoculation of E.G7-OVA cells. Mice were sacrificed when tumor

volumes become over 2,500 mm^3. All treated groups contained four mice.

Statistical Analysis. Tukey-Kramer test was performed for the statistical analysis of the results in Figures 2, 3 and S2. Survival data in Figures 4B and S4B were evaluated using Log-rank test.

RESULTS AND DISCUSSION

Preparation of Antigen-Loaded Liposomes Modified with Hyaluronic Acid Derivatives. pH-Responsive HA derivatives (MGlu-HA-A and CHex-HA-A, Figure 1) with different contents of carboxylated groups were synthesized according to earlier reports and were characterized using ^1H NMR as shown in Tables S1 and S2.[14] OVA, the model antigenic protein, was encapsulated into liposomes using thin film hydration method. Table 1 presents the size, zeta potential, and OVA contents for the liposomes. All liposomes showed a narrow size distribution. The average sizes were about 120–140 nm, which corresponds to the pore size of the polycarbonate membrane during extrusion processes. Liposomes containing HA derivatives showed highly negative zeta potentials, indicating the modification of HA derivatives having carboxy groups onto the liposome surface. To assess the modification efficacy of HA derivatives on the surface of liposomes, sugar content detection method (phenol-sulfuric acid assay) was used. As shown in Table

S3, 33%, 88% and 73% of HA-A6, MGlu92-HA-A14 and CHex94-HA-A14 within the in feed were respectively modified onto the liposomal surface. HA-A6 showed low modification amount compared with MGlu92-HA-A14 and CHex94-HA-A14. This might result from low anchor group contents to fix the polymer onto the liposomes, which is almost consistent with earlier reports from the literature for chondroitin derivative-modified liposomes.[29] OVA contents in liposomes were approximately 100–200 g/mol lipid, which is almost consistent with earlier results obtained for carboxylated polyglycidol derivative-modified liposomes.[13] OVA retention in the liposomes was evaluated by incubation of OVA-loaded liposomes at pH 7.4 and 4.5, which corresponds to lysosomal pH after internalization to the cells (Figure S1). When liposomes were incubated at pH 7.4, both unmodified liposome and CHex94-HA-A14-modified liposome retained 80% of OVA after 4 h incubation. In contrast, OVA retention in CHex94-HA-A14-modified liposome at pH 4.5 decreased significantly compared with unmodified liposomes. These results indicate that CHex94-HA-A14 on the liposome surface disrupted the liposomal membrane via hydrophobic interaction after protonation of carboxy groups next to the cyclohexyl unit and induced the release of OVA from liposomes, which is consistent with the case of fluorescent-dye-based pH-responsiveness assessment from our earlier study of HA derivative-modified liposomes.[14]

Table 1. Particle Sizes, Zeta Potentials and OVA contents for Liposomes with or without HA Derivatives

Liposome	Size (nm)	PdI	Zeta potential (mV)	OVA/lipid (g/mol)
Unmodified	125 ± 15	0.106	−7.6 ± 1.0	182 ± 22
HA-A6	122 ± 1	0.145	−7.0 ± 2.3	194 ± 43
MGlu32-HA-A4	130 ± 12	0.139	−31.5 ± 3.5	101 ± 21
MGlu92-HA-A14	136 ± 9	0.131	−31.4 ± 7.6	182 ± 31
CHex50-HA-A10	124 ± 18	0.169	−22.2 ± 6.8	110 ± 28
CHex94-HA-A14	116 ± 14	0.163	−27.3 ± 5.6	122 ± 26

Antigen Delivery Performance of Liposomes Modified with Hyaluronic Acid Derivatives. Next, cellular association of HA derivative-modified liposomes was investigated to evaluate their antigen delivery performance to APCs. Our earlier study revealed that liposomes modified with CHex group-introduced HA derivatives enhanced the cellular association of liposomes to CD44-expressing tumor cells to a considerably greater degree than HA-coated liposomes did because of strong interaction with CD44 by hydrophobic interaction and/or the cyclohexyl structure in the CHex unit.[14] Figure 2A shows CD44 expression on various murine cell lines. Compared to fibroblast (NIH3T3 cell), results show that dendritic cell (DC2.4 cell) and macrophage (RAW264.7 cell) cell lines showed much higher CD44 expression. Such a difference in CD44 expression

between fibroblasts and APCs is expected to change the cellular association of HA derivative-modified liposomes. Actually, cellular association of liposomes to NIH3T3 cell was quite low; HA derivative modification showed no effect on cellular association (Figure 2B). By contrast, CHex-HA-A-modified liposomes showed greater than five times higher cellular association to DC2.4 cells and RAW264.7 cells than unmodified liposomes showed (Figures 2C and 2D). These findings suggest that CHex-HA-A-modified liposomes can achieve selective antigen delivery to APCs after subcutaneous injection by avoidance of non-specific association to fibroblasts or other resident cells in skin tissues. To reveal the role of CD44 on cellular association of liposome, knockdown of CD44 expression on APC was performed using CD44-specific siRNA. Decrease in CD44 expression on DC2.4 cells and RAW264.7 was confirmed in mRNA level (Figure S2). Subsequently, fluorescence-labeled liposomes modified with or without HA derivatives were applied to CD44-knockdown cells. As Figure S3 shows, knockdown of CD44 significantly reduced the cellular association of HA-A-modified liposomes and CHex-HA-A-modified liposomes. These results indicate clearly that not only HA-A-modified liposomes but also CHex-HA-A-modified liposomes are recognized by CD44 on APCs. Furthermore, decreased extent of cellular association of CHex-HA-A-modified liposomes by CD44 knockdown was much higher than that of HA-A-modified liposomes.

These results also suggest the contribution of CHex units to strong interaction with CD44. MGlu unit-introduced HA-modified liposome showed high cellular uptake by RAW264.7 cells (Figure 2D), but the same liposome was not taken up by DC2.4 cells (Figure 2C). As reported earlier, introduction of MGlu units to HA suppressed the cellular association to CD44-expressing tumor cells.[14] Macrophages are known to have scavenger receptors to recognize the apoptotic cells and/or anionic molecules.[30,31] MGlu-HA-A-modified liposomes might be recognized by RAW264.7 cells via scavenger receptors because of their negative zeta potential (Table 1), leading to high cellular association. Although dendritic cells also have scavenger receptors and CHex-HA-A-modified liposomes possess highly negative zeta potentials, HA derivative-modified liposomes might be recognized mainly by CD44 on DC2.4 cells considering the low cellular association of MGlu-HA-A-modified liposomes and the high cellular association of CHex-HA-A-modified liposomes to these cells (Figure 2C). Therefore, CHex-HA-A-modified liposomes were used as CD44-targeting antigen carriers in the following experiments.

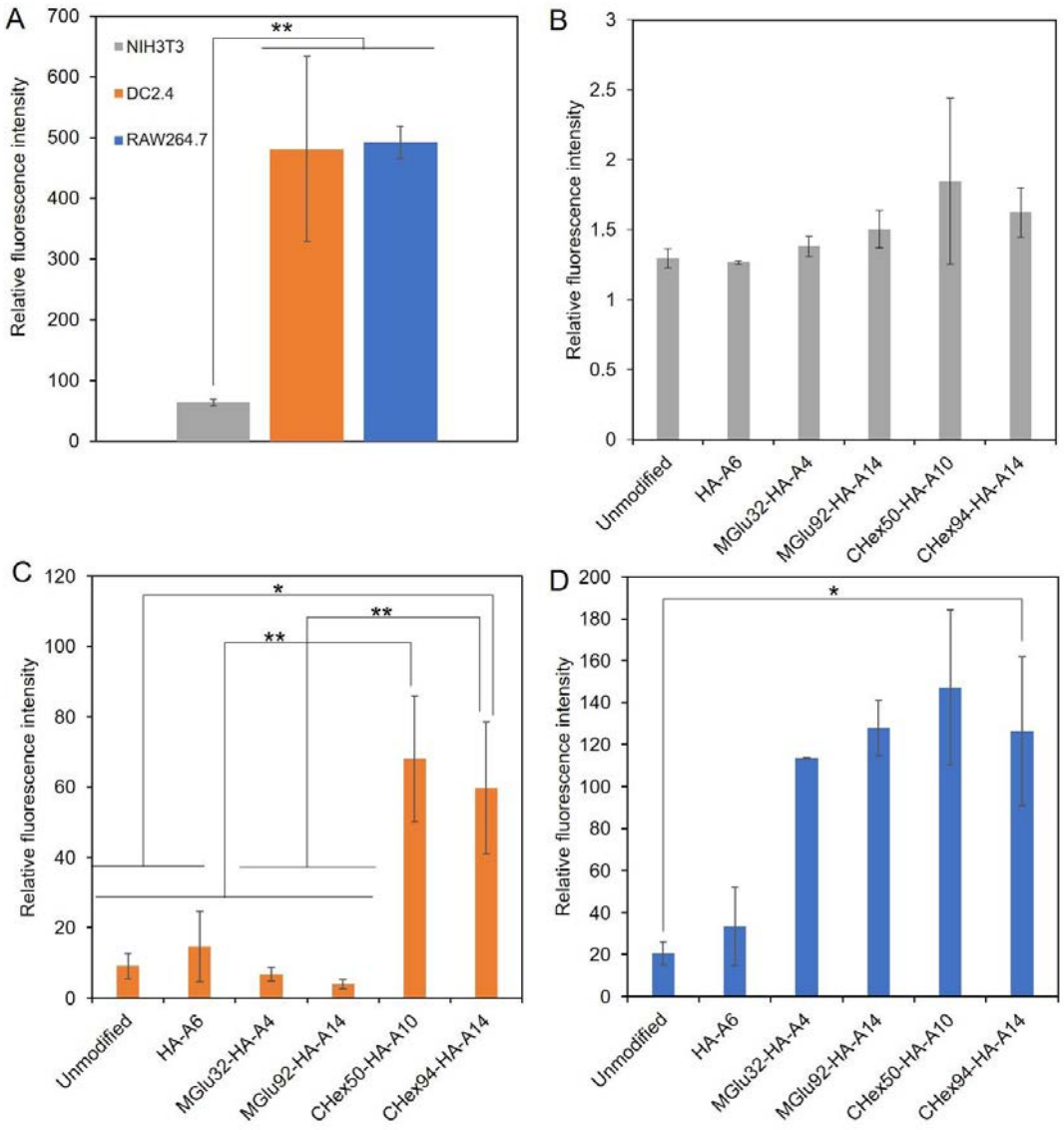

Figure 2. (A) Fluorescence intensity for cells treated with anti-mouse PE-CD44 antibody. Fluorescence intensity for NIH3T3 cells (B), DC2.4 cells (C), and RAW264.7 cells (D) treated with DiI-labeled liposomes modified with or without HA derivatives for 4 h at 37 °C in serum-free medium (0.5 mM lipid concentration). Data are shown as relative fluorescence intensity per cellular autofluorescence. They were corrected by DiI fluorescence intensity per lipid in each liposome. *$p < 0.01$, **$p < 0.05$.

The intracellular distribution of liposomes and model antigen was further examined. FITC-OVA-loaded and DiI-labeled liposomes were applied to DC2.4 cells. Then DiI and FITC-OVA fluorescence in the cells was detected using confocal laser scanning microscopy (Figure S4). For cells treated with unmodified liposomes and HA-A-modified liposome, aggregates absorbed on the cell surface and weak red fluorescence dots were observed within cells. In addition, green fluorescent dots and very weak diffused fluorescence were detected from these cells. Both liposomes might be unstable under these experimental conditions: some liposomes might be trapped mainly in endo/lysosome after internalization; a small portion of FITC-OVA might be leaked to cytosol via degradation of liposomes in lysosomes. For cells treated with CHex-HA-A-modified liposomes, not only dotted red/green fluorescence but also strong green fluorescence diffused into whole cells were observed. These results indicate that CHex-HA-A-modified liposomes were taken up efficiently by the cells and that they induced FITC-OVA release into cytosol from endo/lysosomes via pH-responsive membrane destabilization properties of CHex-HA-A-modified liposomes, as shown in Figure S1 and as described in an earlier report.[14] Therefore, modification of liposomes by CHex-HA-A can achieve not only selective association to APCs but also cytoplasmic delivery of

antigen, which is expected to lead to cross-presentation.

Adjuvant Properties of Hyaluronic Acid Derivatives. In the antigen presentation process, APCs become mature states. Also, MHC molecules and co-stimulatory molecules are up-regulated and various cytokines are secreted from activated APCs.[32,33] Therefore, antigen carriers should have not only antigen delivery performance but also adjuvant properties to induce maturation of APCs. Upon tissue injury, high molecular weight HA distributed in extracellular matrix is degraded into low molecular weight fragments, which activate inflammatory responses via recognition of low molecular weight HA by CD44 and/or TLR2/4.[21–26] In this study, low molecular weight HA (Mw: 8,000–50,000) was used for liposome modification. Therefore, adjuvant properties of HA derivatives were investigated by measuring Th1 cytokine (TNF-α and IL-12) production from DC2.4 cells treated with HA derivatives (Figures 3A and 3B). Results show that CHex-HA promoted TNF-α production from DC2.4 cells better than the parent HA. Moreover, introduction of a great amount of CHex units (more than 100 CHex units/100 sugar units) markedly increased cytokine production (Figure 3A), indicating that the introduction of CHex units is effective to increase adjuvant activities of HA, probably because hydrophobic CHex units increase interaction with CD44 and TLR2/4 on

dendritic cells.[14,21–26] For the case of IL-12, production levels of IL-12 by HA derivatives having low CHex units were almost identical to those of parent HA, although markedly high IL-12 production was observed by CHex124-HA (Figure 3B). These results suggest that obtaining strong adjuvant activity by HA derivatives requires more than 100 CHex units/100 sugar units. Our earlier reports also described that the introduction of MGlu or CHex units to dextran, curdlan or mannan increased the adjuvant activities of parent polysaccharides, probably because of interaction with scavenger receptors or other lectins.[8,9] Although the effect of CHex unit introduction to HA on their adjuvant activity was almost identical to these earlier reports, further evaluations should be conducted to reveal the activation mechanism of carboxylated polysaccharide derivatives by measuring the interaction of polysaccharide derivatives with corresponding receptors.

Adjuvant properties of HA derivative-modified liposomes were also evaluated (Figures 3C and 3D). High amounts of CHex unit-introduced HA derivative-modified liposomes showed markedly high IL-12 and IL-2 production from DC2.4 cells compared with other examined liposomes, which is almost consistent with results obtained for adjuvant properties of HA derivatives. Therefore, HA derivatives showed adjuvant effects even after modification on liposomes. IL-12 and IL-2 cytokines activate cellular immunity and promote the proliferation of T lymphocytes.[34,35] Therefore, CHex94-HA-A-modified

liposomes are expected to activate cellular immune responses effectively.

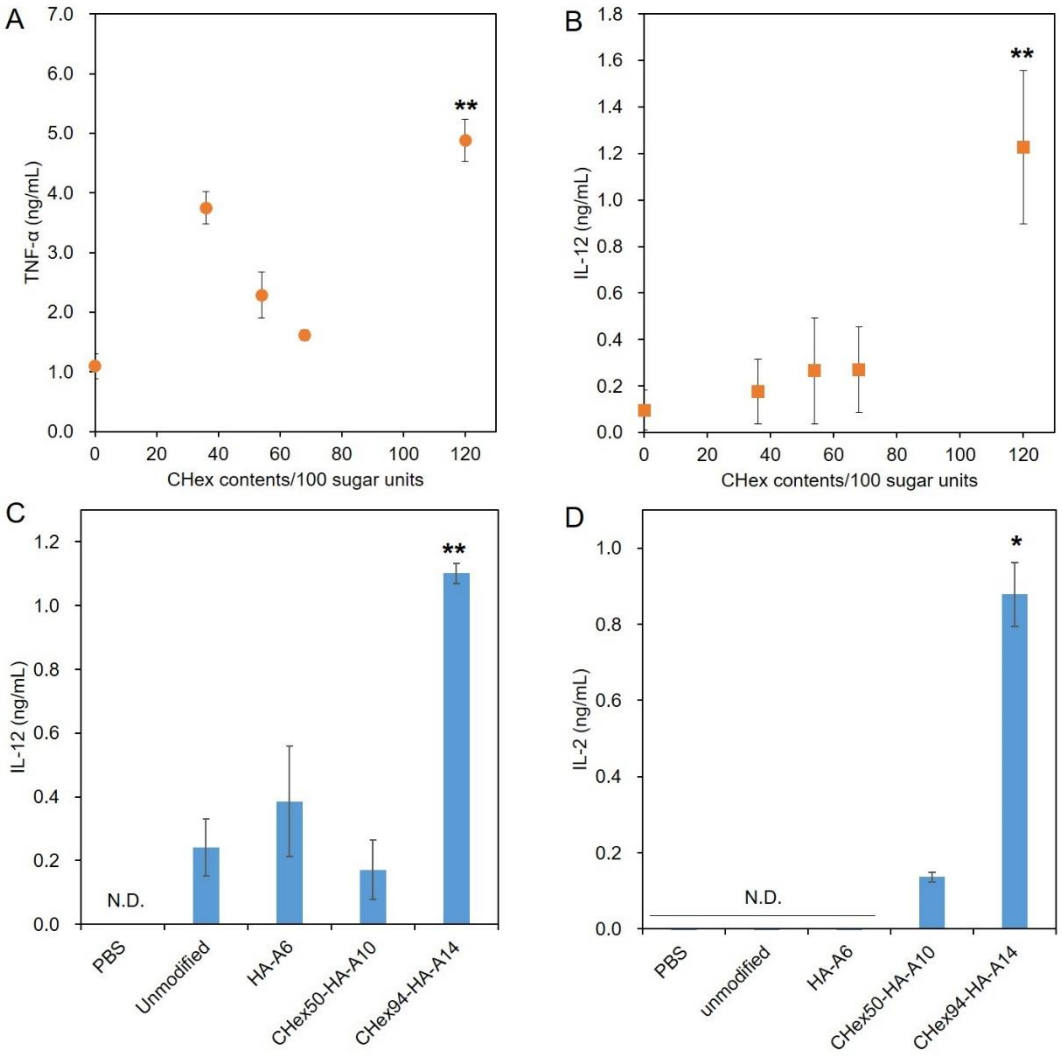

Figure 3. TNF-α (A) and IL-12 (B) production from DC2.4 cells treated with HA derivatives (0.5 mg/mL) for 24 h. IL-12 (C) and IL-2 (D) production from DC2.4 cells treated with liposomes modified with or without HA derivatives (0.5 mM) for 24 h. *$p <$ 0.01 and **$p < 0.05$ for other groups.

***In Vivo* Immunity-Inducing Functions of Liposomes Modified with Hyaluronic Acid Derivatives.** Considering the intracellular antigen delivery performance and adjuvant functions of HA derivative-modified liposomes, *in vivo* immunity-inducing properties of these liposomes were examined. After OVA-loaded liposomes were administered subcutaneously to mice, splenocytes were collected from the mice. Splenocytes were then cultured in the presence or absence of OVA for 4 days. The production of IFN-γ in supernatants was evaluated using ELISA (Figure S5). Splenocytes from mice immunized with CHex94-HA-A-modified liposomes showed considerably high IFN-γ production only in the presence of *in vitro* OVA stimulation compared with PBS- or HA-A-modified liposome-treated groups. There results indicate that OVA-specific Th1 response was induced in the spleen of mice treated with CHex94-HA-A-modified liposomes and CTLs or Th1 cells secreted IFN-γ in response to *in vitro* antigen stimulation.

Finally, we investigated the therapeutic effects of HA derivative-modified liposomes on tumor-bearing mice (Figure 4). OVA-expressing murine T lymphoma E.G7-OVA cells were inoculated to the backs of mice. Then OVA-loaded liposomes were administered subcutaneously into the tumor-bearing mice on 7 and 14 days after tumor inoculation. Subsequently, the tumor volumes and survival of mice were monitored.

When mice were administered PBS and HA-A-modified liposomes, the tumor volume increased with time and reached the endpoint within 22 days (Figure 4). Tumor volumes decreased during 14 days after administration of CHex94-HA-A-modified liposomes (Figure 4A), indicating that OVA-specific cellular immune responses induced by administration of the CHex94-HA-A-modified liposomes (Figure S5) killed OVA-expressing tumor cells efficiently. Survival of mice was also extended considerably by the administration of CHex94-HA-A-modified liposomes. In fact, 25% of mice became completely tumor-free (Figure 4B). During the experiments, no significant reduction of mice body weight was observed from treatment (Figure S6). To improve the therapeutic effects of CHex94-HA-A-modified liposomes, conventional lipid adjuvant (MPLA) was incorporated to the liposomes. Their therapeutic effects on tumor-bearing mice were compared with liposome without MPLA (Figure S7). The extent of the tumor volume decrease was improved slightly by MPLA inclusion (Figure S7A), but no significant difference was found in the survival rate between cases using liposome with and without MPLA (Figure S7B, $p = 0.801$). These results suggest that CHex-HA-A-modified liposomes possess sufficient adjuvant properties irrespective of the presence of MPLA. Another explanation is that CHex-HA might interfere with the interaction of MPLA with its receptor (TLR4)[36,37]. Therefore, other adjuvant molecules such as CpG-DNA or

poly(I:C) would be preferable to obtain the synergetic adjuvant effects with HA derivatives. Although further optimization in polymer structures, adjuvant molecules, administration times and antigen amounts must be done to obtain stronger therapeutic effects, these data revealed the effectiveness of pH-responsive hyaluronic acid derivative-modified liposomes to induce antigen-specific cellular immunity and cancer immunotherapeutic effects.

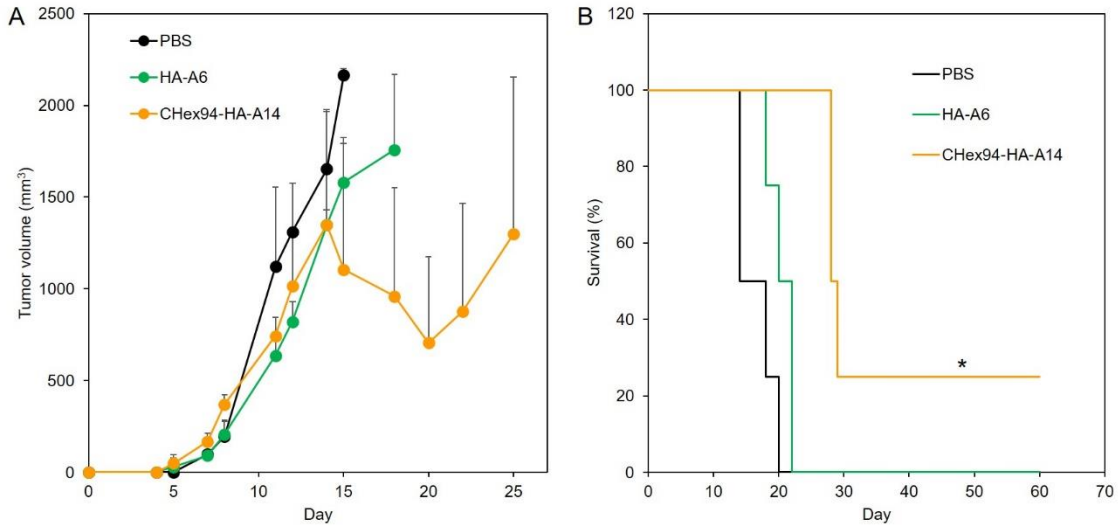

Figure 4. Antitumor effects induced by subcutaneous administration of OVA-loaded liposomes. C57BL/6 mice were immunized subcutaneously on days 7 and 14 with PBS, HA-A-modified liposomes, and CHex94-HA-A-modified liposomes. Changes in tumor volume (A) and survival (B) of mice were monitored after E.G7-OVA cell (1×10^6 cells/mouse) inoculation. All treated groups included four mice. *$p < 0.01$ for other groups.

CONCLUSIONS

This study examined the antigen delivery performance of pH-sensitive HA derivative-modified liposomes. CHex-HA-A-modified liposomes showed efficient uptake in a CD44-specific manner by antigen presenting cells compared with fibroblasts. In addition, CHex-HA-A-modified liposomes achieved cytoplasmic delivery of antigenic proteins into dendritic cells and promoted Th1 cytokine production from these cells. Subcutaneous administration of CHex-HA-A-modified liposomes containing model antigen to mice induced antigen-specific Th1 responses in spleen and showed strong therapeutic effects in tumor-bearing mice. Therefore, pH-sensitive HA derivative-modified liposomes are promising antigen carriers to achieve specific delivery of antigen into antigen-presenting cells and to activate antigen-specific cellular immunity for cancer immunotherapy.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: XXX.

Synthesis and characterization of HA derivatives (Tables S1 and S2); HA derivative contents in liposomes (Table S3); OVA retention in liposomes after incubation at pH

7.4 or 4.5 (Figure S1); Relative CD44 mRNA expression in APCs after siRNA transfection (Figure S2); Effect of CD44 knockdown on cellular association of liposomes (Figure S3); Intracellular distribution of DiI-labeled liposomes containing FITC-OVA in DC2.4 cells (Figure S4); Evaluation of Th1 response in spleen of mice immunized with liposomes (Figure S5); Change in body weight of mice in Figure 4 (Figure S6); and Effect of conventional adjuvant inclusion into liposomes on therapeutic effect of tumor-bearing mice (Figure S7) (PDF)

AUTHOR INFORMATION

Author Contributions

M.M., K.K. and E.Y. conceived and designed the research. M.M. performed all experiments. E.Y. performed *in vivo* experiments. H.H., A.H. and K.K. analyzed the data. M.M. and E.Y. wrote the paper. All authors have given approval to the final version of the manuscript.

Funding

This work was supported by Grants-in-aid for Scientific Research from the Ministry of Education, Science, Sports, and Culture in Japan (15H03024) and Grant from The Kao Foundation for Arts and Sciences.

Notes

The authors declare no competing financial interest.

ACKNOWLEDGEMENTS

We would like to thank Ms. Minori Okubo, Mr. Yoshiki Fukaya, Mr. Shin Yanagihara, Ms. Nozomi Kasyo and Mr. Takumi Tsujimura for their supports for *in vivo* experiments.

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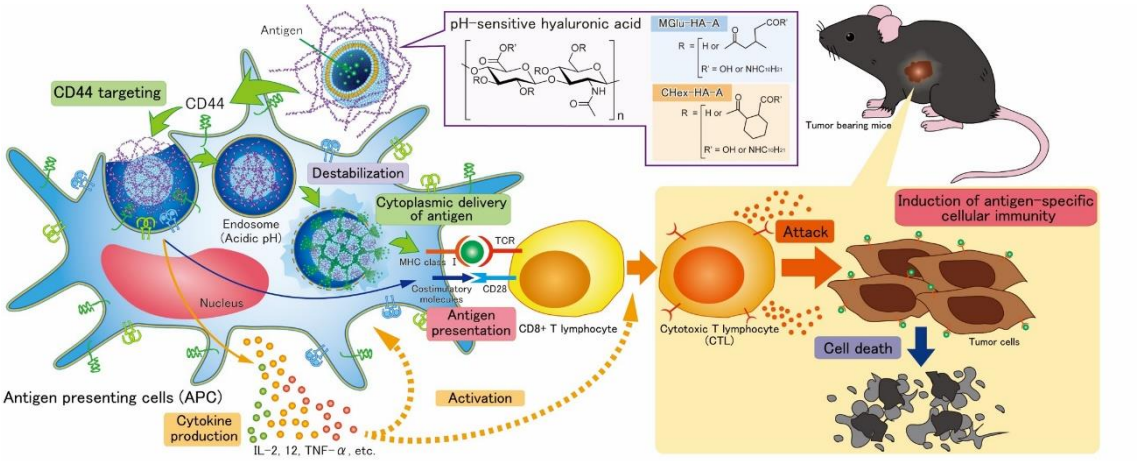

Development of pH-Responsive Hyaluronic Acid-Based Antigen Carriers for Induction of Antigen-Specific Cellular Immune Responses

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