Exosome-based tumor antigens—adjuvant co-delivery utilizing genetically engineered tumor cell-derived exosomes with immunostimulatory CpG DNA

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Abstract
For cancer immunotherapy via tumor antigen vaccination in combination with an adjuvant, major challenges include the identification of a particular tumor antigen and efficient delivery of the antigen as well as adjuvant to antigen-presenting cells. In this study, we proposed an efficient exosome-based tumor antigens-adjuvant co-delivery system using genetically engineered tumor cell-derived exosomes containing endogenous tumor antigens and immunostimulatory CpG DNA. Murine melanoma B16BL6 cells were transfected with a plasmid vector encoding a fusion streptavidin (SAV; a protein that binds to biotin with high affinity)-lactadherin (LA; an exosome-tropic protein) protein, yielding genetically engineered SAV-LA-expressing exosomes (SAV-exo). SAV-exo were combined with biotinylated CpG DNA to prepare CpG DNA-modified exosomes (CpG-SAV-exo). Fluorescent microscopic observation revealed the successful modification of exosomes with CpG DNA by SAV-biotin interaction. CpG-SAV-exo showed efficient and simultaneous delivery of exosomes with CpG DNA to murine dendritic DC2.4 cells in culture. Treatment with CpG-SAV-exo effectively activated DC2.4 cells and enhanced tumor antigen presentation capacity. Immunization with CpG-SAV-exo exhibited stronger in vivo antitumor effects in B16BL6 tumor-bearing mice than simple co-administration of exosomes and CpG DNA. Thus, genetically engineered CpG-SAV-exo is an effective exosome-based tumor antigens-adjuvant co-delivery system that will be useful for cancer immunotherapy.

1. Introduction

Tumor antigen-based cancer immunotherapy can induce tumor antigen-specific cytotoxic T lymphocytes (CTLs), which eradicate or suppress tumor growth [1]. However, the identification and purification of particular tumor antigens, which are required to achieve antigen-specific cancer immunotherapy, have been recognized as major challenges [2,3]. In addition, the efficient delivery of tumor antigens to antigen-presenting cells (APCs) and APC activation via adjuvants are essential steps in the induction of a potent antitumor immune response [4–7]. Therefore, utilization of novel materials that can be used as an endogenous tumor antigens as well as delivery carrier for adjuvant is indispensable for the development of cancer immunotherapy. Exosomes are cell-derived membrane vesicles with diameters of 30–120 nm that serve as endogenous delivery carriers for cargo, such as proteins and nucleic acids [8–10]. Tumor cell-derived exosomes are known to contain endogenous tumor antigens and can be used to induce antitumor immunity by transferring tumor antigens to APCs, such as dendritic cells (DCs) [11, 46–48]. Moreover, recent clinical trials for cancer immunotherapy using tumor cell-derived exosomes from cancer patients have demonstrated the effectiveness of exosomes as a cancer vaccine [26,49]. Therefore, the application of tumor cell-derived exosomes as cancer vaccine that can induce tumor antigen-specific immune responses without requiring the identification or purification of particular tumor antigens is expected. Moreover, therapeutic cargo, including small interfering RNAs or anti-inflammatory agents, can be loaded into exosomes and efficiently delivered to targeting cells [12–15], suggesting that exosomes could be used as adjuvant carriers. As the activation of APCs incorporating tumor antigens is an important step in the induction
of a tumor antigen-specific immune response [16–18], the efficient and simultaneous delivery of tumor cell-derived exosomes and adjuvant to the same APC could effectively induce a potent tumor-specific immune response. Based on these considerations, we hypothesized that adjuvant-modified tumor cell-derived exosomes is a viable approach for the efficient and simultaneous delivery of tumor antigens with adjuvant to the same APC and could thus induce potent tumor antigen-specific immune responses.

Here, we present an effective exosome-based tumor antigens—adjuvant co-delivery system for cancer immunotherapy. We genetically engineered murine melanoma B16BL6 tumor cells to generate exosomes that express SAV–LA [19], a fusion protein of streptavidin (SAV; a protein that binds to biotin with strong affinity) and lactadherin (LA; a protein that locates on the exosomes membrane), and subsequently modified these exosomes with biotinylated immunostimulatory CpG DNA by SAV-biotin interaction (Fig. 1). These CpG DNA-modified exosomes (CpG–SAV-exo) efficiently and simultaneously delivered exosomes with CpG DNA to the same APC, leading to a high capacity of these cells to present tumor antigens. Moreover, when compared with the simple co-administration of exosomes and CpG DNA, CpG–SAV-exo more effectively induced tumor antigen-specific immune response and inhibited B16BL6 tumor growth in mice.

2. Materials and methods

2.1. Chemicals

Dulbecco’s modified Eagle’s medium (DMEM) and Roswell Park Memorial Institute (RPMI) medium were obtained from Nissui Pharmaceutical, Co., Ltd. (Tokyo, Japan). Opti-modified Eagle’s medium (Opti-MEM), penicillin/streptomycin/L-glutamine (PSG), and sodium pyruvate was purchased from Thermo Fisher Scientific (Waltham, MA, USA). Fetal bovine serum (FBS) was obtained from HyClone Laboratories, Inc. (South Logan, UT, USA). MEM non-essential amino acids (NEAA) were purchased from Nacalai Tesque, Inc. (Kyoto, Japan). Monothioglycerol (MTG) and mitomycin C were purchased from Wako Pure Chemical (Osaka, Japan). The fluorescent membrane dyes used to label exosomes, PKH26 and PKH67, were purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Oligodeoxynucleotides (ODNs)

A fully phosphorothioate-modified ODN containing a CpG motif (ODN 1668: 5’-TCCATGACGTCTCTGATGCT-3’) labeled with tri-ethylene glycol (TEG)-biotin at the 3’ end was purchased from Integrated DNA Technologies, Inc. (Coralville, IA, USA). The CpG motif of the ODN is underlined. CpG DNA labeled with 6-carboxy-fluorescein at the 5’ end and TEG-biotin at the 3’ end was also purchased from Integrated DNA Technologies and used to evaluate the modification efficiency as well as cellular uptake.

2.3. Cell culture

Murine melanoma B16BL6 cells were obtained from the Riken BRC (Tsukuba, Japan). Mouse dendritic DC2.4 cells were kindly provided by Dr. K. L. Rock (University of Massachusetts Medical School, Worcester, MA, USA). EG7 cells (OVA-transfected EL4 cells) were purchased from the American Type Culture Collection (Manassas, VA, USA). BUSA14 cells (mouse T cell hybridoma cells specific for mouse melanoma antigen gp100) were a generous gift from Prof. Lea Eisenbach (The Weizmann Institute of Science, Rehovot, Israel) [20].

2.4. Animals

Five-week-old male C57BL/6J mice and BALB/c nu/nu mice were purchased from Japan SLIC, Inc. (Shizuoka, Japan). All protocols for the animal experiments were approved by the Animal Experimentation Committee of the Kyoto University Graduate School of Pharmaceutical Sciences.

2.5. Collection of SAV-exo from B16BL6 cells

pCMV-SAV–LA and pCMV-Gaussia luciferase (gLuc)–LA were constructed as described in a previous report [19,21]. SAV–LA expressing exosomes (SAV-exo) were purified from the culture supernatants of B16BL6 cells transfected with pCMV-SAV–LA as previously described (19). In brief, 1 μg of pDNA was mixed with 2.58 μg of PEI “Max” (Polysciences, Warrington, PA, USA) at a final concentration of 16 μg of pDNA/mL. The resulting complex was added to B16BL6 cells seeded on culture plates, followed by 1-h incubation. Subsequently, the remaining complex was removed, and cells were cultured with medium supplemented with exosome-depleted FBS for an additional 24 h. The culture supernatant was cleared by sequential centrifugation and filtration through a 0.2-μm filter and ultracentrifuged at 100,000 × g for 1 h to sediment the exosomes. Exosomes were washed twice with PBS. Exosomes labeled with gLuc–LA (gLuc-exo) were obtained by
collecting exosomes from cells transfected with pCMV-gLuc–LA as described above. Exosome amounts were estimated by measuring protein concentrations using the Bradford assay.

2.6. Preparation of CpG DNA-modified exosomes (CpG-SAV-exo)

To obtain CpG-SAV-exo, SAV-exo (1 μg of protein) was incubated with 100 pmol of biotinylated CpG DNA for 10 min at room temperature. Subsequently, samples were washed with PBS and ultracentrifuged to remove free CpG DNA. To prepare fluorescently labeled CpG-SAV-exo, fluorescently labeled biotinylated CpG DNA was incubated with SAV-exo as described above. After a PBS wash and ultracentrifugation, exosome membranes were stained with PKH26 or PKH67 dye for 5 min. Thereafter, the exosomes were washed with PBS and ultracentrifuged to remove free dye.

2.7. Particle number, size, zeta potential, and electron microscopic observation of exosomes

A qNano instrument (Izon Science Ltd., Christchurch, New Zealand) was used to measure the number of exosome particles and particle size distribution, using a NP100 nanopore according to the manufacturer’s instructions. Collected data were processed using Izon Control Suite software, version 3.2. A Zetasizer Nano ZS (Malvern Instruments, Malvern, UK) was used to determine the zeta potential of the exosomes.

To observe exosome morphology, an exosome suspension was mixed with an equal volume of 4% paraformaldehyde; this mixture was subsequently applied to a carbon/Formvar mixed with an equal volume of 4% paraformaldehyde; this mixture was incubated with SAV-exo as described above. After a PBS wash and ultracentrifugation, exosome membranes were stained with PKH26 or PKH67 dye for 5 min. Thereafter, the exosomes were washed with PBS and ultracentrifuged to remove free dye.

2.8. Western blotting

Cell lysates were prepared by freezing and thawing four times, followed by centrifugation at 15,000×g for 15 min to remove cell debris. Exosomes and cell lysates (5 μg of protein) were reduced with 0.1-M dithiothreitol and heat treatment at 95 °C for 3 min. The samples were then subjected to 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and electrophoretically transferred to a polyvinylidene fluoride transfer membrane. The membrane was blocked with Blocking One solution (Nacalai Tesque, Japan) and incubated for 20 min. The sample was then washed twice with PBS and fixed with 1% glutaraldehyde for 5 min. After another wash with PBS, the sample was incubated with 1% uranyl acetate for 1 min. The sample was finally observed under a TEM (Hitachi H-7650; Hitachi High-Technologies Corporation, Tokyo, Japan).

2.9. Cellular uptake of CpG-SAV-exo by DC2.4 cells

DC2.4 cells were seeded into 96-well plates at a density of 5 × 10^4 cells/well. To evaluate CpG DNA uptake, CpG-SAV-exo prepared with fluorescently-labeled biotinylated CpG DNA were added to cells and incubated for 8 h. To evaluate exosome uptake, PKH67-labeled CpG-SAV-exo was added and incubated for 8 h. Cells were then washed twice with PBS and harvested. Subsequently, the fluorescence intensity of the cells was determined by flow cytometry (Gallios Flow Cytometer, Beckman Coulter, CA, USA) with Kaluza software (version 1.0, Beckman Coulter), and the MFI was calculated as an indicator of cellular uptake.

2.10. Fluorescence microscopic observation

DC2.4 cells were seeded on 13-mm diameter glass coverslips and incubated at 37 °C for 4 h. The culture medium was replaced with Opti-MEM containing fluorescently labeled CpG-SAV-exo. After 24 h incubation at 37 °C, cells were washed twice with PBS, fixed with 4% paraformaldehyde for 10 min, and washed again twice with PBS. Subsequently, 300-nM 4,6-diamino-2-phenylindole (DAPI) was added for a 10-min incubation. Next, the cells were washed twice with PBS, and the coverslips were mounted on glass slides using SlowFade Gold (Thermo Fisher Scientific). Cells were observed with a fluorescence microscope (Biozero BZ-X710, Keyence, Osaka, Japan).

2.11. Cytokine release from DC2.4 cells

DC2.4 cells were seeded into 96-well plates at a density of 5 × 10^4 cells/well and incubated for 24 h before treatment. Exosomes (Exo), biotinylated CpG DNA (CpG), SAV-exo, Exo mixed with CpG (Exo + CpG), gLuc-exo mixed with CpG (gLuc-exo + CpG), or CpG-SAV-exo were diluted in 0.1 mL of Opti-MEM and added to each well. DC2.4 cells treated with LPS or Opti-MEM were prepared as a positive and negative control, respectively. The cells were incubated at 37 °C for 8 or 24 h, after which supernatants were collected. The levels of TNF-α, IL-6 and IL-12p40 in the supernatants were determined by enzyme-linked immunosorbent assay (ELISA) using OptiEIA sets (Pharmingen, San Diego, CA, USA).

2.12. Antigen presentation assay

DC2.4 cells were seeded into 96-well plates at a density of 5 × 10^4 cells/well and incubated for 4 h. Next, Exo, SAV-exo, Exo + CpG, gLuc-exo + CpG, or CpG-SAV-exo and 5 × 10^4 of BUSA14 cells were added to each well. BUSA14 cells co-cultured with DC2.4 cells treated with mouse gp10025-33; EGSRNQDWL (Anaspec Inc., Fremont, CA, USA) or Opti-MEM were prepared as a positive and negative control, respectively. After 24-h incubation at 37 °C, the concentration of IL-2 in the supernatant was determined using OptiEIA ELISA sets (Pharmingen).

2.13. Evaluation of cytokine induction from splenocytes and CTL assay

PBS, Exo, CpG-SAV-exo, Exo + CpG, or CpG-SAV-exo (1-pmol DNA and 1-μg exosomes/50-μL PBS/mouse) was intradermally injected into the left flank of a C57BL/6 mouse. Mice were immunized three times at 3-day intervals. Seven days after the last immunization, 5 × 10^5 splenocytes collected from immunized mice were incubated with mitomycin C-treated B16BL6 cells or EG7 cells at a ratio of 10:1 in a 48-well culture plate for 3 days. The concentrations of IFN-γ, IL-4, IL-10, and TGF-β1 in the culture supernatants were measured by ELISA. For the CTL assay, splenocytes
collected from immunized mice were restimulated with mitomycin C-treated B16BL6 cells for 4 days and used as effector cells. B16BL6 cells and EG7 cells were separately labeled with 51Cr by incubation with Na2CrO4 in culture medium for 45 min at 37 °C. After washing, 2 × 104 of the 51Cr-labeled target cells and serially diluted effector cells were co-incubated in 200 μL of culture medium for 4 h at 37 °C. The spontaneous release of 51Cr in the absence of effector cells and the maximal release of 51Cr in the presence of 1% TritonX-100 were also evaluated as controls. Cells were centrifuged at 300 × g for 5 min, and 100 μL of supernatant from each sample was collected to measure radioactivity. The cytolytic activity of CTL was calculated on the basis of the following equation:

\[
\% \text{ of killing} = \frac{(\text{observed release} - \text{spontaneous release})}{(\text{maximal release} - \text{spontaneous release})} \times 100
\]

2.14. Measurement and isotyping of antibody production

Seven days after the last immunization, serum samples were collected from mice via tail veins. B16BL6 cells or EG7 cells were suspended in 0.1-M carbonate buffer and disintegrated by freezing and thawing six times, followed by centrifugation to remove cell debris. Cell lysates (1 mg/mL) were used to coat each well of 96-well ELISA plates during an overnight incubation at 4 °C. After three washes, the plates were blocked with 5% bovine serum albumin (BSA) for 1 h at room temperature. Next, the plates were washed three times, and 100-μL aliquots of serial serum dilutions were added to each well. After 2-h incubation, the plates were washed five times, and 100 μL of HRP-conjugated anti-mouse IgG, anti-mouse IgG1, or anti-mouse IgG2 antibody (1:3000; Bethyl Laboratories, Montgomery, TX, USA) was added to each well. After 1-h incubation, each well was washed five times and 200 μL of freshly prepared 0-phenylenediamine dihydrochloride (Wako Pure Chemical) solution containing 0.04% hydrogen peroxide in phosphate-citrate buffer (Nacalai Tesque) was added to each well. After 10-min incubation, 50 μL of 10% H2SO4 was added, and the absorbance of each well was measured at 450 nm.

2.15. Treatment of tumor-bearing mice

In the preventive model, mice were intradermally immunized three times with PBS, CpG-SAV-exo, SAV-exo, Exo + CpG, or CpG-SAV-exo (1-pmol DNA and 1 μg exosomes/50 μL PBS/mouse) at 3-day intervals. Seven days after the last immunization, mice were subcutaneously inoculated with 5 × 10^5 of B16BL6 cells. In the therapeutic model, mice were subcutaneously inoculated with 5 × 10^5 of B16BL6 cells. When the tumor volume exceeded 100 mm³, the mice received intratumoral or intradermal injection of PBS, CpG, SAV-exo, Exo + CpG, or CpG-SAV-exo. Immunization were repeated three times at 3-day intervals. Tumor size was measured using a slide caliper, and tumor volume was calculated using the following formula:

\[
\text{Tumor volume} (\text{mm}^3) = (\text{longer length} \times \text{shorter length}^2) \times 0.5
\]

2.16. Quantification of mRNA

When the tumor volume of mice exceeded 100 mm³, PBS, Exo, CpG, SAV-exo, Exo + CpG, or CpG-SAV-exo were directly administered into tumor tissue. The intratumoral injection was repeated three times with 3-days intervals and total mRNA was extracted from tumor tissue at 1 day after the last immunization using Sepasol-RNA I Super G (Nacalai Tesque). Reverse transcription was performed using a ReverTra Ace qPCR RT Kit (TOYOBO, Osaka, Japan), followed by RNaseH treatment (Ribonuclease H; TAKARA BIO INC., Shiga, Japan). To determine the mRNA level of VEGF and TGF-β1, real-time PCR was conducted using KAPA SYBR FAST qPCR Kit Master Mix (2 ×) ABI Prism (Kapa Biosystems, Boston, MA, USA). The sequence of the primers used for amplification were: GAPDH forward, 5'-AGGTCGTGGTGAACGGATTTG-3'; reverse, 5'-TGTAGACATTGTAGTGTGCATA-3'; VEGF forward, 5'-CATCTTCAAGCCCCTCCTGTG-3'; reverse, 5'-CAGGCGCTCATGTTACAGCA-3'; TGF-β1 forward, 5'-TTGCTTACGTCACAGA-3'; reverse, 5'-TGGTTGTAAGGGCAAGGAC-3'. Amplified products were detected on-line via intercalation of the fluorescent dye using the StepOnePlus Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). The mRNA expression of VEGF and TGF-β1 gene were normalized by using the mRNA level of GAPDH.

2.17. Histological analysis

Tumor-bearing mice received three intratumoral injections of PBS, CpG, Exo, SAV-exo, Exo + CpG, or CpG-SAV-exo with 3-days intervals. At 1 day after the last immunization, tumor tissue was collected and frozen at −80 °C. Tumor sections of 10 μm thickness were prepared by using cryostat and were fixed in 4% paraformaldehyde in PBS. After washing with PBS, sections were incubated with 20% FBS/PBS for 1 h at 37 °C. The tumor sections were incubated with anti-mouse CD31 antibody (1:50; Biologend, San Diego, CA, USA) and followed by incubation with Alexa Fluor 488 Anti-Rat IgG (1:200; Molecular probes, Invitrogen) for 1 h. The specimens were washed with PBS, embedded in SlowFade Antifade Kit with DAPI (Thermo Fisher Scientific) and observed under a confocal microscope (A1R MP, Nikon Instech Co., Ltd., Tokyo, Japan).

2.18. Evaluation of pulmonary metastasis of B16BL6 cells

When the tumor volume of mice exceeded 100 mm³, PBS, CpG, Exo, SAV-exo, Exo + CpG, or CpG-SAV-exo were directly administered into tumor tissue. Intratumoral injections were repeated three times with 3-day intervals. At 1 day after the last immunization, lungs were removed from the mice of the pulmonary tumor nodules were counted.

2.19. Statistical analysis

Statistical differences were evaluated using a one-way analysis of variance (ANOVA), followed by the Tukey–Kramer multiple comparison test. P values of <0.05 were considered statistically significant.

3. Results

3.1. Preparation of CpG DNA-modified exosomes

We collected exosomes from B16BL6 cells that had been transduced with a plasmid vector encoding SAV-IA and conducted a western blotting analysis (Fig. 2A). The exosome marker proteins Alix, HSP70, and CD81 were detected in exosomes collected from B16BL6 cells [22–24]. The exosome samples contained little contamination from cell debris. SAV was detected in exosomes collected from SAV-IA-transfected B16BL6 cells, but not in exosomes collected from untransfected cells. Moreover, B16BL6 cell-derived exosomes also contained the
well-known melanoma antigens gp100 and TRP2 (Fig. 2A). To confirm CpG DNA modification of the exosomes, CpG-SAV-exo prepared by mixing fluorescein-labeled biotinylated CpG DNA and PKH26-labeled SAV–LA-expressing exosomes (SAV-exo) was observed under a fluorescent microscope (Fig. 2B). When SAV-exo was mixed with CpG DNA, green signals representing fluorescein-labeled biotinylated CpG DNA were found to co-localize with red signals from PKH26-labeled exosomes. In contrast, only red signals were observed when Exo or Gaussia luciferase-LA expressing exosomes (gLuc-exo) were mixed with fluorescein-labeled biotinylated CpG DNA, indicating that neither gLuc nor lactadherin (LA) on the exosomes interacted with biotinylated CpG DNA. These findings demonstrated that CpG DNA-modified exosomes were successfully prepared through the interaction of SAV and biotinylated CpG DNA.

The dose of CpG DNA alone or mixture with exosomes in further experiments was determined based on this modification efficiency. We further evaluated the influence of CpG DNA modification on the physicochemical properties of exosomes. Both the particle size distribution and morphology, as observed with a transmission electron microscope (TEM), were similar among Exo, SAV-exo, and CpG-SAV-exo (Fig. 2C).

### Table 1

<table>
<thead>
<tr>
<th>Exosome Type</th>
<th>Particle Size (nm)</th>
<th>Zeta Potential (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Exo</td>
<td>112 ± 12</td>
<td>-33 ± 0.2</td>
</tr>
<tr>
<td>SAV-exo</td>
<td>119 ± 9</td>
<td>-32 ± 0.5</td>
</tr>
<tr>
<td>CpG-SAV-exo</td>
<td>109 ± 10</td>
<td>-32 ± 1.6</td>
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The mean fluorescence intensity (MFI) values of DC2.4 cells after the addition of 1 pmol/mL fluorescein-labeled CpG DNA (F-CpG),...
1 μg/mL Exo mixed with 1 pmol/mL F-CpG, 1 μg/mL gLuc-exo mixed with 1 pmol/mL F-CpG, F-CpG-SAV-exo (1 pmol/mL DNA and 1 μg/mL exosomes) were evaluated by flow cytometry (Fig. 3A–B). The MFI values were significantly higher for DC2.4 cells treated with F-CpG-SAV-exo than for those treated with corresponding F-CpG alone, indicating that cellular uptake of CpG DNA was increased by exosome modification. On the other hand, cells treated with a mixture of Exo or gLuc-exo with F-CpG exhibited similar MFI values to those obtained with F-CpG alone. In contrast to the cellular uptake of CpG DNA, the uptake of PKH67-labeled exosomes by DC2.4 was comparable among all samples (Fig. 3C–D), suggesting that gLuc-LA fusion protein and CpG DNA on the exosomes hardly influenced the cellular uptake of exosomes by DC2.4 cells. We further observed the cellular uptake of CpG-SAV-exo by DC2.4 cells under a fluorescent microscope (Fig. 3E). Simultaneous uptake of F-CpG and PKH26-labeled exosomes by the same DC2.4 cell was observed after the addition of CpG-SAV-exo. On the other hand, only exosomes were taken up by DC2.4 cells after the addition of Exo + CpG.

3.3. Cytokine release and tumor antigen presentation by DC2.4 cells

Next, the release of tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6) and interleukin-12 p40 (IL-12p40) from DC2.4 cells treated with Exo, SAV-exo, Exo + CpG, gLuc-exo + CpG, or CpG-SAV-exo were evaluated (Fig. 4A–C and Supplementary Fig. 2). Significantly higher amounts of TNF-α, IL-6 and IL-12p40 were released from DC2.4 cells treated with CpG-SAV-exo than those from the cells treated with Exo + CpG or gLuc-exo + CpG; the amounts of cytokines from the latter were comparable to those induced by treatment with Exo or SAV-exo. To evaluate the tumor antigen presentation capacity of DC2.4 cells, we measured the concentration of IL-2 released from BUSA14 cells. T cell hybridoma cells specific for the melanoma antigen gp100, after co-culture with DC2.4 cells treated with B16BL6 exosomes (Fig. 4D). CpG-SAV-exo treatment significantly increased the IL-2 secretion from BUSA14 cells co-cultured with DC2.4 cells. However, neither treatment with Exo + CpG nor gLuc-exo + CpG increased IL-2 production by BUSA14 cells co-cultured with DC2.4 cells.

3.4. Induction of B16BL6-specific immune response by CpG-SAV-exo

Next, mice were intradermally injected with phosphate-buffered saline (PBS), CpG-SAV-exo, SAV-exo, Exo + CpG, or CpG-SAV-exo. Th-1 type cytokine interferon-γ (IFN-γ) secretion from murine splenocytes after re-stimulation with mitomycin C-treated B16BL6 or EG7 cells was measured (Fig. 5A). After re-stimulation with mitomycin C-treated B16BL6 cells, small amounts of IFN-γ were secreted from the splenocytes of mice immunized with Exo, SAV-exo, or Exo + CpG. In contrast, the splenocytes of mice immunized with CpG-SAV-exo induced higher amounts of IFN-γ than that in the other samples after re-stimulation. No significant amounts of IFN-γ were released from splenocytes after re-stimulation with mitomycin C-treated EG7 cells. In contrast to IFN-γ, no significant differences in IL-4, IL-10, and transforming growth factor-β1 (TGF-β1) production from splenocytes were observed between groups (Supplementary Fig. S3A–C). We further evaluated the B16BL6-specific humoral immune response (Fig. 5B–G). Immunization with CpG-SAV-exo increased the amount of total B16BL6-specific IgG antibodies in mice than that with Exo + CpG immunization. Furthermore, B16BL6-specific antibody isotyping revealed an increase in the Th-1-related IgG2a isotype in mice immunized with CpG-SAV-exo (Fig. 5D and E). On the other hand, enhancement of the Th-2-related IgG1 isotype was...
not observed after the immunization with CpG-SAV-exo, which were comparable to those with Exo or Exo + CpG (Fig. 5F and G). Treatment with CpG-SAV-exo resulted in higher IgG2a/IgG1 ratio compared with the other groups (Fig. 5H), indicating that CpG-SAV-exo induced potent Th-1 type humoral immune response against the B16BL6.

Next, we examined B16BL6-specific CTL responses using B16BL6 cells and EG7 cells as target cells (Fig. 6A and B). Splenocytes from mice immunized with CpG-SAV-exo exhibited a higher level of CTL activity against B16BL6 cells than those from mice immunized with Exo, SAV-exo, or Exo + CpG did. No significant EG7-specific CTL activity was observed, indicating that CTL activity was specific to B16BL6 cells.

3.5. Protective antitumor immunity induced by CpG-SAV-exo

Mice were intradermally immunized three times with PBS, CpG-SAV-exo, SAV-exo, Exo + CpG, or CpG-SAV-exo (1-pmol DNA and 1 µg exosomes/50 µL PBS/mouse) and subsequently challenged with B16BL6 cells 7 days after the last immunization. Immunization with CpG-SAV-exo markedly inhibited tumor growth in mice (Fig. 7A and Supplementary Fig. S4 and S5), whereas immunization with Exo + CpG induced only a slight antitumor effect, comparable to that observed in the Exo and SAV-exo groups. In accordance with the tumor growth profile, the survival of mice challenged with B16BL6 cells was significantly prolonged by immunization with CpG-SAV-exo, whereas immunization with Exo + CpG had little effect (Fig. 7B).

3.6. Therapeutic antitumor immunity induced by CpG-SAV-exo

We next examined the therapeutic antitumor immunity induced by CpG-SAV-exo in a pre-established tumor model. Tumor-bearing mice were intratumorally immunized three times with PBS, CpG-SAV-exo, SAV-exo, Exo + CpG, or CpG-SAV-exo (1-pmol DNA and 1 µg exosomes/50 µL PBS/mouse). Immunization with Exo and SAV-exo, as well as with Exo + CpG, hardly retarded tumor growth. In contrast, immunization with CpG-SAV-exo significantly inhibited tumor growth, relative to that with the other treatments (Fig. 7C and Supplementary Fig. S6 and S7). Furthermore, intratumoral immunization with CpG-SAV-exo significantly increased the survival of tumor-bearing mice, whereas no prolonged survival was observed in mice immunized with Exo + CpG (Fig. 7D). Intra-tumoral administration of CpG-SAV-exo was more effective in inhibiting tumor growth than the treatment with intradermal administration of CpG-SAV-exo (Supplementary Fig. S8). In order to investigate the involvement of T cells in antitumor immunity induced by CpG-SAV-exo, athymic BALB/c nu/nu mice were inoculated with B16BL6 cells and antitumor effect after immunization with CpG-SAV-exo was evaluated by measuring the tumor growth in mice (Supplementary Fig. S9). In contrast with the potent antitumor effect induced by CpG-SAV-exo in wild-type tumor-bearing mice, immunization with CpG-SAV-exo hardly inhibited the tumor growth in BALB/c nu/nu tumor-bearing mice, suggesting that T cells played important role in antitumor immunity induced by CpG-SAV-exo.

3.7. Influence of CpG-SAV-exo on the tumor microenvironment and metastasis

To investigate whether the administration of CpG-SAV-exo affect tumor microenvironment, we evaluated the mRNA expression of VEGF and TGF-β1 as well as angiogenesis in tumor tissue after the treatment with CpG-SAV-exo. The mRNA level of VEGF and TGF-β1 in tumor tissue were hardly changed by any treatments (Supplementary Fig. 10A and B). Moreover, treatment with CpG-SAV-exo hardly induced angiogenesis at tumor tissue (Supplementary Fig. 10C). In order to evaluate the effect of CpG-SAV-exo administration on tumor metastasis, we collected the lung from tumor-bearing mice treated with CpG-SAV-exo and counted the number of tumor nodules. (Fig. 8). The number of lung metastasis of mice treated with Exo or Exo + CpG were comparable to those with PBS. On the contrary, lung metastasis of B16BL6 cells was reduced by the treatment with CpG-SAV-exo. These findings indicated that immunization with CpG-SAV-exo hardly affected the tumor microenvironment and rather suppressed tumor metastasis.

4. Discussion

As few tumor antigens have been identified and purified, cancer immunotherapy via vaccination with tumor cell-derived exosomes, which does not require identification and purification, would be
bene
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cial for the induction of tumor antigen-specific CTL responses
against a variety of cancers [25,46,49]. In this study, we proposed a
potent exosome-based tumor antigens
eadjuvant delivery system
and have demonstrated the usefulness of this system for cancer
immunotherapy. Clinical trials to evaluate tumor cell-derived exo-
somes together with adjuvants for cancer immunotherapy have
been conducted [11,26]. However, the simple co-administration of
tumor antigen-containing exosomes with adjuvant, as performed
in these trials, would lead to the insuf
fi
cient delivery of antigens
and adjuvant to the same APC after administration, thus inducing
incomplete antitumor immunity. In the present study, the modi-
fication of tumor cell-derived exosomes with immunostimulatory
CpG DNA via gene engineering technology yielded the ef
fi
cient and
simultaneous delivery of tumor antigens together with adjuvant to
the same APC and induced potent antitumor immunity (Figs. 3 and
7), suggesting that our antigens
eadjuvant delivery system repre-
sents a valuable approach to the further development of exosome-
based cancer immunotherapies.

The results of western blotting and fluorescent microscopy
revealed that exosomes collected from B16BL6 cells transfected
with a plasmid encoding SAV—LA indeed expressed SAV and
possessed a capacity for binding to biotinylated CpG DNA (Fig. 2A
and B). We utilized the strong affinity of SAV for biotin
(Kd = 10
−15 M) to modify exosomes with CpG DNA [27–29].
Furthermore, our previous studies demonstrated that LA could be
used to stably label molecules of interest on exosomes [19,30].
These characteristics pertaining to the stability of binding between
exosomes and CpG DNA are expected to contribute to the ef
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cient
delivery of these components to APCs (Fig. 3). Whereas CpG DNA
modification conferred activation potency on exosomes, negatively
charged CpG DNA could potentially have altered the zeta potential
of the exosomes and thus in
fl
uenced the delivery ef
fi
ciency of
exosomes to APCs. However, we con
fi
rmed that the number of CpG
DNA molecules bound to exosomes were obviously fewer than
those of other negatively charged components such as phosphati-
dylserine that mainly provide negative charge to exosomes [9].
Moreover, the possibility was experimentally ruled out by
measuring physicochemical properties and evaluating the cellular
uptake of exosomes by DC2.4 cells (Figs. 2C and 3, Table 1).

Regarding the efficient and simultaneous delivery of CpG DNA

![Fig. 5. Induction of potent B16BL6-specific cellular and humoral immunity by CpG DNA-modified exosomes (CpG-SAV-exo). Mice were intradermally immunized three times with PBS, CpG-SAV-exo, SAV-exo, Exo + CpG, or CpG-SAV-exo (1-pmol DNA and 1-μg exosomes/50 μL-PBS/mouse) at 3-day intervals. (A) Cellular immune response induced by CpG-SAV-exo. Splenocytes were collected 7 days after the last immunization and stimulated with mitomycin C-treated B16BL6 or EG7 cells for 3 days. Levels of interferon-γ (IFN-γ) in the culture media were measured. (B-H) Humoral immune responses after immunization with CpG-SAV-exo. Plates were coated with B16BL6 or EG7 lysates and incubated with serum from immunized mice. Total mouse immunoglobulin G (IgG) (B, C), mouse IgG2a (D, E), and mouse IgG1 (F, G) were detected. (H) Ratio of IgG2a/IgG1 antibodies titers against B16BL6. Results are expressed as the means ± standard deviations of data from five mice. Data shown are representative of two independent experiments. *P < 0.05 compared with the Exo + CpG group.](image-url)
via exosomes to DC2.4 cells, as demonstrated by a flow cytometric analysis and fluorescent microscopic observation, DC2.4 cells treated with CpG DNA-modified exosomes exhibited a high antigen presentation capacity (Figs. 3 and 4). By modifying exosomes with CpG DNA, a strong Th-1 antigen-specific immune response and antitumor effects were induced via vaccination with the modified exosomes in 1-μg doses [31–33]. This exosome dose setting is obviously smaller than the doses used in preceding studies of tumor cell-derived exosomes, which ranged from 10 to 200 μg/dose [34–36] (Fig. 7). Clinical trials of tumor cell-derived exosomes for cancer immunotherapy require the repeated co-administration of large amounts of tumor cell-derived exosomes and adjuvant [26]. The tumor cell-derived exosome yield has been reported to be a crucial factor in exosome-based vaccination [37,38]; accordingly, a reduction in the required dose of exosomes via adjuvant modification would facilitate the further practical application of these therapies.

Treatment of B16BL6 tumor-bearing mice revealed that intratumoral immunization with CpG-SAV-exo elicited stronger antitumor immunity than that induced by intradermal immunization with CpG-SAV-exo (Fig. 7C–D, Supplementary Fig. S8). It has been demonstrated that the direct injection of CpG DNA into tumor tissues can effectively inhibit tumor growth, as the production of proinflammatory cytokines, such as TNF-α that induce hemorrhagic necrosis in solid tumors is enhanced by CpG DNA treatment [39]. Moreover, the intratumoral injection of CpG DNA has been found to elicit strong tumor-specific CD4+ and CD8+ T cell responses [40]. Furthermore, CpG DNA can inhibit the suppressive activity of myeloid-derived suppressor cells that contribute to the inhibitory tumor microenvironment [41]. In this study, both immunostimulatory activity and the delivery of CpG DNA to APCs were remarkably enhanced by exosome modification (Figs. 3 and 4), suggesting that the intratumoral injection of CpG DNA-modified exosomes is a
Pulmonary metastasis of B16BL6 cells in tumor-bearing mice after the treatment with CpG-SAV-exo. At 1 day after the last immunization, lung of tumor-bearing mice was isolated and the number of pulmonary tumor nodules were counted. Photographs of lungs after the treatment with (A) Phosphate-buffered saline (PBS), (B) CpG, (C) exosome (Exo), (D) SAV-LA-expressing exo (SAV-exo), (E) Exo + CpG, and (F) CpG DNA-modified exo (CpG-SAV-exo). (G) Number of pulmonary tumor nodules. Results are expressed as the means ± standard deviations of data from four mice. *P < 0.05 compared with the Exo + CpG group.

Fig. 8. Pulmonary metastasis of B16BL6 cells in tumor-bearing mice after the treatment with CpG-SAV-exo. At 1 day after the last immunization, lung of tumor-bearing mice was isolated and the number of pulmonary tumor nodules were counted. Photographs of lungs after the treatment with (A) Phosphate-buffered saline (PBS), (B) CpG, (C) exosome (Exo), (D) SAV-LA-expressing exo (SAV-exo), (E) Exo + CpG, and (F) CpG DNA-modified exo (CpG-SAV-exo). (G) Number of pulmonary tumor nodules. Results are expressed as the means ± standard deviations of data from four mice. *P < 0.05 compared with the Exo + CpG group.


