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RESEARCH PAPER

Co-delivery of Adenovirus and Carmustine by Anionic Liposomes with Synergistic Anti-tumor Effects

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ABSTRACT

Purpose To improve gene transducibility mediated by adenovirus (Ad) in cancer cells and further enhance anti-tumor effects by co-delivery.

Methods Calcium-induced phase change method was used to prepare the complex of anionic liposomes and adenovirus (AL/Ad5). Gene expression was qualitatively detected by X-gal staining and quantitatively detected by ELISA. Taking adenovirus-mediated stromal cell-derived factor-1α (Ad5-SDF1α) as therapeutic gene and carmustine (BCNU) as chemotherapeutic agent, a co-delivering system of AL/Ad5-SDF1α/BCNU was prepared and administered to tumor-bearing mice by intratumor injection.

Results Enhanced LacZ gene transduction was obtained in B16 and Lewis lung carcinoma cells *in vitro* and *in vivo*. Complexes of AL/Ad5-SDF1α improved SDF1α gene expression and led to accumulation of dendritic cells among the murine B16 melanoma cells *in vivo*. This co-delivery system of AL/Ad5-SDF1α/BCNU could significantly suppress tumor growth and prolong survival of tumor-bearing mice.

Conclusions Through the co-delivering system, AL/Ad5-SDF1α could synergize with BCNU to improve the antitumor effect. It may be a promising strategy for solid tumor therapy.

KEY WORDS adenovirus · anionic liposomes · cancer gene therapy · carmustine · SDF1α

ABBREVIATIONS

Ad5-SDF1α	adenovirus-mediated stromal cell-derived factor-1α
Ad-SOD	adenovirus-mediated superoxide dismutases
AL	anionic liposomes
AL/Ad5	complexes of anionic liposomes and adenovirus
AL/Ad5-SDF1α/ BCNU	complexes of adenovirus and carmustine (BCNU) co-delivered by aninoic liposomes
BCNU	carmustine [1, 3-bis(2-chloroethyl)-1-nitro-sourea]
CAR	coxsackie-adenovirus receptor
DCs	dendritic cells
ELISA	enzyme-linked immunosorbent assay
LacZ	β-galactosidase gene Z
LLC	Lewis lung carcinoma
MOI	multiplicity of infection
Pfu	plaque-forming unit
Q-PCR	quantitative-PCR
RGD	arginine-glycine-aspartate
X-gal	5-bromo-4-chloro-3-indolyl β-D-galactopyranoside

INTRODUCTION

Gene therapy represents an exciting biotechnological advancement that may revolutionize conventional cancer treatment.

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At present, viral and non-viral methods of gene transfer have been used for clinical trials. The most widely used viral vectors are retroviruses, adeno-associated viruses, herpes viruses, and adenoviruses (1). Among them, adenoviral (Ad) vector is one of the promising vectors in the gene therapy for its high transduction efficiency in many coxsackie-adenovirus receptor (CAR) sufficient cell lines (2). However, some researchers have shown that the CAR expression is highly variable on tumor cells (3). Down-regulation of the CAR protein level in cancer cells results in their insensitivity to adenovirus infection, because the presence of this receptor is important for efficient Ad uptake and high transduction efficacy (4). Moreover, Ad vectors have high immunogenicity resulting from the capsid domains, which limits the success of gene transfer or re-administration (5).

To address the above-mentioned issues, numerous strategies have been developed to modify adenoviral tropism and to reduce vector-related toxicity, which often involve genetic modifications of the Ad (4), the chemical introduction of a linker molecule conjugated onto the viral surface (6), and physical modification of the viral capsid (7,8). Among those strategies, complexing Ad with cationic liposomes or polymers to mask the surface of the Ad has been demonstrated to broaden vector tropism, improve Ad transfectivity in the presence of neutralizing immunity, reduce the immunogenicity (5) and alter vector biodistribution *in vivo* (9). However, these vectors with positive charged surface are prone to aggregation, leading to widely variable and inherently unstable complexes that are difficult to translate to a clinical setting (10). Anionic liposomal DNA delivery vectors as an alternative to cationic liposomes have been reported in recent years (11,12). Anionic lipids in general are less cytotoxic (13) and some of them are the endogenetic components of eukaryotic cell membranes (14). It was demonstrated that a novel anionic lipoplex DNA delivery system encoding a gene of interest resulted in high transfection efficiency, good serum stability and low toxicity *in vitro* (15). In a previous study, we had prepared a kind of complex with a particle size of 211 ± 10 nm and a zeta potential of -41.2 ± 2.2 mv by the combination of Ad5 and anionic liposomes (AL), referred to as AL/Ad5, which was taken as a vehicle to mediate the interested gene to CAR-deficient MDCK and CHO cells and achieved higher gene transfer efficacy compared to the naked adenovirus (16). We hypothesized that AL/Ad5 could also enhance and prolong the Ad5-mediated gene expression in Lewis lung carcinoma (LLC) cells, in which the gene transduction efficacy was poor (17), and in B16 melanoma cells, which are deficient of CAR (18).

For efficient cancer treatment, the therapeutic strategy is very important. Immunogenic gene therapy is one of the specific, potent approaches to cancer therapy (19). For the initiation of antigen-specific immune responses, dendritic cells (DCs) are the most potent specialized antigen-presenting cells (20,21). After encountering antigens, the dendritic cells migrate to lymphoid tissue, a process that matures the dendritic cells to a stage capable of presenting the antigens to the immune system. The attraction of dendritic cells to sites of foreign antigens, and the subsequent migration of dendritic cells to lymphoid tissues, is mediated by chemokines which induce directional migration of the dendritic cells. Among these cytokines, as one member of CXC chemokine family, stromal cell-derived factor-1 (SDF1α), also called pre-B-cell growth-stimulating factor and CXCL12, was initially identified as a growth factor for B-cell progenitors (22). The chemokine of SDF1α is thought to play a role in the migration and homing of circulating hematopoietic progenitor cells to the bone marrow. It was reported that the anti-tumor effect was observed after administering the adenovirus vectors encoding SDF1α (Ad5-SDF1α) to LLC and B16 tumor-bearing mice (23).

Due to the highly heterogeneous and continuously evolving nature of solid tumors, combination therapy is often necessary for efficient treatment. The recent publications in experimental breast cancer models suggest that a combination of chemotherapy and plamid DNA-mediated immunogene therapy could lead to prolonged survival of and potentially cure mice bearing established tumors (24). To further explore the combination effect of immunogene therapy and chemotherapy, in the present work, Ad5-SDF1α and a chemotherapy agent, Carmustine (BCNU), were co-delivered by aninoic liposomes (AL/Ad5-SDF1α/BCNU). The antitumor effect of this co-delivery system of AL/Ad5-SDF1α/BCNU was evaluated and compared with Ad5-SDF1α or BCNU alone in B16 and LLC tumor-bearing mice.

MATERIALS AND METHODS

Mice and Adenoviral Vectors

Specific pathogen-free C57BL/6N male mice, 5 to 7 weeks old, were purchased from the Laboratory Animal Center of Sichuan University. The study was approved by the Animal Ethics committee of Sichuan University West China Medical School, and all procedures with animals were conducted according to the guidelines of the local Animal Use and Care Committees of Chengdu and executed according to the National Animal Welfare Law of China.

The adenovirus vectors used for this study are based on the Ad5 backbone, E1/E3-deleted. Ad5-LacZ expressing β-galactosidase was purchased from the Vector Gene Technology Company Limited (VGTC, Beijing, China), and

Ad5-SDF1α encoding the mouse stromal cell-derived factor-1α (SDF1α, CXCL12) was obtained from Cell Biolabs (San Diego, CA, USA). Viruses were amplified in 293 cells and purified by cesium chloride gradient centrifugation. The genome copy number of virus was determined by quantitative (Q)-PCR according to the previous report (16), and virus was titered using plaque-forming assay on 293 cells.

Preparation of Co-delivery System, Complexes of Adenoviral Anionic Liposomes, Carmustine (AL/Ad5/BCNU)

The complexes of anionic liposomes/adenovirus (AL/Ad5) were prepared by the calcium-induced phase changes method according to the previous report (16). Complexes of anionic liposomes/adenovirus with carmustine ([1, 3-bis (2-chloroethyl)-1-nitrosourea, BCNU]), referred to as AL/Ad5/BCNU, were prepared by the same way, except that BCNU (from Shandong Lanjin, China) powder was added to the lipids component at the beginning of preparing thin film. First, BCNU-encapsulated anionic liposomes were prepared using thin film hydration and sonication dispersion technique. Ca^{2+} was introduced by direct addition of 100 mM $CaCl_2$ to the preformed anionic liposomes and incubated at 25°C for 1 h. The resulting precipitates were pelleted by centrifuging and resuspended with a concentrated viral solution or TES buffer by vortexing for 10 min at room temperature. EDTA was added directly to this solution (15 mM), and NaOH was added to adjust the pH to 7.4. The solution was vortexed for 10 min followed by incubation for 30 min.

Evaluation of Transgene Expression *In Vitro*

LLC or B16 cells (from Chinese Academy of Sciences, Shanghai, China) were seeded at 1×10^5 cells per well on 24-well plates with complete DMEM medium and incubated at 37°C, 5% CO_2 until confluence reached ~70%. The medium was removed, and the cells were washed twice with PBS. Then 0.5 mL DMEM medium containing Ad5-LacZ with a multiplicity of infection (MOI) of 40 for each well which were alone or complexed with anionic liposomes (with or without BCNU) were added dropwise to each well and incubated with the cells for 4 h at 37°C, 5% CO_2. Then the infection solution was replaced by fresh medium, and cells were incubated for another 40 h and subsequently were allowed to assess transduction efficiency. Independent triplicate wells were analyzed for each sample.

Quantification of β-galactosidase expression was determined by assay for total protein and activity of β-galactosidase in cells according to previous report (16). The results were standardized for total protein concentrations in the samples using a BCA kit (Pierce, USA) according to manufacturer's instructions.

In Vivo LacZ Gene Expression in Tumors

LLC tumor cells (1.0×10^6) were injected s.c. to the right flank of C57BL/6N mice. After 8 days, the tumors were injected with 100 μL PBS or 10^9 pfu (plaque forming unit) of naked Ad5-LacZ (Adenovirus 5, carrying the reporter gene of β-galactosidase gene Z), AL/Ad5-LacZ complexes in 100 μL PBS. Mice were sacrificed at 1, 2, 3, and 4 weeks following intratumoral injection. The tumor was resected for assessing the LacZ gene expression. Blood was collected via a retro-orbital venipuncture utilizing a heparinized (1%) capillary tube to prepare antisera for the following determination of neutralizing antibody titers.

For histochemical analysis, the collected tumors were made into cryostat sections (8 μm), fixed in 4% PFA in PBS (pH 7.5) for 20 min on ice, and then incubated with X-gal reaction mixture containing 5 mM $K_4Fe(CN)_6$, 5 mM $K_3Fe(CN)_6$, and 2 mM magnesium chloride in PBS for overnight at 37°C. After staining, the tissue biopsies were rinsed with PBS, dehydrated in a graded series of alcohol, and stained with eosin. The β-galactosidase-stained cells were identified by light microscopy (Olympus AX-70).

To quantify β-galactosidase expression, the tissues were homogenized in lysis buffer, and β-gal levels were measured using a sensitive (detection limit 40 pg/mL) enzyme-linked immunosorbent assay (ELISA) kit according to the manufacturer's instructions (Boehringer Mannheim, Mannheim, Germany). Protein concentrations in the homogenates were measured with a bicinchoninic acid (BCA) protein assay solution (Pierce, Rockford, USA). Results are expressed as pictogram of β-galactosidase per milligram of total protein.

Quantitative Analysis of Adenovirus Copy Number in Solid Tumors *In Vivo*

To quantify the genome copy number of adenovirus by Q-PCR, the tumor tissue samples from C57BL/6N mice were collected (4-week), homogenized in lysis buffer, and centrifugated at 10,000 g for 15 min. Total DNA was isolated from the tissue extracts using the DNeasy tissue kit (Qiagen, Germany).The forward primer (CAAA GACTGGTTCCTGGTACAAATG), reverse primer (GTGTAGGATGCCCACCTGTTG) and probe (5′-FAM AGTATCATCCACCACCTGACGGCTCBHQ1-3′) were designed and synthesized according to a conserved region of the species C adenovirus hexon gene (nucleotides 21127 to 21304 of Ad5; GenBank accession number NC_001406). The Q-PCR amplification was carried out according to the previous report (16).

Measurement of Ad5 Neutralizing Antibody Titer Using Transgene Inhibition Method

To determine the neutralizing capacity of antisera from tumor-bearing C57BL/6N mice, the ability of antisera to prevent the capacity of recombinant adenoviral vectors to infect 293T cells (80% subconfluent) was assayed according to the previously reported procedure (16). The result was expressed as the relative transduction efficiency at a dilution of 1/80.

Detection of SDF1α Gene Expression and Presence of Dendritic Cells by Immunohistochemistry

B16 tumor cells (1.0×10^6) were given s.c. to C57BL/6N mice. After 8 days, the tumors were injected with PBS, naked Ad5-LacZ, naked Ad5-SDF1α and AL/Ad5-SDF1α. Three days later, the tumors were harvested and frozen in OCT compound for immunohistochemistry. Cryostat sections (8 μm) were placed on slides, air-dried, and fixed in acetone for 10 min and air-dried. After washing in PBS/0.01% Triton X-100, the slides were incubated with PBS/0.01% Triton X-100/5% normal goat serum for 60 min.

For detecting the gene expression of SDF1α, the slides were incubated overnight at 4°C with a 1:50 dilution of monoclonal anti-human/mouse CXCL12/SDF1α (R&D Systems, Inc.). After washing in PBS/0.01% Triton X-100, the slides were incubated with a 1:100 dilution of fluorescein-labeled affinity purified antibody to mouse IgG (H+L). For detecting the presence of immune-related cells known to be attracted by SDF1a *in vitro*, the slides were incubated overnight at 4°C with a 1:25 dilution of rat anti-mouse dendritic cell antibody (anti-DEC205, BD Pharmingen, USA), 1:50 dilution of hamster anti-mouse CD11c (AbD, serotec, USA). After washing in PBS/0.01% Triton X-100, the slides were incubated with a 1:50 dilution of fluorescein-labeled affinity purified antibody to mouse IgG (H+L) or fluorescein-labeled affinity purified antibody to hamster IgG (KPL, Gaithersburg, MD, USA).

After being washed in PBS, all the specimens were stained with 4,6-diamidino-2-phenylindole nuclear stain (DAPI, ROCHE, American) to detect the positions of nuclei and mounted on glass slides with antifade mounting medium (Sigma). Specimens were viewed with a Leica TCS SP5 AOBS Confocal microscopy system (Leica, Germany).

Tumor Growth Inhibition by Co-delivery System

To prepare the BCNU solution, Carmustine (BCNU) was dissolved in absolute alcohol at a concentration of 100 mg/ml (25). This solution was diluted in PBS just before intratumoral injection for doses of 25 mg/kg (26).

The tumor cells, 1×10^6 LLC or B16 cells, were injected s.c. in right flank of each C57BL/6N mouse on day 0. When the tumors had grown to 70 to 100 mm^3 (day 8), 100 μL PBS or 10^9 pfu of naked Ad5-SDF1α, AL/Ad5-SDF1α complexes, AL/Ad5-SDF1α/BCNU complexes, AL/Ad5-LacZ complexes, BCNU solution were intratumorally injected in each group (At least $n=5$). The long and short diameters of tumors were monitored every other day. Tumor volume was calculated by the formula length×-width×width/2 and expressed as average ± S.D. The death date of mice was recorded for survival studies.

Statistical Analysis

The data are shown as the mean ± S.D. Comparisons between groups were carried out by a paired samples *t*-test or one-way analysis of variance using SPSS, version 16.0 (SPSS Inc., Chicago, IL, USA). Differences in survival and tumor growth of mice between different groups were analyzed using the Kaplan-Meier method. In all cases, $p<0.05$ was considered statistically significant difference.

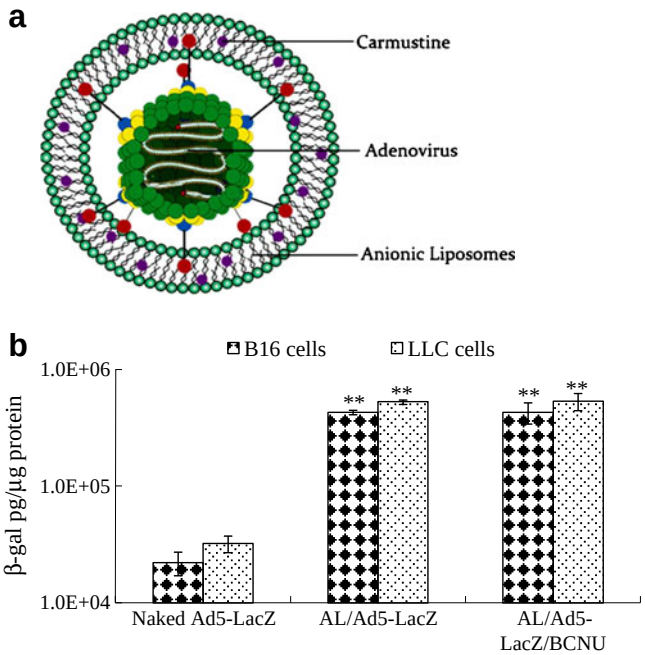


Fig. 1 Schematic structure representation of co-delivery system (**a**) and gene transfer efficiency assay upon B16 and LLC cells (**b**). The cells were treated with 300 μL of three different formulations: the naked Ad5-LacZ, Ad5-LacZ complexed with anionic liposomes (AL/Ad5-LacZ) and the co-delivery system of AL/Ad5-LacZ/BCNU at MOI of 40. The transduction efficiencies of each formulation were quantificationally assessed by using the β-galactosidase enzyme assay system. Data are presented as mean ± standard deviation (S.D.) values ($n=5$); $^{**}P<0.01$, AL/Ad5-LacZ and AL/Ad5-LacZ/BCNU *vs.* the naked Ad5-LacZ, respectively.

RESULTS

The Characterization of Complexes of AL/Ad5/BCNU

Fig. 1a shows the schematic structure representation of the complexes of adenovirus and carmustine (BCNU) co-delivered by aninoic liposomes (AL/Ad5/BCNU), which were prepared by the calcium-induced phase changes method. The resulting particle size of AL/Ad5/BCNU detected by Malvern Zetasizer Nano ZS90 (Malvern instruments Ltd., UK) is 215 ± 12 nm, which is almost the same size as AL/Ad5 (211 ± 10 nm, $p > 0.05$), and there is no difference in zeta potential between AL/Ad5 and AL/Ad5/BCNU ($p > 0.05$). The HPLC result demonstrated the drug loading of BCNU was $55 \pm 2.8\%$, which indicated that the co-delivery strategy could improve the aqueous solubility of BCNU.

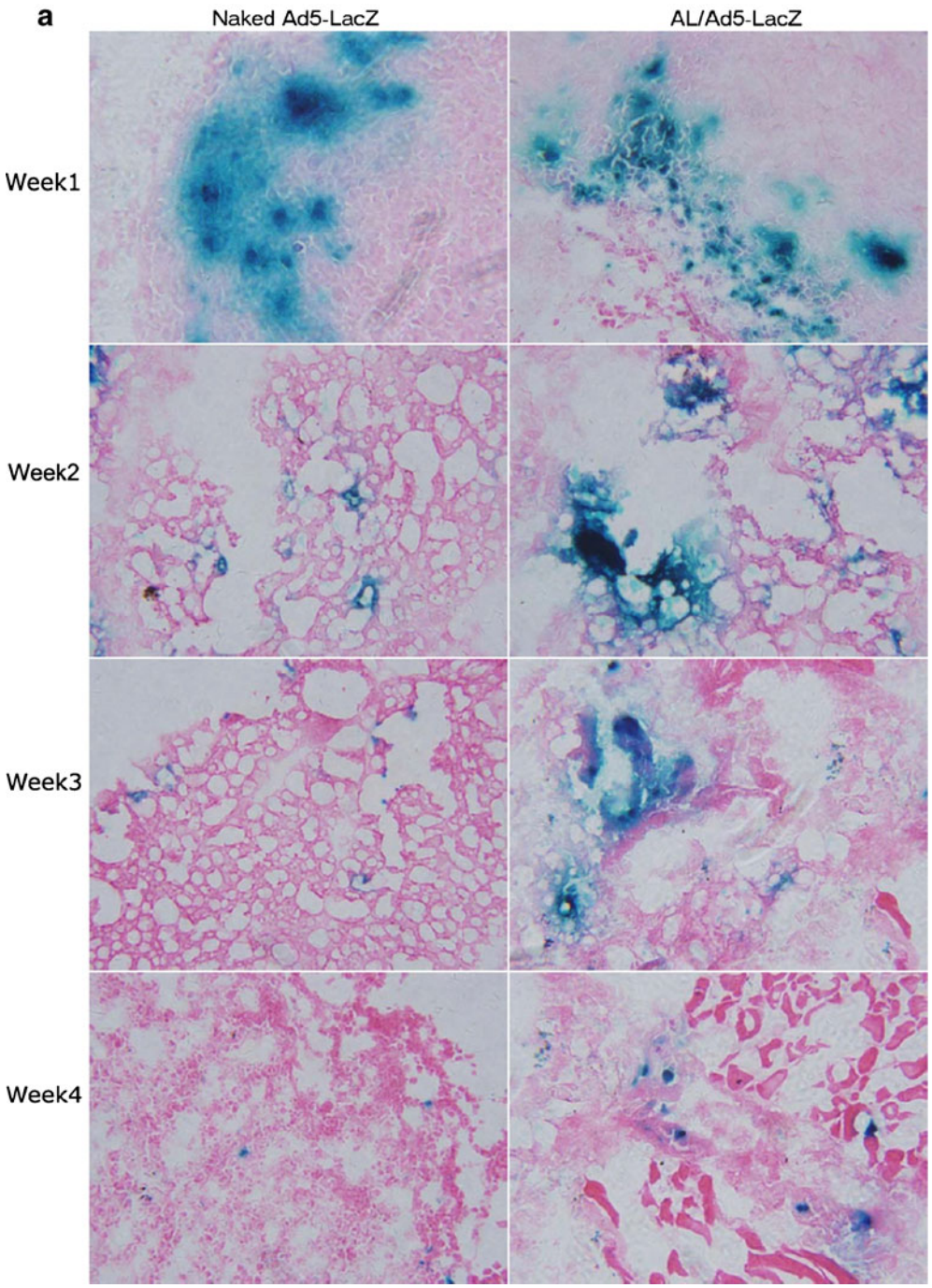


Fig. 2 Characterization of the formulated Ad5-LacZ *in vivo* including LacZ gene expression detected by X-gal staining (**a**) or by ELISA (**b**), vector genome quantification by Q-PCR (**c**), and antisera neutralization assay after intratumor injection of different formulation (**d**). Results are presented as mean ± standard deviation (S.D.) ($n = 5$); $^{*}P < 0.05$, $^{**}P < 0.01$, the treated group *vs.* controlled group.

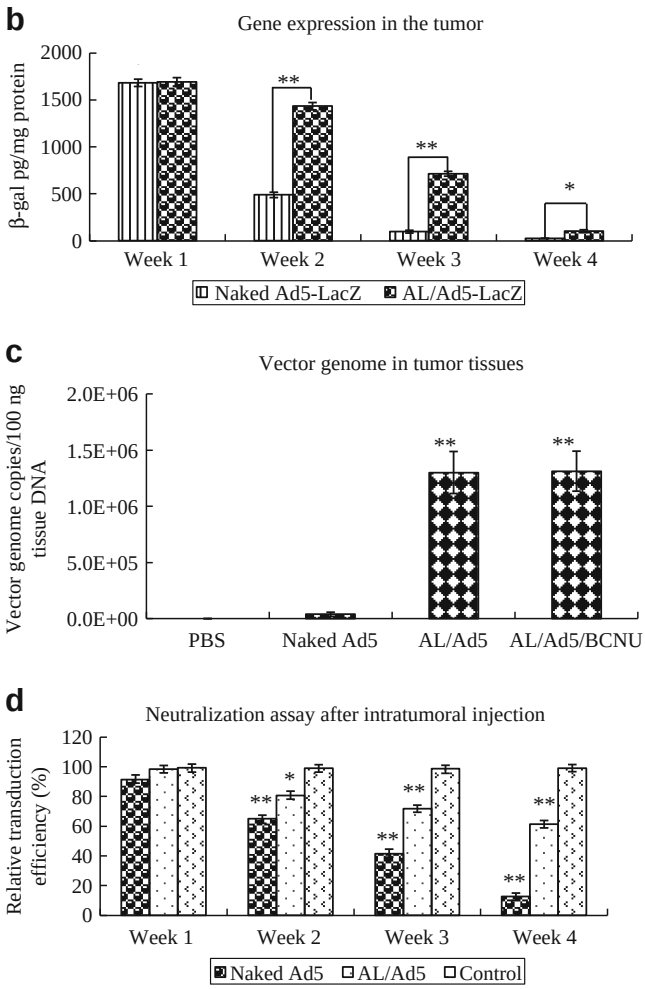


Fig. 2 (continued)

Complexes of Anionic Liposomes and Adenovirus Enhancing Gene Transfer *In Vitro*

The tumor cells of B16 and LLC were given the formulation of naked Ad5, AL/Ad5 and AL/Ad5/BCNU, respectively. Fig. 1b indicates that the average amounts of β-galactosidase in B16 cells transduced by AL/Ad5-LacZ or AL/Ad5-LacZ/BCNU was $(4.30\pm0.08)\times10^5$ and $(4.31\pm0.07)\times10^5$ pg β-gal/μg protein, respectively. Their expression levels were ~18-fold higher than that of β-galactosidase transduced by naked Ad5, which is $(2.22\pm0.05)\times10^4$ pg β-gal/μg protein. Fig. 1b also showed similar results of gene expression in LLC cells with that of in the B16 cells. The gene expression of Ad5 was improved 16-fold by the incorporation of AL. There is no difference in gene expression between AL/Ad5-LacZ and AL/Ad5-LacZ/BCNU, which indicated BCNU (10 μM) did not affect the gene expression when formulated with AL/Ad5-LacZ. The result that LacZ gene expression mediated by naked Ad5 was poor might be due to the lack of CAR/integrin expression on the cell surface (16). Encouragingly, AL/Ad5-LacZ could significantly improve the adenovirus-mediated gene transduction in both B16 cells and LLC cells, which implied the *in vivo* therapeutic potential of combination strategy in B16 and LLC solid tumors.

Improvement of Gene Transduction *In Vivo* and Vector Genome Detection Result by Q-PCR

In vivo transduction efficiency and vector genome detection results are shown in Fig. 2 (a–c) taking the naked adenovirus as controls, which demonstrated that qualitative detection result by X-gal staining (Fig. 2a) was consistent with quantitative detection result by ELISA (Fig. 2b). Fig. 2(b) demonstrates that at week 1, the amounts of β-galactosidase in the tumor of mice treated with naked adenovirus and AL/Ad5-LacZ were 1682.35 ± 38.9 and 1692.58 ± 28.7 pg β-gal/mg protein, respectively, with no obvious difference between them ($P>0.05$). At week 2, the gene expression transduced by the naked adenovirus was significantly decreased by 71%, but that transduced by AL/Ad5-LacZ formulations decreased by only 15%, exhibiting ~3-fold higher galactosidase expression than those receiving naked Ad5. At week 3, compared with the formulation of naked Ad5, the AL/Ad5-LacZ led to a ~7.4-fold enhancement of gene expression in tumors. At week 4, LacZ expression in the tumors of mice treated with AL/Ad5-LacZ was much weaker, but it was still relatively stronger than those treated with naked Ad5. This result demonstrated that the formulation of AL/Ad5-LacZ, compared with the formulation of naked Ad5, could significantly enhance and prolong the adenovirus-mediated gene expression in solid tumors.

On the other hand, the result (Fig. 2c) detected by Q-PCR at week 4 showed that the adenovirus DNA was detected in the tumors of mice treated with naked Ad5-LacZ, AL/Ad5-LacZ and AL/Ad5-LacZ/BCNU but not in mice treated with PBS, which indicated that this method of Q-PCR used in this study was reliable. Compared with the genome copy in the naked Ad5 group, much higher genome copy number was observed in the AL/Ad5-LacZ and AL/Ad5-LacZ/BCNU group, indicating BCNU did not affect the location of Ad5 particles and the anionic liposomes can partially protect the adeno-vectors from elimination by immune cells, which was consistent with the gene expression result *in vivo*.

Decrease of Neutralizing Antibody Titers *In Vivo*

In Fig. 2d, we show the results of a neutralization assay upon the serum collected at the indicated time point from C57BL/6N mice treated with the different formulations by intratumoral injection. The relative transduction efficiency was taken to demonstrate the titre of neutralizing antibody,

which was reverse consensus. One week post-injection, all of the tested groups displayed little inhibition of gene expression, which suggested the titers of anti-adenoviral antibodies were very low. At 2-week time point, on comparing the relative transduction efficiency in AL/Ad5-LacZ complexes and naked adenovirus groups, we found that the AL/Ad5-LacZ group had 24% greater average gene transduction (80.5±2.4% *versus* 64.8±2.5%) than the naked adenovirus. At week 3, the gene transduction in naked Ad5 group decreased to 41.5%, but in the AL/Ad5-LacZ group it still remained at 71.7% of relative transduction efficiency. At week 4, the relative transduction efficiency decreased to 12.6±2.3% in naked adenovirus group, while it was 61.3±2.5% in the AL/Ad5-LacZ group. This result indicated that naked adenovirus induced significantly higher titers of adenoviral-specific antibodies compared to the AL/Ad5-LacZ complexes.

Enhanced Ad5-SDF1α Gene Expression Transduced by Combination Strategy

The chemokine CXCL12/SDF1α and its receptor CXCR4 play a major role in tumor invasion, proliferation, and metastasis. Recent research (23) has administered the adenovirus vectors encoding SDF1α to LLC or B16 tumor-bearing mice, and anti-tumor effect was observed. To test whether SDF1α mediated by AL/Ad5-SDF1α could be expressed after intra-tumor administration, a monoclonal anti-human/mouse CXCL12/SDF1α primary was used in immunohistochemistry detection taking PBS, AL/Ad5-LacZ or naked Ad5-SDF1α as control. Fig. 3 showed that 3 days after the administration, AL/Ad5-SDF1α strongly induced the expression of SDF1α, and weaker SDF1α expression was observed in naked Ad5-SDF1α group, with no positive detection in PBS and AL/Ad5-LacZ groups.

Ad5-SDF1α Inducing the Accumulation of Dendritic Cells

The chemotactic function of SDF1α is mediated by the chemokine receptor CXCR-4, which is expressed on mononuclear leukocytes, $CD34^+$ hematopoietic progenitor cells, megakaryocytes, and dendritic cells (27–29). To confirm the accumulation of dendritic cells in tumors, B16 tumors were examined 3 days after intratumoral administration of the formulations including AL/Ad5-SDF1α and

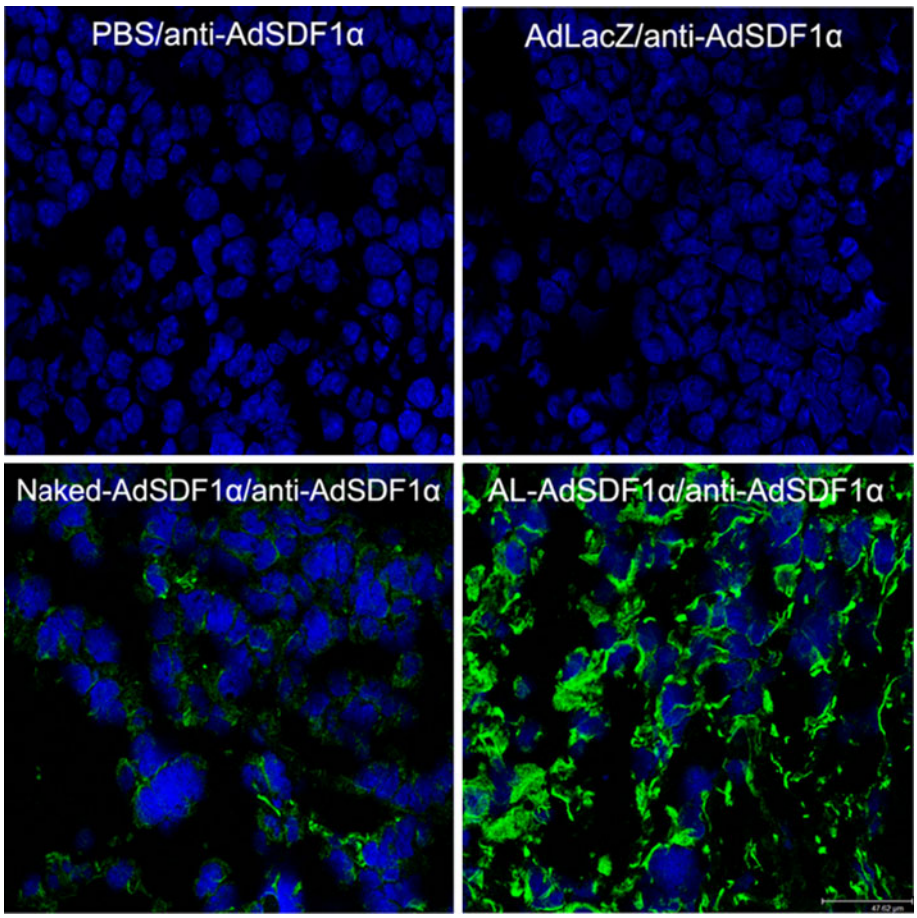


Fig. 3 Gene expression of SDF1α in the tumors. Fluorescence immunohistochemistry for SDF1α was performed on the tumor established by injections of 1.0×10^6 cells in C57BL/6N mice. Eight days later, the tumors were injected with PBS, AL/Ad5-LacZ, Naked Ad5-SDF1α and AL/Ad5-SDF1α. After 3 days, the tumor tissue was harvested and frozen in OCT compound. Frozen 8 μm sections were fixed in acetone for SDF1α gene. Bar, 47.62 μm. Positive FITC signal is represented in green, with DAPI nuclear stain in blue.

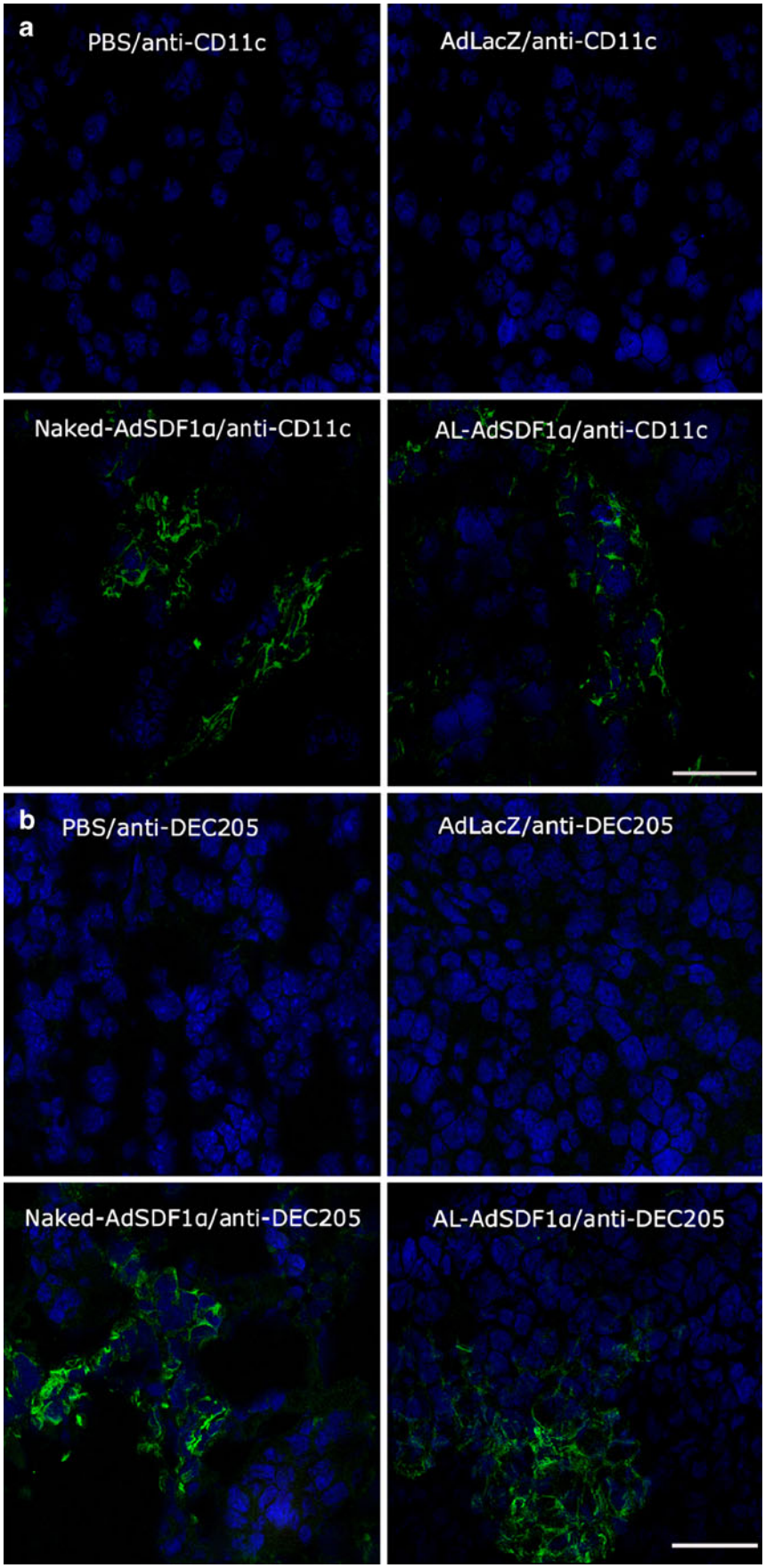


Fig. 4 Function of Ad5-SDF1α *in vivo*. Accumulation of dendritic cells in s.c. B16 tumors detected by immunohistochemistry using anti-CD11c (**a**) and anti-DEC205 (**b**) antibodies. B16 cells (1.0×10^6) were injected s.c. to C57BL/6N mice. About 8 days later, the tumors were injected with indicated formulations. After 3 days, the tumor was resected and examined for the presence of dendritic cells. Bar, 47.62 μm. (**c**) Quantification of the number of dendritic cells in the tumors. Columns, mean number of dendritic cells observed per high-power field. Results are presented as mean ± standard deviation (S.D.) ($n = 5$); $^*P < 0.05$, treated group *vs*. PBS group.

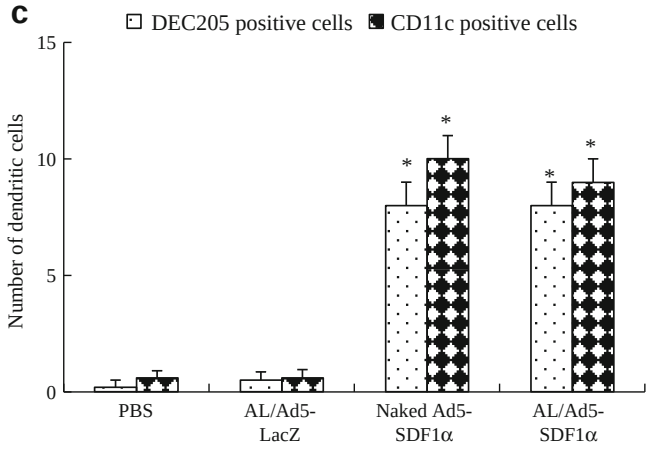


Fig. 4 (continued)

naked Ad5-SDF1α or controls by immunohistochemistry using anti-CD11c and anti-DEC205 antibodies. Fig. 4 shows that in B16 tumors infected with AL/Ad5-SDF1α and naked Ad5-SDF1α but not in controls, there were increased average numbers of dendritic cells per high power field stained with both anti-CD11c (Fig. 4a) and anti-DEC205 (Fig. 4b). By counting the average number of positive cells in each randomly chosen fields ($n>10$), the result (Fig. 4c) indicated there was no difference in dendritic cell numbers between AL/Ad5-SDF1α and naked Ad5-SDF1α groups at day 3.

Tumor Suppression and Survival Prolongation by Co-delivery System of AL/Ad5-SDF1α/BCNU

The complexes of AL/Ad5-SDF1α/BCNU taking stromal cell-derived factor-1 combined with BCNU as therapeutic agents were investigated for their anti-tumor potential in mice with solid B16 and LLC tumors, respectively. The tumor model was established with C57BL/6N mice that were subcutaneously injected with LLC and B16 carcinoma cells, respectively. When the tumors had grown to 70–100 mm^3 (day 8), mice were randomly divided into six groups ($n=5$). For the therapeutic study, a single injection of each formulation was administered intratumorally.

In LLC tumor model, as shown in Fig. 5a, tumor growth in the mice treated with AL/Ad5-SDF1α/BCNU was inhibited significantly compared to the control animals that were treated with PBS, naked Ad5-SDF1α, AL/Ad5-LacZ, or BCNU alone, respectively ($p<0.01$). The mice treated with AL/Ad5-SDF1α appeared to exhibit moderately inhibited tumor growth. Clear difference between AL/Ad5-SDF1α/BCNU and naked Ad5/SDF1α-treated groups was observed from day 15 post-injection. Concerning the survival time, Fig. 5b and e showed that in LLC tumor-bearing mice, compared to the PBS group, naked Ad5-SDF1α, AL/Ad5-LacZ and AL/Ad5-SDF1α groups failed to increase the means survival time, but the BCNU group significantly prolonged it ($p<0.05$). Moreover, the co-delivery system of AL/Ad5-SDF1α/BCNU greatly increased the mean survival time to 34.6 days, which had significant difference with PBS group ($p<0.01$). When the AL/Ad5-SDF1α or BCNU groups were compared with the combination group respectively, significant differences were also observed ($p<0.05$, Fig. 5e).

In B16 tumors, the treatment with AL/Ad5-SDF1α/BCNU resulted in a significant regression of established tumors (Fig. 5c) compared with PBS, naked Ad5-SDF1α, AL/Ad5-LacZ or BCNU alone, respectively. There was no therapeutic effect in the control groups of PBS group. Compared to the naked Ad5/SDF1α group, the AL/Ad5-SDF1α/BCNU group induced significant inhibition on the tumor growth ($p<0.01$). For the mean survival time, Fig. 5d and e showed that neither naked Ad5-SDF1α nor AL/Ad5-SDF1α had a significant effect on prolongation of survival time compared with the PBS, but both BCNU and the combination therapy group of AL/Ad5-SDF1α/BCNU significantly prolonged it with $p<0.05$ and $p<0.01$, respectively. In comparisons with AL/Ad5-SDF1α or BCNU, AL/Ad5-SDF1α/BCNU also showed significant difference in survival time ($p<0.01$, Fig. 5e).

These results indicate that in both B16 and LLC tumors, AL/Ad5-SDF1α can synergize with BCNU to improve the antitumor effect and prolong the survival time of tumor-bearing mice by intra-tumor administration.

DISCUSSION

Recombinant adenoviruses have been well studied as a model system in viral gene delivery and recently received much attention in cancer therapy for their practical advantages and application potentials. Among over 50 immunologically distinct serotypes of adenoviral family, the most frequently used vectors, Adenovirus type 5 (Ad5), initiate infection by binding of the fiber knob to the CAR, which is followed by lower affinity interaction between arginine-glycine-aspartate (RGD) domain in the penton base and cellular $\alpha_v\beta$ integrins, inducing receptor-mediated endocytosis within a clathrin-coated endosom. Subsequently, the viruses escape to cytosol and transport to the nucleus, where viral genes or transgenes are expressed (30). The fiber is the key component that interacts with cell at the first step of intracellular trafficking; meanwhile, its binding to CAR may be an important rate-limiting step for cell entering (4). Some research has shown that the Ad5 viral particle has a negative net charge on both fiber and hexon that can hardly attach to cell surface, which is also predominated by anionic motif. Arcasoy *et al.* (31) have demonstrated that negative

charged cell membrane sialoglycoconjugates present a barrier to the adsorption of adenovirus. These findings suggested that modification of the virion capsid could increase its binding affinity with the cell, thus enhancing the cellular uptake rate.

As an alternative to viral vector, non-viral gene delivery system has drawn rapidly growing interests because it offers several advantages over the viral counterparts (32). Cationic materials including cationic liposome, peptides, polymers, *et al.*, as non-viral carriers could condense DNA, forming a positive charged complex that could readily bind and enter the cell. Nevertheless, poor nuclear import and absence of transcriptional enhancer remain as critical hurdles in non-viral DNA delivery (33).

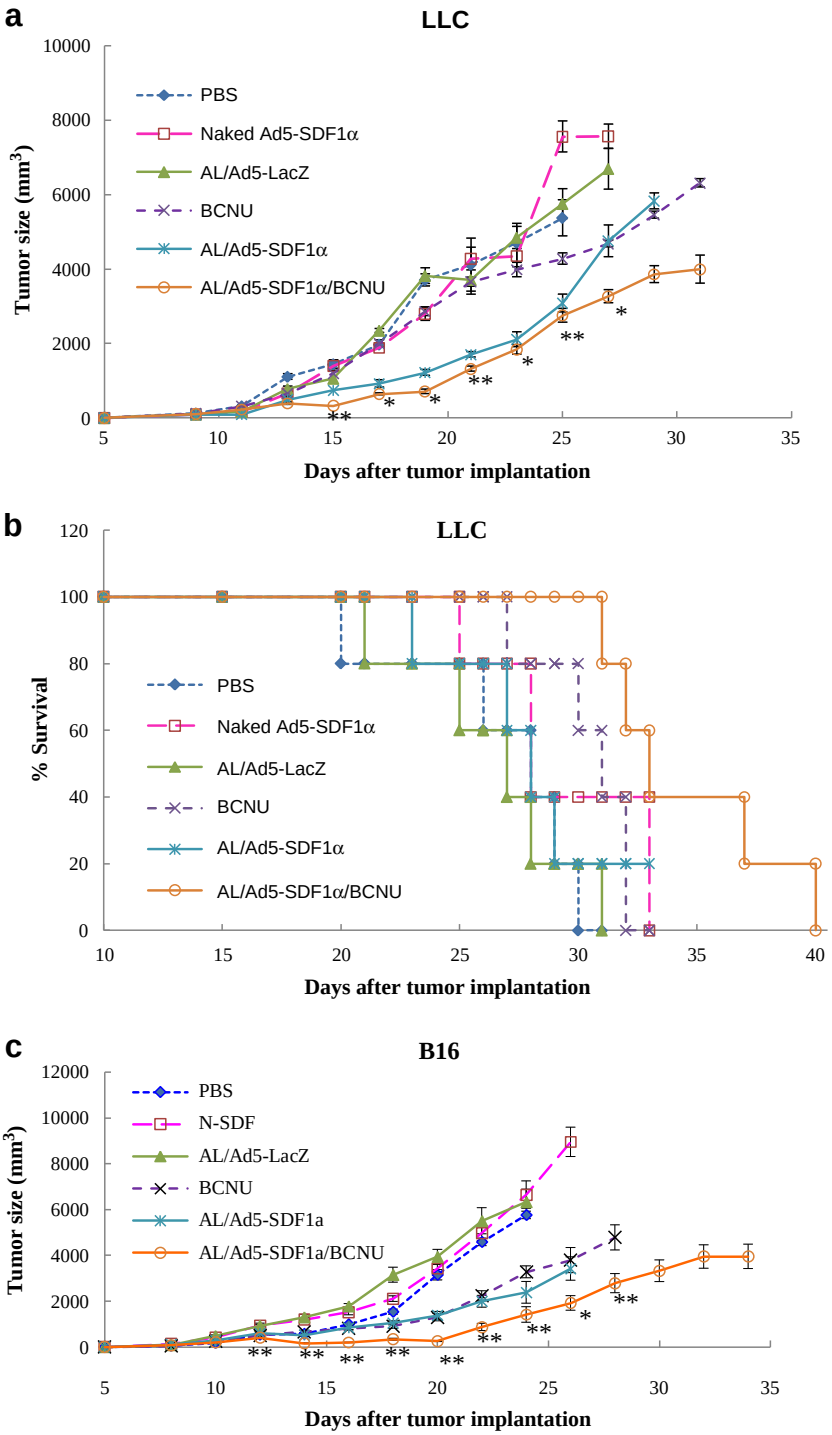
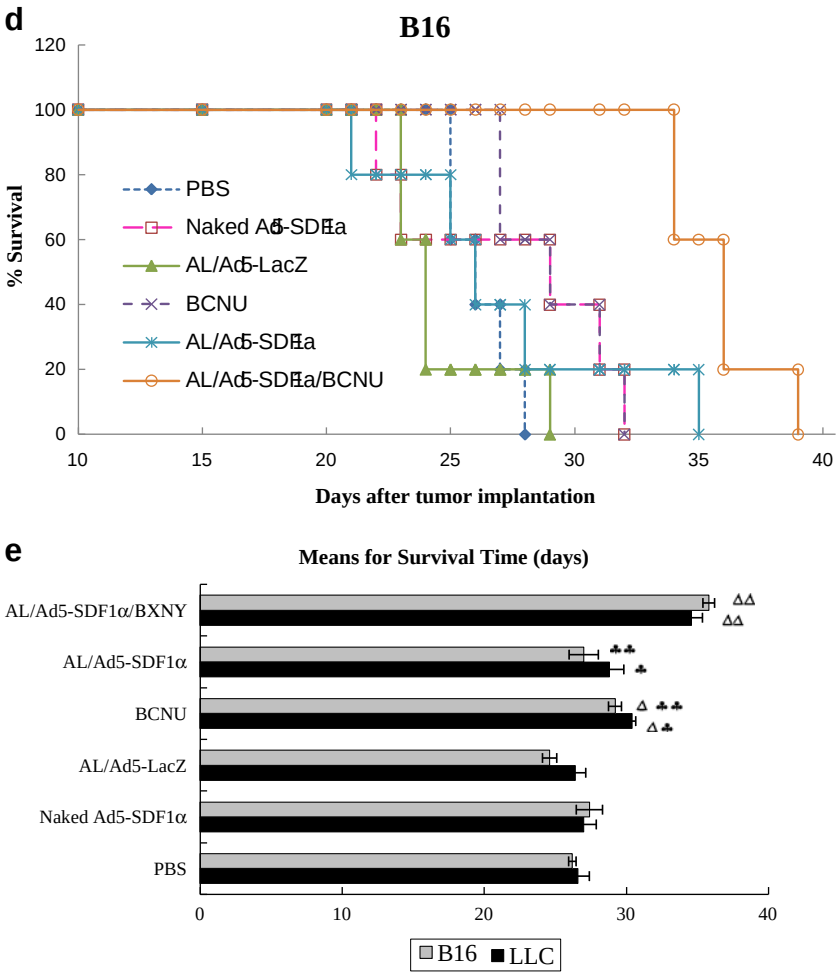


Fig. 5 Effects of combination therapy on suppression of pre-existing subcutaneous tumors and elongation of survival. Mice were injected s.c. on day 0 with 1.0×10^6 LLC or B16 cells. Eight days later, 100 μL of each tested formulations were intratumorally injected in the mouse from each group ($n = 5$), respectively. Tumor volume was monitored every other day and the death date of each mouse was recorded. (**a**, **c**) Mean tumor size (mm^3); *error bars* indicate standard error (S.D.); $^*p < 0.05$, $^{**}p < 0.01$ naked Ad5-SDF1α group *vs* AL/Ad5-SDF1α/BCNU group. (**b**, **d**, **e**) Survival rates of mice and means for survival time in each group. Data shown as mean ± S.D. $^{\Delta}p < 0.05$, $^{\Delta\Delta}p < 0.01$ PBS group *vs*. the treated groups; $^{\clubsuit}P < 0.05$, $^{\clubsuit\clubsuit}p < 0.01$, BCNU and AL/Ad5-SDF1α *vs* AL/Ad5-SDF1α/BCNU, respectively.

Fig. 5 (continued)



In the present study, the complex of anionic liposomes and adenovirus (AL/Ad5) not only compensate for the limitation of viral and non-viral vectors but also take advantage of the unique features of these two classical gene carriers. In our previous study, we reported that AL/Ad5 could improve the efficiency of adenovirus-mediated gene transfer to CAR-deficient cells such as MDCK and CHO (16). Here, we also demonstrated that this strategy could increase the gene expression mediated by adenovirus in cancer cells, LLC and B16 cell lines.

It was reported that neutralizing antibodies could inactivate adenovirus vector, resulting in failure of repeated administrations of adenovirus recombinant. To test whether AL/Ad5 could reduce the induction of the humoral antibody response, the anti-adenoviral antibodies titer (neutralizing antibody) was assessed by transgene inhibition method. We found that the neutralizing antibody titer increased gradually over time from 1 week to 4 weeks. Importantly, the neutralizing antibody titer was much higher in the serum of mice treated with the naked Ad5 alone than that in the groups receiving liposome-vector complexes, AL/Ad5. It was in agreement with the findings by Natsume *et al.* (34) and Steel *et al.* (35), whose study developed complexes of adenovirus and cationic liposomes. Our results suggested the combination of anionic liposomes with adenovirus could significantly decrease the generation of neutralizing antibody.

It has been reported that dendritic cells play a critical role in the induction of cellular immunity in malignant disorders, and dendritic cell cellular therapy is a useful tool for eliciting antitumor immunity (21,36). To play the full function of stromal cell-derived factor-1α (SDF1α) to attract dendritic cells and present antigens to the immune system, we developed a novel delivery system of AL/Ad5 prepared by the calcium-induced phase-change method to transfer and further express the SDF1α cDNA in murine s.c. tumors. The results showed that SDF1α could induce local accumulation of dendritic cells (Fig. 4) and suppress the growth of pre-existing tumors (Fig. 5). However, the function of SDF1α gene therapy alone on suppressing tumor growth and elongating survival time was limited. To achieve an improved anticancer therapeutic effect, a combination of two or more different medicines according to their individual mechanisms has already proved itself to be a popular and effective therapeutic strategy (37). Thus, a chemotherapy

agent, Carmustine (BCNU), was also incorporated into Ad5-loaded liposomes. BCNU, a nitrosourea which undergoes a complex chemical breakdown resulting in DNA alkylation and strand-breaking, could induce tumor growth delay of the Lewis lung carcinoma (38) and B16 melanoma (25). Weydert *et al.* also reported that combining BCNU chemotherapy with adenovirus-mediated superoxide dismutases (Ad-SOD) gene therapy could be extremely successful in the treatment of breast cancer (39). However, BCNU is not easy for administration because of its lipophilicity and low aqueous solubility. Therefore, it was often given as an i.p. infusion after being dissolved in sterile ethanol and diluted in saline prior to use, which showed some extent of toxicity to normal cells besides tumor cells (25). In the previous published combination therapy containing BCNU and therapeutic gene mediated by adenovirus, BCNU and the therapeutic gene were administered separately at different times (25,39), which was inconvenient for injection, and their synergistic effects might be hampered. In our study, BCNU and Ad5-SDF1α were co-encapsulated into liposomes to form a co-delivery system. Furthermore, it was demonstrated that incorporation of BCNU would not compensate the transduction ability of AL/Ad5 *in vitro* (Fig. 1b).

To directly investigate the growth inhibition, we developed the s.c. tumor model *in vivo*, and the formulation solution was given by intratumoral administration. The intra-tumor injection results *in vivo* indicated that both Ad5-SDF1α and BCNU alone could only moderately inhibit the tumor growth. However, the combination therapy of Ad5-SDF1α and BCNU co-delivered by anionic liposomes could significantly suppress the growth of both B16 and LLC tumors and prolong the survival of tumor-bearing mice. The present study has, to our knowledge, first combined SDF1α-mediated immunological gene therapy with BCNU for cancer treatment. But the obtained result is still far from our goal of complete inhibition of tumor growth. In future studies, we would further optimize the ratio between the particle number of Ad5 and BCNU, further investigate the application of combination strategies in other tumor models, and elucidate the mechanism of combination therapy.

CONCLUSIONS

Taken together, in the work presented here, we prepared the complexes of AL/Ad5-SDF1α/BCNU, which co-delivered Ad5-SDF1α and chemotherapeutic agent carmustine to tumor cells (Lewis lung carcinoma and B16 melanoma) *in vitro*/*in vivo*. The results showed that AL/Ad5 could enhance and prolong the local tumor gene expression and reduce the generation of neutralization antibody. AL/Ad5-SDF1α could induce local accumulation of dendritic cells, and the co-delivery system of AL/Ad5-SDF1α/BCNU significantly suppressed the growth of pre-existing tumors and prolonged the survival of tumor-bearing mice.

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