sion gene using either an *ex vivo* BCR-ABL transduction method or *in vivo* BCR-ABL direct transduction method. Then, we examined the expression of the fusion oncogene in transduced progenitor cells *in vivo* and monitored the marmosets for leukemogenic events.

With the *ex vivo* BCR-ABL transduction method, BCR-ABL expression was not detected by RT-PCR after day 100 (**Figure 2(a)**). This lack of sustained expression may result from a low gene transduction efficiency of hematopoietic stem/progenitor cells even though the high transduction efficiency at progenitor levels. Additionally, the transduced cells may have been immunologically rejected. Anti-human G-CSF neutralizing antibodies appeared in the transplanted marmosets even with an immunosuppressive pre-conditioning regimen (data not shown). The presence of these antibodies supports the latter possibility. Majority of the marmosets used in this study were four to six years old, which is an immunologically competent and equivalent to an adult human. Treating the marmosets with immune suppressants might solve this problem.

Previously, our group successfully transduced G-CSF-mobilized hematopoietic stem/progenitor cells in a marmoset model with the MDR1 gene using a retroviral vector [21]. In this study, a consistent increase in progenitor cells in the peripheral blood was observed using the same G-CSF mobilization protocol. Retroviral vectors mainly transduce dividing cells. In contrast, lentiviral vectors transduce both dividing and non-dividing cells [12,20]. And recent reports showed that the retroviral vector, but not lentiviral vector, is integrated near transcriptional genes and induce leukemia [23]. Moreover, in a previous study, we successfully transduced cord blood hematopoietic stem/progenitor cells using a lentiviral vector [12]. For these reasons, we chose a lentiviral vector to transduce p190 BCR-ABL in order to obtain a high transduction efficiency. However, the p190 BCR-ABL gene is approximately 6 kilobase pairs in length, and its large size hinders the production of a high-titer lentiviral vector. This limitation may be one reason why BCR-ABL was expressed for a limited time with the *ex vivo* BCR-ABL transduction method (**Figure 2(a)**).

This was our first attempt to directly inject a lentiviral vector *in vivo* into the bone marrow of common marmosets, although a previous study reported efficient gene transduction by direct injection into the central nervous system of rats [24]. BCR-ABL was detected in the plasmaon day 1 but not day 6. It is likely that vectors injected into the bone marrow, which is rich in blood vessels, transiently leaked into the circulation. Of note, we observed long-term BCR-ABL expression in hematopoietic cells (**Figure 2(b)**). Further optimization of the experimental procedures, such as altering the pre-conditioning regimen, using a higher titer vector, and treating with immunosuppressive drugs after injecting the lentiviral vector, might be effective to obtain stable gene expression in hematopoietic stem/progenitor cells *in vivo*.

To date, mouse models have been predominantly used to study human cancers [25]. However, primates are more genetically related to humans than rodents. For this reason, much effort has focused on establishing a primate model that can be used to more precisely evaluate new cancer therapies in pre-clinical studies. However, thus far, there have been no successful reports of a primate model that mimics human cancers, and this study also faced challenges. The primary reason for these difficulties may be because primate and rodent hematopoietic stem/progenitor cells have different susceptibilities to oncogene transduction [26,27]. In gene therapy clinical trials in France for X chromosome-linked severe combined immune deficiency, leukemia developed two to three years after the common γ chain receptor gene was transduced into hematopoietic stem/progenitor cells [26-28]. In contrast, in a mouse model of oncogene transduction, the duration of developing leukemia is generally less than one year [9-11]. Therefore, these findings support the hypothesis mentioned above.

Moreover, the susceptibility to malignant transformation by each oncogene reportedly differs based on age in humans. For example, for the chromosomal translocations in ALL, MLL-AF4 is dominant in infants, TEL-AML1 in children, and BCR-ABL in adults [29]. Furthermore, cord blood cells were used in all previous reports of BCR-ABL transduction into human hematopoietic stem/progenitor cells in a SCID mouse model [30]. There have been no studies that used adult hematopoietic progenitor cells. In this study, all of the marmosets were adults. Considering these findings, it will be important to consider using younger marmosets in future studies.

Furthermore, it is well known that multiple genetic mutations are required for leukemogenesis [31]. The number of required gene mutations may not be the same between primates and rodents. In the marmoset model, subsequent gene mutations in addition to p190 BCR-ABL may be required for malignant transformation [32]. Thus, it may be necessary to test the cotransduction of p190 BCR-ABL and another oncogene, mutated tumor suppressor gene, or anti-apoptosis gene in order to achieve leukemogenesis in the marmoset model.

Unexpectedly, one marmoset that was transduced using the *in vivo* BCR-ABL direct transduction method developed myelofibrosis-like disease (**Figures 3(a)-(c)**). BCR-ABL gene expression was detected in various organs. However, BCR-ABL was not expressed in the lung, lymph nodes and bone marrow. BCR-ABL expression could be detected where extramedullar hematopoiesis

was observed (liver and spleen) and in organs rich in blood perfusion (heart, kidney and peripheral blood). These differences might contribute to the observed tissue specificities, although this is speculative because the data are only from one marmoset. Bone marrow was negative for BCR-ABL. We believe that our inability to detect BCR-ABL expression in this sample was because we could obtain insufficient hematopoietic cells from the fibrotic bone marrow. Recently, a mutation in JAK2 gene was reported to cause myelofibrosis [33]. We hypothesized that the lentiviral vector inserted into the host genome and unregulated the expression of genes such as JAK2. We performed LAM-PCR (linear amplification-mediated PCR), which identifies the sequence flanking the integrated vector genome, to identify the unregulated gene that may be responsible for myelofibrosis [34]. However, due to the insufficient bone marrow samples, this attempt was unsuccessful. If the phenomenon is reproduced in other marmosets, further analyses will be required to understand this pathology.

In conclusion, we stably expressed an oncogene *in vivo* in a marmoset model, although several steps may be required to develop hematological malignancy in this model. The results provide information that can be used to establish a marmoset disease model in which hematopoietic stem/progenitor cells are targeted for oncogene delivery.

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RESEARCH ARTICLE

Intravenous injection of irradiated tumor cell vaccine carrying oncolytic adenovirus suppressed the growth of multiple lung tumors in a mouse squamous cell carcinoma model

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Abstract

Background Although cancer therapy using replication-selective oncolytic adenoviruses has been available for many years, its anti-tumor efficacy is suboptimal as a result of low and nonspecific infectivity that depends on coxsackie adenovirus receptor expression of the target cancer and normal cells, and generation of an anti-adenovirus neutralizing antibody. In addition, concerns of triggering a severe innate immune response against the adenovirus limit the systemic administration. We developed the carrier cell-based oncolytic virus system (CBOVS) using irradiated tumor cells as carrier cells and concealing the adenovirus (Ad-IAI.3B) inside to improve the specific infectivity. We investigated the anti-tumor effect of CBOVS in a multiple lung tumor mouse model.

Methods The ability of CBOVS to infect Ad-IAI.3B to the target cancer cells was examined *in vitro* in the presence of anti-adenovirus antibodies. To evaluate the systemic effect of CBOVS, we intravenously injected CBOVS into mice with lung tumors (KLN205 cell lines).

Results CBOVS enhanced the infectivity of Ad-IAI.3B to tumor cells in the presence of anti-adenovirus antibodies *in vitro*. Intravenous injections of CBOVS produced an accumulation of the adenovirus in the lung-bearing tumors and produced a strong anti-tumor effect *in vivo*. Furthermore, lymphocytes collected from the CBOVS-treated mice induced an increase in cytokines related to the Th1 response (interferon-γ, interleukin-12) by pulsing with KLN205.

Conclusions These findings suggest that CBOVS could protect adenoviruses from neutralizing antibodies and systemically deliver them to lung tumors. Furthermore, CBOVS appears to have potential as a tumor cell vaccine that activates cytotoxic immunity against cancer cells. Copyright © 2011 John Wiley & Sons, Ltd.

Keywords adenovirus; carrier cell; gene therapy; immune therapy; squamous cell carcinoma; tumor cell vaccine

Introduction

Oncolytic adenoviruses capable of replicating specifically in tumor cells have shown early promise as a cancer therapy, and a variety of gene-modified adenoviruses have been constructed [1,2]. However, many clinical and preclinical studies have identified obstacles to adapting adenoviral cancer

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therapy for clinical applications. Because adenovirus infection mainly occurs via the coxsackie adenovirus receptor (CAR) [3], its infectivity is compromised because it depends on CAR expression of the target cancer cells. By contrast, there can be nonspecific infections to normal cells expressing CAR, which results in an undesirable toxicity and risk with such therapies. Also, in the case of primary or metastatic cancer of internal organs, the therapy may need to be via systemic administration, such as by intravenous (i.v.) injection, which may trigger an innate immune response [4,5]. For example, after i.v. injection, the adenoviral vector rapidly accumulates in the liver as a result of to adenoviral *in vivo* tropism [6,7]. Host innate immune responses are considered to result in part from the uptake of adenoviral vectors and the secretion of proinflammatory cytokines by Kupffer cells, which are macrophages located in the liver [8,9].

Recently, as a result of concerns being raised regarding systemic administration, some investigators have suggested the use of the systemic delivery of oncolytic viruses within carrier cells, in which the cells are used as a 'Trojan horse' delivery system, transporting the oncolytic viruses to the tumor site without eliciting a host immune response. Mesenchymal progenitor cells and irradiated tumor cell lines have both been considered as potential carrier cells for replication-selective oncolytic adenoviruses. Several pre-clinical studies have demonstrated the potential of cellular vehicles to protect loaded viruses from neutralizing antibodies and to deliver them to the tumor site by some sort of tropism of the tumor cells themselves [10–13].

We have developed one such novel delivery system, the carrier cell-based oncolytic virus system (CBOVS), using irradiated tumor cells as carrier cells, which serve both as an efficient vehicle for carrying oncolytic viruses and as a tumor cell vaccine to activate anti-tumor immunity. Several studies of tumor cell vaccines are in progress, and some have suggested that an autologous cell vaccine alone could not provoke an immune reaction sufficient to suppress tumor growth. Because developing tumors often trigger an immunosuppressive reaction against the tumor, anti-tumor immune responses are also insufficient, making the elimination of tumors more difficult. An allogeneic tumor cell vaccine is among the most promising methods for overcoming these problems [14–16]. We adapted an allogeneic tumor cells to carrier cells, with the aim of combining oncolytic virus therapy with a tumor cell vaccine.

In the present study, we investigated the *in vivo* anti-tumor effect of the systemic delivery of CBOVS using KLN205 cells, a mouse squamous carcinoma cell line, in a multiple lung tumor syngeneic mouse model. To construct the CBOVS, we used a replication-selective oncolytic adenovirus termed Ad-IAI.3B, containing the *E1A* gene, which is an essential gene for replication, and which is regulated by the human IAI-3B promoter. The *IAI-3B* gene was first isolated from ovarian cancer [17] and Ad-IAI.3B is known to be able to replicate efficiently in human lung carcinoma A549 cells [18]. These characteristics indicated that A549 cells may function well as carrier cells of CBOVS. In previous studies, it the local anti-tumor effect of the intratumoral injection of CBOVS in both xenogenic and syngeneic tumor mouse models was demonstrated, and it was noted that the transfer of the adenovirus from CBOVS to tumor cells was mediated by the virus containing carrier-cell fragments derived from CBOVS, which were engulfed by the target tumor cells [19]. In the present study, we first examined the ability of CBOVS to infect Ad-IAI.3B to target mouse KLN205 tumor cells in the presence of anti-adenovirus antibody *in vitro*. Then, we i.v. injected CBOVS to immunocompetent mice bearing multiple lung syngeneic KLN205 tumors, and evaluated the systemic delivering efficiency and anti-tumor effect of CBOVS *in vivo*.

Materials and methods

Cell culture and adenovirus

Human nonsmall cell lung cancer A549 cells, and mouse squamous cell carcinoma KLN205 cells, were obtained from the Japanese Collection of Research Bioresources Cell Bank (Osaka, Japan). KLN205 cells were grown in minimal essential medium (MEM) medium (Nissui Co., Tokyo, Japan) supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 1% non-essential amino acids. A549 cells were grown in RPMI medium (Nissui Co.) with 10% heat-inactivated FBS. The construction, amplification and purification of Ad-IAI.3B (i.e. the recombinant replication-selective adenovirus containing the *E1A* gene controlled by IAI.3B promoter) are described elsewhere [18]. Ad-WT (wild type adenovirus, adenovirus type 5 reference material) was purchased from ATCC (American Type Culture Collection, Manassas, VA, USA).

Construction of CBOVS

A549 cells were infected with Ad-IAI.3B at a mutliplicity of infection of 200 and cultured in RPMI without FBS for 48 h. Virus-infected A549 cells were then collected and irradiated with 400 Gy of X-rays to inactivate their proliferation activity, and were stored at −80 °C with 5% glycerol (Wako, Osaka, Japan) and 95% albumin (CSL Behring, King of Prussia, PA, USA). A549 cells alone were also irradiated as controls (IR-A549). The constructed CBOVS were titered with a normal plaque assay after re-extracting Ad-IAI.3B from CBOVS by freeze-thaw cycles. It was previously confirmed that Ad-IAI.3B maintained its replication ability after the irradiation [19].

Infection assay with anti-adenovirus antibody

KLN205 cells were cultured in MEM medium with and without anti-adenovirus antibody (dilution 1:1000; Viro Stat, Portland, ME, USA) and infected with Ad-IAI.3B or CBOVS at a titer of 1000 pfu/cell ($n = 5$ per group). Six hours after the infection, KLN205 cells were washed well

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with phosphate-buffered saline (PBS) to remove CBOVS added to the medium. After washing, the plates were checked under the microscope to confirm that there were no residual CBOVS. KLN205 cells were collected and washed again with PBS, and the total DNA was then extracted using DNeasy Blood & Tissue Kits (Qiagen, Valencia, CA, USA).

Replication assay of Ad-WT and Ad-IAI.3B

KLN205 cells were cultured in MEM medium, and infected with Ad-WT or Ad-IAI.3B at a titer of 1000 pfu/cell ($n = 5$ per group). Six, 12, 24 and 48 h after the infection, KLN205 cells were washed well with PBS. After washing, KLN205 cells were collected and washed again with PBS, and the total DNA was then extracted using DNeasy Blood & Tissue Kits (Qiagen).

Real-time polymerase chain reaction (PCR) analysis

The amount of adenovirus DNA (Ad-DNA) was quantified by real-time PCR with the forward primer 5′-GCTGTGCCCCATTAAACCA-3′ and reverse primer 5′-CGCCAGGCTGTGGAATG-3′. SYBR green Real-Time PCR reactions were performed with POWER SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA) using the ABI PRISM 7500 instrument. Amplification of the target sequence was performed in triplicate reactions each containing 2 ng of total DNA, 2 × Master Mix and primers at a final concentration of 50 nM. The cycle conditions were 50 °C for 2 min, 95 °C for 10 min, 40 cycles of 95 °C for 15 s and 64 °C for 1 min. The copy numbers were calculated as the average of a triplicate. Samples in which amplification was not detected within 40 cycles were reported as negative. Quantification of the target sequence was determined using a standard curve of plasmid DNA, as described previously [18].

Animal experiments

Eight-week-old DBA/2 female mice (Clea-Japan, Tokyo, Japan) were used for a biodistribution study ($n = 6$ per group) and a tumor growth inhibition study ($n = 6$ per group). In the biodistribution study, KLN205 cells (5×10^5 cells/animal) were i.v. injected via the tail vein to obtain multiple tumors in the lung. Fourteen days after the inoculation of KLN205 cells, the animals were i.v. injected with PBS, Ad-IAI.3B (7.6×10^{10} pfu/animal) or CBOVS (7.6×10^{10} pfu/5×10^5 cells/animal). The animals were sacrificed 12 h after the treatment and the amount of adenoviral DNA that had infected into the lung, liver, spleen and kidney was quantified by real-time PCR. In the tumor growth inhibition study, the animals were intramuscularly injected with Ad-LacZ, a recombinant replication-deficient adenovirus containing the *Lac-Z* gene (1×10^{11} vp/animal) to immunize against adenovirus at day 0. The animals were then i.v. injected with KLN205 cells (5×10^5 cells/animal) at day 25, and were then treated with PBS, Ad-IAI.3B (7.6×10^{10} pfu/animal), IR-A549 cells (5×10^5 cells/animal) or CBOVS (7.6×10^{10} pfu/5×10^5 cells/animal) at days 35, 40 and 45. At day 50, the animals were sacrificed, the lung weight was measured, and the lung tissues were obtained for histological examination. In addition, lymphocytes from the spleen were collected at day 50 and the cytokine production was measured by an enzyme-linked immunosorbent assay (ELISA). All the procedures were carried out in accordance with the guidelines for animal experimentation at Kobe University School of Medicine.

Histology and immunohistochemistry

Lung tissues were fixed with 10% buffered formalin, embedded in paraffin, and sectioned at a thickness of 4 μm. The sections were used for hemotoxylin and easonin (H&E) staining or immunostaining. For immunostaining, the sections were deparaffinized, heated at 121 °C for 10 min in citrate buffer, and incubated with primary antibodies at 4 °C, overnight. After incubating with biotinylated secondary antibody for 30 min at room temperature, 3,3′-diaminobenzidine staining was carried out using the labeled streptavidin biotin method. The antibodies used were anti-CD45R/B220 for B-cells diluted 1:100 (BD Pharmingen, San Diego, CA, USA) and anti-CD3 for T-cells diluted 1:100 (Santa Cruz Biotechnology, Santa Cruz, CA, USA).

Histological examination and measurement of the tumor mass area of the lung slides were carried out using a BZ-8100 microscope and VH Analyser VH-H1A5 (Keyence, Osaka, Japan). For the measurement of the tumor mass area, two cross sections each were obtained from the right and left lateral posterior lobes, the areas of the lung mass within these four sections in each animal were measured, and a mean value of five animals per group was calculated and displayed as a percentage of the tumor area per total area.

Cytokine production

The lymphocytes were collected using a cell strainer having pore sizes of 100 μm (BD Biosciences, San Jose, CA, USA) and were plated into 24-well plates in triplicate (3×10^6 cells/well). The lymphocytes were incubated at 37 °C in RPMI medium containing 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, 50 μM 2-mercaptoethanol and 2 mM L-glutamine. They were pulsed with KLN205 cells (5×10^4 cells/well) or PBS (control), and, the supernatants were collected 24 h after the stimulation. The concentrations of interferon (INF)-γ and interleukin (IL)-12 were measured by a specific ELISA

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in accordance with the manufacturer's instructions (Pierce Biotechnology, Rockford, IL, USA).

Statistical analysis

Values are presented as the mean ± SD, and were examined with a two-tailed unpaired *t*-test. $p < 0.05$ was considered statistical significant.

Results

Infection efficacy of CBOVS in the presence of anti-adenovirus antibody *in vitro*

Ad-IAI.3B alone or CBOVS containing the same titer of Ad-IAI.3B was infected to KLN205 cells in the presence of anti-adenovirus antibody to determine whether CBOVS could protect against the neutralization of Ad-IAI.3B by the anti-adenovirus antibody. Six hours after the infection, total DNA from KLN205 cells was extracted and the amount of Ad-DNA was evaluated by real-time PCR (Figure 1A). When KLN205 cells were cultured with Ad-IAI.3B alone, the Ad-DNA amount was decreased when the anti-adenovirus antibody was added to the medium. By contrast, CBOVS maintained its infection efficacy even in the presence of the antibody. We also found that, in the absence of the antibody, CBOVS could infect the Ad-IAI.3B to KLN205 cells more efficiently than Ad-IAI.3B alone.

Replication of Ad-IAI.3B in KLN205, mouse squamous cell carcinoma cells *in vitro*

To determine whether Ad-IAI.3B could replicate in KLN205 cells or not, we performed the replication assay. The results obtained demonstrated that Ad-IAI.3B could replicate in KLN205 cells as well as a wild-type adenovirus (Figure 1B).

Distribution of Ad-IAI.3B to multiple lung tumors after CBVOS i.v. injection

Mice with and without multiple lung KLN205 tumors were i.v. injected with Ad-IAI.3B alone or CBOVS and, 12 h after the treatment, the lung, liver, spleen and kidney were removed. Total DNA was extracted from each organ and the quantity of infected Ad-DNA was evaluated by real-time PCR (Figure 2). There was no difference in the amount of Ad-DNA in the lung of mice injected with Ad-IAI.3B alone between mice with lung tumors and mice without lung tumors, indicating that systemic injection could not deliver the Ad-IAI.3B efficiently to lung tumors (Figure 2A). By contrast, the amount of Ad-DNA in the lungs of CBOVS-injected mice with tumors was significantly higher than that in lungs of mice without tumors, demonstrating the capability of CBOVS to deliver the Ad-IAI.3B to the tumors (Figure 2A). There were no differences between the mice with tumors and the mice without tumors in the other organs (Figures 2B to 2D). In addition, the amounts of Ad-DNA in the lungs were relatively low compared to those in the livers and spleens (Figures 2A to 2C), and the amounts of DNA in the livers and spleens of mice injected with Ad-IAI.3B alone were relatively high compared to those of mice injected with CBOVS (Figures 2B to 2C).

Tumor growth inhibitory effect of i.v. injected CBOVS

Mice were first immunized with Ad-LacZ to provoke anti-adenovirus immunity at day 0, and were then inoculated with KLN205 cells to generate multiple lung tumors at day 25. They were then i.v. injected with PBS, Ad-IAI.3B,

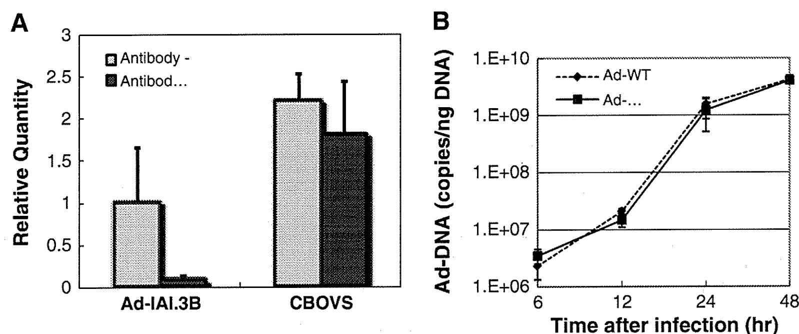

Figure 1. (A) KLN205 cells were infected with Ad-IAI.3B or CBOVS at a titer of 1000 pfu/cell in the presence of anti-adenovirus antibody. Six hours after infection, KLN205 cells were collected, total DNA was extracted and the amount of Ad-DNA infected to KLN205 cells was quantified by real-Time PCR. The amount of Ad-DNA is presented as the relative value compared to the Ad-IAI.3B-infected group without antibody. All data are presented as the mean ± SD ($n = 5$ per group). (B) KLN205 cells were infected with Ad-WT or Ad-IAI.3B at a titer of 1000 pfu/cell. At 6, 12, 24, 48 h after the infection, KLN205 cells were collected, total DNA was extracted, and the amount of Ad-DNA replicated in KLN205 cells was quantified by real-time PCR. The averages of Ad-DNA copies are presented at each point. All data are presented as the mean ± SD ($n = 5$ per group).

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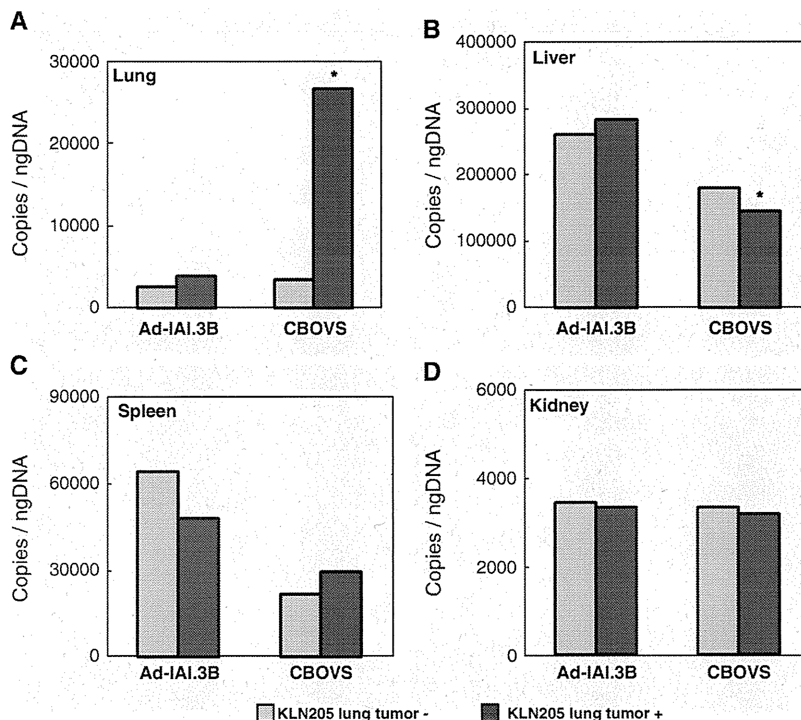

Figure 2. KLN205 cells (5×10^5 cells/animal) were i.v. injected to DBA/2 mice via the tail vein to obtain multiple tumors in the lung. Fourteen days after the inoculation of KLN205 cells, the mice with/without lung tumors were i.v. injected with PBS, Ad-IAI.3B (7.6×10^{10} pfu/animal) or CBOVS (7.6×10^{10} pfu/5×10^5 cells/animal). They were sacrificed after 12 h of treatment and the amount of Ad-DNA infected to (A) lung, (B) liver, (C) spleen and (D) kidney was quantified by real-time PCR. All data are presented as the mean ± SD of five animals. $^*p < 0.05$ versus animals without tumors (Student's t-test).

IR-A549 or CBOVS three times at days 35, 40 and 45. The animals were sacrificed at day 50 and their lungs were examined to determine the tumor-suppressing effects of CBOVS. The appearances of the lung are shown in Figure 3. A large amount of tumor mass was formed in the lungs of the control (Figure 3A) and Ad-IAI.3B-treated (Figure 3B) mice. By contrast, the number and size of the tumor masses in the lungs of the CBOVS-treated (Figure 3D) mice were markedly reduced. Noteworthy, tumor reduction was also observed in the IR-A549-treated group (Figure 3C), although its effect was less than that of CBOVS. The weights of the lungs obtained from the CBOVS-treated mice were significantly lighter than those from the control mice ($p < 0.0001$) and those for the IR-A549-treated group were also lighter than those of the controls ($p < 0.05$) (Figure 4A). Figure 4B shows the percentages of tumor area per total. The results were similar to those of the lung weight measurement, showing a high tumor growth inhibitory effect in the CBOVS-treated group and a mild effect in the IR-A549-treated group.

On histological examination, we observed lymphocytes infiltrating to the tumor mass in the CBOVS-treated group (Figure 5A-IV). Furthermore, immunohistochemical staining revealed that these lymphocytes comprised a small number of CD45R/B220-positive cells (B-cells; Figure 5B-IV) and a large number of CD3-positive cells (T-cells; Figure 5C-IV). These findings were not seen in the control, Ad-IAI.3B- or IA-A549- treated group, which suggests that a strong cytotoxic immune reaction against tumor cell KLN205 was evoked by the i.v. injection of CBOVS.

Production of INF-γ and IL-12 by lymphocytes after stimulation with KLN205 cells

Lymphocytes were collected, pulsed with PBS or KLN205 cells and incubated. The supernatants were collected 24 h after the pulsation and the amounts of INF-γ (Figure 6A) and IL-12 (Figure 6B) were measured using an ELISA. The production of INF-γ increased significantly after the pulsation with KLN205 cells in all groups, although the amount of INF-γ produced by lymphocytes obtained from the CBOVS-treated mice was markedly higher than that of the PBS-treated or Ad-IAI.3B-treated group. Furthermore, the lymphocytes from the IR-A549-treated mice showed high cytokine production, although it was not as high as that of the CBOVS-treated group. The production of IL-12 increased only in the CBOVS-treated group after the pulsation, and there was no change in the PBS-, Ad-IAI.3B- or IR-A549- treated group.

Discussion

To investigate the feasibility of cancer therapy using CBOVS, we evaluated the capability of CBOVS to protect

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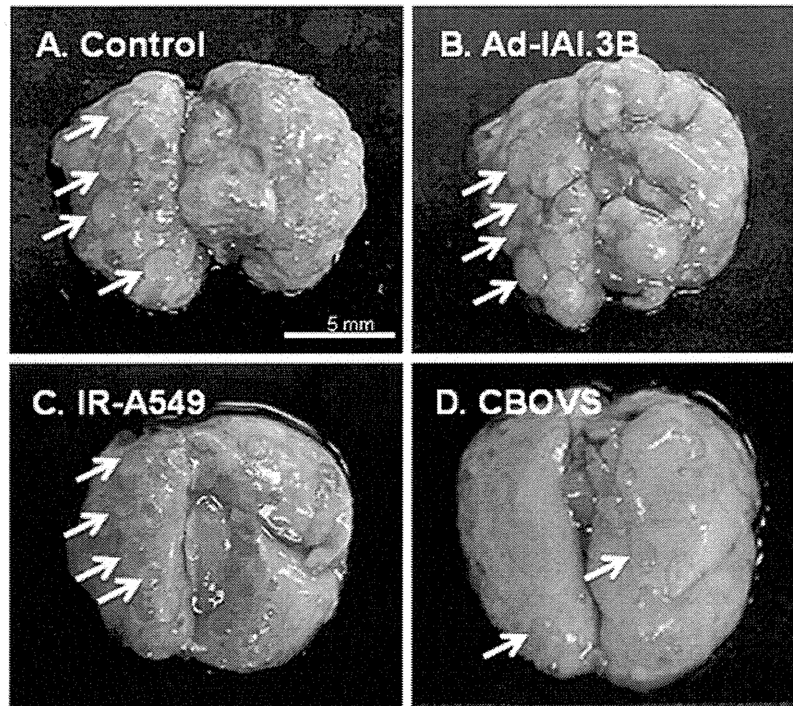

Figure 3. DBA/2 mice were intramuscularly injected with replication-deficient Ad-LacZ (1×10^{11} vp/animal) to immunize against adenovirus at day 0. They were then i.v. injected with KLN205 cells (5×10^5 cells/animal) at day 25, and were then treated with PBS, Ad-IAI.3B (7.6×10^{10} pfu/animal), IR-A549 cells (5×10^5 cells/animal) or CBOVS (7.6×10^{10} pfu/5×10^5 cells/animal) at days 35, 40, and 45. At day 50 (14 days after treatment), the lungs were obtained from animals. (A) Control, (B) Ad-IAI.3B, (C) IR-A549 and (D) CBOVS. White arrows indicate some of the tumor nodules.

an adenovirus from neutralizing antibodies *in vitro*, and to deliver adenoviruses systemically to the tumor sites for the suppression of tumor growth *in vivo*. We showed that CBOVS could maintain the adenoviral infectivity to KLN205 tumor cells in the presence of an anti-adenovirus neutralizing antibody. Moreover, the infective efficiency of CBOVS was higher than that of Ad-IAI.3B alone in the absence of the antibody. These results indicate that carrier cells of CBOVS can enhance the infection of Ad-IAI.3B to tumor cells. The precise mechanism of the protection and maintenance of the infectivity of Ad-IAI.3B was not examined in the present study. However, Hamada *et al.* [19] demonstrated that viral particle-containing small vesicles could be derived from the superficial aspects of CBOVS and were engulfed by the cytoplasm of the target cells. Thus, we suspect that the efficacy of the infection via engulfment exceeded the infection via CAR in the present study.

Although several studies have demonstrated the usefulness of oncolytic adenoviral cancer therapy, their methods of administration were mostly limited to intratumoral injection as a result of issues of nonspecific infection that might cause severe side-effects after systemic administration of an adenovirus [20]. Indeed, the death of a patient was reported during an adenoviral clinical trial in 1999 [5,21]. Yet there remains a long-considered need for the systemic treatment of distant and unresectable tumors, which remain a major cause of cancer death. We see promise in, and continue to seek, a

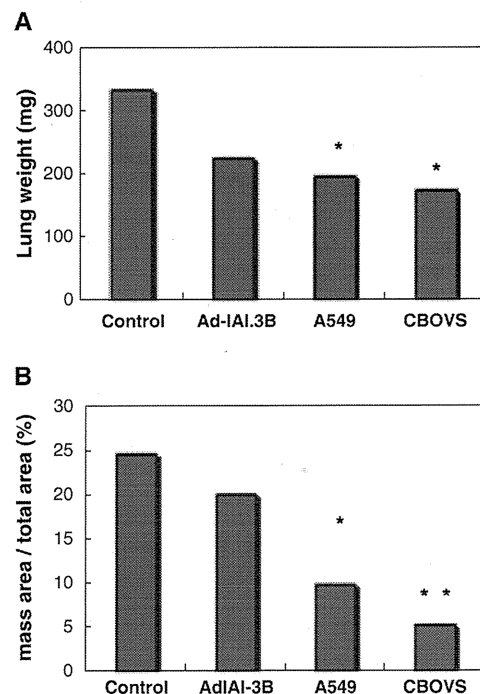

Figure 4. (A) Weights of the lung shown in Figure 3 were measured at the time of sacrifice (day 50). (B) In addition, paraffin sections were obtained from the fixed lung tissue. The sections were H&E stained and the areas of the tumor mass were measured under a light microscope. The ratio of the mass area per total area was calculated. All data are presented as the mean ± SD of five animals. *$p < 0.05$ versus control, **$p < 0.0001$ versus control (Student's *t*-test).

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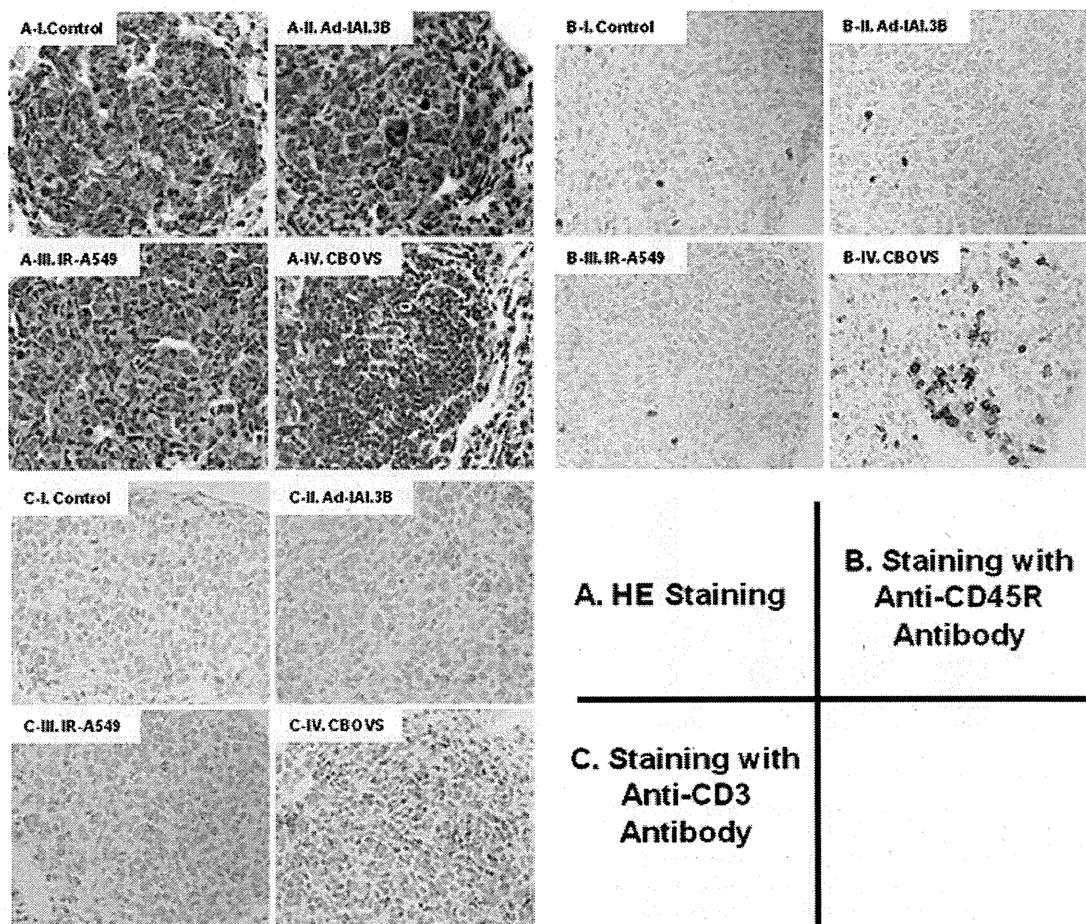

Figure 5. (A) Sections of the lung tumors were H&E stained and observed under a light microscope. Infiltration of lymphocytes was seen in CBOVS-treated mice. (B) Sections of the lung tumors were stained with anti-CD45R (B-cells). Infiltration of B-cells was seen in CBOVS-treated mice. (C) Sections of the lung tumors were stained with anti-CD3 (T-cells). Infiltration of T-cells was seen in CBOVS-treated mice.

systemic adenoviral therapy for primary or metastatic cancer using a carrier cell delivery system. To examine whether CBOVS can deliver adenoviruses to distant lung tumor sites, we i.v. injected Ad-IAI.3B alone or CBOVS to mice that had multiple lung tumors. In our results, the patterns of bisodsitribution of Ads in the liver, spleen, lung and kidney were similar to those reported in previous studies of adenoviral systemic administration to rodents [22–24]. When Ad vector utilizing human adenovirus type 5 is systemically administrated, the clearance of Ad vector from the bloodstream and its accumulation in the liver and spleen occur rapidly [6,25]. Although *in vitro* adenoviral tropism can be well explained with CAR/integrins interaction, the mechanisms of *in vivo* adenoviral tropism are not yet clear [26]. This *in vivo* adenoviral hepatotropism is considered to be related to the interaction between Ad and hepatic heparin sulfate proteoglycans (HSPGs) [26]. In addition, blood coagulation factor IX and complement component C4-binding protein (C4BP) are assumed to bind to the virus fiber and bridge to hepatocytes [27]. In the present study, systemic administration of Ad-IAI.3B with irradiated carrier cells (CBOVS) decreased adenoviral accumulation in the liver and spleen compared to adenoviral administration without carrier cells. Furthermore, CBOVS significantly increased adenoviral accumulation in lung when the lung contained multiple tumors. It is still unclear whether the A549 carrier cells efficiently accumulated in tumors in the lung or whether Ad-IAI.3B replicated in tumors; however, this redirection of adenoviral *in vivo* tropism with CBOVS might have great advantages for establishing the systemic delivery system of oncolytic Ads for the treatment of patients with disseminated tumors.

Although the mechanisms of redirecting *in vivo* tropism with CBOVS remain unknown, there are several proposed hypotheses. Concealing with CBOVS might prevent Ad-IAI.3B from binding to HSPGs, blood factor IX, C4BP and other blood factors, and decrease Ad-IAI.3B accumulation in liver. Cancer cells obtained from the primary tumor site can migrate to metastatic tumor sites after dissemination to the bloodstream. Several molecular interactions between migrating tumor cells and host tissues are assumed to play roles in this tumor cell tropism in a manner that is different from that of natural

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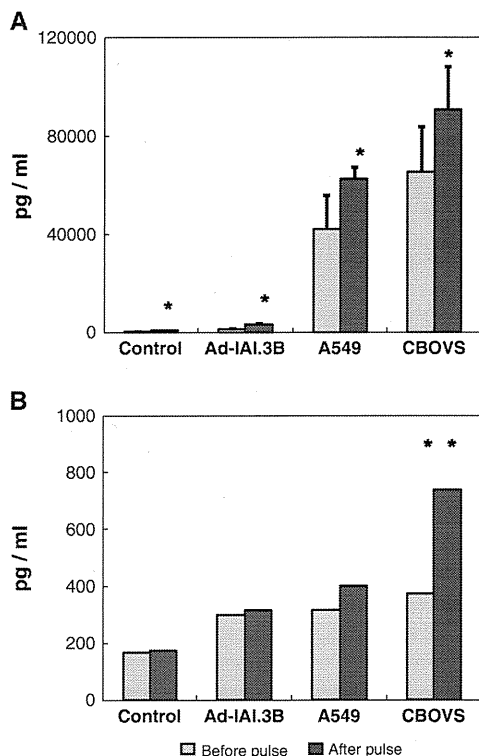

Figure 6. The animals used were described in Figure 3. Lymphocytes of spleen were collected from control group and CBOVS group animals at day 50. The lymphocytes were cultured, stimulated with KLN205 cells and the concentrations of (A) INF-γ and (B) IL-12 were quantified by ELISA, 24 h after stimulation. All data are presented as the mean ± SD of five animals. *$p < 0.05$, **$p < 0.01$ versus without stimulation group (Student's *t*-test).

adenoviral *in vivo* tropism. This suggests that tumor-derived carrier cells can localize effectively at the metastatic site and may be used to deliver oncolytic Ads to targeted cancer cells [10,28,29]. In the present study, we used irradiated human tumor cells to construct CBOVS for a mouse experimental model, and not autologous carrier cells, which is inconsistent with the above-mentioned hypothesis, although it is still possible that some kind of crosstalk between carrier cells and the environment at the metastatic site is involved in the localization of the vehicle. Anatomical factors may also play a significant role. For example, the lung is the site of the first blood capillaries through which the carrier cells pass after i.v. injection, which may increase their accumulation therein. Further studies are required to clarify these and many other details, although the results obtained in the present study indicate that the efficient delivery of Ad-IAI.3B to multiple lung tumors could be achieved using the CBOVS system, without any significant dissemination of adenoviruses and the risk of systemic toxicity.

After three rounds of CBOVS injection, the animals were sacrificed and the lung tumors were examined. The growth of the tumors was significantly suppressed in the CBOVS-injected animals and the infiltration of lymphocytes, especially T-cells, was observed. Furthermore, the ELISA assay revealed that INF-γ and IL-12, which mainly contribute to cytotoxic immunity, were induced from lymphocytes of the mice treated with CBOVS. The observed activation of T-cells and the induction of cytokines in the CBOVS-treated mice suggest that a strong immune reaction against the target cancer cells was evoked by CBOVS treatment because the role of T-cells in anti-tumor immunity has been well documented [30–32]. Although Ad-IAI.3B could replicate in KLN205 tumor cells *in vitro*, we did not observe a tumor-suppressing effect in the animals treated with Ad-IAI.3B alone. However, we were surprised to find that treatment with the empty carrier cells, IR-A549, produced a mild tumor-suppressing effect. The high levels of INF-γ production in splenocytes from the mice treated with xenogenic human IR-A549 cells suggested the strong lung inflammation that might contribute the anti-tumor effect. Nevertheless, KLN205 pulsing stimulated the INF-γ production of splenocytes from mice treated with IR-A549, which might suggest some cross reactivity between A549 and KLN205 antigens. In addition, the secretion of IL-12 was also stimulated by KLN205 pulsing in the case of CBOVS.

Taken together, these findings suggest that the anti-tumor immunity of CBOVS might be induced by at least two mechanisms. First, the carrier cells of CBOVS could act as an antigen to provoke anti-tumor immunity. Second, the encapsulated Ad-IAI.3B could act as an adjuvant for an immunoreaction against target cancer cells infected with Ad-IAI.3B. The first mechanism, which uses tumor cells as a vaccine to induce anti-tumor immunity, has been extensively studied, and non-autologous (allogeneic or xenogenic) cells as the antigen have shown more promise than autologous syngeneic cells because syngeneic antigens can be considered as 'self' by the host's immune system and they also activate less anti-tumor immunity than do non-autologous cells that are completely foreign to the host [14–16]. Our strategy of using non-autologous cells as carriers was effective because the carrier cell can act not only as the vehicle, but also as an antigen to provoke a more robust anti-tumor immune response. The second mechanism, which uses adenoviral vectors as an adjuvant to enhance the immune response against viral gene-transduced cancer cells, has also been investigated, and it has been demonstrated that the anti-adenovirus immune response stimulated by virus administration can lead to the destruction of the virus-infected cancer cells and could induced anti-tumor adaptive immunity [33,34]. We consider that these mechanisms played an important role in the present study because the animals were immunized against adenovirus before the treatments. In our previous study [19], we also demonstrated that pre-immunization with Ad could enhance the anti-tumor effect of CBOVS. Ad-IAI.3B delivered to the target cells evoked an immune reaction against Ad-IAI.3B and the infected tumor cells were then attacked as a collateral effect. In conclusion, as

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a result of these combined immune responses caused by two different antigens (i.e. the carrier cell and the adenovirus), a strong tumor-suppressing effect was achieved by CBOVS treatment.

In conclusion, the results obtained in the present study demonstrate that CBOVS has a great potential for novel combinatory anti-cancer therapeutics where both oncolytic adenoviral therapy and immunotherapy can be achieved by one product. However, further intensive investigation of the safety of i.v. injection of CBOVS is required before clinical translation.

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RESEARCH ARTICLE

Antitumor effect of chondroitin sulfate-coated ternary granulocyte macrophage-colony-stimulating factor plasmid complex for ovarian cancer

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Abstract

Background Although replication-competent viruses have been developed for treating cancers, their cytotoxic effects are insufficient as a result of infection inhibited by the generation of neutralizing antibodies, and systemic administration is difficult as a result of the life-threatening serious side-effects of virus-induced cytokine surge. To overcome these critical problems, we devised a plasmid/polycation/polyanion complex and assessed the potential of ternary plasmid complexes coated with chondroitin sulfate in gene therapy for ovarian cancer. The antitumor effects of chondroitin sulfate-coated complex as an anionic component were compared with those of hyaluronic acid on ovarian cancer.

Methods Plasmid harboring the gene of murine granulocyte macrophage-colony-stimulating factor (mGM-CSF) was complexed with polyethyleneimine (PEI) and hyaluronic acid or chondroitin sulfate. Murine ovarian cancer cells were injected into (C57BL/6 $\times$ C3H/He) F_1 mice to prepare a subcutaneous or intraperitoneal tumor model.

Results DNA/PEI was charged positively and DNA/PEI/chondroitin sulfate or DNA/PEI/hyaluronic acid was charged negatively. Plasmid-green fluorescent protein (GFP)/PEI coated with 10-kilodalton (kDa) chondroitin sulfate increased transfection efficiency compared to coating with chondroitin sulfate of higher-molecular-weight or hyaluronic acid. The transfection efficiency of GFP/PEI/10-kDa chondroitin sulfate in ovarian cancer cells was six-fold higher than that in normal cells. Intraperitoneal injection of mGM-CSF/PEI coated with 10-kDa chondroitin sulfate prolonged survival compared to that coated with hyaluronic acid. Intratumoral injection of mGM-CSF/PEI coated with 10-kDa chondroitin sulfate achieved mouse survival rates of 100%, although that with hyaluronic acid did not.

Conclusions These findings suggest that GM-CSF/PEI coated with 10-kDa chondroitin sulfate has the potential for use in gene therapy of ovarian cancer.
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Keywords cancer gene therapy; chondroitin sulfate; hyaluronic acid; ovarian cancer; polyethyleneimine; ternary plasmid complex: GM-CSF

Introduction

Although replication-competent viruses have been developed for treating cancers, their cytotoxic effects are insufficient because infection with them

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is inhibited by the generation of neutralizing antibodies. A replication-competent virus-infected carrier cell overcomes virus-induced immunogenicity and induces complete tumor reduction [1]. This carrier cell system has asscociated problems, including difficulties of systemic administration as a result of trapping in capillary vessels, life-threatening serious side-effects as a result of replication-competent virus-induced cytokine surge, difficulties with mass industrial production, the need for liquid nitrogen for stocking, instability on long-term storage, and the need for hospitalization and an isolated room as a result of the biodiversity treaty. The development of a safe nonviral vector system is thus clearly required.

Polycations and cationic lipids electrostatically bind to DNA and facilitate gene transfer into target cells *in vitro*. *In vivo* transfection of this binary DNA/cation complex is difficult because the surfaces of the complexes are usually positively charged, and interaction with blood cells, serum proteins and extracellular matrix prevents their efficient delivery [2–4]. A protective polyanion coating was developed to afford negatively-charged DNA complexes [5], which exhibit diminished interaction with serum proteins.

Plasmid/polycation/polyanion ternary complexes have a negative surface charge and exhibit high dispersion stability, even in the presence of blood cells or proteins. Polyanions play roles not only in protective coating, but also as ligands for target cells [4,6] and transcriptional enhancers [6,7], and some degree of improvement in reporter-gene expression in tumor has been observed after their injection into the tail vein of mice [8,9]. Particles with a diameter < 100 nm are required for distribution in the body and accumulation in tumors as a result of enhanced permeability and retention. Mixing of DNA and polycations under highly concentrated conditions usually yields much larger particles (> 200 nm). Moreover, DNA/polycation binary complexes readily aggregate and thereby increase in size [10]. However, DNA/polycation/polyanion ternary complexes can be concentrated by freeze-drying and undergo rehydration without aggregation or inactivation [11].

Hyaluronic acid (HA) and chondroitin sulfate (CS), which are present in tumor matrix, and the receptors of which are CD44 and overexpressed in metastatic cancer tissue, have been mainly used as anionic components of ternary complexes in drug delivery systems for cancer treatment [12,13]. Plasmid-granulocyte macrophage-colony stimulating factor (GM-CSF)/polyethyleneimine (PEI)/HA ternary complex expresses the genes only in tumors and not in normal tissues, and significantly suppresses B16 tumor growth [11], although the antitumor activity of GM-CSF/PEI/CS ternary complex remained to be identified. In the present study, we compared raw materials and molecular weight fractions of CS as an anionic component of ternary complexes and compared the antitumor activity of GM-CSF/PEI/CS with that of GM-CSF/PEI/HA for ovarian cancer.

Materials and methods

Cell lines and culture conditions

Human ovarian adenocarcinoma HEY, OCC1 and OVCAR3 cells from Dr G. Mills (The University of Texas, MD Anderson Cancer Center, TX, USA), human ovarian clear cell carcinoma RMG-1 cells from Dr S. Nozawa (Keio University, Tokyo, Japan) and murine ovarian cancer (OVHM) cells from Dr H. Fujiwara (Osaka University, Osaka, Japan) were obtained. Normal human fibroblast F27 cells were established by Dr K. Hashimoto (Ehime University, Japan). Human ovarian teratocarcinoma PA-1 and murine fibroblast Lcl-1D cells were obtained from the Japanese Collection of Research Bioresources Cell Bank (Osaka, Japan). Murine fibroblast cells (NIH/3 T3) was obtained from Dr Tagawa (Chiba Cancer Institute, Chiba, Japan). Human amniotic stem cells (ASP) were obtained from Dr N. Sakuragawa (Bioresource Application Institute, Aichi, Japan).

Cells were maintained in a humidified 5% CO_2/95% air incubator at 37 °C. All cell lines except human amniotic stem cells were grown in RPMI medium supplemented with 10% fetal bovine serum. Human amniotic stem cells were grown in DMEM/F12 with 10% fetal bovine serum, 10 ng/ml human leukemia inhibitory factor, 10 ng/ml epidermal growth factor and 10 ng/ml basic fibroblast growth factor.

Reagents

HA sodium salt and CS were supplied by Seikagaku Corp. (Tokyo, Japan). Linear PEI 'MAX' (MW 40 000) was purchased from Polyscience, Inc. (Warrington, PA, USA). Green fluorescent protein (GFP)-coding plasmid with cytomegalovirus promoter was obtained from Clontech Laboratories, Inc. (Mountain View, CA, USA). Plasmid coding luciferase gene was constructed by subcloning the *Hind*III/*Xba*I firefly luciferase cDNA fragment from the pGL3-control vector (Promega, Madison, WI, USA) into the pcDNA3.1 vector (Invitrogen, Carlsbad, CA, USA). Plasmid coding murine granulocyte macrophage-colony-stimulating factor (mGM-CSF) was similarly produced with pcDNA3.1 vector and the gene isolated from mouse lymphocytes. They were amplified in Escherichia coli and purified with a Qiagen Plasmid Mega Kit (Tokyo, Japan). Cell culture lysis reagent and luciferase assay substrate were purchased from Promega. The protein assay kit was obtained from Bio-Rad Laboratories (Hercules, CA, USA).

Freeze-dried and rehydrated DNA complex

Typically, an aqueous solution of plasmid DNA (30 μl; 0.792 mg/ml) was mixed with linear PEI solution (30 μl; 2.3 mg/ml) and then with a solution

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of HA (60 μl; 10 mg/ml) or CS (60 μl; 2.3 mg/ml) as previously described [11]. All the solutions were prepared in pure water. After being left to stand for 20 min, they were freeze-dried, and the resulting white spongy complexes were rehydrated with water or 5% glucose to [DNA] = 200 μg/ml.

ζ-Potential and size measurement

The freeze-dried complex containing 1 μg of DNA was rehydrated with water (200 μl). After 30 min, it was diluted with 800 μl of water and the ζ-potential and the size was measured with a particle analyser (Zetasizer Nano ZS, Malvern, UK).

In vitro transfection

OVHM cells were seeded onto 24-well plates at 5×10^4 cells per well, and cultured for 2 days. Lyophilized-and-rehydrated luciferase complex suspensions were prepared in phosphate-buffered saline (PBS) ([DNA] = 200 μg/ml). After 20 min, they were added to the cells (1 μg of plasmid per well). After an additional 20-h incubation at 37 °C, the cells were lysed, and transgene expression and protein content in the lysate were assessed with the corresponding assay kit.

Each cell line was seeded onto 24-well plates at 5×10^4 cells per well, and cultured for ≥ 2 days. Lyophilized-and-rehydrated plasmid-GFP complex suspensions were prepared in PBS ([DNA] = 200 μg/ml). After 20 min, they were added to the cells (1 μg of plasmid per well). Liposomal transfection was carried out using LF2000 reagent (Invitrogen). GFP-positive cells were counted with a fluorescence microscope (Axiovert 40; Carl Zeiss, Hallbergmoos, Germany) and the transfection efficiency was estimated.

Inhibition of subcutaneous ovarian tumor growth in syngeneic mice

To determine inhibition of syngeneic subcutaneous tumor growth, murine OVHM cells (1×10^6) were injected into the left posterior flank of female (C57BL/6 × C3H/He) F_1 mice (CLEA Japan Inc., Tokyo, Japan). Freeze-dried ternary complex of HA or CS was rehydrated with 500 μl of PBS for intratumoral injection. When the size of the subcutaneous tumor reached 5–8 mm in diameter, the resuspended complex containing 100 μg of the plasmid was injected intratumorally every day for 3 days. These animal studies were approved by the Ehime University Review Board.

Inhibition of intraperitoneal ovarian tumor growth in syngeneic mice

To simulate a clinical trial of gene therapy for ovarian cancer, the orthotopic intraperitoneal carcinomatosis model was used because ovarian cancer remains localized within the peritoneal cavity in a large proportion of patients, ultimately causing local morbidity and lethal complications. Murine OVHM cells (1×10^6) were injected into the abdominal cavity of female (C57BL/6 × C3H/He) F_1 mice (CLEA Japan Inc.). Freeze-dried ternary complex of HA or CS was rehydrated with 7 ml of PBS. Four to nine days after intraperitoneal inoculation of OVHM cells, the re-suspended and diluted complex containing 100 μg of plasmid was injected intraperitoneally and subcutaneously every day for 6 days. A 2-ml portion of 7 ml of ternary complex containing PBS was injected into the OVHM cell-injected abdominal skin to prevent local recurrence of OVHM tumor, and the remaining 5 ml of ternary complex containing PBS was injected into the peritoneal space because this subcutaneous injection prevents local recurrence, whereas no subcutaneous injection induced local recurrence in more than 80% of mice, and this excess amount of intraperitoneal injection could increase the antitumor activity of ternary complex. These animal studies were approved by the Ehime University Review Board.

Statistical analysis

Values are reported as the mean ± SD, and were examined with the unpaired *t*-test, Welch test and regression analysis. Survival data were plotted on Kaplan–Meier curves, and examined with the log-rank test using the LIFETEST procedure. $p < 0.05$ was considered statistically significant.

Results

Ten-kilodalton (kDa) CS yields efficient tumor-specific transfection

To obtain strong antitumor activity, high-molecular-weight (730 kDa) HA and the low-molecular-weight CS, which are present in tumor matrix and glycosaminoglycans, were compared as the anionic component of ternary DNA complex. In the ζ-potential assay, DNA and binary and ternary complexes were charged negatively, positively and negatively, respectively (Figure 1A). In the luciferase assay, luciferase activity of ternary complex with 10-kDa shark CS was three-fold higher than that of chicken HA and significantly higher than those of other higher-molecular-weight fractions of shark or cattle CS (Figure 1B). This 10-kDa shark CS was therefore used as an anionic component of ternary complex in this experiment. The size of DNA/PEI/CS was distributed from 59 to 164 nm, which is smaller than that of DNA/PEI/HA from 79 to 531, and the peak size of DNA/PEI/CS was 79 nm, which is 65% of that of DNA/PEI/HA at 122 nm (Figure 1C). Although binary GFP/PEI aggregated red blood cells (Figure 2A), ternary GFP/PEI/CS did not (Figure 2B). GFP plasmid yielded GFP-positive cells in none of the cell lines tested. LF2000 transfection and binary GFP/PEI did not yield significant differences in the numbers of GFP-positive cells between normal and cancer cells. The rate of transfection

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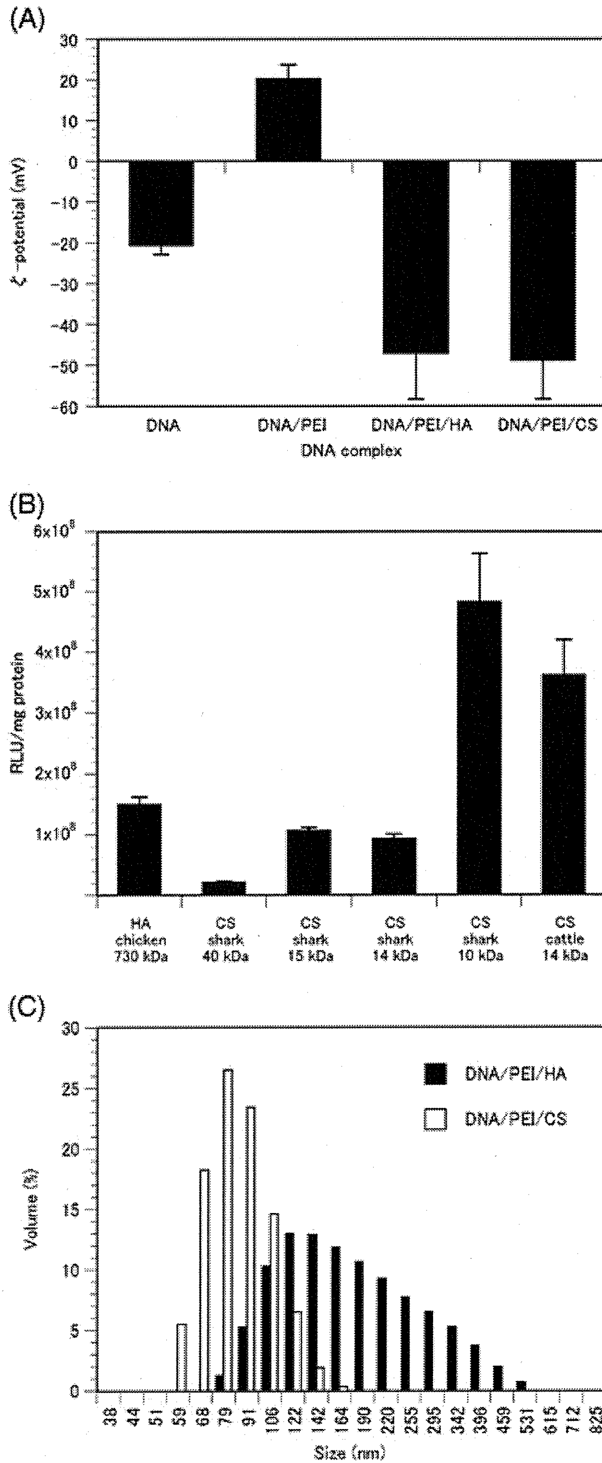

Figure 1. (A) ζ-potentials (mV) of DNA, binary DNA/PEI and ternary DNA/PEI/HA and CS complexes. (B) Luciferase activity of luciferase/PEI coated with HA from chicken and each molecular weight fraction of CS from shark or cow in cultured OVHM cells. RLU, relative light units. (C) The size of ternary DNA/PEI/HA and CS complexes.

with ternary GFP/PEI/HA or GFP/PEI/CS transfection in cancer cells was significantly higher than that in normal cells, and the rate of transfection with ternary GFP/PEI/CS transfection was significantly higher than that with

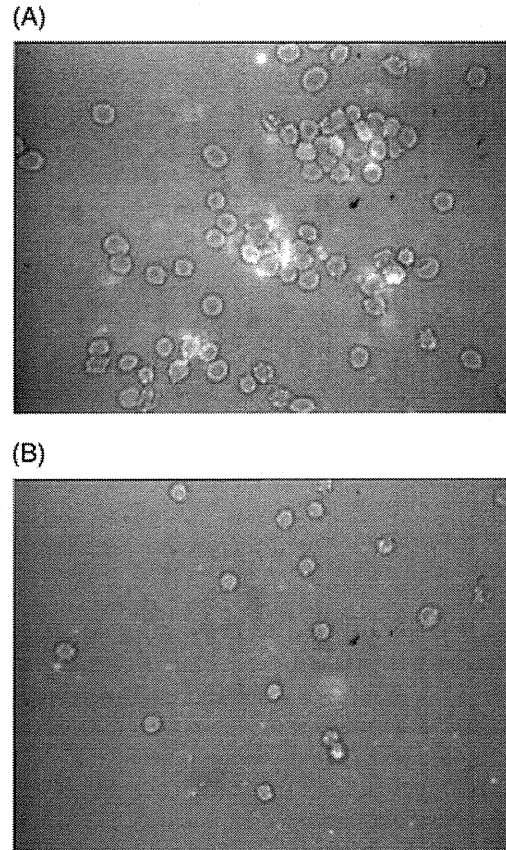

Figure 2. Binary GFP/PEI complex (A) and ternary GFP/PEI/CS complex (B) was incubated with red blood cells. Magnification: × 60.

GFP/PEI/HA transfection in all cancer cell lines examined (Figure 3A). LF2000 was highly toxic for all cell lines compared with binary and ternary complexes. Binary complex was more toxic than ternary complexes for all cell lines tested, except OVHM and ASP (Figure 3B). GFP expression by ternary GFP/PEI/CS or GFP/PEI/HA transfection peaked at days 2 or 3 and persisted for 9–27 days in cancer cells (Figure 4).

Antitumor effect of small mGM-CSF/PEI/CS ternary complex on subcutaneous tumor-bearing mice

Small DNA/PEI/HA or CS complexes were prepared with mGM-CSF-coding plasmid. It was freeze-dried and rehydrated at 200 μg/ml for intratumoral injection. Mice bearing OVHM tumor were injected with the complex three times daily (each injection, 100 μg/500 μl of plasmid per mouse). All control mice injected with medium died within 102 days. Binary mGM-CSG/PEI complex yielded a 20% rate of complete tumor reduction, whereas ternary mGM-CSF/PEI/HA complex and ternary mGM-CSF/PEI/CS complex yielded rates of 82% and 100%, respectively (Figure 5A). These rates of reductions were significantly different among types of treatment.

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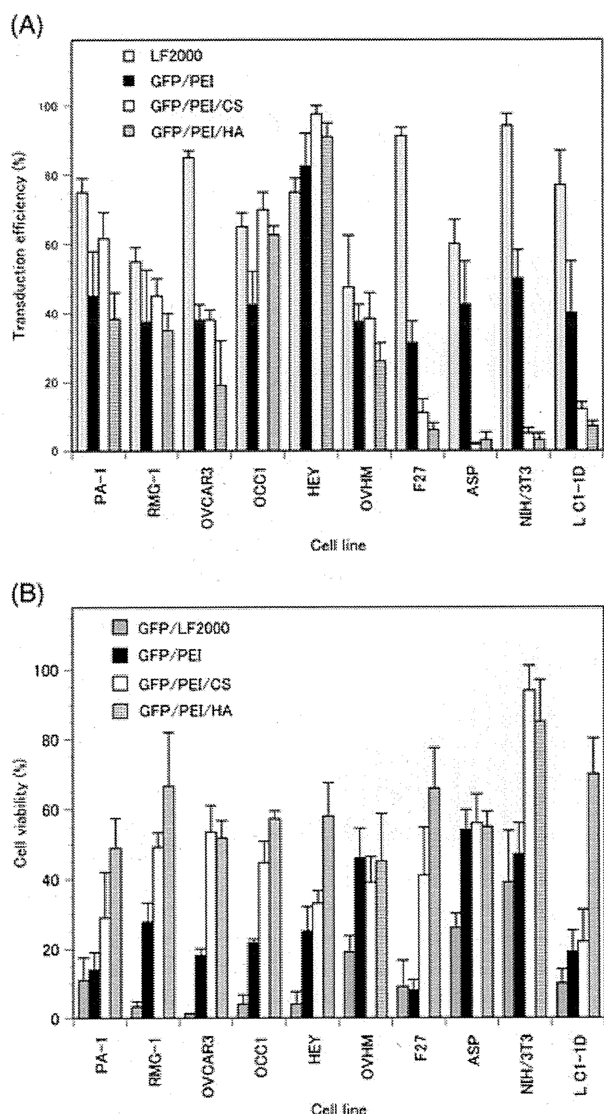

Figure 3. (A) Transfection efficiency of ternary GFP/PEI complex coated with HA or 10-kDa CS after 2 days of incubation. Percentage (%) of GFP-positive cells counted with a fluorescence microscope was considered transfection efficiency. (B) Viability of each cell line was determined by cell count assay after 2 days of incubation of LF2000-GFP, binary GFP/PEI complex, and ternary GFP/PEI complex coated with HA or 10-kDa CS.

Antitumor effect of small mGM-CSF/PEI/CS ternary complex on intraperitoneal tumor-bearing mice

Small DNA/PEI/HA or CS complexes were prepared with mGM-CSF-coding plasmid. It was freeze-dried, rehydrated and diluted in 7 ml of PBS for intraperitoneal and subcutaneous injection. Mice bearing intraperitoneal OVHM tumor were injected with the complex six times daily (each injection, 100 μg of the plasmid per mouse). All control mice injected with medium or binary mGM-CSG/PEI complex died within 31 and 28 days, respectively. Ternary mGM-CSF/PEI/HA complex significantly prolonged the survival of mice compared to mice injected with medium control or binary complex, although all mice died within 71 days. Furthermore, prolongation of survival was more pronounced with ternary mGM-CSF/PEI/CS complex than with mGM-CSF/PEI/HA, although all mice died within 130 days (Figure 5B).

Discussion

The numbers of deaths as a result of ovarian cancer in Japan and the USA are, respectively, 4000 and 14000 per year [14]. Of gynecologic cancers, ovarian cancer is the most important and has the poorest prognosis. Although the rate of recurrence of FIGO III stage ovarian cancer remains 60–70%, ovarian cancer in this stage of intraperitoneal carcinomatosis is curable because metastasis is local and within the abdominal cavity and not distant. Ovarian cancer readily metastasizes to the abdominal cavity because the ovaries abut the abdominal cavity. The present study demonstrated that ternary mGM-CSF/PEI/CS complex treatment significantly prolonged the survival of mice with intraperitoneal carcinomatosis treated with it despite a lack of complete remission, because 100 μg/500 μl of ternary complex is a concentration sufficient to treat 5–8 mm subcutaneous tumors but 100 μg/7 ml is not sufficient to treat tumors in the large intraperitoneal space. We set the upper limit of the dose at 600 μg/body considering safety with intraperitoneal administration because a greater than 1000 μg/body intraperitoneal injection of ternary complex induced 20–30% of mouse death, whereas less than 1000 μg/body that did not. It will be necessary to prolong the half-life of ternary DNA complex in the abdominal cavity because it readily migrates from the peritoneum to the systemic circulation and excess intraperitoneal doses of ternary DNA complex induce fatal side-effects as a result of PEI toxicity. A large amount of liquid solution, 5 ml of 7 ml, was used to treat intraperitoneal tumors because the 500 μl volume of liquid solution used for subcutaneous tumors cannot reach all of the intraperitoneal space. A 2 ml portion of 7 ml of ternary complex solution was injected into the OVHM cell-injected skin in the present study. This subcutaneous injection completely prevented the local recurrence of OVHM tumors in the injected skin because more than 80% of mice exhibited recurrence in the injected skin without any subcutaneous injection of ternary complex solution. It appears likely that the treatment with this ternary GM-CSF/PEI/CS complex only cannot achieve cure but may furthermore improve survival in cases of intraperitoneal ovarian carcinomatosis, together with anticancer agents such as taxols, cisplatin, and others.

GM-CSF, which has broad biological activity, was one of the first cytokines to be identified [15]. GM-CSF regulates the viability, proliferation, differentiation and function of hematopoietic progenitor cells at the same time as increasing the viability and function of dendritic cells, the differentiation and growth of dermal Langerhans cells, and the ability of antigen-presenting cells to capture

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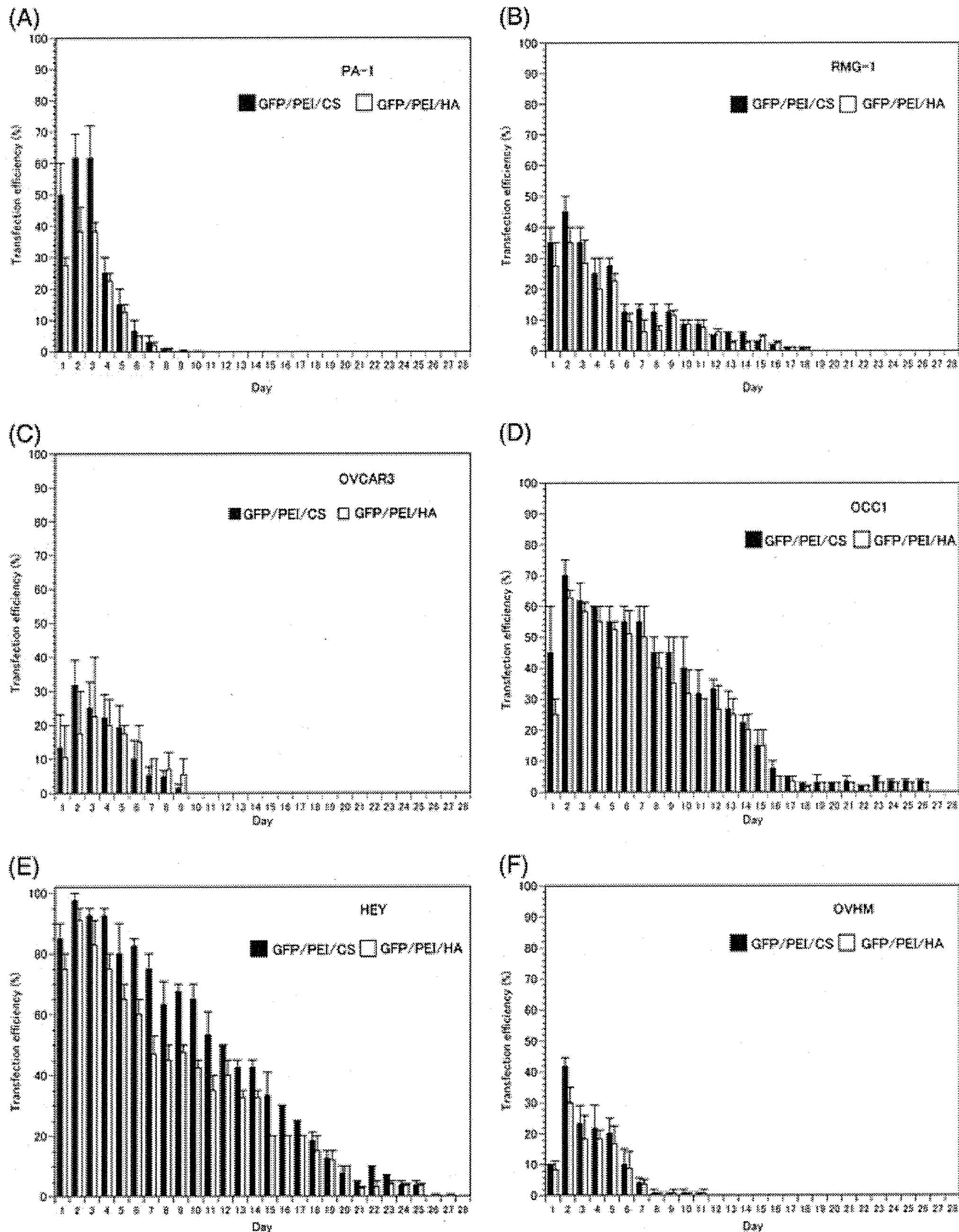

Figure 4. Time course of transfection efficiency of HA or 10-kDa CS in human ovarian carcinoma PA-1 (A), RMG-1 (B), OVCAR3 (C), OCC1 (D), HEY (E) and murine ovarian carcinoma OVHM (F) cells. Percentage (%) of GFP-positive cells was determined with a fluorescence microscope as the transfection efficiency.

foreign antigens [16]. GM-CSF alone or in combination with other cytokines has been reported to enhance immunity in cancer treatment [17,18]. Despite these pharmacological properties, GM-CSF has a very short biological half-life and therefore requires frequent injection throughout treatment, which results in problematic side-effects. In addition, the very limited serum stability of cytokines and easy destruction and inactivation of body proteins indicate the need for the investigation of new routes of administration of GM-CSF. We therefore investigated the efficacy of systems providing long durations of action in treatment with plasmid DNA coding GM-CSF.

Binary GM-CSF/chitosan and GM-CSF/PEI complexes were previously reported to induce immune responses to *Staphylococcus aureus* infection in dairy cattle [19] and to enhance antigen presentation in dendritic cell-based vaccination [17], respectively. Although ternary mGM-CSF/PEI/HA complex has been reported to inhibit the growth of mouse melanoma cell tumors [11], this has not yet been reported for ternary mGM-CSF/PEI/CS. We used a gene delivery system prepared with PEI in the

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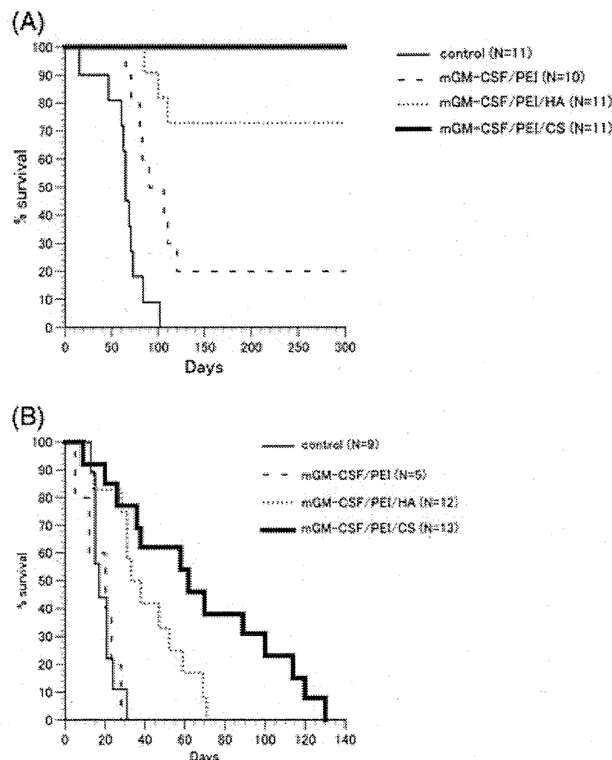

Figure 5. (A) Kaplan–Meier survival curve of antitumor effects of mGM-CSF/PEI/HA or 10-kDa CS ternary complex in mice bearing subcutaneous OVHM tumors. The complex (100 μg/500 μl) was intratumorally administered into 5–8 mm diameter subcutaneous tumors over 3 consecutive days after subcutaneous inoculation of OVHM cells (1×10^6 cells). (B) Kaplan–Meier survival curve of antitumor effect of mGM-CSF/PEI/HA or 10-kDa CS ternary complex in mice bearing intraperitoneal OVHM tumors. The complex (100 μg/7 ml) was intraperitoneally and subcutaneously administered six times daily after the intraperitoneal inoculation of OVHM cells (1×10^6 cells).

present study because that with chitosan is lower in transfection efficiency [19,20]. Ternary complexes coated with seamless, bovine or shark ≥ 14-kDa CSs were reported previously [21–23]. We have reported here for the first time use of a 10-kDa fraction of CS as an anionic component of ternary complex, and have shown that ternary complex coated with this fraction exhibited higher luciferase activity than when fractions of CS ≥ 14-kDa or high-molecular-weight HA were used. Furthermore, the 10-kDa fraction CS-coated mGM-CSF complex inhibited the growth of intraperitoneal and subcutaneous tumor in a syngeneic mouse model to a greater extent than did ternary mGM-CSF/PEI/HA complex. In the present study, the CS polymer was higher in gene expression efficiency than the HA polymer, and the size of the CS polymer was approximately two-thirds of that of the HA polymer. Regarding the size after the polymer processing being small, it is suggested to be advantageous for uptake in the cell after attachment to the CD44 receptor on the cell membrane. Furthermore, as we reported previously [24,25], addition of polyanions, such as HA and polyethylene glycol derivatives, to the DNA/polycation complex caused the loosening of the complex particles and improved transcription efficiency by the effect of the charge balance in the polyampholyte. The addition of CS would thus also loosen the complex. The loosening effect of CS would be stronger than that with HA because CS has many strong acid (sulfuric acid) pendants along the chain.

Three intratumoral injections of 100 μg of ternary mGM-CSF/PEI/CS complex induced complete tumor reduction in all subcutaneous tumors. There are many types of tumors that can be directly injected in intratumoral fashion, such as head and neck cancers, thyroid cancer, esophageal cancer, breast cancer and skin cancer, as well as gynecological cancers, such as vaginal cancer, vulvar cancer and cervical cancer. Deep organ cancers such as lung cancer, liver cancer, brain tumor and others and digestive organ cancers such as gastric cancers, colon cancers, and others are, respectively, computed tomography- or echo-guided and endoscopically-injectable tumors. These tumors can therefore be radically treated only with three intratumoral injections of ternary GM-CSF/PEI/CS complex. This local treatment procedure appears promising because radical surgery for these tumors often features severe postoperative complications, such as behavioural disorders, dysphagia, eating disorders, weight loss, incontinence and cosmetic problems, which often decrease quality of life and make full social rehabilitation impossible. Our method of ternary complex gene therapy by intratumoral injection does not require hospitalization

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