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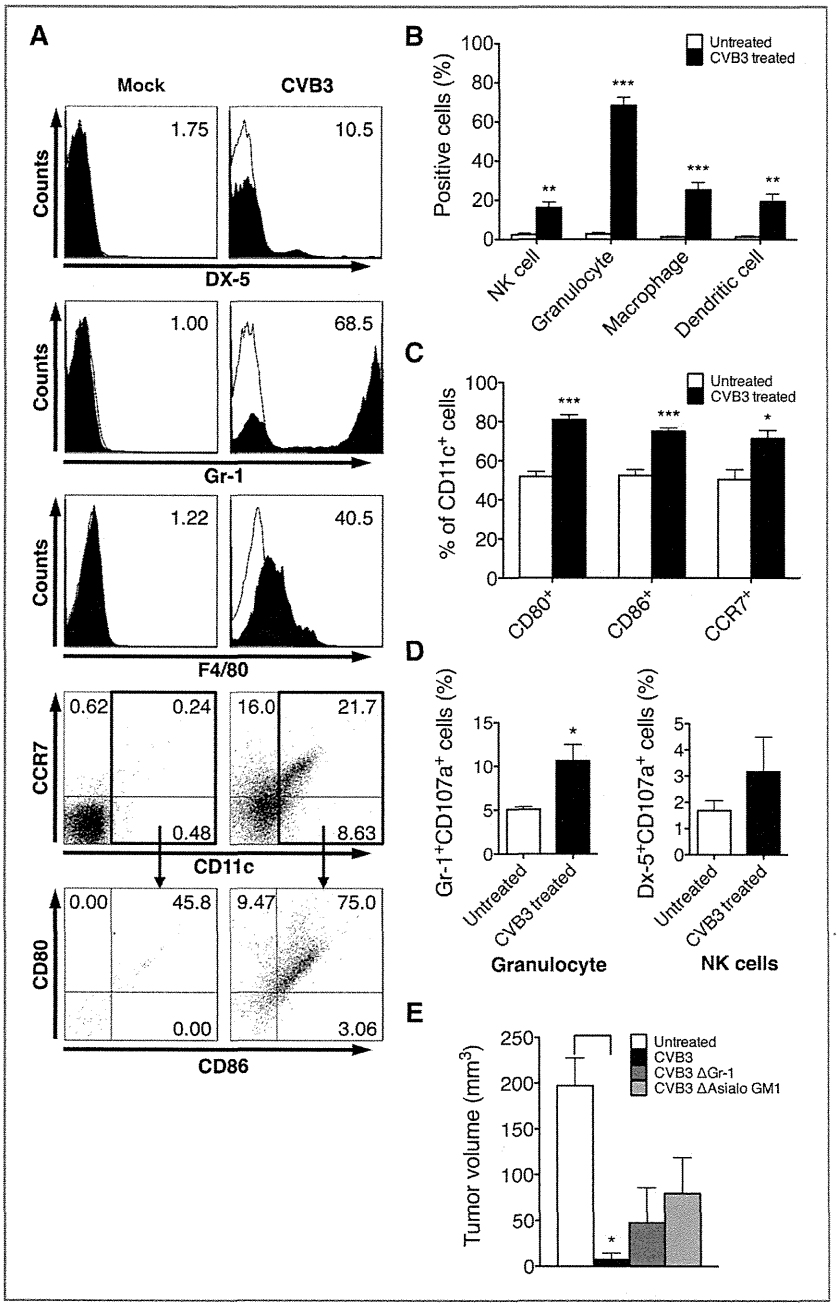

Figure 7. Immunostimulatory effects of intratumoral CVB3 administration on tumor-infiltrating lymphocytes (TIL). TILs obtained on day 2 after intratumoral CVB3 administration were subjected to flow cytometry analysis. A, numbers presented in the histogram reflect the percentage of DX-5$^+$, Gr-1$^+$, F4/80$^+$, and CD11c$^+$ cells relative to the total cell number of TILs (top 3 rows). The numbers presented in 2-dimensional dot plots reflect the percentage of different mature DC subsets relative to the total number of DCs (bottom 2 rows). B, percentages of innate immune subpopulations of NK cells, granulocytes, macrophages, and DCs relative to the total cell count of TILs are shown ($n = 5$). **, $P < 0.01$; ***, $P < 0.001$. C, percentages of 3 different subtypes of mature DCs relative to the total count of DCs are shown. Bar graphs depict the means ± SEM ($n = 5$). *, $P < 0.05$; ***, $P < 0.001$. D, percentages of granulocytes or NK cells expressing CD107a in enriched TILs relative to total TILs are shown. *, $P < 0.05$. E, nude mice ($n = 4$) bearing A549 cells were injected intraperitoneally with anti–Gr-1 antibody on days 1, 4, 7, 10, 13, 16, and 19, or anti-asialo GM1 antibody on days 1, 7, 13, and 19 posttumor challenge. CVB3 virus was injected into the right lateral tumor every other day for a total of 5 doses (5×10^6 $TCID_{50}$/dose). The tumor volumes measured on day 18 are shown ($n = 4$). *, $P < 0.05$.

(Supplementary Fig. S4B). There have been no reports of human hepatitis caused by CVB3 infection. Elevated creatinine kinase was found in the sera of CVB3-treated mice, with histologic evidence of mild myocarditis, but no cytopathic damage in the lungs or kidneys (Supplementary Fig. S4B). Although human CAR mRNA is expressed in the heart (44), CVB3 possibly causes severe myocarditis in infants only (45), suggesting its relatively mild side effects in use for adult patients.

As oncolytic virotherapy is expected to promote inflammatory DAMP within the tumor, which is beneficial for effective antitumor immune responses, it is important to assess

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whether oncolytic viruses generate immunogenic cell death (18). CVB3 infection induced these immunogenic changes such as CRT exposure, release of extracellular ATP, and HMGB1 translocation in NSCLC cells (Fig. 6). Upon propagation of oncolytic virus, alterations in the repertoire of immune cells in tumor microenvironment can restore inherent antitumor immunity (46), through inductions of interferons and/or cytokines that activate NK cells and mature DCs (47, 48). Tumor-infiltrating DCs have been shown to be impaired at maturation (49). Media from malignant cells infected with reovirus promoted maturation of DCs (46). Likewise CVB3 may alter the immunologic microenvironment by accumulating diverse mature DCs into tumors (Fig. 7C), probably because the single-stranded RNA genome converts dysfunctional DCs into functional DCs after ligation of retinoic acid–inducible gene-1 (RIG-1)-like receptors (50). Indeed, CVB3 intervention was shown to shape NK cell polarization with antitumor effects, possibly through promoted recruitment of cytolytic $CD107a^+$ NK cells into xenografts (Fig. 7D and E). Contribution of neutrophils to the antitumor effects may be partially due to *IFN-β* production following CVB3 administration, as *IFN-β* can instruct neutrophils to possess an antitumor phenotype (51). These immunostimulatory properties of CVB3 on innate immunity may subsequently prime effective generation of adaptive immunity synergizing with direct oncolytic activities. After showing that TC-1 syngeneic mouse NSCLC cells were susceptible to *in vitro* CVB3 infection (Supplementary Fig. S5A), to further evaluate the effects of T cell–mediated cellular immunity on the oncolytic effects of CVB3, we treated immunocompetent mice with TC-1 tumors with a single CVB3 intratumoral administration, which significantly inhibited TC-1 tumor development in a dose-dependent manner ($P < 0.05$; Supplementary Fig. S5B), with significantly prolonged survival (Supplementary Fig. S5C). These findings indicate that T cell–mediated antiviral immunity is not a significant barrier for intratumoral replication of CVB3.

Notably, in all experiments, no mice manifested lethality during CVB3 treatments, highlighting acceptable safety characteristics as an oncolytic agent.

In summary, our large-scale 2-step screening identified CVB3 as a tumor-specific virus, which depends on apoptotic and survival signaling pathways. Furthermore, systemic and immunostimulatory antitumor effects of CVB3 provide an encouraging avenue for future preclinical and clinical development as a promising viral agent for the treatment of NSCLC patients.

Disclosure of Potential Conflicts of Interest

K. Tani: minor stock, Oncolys BioPhama Inc. No potential conflicts were disclosed by the other authors.

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References

1. Russell SJ, Peng KW. Viruses as anticancer drugs. Trends Pharmacol Sci 2007;28:326–33.
2. Aghi M, Martuza RL. Oncolytic viral therapies—the clinical experience. Oncogene 2005;24:7802–16.
3. Park BH, Hwang T, Liu TC, Sze DY, Kim JS, Kwon HC, et al. Use of a targeted oncolytic poxvirus, JX-594, in patients with refractory primary or metastatic liver cancer: a phase I trial. Lancet Oncol 2008;9:533–42.
4. Kumar S, Gao L, Yeagy B, Reid T. Virus combinations and chemotherapy for the treatment of human cancers. Curr Opin Mol Ther 2008;10:371–9.
5. Morton CL, Houghton PJ, Kolb EA, Gorlick R, Reynolds CP, Kang MH, et al. Initial testing of the replication competent Seneca Valley virus (NTX-010) by the pediatric preclinical testing program. Pediatr Blood Cancer 2010;55:295–303.
6. Shafren DR, Sylvester D, Johansson ES, Campbell IG, Barry RD. Oncolysis of human ovarian cancers by echovirus type 1. Int J Cancer 2005;115:320–8.
7. Au GG, Beagley LG, Haley ES, Barry RD, Shafren DR. Oncolysis of malignant human melanoma tumors by Coxsackieviruses A13, A15 and A18. Virol J 2011;8:22.
8. Shafren DR, Au GG, Nguyen T, Newcombe NG, Haley ES, Beagley L, et al. Systemic therapy of malignant human melanoma tumors by a common cold-producing enterovirus, coxsackievirus a21. Clin Cancer Res 2004;10:53–60.
9. Skelding KA, Barry RD, Shafren DR. Enhanced oncolysis mediated by Coxsackievirus A21 in combination with doxorubicin hydrochloride. Invest New Drugs 2012;30:568–81.
10. Feuer R, Mena I, Pagarigan R, Slifka MK, Whitton JL. Cell cycle status affects coxsackievirus replication, persistence, and reactivation *in vitro*. J Virol 2002;76:4430–40.
11. Evans DJ. Reverse genetics of picornaviruses. Adv Virus Res 1999;53:209–28.
12. Michos AG, Syriopoulou VP, Hadjichristodoulou C, Daikos GL, Lagona E, Douridas P, et al. Aseptic meningitis in children: analysis of 506 cases. PLoS One 2007;2:e674.
13. Skelding KA, Barry RD, Shafren DR. Systemic targeting of metastatic human breast tumor xenografts by Coxsackievirus A21. Breast Cancer Res Treat 2009;113:21–30.
14. Kelly EJ, Hadac EM, Greiner S, Russell SJ. Engineering microRNA responsiveness to decrease virus pathogenicity. Nat Med 2008;14:1278–83.
15. Diaz RM, Galivo F, Kottke T, Wongthida P, Qiao J, Thompson J, et al. Oncolytic immunovirotherapy for melanoma using vesicular stomatitis virus. Cancer Res 2007;67:2840–8.
16. Matzinger P. Tolerance, danger, and the extended family. Annu Rev Immunol 1994;12:991–1045.
17. Prestwich RJ, Errington F, Ilett EJ, Morgan RS, Scott KJ, Kottke T, et al. Tumor infection by oncolytic reovirus primes adaptive antitumor immunity. Clin Cancer Res 2008;14:7358–66.
18. Zitvogel L, Kepp O, Senovilla L, Menger L, Chaput N, Kroemer G. Immunogenic tumor cell death for optimal anticancer therapy: the calreticulin exposure pathway. Clin Cancer Res 2010;16:3100–4.
19. Kuninaka S, Yano T, Yokoyama H, Fukuyama Y, Terazaki Y, Uehara T, et al. Direct influences of pro-inflammatory cytokines (IL-1beta, TNF-

Published OnlineFirst March 29, 2012; DOI: 10.1158/0008-5472.CAN-11-3185

alpha, IL-6) on the proliferation and cell-surface antigen expression of cancer cells. Cytokine 2000;12:8–11.
20. Karber G. 50% end-point calculation. Arch Exp Pathol Pharmak 1931;162:480–3.
21. Meng X, Nakamura T, Okazaki T, Inoue H, Takahashi A, Miyamoto S, et al. Enhanced antitumor effects of an engineered measles virus Edmonston strain expressing the wild-type N, P, L genes on human renal cell carcinoma. Mol Ther 2010;18:544–51.
22. Tesniere A, Schlemmer F, Boige V, Kepp O, Martins I, Ghiringhelli F, et al. Immunogenic death of colon cancer cells treated with oxaliplatin. Oncogene 2010;29:482–91.
23. Inoue H, Iga M, Xin M, Asahi S, Nakamura T, Kurita R, et al. TARC and RANTES enhance antitumor immunity induced by the GM-CSF-transduced tumor vaccine in a mouse tumor model. Cancer Immunol Immunother 2008;57:1399–411.
24. Inoue H, Iga M, Nabeta H, Yokoo T, Suehiro Y, Okano S, et al. Non-transmissible Sendai virus encoding granulocyte macrophage colony-stimulating factor is a novel and potent vector system for producing autologous tumor vaccines. Cancer Sci 2008;99:2315–26.
25. Shafren DR, Williams DT, Barry RD. A decay-accelerating factor-binding strain of coxsackievirus B3 requires the coxsackievirus-adenovirus receptor protein to mediate lytic infection of rhabdomyosarcoma cells. J Virol 1997;71:9844–8.
26. Esfandiarei M, Luo H, Yanagawa B, Suarez A, Dabiri D, Zhang J, et al. Protein kinase B/Akt regulates coxsackievirus B3 replication through a mechanism which is not caspase dependent. J Virol 2004;78:4289–98.
27. Alter G, Malenfant JM, Altfeld M. CD107a as a functional marker for the identification of natural killer cell activity. J Immunol Methods 2004;294:15–22.
28. Grote D, Cattaneo R, Fielding AK. Neutrophils contribute to the measles virus-induced antitumor effect: enhancement by granulocyte macrophage colony-stimulating factor expression. Cancer Res 2003; 63:6463–8.
29. Jemal A, Bray F, Center MM, Ferlay J, Ward E, Forman D. Global cancer statistics. CA Cancer J Clin 2011;61:69–90.
30. Fidias P, Novello S. Strategies for prolonged therapy in patients with advanced non-small-cell lung cancer. J Clin Oncol 2010;28:5116–23.
31. Wang Y, Wang S, Bao Y, Ni C, Guan N, Zhao J, et al. Coxsackievirus and adenovirus receptor expression in non-malignant lung tissues and clinical lung cancers. J Mol Histol 2006;37:153–60.
32. Li L, Spendlove I, Morgan J, Durrant LG. CD55 is over-expressed in the tumour environment. Br J Cancer 2001;84:80–6.
33. Dean M, Fojo T, Bates S. Tumour stem cells and drug resistance. Nat Rev Cancer 2005;5:275–84.
34. Carthy CM, Yanagawa B, Luo H, Granville DJ, Yang D, Cheung P, et al. Bcl-2 and Bcl-xL overexpression inhibits cytochrome c release, activation of multiple caspases, and virus release following coxsackievirus B3 infection. Virology 2003;313:147–57.
35. Martin U, Jarasch N, Nestler M, Rassmann A, Munder T, Seitz S, et al. Antiviral effects of pan-caspase inhibitors on the replication of coxsackievirus B3. Apoptosis 2007;12:525–33.
36. Tang JM, He QY, Guo RX, Chang XJ. Phosphorylated Akt overexpression and loss of PTEN expression in non-small cell lung cancer confers poor prognosis. Lung Cancer 2006;51:181–91.
37. Balsara BR, Pei J, Mitsuuchi Y, Page R, Klein-Szanto A, Wang H, et al. Frequent activation of AKT in non-small cell lung carcinomas and preneoplastic bronchial lesions. Carcinogenesis 2004;25:2053–9.
38. Zhou J, Wulfkuhle J, Zhang H, Gu P, Yang Y, Deng J, et al. Activation of the PTEN/mTOR/STAT3 pathway in breast cancer stem-like cells is required for viability and maintenance. Proc Natl Acad Sci U S A 2007;104:16158–63.
39. Lee HE, Kim JH, Kim YJ, Choi SY, Kim SW, Kang E, et al. An increase in cancer stem cell population after primary systemic therapy is a poor prognostic factor in breast cancer. Br J Cancer 2011;104:1730–8.
40. Vicent S, Garayoa M, Lopez-Picazo JM, Lozano MD, Toledo G, Thunnissen FB, et al. Mitogen-activated protein kinase phosphatase-1 is overexpressed in non-small cell lung cancer and is an independent predictor of outcome in patients. Clin Cancer Res 2004;10:3639–49.
41. Vicent S, Lopez-Picazo JM, Toledo G, Lozano MD, Torre W, Garcia-Corchon C, et al. ERK1/2 is activated in non-small-cell lung cancer and associated with advanced tumours. Br J Cancer 2004;90: 1047–52.
42. Guo WF, Lin RX, Huang J, Zhou Z, Yang J, Guo GZ, et al. Identification of differentially expressed genes contributing to radioresistance in lung cancer cells using microarray analysis. Radiat Res 2005;164:27–35.
43. Janmaat ML, Rodriguez JA, Gallegos-Ruiz M, Kruyt FA, Giaccone G. Enhanced cytotoxicity induced by gefitinib and specific inhibitors of the Ras or phosphatidyl inositol-3 kinase pathways in non-small cell lung cancer cells. Int J Cancer 2006;118:209–14.
44. Tomko RP, Xu R, Philipson L. HCAR and MCAR: the human and mouse cellular receptors for subgroup C adenoviruses and group B coxsackieviruses. Proc Natl Acad Sci U S A 1997;94:3352–6.
45. Dan M, Chantler JK. A genetically engineered attenuated coxsackievirus B3 strain protects mice against lethal infection. J Virol 2005;79: 9285–95.
46. Errington F, Steele L, Prestwich R, Harrington KJ, Pandha HS, Vidal L, et al. Reovirus activates human dendritic cells to promote innate antitumor immunity. J Immunol 2008;180:6018–26.
47. Zhang Y, Chirmule N, Gao GP, Qian R, Croyle M, Joshi B, et al. Acute cytokine response to systemic adenoviral vectors in mice is mediated by dendritic cells and macrophages. Mol Ther 2001;3: 697–707.
48. Benencia F, Courreges MC, Conejo-Garcia JR, Mohamed-Hadley A, Zhang L, Buckanovich RJ, et al. HSV oncolytic therapy upregulates interferon-inducible chemokines and recruits immune effector cells in ovarian cancer. Mol Ther 2005;12:789–802.
49. Vicari AP, Chiodoni C, Vaure C, Ait-Yahia S, Dercamp C, Matsos F, et al. Reversal of tumor-induced dendritic cell paralysis by CpG immunostimulatory oligonucleotide and anti-interleukin 10 receptor antibody. J Exp Med 2002;196:541–9.
50. Pichlmair A, Reis e Sousa C. Innate recognition of viruses. Immunity 2007;27:370–83.
51. Jablonska J, Leschner S, Westphal K, Lienenklaus S, Weiss S. Neutrophils responsive to endogenous IFN-beta regulate tumor angiogenesis and growth in a mouse tumor model. J Clin Invest 2010;120: 1151–64.

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Coxsackievirus B3 Is an Oncolytic Virus with Immunostimulatory Properties That Is Active against Lung Adenocarcinoma

Shohei Miyamoto, Hiroyuki Inoue, Takafumi Nakamura, et al.

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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 × 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

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

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

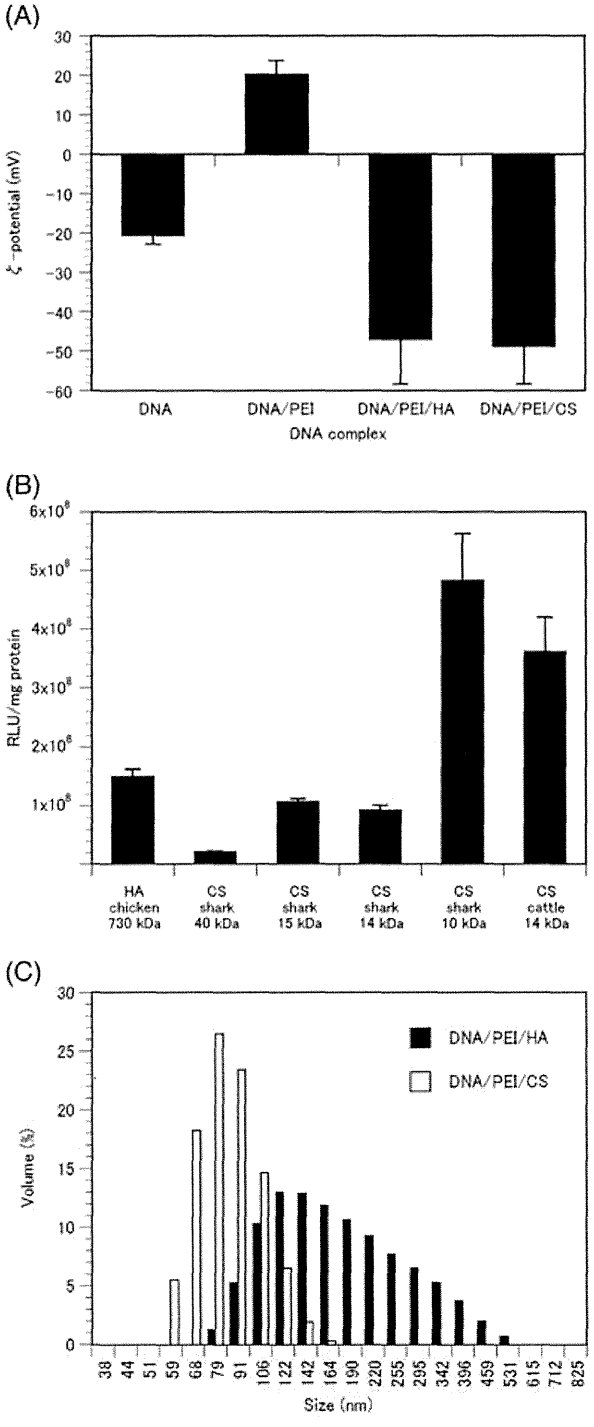

Figure 1. (A) ζ-potentials (mV) of DNA, binary DNA/PEI and ternary DNA/PEI/HA and CS complexes. (B) Luciferase activity of luciferase/PEI coated with HA from chicken and each molecular weight fraction of CS from shark or cow in cultured OVHM cells. RLU, relative light units. (C) The size of ternary DNA/PEI/HA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

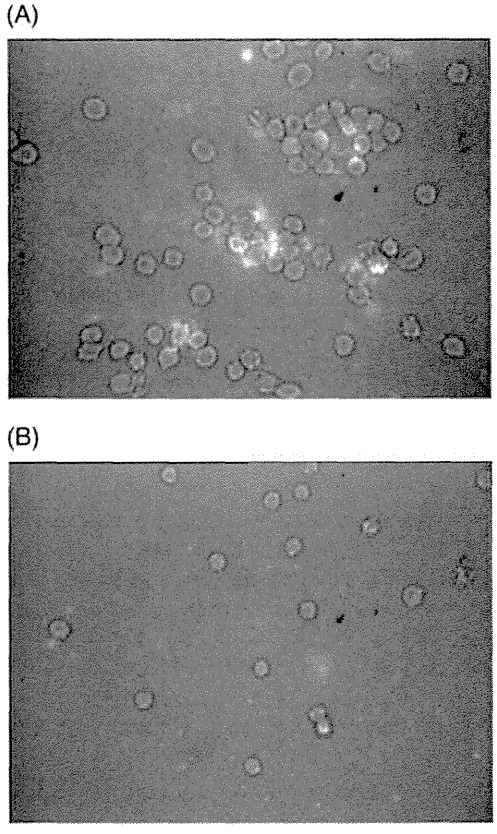

Figure 2. Binary GFP/PEI complex (A) and ternary GFP/PEI/CS complex (B) was incubated with red blood cells. Magnification: × 60.

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 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.

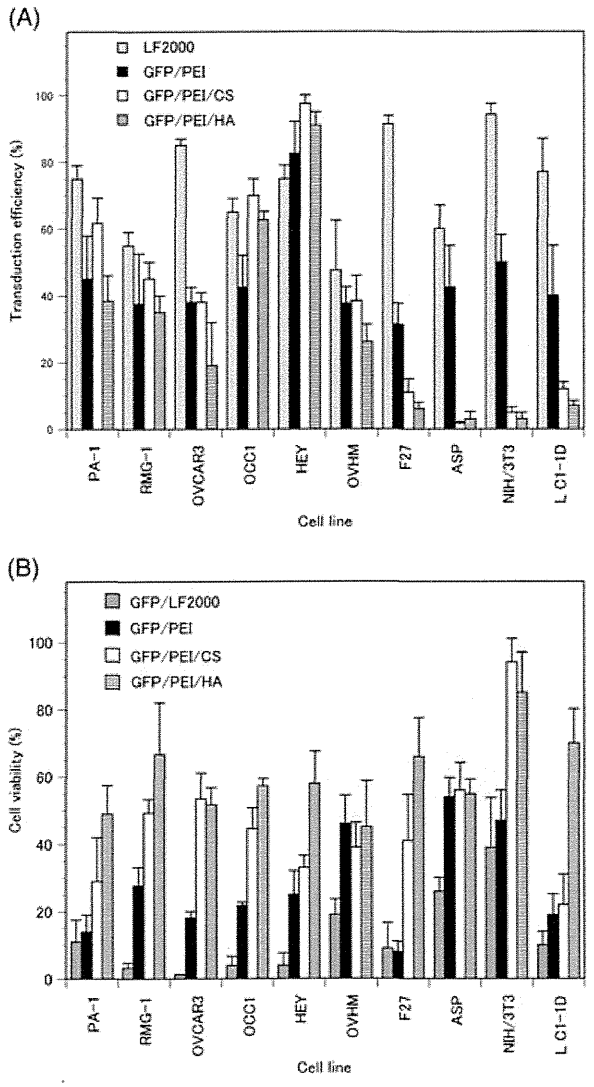

Figure 3. (A) Transfection efficiency of ternary GFP/PEI complex coated with HA or 10-kDa CS after 2 days of incubation. Percentage (%) of GFP-positive cells counted with a fluorescence microscope was considered transfection efficiency. (B) Viability of each cell line was determined by cell count assay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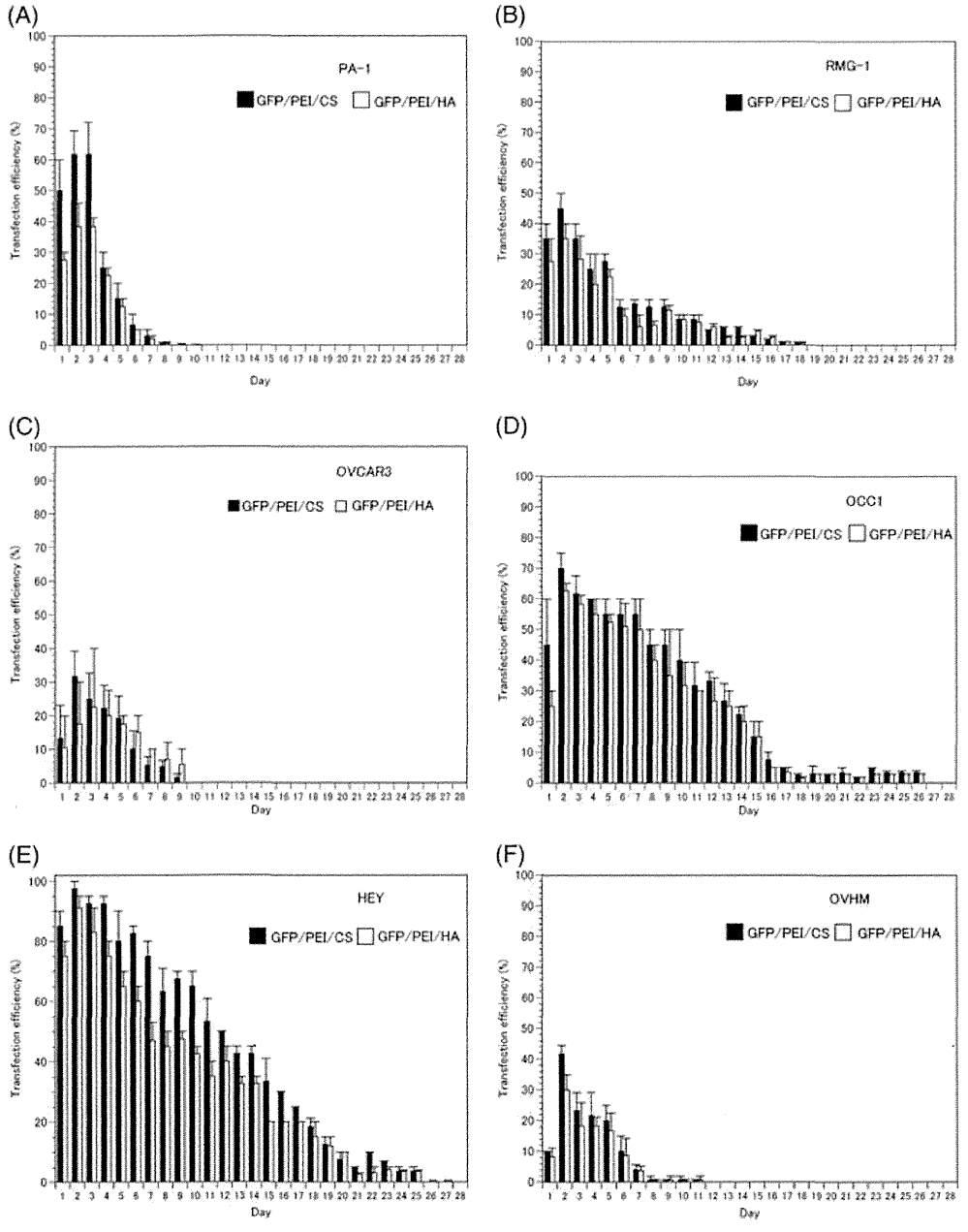

Figure 4. Time course of transfection efficiency of HA or 10-kDa CS in human ovarian carcinoma PA-1 (A), RMG-1 (B), OVCAR3 (C), OCC1 (D), HEY (E) and murine ovarian carcinoma OVHM (F) cells. Percentage (%) of GFP-positive cells was determined with a fluorescence microscope as the transfection efficiency.

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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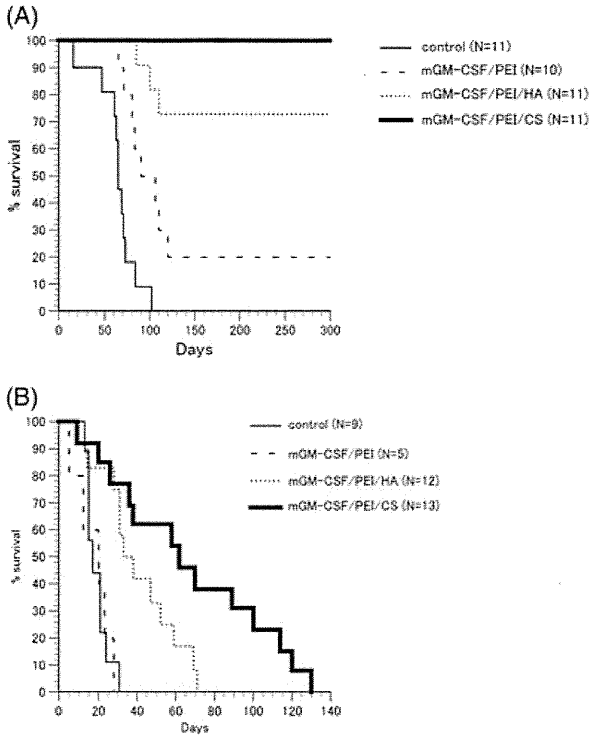

Figure 5. (A) Kaplan–Meier survival curve of antitumor effects of mGM-CSF/PEI/HA or 10-kDa CS ternary complex in mice bearing subcutaneous OVHM tumors. The complex (100 μg/500 μl) was intratumorally administered into 5–8 mm diameter subcutaneous tumors over 3 consecutive days after subcutaneous inoculation 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

and could radically cure any cancer subject to monitoring visually or by echographic, endoscopic, computed tomography or other types of monitoring in the outpatient clinic. This gene therapy could reduce the physical and financial burden on patients, as well as national medical expenses. Local intratumoral injection by GM-CSF/PEI/CS ternary complex thus has the potential to replace surgery for locally injectable tumors in the near future. Furthermore, it appears very likely that it will become an important method of treatment in place of anticancer drugs with the further development of systemic treatment of cancers.

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References

1. Hamada K, Desaki J, Nakagawa K, *et al.* Carrier cell-mediated infection of a replication-competent adenovirus for cancer gene therapy. *Mol Ther* 2007; **15**: 1121–1128.
2. Plank C, Mechtler K, Szoka FC Jr, Wagner E. Activation of the complement system by synthetic DNA complexes: a potential barrier for intravenous gene delivery. *Hum Gene Ther* 1996; **12**: 1437–1446.
3. Maruyama K, Iwasaki F, Takizawa T, *et al.* Novel receptor-mediated gene delivery system comprising plasmid/protamine/sugar-containing polyanion ternary complex. *Biomaterials* 2004; **16**: 3267–3273.
4. Koyama Y, Yamada E, Ito T, Mizutani Y, Yamaoka T. Plasmid/polycation complexes for receptor-mediated gene delivery. *Macromol Biosci* 2002; **2**: 251–256.
5. Finsinger D, Remy JS, Erbacher P, Koch C, Plank C. Protective copolymers for nonviral gene vectors: synthesis, vector characterization and application in gene delivery. *Gene Ther* 2000; **7**: 1183–1192.
6. Ito T, Iida-Tanaka N, Niidome T, *et al.* Hyaluronic acid and its derivative as a multi-functional gene expression enhancer: protection from non-specific interactions, adhesion to targeted cells, and transcriptional activation. *J Control Release* 2006; **112**: 382–388.
7. Koyama Y, Yamashita M, Iida-Tanaka N, Ito T. Enhancement of transcriptional activity of DNA complexes by amphoteric PEG derivative. *Biomacromolecules* 2006; **7**: 1274–1279.
8. Sakae M, Ito T, Yoshihara C, *et al.* Highly efficient in vivo gene transfection by plasmid/PEI complexes coated by anionic PEG derivatives bearing carboxyl groups and RGD peptide. *Biomed Pharmacother* 2008; **62**: 448–453.
9. Ito T, Iida-Tanaka N, Koyama Y. Efficient in vivo gene transfection by stable DNA/PEI complexes coated by hyaluronic acid. *J Drug Target* 2008; **16**: 276–281.
10. Trubetskoy VS, Loomis A, Slattum PM, Hagstrom JE, Budker VG, Wolff JA. Caged DNA does not aggregate in high ionic strength solutions. *Bioconjug Chem* 1999; **10**: 624–628.
11. Ito T, Yoshihara C, Hamada K, Koyama Y. DNA/polyethyleneimine/hyaluronic acid small complex particles and tumor suppression in mice. *Biomaterials* 2010; **31**: 2912–2918.
12. Sugahara KN, Hirata T, Tanaka T, *et al.* Chondroitin sulfate E fragments enhance CD44 cleavage and CD44-dependent motility in tumor cells. *Cancer Res* 2008; **68**: 7191–7199.
13. Henke CA, Roongta U, Mickelson DJ, Knutson JR, McCarthy JB. CD44-related chondroitin sulfate proteoglycan, a cell surface receptor implicated with tumor cell invasion, mediates endothelial cell migration on fibrinogen and invasion into a fibrin matrix. *J Clin Invest* 1996; **97**: 2541–2452.
14. Hirabayashi Y, Marugame T. Comparison of time trends in ovary cancer mortality (1990–2006) in the world, from the WHO mortality database. *Jpn J Clin Oncol* 2009; **39**: 860–861.
15. Grzybowski J, Oldak E, Antos-Bielska M, Janiak MK, Pojda Z. New cytokine dressing kinetics of the in vitro rhG-CSF, rhGM-CSF, and rhEGF release from the dressings. *Int J Pharm* 1999; **184**: 173–178.
16. Hamilton JA. Colony-stimulating factors in inflammation and autoimmunity. *Nat Rev Imunol* 2008; **8**: 533–544.
17. Bradbury PA, Shepherd FA. Immunotherapy for lung cancer. *J Thorac Oncol* 2008; **3**: 164–170.
18. Holt GE, Disis ML. Immune modulation as a therapeutic strategy for non-small-cell lung cancer. *Clin Lung Cancer* 2008; **9**: 13–19.
19. Nour El-Din AN, Shkareta L, Talbot BG, Diarra MS, Lacasse P. DNA immunization of dairy cows with the clumping factor A of *Staphylococcus aureus*. *Vaccine* 2006; **24**: 1997–2006.
20. Sezer AD, Akbuga J. Comparison on in vitro characterization of fucospheres and chitosan microspheres encapsulated plasmid DNA (pGM-CSF): formulation design and release characteristics. *AAPS PharmSciTeck* 2009; **10**: 1193–1199.
21. Guilherme MR, Reis AV, Alves BRV, Kunita MH, Rubira AF, Tambourgi EB. Smart hollow microspheres of chondroitin sulfate conjugates and magnetite nanoparticles for magnetic vector. *J Colloid Int Sci* 2010; **352**: 107–113.
22. Lim JJ, Hammoudi TM, Bratt-Leal AM, *et al.* Development of nano- and microscale chondroitin sulfate particles for controlled growth factor delivery. *Acta Biomat* 2011; **7**: 986–995.
23. Bhadra D, Bhadra S, Jain NK. PEGylated peptide dendrimeric carriers for the delivery of antimalarial drug chloroquine phosphate. *Pharm Res* 2006; **23**: 623–633.
24. Yoshihara C, Shew CY, Ito T, Koyama Y. Loosening of DNA/polycation complexes by synthetic polyampholyte to improve the transcription efficiency: effect of charge balance in the polyampholyte. *Biophys J* 2010; **98**: 1257–1266.
25. Ito T, Iida-Tanaka N, Niidome T, *et al.* Hyaluronic acid and its derivative as a multi-functional gene expression enhancer: protection from non-specific interactions, adhesion to targeted cells, and transcriptional activation. *J Control Release* 2006; **112**: 382–388.