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H. 知的財産権の出願・登録状況

なし

研究成果の刊行に関する一覧表

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Mima, H., Yamamoto, S., Ito, M., Tomoshige, R., Tabata, Y., Tamai, K., and **Kaneda, Y.**	Targeted chemotherapy against intraperitoneally disseminated colon carcinoma using a cationized gelatin-conjugated HVJ envelope vector.	Mol. Cancer Ther.			in press
Mima, H., Tomoshige, R., Kanamori, T., Tabata, Y., Yamamoto, Ito, S., Tamai, K., and **Kaneda, Y.**	Biocompatible polymer enhances the in vitro and in vivo transfection efficiency of HVJ envelope vector	J. Gene Medicine	7	888-897	2005
Kim, YD, Park, K-G, Morishita, R., **Kaneda, Y.,** Kim, S-Y., Song, D-K., Kim, S-D., Nam1, C-W., HC Lee, HC., Lee, K-U., Park, J-Y., Kim, B-W., Kim, J-G. and Lee, I-K.	Liver-directed gene therapy of diabetic rats using an HVJ-E vector containing EBV plasmids expressing insulin and GLUT 2 transporter.	Gene Therapy			in press.
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Targeted chemotherapy against i.p. disseminated colon carcinoma using a cationized gelatin–conjugated HVJ envelope vector

Hidetoshi Mima,[1,3] Seiji Yamamoto,[1] Makoto Ito,[1] Ryuji Tomoshige,[2] Yasuhiko Tabata,[2] Katsuto Tamai,[1] and Yasufumi Kaneda[1]

[1]Division of Gene Therapy Science, Graduate School of Medicine, Osaka University, Osaka, Japan; [2]Department of Biomaterials, Fields of Tissue Engineering Institute for Frontier Medical Sciences, Kyoto University, Kyoto, Japan; and [3]Department of Digestive and Cardiovascular Medicine, University of Tokushima, Graduate School, Tokushima, Japan

Abstract

The hemagglutinating virus of Japan envelope (HVJ-E; Sendai virus) vector derived from inactivated HVJ particles can be used to deliver DNA, proteins, and drugs into cells both *in vitro* and *in vivo*. HVJ-E is capable of delivering bleomycin, an anticancer drug, to various cancer cell lines, thereby producing 300-fold greater cytotoxicity than administration of bleomycin alone. In a mouse model of peritoneally disseminated colon cancer, we injected HVJ-E containing the *luciferase* gene into the peritoneum. Unexpectedly, *luciferase* gene expression was not observed within the tumor deposits or any organs. However, when combined with cationized gelatin (CG), CG-HVJ-E produced a high level of *luciferase* gene expression primarily within the tumor deposits. Forty-eight hours after introducing colon cancer cells into the peritoneum of experimental mice, CG-HVJ-E with or without bleomycin was injected into the abdominal cavity. Following six injections of bleomycin-incorporated CG-HVJ-E, complete responses were observed in 40% of the mice examined. All of the mice that received either empty CG-HVJ-E or bleomycin alone died within 40 days of having cancer cells introduced into the peritoneum. When the mice with complete responses were rechallenged with colon cancer cells from the same cell line, no tumors developed. Thus, CG-HVJ-E may suppress peritoneal dissemination of cancer. [Mol Cancer Ther 2006;5(4):1–8]

Received 9/2/05; revised 12/20/05; accepted 1/25/06.

Grant support: Ministry of Health, Labour, and Welfare of Japan.

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Requests for reprints: Yasufumi Kaneda, Division of Gene Therapy Science, Graduate School of Medicine, Osaka University, 2-2 Yamada-oka, Suita, Osaka 565-0871, Japan. E-mail: kaneday@gts.med.osaka-u.ac.jp

doi:10.1158/1535-7163.MCT-05-0352

Introduction

Although improved surgical, chemotherapy, and radiotherapy methods have been developed to treat patients with cancer, it remains difficult to eradicate cancer completely. In particular, metastatic cancer involving multiple foci and microscopic cancers are hard to treat, and prevention of recurrence remains a difficult problem in cancer therapy (1). Antitumor immunotherapy holds great promise; however, a number of obstacles have been encountered in the course of its development (2, 3). Although immunotherapy can reduce, delay, and sometimes prevent tumor recurrence, a number of tumors progress after developing mechanisms to avoid recognition and elimination by the immune system (4, 5). A number of studies suggest that both tumor regression and induction of antitumor immunity are indispensable for complete tumor eradication. For this reason, immunotherapy is often combined with other therapies, such as surgical resection, radiotherapy, and chemotherapy (6, 7). Drug delivery systems have the potential to overcome the problem of escape from immune recognition and to increase the efficiency of killing of unresectable tumors (8).

One important issue in drug delivery vectors is how to cross the cell membrane to introduce therapeutic molecules (9). There are several ways to bypass the cell membrane. Liposome-mediated delivery results in drug uptake by endocytosis or phagocytosis. However, this requires rapid penetration of the endosome or phagosome membrane by the foreign molecules before degradation (10). Viruses can enter cells; thus, viral vectors are capable of penetrating cell membranes (11). Adenovirus can escape from the endosome by disrupting the endosomal membrane with penton fibers (12). This capability has been used to enhance the efficiency of gene transfer using transferrin-polylysine-DNA complexes (13). Other viruses fuse with the cell membrane, thereby introducing their genomes into the cytoplasm. There are two different mechanisms of virus-cell fusion: pH dependent and pH independent. Influenza virus (14), Semliki Forest virus (15), and vesicular stomatitis virus (16) exhibit pH-dependent fusion, whereas hemagglutinating virus of Japan (HVJ, Sendai virus; ref. 17) and retrovirus (18) fuse with the cell membrane at both acidic and neutral pH. Viral fusion proteins have been identified, and synthetic vectors expressing viral fusion proteins can transfer foreign genes efficiently into the cytoplasm (19). We attempted to use the fusion capabilities of Sendai virus for drug delivery. First, we developed HVJ-liposomes, in which drug-incorporated liposomes were fused with inactivated Sendai virus (20). HVJ-liposomes are capable

of delivering genes and synthetic oligodeoxynucleotides into cells in various animal models (21). To further simplify drug delivery using HVJ-liposomes, we tried to develop a nonviral delivery system. Finally, we developed an HVJ envelope (HVJ-E) vector (22, 23). Using this system, macromolecules, such as plasmid DNA, RNA, synthetic oligonucleotides, proteins, and peptides, get incorporated into inactivated HVJ particles by treatment with a mild detergent and centrifugation, after which they can be delivered to cells *in vitro* and *in vivo*. To enhance drug delivery, we combined HVJ-E with cationized gelatin (CG; ref. 24). CG-conjugated HVJ-E (CG-HVJ-E) was still capable of fusion. In CT-26 cells, CG-HVJ-E–mediated *luciferase* gene expression was ~10 to 20 times greater than that achieved using HVJ-E without conjugation to a polymer. Furthermore, the stability of HVJ-E in fresh mouse serum was greatly enhanced by conjugation with CG.

Herein, we show that CG-HVJ-E is a very effective vehicle for delivering bleomycin to tumor cells after peritoneal dissemination. Multiple injections of CG-HVJ-E/bleomycin produced complete responses in 40% of the mice examined, all of which were further resistant to tumor rechallenge.

Materials and Methods

HVJ

HVJ was amplified in chorioallantoic fluid from 10- to 14-day-old chick eggs, after which it was purified by centrifugation and inactivated by UV irradiation (99 mJ/cm^2), as previously described (22). Inactivated virus cannot replicate, but its capacity for viral fusion remains intact.

Cell Culture

Human cancer cells and mouse colon cancer CT-26 cells were maintained in DMEM supplemented with 10% fetal bovine serum and antibiotics.

Preparation of HVJ-E/bleomycin

Inactivated HVJ (1.8×10^{10} particles) was mixed with 60 μL of 5 or 40 mg/mL bleomycin and 2 μL of 3% Triton X-100, as previously described (22). After 15 minutes of incubation at 4°C, the suspension was washed with 500 μL of PBS (pH 7.4) and centrifuged (18,500 × *g*) for 15 minutes at 4°C. After this, the suspension was again washed twice with 500 μL of PBS to completely remove the detergent and any unincorporated bleomycin. After centrifugation, the HVJ-E/bleomycin was suspended in 180 μL of PBS.

Preparation of CG-HVJ-E/Bleomycin, Polymer-Conjugated HVJ-E, and CG/Bleomycin

Cationization of gelatin was done by introducing ethylene diamine into the carboxyl groups of low molecular weight gelatin (MW = 5,000), as previously described (24), and the mole-to-mole ratio of amino groups to carboxyl groups within the gelatin was 48.7. A 5-mg amount of CG was added to 300 μL of PBS containing 3×10^{10} particles of HVJ-E vector containing bleomycin. The solution was mixed by tapping several times. After this, the solution was incubated on ice for 30 minutes, during which it formed CG-conjugated HVJ-E vector containing bleomycin, which was purified by centrifugation. CG-conjugated (MW = 100,000), dextran-conjugated, and pullulan-conjugated HVJ-E vectors were prepared by mixing five of these polymers (25–27) with 3×10^{10} particles of HVJ-E vector containing the *luciferase* gene. For *in vivo* use, 1.5×10^{10} particles of polymer-conjugated HVJ-E vector was suspended in 500 μL of PBS. CG/bleomycin without HVJ-E was prepared by mixing 5 mg CG with 100 μL of 5 or 40 mg/mL bleomycin followed by centrifugation. CG-*luciferase* gene without HVJ-E vector was also prepared according to the previous method (25).

Quantification of Bleomycin in HVJ-E

A total of eight HVJ-E/bleomycin suspensions were prepared using eight different concentrations of bleomycin (0, 5, 10, 20, 30, 40, 50, and 100 mg/mL). Each HVJ-E/bleomycin suspension (200 μL) was dissolved in an equal volume of chloroform. After being vortexed and centrifuged (18,500 × *g* for 15 minutes at 4°C), the aqueous layer was recovered and added to 800 μL of PBS. The bleomycin content was assessed by high-performance liquid chromatography. Peaks representing bleomycin A2 and B2 at 245 nm were added, after which the concentration of bleomycin was determined from the standard curve. The efficacy of bleomycin inclusion into this vector was quantitatively measured with high-performance liquid chromatography. The amount of bleomycin in 1,000 HAU of HVJ-E/bleomycin was 0.18 μg when 5 mg/mL bleomycin was used, 0.41 μg with 10 mg/mL bleomycin, 0.84 μg with 20 mg/mL bleomycin, 1.12 μg with 30 mg/mL bleomycin, 1.32 μg with 40 mg/mL bleomycin, 1.34 μg with 50 mg/mL bleomycin, and 1.52 μg with 100 mg/mL bleomycin. Thus, the amount of bleomycin incorporated into the HVJ-E vector increased in proportion to the bleomycin concentration up to 40 mg/mL of bleomycin, at which a plateau was reached. Therefore, 40 mg/mL of bleomycin was used to prepare the HVJ-E/bleomycin used for the *in vivo* experiments.

In vitro Experiments

To perform *in vitro* transfection of HVJ-E/bleomycin, 5×10^4 CT-26 cells were seeded into six-well plates 1 day before transfection. Cells were maintained in DMEM (Nakalai Tesque, Kyoto, Japan) supplemented with 10% fetal bovine serum, penicillin (50 units/mL), and streptomycin (50 μg/mL) and incubated at 37°C in 5% CO_2. Tissue culture medium and supplements were purchased from Nakarai Tesque. HVJ-E/bleomycin was prepared with 5 or 40 mg/mL bleomycin. A 5-μL aliquot of 5 mg/mL protamine sulfate (Nakarai Tesque) and 500 μL of medium were added to 30 μL of HVJ-E/bleomycin (3×10^9 particles). This HVJ-E/bleomycin solution was added to each well after removal of the cell culture medium. HVJ-E alone and five different concentrations of bleomycin alone (0, 1, 10, 25, and 100 μg/mL) were then added to some of the wells. After 30 minutes of incubation, the medium was changed to fresh medium. After 2 days of incubation, the cells were counted with a Coulter particle counter (Beckman Coulter).

Assessment of the Effect of Fusion and Endocytosis on HVJ-E and CG-HVJ-E Vector

For the evaluation of fusion-mediated delivery, we used antiserum against the F protein of HVJ prepared in our laboratory by immunizing a rabbit with purified F protein (24). The concentration of anti-F antibodies in the antiserum was ~30 μg/mL. Aliquots of antiserum were stored at −80°C. The antiserum was diluted with saline. HVJ-E (3×10^9 particles) and CG-HVJ-E containing the *luciferase* gene were preincubated with diluted or undiluted antiserum (20 μL) for 30 minutes at 37°C. This mixture was then added to cultured cells. Preimmune rabbit serum was used as the control. Luciferase activity was measured 24 hours after transfection.

To evaluate the effect of endocytosis-mediated delivery, wortmannin (Sigma Chemical Co., St. Louis, MO), an inhibitor of phosphatidylinositol-3-kinase that inhibits endocytosis (28), was used. The reagent was dissolved in DMSO to a final concentration of 10 mmol/L, dispensed into 5-μL aliquots, and stored at −80°C. Before use, the aliquots were thawed and diluted in serum-free DMEM. Care was taken to shield the aliquots from light. Before transfection, cells were washed with serum-free DMEM and incubated with various concentrations of wortmannin for 15 minutes. The cells were then subjected to *in vitro* transfection, as described above.

***In vivo* Experiments**

All animal experiments were approved by the Animal Committee of Osaka University and conducted in a humane fashion according to their guidelines. Male BALB/c mice, 6 to 7 weeks of age, were obtained from Charles River Japan, Inc. (Yokohama, Japan). Mice were housed for 7 to 14 days and allowed *ad libitum* access to food and water. For tumor cell implantation, CT-26 cells were enzymatically detached from their culture flasks and counted. Viable cells (1.5×10^6) were resuspended in 500 μL of PBS and injected into the peritoneal cavity of each mouse. Two days after tumor inoculation, 500 μL of CG-HVJ-E/bleomycin (1.5×10^{10} particles) or CG-bleomycin were injected six times every third day, and animal survival was monitored.

Rechallenge Experiment

On day 115 after tumor inoculation, two surviving mice and three age-matched naive mice were rechallenged by i.d. injection of 5×10^6 parental cells into both sides of their trunk. In addition, two other surviving mice from the same experiment were rechallenged by i.p. injection of 1.5×10^6 parental cells.

Results

HVJ-E Vector Delivers Molecular Agents into Cells

The HVJ-E vector delivers molecules, such as anticancer drugs, into cells by membrane fusion, as illustrated in Fig. 1. We investigated the delivery mechanism using anti-F antibody or wortmannin. As shown in Supplementary Fig. S1A and B, undiluted anti-F antibody reduced the transfection efficiency of HVJ-E to 20% of that of the control group, whereas 100 nmol/L wortmannin did not significantly decrease transfection efficiency. These results suggest that drug delivery by HVJ-E vector occurs mainly by fusion. Therefore, agents delivered by the HVJ-E vector are released directly into the cytoplasm, bypassing endocytosis, the mechanism by which liposome-mediated and naked DNA-mediated delivery are achieved.

HVJ-E Vector Enables Effective Delivery of Bleomycin into Cells

We attempted to incorporate anticancer drugs into the HVJ-E vector to enhance its cytotoxicity following fusion-mediated delivery. We used bleomycin as the anticancer agent to be incorporated into HVJ-E. Bleomycin has a marked antineoplastic effect but limited cell permeability (29). To evaluate the potential of the HVJ-E vector to effectively deliver bleomycin into cancer cells, we incorporated bleomycin into the vector and assessed its cytotoxicity

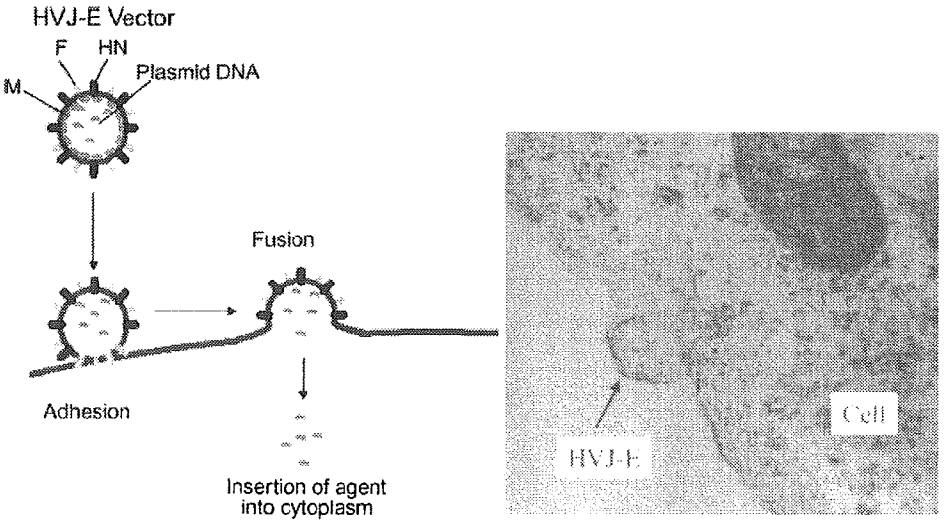

Figure 1. Schematic diagram and electron microscopy images of HVJ-E vector–mediated membrane fusion and drug delivery. The HVJ-E vector effectively encloses and delivers drugs into cells by membrane fusion.

against various cancer cell lines *in vitro*. We prepared HVJ-E/bleomycin using 5 or 40 mg/mL of bleomycin in the HVJ-E inclusion reaction and tested its cytotoxicity against CT-26 mouse colon adenocarcinoma cells. HVJ-E/bleomycin or bleomycin alone were incubated with cultured cells for 30 minutes, and the cells were further cultivated for 48 hours. Bleomycin alone was not particularly cytotoxic, killing only 60% of CT-26 cells at 100 μg/mL of BML. Although HVJ-E alone was not toxic to CT-26 cells, HVJ-E/bleomycin killed >90% of CT-26 cells in the culture (Fig. 2A). The bleomycin content of HVJ-E/bleomycin prepared using 5 and 40 mg/mL of bleomycin in the inclusion reaction was 0.36 and 2.54 μg/mL, respectively. Therefore, HVJ-E/bleomycin was 300-fold more cytotoxic than bleomycin alone. HVJ-E/bleomycin exhibited similar effects on a number of other human cancer cell lines (Fig. 2B). Although the cytotoxicity of 100 μg/mL bleomycin varied among different human cancer cell lines, HVJ-E/bleomycin caused a marked reduction in cell survival in all cell lines tested. In addition, treatment with HVJ-E/bleomycin reduced the proportion of cells in the G_0-G_1 phase of the cell cycle and increased the proportion of cells in the G_2-M phase (data not shown). These cell cycle changes are a characteristic of bleomycin treatment. Thus, it seems that the HVJ-E vector efficiently delivered bleomycin into cells by fusion with the plasma membrane.

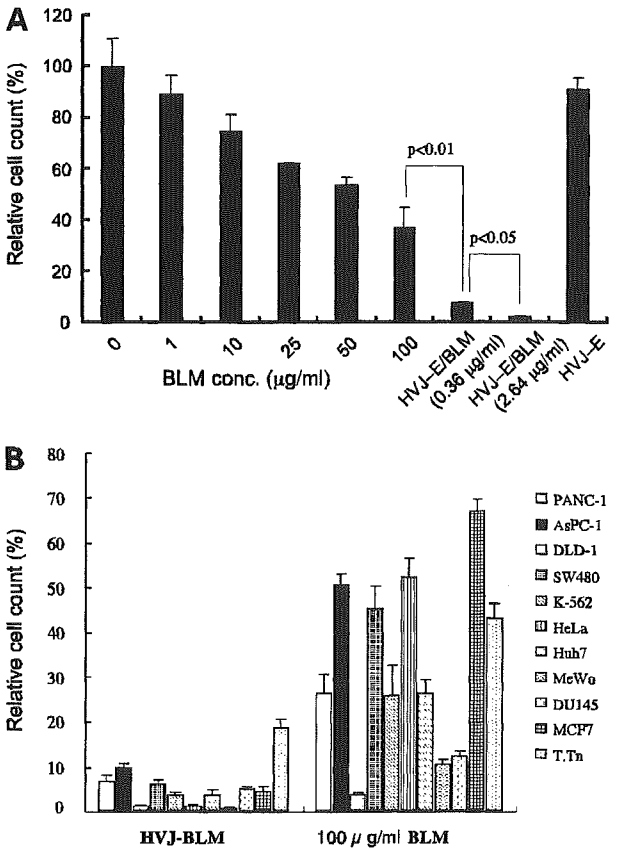

Figure 2. Efficient delivery of an antitumor agent (bleomycin, *BLM*) into cancer cells using the HVJ-E vector. **A**, after 30 min of HVJ-E/bleomycin transfection, cells were cultured for 2 d. After this, viable CT-26 cells were counted. When HVJ-E/bleomycin (5 and 40 mg/mL) was used, the concentration of bleomycin in the medium was 0.36 and 2.64 μg/mL, respectively. HVJ-E/bleomycin (5 mg/mL) significantly suppressed cell viability compared with bleomycin alone ($P < 0.01$). HVJ-bleomycin (40 mg/mL) also showed significantly higher cytotoxicity than HVJ-bleomycin (5 mg/mL; $P < 0.05$). **B**, investigation of HVJ-E/bleomycin cytotoxicity against various human cancer cell lines. PANC-1, pancreatic cancer; AsPC-1, pancreatic cancer; DLD-1, colon adenocarcinoma; SW480, colon cancer; K562, erythroleukemia; HeLa, uterocervical cancer; Huh-7, hepatocellular carcinoma; MeWo, malignant melanoma; DU145, prostate cancer; MCF7, mammary carcinoma; T.Tn, esophageal squamous cell carcinoma. In all tumor cells, HVJ-E/bleomycin showed significantly higher cytotoxicity compared with bleomycin alone ($P < 0.01$).

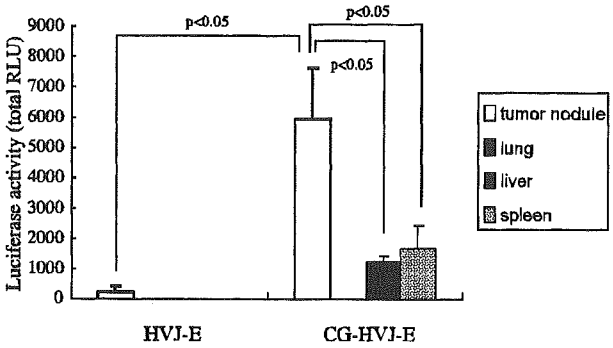

Figure 3. Luciferase expression in tumor nodules, liver, spleen and lung, following i.p. injection of HVJ-E or CG-HVJ-E containing the *luciferase* gene. Seven days after i.p. injection of CT-26 cells, HVJ-E or CG-HVJ-E containing the *luciferase* gene were injected into the peritoneal cavities of tumor-bearing mice. Luciferase activity in various organs was investigated 24 h after gene transfer. Significantly higher luciferase activity was obtained in tumor nodules using CG-HVJ-E than using HVJ-E alone ($P < 0.05$). Using CG-HVJ-E, luciferase activity was significantly higher in tumor nodules than in other organs ($P < 0.05$).

In vivo Gene Transduction for the Treatment of Peritoneally Disseminated Tumors

A major challenge in cancer chemotherapy is finding a way to treat inoperable and invisible cancer lesions and hence the need for cancer-specific drug delivery vectors. Here, we attempted to treat peritoneal deposits of cancer cells using the HVJ-E vector system. First, we confirmed the presence of peritoneal tumor deposits 1 week after i.p. injection of CT-26 tumor cells (10^7 cells). As previously reported (30), a number of tumor deposits are observed to develop within the i.p. cavity following the introduction of CT-26 tumor cells. In particular, metastasis to lymph nodes around the root of the mesentery are frequently detected in the i.p. cavity.

We used the *luciferase* gene as a reporter gene to measure gene expression. To evaluate the transfection efficiency of HVJ-E into peritoneally disseminated colon tumor cells, HVJ-E containing pcLuc plasmid was injected into the peritoneal cavities of mice 7 days after i.p. injection of CT-26 tumor cells. Twenty-four hours after

injection of HVJ-E, the mice were killed, and all tumor deposits and nonaffected organs were removed and examined for luciferase activity. As shown in Fig. 3, *luciferase* gene expression was not detected in either the tumor deposits or other organs, such as the lung, liver, or spleen. No significant luciferase activity was detected in any tissue when either naked plasmid DNA encoding the *luciferase* gene or CG-conjugated *luciferase* gene was i.p. injected (data not shown).

Next, we combined HVJ-E with CG (MW = 5,000 and 100,000), dextran, and pullulan. When CG (MW = 5,000)-HVJ-E containing the *luciferase* gene was i.p. injected, a high level of expression of the *luciferase* gene was preferentially detected in the tumor deposits compared with other polymers, such as CG (MW = 100,000), dextran, and pullulan, with limited expression in the spleen and liver and no expression in lung (Supplementary Fig. S2).

Treatment of Peritoneally Disseminated Colon Cancer Using CG-HVJ-E/Bleomycin

Then, we combined HVJ-E containing bleomycin with CG (CG-HVJ-E/bleomycin) and compared its cytotoxicity against cultured CT-26 cells with that of HVJ-E/bleomycin. As shown in Fig. 4, CG-HVJ-E/bleomycin killed CT-26 cells as efficiently as HVJ-E/bleomycin. No cytotoxicity was observed when CG was combined with bleomycin alone (CG/bleomycin). We then investigated the mechanism of CG-HVJ-E–mediated delivery using anti-F antibody or wortmannin. As shown in Supplementary Fig. S3A and B, both undiluted anti-F antibody and 100 nmol/L wortmannin significantly reduced the transfection efficiency of CG-HVJ-E. Thus, the delivery mechanism of CG-HVJ-E vector seems to depend on both membrane fusion and endocytotic uptake.

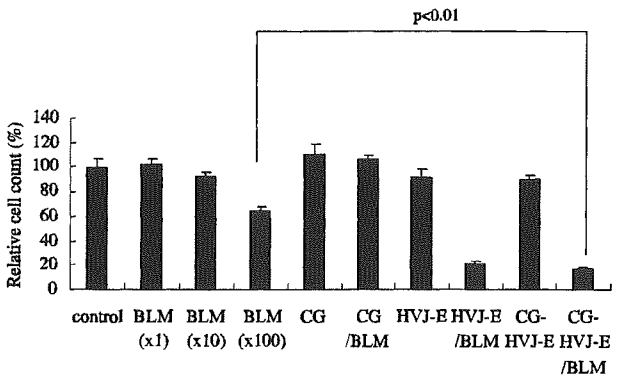

Figure 4. Cytotoxic effects of bleomycin (*BLM*) against CT-26 cells delivered by CG, HVJ-E or CG-HVJ-E. The concentration of bleomycin in (×1), (×10), (×100), CG/bleomycin, HVJ-E/bleomycin, and CG-HVJ-E/bleomycin was 0.36, 3.6, 36, 0.36, 0.36, and 0.36 μg/mL, respectively. Following 30 min of vector treatment, CT-26 cells were cultured for 2 d. After this, viable CT-26 cells were counted. As a control, cell viability without treatment was also evaluated. Significant cytotoxicity was obtained using CG-HVJ-E/bleomycin compared with bleomycin (×100) alone ($P < 0.01$). No significant differences in cytotoxicity were seen between CG-HVJ-E/bleomycin and HVJ-E/bleomycin.

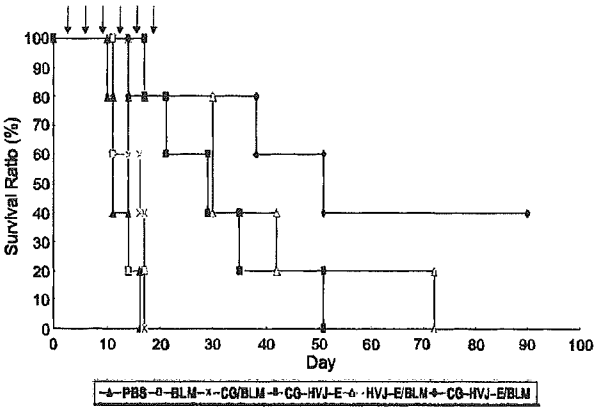

Figure 5. Survival of tumor-bearing mice. Two days after i.p. injection of CT-26 cells, CG or CG-HVJ-E containing bleomycin (*BLM*) was i.p. injected six times every third day. An empty vector without bleomycin, bleomycin alone, or PBS were also i.p. injected in a similar manner. The survival of tumor-bearing mice was monitored. Arrows indicate the timing of i.p. injection (on days 2, 5, 8, 11, 14, and 17) after tumor inoculation.

Next, we injected CG-HVJ-E/bleomycin into the peritoneal cavity 48 hours after the introduction of CT-26 cells. Three injections were given every third day. Although mouse survival was prolonged by administration of either CG-HVJ-E or CG-HVJ-E/bleomycin, compared with bleomycin alone, all of the mice died within 40 days (data not shown). Next, we evaluated the effects of i.p. injection of the vectors six times every third day. As shown in Fig. 5, complete responses were observed in 40% of the mice injected with CG-HVJ-E/bleomycin. The mice that received CG-HVJ-E without bleomycin showed prolonged survival, compared with those administered bleomycin alone, but still died within 50 days. The mice showing complete responses survived >90 days and experienced complete remission.

Rechallenge of Surviving Mice with CT-26

Next, we injected CT-26 cells into the surviving mice i.d. or i.p. Approximately 5×10^6 CT-26 cells were i.d. injected into both sides of the mouse trunk. Although the tumor masses were observed to grow up to 3 to 4 mm in diameter within 5 days of the rechallenge in both control mice and those showing complete responses, tumor masses among the mice previously showing complete responses then underwent a rapid reduction in size within 11 days of the rechallenge (Fig. 6). However, tumor masses among the control mice continued to grow up to 8 to 10 mm in diameter within 11 days of the rechallenge (Fig. 6). In addition, $\sim 1.5 \times 10^6$ CT-26 cells were also injected directly into the i.p. cavities of mice showing complete responses, as well as control mice. Within 11 days of the rechallenge, control mice were emaciated and had diarrhea, and a number of tumor deposits were observed in the peritoneal cavity, whereas the mice previously showing complete responses remained healthy with no tumor deposits observed within the peritoneal cavity (data not shown).

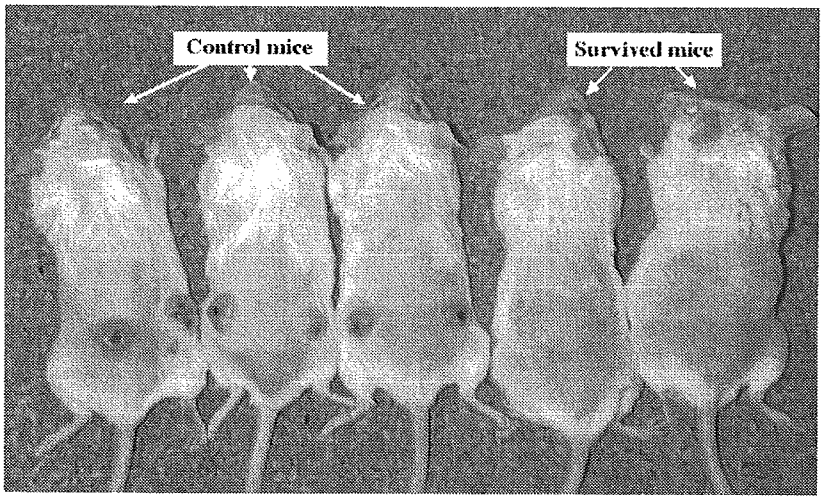

Figure 6. Resistance to rechallenge with parental CT-26 cells among mice previously showing complete responses. The two on the right previously treated with CG- HVJ-E/bleomycin (*BLM*) were rechallenged with i.d. injection of 5×10^6 parental CT-26 cells into both sides of the mouse trunk 115 d after initial tumor inoculation. The three mice on the left are age-matched naive mice. Within 11 d of tumor rechallenge, intradermal tumor formation was observed in each mouse.

However, when Meth-A cells, which are fibrosarcoma cells derived from BALB/c mouse, were i.d. injected into the surviving mice, tumors arose in those mice as well as in naive mice (data not shown).

Thus, multiple i.p. injection of CG-HVJ-E/bleomycin not only inhibits the tumorigenesis of CT-26 cells following i.p. dissemination but also induces an effective and long-lasting anti-CT-26 memory in mice.

Discussion

In this study, we showed the feasibility of using the HVJ-E vector for cancer treatment. One of the advantages of the HVJ-E vector is fusion-mediated drug delivery and thereby enhancing drug efficacy. Bleomycin is ~1,500 Da and thus has limited permeability into cells and low bioavailability following oral, i.m., or i.v. administration (29). In the present study, *in vivo*, cell culture experiments showed enhanced cytotoxicity of bleomycin delivered by the HVJ-E vector against a variety of cancer cells. Fusion between the HVJ-E vector and cancer cell membranes might overcome the problem of limited bleomycin cell permeability. As shown in Supplementary Fig. S3, the delivery by CG-HVJ-E was mediated by both fusion and endocytosis. CG-HVJ-E may be more effective for drug delivery than HVJ-E alone because HVJ can fuse with the cell membrane under neutral conditions and also with the endosomal membrane under acidic conditions (17).

A disadvantage of the HVJ-E vector is rapid degradation in the presence of fresh serum, although the *in vitro* transfection efficiency of HVJ-E was not inhibited by culture medium containing 10% fetal bovine serum (22). However, we previously showed that CG-HVJ-E is remarkably stable in 50% fresh mouse serum. Although it is unproven that HVJ is degraded by complement lysis in mouse serum, as known to be the case for retrovirus and HIV (31, 32), the interaction between serum proteins and HVJ-E may contribute to reduced transfection efficacy of HVJ-E. Conjugation with CG seems to protect the surface molecules of HVJ-E from the detrimental effects of serum proteins (24). In this report, CG-HVJ-E showed a proclivity toward i.p. tumor deposits. In a previous report, we showed that HVJ-cationic liposomes also have a proclivity for tumor deposits when injected into peritoneal cavity (30). We speculate that HVJ-cationic liposomes might be absorbed into lymph vessels and fuse with tumor cells when they reach lymph nodes enlarged with tumor cells. We observed enhanced fusion of HVJ with tumor cells than normal cells.[4] Therefore, it is likely that positively charged CG-HVJ-E (ζ potential = 11.30 mV; ref. 24) results in preferential delivery of bleomycin to tumor cells within the peritoneum.

Another advantage of the HVJ-E vector is its capacity for repeated injection. Gene transfer to mouse muscle was not inhibited by repeated injections (23). Similar results were obtained when HVJ-liposomes was repeatedly injected into rat liver. After repeated injections, the anti-HJV antibodies generated in mice were not sufficient to neutralize HVJ-liposomes (33). Presumably, this is because fusion occurs more rapidly than recognition by neutralizing antibodies. Indeed, fusion between the HVJ-E vector and the cell membrane occurs in 10 seconds (23).

In this experiment, complete responses were observed in 40% of tumor-inoculated mice. Furthermore, these mice were resistant against tumor rechallenge. This result indicates that antitumor immunity was achieved in mice treated with CG-HVJ-E/bleomycin. Some reports suggest that antitumor immunity might be induced in mice following a variety of forms of treatment, including chemotherapy, radiotherapy, or gene therapy (34–36). On the other hand, it is well known that viruses and bacteria can induce robust and long-lasting immune responses through Toll-like receptors (37, 38). Some

[4] Kaneda, unpublished data.

reports suggest that live HVJ infection induces the secretion of various cytokines, including IFN-α, IFN-β, IFN-γ, interleukin-6, interleukin-12, and tumor necrosis factor in dendritic cells, while also inducing a maturation of dendritic cells (39, 40). Moreover, inactivated HVJ can induce dendritic cell maturation as effectively as live HVJ (39, 41). We observed a retained capability of dendritic cell maturation by the HVJ-E vector, and i.t. injection of the HVJ-E vector into mice mildly enhanced IFN-γ and interleukin-5 production by antigen stimulated splenocytes.[5] This explains why CG-HVJ-E prolonged mouse survival even without bleomycin. When rechallenged with tumor cells, the mice treated with CG-HVJ-E/bleomycin rejected the same CT-26 tumor not Meth-A. This suggests that tumor-specific cytotoxic T cells, not natural killer cells, were generated in those mice by CG-HVJ-E/bleomycin treatment. Therefore, CG-HVJ-E/bleomycin might induce antitumor immunity and via direct toxicity to tumor cells by bleomycin delivery. Given its dual function, the HVJ-E vector might be especially useful for tumor eradication. Currently, no drug delivery system has shown similar capabilities specific to the treatment of cancer.

Thus, the HVJ-E vector system shows potential for the treatment of cancer in the future.

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THE JOURNAL OF GENE MEDICINE
J Gene Med 2005; 7: 888–897.
Published online 14 February 2005 in Wiley InterScience (www.interscience.wiley.com). **DOI:** 10.1002/jgm.735

RESEARCH ARTICLE

Biocompatible polymer enhances the *in vitro* and *in vivo* transfection efficiency of HVJ envelope vector

Hidetoshi Mima[1,3]
Ryuji Tomoshige[2]
Toshihide Kanamori[1]
Yasuhiko Tabata[2]
Seiji Yamamoto[1]
Susumu Ito[3]
Katsuto Tamai[1]
Yasufumi Kaneda[1]*

[1]*Division of Gene Therapy Science, Graduate School of Medicine, Osaka University, 2-2 Yamada-oka, Suita, Osaka 565-0871, Japan*

[2]*Department of Biomaterials, Fields of Tissue Engineering Institute for Frontier Medical Sciences, Kyoto University, 53 Kawara-cho, Shogoin, Sakyo-ku, Kyoto 606, Japan*

[3]*Department of Digestive and Cardiovascular Medicine, University of Tokushima Graduate School, 2-50 Kuramoto-cho, Tokushima 770-8503, Japan*

*Correspondence to:
Yasufumi Kaneda, Division of Gene Therapy Science, Graduate School of Medicine, Osaka University, 2-2 Yamada-oka, Suita, Osaka 565-0871, Japan.
E-mail:
kaneday@gts.med.osaka-u.ac.jp

Received: 18 August 2004
Revised: 29 November 2004
Accepted: 1 December 2004

Abstract

Background Vector development is critical for the advancement of human gene therapy. However, the use of viral vectors raises many safety concerns and most non-viral methods are less efficient for gene transfer. One of the breakthroughs in vector technology is the combination of the vector with various polymers.

Methods HVJ (hemagglutinating virus of Japan) envelope vector (HVJ-E) has been developed as a versatile gene transfer vector. In this study, we combined HVJ-E with cationized gelatin to make it a more powerful tool and assessed its transfection efficiency *in vitro* and *in vivo*. In addition, we investigated the mechanism of the gene transfer by means of the inhibition of fusion or endocytosis.

Results The combination of both protamine sulfate and cationized gelatin with HVJ-E, referred to as PS-CG-HVJ-E, further enhanced the *in vitro* transfection efficiency. In CT26 cells, the luciferase gene expression of PS-CG-HVJ-E was approximately 10 times higher than that of the combination of protamine sulfate with HVJ-E or the combination of cationized gelatin with HVJ-E, referred to as PS-HVJ-E or CG-HVJ-E, respectively. Furthermore, the luciferase gene expression in liver mediated by intravenous administration of CG-HVJ-E was much higher than the luciferase gene expression mediated by PS-HVJ-E or PS-CG-HVJ-E and approximately 100 times higher than that mediated by HVJ-E alone.

Conclusions Cationized gelatin-conjugated HVJ-E enhanced gene transfection efficiency both *in vitro* and *in vivo*. These results suggest that low molecular weight cationized gelatin may be appropriate for complex formation with various envelope viruses, such as retrovirus, herpes virus and HIV.

Keywords non-viral vector; gene transfer; polymer; fusion-mediated delivery

Introduction

The success of gene therapy is largely dependent on the development of a vector. So far, numerous viral and non-viral (synthetic) methods of gene transfer have been developed and improved upon. The use of viral vectors raises many safety concerns because of the possible co-introduction of genetic elements from parent viruses, leaky expression of viral genes, immunogenicity and changes in the host genome structure [1,2]. Non-viral vectors are less toxic and less immunogenic alternatives to viral vectors [3,4]. However, most non-viral methods are less efficient for gene transfer, especially *in vivo*. Thus,

a breakthrough in vector technology is required for the development of highly efficient vectors with low toxicity.

One promising development in vector technology is the combination of the vector with various polymers [5,6]. Biocompatible polymers have been combined with viral and non-viral vectors to enhance gene transfer efficiency both *in vitro* and *in vivo* [7–12]. Adenovirus vector combined with atelocollagen increased stability in tissues and reduced the toxicity [13,14]. The mixture of adeno-associated vector with heparin increased transfection efficiency [15]. The most popular polymers to enhance transfection efficiency are cationic polymers, such as polyethylenimine [16–19] and cationized gelatin [20–22]. Cationic polymers assemble with vectors and form small composite particles that interact with the cell surface and are internalized by endocytosis. The polymer must be positively charged to increase the transfection efficiency of the polymer–DNA complex (polyplex) [23]. However, cationic polymer-based gene delivery systems have faced limitations in the systemic delivery of therapeutic genes due to difficulties in formation, *in vivo* stabilization, toxicity and low transfection efficiency [24–28]. Moreover, positively charged polyplexes aggregate more readily as their concentration increases, and they quickly precipitate out of solution above their critical flocculation concentration or in the presence of salt or serum. These drawbacks have limited the progress of polyplexes in clinical trials. Recent efforts to solve the limitations of polymers have focused on the development of low molecular weight polymers, biodegradable polymers and polymers with reduced positive charge [29]. Gelatin is a biodegradable polymer with various sizes ranging from high (MW 100 000 Da) to low molecular weight (MW 3000 Da) [30]. By conjugation with cationic molecules (Figure 1), such as ethylenediamine, spermine or spermidine, the positive charge ratio per gelatin molecule can be controlled [20,22].

In the present study, we combined HVJ (hemagglutinating virus of Japan) with cationized gelatin. HVJ envelope vector (HVJ-E) is a unique non-viral vector which incorporates plasmid DNA into inactivated HVJ particles. HVJ, also known as Sendai virus, can fuse with cell membranes [31]. Two distinct glycoproteins on the viral envelope are required for cell fusion. The HVJ RNA genome is approximately 15 kb. When the viral genome is intact, highly immunogenic viral proteins are produced in the infected cells. Therefore, we inactivated HVJ with UV irradiation and incorporated plasmid DNA into inactivated viral particles by mild detergent treatment and centrifugation. The resulting HVJ-E can fuse with cell membranes to directly introduce plasmid DNA into cells both *in vitro* and *in vivo* [32]. The major limitation of HVJ-E is the instability of viral particles in fresh blood. Although this characteristic of HVJ-E is an advantage in terms of safety, it is an obvious defect in terms of efficacy.

In this manuscript, we report that cationized gelatin-conjugated HVJ-E enhances gene transfection efficiency both *in vitro* and *in vivo*.

Materials and methods

Reagents, cells and preparation of DNA

Triton-X 100 was purchased from Nakalai Tesque (Kyoto, Japan) and used as a detergent diluted with TE solution (10 mM Tris-Cl, pH 8.0, 1 mM EDTA) to 3% concentration when we incorporated plasmid DNA into HVJ-E. Gelatin was prepared through an acid process of pig skin type I collagen and was kindly supplied by Nitta Gelatin Co. (Osaka, Japan). Ethylenediamine (ED), glutaraldehyde, 2,4,6-trinitrobenzenesulfonic acid, β-alanine and the protein assay kit (lot no. L8900) were purchased from Nakalai Tesque (Kyoto, Japan) and used according to the manufacturer's instructions. As a coupling agent, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride salt (EDC) was obtained from Dojindo Laboratories (Kumamoto, Japan).

Primary human aortic endothelial cells (HAEC) were purchased from Sanko-Junyaku (Tokyo, Japan). All other cell lines were purchased from the American Type Culture Collection (Rockville, MD, USA). Adherent and primary cells were cultured in Dulbecco's modified Eagle's medium

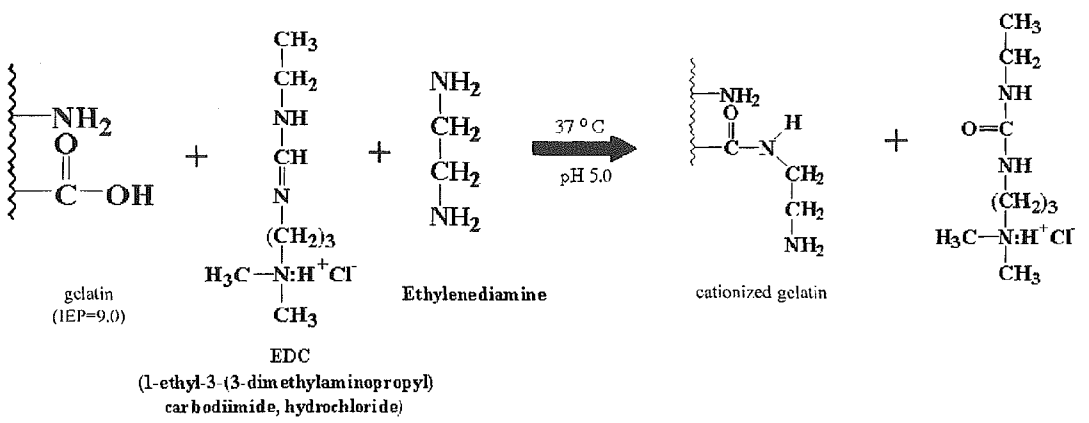

Figure 1. Synthesis of cationized gelatin. Cationized gelatin was mixed with HVJ-E containing a marker gene. The complex was isolated by centrifugation and used for transfection experiments. (IEP; isoelectric point)

(DMEM) and RPMI 1640, respectively, supplemented with 10% fetal bovine serum (FBS).

Luciferase expression plasmid driven by the cytomegalovirus promoter was purchased from Promega (Madison, WI, USA). Qiagen columns (Hilden, Germany) were used to purify DNA.

Preparation of cationized gelatin combined with HVJ-E

HVJ was prepared as previously described [31]. HVJ was propagated in chick eggs, purified by centrifugation, inactivated by UV irradiation and stored at −20 °C as previously described [32]. Stored virus was suspended in 40 μl of TE solution (10 mM Tris-Cl, pH 8.0, 1 mM EDTA). The virus suspension was mixed with plasmid DNA (200 μg/50 μl) and 5 μl of 3% Triton X-100. The mixture was centrifuged at 18 500 *g* for 15 min at 4 °C. After washing the pellet with 1 ml of balanced salt solution (10 mM Tris-Cl, pH 7.5, 137 mM NaCl and 5.4 mM KCl) to remove the detergent and unincorporated DNA, the envelope vector was suspended in 300 μl of phosphate-buffered saline (PBS). The vector was stored at 4 °C until use.

Cationization of gelatin was performed by introducing ethylenediamine (ED) into the carboxyl groups of low molecular weight gelatin (MW 5000) (Figure 1). Briefly, 13.98 g of ED and 2.67 g of EDC were added to 250 ml of 0.1 M phosphate buffer (pH 5.0) containing 5.00 g of low molecular weight gelatin. The reaction mixture was agitated at pH 5.0 at 37 °C for various time periods and then dialyzed against double-distilled water for 48 h at 25 °C by use of a dialysis membrane tube (lot no. 131 096, cut-off MW 1000, Spectra/PorCE, SPECTRUM) to separate residual ED- and EDC-degraded product from cationized gelatin prepared. The dialyzed solution was freeze-dried to obtain powdered cationized gelatin. The percentage of amino groups introduced into this gelatin, referred to as cationized gelatin, was determined by the trinitrobenzenesulfonate method based on the calibration curve prepared by using β-alanine [22]. The percentage of amino groups introduced into gelatin was 48.7 mole/mole carboxyl groups of gelatin.

A complex was formed between the HVJ-E vector and cationized gelatin by simply mixing the two materials in aqueous solution. Briefly, 5 mg of cationized gelatin were added to 300 μl of 0.1 M PBS (pH 7.4) containing 3×10^{10} particles of HVJ-E vector. The solution was mixed by tapping several times. Then, the solution was incubated on ice for 30 min to form cationized gelatin-conjugated HVJ-E vector. The optimal ratio of cationized gelatin and HVJ-E was determined by the measurement of luciferase activity *in vitro*. Cationized gelatin-conjugated HVJ-E vector was purified by centrifugation.

Measurement of zeta potential and apparent molecular size

The zeta potential was measured by an electrophoretic light scattering (ELS) assay. This assay was performed with an ELS-7000AS instrument (Otsuka Electric Co. Ltd., Osaka, Japan) at 37 °C with an electric field strength of 100 V/cm [20]. The ELS measurement was performed 3 to 5 times for each sample. The particle size of HVJ-E or polymer-conjugated HVJ-E was measured by dynamic light scattering (DLS) assay, as previously described [20]. The DLS measurement was performed 3 to 5 times for each sample.

Gene transfer *in vitro* and *in vivo*

For *in vitro* transfection, approximately 5×10^5 cells were prepared 1 day before transfection. HVJ-E ($3–6 \times 10^9$ particles) or cationized gelatin-conjugated HVJ-E was mixed with various concentrations of protamine sulfate. This mixture was added to cells cultured in medium supplemented with 10% FBS. After incubation for 10 min at 37 °C and 5% CO_2, the medium was replaced. The cells were cultured overnight before the gene expression was assayed. For *in vitro* transfection with anionic liposomes, the procedure was as previously described [33]. Luciferase activity was measured with a luciferase assay kit (Promega), and the protein content of the samples was assayed by the Bradford method as previously described [32].

HVJ-E (6×10^9 particles) or cationized gelatin-conjugated HVJ-E containing the luciferase gene (6 μg) was suspended in 100 μl PBS with or without protamine sulfate (200 μg) and injected into the tail veins of BALB/c mice (8 weeks of age). Mice were euthanized 24 h after the injection. The organs including lung, liver, spleen, heart and kidney were removed and cut into small pieces in 5-times volume of diluted luciferase cell culture lysis reagent (Promega). All steps were performed on ice. After centrifugation at 2380 *g* at 4 °C for 10 min, 20 μl of the supernatant were assayed for luciferase activity. All animals were handled in a humane manner in accordance with the guidelines of the Animal Committee of Osaka University.

Assessment of the effect of fusion and endocytosis on transfection efficiency

We prepared antiserum against F protein of HVJ by immunizing a rabbit with purified F protein. The concentration of anti-F antibodies in the antiserum was approximately 30 μg/ml. The aliquots of antiserum were stored at −80 °C. The antiserum was diluted with saline. Polymer-combined HVJ-E (3×10^9 particles) that contained the luciferase gene was preincubated with diluted or undiluted antiserum (20 μl) for 30 min at 37 °C. Then, this mixture was added to cultured

cells. Preimmune rabbit serum was used as a control. Luciferase activity was measured 24 h after the transfection.

Wortmannin (Sigma Chemical Co.) was dissolved in dimethyl sulfoxide to a final concentration of 10 mM, dispensed into 5-μl aliquots and stored at −80 °C. Prior to use, wortmannin aliquots were thawed and diluted in serum-free DMEM. Care was taken to shield the aliquots from light. Before transfection, cells were washed with serum-free DMEM and incubated with various concentrations of wortmannin for 15 min [34,35]. The cells were then subjected to *in vitro* transfection, as described above.

Assessment of the effect of fresh mouse serum on gene transfection with HVJ-E and polymer-conjugated HVJ-E

HVJ-E, PS-HVJ-E, CG-HVJ-E and PS-CG-HVJ-E containing luciferase expression plasmid were separately suspended in 100 μl PBS. The suspensions were mixed with 100 μl of fresh mouse serum. The mixture was incubated at 37 °C for 5 min. Then, after the serum had been removed by centrifugation, the vector, suspended in 30 μl of PBS, was added to cultured cells, and the cells were incubated at 37 °C for 10 min in a 5% CO_2 incubator. The medium was replaced with fresh medium containing 10% FBS. The luciferase activities of each sample were measured 24 h after transfection.

Statistical analysis

The Bonferroni/Dunn test was used to determine whether differences were statistically significant. A value of $P < 0.05$ was considered significant.

Results

Measurement of zeta potential and apparent molecular size

First, we examined the zeta potential and particle size of these complexes (Table 1). HVJ-E was anionic (−3.87 mV), and the diameter was approximately 350 nm. With protamine sulfate, the zeta potential became cationic (4.51 mV), and the diameter was six times larger (2114 nm). The cationized gelatin complex was more cationic (11.30 mV) and smaller (777 nm) than PS-HVJ-E. The zeta potential and size of PS-CG-HVJ-E were intermediate (9.53 mV, 1927 nm) between those of PS-HVJ-E and CG-HVJ-E.

Table 1. Apparent molecular size and Zeta potential of HVJ-envelope vector and its complexes

Complex	Apparent molecular size (nm)	Zeta potential (mV)
HVJ-E	355 ± 35	−3.87 ± 0.69
PS-HVJ-E	2114 ± 207	4.51 ± 0.86
CG-HVJ-E	777 ± 140	11.30 ± 2.52
PS-CG-HVJ-E	1927 ± 292	9.53 ± 1.47

Evaluation of the *in vitro* transfection efficiency of HVJ-E conjugated to cationized gelatin, protamine sulfate or both

Then, we examined the *in vitro* transfection efficiency of HVJ-E, CG-HVJ-E, PS-HVJ-E and PS-CG-HVJ-E. Low molecular weight cationized gelatin (MW 5000 Da) increased the HVJ-E transfection efficiency, but high molecular weight cationized gelatin (MW 100 000 Da) was not effective for gene transfer with HVJ-E (data not shown). As shown in Figure 2, cationized gelatin increased transfection efficiency to the same level as protamine sulfate when compared with HVJ-E alone. An amount of 500 μg of cationized gelatin added to 3×10^9 HVJ-E particles resulted in the highest gene transfection efficiency of CG-HVJ-E without affecting cytotoxicity. When protamine sulfate was added to CG-HVJ-E, the resulting luciferase gene expression in CT26 cells was approximately 10 times higher than the luciferase gene expression mediated by PS-HVJ-E or CG-HVJ-E (Figure 2). The enhanced transfection efficiency resulting from CG-HVJ-E combined with protamine sulfate was also observed in other cell lines (B16-F1) and primary cells (HAEC, human aortic endothelial cells), although the enhancement ratio varied among the different types of cells (Table 2).

Assessment of the effect of fusion and endocytosis on transfection efficiency

Next, the mechanism of transfection by PS-CG-HVJ-E was investigated. To test the effect of fusion protein of HVJ-E on transfection efficiency, the complex was incubated with anti-F protein antibody, and then the mixture was added to cells. As shown in Figure 3A, HVJ-E or CG-HVJ-E was preincubated with anti-F protein antiserum, and the mixture of the vector and serum was added to cultured cells. Luciferase gene expression was hardly detected. Preimmune serum did not cause inhibition. When diluted anti-F serum was used, the luciferase gene expression recovered in a dilution-dependent manner. Dot-blot analysis revealed that 1 μg anti-F antibody bound to 9.7×10^6 HVJ-E particles. From this data, the undiluted antiserum (20 μl) could bind to 5.8×10^9 PS-CG-HVJ-E particles. Therefore, it was anticipated that the undiluted antiserum contained an excess amount of anti-F antibody recognizing all the PS-CG-HVJ-E

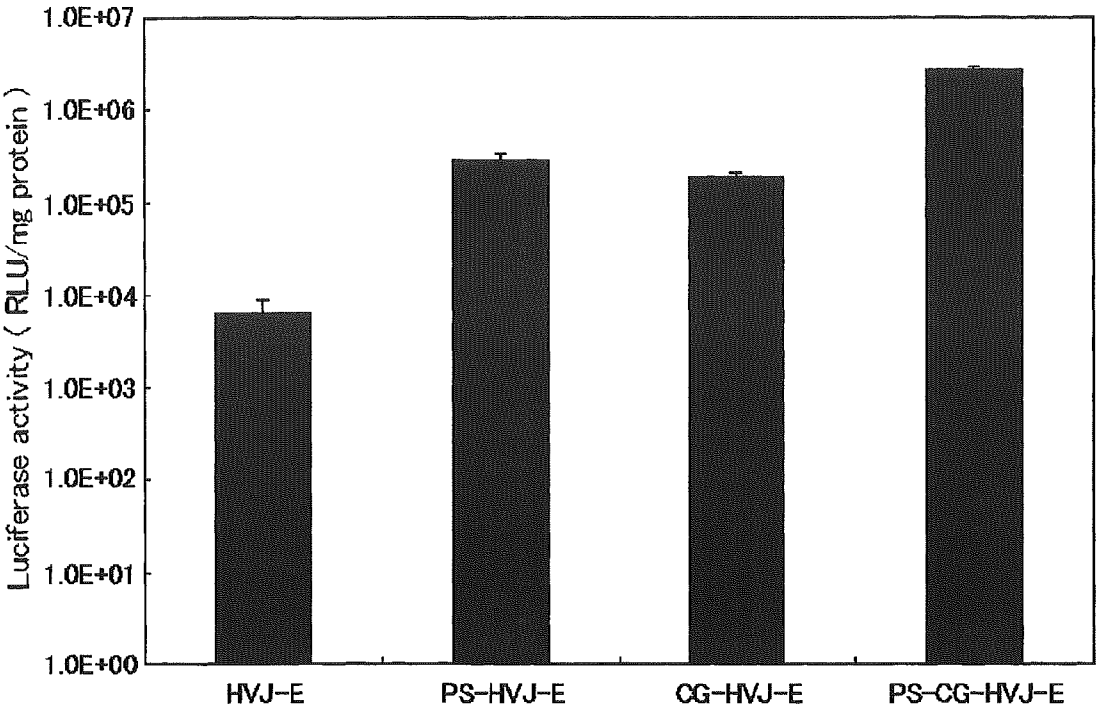

Figure 2. Luciferase gene expression in CT26 cells transfected with HVJ-E, PS-HVJ-E, CG-HVJ-E or PS-CG-HVJ-E. The vectors were incubated with cells for 10 min, and the luciferase activity was measured 24 h after removal of the vector. Results are shown as mean ± s.d. (n = 3). Similar results were obtained in three experiments

Table 2. Results of *in vitro* transfer with Cationized Gelatin conjugated HVJ-envelope vector

Cell line	HVJ-E	PS-HVJ-E	CG-HVJ-E	PS-CG-HVJ-E
Adherent cells				
B16-F1	$7.36 \pm 0.09 \times 10^5$	$8.15 \pm 0.40 \times 10^6$	$7.56 \pm 1.92 \times 10^6$	$1.16 \pm 0.04 \times 10^7$
BHK21	$3.49 \pm 0.38 \times 10^6$	$1.43 \pm 0.05 \times 10^7$	$3.71 \pm 0.18 \times 10^7$	$3.20 \pm 0.30 \times 10^7$
Primary cell				
HAEC	$8.94 \pm 0.88 \times 10^4$	$7.62 \pm 0.55 \times 10^4$	$1.54 \pm 0.06 \times 10^5$	$2.47 \pm 0.82 \times 10^5$

Luciferase activity (RLU/mg protein)

particles used in the experiment, but the antiserum diluted more than 2-fold failed to recognize all the particles. This result was consistent with the data shown in Figure 3A.

Then, the possibility of endocytotic uptake of the complex was assessed using wortmannin, which inhibits endocytosis [34,35]. Wortmannin inhibited the luciferase gene expression in a dose-dependent manner (Figure 3B). Wortmannin at a concentration of 100 nM inhibited gene transfection efficiency by 40%. The inhibition with wortmannin was much smaller than that with anti-F antibody. At the same time, although we tested the affecting cytotoxicity of wortmannin, no significant difference was observed between the group of 100 nM wortmannin and the control group (data not shown). From these results, we hypothesized that fusion was necessary for the transfection ability of PS-CG-HVJ-E, which was enhanced by endocytotic uptake.

Evaluation of the *in vitro* transfection efficiency of anionic liposome with or without HVJ, conjugated to cationized gelatin

To confirm this hypothesis, both anionic and HVJ-anionic liposomes were combined with cationized gelatin and protamine sulfate. When anionic liposomes without fusion protein were combined with protamine sulfate or cationized gelatin, the transfection efficiency increased compared with that of liposomes alone (Figure 4A). The combination of cationized gelatin–liposomes with protamine sulfate further enhanced transfection efficiency. A similar enhancement of transfection by protamine sulfate and cationized gelatin was seen in HVJ–liposomes (anionic liposomes with fusion proteins) (Figure 4B). However, the absolute value of luciferase gene expression by protamine sulfate–cationized gelatin–HVJ-liposomes was approximately 20 times higher than that by protamine

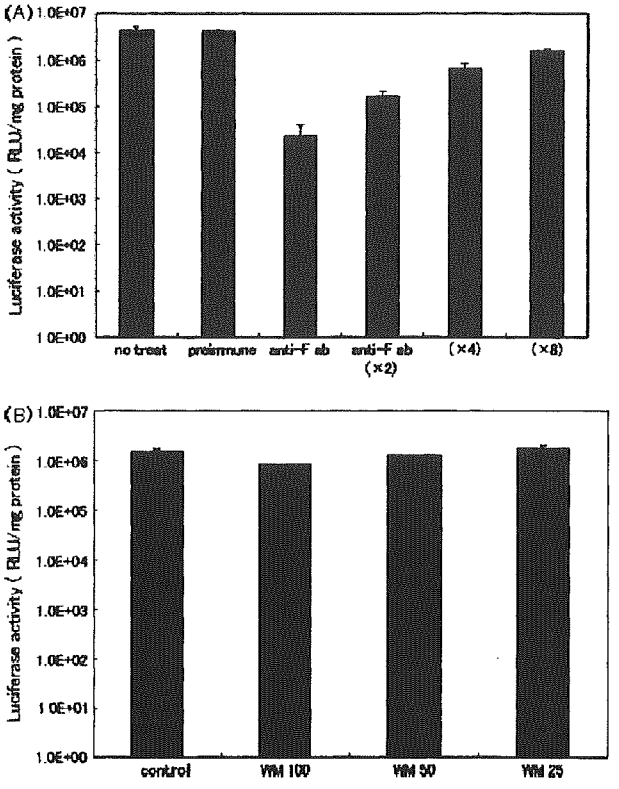

Figure 3. Effects of anti-F protein antibody (anti-F ab) (A) and wortmannin (WM) (B) on gene expression by PS-CG-HVJ-E. (A) After incubation of PS-CG-HVJ-E with antiserum, the mixture was added to CT26 cells and incubated for 10 min. Luciferase activity was measured 24 h after the removal of the mixture. Preimmune rabbit serum was used as a control. (B) CT26 cells were pretreated with various concentrations of wortmannin for 15 min. Then, the cells were subjected to gene transfer with PS-CG-HVJ-E. Luciferase activity was measured 24 h after transfer. Results are shown as mean ± s.d. (n = 3). Similar results were obtained in three independent experiments

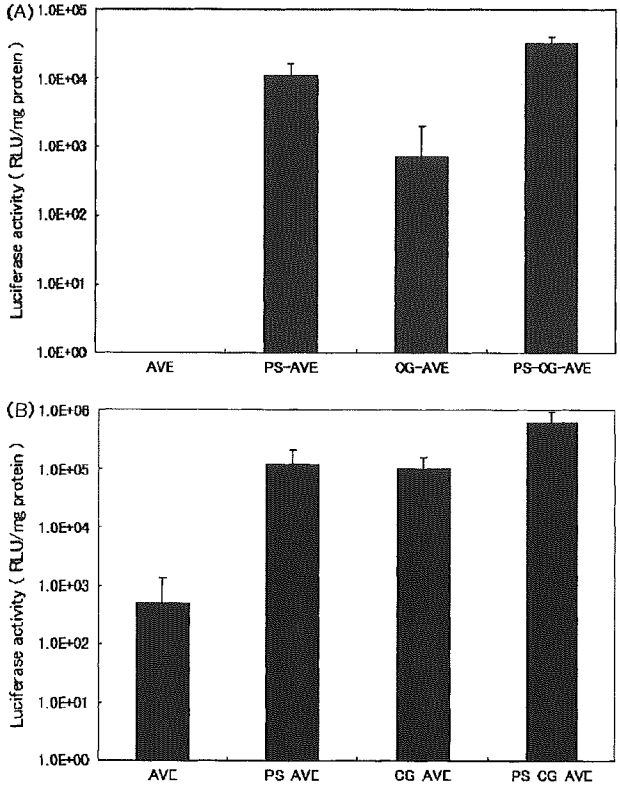

Figure 4. The effect of protamine sulfate, cationized gelatin or both on transfection efficiency by anionic liposomes (A) and anionic liposomes fused with HVJ (B). Vectors were incubated with CT26 cells for 1 h, and the luciferase activity was assessed after 24 h. AVE means anionic liposome with the same lipid components as the HIV envelope [51]. Results are shown as mean ± s.d. (n = 3). Similar results were obtained in three independent experiments

sulfate–cationized gelatin–liposomes without HVJ. Thus, gene transfer by PS-CG-HVJ-E appeared to be mediated by fusion and enhanced by endocytosis.

Specific localization of cationized gelatin-conjugated HVJ-E via intravenous administration

Next, the effect of polymer conjugation with HVJ-E on gene transfection *in vivo* was investigated (Figure 5). When HVJ-E alone was intravenously injected into the mouse tail vein, gene expression was mainly detected in the spleen. However, the gene expression was low. To enhance gene expression, HVJ-E combined with either protamine sulfate or cationized gelatin was injected into the mouse tail vein. Conjugation with protamine sulfate slightly increased luciferase expression in the liver, spleen and lung. However, CG-HVJ-E specifically enhanced gene expression in the liver approximately 100 times more than HVJ-E alone and approximately 10 times more than PS-HVJ-E. In the lung and spleen, very low levels of gene expression were observed, but no expression was detected in other organs, such as the kidney and heart. In this case, injection of PS-CG-HVJ-E resulted in lower luciferase gene expression in liver than injection of CG-HVJ-E.

Assessment of the stability of HVJ-E conjugated to cationized gelatin mixed with mouse fresh serum in comparison with HVJ-E alone

Finally, to clarify the role of cationized gelatin in enhanced *in vivo* gene transfection efficiency, CG-HVJ-E containing the luciferase gene was added to cultured cells to assess transfection efficiency after incubation with fresh mouse serum for 5 min. The transfection efficiency of HVJ-E was attenuated by incubation with mouse serum. Luciferase gene expression after the incubation of HVJ-E with fresh mouse serum at 37 °C decreased to 20% of the luciferase gene expression in the absence of mouse serum. On the other hand, luciferase gene expression after the incubation of PS-HVJ-E, CG-HVJ-E and PS-CG-HVJ-E with fresh mouse serum at 37 °C was 52.9, 72.5 and 56.7%, respectively, of the luciferase gene expression in the absence of mouse serum (Figure 6). CG-HVJ-E was

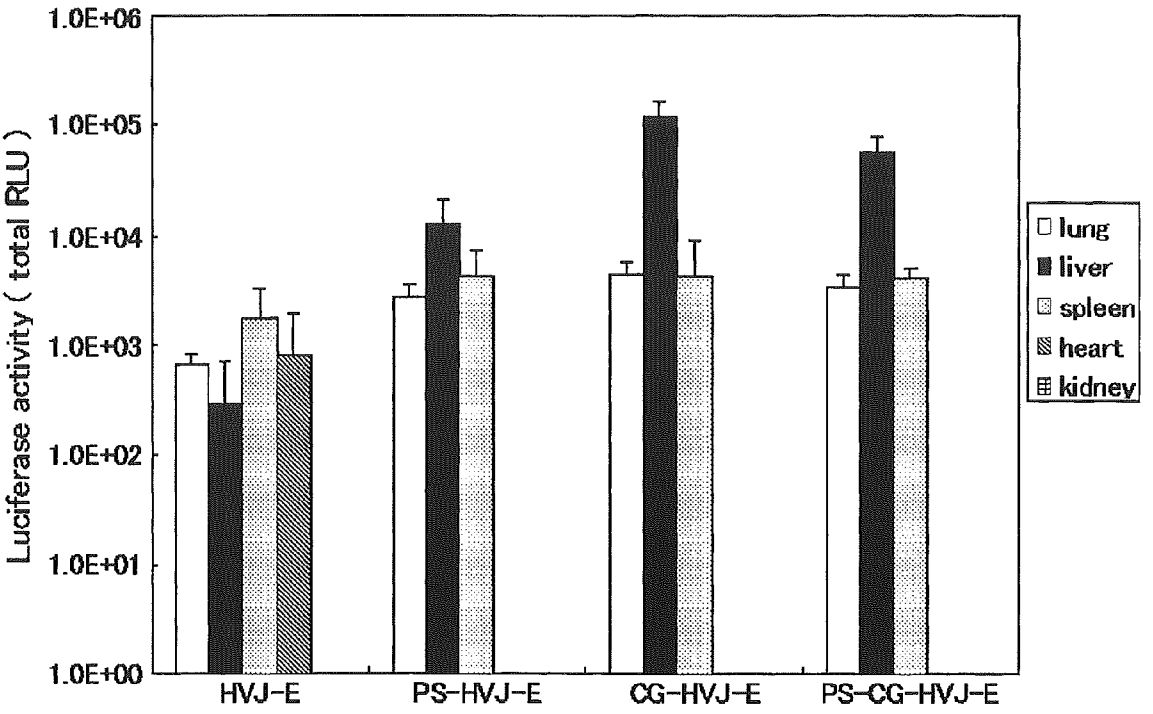

Figure 5. *In vivo* gene transfection efficiency of HVJ-E, PS-HVJ-E, CG-HVJ-E and PS-CG-HVJ-E after injection into mouse tail vein. Luciferase activity was measured in organ lysates 24 h after injection and the results are expressed as mean ± s.d. of luciferase activity of each organ from 5 to 6 mice. The group of CG-HVJ-E showed significantly higher gene expression in liver than all other groups ($P < 0.05$). Similar results were obtained in four independent experiments

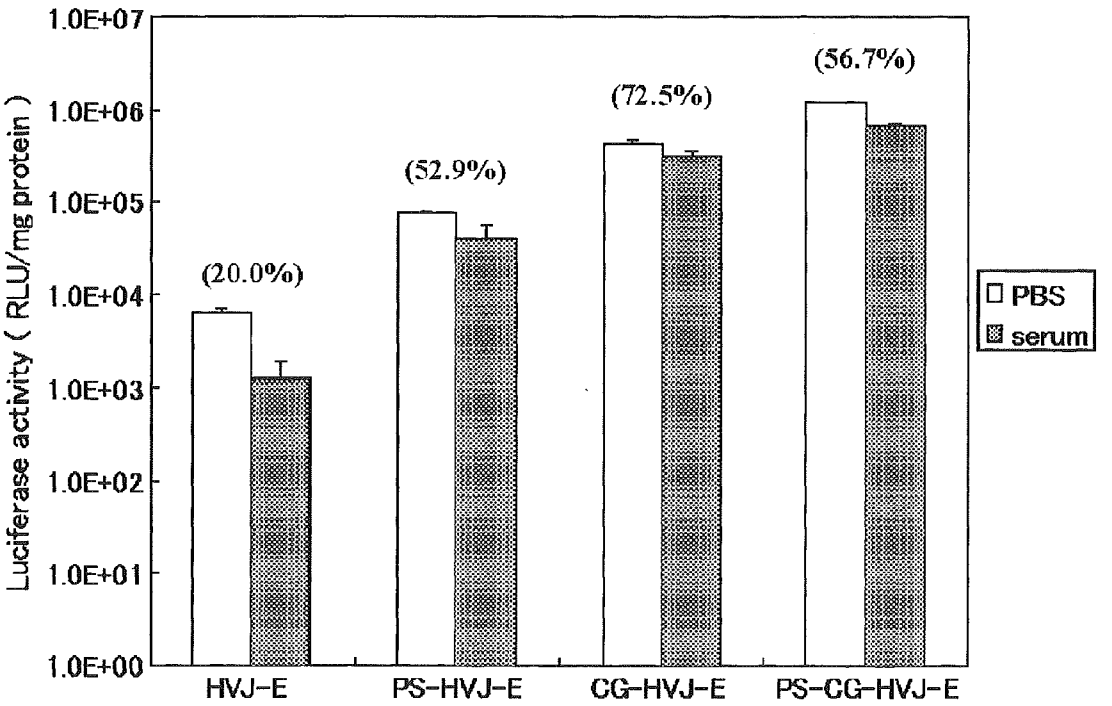

Figure 6. The effect of fresh serum on the transfection efficiency of HVJ-E or polymer-conjugated HVJ-E. After incubation of HVJ-E or polymer-conjugated-HVJ-E with fresh mouse serum, the serum was removed by centrifugation and added to CT26 cells. Luciferase activity was measured 24 h after removal of the vector. The percentage indicates the ratio of luciferase gene expression after incubation with serum (n = 3) to the luciferase gene expression after incubation with PBS (n = 3). Results are shown as mean ± s.d., respectively. Similar results were obtained in three independent experiments

the most resistant to mouse serum. Thus, we succeeded in developing a serum-resistant vector system.

Discussion

We succeeded in enhancing the transfection efficiency of HVJ-E by combining it with cationic polymers. For cultured cells *in vitro*, the most efficient transfection was obtained by combining HVJ-E with both cationized gelatin and protamine sulfate. However, for *in vivo* transfection, CG-HVJ-E without protamine sulfate resulted in the highest gene expression. These findings are consistent with our previous report indicating that the particle size of cationic liposomes may affect gene transfection efficiency [36]. By adding both protamine sulfate [37] and cationized gelatin to HVJ-E, the size and charge of the resulting complex may have been the most suitable for *in vitro* transfection. Protamine sulfate and cationized gelatin affected gene transfection efficiency in a variety of cell lines as well as in primary cells, although the efficiency was varied among cell types. The ratio of protamine sulfate and cationized gelatin used for these experiments was determined by gene transfection experiments with CT26 cells. Thus, gene expression in the other cell types may be enhanced when the conditions are optimized for each cell type.

We determined that cell fusion is the mechanism responsible for a PS-CG-HVJ-E-mediated gene transfer system. Although endocytosis appeared to be involved in gene transfection based on the wortmannin experiments, transfection was completely inhibited by antibody against the fusion protein of HVJ. Since the fusion activity of HVJ is pH-independent [31], HVJ can fuse with the cell membrane both on the cell surface and in endocytotic vesicles. Even for the HVJ-E complex with protamine sulfate and cationized gelatin, the F protein of HVJ appeared to associate with the cell membrane, and fusion activity appeared to be necessary for gene transfection.

As shown in Figure 5, HVJ-E complexed with cationized gelatin targeted the liver. With protamine sulfate, gene expression in the liver after intravenous injection was lower than with CG-HVJ-E. We speculate that larger particles with positive charge are less mobile when intravenously administered. Comparison with PS-HVJ-E and PS-CG-HVJ-E suggests that CG-HVJ-E may have the appropriate size and potential for targeting the liver after intravenous injection.

Numerous biocompatible polymers have been developed to enhance gene delivery systems [38–45]. Pullulan complexed with naked DNA targets the liver [46,47]. However, pullulan–HVJ-E complexes failed to transfect tissues, including the liver. Dextran–HVJ-E was also not an efficient complex for gene transfer. Only low molecular weight cationized gelatin has formed an effective complex with HVJ-E that enhances transfection efficiency both *in vitro* and *in vivo*, although the precise mechanism is still unknown.

Our results suggest that the CG-HVJ-E vector may be effective and practical for the treatment of liver diseases, such as liver cirrhosis and hepatitis, when therapeutic genes encoding secreted proteins, such as HGF, soluble TGF-β receptor and decorin, are employed. Moreover, long-term gene expression in the liver can be achieved with Epstein-Barr virus replicon plasmid [33] and the Sleeping Beauty transposon system [48]. CG-HVJ-E may be clinically tested in the near future because it does not require a large volume of solution to be injected (as used in the hydrodynamic method) [48,49]. An adverse effect of this treatment is that coagulation function is transiently decreased by CG-HVJ-E in mice, although it recovered in 1 day (H. Mima and Y. Kaneda, unpubl. obs.). This adverse effect is probably caused by HVJ hemagglutinating protein, which is necessary for binding with sialic acid, a virus receptor [32]. When HVJ-E is complexed with cationized gelatin, cationized gelatin may perform the function of hemagglutinating protein and enhance the association with cell membranes. If HVJ-E without hemagglutinating protein is combined with cationized gelatin, the complex may reduce adverse effects to a much lower level.

An additional advantage of cationized gelatin is that it protects HVJ-E from degradation in fresh mouse serum. Although the *in vitro* transfection efficiency of HVJ-E was not inhibited by culture medium containing 10% FBS [32], the activity of HVJ-E was rapidly lost in the presence of fresh mouse serum (Figure 6). However, CG-HVJ-E was significantly stable in 50% fresh mouse serum. The high transfection activity of CG-HVJ-E after intravenous injection appears to be mediated by the stability of the vector in fresh serum. Retrovirus [50] and HIV [51] are degraded in human serum due to complement lysis. Liposomes composed of hydrogenated egg phosphatidylcholine and cholesterol activate the complement system in rats by interacting with IgG and IgM [52]. Although it is unproven that HVJ is degraded by complement lysis in mouse serum, the interaction of serum proteins with HVJ-E may be involved in the loss of transfection activity of HVJ-E. Conjugation to cationized gelatin appears to protect the surface molecules of HVJ-E from the detrimental effects of serum proteins.

The results of this study suggest that low molecular weight cationized gelatin may be appropriate for complex formation with various envelope viruses, such as retrovirus, herpes virus and HIV, and that the cationized gelatin–envelope virus vector may enhance transfection efficiency both *in vitro* and *in vivo*. This technology may lead to the achievement of an ideal vector system with high efficiency and minimal toxicity.

References

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