polymer mass remaining by day 63, indicating complete degradation (Fig. 6). However, none of the samples from the OPF 3K groups had completely degraded by day 63, with ~35–45% of the initial polymer mass remaining (Fig. 6). The negative values observed for some groups stem from the determination of the fraction of the initial polymer mass remaining using separate samples at each time point and an average value for the initial polymer mass. Thus, the actual initial mass of a sample from a given time point may have deviated from the value determined experimentally. As a result, the swelling ratio values provide a suitable alternative to monitor hydrogel degradation, as they allow for nondestructive tracking of individual release samples over the course of the study.

The concentration of plasmid DNA in each release solution was quantified fluorescently through use of the PicoGreen and OliGreen reagents. PicoGreen and OliGreen have been applied previously toward the quantification of double-stranded DNA (dsDNA) and total DNA (both dsDNA and single-stranded DNA (ssDNA)), respectively, in release solutions.[17] The difference between the detection of DNA within a solution by the OliGreen reagent (total DNA) and PicoGreen reagent (dsDNA) can be attributed to ssDNA.[17] As expected, the amount of DNA quantified by the OliGreen reagent (total DNA) was greater than or equal to the amount quantified by the PicoGreen reagent (dsDNA) for each group at each time point in the study. This difference between the amount of DNA detected by OliGreen and PicoGreen was only significant at the time points just prior to complete degradation of the respective hydrogels in the OPF 3K and OPF 10K groups and composite groups in which DNA was loaded into the OPF component. The increased fraction of ssDNA released with time may reflect damage induced to DNA by acidic degradation products in the microenvironment as the hydrogel degrades. OPF hydrogel networks have been shown to degrade through hydrolysis of the ester bonds to form acrylic and fumaric acids among other products.[26] Indeed, similar increases in DNA damage with time of release have been demonstrated in other studies involving the release of plasmid DNA from degradable hydrogels.[17,27,28] Interestingly, the difference between the amount of DNA detected by OliGreen and PicoGreen in the composite group in which DNA was loaded into the CGMS was not statistically significant at any time point ($p < 0.05$). Potential complexation of plasmid DNA with the cationic gelatin microspheres and degradation products thereof may serve in a limited capacity to protect the DNA from degradation by acidic products of polymer degradation.[4] Further investigation, however, is required to more fully understand this observation. Although the fraction of ssDNA released from each group increased with time, the majority of DNA released from each group at every time point was dsDNA (Fig. 2).

The results clearly indicate the ability to control the release of plasmid DNA from OPF composites *in vitro*, with the majority of released DNA being double-stranded. Indeed, the structural integrity of the DNA following release is of great importance to the potential of the DNA to transfect cells and to be expressed. As a result, the structural integrity of plasmid DNA released from the materials was assessed through agarose gel electrophoresis. The results indicate that plasmid DNA converts predominately to the open-circular conformation when released from both OPF alone and from composites of OPF and CGMS (Figs. 3 and 4). DNA bands were visible for the OPF 3K group on the agarose gels, but not at every time point. This likely stems from the small amount of DNA being released from this group between days 7 and 63, relative to the other material groups. The results from PicoGreen and OliGreen clearly indicate that DNA was indeed being released from OPF 3K gels during this time frame. For some groups, bands of linear DNA were observed at later time points and can be explained by damage induced by acidic polymer degradation products. However, the majority of DNA from each group was released in either the open-circular conformation or super-coiled conformation. Although some conversion of plasmid DNA from the super-coiled to the open-circular conformation was observed, the literature suggests that little difference exists between the efficiency in transfection or transformation protocols of super-coiled or open-circular DNA, whereas linear DNA is much less efficient.[29,30] Thus, the majority of plasmid DNA released from hydrogel composites of OPF and CGMS retains potential for bioactivity.

CONCLUSIONS

Novel hydrogel composites of oligo(poly(ethylene glycol) fumarate) (OPF) and cationized gelatin microspheres (CGMS) were fabricated and studied toward the controlled release of plasmid DNA *in vitro*. Control of the release of plasmid DNA from hydrogels and hydrogel composites appeared to be dominated by the degradation of the OPF network. Additionally, the manner in which the plasmid DNA was loaded into the composites appeared to affect the DNA release kinetics, with loading into the CGMS prolonging the DNA release. The released DNA maintained suitable structural form for transfection based upon results of electrophoretic analysis. The controlled release of DNA from composites of OPF and CGMS, coupled with the potential for the creation of a porous hydrogel network upon CGMS degradation, demonstrates

the potential of the composites for controlled gene delivery in tissue engineering applications.

The authors also thank Dr. Samuel Campos for creation of the pCMV-hBMP2 plasmid.

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Biochemical and Biophysical Research Communications 334 (2005) 1121–1126
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Magnetic nanoparticles with surface modification enhanced gene delivery of HVJ-E vector

Norio Morishita [a,b], Hironori Nakagami [c,*], Ryuichi Morishita [b], Shin-ichi Takeda [d], Fumihito Mishima [d], BungoTerazono [d], Shigehiro Nishijima [d], Yasufumi Kaneda [c], Noriaki Tanaka [a]

[a] *Department of Gastroenterological Surgery, Transplant, and Surgical Oncology, Graduate School of Medicine and Dentistry, Okayama University, Okayama, Japan*
[b] *Division of Clinical Gene Therapy, Graduate School of Medicine, Osaka University, Osaka 565-0871, Japan*
[c] *Division of Gene Therapy Science, Graduate School of Medicine, Osaka University, Osaka 565-0871, Japan*
[d] *Division of Sustainable Energy and Environmental Engineering, Graduate School of Engineering, Osaka University, Osaka 565-0871, Japan*

Received 24 June 2005
Available online 14 July 2005

Abstract

To enter the realm of human gene therapy, a novel drug delivery system is required for efficient delivery of small molecules with high safety for clinical usage. We have developed a unique vector "HVJ-E (hemagglutinating virus of Japan-envelope)" that can rapidly transfer plasmid DNA, oligonucleotide, and protein into cells by cell-fusion. In this study, we associated HVJ-E with magnetic nanoparticles, which can potentially enhance its transfection efficiency in the presence of a magnetic force. Magnetic nanoparticles, such as maghemite, with an average size of 29 nm, can be regulated by a magnetic force and basically consist of oxidized Fe which is commonly used as a supplement for the treatment of anemia. A mixture of magnetite particles with protamine sulfate, which gives a cationic surface charge on the maghemite particles, significantly enhanced the transfection efficiency in an in vitro cell culture system based on HVJ-E technology, resulting in a reduction in the required titer of HVJ. Addition of magnetic nanoparticles would enhance the association of HVJ-E with the cell membrane with a magnetic force. However, maghemite particles surface-coated with heparin, but not protamine sulfate, enhanced the transfection efficiency in the analysis of direct injection into the mouse liver in an in vivo model. The size and surface chemistry of magnetic particles could be tailored accordingly to meet specific demands of physical and biological characteristics. Overall, magnetic nanoparticles with different surface modifications can enhance HVJ-E-based gene transfer by modification of the size or charge, which could potentially help to overcome fundamental limitations to gene therapy in vivo.

Keywords: Magnetic nanoparticle; HVJ-E; Drug delivery system; Gene therapy

Progress in gene therapy requires a novel drug delivery system (DDS). To enhance the transfection efficiency, we developed a hybrid vector utilizing the envelop of HVJ (hemagglutinating virus of Japan). The HVJ-E vector rapidly transfers plasmid DNA, oligonucleotide or protein into cells by cell fusion [1]. However, this modality of targeting is still insufficient for rapid and specific accumulation of active vectors in target tissues. One solution is to engineer the surface proteins of viral vectors or to couple targeting ligands to viral as well as non-viral vectors, which might further improve tissue selectivity [2–5]. Thus, we examined modification of the membrane surface of HVJ to achieve an improvement.

In this study, we focused on magnetic nanoparticles, such as magnetite and maghemite, with an average size

* Corresponding author. Fax: +81 6 6879 3909.
E-mail address: nakagami@gts.med.osaka-u.ac.jp (H. Nakagami).

0006-291X/$ - see front matter © 2005 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2005.06.204

from 20 to 50 nm, which can be positionally regulated by a magnetic force. Magnetic nanoparticles basically consist of oxidized Fe, which is commonly used as a supplement for the treatment of anemia. We hypothesized that the association of magnetite and HVJ-E technology could allow the rapid attachment of HVJ-E and cells by application of a magnetic force, leading to enhanced transfection efficiency. Moreover, magnetic nanoparticles can be modified with several chemical compounds to allow modification of the charge, size, and affinity. In addition, there is increasing interest in using magnetic resonance imaging (MRI) to monitor the in vivo behavior of proteins labeled with magnetic nanoparticles [6,7]. Indeed, magnetic nanoparticles were recently used for gene transfection, because magnetic targeting exploits paramagnetic particles as drug carriers, guiding their accumulation in target tissues with strong local magnetic fields [8,9]. Here, the present study demonstrated that modification of magnetic nanoparticles with protamine sulfate or heparin enhanced the transfection efficiency associated with HVJ-E system in cultured cells in vitro and in murine liver in vivo.

Materials and methods

Preparation of HVJ-E vector and plasmid DNA. HVJ-E vector was obtained from Ishihara (Osaka, Japan). Basically, aliquots of the inactivated virus (6 AU) were suspended in 40 μl TE solution (10 mM Tris–Cl, pH 8.0, 1 mM EDTA) and mixed with plasmid DNA (30 μg), and 0.3% Triton X. The mixture was centrifuged at 18,500*g* for 15 min at 4 °C. After washing the pellet with 1 ml balanced salt solution (BSS; 10 mM Tris–Cl, pH 7.5, 137 mM NaCl, and 5.4 mM KCl) to remove the detergent and unincorporated DNA, the envelop vector was suspended in 300 μl BSS. The titer of the inactivated virus was modified according to each experiment. pEGFP-C1 was purchased from Clontech (CA, USA). pCMV-luciferase-GL2 or GL3 was constructed by cloning the luciferase gene from the pGL2 (for in vitro culture) or pGL3 (for in vivo experiment)-promoter vector (Promega, Madison, WI, USA) into pcDNA3 (5.4 kb) (Invitrogen, San Diego, CA, USA). Plasmids were purified with a Qiagen plasmid isolation kit (Hilden, Germany). FITC-oligodeoxynucleotides (ODN) (random sequence) were purchased from Gene Design (Osaka, Japan).

Preparation of magnetic HVJ-E vector. Commercially available maghemite particle (NanoTek γ-Fe_{23}, C.I. KASEI) was used for the preparation of magnetic HVJ-E vector and plasmid DNA in the present study. The averaged primary size of maghemite particle used in this study was 29 nm. The particles were mixed with protamine sulfate or haparin solution to prepare magnetic nanoparticles modified with bio-polymers, which consist of cationic polymer adsorbed with ferromagnetic nanosized particles. The prepared magnetic nanoparticles were then mixed with HVJ-E vector to provide ferromagnetism to the HVJ-E vector.

Measurement of zeta potential of maghemite particles. Zeta potential is an important parameter in understanding electrostatic colloidal dispersion stability. Zeta potential is the charge that a particle acquires in a particular medium. It is dependent upon the pH, ionic strength and concentration of a particular component. The mobility of particles undergoing electrophoresis is measured by the technique of laser Doppler electrophoresis. This measured electrophoretic mobility is then converted to zeta potential using established theories. In order to know the surface property of the magnetic vector, the zeta potential of the particles with and without surface modification was determined using a capillary cell electrophoresis technique (Zetasizer Nano ZS, Malvern, UK). Mixtures of magnetite particles with and without surface modification with protamine sulfate or heparin and the prepared magnetic HVJ-E vector and plasmid DNA were used.

Gene transfer in vitro and in vivo. For in vitro transfection, 5×10^5 cells were prepared in 6-well culture dishes at 1 day before transfection. HVJ-E vector (1 or 0.2 AU) containing luciferase plasmid (GL2: 5 or 1 μg) or FITC-ODN (2 or 0.4 μg) was mixed with various concentrations of magnetite and DMEM containing 10% FCS, and added to cells cultured in DMEM supplemented with 10% FBS on a magnetic sheet. The magnetic sheet was placed under the 6-well culture dish. The magnetic field gradient is one of the parameters affecting the strength of the magnetic force applied to the magnetic vectors to sediment to the cell surface. Magnetic field measurements were performed by Hall detector to calculate the magnetic field gradient. A 3-axis Hall effect teslameter (MetroLab THM 7025, METROLAB Instruments SA, Switzerland) was used to measure the magnetic field. The averaged value of the measured magnetic field at the sheet surface was approximately 1.2 mT and the calculated value of the field gradient was 1.7 mT/cm. After 10 min of incubation at 37 °C under 5% CO_2, the medium was replaced and the cells were cultured overnight before examination of gene expression. GFP expression or FITC ODN-transfected cells were observed under a fluorescence microscope.

For in vivo transfection, HVJ-E vector (2 AU) containing luciferase gene or GFP (10 μg) suspended in 100 μl PBS was injected directly into the liver of BALB/c mice (8 weeks of age; $n = 5$–9) with several kinds of magnetite. All animals were handled in a humane manner in accordance with the guidelines of the Animal Committee of Osaka University. Frozen sections of liver were stained with hematoxylin or immunostained with anti-GFP antibody (Molecular Probes, Eugene, OR).

Luciferase activity and LDH release. Luciferase activity was measured following the manufacturer's instructions (Promega Madison) [10]. Twenty-four hours after transfection, cells were washed once with PBS and then incubated with lysis buffer. The protein concentration was determined using the Bio-Rad protein assay adapted for use in a 96-well plate. After 20 μl cell extract was mixed with 100 μl luciferase assay reagent (Promega, Madison), the light produced was measured for 2 s using a luminometer.

The extent of cell death was assessed using a commercially available kit (Wako, Osaka) to measure released LDH activity from dead cells according to previous reports [11,12], because loss of cell membrane integrity was observed in both necrotic and apoptotic cells. The percentage of LDH release was calculated relative to the maximum cell death of cells treated with 1% Triton X-100 for 10 min.

Electronmicroscopy. Electron microscopic observation was performed as previously described [1]. After mixing HVJ-E vector and PS-coated magnetite, they were fixed at 4 °C with 1% glutaraldehyde for 2 h and washed twice with 0.1 M phosphate buffer, 10% sucrose. They were further fixed with 1% osmium tetroxide for 30 min and washed twice with 0.1 M phosphate buffer 10% sucrose, and dehydrated through a graded ethanol series (70–100%) and propylene oxide. They were then embedded in epoxy resin (Quetol 812). For transmission electron microscopic (TEM) observation, ultrathin sections were obtained with an ultramicrotome, stained doubly with uranyl acetate and lead citrate, and observed under an electron microscope (H-7100 Hitachi) at an accelerating voltage of 75 kV.

Statistical analysis. All values are expressed as means ± SEM. Analysis of variance with subsequent Bonferroni's/Dunnet's test was employed to determine the significance of differences in multiple comparisons. Values of $P < 0.05$ were considered statistically significant.

Results

Basic characteristics of magnetic nanoparticle, maghemite

To evaluate the HVJ-E vector system mixed with maghemite, we measured luciferase activity to evaluate

transfection activity and LDH release to evaluate cell toxicity after transfection. We mixed several doses of maghemite (from 100 ng/ml to 1 mg/ml) with HVJ-E vector infusing luciferase plasmid. However, unexpectedly, we did not find any improvement in transfection efficiency as assessed by luciferase activity in BHK-21

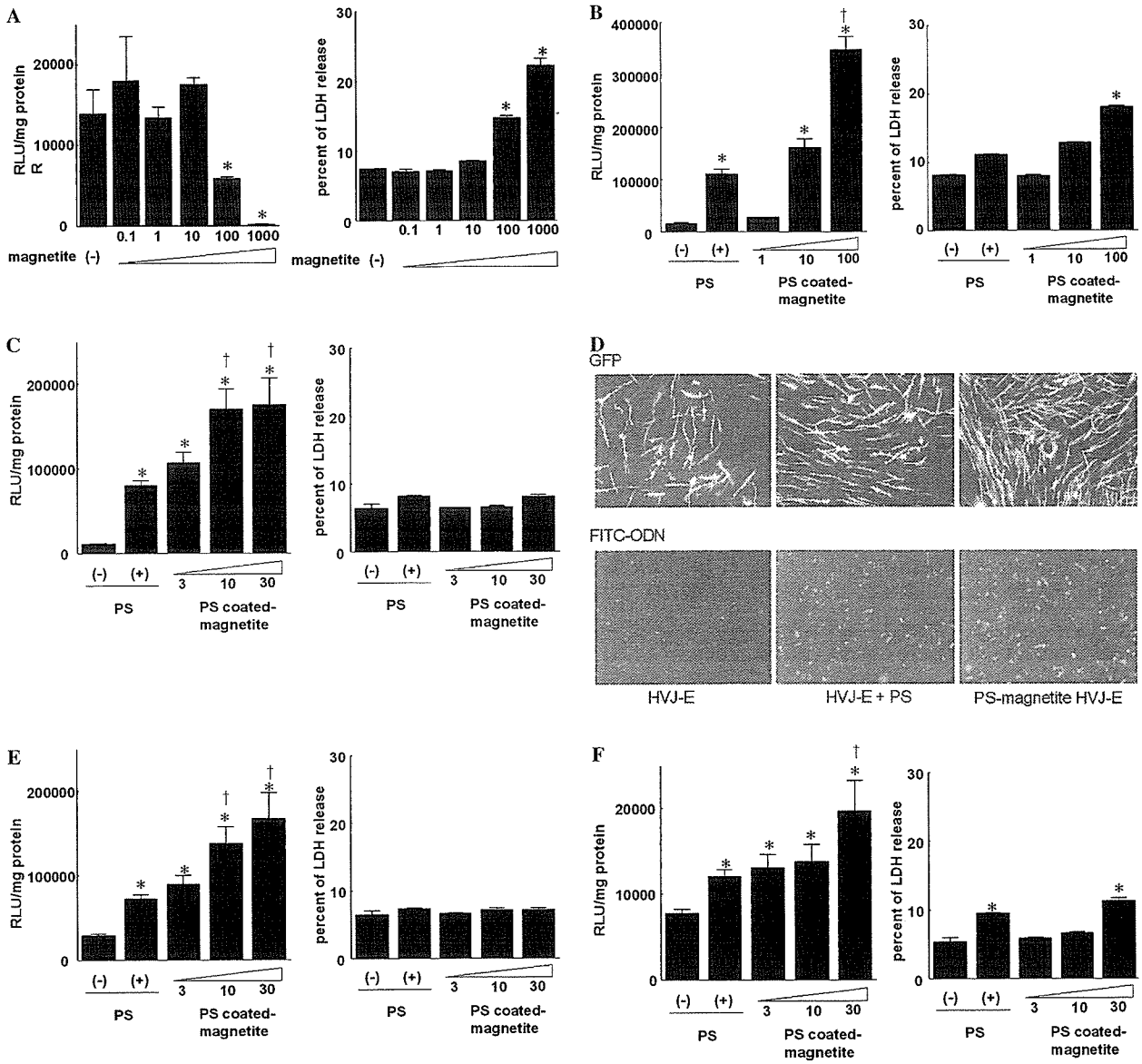

Fig. 1. Transfection efficiency (luciferase activity) and cell toxicity (LDH release) of maghemite-associated HVJ-E vector. (A) HVJ-E vector (1 AU) containing the luciferase expression plasmid (5 μg) was transferred to BHK-21 cells seeded in 6-well dishes with various concentrations of non-coated maghemite and incubated for 10 min. The final concentrations of maghemite were as follows: 0, 0.1, 1, 10, 100, and 1000 μg/ml non-coated maghemite. $^{*}P < 0.01$ vs. magnetite (−). (B) HVJ-E vector (1 HAU) containing the luciferase expression plasmid (5 μg) was transferred to BHK-21 cells seeded in 6-well dishes with various concentrations of protamine sulfate-coated maghemite and incubated for 10 min. The final concentrations of PS and PS-coated maghemite were as follows: 100 μg/ml PS, 1, 10, and 100 μg/ml PS-coated maghemite. (C) HAU HVJ-E vector (0.2 HAU) containing the luciferase expression plasmid (1 μg) was transferred to BHK-21 cells with various concentrations of magnetite and incubated for 10 min. The final concentrations of PS and PS-coated maghemite were as follows; 100 μg/ml PS, 3, 10, and 30 μg/ml PS-coated maghemite. Luciferase gene expression was examined 24 h after transfer. Results are shown as means + SE. $^{*}P < 0.01$ vs. PS(−), $^{\dagger}P < 0.01$ vs. PS(+). (D) Representative example of BHK-21 cells transfected with the EGFP plasmid (1 μg) or FITC-ODN (1 μg) using PS-coated maghemite (30 μg/ml) with HVJ-E vector (0.2 AU) under fluoromicroscopy. Upper panels show EGFP overexpressing cells and lower panels show FITC ODN-overexpressing cells (magnification 100×). (E,F) HVJ-E vector (0.2 AU) containing luciferase expression plasmid was transferred to (E) COS7 and (F) Bovine aortic endothelial cells seeded in 6-well dished with various concentrations of maghemites and incubated for 10 min. The final concentration of PS or PS-coated maghemite are as follows: 100 μg/ml PS, 3, 10, and 30 μg/ml PS-coated maghemite. Luciferase gene expression was examined 24 h after transfer. Results are shown as means + SE. $^{*}P < 0.01$ vs. PS(−), $^{\dagger}P < 0.01$ vs. PS(+).

cells, whereas a high dose of maghemite induced cell death as assessed by LDH release (Fig. 1A). From these results, we speculated that there might be a close association, such as electrostatic interaction, between HVJ-E and maghemite. Since we have previously reported that protamine sulfate (PS), a low-molecular-weight naturally polycationic peptide (~4000 Da), enhanced the transfection efficiency based on the HVJ-E vector in an in vitro culture system [1], we coated the surface of maghemite with PS. After modification with PS, the zeta potential of maghemite was changed to 23.8 ± 1.8 mV from 17.5 ± 1.6 mV, which suggests that surface coating of maghemite enhanced its cationic charge. Interestingly, a mixture of PS-coated maghemite with HVJ-E vector significantly enhanced the transfection efficiency in a dose-dependent manner (Fig. 1B, $P < 0.01$). However, induction of cell death was still detected at a high dosage. To avoid cell toxicity, we reduced the PS-coated maghemite dose and titer of HVJ-E. Finally, we found a suitable dose of PS-coated maghemite (30 μg/ml) and HVJ-E titer (0.2 AU) for BHK cells (Fig. 1C). Using this combination, addition of PS-coated maghemite to the HVJ-E vector significantly enhanced transfection efficiency without apparent cell toxicity (Fig. 1C, $P < 0.01$). Using the same system, high transfection efficiency into BHK cells was also achieved using EGFP gene or FITC-oligodeoxynucleotide (Fig. 1D). We also examined the transfection efficacy of this novel vector system in COS7 cells and bovine aortic endothelial cells. Although the absolute values of transfection efficiency were different in each cell type, high transfection efficiency could be obtained using the luciferase gene in COS7 cells without apparent cell toxicity (Fig. 1E, $P < 0.01$). Similarly, in bovine aortic endothelial cells, a mixture of PS-coated magnetite with the HVJ-E vector significantly enhanced the transfection efficiency in a dose-dependent manner (Fig. 1F, $P < 0.01$). However, in bovine aortic endothelial cells, treatment with PS (100 ng/ml) or high-dose PS-coated maghemite (30 μg/ml) induced cell toxicity assessed by LDH release. Thus, the optimum

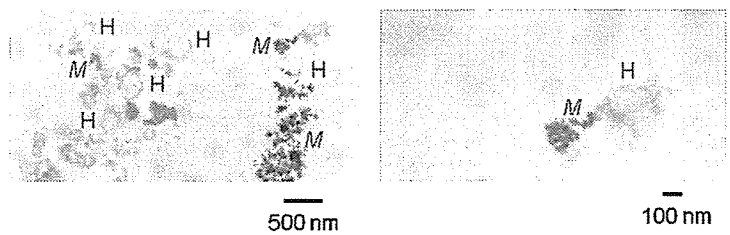

Fig. 2. Representative transmission electron microscopic (TEM) views showing co-existence of HVJ-E vector and PS-coated maghemite: H, HVJ-E vector; M, PS-coated maghemite. Bar = 500 nm in the left panel, and 100 nm in the right panel.

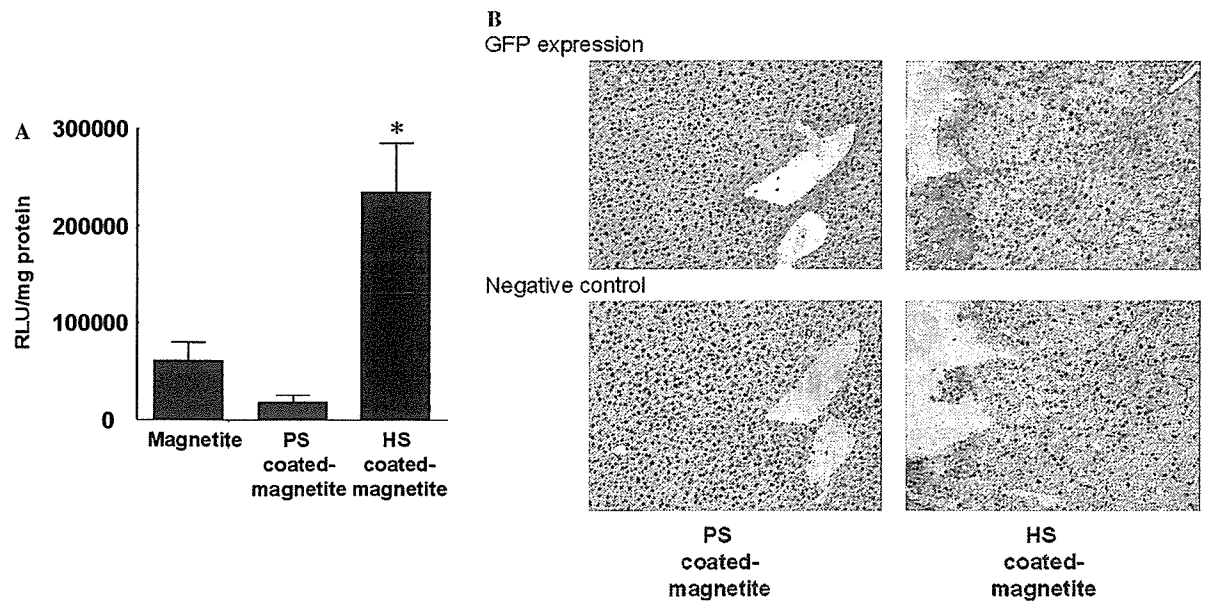

Fig. 3. Transfection efficiency (luciferase activity or EGFP expression) of protamine sulfate (PS)- or heparin sulfate (HS)-coated maghemite with HVJ-E vector. HVJ-E vector (2 AU) containing luciferase (A) or EGFP (B) was injected into the liver with non-coated maghemite, 30 μg/ml PS-coated maghemite, or 30 μg/ml heparin sulfate-coated maghemite.

Table 1
Serum AST, ALT, and LDH levels with each type of transfection

	AST (U/L)	ALT (U/L)	LDH (IU)
HVJ-E only	266 ± 32	205 ± 43	804 ± 79
HVJ-E-PS-coated magnetite	411 ± 95	147 ± 21	1113 ± 166
HVJ-E-HS-coated magnetite	297 ± 45	161 ± 91	803 ± 55

PS, protamine sulfate; HS, heparin sulfate.

dose of magnetic particles should be evaluated in each cell type.

To confirm the interaction of magnetite and the HVJ-E vector, we performed electron microscopy. PS-coated maghemite and the HVJ-E vector co-existed even though a number of maghemite particles were aggregated around the HVJ-E vector (Fig. 2).

To explore transfection in vivo, we further evaluated the maghemite-attached HVJ-E vector by direct injection into mouse liver. Unexpectedly, a mixture of maghemite surface-coated with PS did not enhance the transfection efficiency in the analysis of luciferase activity, differing from the in vitro system. Since co-treatment with heparin has been reported to enhance the transfection efficiency of the HVJ-E vector in brain tissue [13], we next coated the surface of maghemite with heparin, which might antagonize the function of PS. The zeta potential of maghemite was changed to -10.1 ± 2.3 mV from 17.5 ± 1.6 mV, which suggests that the surface coating of maghemite was changed to a fairly negative net charge. A mixture of heparin-coated maghemite with HVJ-E vector significantly enhanced the transfection efficiency in the liver (Fig. 3A, $P < 0.01$). In contrast, in BHK-21 cells, a mixture of heparin-coated maghemite with HVJ-E vector did not increase the luciferase activity (data not shown). These data suggest that suitable modifications for gene transfection differ between in vitro and in vivo. Analysis of EGFP expression in the liver by immunostaining, also confirmed marked expression obtained with heparin-coated maghemite with HVJ-E vector containing the EGFP gene as compared to PS-coated maghemite (Fig. 3B). For safety evaluation, the serum levels of AST and ALT were not further elevated in the heparin-coated maghemite group compared to the control group (Table 1). Furthermore, we tried to enhance the transfection efficiency under the influence of a magnetic field by placing a magnet on the surface of the liver. However, no obvious improvement in luciferase activity in the liver was observed (data not shown). Probably, a stronger magnetic field or more accurate control of magnetite might be necessary to enhance it.

Discussion

In this study, we demonstrated that magnetic nanoparticles with different surface modifications enhanced HVJ-E-based gene transfer by modification of the surface charge. The fundamental principle of magnetically enhanced transfection is simple and comprises the steps of formulating a magnetic vector as described above, adding it to the medium covering cultured cells or injecting it locally into a target tissue, and in addition applying a magnetic field in order to direct the vector towards the target cells. Interestingly, PS as the coating material of nanoparticles was suitable in an in vitro cell culture system, whereas heparin was suitable for gene transfer into the mouse liver in vivo.

Recently, Plank et al. proposed a new method, magnetofection, which comprised the association of vectors with superparamagnetic iron oxide nanoparticles under the application of a magnetic field [8,14]. In this system, the gene carrier itself represents a hybrid system characterized by an iron oxide inner core and a coat consisting of the cationic polymer, polyethyleminine (PEI). Using this system, the cellular uptake by magnetofection proceeds obviously by endocytosis. Interestingly, PEI might also assist the endosomal escape of a gene after transfection into cells. In contrast, in our system, transfection was mediated by a cell fusion process of the HVJ-E vector, which did not require endosomal escape, was not by endocytosis. Thus, we selected PS as the coating material of maghemite in combination with the HVJ-E system, as PS is a low-molecular-weight polycationic peptide (~4000 Da) that is FDA approved as an antidote to heparin anticoagulation [15,16]. Since both the surface of HVJ-E and the cell membrane have a negative net charge, cationic charged PS-coated maghemite can be speculated to enhance the association of HVJ-E with the cell membrane. Thus, PS-coated maghemite would actively carry HVJ-E to cultured cells under a magnetic force.

However, in an in vivo system, heparin, but not PS, was suitable as the coating material in combination with the HVJ-E vector consistent with a previous in vivo report [13] [17]. Of special interest regarding this result was a previous report showing that co-infusion of trophic factors and heparin into the rat brain significantly enhanced the volume of distribution [18]. Thus, we speculate that the distribution of these vectors may be a key to explaining this discrepancy between in vitro and in vivo gene transfer. In addition, no immunohistochemical data has been presented on potential toxic effects of heparin. The size and surface chemistry of magnetic particles could be tailored accordingly to meet specific demands of physical and biological characteristics.

Magnetically targeted drug delivery by particulate carriers to a localized disease site may be efficient as drugs, and very high concentrations of chemotherapeutic or radiological agent can be achieved near the target site, such as a tumor, without any toxic effects on normal surrounding tissue or the whole body. Magnetic carriers receive their magnetic responsiveness to a

magnetic field from incorporated materials such as magnetite, iron, nickel, cobalt, neodymium–iron–boron or samarium–cobalt. As for biomedical applications, magnetic carriers must be water-based, biocompatible, non-toxic, and non-immunogenic. The first medical application directly applied magnetite or iron powder. The first clinical cancer therapy trial was performed in Germany for the treatment of advanced solid cancer in 14 patients using magnetic microspheres that were about 100 nm in diameter and filled with 4′-epidoxorubicin [19]. The phase I study clearly showed low toxicity of the method and accumulation of MMS in the target area. However, MRI measurements indicated that more than 50% of MMS ended up in the liver [20]. Conceptually, magnetic targeting is a very promising approach. However, there are a number of physical, magnetism-related properties which require careful attention. First, more responsive magnetic materials with defined and homogenous material properties in a stable and defined oxidation state need to be synthesized. Second, the size must be small enough that they do not clog the blood vessels through which they are guided to the target organ. Third, altering the surface of magnetite with appropriate molecules should be considered, to either increase or decrease the interaction of magnetite with tissues or organs. Fourth, the magnetite size must be uniform enough to provide an equal probability of magnetic capture for each magnetite particle. Finally, the fate of the particles in the body is an important consideration both for local and systemic short- and long-term toxicity. Thus, further improvements of this hybrid vector are required in future studies.

Overall, we have developed a novel hybrid vector of HVJ-E and magnetic nanoparticles with different surface modifications in an in vitro culture system as well as in vivo. Further modification of this system with MRI might provide new therapeutic potential to achieve tissue targeting.

Acknowledgments

We thank Koichi Mori (Anges MG. Inc.), Naho Suzuki (Genomidea, Inc.), and Naoko Saito (Osaka University) for their excellent technical assistance. This work was partially supported by a Grant-in-Aid from the Organization for Pharmaceutical Safety and Research, a Grant-in-Aid from The Ministry of Public Health and Welfare, a Grant-in-Aid from Japan Promotion of Science, and The Ministry of Education, Culture, Sports, Science and Technology, the Japanese Government.

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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

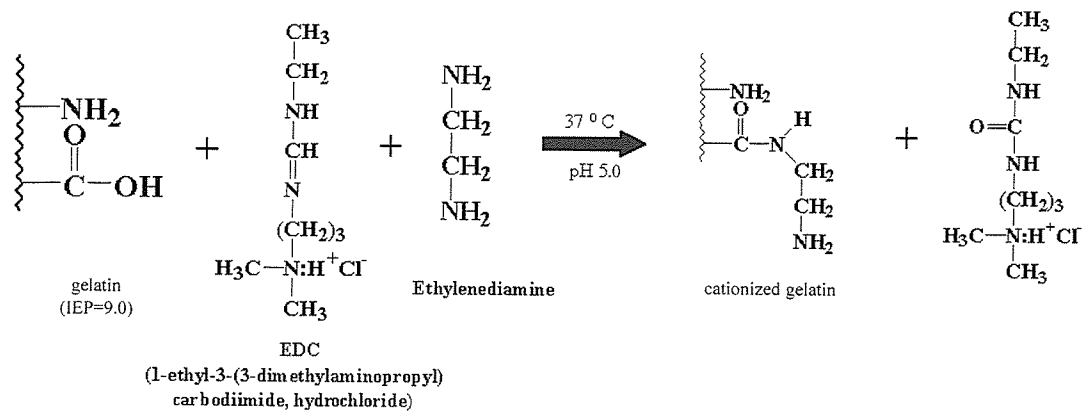

Figure 1. Synthesis of cationized gelatin. Cationized gelatin was mixed with HVJ-E containing a marker gene. The complex was isolated by centrifugation and used for transfection experiments. (IEP; isoelectric point)

(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

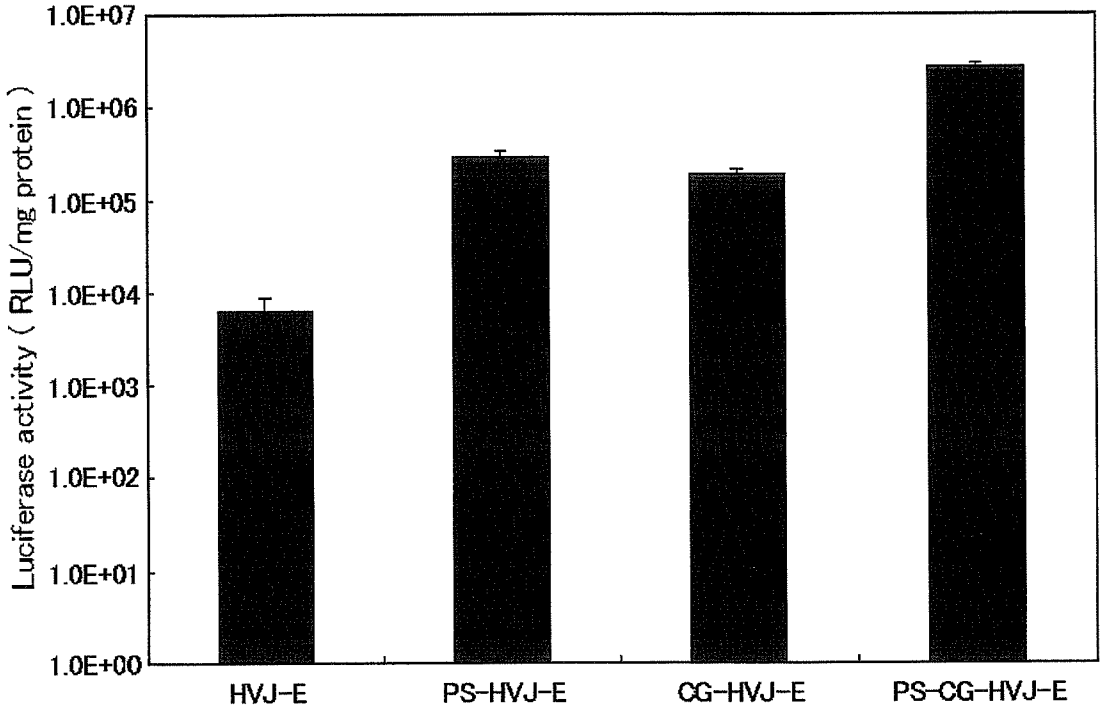

Figure 2. Luciferase gene expression in CT26 cells transfected with HVJ-E, PS-HVJ-E, CG-HVJ-E or PS-CG-HVJ-E. The vectors were incubated with cells for 10 min, and the luciferase activity was measured 24 h after removal of the vector. Results are shown as mean ± s.d. (n = 3). Similar results were obtained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

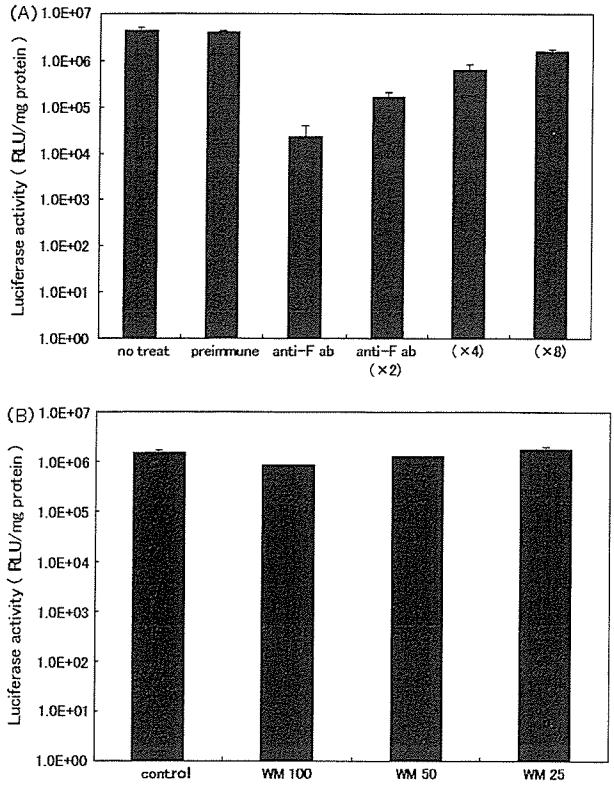

Figure 3. Effects of anti-F protein antibody (anti-F ab) (A) and wortmannin (WM) (B) on gene expression by PS-CG-HVJ-E. (A) After incubation of PS-CG-HVJ-E with antiserum, the mixture was added to CT26 cells and incubated for 10 min. Luciferase activity was measured 24 h after the removal of the mixture. 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

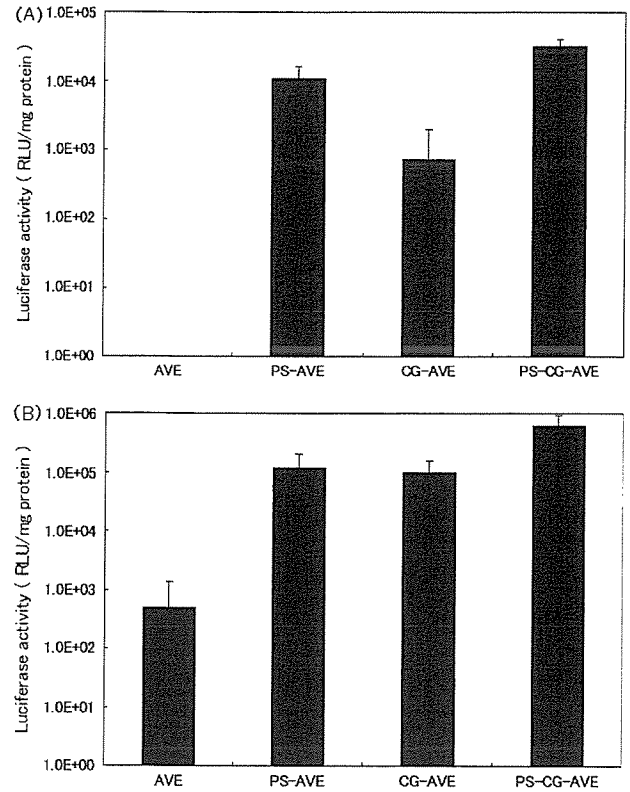

Figure 4. The effect of protamine sulfate, cationized gelatin or both on transfection efficiency by anionic liposomes (A) and anionic liposomes fused with HVJ (B). Vectors were incubated with CT26 cells for 1 h, and the luciferase activity was assessed after 24 h. AVE means anionic liposome with the 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

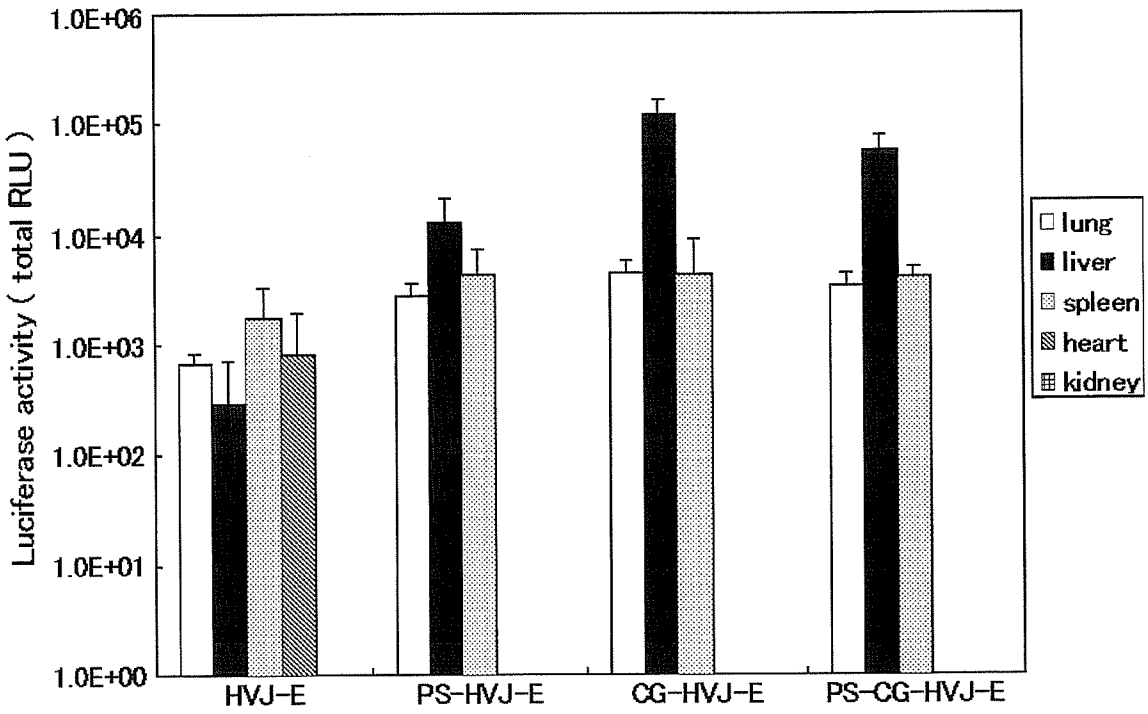

Figure 5. *In vivo* gene transfection efficiency of HVJ-E, PS-HVJ-E, CG-HVJ-E and PS-CG-HVJ-E after injection into mouse tail vein. Luciferase activity was measured in organ lysates 24 h after injection and the results are expressed as mean ± s.d. of luciferase activity of each organ from 5 to 6 mice. 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

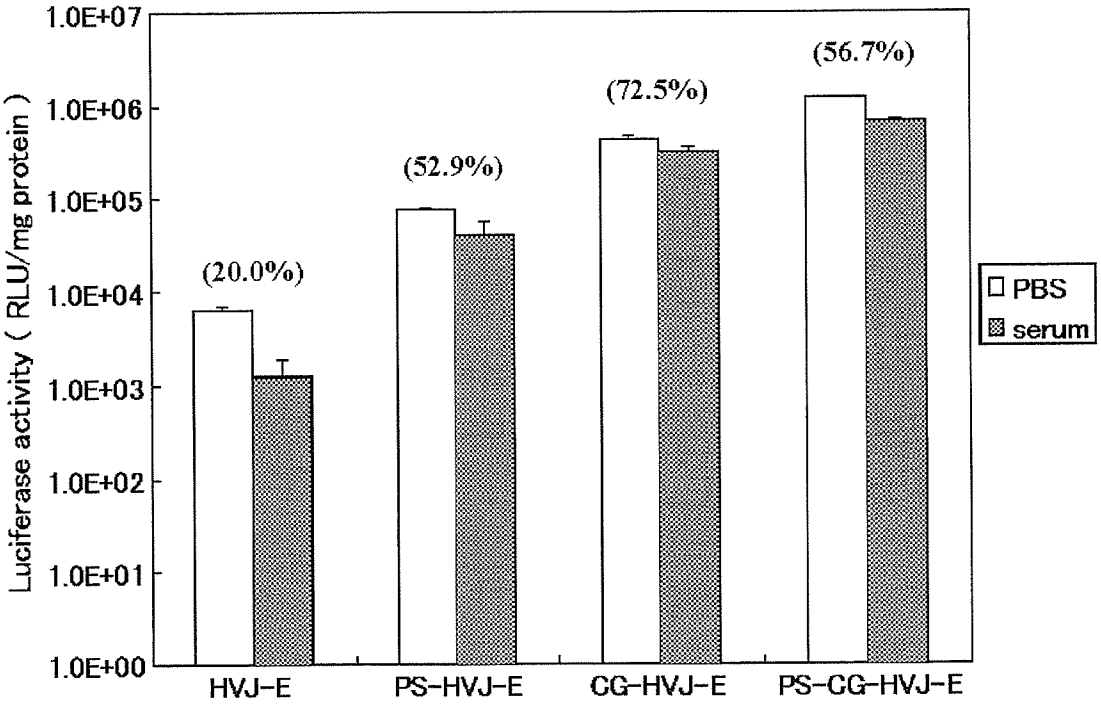

Figure 6. The effect of fresh serum on the transfection efficiency of HVJ-E or polymer-conjugated HVJ-E. After incubation of HVJ-E or polymer-conjugated-HVJ-E with fresh mouse serum, the serum was removed by centrifugation and added to CT26 cells. Luciferase activity was measured 24 h after removal of 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.

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THE JOURNAL OF GENE MEDICINE
J Gene Med 2005; 7: 1044–1052.
Published online 8 March 2005 in Wiley InterScience (www.interscience.wiley.com). **DOI:** 10.1002/jgm.753

RESEARCH ARTICLE

Rad51 siRNA delivered by HVJ envelope vector enhances the anti-cancer effect of cisplatin

Makoto Ito
Seiji Yamamoto
Keisuke Nimura
Kazuya Hiraoka
Katsuto Tamai
Yasufumi Kaneda*

Division of Gene Therapy Science, Graduate School of Medicine, Osaka University, 2-2 Yamada-oka, Suita, Osaka 565 -0871, 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: 27 September 2004
Revised: 4 December 2004
Accepted: 4 January 2005

Abstract

Background Every cancer therapy appears to be transiently effective for cancer regression, but cancers gradually transform to be resistant to the therapy. Cancers also develop machineries to resist chemotherapy. Short interfering RNA (siRNA) has been evaluated as an attractive and effective tool for suppressing a target protein by specifically digesting its mRNA. Suppression of the machineries using siRNA may enhance the sensitivity to chemotherapy in cancers when combined with an effective delivery system.

Methods To enhance the anti-cancer effect of chemotherapy, we transferred siRNA against Rad51 into various human cancer cells using the HVJ (hemagglutinating virus of Japan, Sendai virus) envelope vector in the presence or absence of cis-diamminedichloroplatinum(II) (CDDP, cisplatin). The inhibition of cell growth was assessed by a modified MTT assay, counting cell number, or fluorescence-activated cell sorting (FACS) analysis after Annexin V labeling. The synthetic Rad51 siRNA was also introduced into subcutaneous tumor masses of HeLa cells in SCID mice with or without intraperitoneal injection of CDDP, and tumor growth was monitored.

Results When synthetic Rad51 siRNA was delivered into HeLa cells using the HVJ envelope vector, no Rad51 transcripts were detected on day 2, and Rad51 protein completely disappeared for 4 days after siRNA transfer. When HeLa cells were incubated with 0.02 μg/ml CDDP for 3 h after siRNA transfer, the number of colonies decreased to approximately 10% of that with scrambled siRNA. The sensitivity to CDDP was enhanced in various human cancer cells, but not in normal human fibroblasts. When Rad51 siRNA was delivered into tumors using the HVJ envelope vector, the Rad51 transcript level was reduced to approximately 25%. Rad51 siRNA combined with CDDP significantly inhibited tumor growth when compared to siRNA or CDDP alone.

Conclusions Rad51 siRNA could enhance the sensitivity to CDDP in cancer cells both *in vitro* and *in vivo*. Our results suggest that the combination of CDDP and Rad51 siRNA will be an effective anti-cancer protocol.

Keywords chemotherapy; siRNA; Rad51; non-viral vector; drug delivery; cancer therapy

Introduction

Although many different therapeutic strategies or regimens have been developed, there is no definitive treatment for cancer. Every cancer therapy appears to be transiently effective for cancer regression, but cancers gradually

transform to be resistant to the therapy. Although strategies have been developed to reverse the resistance, cancer cells develop mechanisms to escape the immune system and anti-neoplastic treatments [1–3]. cis-Diamminedichloroplatinum(II) (CDDP) is one of the most widely used anti-cancer drugs [4–6]. CDDP inhibits cellular growth by inducing DNA double-strand breaks [7–9]. However, cells can use DNA repair machinery to respond to the DNA damage. The levels of DNA repair proteins correlate with resistance to anti-cancer drugs, especially alkylating agents, in human cancer cell lines [10]. Two pathways, homologous recombination and non-homologous end joining, are used to repair DNA double-strand breaks [11,12]. BRCA 1 and 2 in a complex with Rad51 are involved in homologous recombination [11–13]. Non-homologous repair is performed by the complex of NBS1, MRE11, and Rad50 with the aid of Ku 70, Ku 80, the DNA-dependent protein kinase catalytic subunit, DNA ligase IV, and XRCC4 [11,14]. Different studies have drawn conflicting conclusions regarding the pathway used to repair CDDP-induced DNA double-strand breaks in mammalian cells. Initially, non-homologous end joining was believed to responsible for the repair of CDDP-induced DNA damage [15–17]. However, CDDP sensitivity was not affected by the level of the Ku70, which is needed for non-homologous end joining repair [18]. However, sensitivity to other DNA-damaging agents, such as bleomycin and methyl methanesulfonate, was elevated by suppression of Ku70 [18]. These findings suggest that non-homologous end joining is not used to repair DNA damage induced by CDDP. Recent evidence suggests that homologous recombination is involved in the repair of DNA double-strand breaks generated by CDDP [19–21]. Cancer cells may become resistant to CDDP by increasing the activity of homologous recombination repair machinery. Indeed, a high level of Rad51 is consistent with tumor progression and tumor resistance to cancer therapy [22]. Conversely, disabling the DNA repair machinery may enhance the sensitivity of cancers to CDDP.

The present study focuses on the function of Rad51 as a regulator of CDDP sensitivity. We tested the ability of short interfering RNA (siRNA) to inhibit the expression of Rad51. siRNA has been evaluated as an attractive and effective tool for suppressing the target protein by specifically digesting its mRNA [23,24]. siRNA is superior to antisense oligonucleotides and ribozymes in terms of efficiency and specificity [25,26]. However, finding a suitable delivery system for siRNA has been problematic [27]. We have been developing a highly efficient gene delivery system with minimum toxicity by converting viruses into non-viral vectors. We incorporated plasmid DNA into inactivated HVJ (hemagglutinating virus of Japan, Sendai virus) particles to form a HVJ envelope vector. By the strong fusion activity, DNA inside the envelope vector can be directly introduced into the cytoplasm of various types of cells both *in vitro* and *in vivo*. The HVJ envelope vector is also very effective for drug delivery [28,29]. siRNA was successfully introduced into pancreatic islet cell lines using the HVJ envelope vector [30]. In the present study, siRNA against human Rad51 enhanced the sensitivity of cancers to CDDP both *in vitro* and *in vivo*.

Materials and methods

HVJ

HVJ was amplified in chorioallantoic fluid of 10- to 14-day-old chick eggs and was purified by centrifugation and inactivated by UV irradiation (99 mJ/cm^2) as previously described [28]. Inactivated virus cannot replicate, but its capacity for viral fusion remains intact.

Cell culture

Human cancer cells and normal human diploid fibroblasts (NHDF) were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and antibiotics.

Rad51 cDNA transfer and cell survival assay

The Rad51 open reading frame sequence was subcloned into the expression vector using the Gateway system (Invitrogen, San Diego, CA, USA), amplified, and transfected into HeLa cells (3×10^5 cells) using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's instructions. The next day, the cells were passaged in 12-well plates (2×10^4 cells/well). Forty-eight hours after transfection, the cells were treated with 0–4 μg/ml CDDP (Nihon Kayaku, Tokyo, Japan) for 3 h. Then, 48 h later, cell survival was assessed by a modified MTT assay (Dojindo, Tokyo, Japan) as described elsewhere [31].

HVJ envelope vector-mediated siRNA transfection *in vitro*

An inactivated HVJ suspension (6×10^9 particles) was mixed with 60 μl of 40 μM Rad51 siRNA (5′-GAGCUUGACAAACUACUUC-3′) solution (Dharmacon, Lafayette, CO, USA) and 6 μl of 2% Triton X-100. Scrambled siRNA (5′-GCGCGCUUUGUAGGATTCG-3′) solution (Dharmacon) was used as a control. After centrifugation (18 500 *g*, 15 min) at 4 °C, the supernatant was removed and HVJ envelope vector that included siRNA was suspended in 120 μl of phosphate-buffered saline (PBS). The incorporation rate of siRNA was approximately 20% of total siRNA initially used. Unincorporated siRNA was reduced to an undetectable level by this process. For *in vitro* transfection of HVJ that contained siRNA, 1×10^5 cancer cells were seeded in 6-well plates 1 day before transfection. Protamine sulfate (5 μl, 5 mg/ml; Nacalai Tesque, Kyoto, Japan) and 500 μl of medium were added to 20 μl (1×10^9 particles) of HVJ that contained