New Vector Innovation for Drug Delivery: Development of Fusigenic Non-Viral Particles

Yasufumi Kaneda*

Division of Gene Therapy Science, Graduate School of Medicine, Osaka University, Suita, Osaka 565-0871, Japan

Abstract: Efficient and minimally invasive drug delivery systems have been developed to treat intractable human diseases. One approach has been the development of chimeric vector systems combining at least two different vector systems. Based on this concept, chimeric drug delivery systems that combine viral and non-viral features have been developed. Fusigenic non-viral particles have been constructed by conferring viral fusion proteins onto non-viral vectors. HVJ (hemagglutinating virus of Japan; Sendai virus)-liposomes were constructed by the combination of DNA-loaded liposomes with a fusigenic envelope derived from HVJ (hemagglutinating virus of Japan, Sendai virus). Reconstituted HVJ-liposomes were also developed by the insertion of isolated fusion proteins of HVJ into DNA-loaded liposomes. Recently, the technology has been developed to incorporate macromolecules directly into inactivated HVJ particles without liposomes. The resulting HVJ envelope vector introduced plasmid DNA, efficiently and rapidly into both cultured cells *in vitro* and organs *in vivo*. Furthermore, proteins, synthetic oligonucleotides and drugs have also been effectively introduced into cells using the HVJ envelope vector. The HVJ envelope vector will be a promising tool for both *ex vivo* and *in vivo* gene therapy experiments.

Key Words: chimeric vector, non-viral vector, HVJ, cell fusion, HVJ-liposomes, HVJ envelope vector, gene therapy.

I. INTRODUCTION

Gene therapy appears to be promising for treating intractable human diseases [1], but further development of effective gene transfer vector systems is key to the advancement of human gene therapy [2]. Efficient and minimally invasive vector systems appear to be most appropriate for both gene therapy and drug delivery. Numerous viral and non-viral (synthetic) methods for gene transfer have been developed [3-6], and in general, viral methods are more efficient than non-viral methods for the delivery of genes to cells. However, viral vectors are not available for drug delivery. Moreover, the safety of viral vectors is of concern due to the concomitant introduction of genetic elements from parent viruses, leaky expression of viral genes, immunogenicity, and changes in the host genome structure, whereas non-viral vectors are less toxic and less immunogenic [5, 6]. From these perspectives, much attentions have been paid on the development of non-viral vector systems. Nevertheless, most non-viral methods are less efficient for transfer of macromolecules, particularly *in vivo*. One approach to deal with these issues is the chimeric combination of viral and non-viral vectors.

Various modifications have been made to enhance the efficiency of gene delivery by non-viral vectors. Although liposomes have been used to target and introduce macromolecules into cells, gene transfer efficiency was low and varied during the early days of liposome development. The synthesis of cationic lipids produced a revolutionary improvement in gene transfer efficiency in 1987 [7]. Felgner *et al.* also developed a new model of liposome/DNA complex called a "lipoplex". Until then, DNA had been incorporated into liposomes, but, with lipoplex, an electrostatic complex was made between negatively charged DNA and positively charged cationic liposomes. Numerous cationic lipids have been synthesized to further improve transfection efficiency and to reduce cytotoxicity of lipoplex [6]. Nevertheless, in lipoplex-mediated transfection DNA is still taken up into cells by phagocytosis or endocytosis, not by fusion.

To solve the problem of degradation of the molecules before reaching the cytoplasm, fusion-mediated delivery systems have been developed. A fusigenic viral liposome with a fusigenic envelope derived from hemagglutinating virus of Japan (HVJ; Sendai virus) was constructed [8, 9]. HVJ has been shown to fuse with cell membrane at neutral pH, and HN and F-fusion proteins of the virus, contributes to the cell fusion [10]. For fusion-mediated gene transfer, DNA-loaded liposomes were fused with UV-inactivated HVJ to form the fusigenic viral-liposome, HVJ-liposome, which is 400 to 500 nm in diameter. This fusion-mediated delivery resulted in the advantageous protection of the molecules in the endosomes and lysosomes from degradation [11].

A similar approach has been performed to enhance gene transfer efficiency of receptor-mediated gene delivery system by combining fusion peptide derived from influenza virus hemagglutinin [12]. A tissue-specific gene delivery system has been developed by binding tissue-specific molecules to a poly-L-lysine/DNA complex. Binding asialoglycoprotein and transferrin to a poly-L-lysine/DNA complex successfully targets DNA to hepatocytes and cancer cells, respectively [13, 14]. However, the limitation of this system is the degradation of the DNA in the lysosomes. To

*Address correspondence to this author at the Division of Gene Therapy Science, Graduate School of Medicine, Osaka University, Suita, Osaka 565-0871, Japan; Tel: 81-6-6879-3900; Fax: 81-6-6879-3909;
E-mail: kaneday@gts.med.osaka-u.ac.jp

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avoid such degradation, a fusion-mediated gene delivery system has been investigated. Influenza virus is known to fuse with cell membranes at acidic pH, and hemagglutinin (HA) protein on the viral envelope is known to be involved in the fusion activity. It has also been elucidated that an N-terminal peptide of influenza HA subunit HA-2 can fuse with cell membranes. The transferrin/poly-L-lysine/DNA complex bound with the HA-2 peptide has been shown to increase gene transfer efficiency in cultured cancer cells more than 1,000 fold compared with that in the absence of the peptide [12].

A more direct and practical approach is the conversion of a fusigenic virion to a non-viral gene delivery particle. Numerous viruses such as influenza, VSV and HVJ are known to induce cell fusion. We have recently succeeded in developing an HVJ envelope vector system [15]. In this review article, we will also explain the new vector system

II. DEVELOPMENT OF HVJ-LIPOSOMES

One approach to improve the vector systems involves the insertion of fusion proteins into liposomes to enhance gene delivery [8, 9]. HVJ, also known as Sendai virus, is able to fuse with cell membranes and also with liposomes [10]. DNA-loaded liposomes are fused with UV-inactivated HVJ to form HVJ-liposomes (Fig. 1). The resulting vesicle, the HVJ-liposome, consists fusion proteins on the envelope and DNA on the inside. The resulting HVJ-liposome is approximately 400 to 500 nm in diameter. These liposomes are able to encapsulate DNA smaller than 100 kb, with a DNA trapping efficiency of approximately 20%. RNA, oligodeoxynucleotides (ODN), proteins, and drugs can also be enclosed and delivered to cells. It has been suggested that the advantage of fusion-mediated introduction of macromolecules may be the protection of macromolecules from degradation in the endosome and lysosome before reaching the cytoplasm. Nakamura *et al.* have clearly demonstrated this hypothesis using FRET (fluorescence resonance energy transfer) in the introduction of antisense oligonucleotides into cell nucleus [11].

HVJ-liposomes have been shown to be useful for *in vivo* gene transfer. When HVJ-liposomes containing the LacZ gene were injected directly into one lobe of a rat liver, approximately 70% of cells expressed LacZ gene activity, and no pathological hepatic changes were observed [16]. In this experiment, gene transfer to rat liver cells was not inhibited by repeated injections. After repeated injections, the anti-HVJ antibody generated was not sufficient to neutralize HVJ-liposomes. Cytotoxic T cells recognizing HVJ were not detected in rats transfected repeatedly with HVJ-liposomes. Thus, one advantage of HVJ-liposomes would be allowance of repeated administration [16].

To improve gene transfer efficiency, lipid components of liposomes have been investigated. Subsequently, new anionic liposomes called HVJ-AVE liposomes; i.e., HVJ-

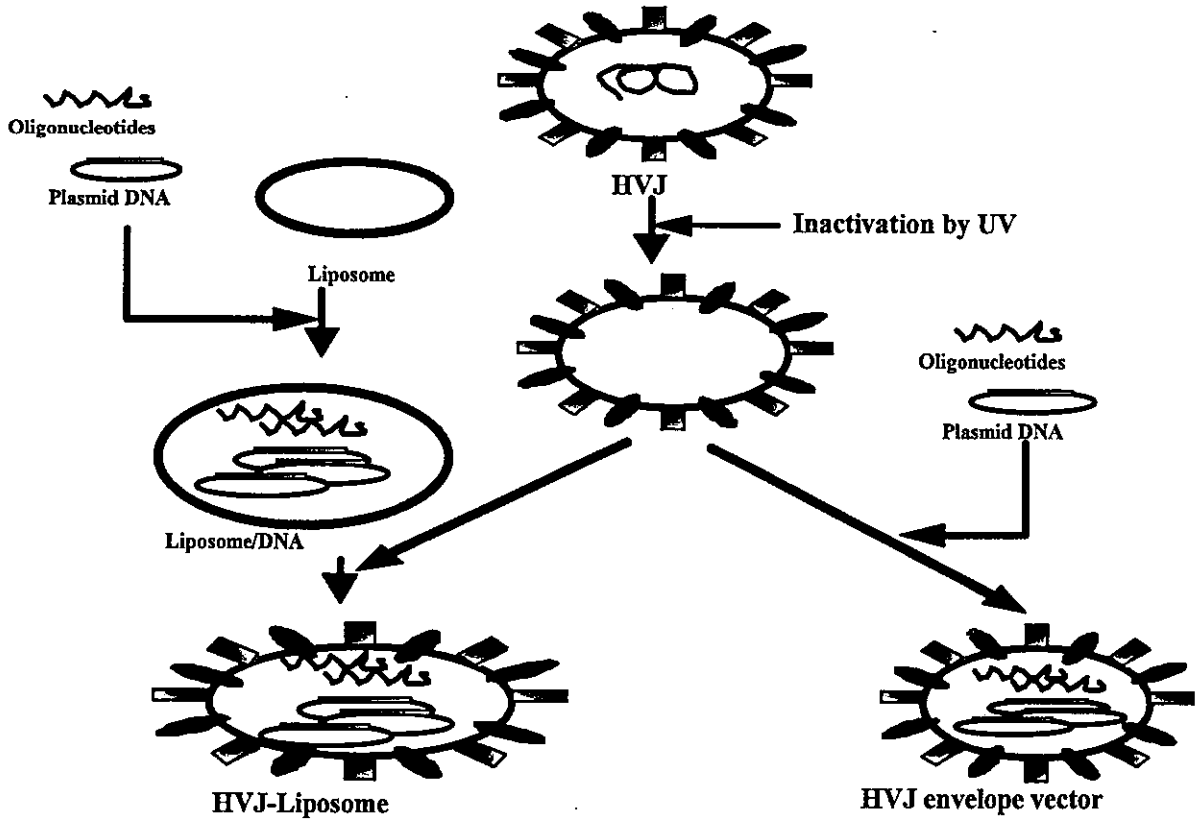

Fig. (1). Development of HVJ-liposomes and HVJ envelope vector. Either plasmid DNA or synthetic oligonucleotides are able to be incorporated into both vectors and effeiciently delivered to cells by the fusion activity of HVJ. However, since viral genome is completely inactivated and degraded, viral proteins are never produced in the cells.

artificial viral envelope liposomes have been developed. The lipid components of AVE liposomes have been to be very similar to the HIV envelope and mimic the red blood cell membrane [17]. HVJ-AVE liposomes have yielded gene expression in liver and muscle 5 to 10 times higher than that observed with conventional HVJ-liposomes [18]. Additional improvements have been made through the construction of cationic-type HVJ-liposomes using cationic lipids. Of the cationic lipids, positively charged DC-cholesterol (DC) [19] has been the most efficient for gene transfer. For luciferase expression, HVJ-cationic DC liposomes were 100 times more efficient than conventional HVJ-anionic liposomes [17]. HVJ-cationic DC liposomes have been shown to be more appropriate for gene transfer to cancer cells. In gene transfer to intraperitoneally disseminated colon cancers, HVJ-cationic liposomes introduced either luciferase DNA or FITC-ODN predominantly to tumor nodules in the mouse abdomen.

III. RECONSTITUTED FUSION PARTICLES

To promote fusion-mediated gene delivery, reconstituted particles containing fusion proteins of HVJ have been developed [20-22]. It has been possible to construct HVJ-liposomes using inactivated whole HVJ virion and isolated fusion proteins can be used instead of the whole virion. HVJ virion was completely lysed with detergent, and the lysates were mixed with DNA solution. In some cases, several lipids were added to the mixture. By removing the detergent using dialysis or a column procedure, reconstituted HVJ particles containing DNA were constructed. Instead of the whole virion of HVJ, fusion proteins (F and HN) isolated from the virion were mixed with the lipids/DNA mixture in the presence or absence of detergent. Since F protein is recognized by the asialogycoprotein receptor on hepatocytes, reconstituted HVJ particles containing only F protein have been constructed to specifically target hepatocytes *in vivo*[22]. In another approach, fusion proteins, F and HN, have been purified from the HVJ virion and liposomes containing F and HN were constructed by the detergent-lysis-dialysis method [23, 24]. The resulting fusion liposomes were fused with DNA-loaded liposomes to form reconstituted HVJ-liposomes [24]. These reconstituted fusion liposomes were as effective as conventional HVJ-liposomes with the fully intact HVJ virion in terms of delivery of both FITC-ODN and the luciferase gene to cultured cells. LacZ gene was also transferred directly to mouse skeletal muscle *in vivo* using these reconstituted fusion particles.

IV. DEVELOPMENT OF HVJ ENVELOPE VECTOR SYSTEM

The disadvantage of HVJ-liposome is the complicated procedure to isolate and produce both inactivated HVJ and DNA-loaded liposomes. Another limitation is that thefusion activity of the HVJ-liposomes decreases to approximately 2% of native HVJ because of the reduction of density of fusion proteins on the surface of HVJ-liposomes. To solve these problems, the HVJ envelope vector system has been developed as illustrated in Fig. (**1**) [15]. HVJ is completely inactivated by either UV-irradiation or ß-propiolactone treatment. Exogenous plasmid DNA is incorporated into the inactivated HVJ by treatment with mild detergent and centrifugation. By this procedure, approximately 15 - 20% of added DNA is able to be incorporated into the inactivated HVJ envelope. Electronmicroscopy confirms that DNA is incorporated into all of the particles of inactivated HVJ. The largest DNA tested was a 14 kb plasmid DNA, with a resultant trapping efficiency of approximately 18%. Without centrifugation, the DNA trapping efficiency is reduced to approximately 3% - 5%. Without detergent treatment, no DNA becomes incorporated into the viral particle. Synthetic oligonucleotides, proteins and peptides can be incorporated into the HVJ envelope by a similar strategy.

The HVJ envelope vector differs from the reconstituted HVJ particles that are prepared by reassembling lipids and fusion proteins after solubilization of the virus particle. In the preparation of the HVJ envelope vector, plasmid DNA is incorporated into inactivated HVJ particles by treatment with mild detergent without destruction of the virion and without the dialysis, purification or addition of lipids or proteins which are used for the preparation of reconstituted HVJ particles [20-22]. Therefore, the composition of the HVJ envelope vector is very similar to that of native HVJ.

For *in vitro* transfection, the HVJ envelope vector containing luciferase expression plasmid was mixed with protamine sulfate, and this mixture was added to cultured cells. Protamine sulfate was absolutely necessary for *in vitro* gene transfer with the HVJ envelope vector to augment attachment of the HVJ envelope vector to the cell surface by providing a cationic charge. The HVJ envelope vector was useful for gene transfer to various cell lines, and a short incubation period (i.e., a 10-min incubation) was sufficient for high expression of the target gene. When the HVJ envelope vector containing the GFP expression plasmid was added to BHK-21 cells, GFP expression was approximately 80%, as determined by flow cytometry. Under such conditions, little cell damage was observed. Fluorescence isothiocyanate-labeled oligodeoxynucleotides (FITC-ODN), proteins such as IgG, bovine serum albumin and human insulin were also transferred to cultured cells at an efficiency of more than 95%. The HVJ envelope vector is much more efficient in gene transfer to primary culture cells, such as rat neuronal cells, human aortic endothelial cells, mouse dendritic cells and rat cardiac myocytes, than other lipofection reagents. Additionally, cells in a suspension are also appropriate targets for HVJ envelope vector. Thus, the HVJ envelope vector should be useful for *ex vivo* gene therapies. Another advantage of the present HVJ envelope vector system is its utility for *in vivo* application. The HVJ-envelope vector is more effective than HVJ-liposomes for *in vivo* gene transfer. LacZ or luciferase gene transfer to lung, liver, uterus, eye, skin, muscle, and brain of animals such as mouse, rat, rabbit and monkey are achieved by direct injection of the HVJ envelope vector. FITC-ODN were also efficiently delivered to rat lung, cartilage of monkey joints and tumor masses. Among the organs we have tested, the HVJ envelope vector is more effective than HVJ-liposomes for gene transfer to liver, uterus, brain, eye, and lung with similar levels of expression detected in muscle and skin. This predominance of the HVJ envelope vector over HVJ-liposomes may be due to the stronger fusion activity of the HVJ envelope vector in comparison to HVJ-liposomes.

Consecutive injection of DNA-loaded HVJ envelope vector supports that no inhibition of gene transfection occurs in mouse skeletal muscle. Thus, the HVJ envelope vector appears to be much less immunogenic than native HVJ.

By intravenous injection of the HVJ envelope vector in mice, the HVJ envelope vector targeted mainly spleen. FITC-ODN were detected in the cells of the marginal zone of mouse spleen at the efficiency of approximately 6%. Although colloidal particles are trapped in reticuloendothelial cells [25], predominant target tissues are variable among vectors. When reconstituted HVJ particles containing only F protein without HN protein are injected into mouse tail vein, gene expression is observed mainly in liver [22] as the galactose residues of F protein are recognized by hepatocytes [22]. HVJ-liposomes containing both F and HN proteins target mainly liver, but also spleen and lung to a lesser degree, when the vector is injected into the saphenous veins of monkeys [26] probably because phospholipids such as phosphatidylserine [25] present on the envelope are recognized by reticuloendothelial cells. The LPD (liposome-protamine sulfate-plasmid DNA) vector targets the lung, kidney, heart, liver, and spleen with highest level of gene expression in the lung [27, 28]. Analysis of the effects of mutations in the fusion glycoproteins of HVJ and alteration in the lipid profile of the envelope will clarify the mechanism underlying the spleen-specific targeting by the HVJ envelope vector. Apart from the mechanism of tissue targeting, the spleen targeting ability of the HVJ envelope vector may be very effective for inducing immunity against infectious diseases and cancers because the vector targets the marginal zone of spleen in which the antigen-presenting cells ccumulated. We have previously reported that strong anti-tumor immunity results when HVJ-liposomes containing melanoma-associated antigen gp100 mRNA are injected directly into mouse spleen [29]. Because direct injection into spleen is not practical for human gene therapy, intravenous administration of the HVJ envelope vector containing tumor-associated antigen genes may yield an effective and practical strategy for cancer treatment.

CONCLUSION

Thus, fusion-mediated non-viral gene delivery systems can achieve safe and efficient gene delivery to many kinds of cells both *in vitro* and *in vivo*. Besides gene delivery, the systems can be also applied to transfer proteins, synthetic oligonucleotides and drugs. The problem of the use of these vectors remains the large scale production of homogeneous vectors for clinical trials. In this perspective, however, the HVJ envelope vector has distinct advantages over other vectors because of the simple means of preparation. In fact, we have recently succeeded in the large scale-production of the HVJ envelope vector. Clinical trials to treat human diseases will begin in the near future using the HVJ envelope vector. The techniques utilized to prepare the HVJ envelope vector will be used to prepare other virus envelope vectors. Using the tissue tropism of various viruses, tissue-specific targeting vectors will be developed such as the herpes virus envelope vector for neuronal cell targeting and hepatitis B virus envelope for hepatocyte targeting.

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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

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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

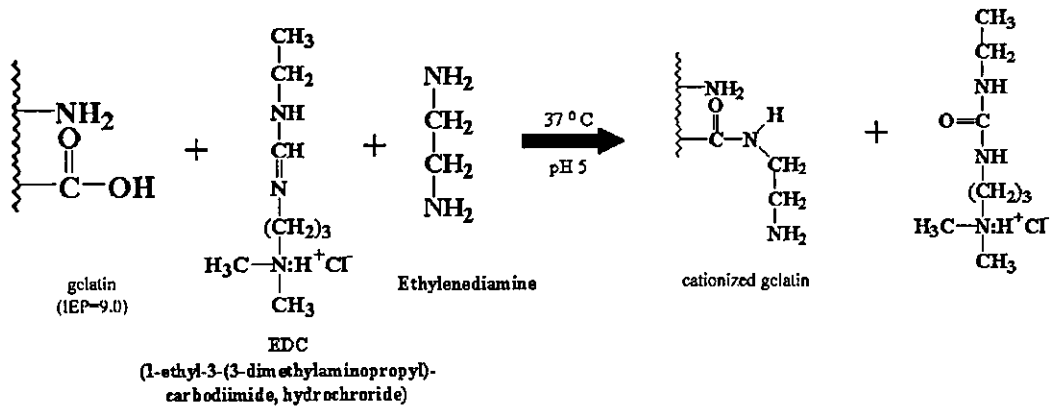

Figure 1. Synthesis of cationized gelatin. Cationized gelatin was mixed with HVJ-E containing a marker gene. The complex was isolated by centrifugation and used for transfection experiments

(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

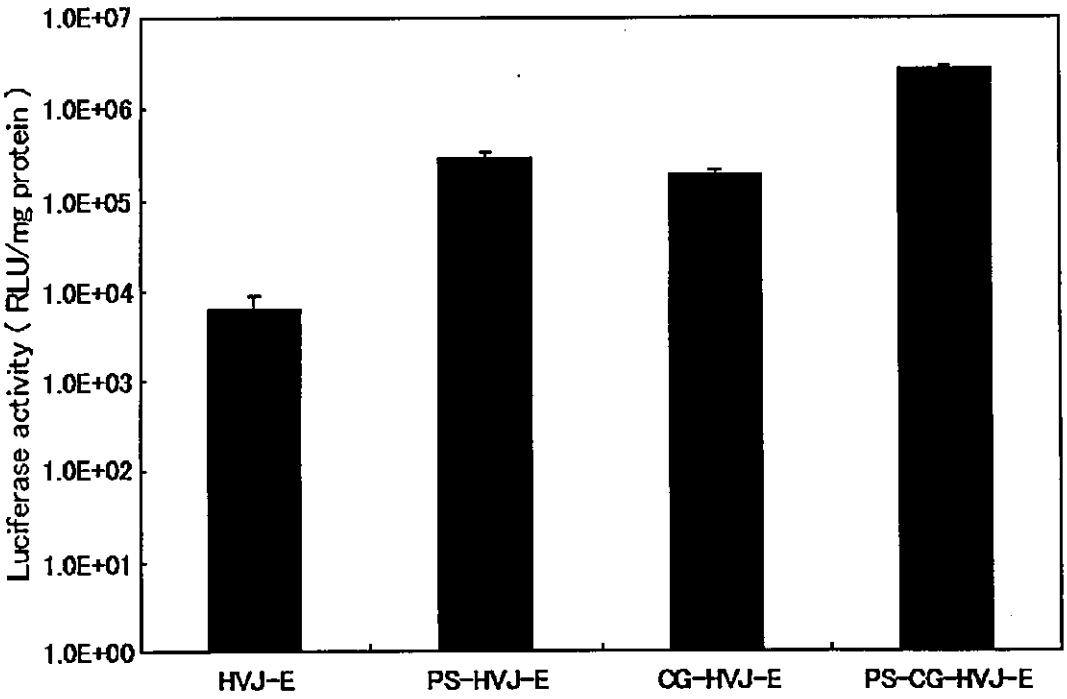

Figure 2. Luciferase gene expression in CT26 cells transfected with HVJ-E, PS-HVJ-E, CG-HVJ-E or PS-CG-HVJ-E. The vectors were incubated with cells for 10 min, and the luciferase activity was measured 24 h after removal of the vector. Results are shown as mean ± s.d. (n = 3). Similar results were obtained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

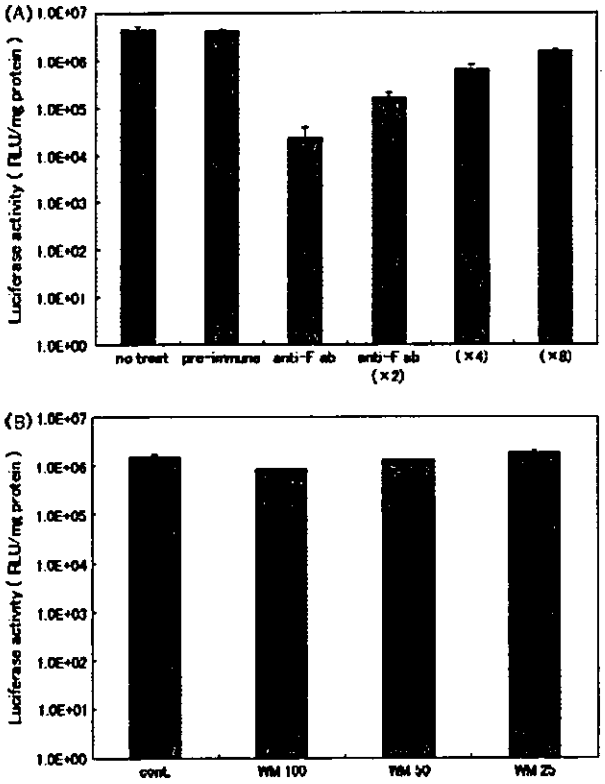

Figure 3. Effects of anti-F protein antibody (A) and wortmannin (B) on gene expression by PS-CG-HVJ-E. (A) After incubation of PS-CG-HVJ-E with antiserum, the mixture was added to CT26 cells and incubated for 10 min. Luciferase activity was measured 24 h after the removal of the mixture. Preimmune rabbit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

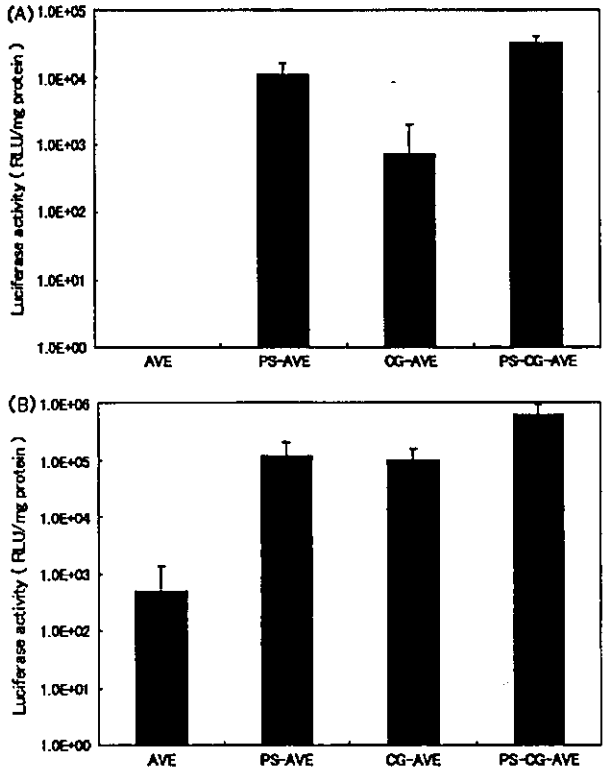

Figure 4. The effect of protamine sulfate, cationized gelatin or both on transfection efficiency by anionic liposomes (A) and anionic liposomes fused with HVJ (B). Vectors were incubated with CT26 cells for 1 h, and the luciferase activity was assessed after 24 h. AVE means anionic liposome with the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

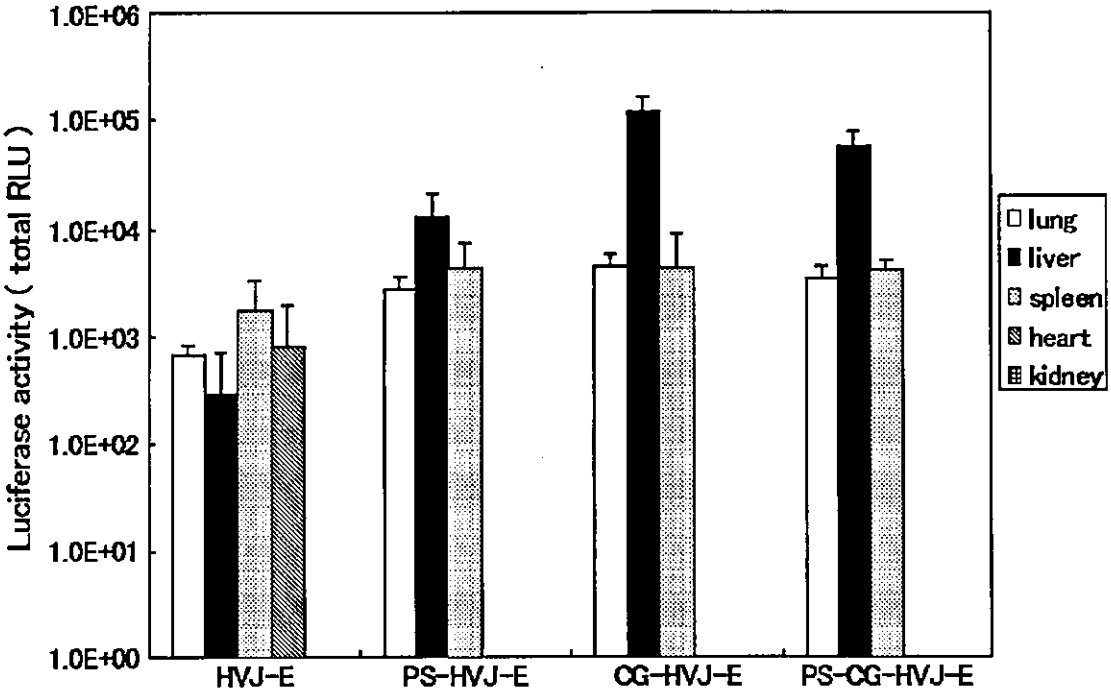

Figure 5. *In vivo* gene transfection efficiency of HVJ-E, PS-HVJ-E, CG-HVJ-E and PS-CG-HVJ-E after injection into mouse tail vein. Luciferase activity was measured in organ lysates 24 h after injection and the results are expressed as mean ± s.d. of luciferase activity of each organ from 5 to 6 mice.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

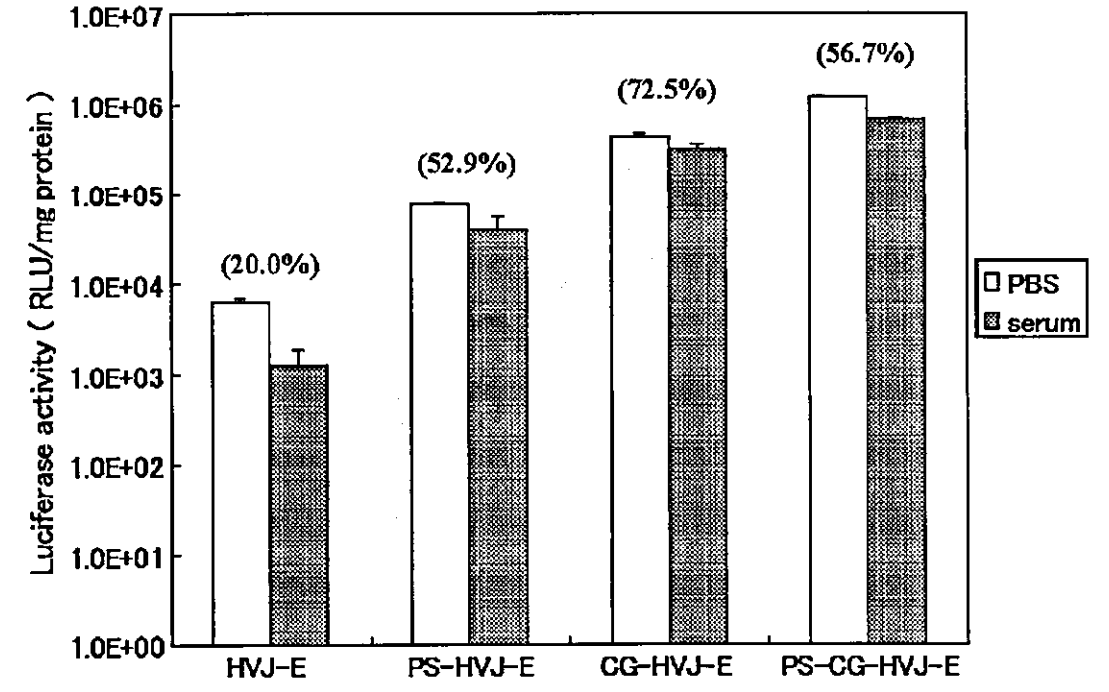

Figure 6. The effect of fresh serum on the transfection efficiency of HVJ-E or polymer-conjugated HVJ-E. After incubation of HVJ-E or polymer-conjugated-HVJ-E with fresh mouse serum, the serum was removed by centrifugation and added to CT26 cells. Luciferase activity was measured 24 h after removal of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

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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. Copyright © 2005 John Wiley & Sons, Ltd.

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

siRNA. Approximately 80 pmol siRNA were delivered to 1×10^5 cells. The cell culture medium was removed, and the HVJ envelope vector was added to each well. Thirty minutes later, the medium containing the vector was replaced with fresh medium.

Western blot analysis

The harvested human cancer cells were lysed in lysis buffer (1% SDS, 20 mM Tris-HCl (pH 8), 135 mM NaCl, 10% glycerol, and a protease inhibitor mixture (Roche, Basel, Switzerland)). After adding 2× sample buffer (0.1 M Tris-HCl (pH 6.8), 4% SDS, 12% 2-mercaptoethanol, 20% glycerol, and 0.01% bromphenol blue), 30 μg of protein were separated by 10% sodium dodecyl sulfate/polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto a polyvinylidene fluoride membrane (Millipore, Bedford, MA, USA). The membrane was blocked with 5% skim milk and subsequently probed with antibodies, anti-human Rad51 (Santa Cruz, Santa Cruz, CA, USA), anti-β-actin (Abcam, Cambrige, UK), and anti-GAPDH (Ambion, Austin, TX, USA). Proteins were detected with horseradish peroxidase labeled anti-goat (Santa Cruz) or anti-mouse (Amersham, Piscataway, NJ, USA) antibodies and the enhanced chemiluminescence reagent (Amersham).

Northern blot analysis

Total RNA was isolated from HeLa cells using ISOGEN (Nippon Gene, Toyama, Japan) according to the manufacturer's instructions. Total RNA (15 μg/lane) was separated in a formaldehyde/1.5% agarose gel, transferred to Hybond N+ membrane (Amersham), and then hybridized with ^{32}P-labeled Rad51 and G3PDH cDNA probes.

Colony forming assay

Twenty-four hours after HVJ envelope vector-mediated siRNA transfection to HeLa cells *in vitro*, the cells were seeded in a 6-cm dish at a density of 10^3 cells/dish and treated with 0–0.1 μg/ml CDDP for 3 h. After 7 days, the colonies were fixed with methanol and stained with Giemsa (Nacalai Tesque). Then, the colonies were counted. The percentage of colony-forming cells after CDDP treatment was calculated and compared to the untreated control group.

CDDP sensitivity in cultured cells by Rad51 siRNA transfer

Forty-eight hours after transfer of siRNA, the cells were treated with 0.1, 0.3 and 1.0 μg/ml CDDP for 3 h. Then, 48 h later, cell number was counted using a particle counter (Coulter Corporation, Miami, FL, USA). To assess apoptosis, cells treated with Rad51 siRNA and CDDP were harvested and stained with fluorescent isothiocyanate-labeled Annexin V (Becton Dickinson, San Diego, CA, USA) for 20 min at room temperature. The labeled cells were analyzed with FACScan (Becton Dickinson).

In vivo experiments

Viable HeLa cells (5×10^6 cells) were resuspended in 100 μl of PBS and intradermally injected into the right flanks of 6-week-old male SCID mice (Charles River Japan, Yokohama, Japan). The inactivated HVJ suspension (6×10^9 particles) was mixed with 60 μl of 250 μM Rad51 siRNA solution and 6 μl of 2% Triton X-100. Scrambled siRNA solution was used as a control. After centrifugation (18 500 *g*, 15 min) at 4 °C, the supernatant was removed and the HVJ envelope vector containing siRNA was suspended in 120 μl of PBS. Seven days after tumor inoculation, 100 μl (5×10^9 particles) of HVJ envelope vector containing siRNA were injected into the tumor. Approximately 2.5 nmol siRNA were delivered to the tumor mass in a mouse. The injection

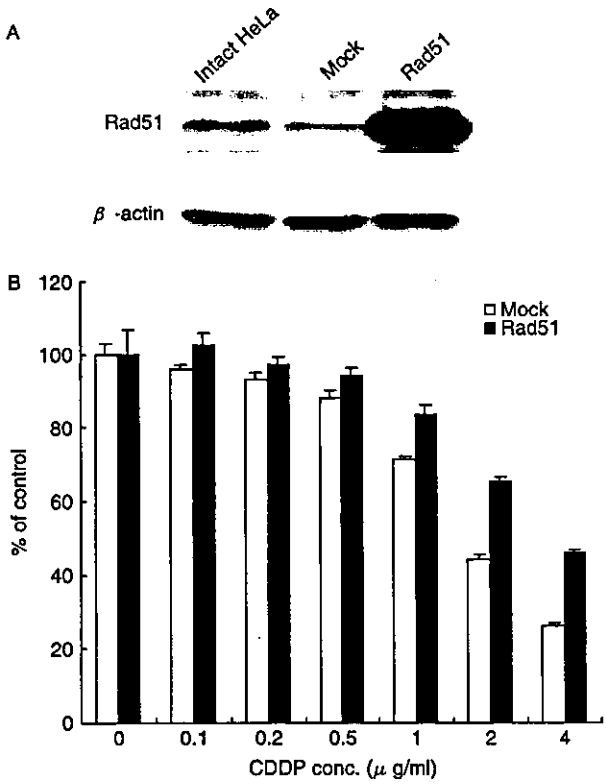

Figure 1. (A) Detection of human Rad51 transcript 48 h after the transfection of human Rad51 cDNA driven by the CMV promoter. Mock sample indicates HeLa cells transfected with a plasmid that did not contain Rad51 cDNA. Intact HeLa indicates HeLa cells that were not transfected. (B) Cell survival was detected by a modified MTT assay after treatment with 0–4 μg/ml CDDP for 3 h. The ordinate indicates the ratio of viable cells treated with various concentrations of CDDP to initial cell number. The mean value ± standard deviation from triplicate samples is shown

was repeated at 2-day intervals until each mouse received a total of three injections. At the time of the second siRNA injection, 200 μg of CDDP were intraperitoneally injected. Tumor size was measured every 2 days, and the tumor volume was calculated using the simplified formula for a rotational ellipse ($l \times w^2 \times 0.5$). All animals were treated in a humane fashion in accordance with the guidelines of the Animal Committee of Osaka University.

Results

To determine what factors induced by CDDP contribute to the repair of DNA damage, we examined the gene expression of repair genes in cells treated with CDDP. The protein level of Rad51, which is involved in homologous recombination repair, increased 1.57 ± 0.4 times more with CDDP than that without CDDP (data not shown). However, the expression level of Ku70, which is involved in non-homologous end joining, was not changed (0.9 ± 0.3 times) by CDDP treatment.

We examined whether Rad51 expression resulted in resistance to CDDP. To increase the expression of Rad51, HeLa cells were transfected with the human Rad51 gene driven by the cytomegalovirus (CMV) promoter (Figure 1A). When cell proliferation was measured by a modified MTT assay, Rad51-transfected HeLa cells cultured with various concentrations of CDDP were more viable than control cells that had undergone only a mock transfection (Figure 1B). The experiment was repeated three times, and similar results were obtained.

To enhance sensitivity to CDDP, we attempted to suppress Rad51 expression with siRNA. When Cy3-labeled siRNA was delivered to HeLa cells using the HVJ envelope vector, the efficiency was 80–100% (data not shown). Rad51 transcripts were not detected by Northern blot analysis 1 day after siRNA delivery, whereas scrambled siRNA did not reduce the transcript level (Figure 2A). We tested five different siRNAs for Rad51, but the only effective siRNA was a 19-mer from no. 321 of the Rad51 mRNA sequence. The other four siRNAs (19-mers from nos. 89, 462, 828, and 989) did not suppress Rad51 expression (data not shown). Two different antisense oligonucleotides against human Rad51 did not reduce the expression of human Rad51 (Figure 2B). These oligonucleotides had the same sequence as mouse Rad51 antisense oligonucleotides that had been used for suppression of Rad51 [32]. Rad51 protein was not detected by Western blots for 4 days after siRNA transfer. A small amount of Rad51 protein began to reappear on day 5 (Figure 2C). When Rad51 siRNA was introduced into HeLa cells, the growth of the cells was suppressed and the viability was 70% less than cells treated with scrambled siRNA (Figure 3A). The growth of cells treated with scrambled siRNA was not significantly different compared to that of cells treated with HVJ-E containing PBS. When HeLa cells were incubated with 0.02 μg/ml CDDP for 3 h after the delivery of Rad51 siRNA, the survival of the cells was reduced by 90% when compared to equivalent cells that were not exposed to CDDP (Figure 3B). More than 90% of colonies were formed with the same concentration of CDDP when scrambled siRNA was transferred into HeLa cells. Accordingly, with Rad51

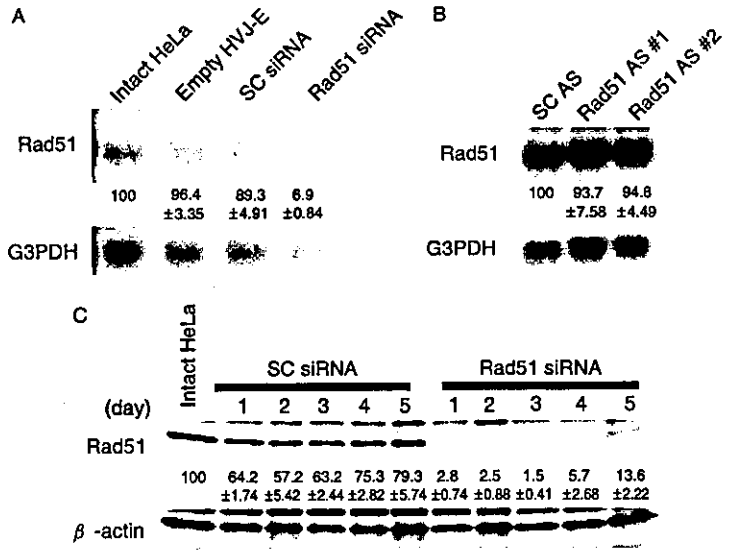

Figure 2. (A) Rad51 transcripts detected by Northern blot analysis 1 day after the delivery of Rad51 siRNA or scrambled (SC) siRNA. Rad51 mRNA in intact HeLa cells and HeLa cells treated with empty HVJ envelope vector were also measured. (B) Rad51 detection by Northern blot analysis 1 day after the delivery of two different antisense oligonucleotides (#1 and #2) against human Rad51 (Rad51 AS) or scrambled oligonucleotides (SC AS). (C) Rad51 protein detected by Western blot on days 1 to 5 after the delivery of either Rad51 siRNA or SC siRNA. These experiments were repeated twice and similar results were obtained. The ratio of Rad51 expression to G3PDH or β-actin expression was calculated by measuring the density of each band using the NIH imager. The percentage of Rad51 expression (mean ± standard deviation) is shown below each lane

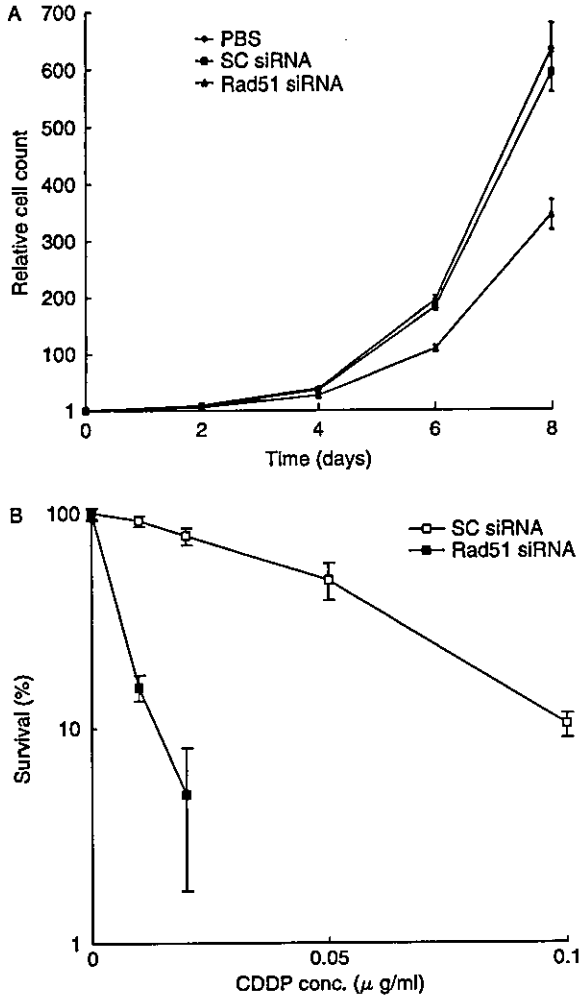

Figure 3. (A) The growth of HeLa cells detected by cell count on days 0 to 8 after the delivery of Rad51 siRNA, scrambled (SC) siRNA or PBS using the HVJ envelope vector. (B) The colony formation of HeLa cells after the delivery of either Rad51 siRNA or SC siRNA. The ordinate indicates the ratio of the number of colonies in the presence of various concentrations of CDDP to the number of colonies without CDDP after the delivery of siRNA. The mean value ± standard deviation from triplicate samples is shown at each point of both experiments. No colonies were observed at 0.05 and 0.1 μg/ml CDDP when Rad51 siRNA was delivered

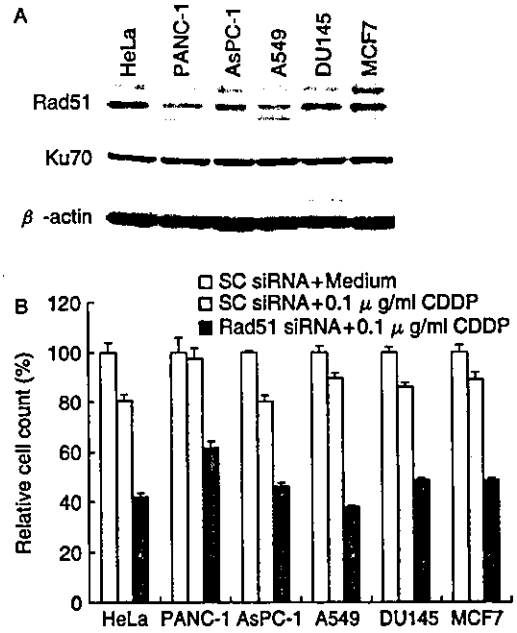

Figure 4. The increase in CDDP sensitivity in various cancer cell lines with Rad51 siRNA. (A) Rad51 and Ku70 protein levels in various cancer cell lines were detected by Western blotting. (B) siRNA was introduced into the human cancer cells using the HVJ envelope vector on day 1 after the inoculation of 10^5 cells in a 6-well plate. On day 3, cells were incubated with CDDP (0.1 μg/ml) for 3 h, and cell number was counted using a particle counter on day 5. Relative cell count indicates the ratio of cell number (the mean value of triplicate samples) treated with either scrambled (SC) or Rad51 siRNA + CDDP to that treated with SC siRNA + medium

siRNA, the number of colonies decreased to approximately 10% of that with scrambled siRNA.

We tested the effect of Rad51 siRNA on the sensitivity of CDDP in various human cancer cell lines including PANC-1 (pancreatic cancer), AsPC-1 (pancreatic cancer), A549 (lung cancer), DU145 (prostate cancer), MCF7 (mammary carcinoma), and HeLa S-3 (cervical cancer). First, the amounts of Rad51 and Ku70 in these human cancer cells were detected by Western blotting. The protein levels of Rad51 varied among cell lines while Ku70 protein levels were almost similar (Figure 4A). Then, on day 2 after the treatment with CDDP (0.1 μg/ml), the ratio of cell numbers of these cancer cell lines was examined in the presence of Rad51 siRNA or scrambled siRNA introduced using the HVJ envelope vector. Without Rad51 siRNA, more than 80% of the cells were still alive in all the cancer cell lines. Scrambled siRNA did not induce any toxicity in all the cell lines. However, with Rad51 siRNA, Rad51 protein level was reduced to less than 10% of that without siRNA in all the cell lines (data not shown), and all the cell lines were much more sensitive to CDDP. The sensitivity to CDDP increased more than 30% in all cases (Figure 4B). Thus, the enhancement of CDDP sensitivity by Rad51 siRNA appeared to be generally applicable to many cancer cells.

Next, we examined the sensitivity to CDDP in non-cancerous human cells after transfer of Rad51 siRNA. As shown in Figure 5A, the sensitivity to CDDP was not enhanced in NHDF when the concentration of CDDP increased. Then, we compared the apoptosis of NHDF to that of HeLa cells by the treatment with Rad51 siRNA in the presence or absence of 0.1 μg/ml CDDP (Figure 5B). The apoptotic cell ratio was not significantly different between HeLa cells (4.0 ± 1.1%) and NHDF (3.2 ± 0.5%) with Rad51 siRNA in the absence of CDDP. However, in the presence of CDDP, the apoptosis increased to 15.0% in HeLa cells, while it was 4.9% in NHDF.

We examined the ability of CDDP and Rad51 siRNA to suppress tumor growth in SCID mice. First, to test the gene delivery efficiency *in vivo*, we injected the HVJ

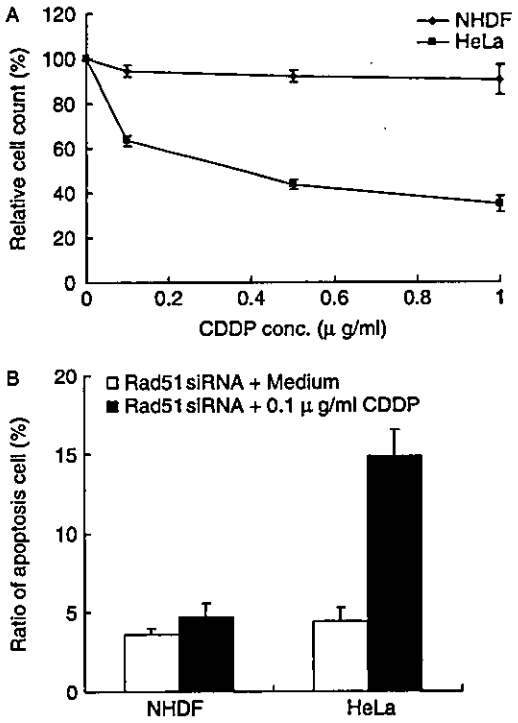

Figure 5. Rad51 siRNA did not enhance the sensitivity to CDDP in NHDF. (A) Forty-eight hours after transfer of Rad51 siRNA, the cells were treated with 0.1, 0.3 and 1.0 μg/ml CDDP for 3 h. Then, 48 h later, cell number was counted using a particle counter. Relative cell count indicates the ratio of cell number (the mean value of triplicate samples) treated with CDDP to that treated with medium alone. (B) To assess apoptosis, cells treated with Rad51 siRNA and CDDP were stained with fluorescein isothiocyanate (FITC)-labeled Annexin V and analyzed with FACScan. The ordinate indicates the ratio of labeled cells treated with Rad51 siRNA + medium or Rad51 + CDDP to that with scrambled siRNA + medium

envelope vector containing fluorescein isothiocyanate (FITC)-labeled oligodeoxynucleotides (FITC-ODN) into HeLa cell-derived tumors. As shown in Figure 6, the number of FITC-labeled cells and cells stained with Hoechst in randomly selected fields of three independent experiments were counted. They were 1227/2256, 616/1360, and 769/1424 cells. Thus, the delivery efficiency of FITC-ODN to HeLa cell tumors *in vivo* was 51.5 ± 5.2% (mean ± standard deviation). Next, Rad51 siRNA was delivered to tumors using the HVJ envelope vector. Western blot analysis showed that the level of Rad51 transcript was reduced to approximately 25% of that in intact HeLa tumors (Figure 7). Intraperitoneal injection of 200 μg of CDDP on day 2 transiently suppressed tumor growth, but tumors began to grow again 8 days after the treatment. To enhance the anti-tumor effect of CDDP, Rad51 siRNA delivered by the HVJ envelope vector was injected into the tumors on days 0 and 2. However, the suppression of tumor growth was not significant when compared to CDDP treatment alone (data not shown). Finally, Rad51 siRNA was injected into tumor mass on days 0, 2, and 4, and CDDP was injected into the abdominal cavity on day 2. This combination treatment significantly reduced the growth of HeLa tumors when compared to other treatment groups (Figure 8). Thus, the combination of CDDP and Rad51 siRNA is an effective anti-cancer protocol.

Discussion

We enhanced the sensitivity of cancer cells to CDDP by completely suppressing Rad51 with siRNA. The combination of CDDP and siRNA caused the regression of human tumors in mice. These results support the theory that DNA damage induced by CDDP can be repaired by Rad51. Our results suggest that CDDP-induced DNA damage can be repaired by homologous recombination of DNA double-strand breaks. We succeeded in suppression of Ku70 proteins in HeLa cells using Ku70 siRNA, but the sensitivity to CDDP was not enhanced in HeLa cells (data not shown). An antisense Ku70 study supports our observation [18]. Although we have not applied siRNA technology to suppress another factors such as Ku80 and DNA protein kinase (DNA-PK) which are also involved in non-homologous DNA end joining, it has been reported that silencing of DNA-PK or Ku86 by siRNA enhances sensitivity to radiation and anti-cancer drugs such as methyl methanesulfonate and bleomycin, but not to DNA cross-linking agents such as cisplatin and chlorambucyl [32–34]. Moreover, cisplatin killing is mediated by kinase activity of the Ku70, Ku80 and DNA-PK complex [35]. However, another report indicates that novel inhibitors of DNA-PK, vanillins, sensitize cells to cisplatin [36]. Thus, the involvement of DNA-PK in cisplatin sensitivity is still controversial. A comparative study of Rad51 siRNA and DNA-PK siRNA in cisplatin sensitivity should be conducted.

siRNA very effectively suppressed Rad51 expression. A previous study found that antisense oligodeoxynucleotides against mouse Rad51 enhanced the radiosensitivity of malignant glioma [37]. Although the target sequence of the antisense oligonucleotides is the same in humans and mice, the antisense oligonucleotides to human Rad51 did not suppress human Rad51 mRNA (Figure 2). As shown in Figure 2, Rad51 protein completely disappeared for 4 days after the siRNA transfer. We have never observed such complete loss of target protein using either antisense oligonucleotides or ribozymes. However, only one of five siRNA constructs effectively suppressed Rad51 expression. The system for predicting effective siRNA sequences should be improved.

When siRNA was delivered using the HVJ envelope vector, the efficiency was almost 100% in cultured cells, and Rad51 expression was completely prevented for 4 days after the delivery. siRNA very effectively suppresses gene expression, especially when an efficient delivery system is used. However, even when the HVJ envelope vector was used, the efficiency of a single siRNA injection into a tumor was only 50%. One limitation of synthetic siRNA is that its effect is transient, probably because