2．学会発表

- 「Development of HVJ envelope vector for in vitro and in vivo transfer of plasmid DNA, oligonucleotides and proteins.」 Kaneda. Y.: 5th Annual meeting of the American Society of Gene Therapy 2002年6月7日，米国・ボストン

- 「Development and Manufacturing of HVJ (hemagglutinating virus of Japan) - Envelope Vector for Gene Therapy.」 Nakajima, T. et al.: 5th Annual meeting of the American Society of Gene Therapy 2002年6月6日，米国・ボストン

- 「Novel Nonviral Vector of Dendtric Cells To Induce Anti-Melanoma Immunity.」 Kaneda. Y, et al.: 6th Annual meeting of the American Society of Gene Therapy 2003年6月6日，米国・ワシントンDC.

- 「 Development of an Innovative Non-Viral Vector for Cardiovascular Disease. 」 Nakajima.T, Fukummura.M, Miyaji.K, Yoshida.K, Kaneda. Y, Kotani.H.: 6th Annual meeting of the American Society of Gene Therapy 2003年6月6日，米国・ワシントンDC.

- 「Development of HVJ-E High Throughput Gene Function Screening System: Screening of Endothelial Cell Growth Factors」、Tomoyuki Nishikawa, Hironobu Nakagami, Chiah Yian Yo, Yasufumi Kaneda、The American Society of Gene Therapy’s 7th Annual Meeting、2004年6月2日-6日、米国・ミネアポリス

- 「A Novel Therapeutic Strategy To Treat Brain Ischemia:Over-Expression of Hepatocyte Growth Factor Gene Reduced Ischemic Injury without Cerebral Edema in Rat Model」 Munehisa Shimamura, Naoyuki Sato, Toshio Ogihara, Yasufumi Kaneda, Ryuichi Morishita、The American Society of Gene Therapy’s 7th Annual Meeting、2004年6月2日-6日、米国・ミネアポリス

- 「 Improvement of Myocardial Dysfunction by Therapeutic Angiogenesis Induced by Transfection of HGF Gene Using Left Ventricular Electromechanical Mapping in Porcine Chronic Myocardial Infarction Model」、Junya Azuma, Yosiaki Taniyama, Motokuni Aoki, Yasuhumi Kaneda, Toshio Ogihara, Ryuichi Morisita 、 The American Society of Gene Therapy’s 7th Annual Meeting、2004年6月2日-6日、米国・ミネアポリス

G. 知的所有権の出願・登録状況（予定を含む）

1. 特許取得（3件）

- “ウイルスエンベロープからなる免疫アジュバント” 特願 2004-108599(国内出願中)

- “ゲノムライブラリーの迅速スクリーニングおよび高効率遺伝子機能解析方法” 特願 2002-337545

- “化学療法剤を封入した医薬製剤” 特願 2002-320577

2. 実用新案登録

特になし

III. 研究成果の刊行に関する一覧表

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Yasufumi Kaneda	Hemagglutinating virus of Japan (HVJ) envelope vector as a versatile gene delivery system.	Mol Ther.	6(2)	219-226	2002
Yasufumi Kaneda	A novel combination of suicide gene therapy and histone deacetylase inhibitor for treatment of malignant melanoma	Cancer Gene Therapy	10	179-186	2003
Yasufumi Kaneda	HVJ-envelope vector for gene transfer into central nervous system	Biochem Biopys Res Commun	300(2)	464-471	2003
Yasufumi Kaneda	Enhanced angiogenesis and improvement of neuropathy by cotransfection of human hepatocyte growth factor and prostacyclin synthase gene	FASEB J.	17(6)	779-781	2003
Yasufumi Kaneda	Novel Therapeutic Strategy to Treat Brain Ischemia:Overexpressio n of Hepatocyte Growth Factor Gene Reduced Ischemic Injury Without Cerebral Edema in Rat Model	Circulation	109	424-431	2004
Yasufumi Kaneda	No influence of tumor groeth by intramuscular injection of hepatocyte growth factor plasmid DNA: safety evaluation of therapeutic angiogenesis gene therapy in mice	Biochem Biopys Res Commun	315(1)	59-65	2004
Yasufumi Kaneda	Intrathecal injection of HVJ-E containing HGF gene to cerebrospinal fluid can prevent and ameliorate hearing impairment in rats.	The FASEB Journal	18(1)	212-214	2004
Yasufumi Kaneda	New vector innovation for drug delivery: development of fusigenic non-viral particle.	Current Drug Targets	4	599-602	2003
Kotani Hitoshi	The HVJ-envelope as an innovative vector system for cardiovascular disease.	Current Gene Therapy	4(2)	183-194	2004

Yasufumi Kaneda	Biocompatible polymer enhances the *in vitro* and *in vivo* transfection efficiency of HVJ envelope vector.	The Journal of Gene Medicine			in press
Yasufumi Kaneda	Rad51 siRNA delivered by HVJ envelope vector enhances the anti-cancer effect of cisplatin.	The Journal of Gene Medicine			in press

Ⅳ.研究成果の刊行物・別刷

doi:10.1006/mthe.2002.0647, available online at http://www.idealibrary.com on IDEAL

ARTICLE

Hemagglutinating Virus of Japan (HVJ) Envelope Vector as a Versatile Gene Delivery System

Yasufumi Kaneda,[1,*] Toshihiro Nakajima,[2] Tomoyuki Nishikawa,[1] Seiji Yamamoto,[1] Hiroyuki Ikegami,[1] Naho Suzuki,[2] Hitomi Nakamura,[3] Ryuichi Morishita,[1] and Hitoshi Kotani[2]

[1]*Division of Gene Therapy Science, Graduate School of Medicine, Osaka University, Suita, Osaka 565-0871, Japan*
[2]*Anges MG, Inc., 1-8-31 Midorigaoka, Ikeda, Osaka 563-8577, Japan*
[3]*Department of Obstetrics and Gynecology, Graduate School of Medicine, Osaka University, Suita, Osaka 565-0871, Japan*

**To whom correspondence and reprint requests should be addressed. Fax: 81-6-6879-3909. E-mail: kaneday@gts.med.osaka-u.ac.jp.*

We have developed a simple method for converting the lipid envelope of an inactivated virus to a gene transfer vector. Hemagglutinating virus of Japan (HVJ; Sendai virus) envelope vector was constructed by incorporating plasmid DNA into inactivated HVJ particles. This HVJ envelope vector introduced plasmid DNA efficiently and rapidly into various cell lines, including cancer cells and several types of primary cell culture. Efficiency of gene transfer was greatly enhanced by protamine sulfate and centrifugation. Fluorescein isothiocyanate-labeled oligodeoxynucleotides (FITC-ODN) were also delivered to cells at > 95% efficiency. When HVJ envelope vector was injected into organs directly, reporter gene expression was observed in organs including liver, brain, skin, uterus, tumor masses, lung, and eye. When HVJ envelope vector containing luciferase gene was injected into mouse tail vein, luciferase gene expression was detected primarily in spleen. FITC-ODN were also delivered to spleen cells by intravenous injection of HVJ envelope. These results suggest that HVJ envelope vector will be useful for both *ex vivo* and *in vivo* gene therapy experiments.

Key Words: HVJ envelope, gene transfer, drug delivery, gene therapy, targeting

INTRODUCTION

Efficient and minimally invasive vector systems are needed for both gene therapy and drug delivery. Numerous viral and nonviral (synthetic) methods of gene transfer have been developed and improved [1,2]. The safety of viral vectors is of concern because cointroduction of genetic elements from parent viruses, leaky expression of viral genes, immunogenicity, and changes in the host genome structure can occur, whereas nonviral vectors are less toxic and less immunogenic [3]. However, most nonviral methods provide less efficient gene transfer, especially *in vivo*. There are three basic approaches to development of an *in vivo* gene transfer vector with high efficiency and low toxicity. The first approach is to correct for the limitations of current vector systems. For example, gutless adenoviral vectors have been developed to reduce cytotoxicity [4,5], and a novel lipid has been used to enhance liposome-mediated gene delivery [6,7]. The second approach is to improve gene delivery methods. Viruses such as foamy virus [8], baculovirus [9], and simian virus 40 [10], and polymers such as polyethyleneimine [11] have been adapted as gene transfer vectors. The third approach is to construct chimeric vectors that compensate for the limitations of one vector system by the strengths of another. Examples of chimeric systems currently available include a pseudotyped retrovirus vector with vesicular stomatitis virus (VSV) G-protein envelope [12] and a lentivirus vector containing human immunodeficiency virus (HIV) proteins decorated with pseudotype retrovirus envelope-containing VSV-G protein [13]. A similar strategy has been used to develop nonviral gene delivery vectors, such as cationic lipid–protamine–DNA complexes with enhanced *in vivo* gene transfer efficiency [14,15].

We have developed chimeric gene transfer systems that combine viral and nonviral features. We have constructed HVJ-liposomes by combining DNA-loaded liposomes with a fusigenic envelope derived from hemagglutinating virus of Japan (HVJ; Sendai virus) [16,17]. DNA, RNA, and oligonucleotides can be incorporated into liposomes with this system and delivered efficiently both *in vitro* and *in vivo* by virus–cell fusion, which protects the contents from degradation by endosomes and lysosomes. The safety of HVJ-liposomes was evaluated in monkeys, and no critical cell damage was observed [18]. Numerous gene therapy strategies that use the HVJ-liposome system have been used successfully in animals [19,20]. However, this system

1525-0016/02 $35.00

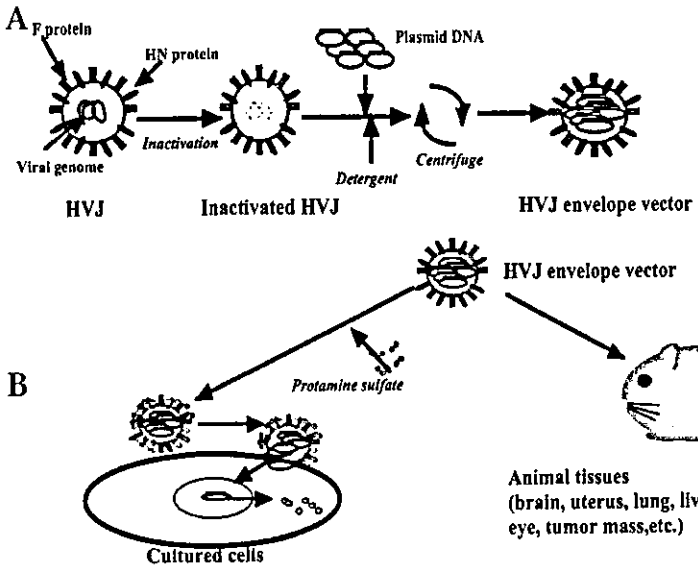

FIG. 1. HVJ envelope vector system. (A) Construction of HVJ envelope vector. Plasmid DNA was incorporated into inactivated HVJ particles by treatment with detergent and centrifugation. (B) Gene transfer with HVJ envelope vector. For *in vitro* gene transfer, HVJ envelope vector was mixed with protamine sulfate, and the mixture was added to cultured cells. For *in vivo* gene transfer to tissues such as brain, liver, and lung, HVJ envelope vector was injected directly into animal tissues without protamine sulfate.

is limited because the fusion activity of HVJ-liposomes is greatly decreased compared with that of native HVJ as a result of the reduced density of fusion proteins on HVJ-liposomes. Actually, hemagglutinating activity, one of the indicators of fusion activity, of HVJ-liposomes was reduced to ~ 2% that of HVJ itself (Y.K., unpublished data). Another limitation is that vector production is complicated by the presence of two distinct vesicles, HVJ particles and liposomes. Reconstituted HVJ particles have been prepared in several laboratories [21–25], but they differ from native HVJ particles in the contents of fusion proteins [23,25] and lipid components [21,22]. The ratio of fusion proteins F and HN in reconstituted HVJ particles also differs from that in native HVJ [24], and this greatly affects fusion activity [26,27]. Furthermore, the methods of preparation of reconstituted particles are generally complicated.

To address these problems, we developed a simple method to convert an inactivated virus containing lipid envelope into a gene transfer vector. We used this technology to introduce plasmid DNA directly into inactivated HVJ envelope. We then investigated the feasibility of using this HVJ envelope vector for *in vitro* and *in vivo* gene transfer.

RESULTS

Incorporation of Plasmid DNA into Inactivated HVJ Particles

Development of the HVJ envelope vector is illustrated in Fig. 1A. Exogenous plasmid DNA was incorporated into inactivated HVJ by treatment with mild detergent and centrifugation. Inactivated HVJ of 3×10^{10} particles was mixed with 0.24–0.80% Triton X-100 in the presence of plasmid DNA (200 μg) on ice, and the mixture was centrifuged at 18,500*g* for 15 minutes to move the DNA into the HVJ particle. The amount of DNA resistant to DNase I digestion was 34.8 ± 5.3 μg. Therefore, the DNA-trapping efficiency of HVJ envelope vector with this method was ~ 15–20%. The largest DNA tested was a 14-kb plasmid DNA, and the trapping efficiency was ~ 18%. Without centrifugation, the DNA-trapping efficiency was ~ 3–5%. Without detergent treatment, no DNA was entrapped in the viral particle. We tested several detergents for preparation of HVJ envelope vector. The optimal concentrations of detergent were 0.24–0.80% for Triton X-100, 0.04–0.12% for Nonidet P-40, 1.2–2.0% for CHAPS, and 0.55–0.80% for octylglucoside.

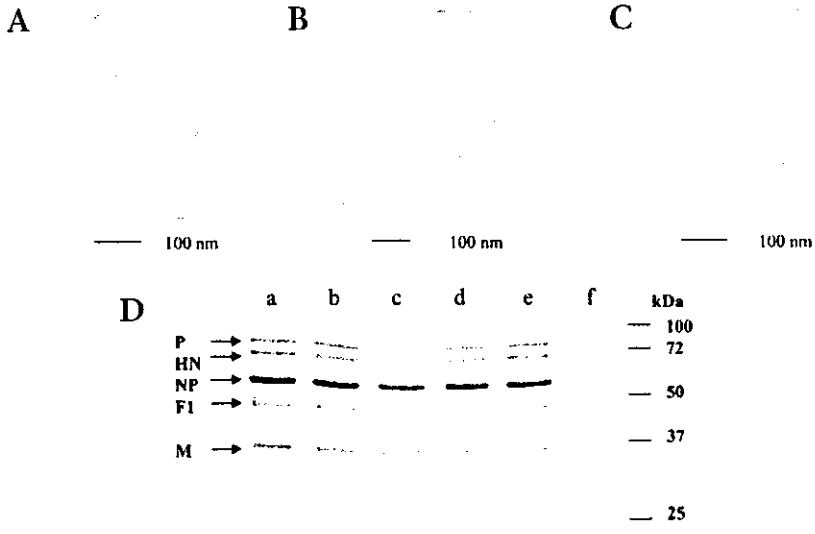

FIG. 2. Electron microscopy of native HVJ (A) and of inactivated HVJ treated with mild detergent in the absence (B) or in the presence (C) of plasmid DNA. (D) SDS–PAGE analysis of proteins of native HVJ (a), inactivated HVJ (b), HVJ envelope vector containing plasmid DNA (c), HVJ envelope vector without plasmid DNA (empty HVJ envelope vector) (d), and mixture of inactivated HVJ and plasmid DNA without detergent (e). Lane f is a molecular weight marker. Arrows indicate the positions of HVJ proteins deduced from electrophoretic mobility as described [27]. F1 is a cleaved fragment of F protein.

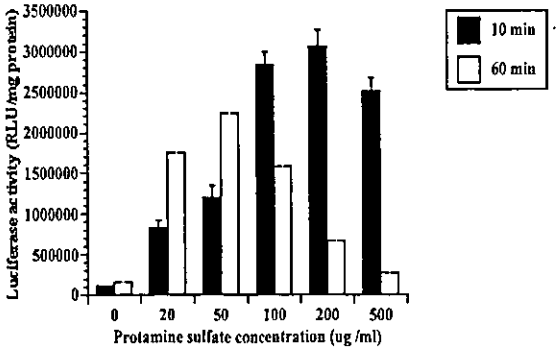

FIG. 3. Enhanced transfection by HVJ envelope vector with protamine sulfate. HVJ envelope vector (3×10^9 particles) containing luciferase expression plasmid was transferred to HCT116 cells with various concentrations of protamine sulfate and incubated for 10 (black bar) or 60 (gray bar) minutes. Luciferase gene expression by HCT116 cells was examined 24 hours after transfer. Results are shown as mean ± SD ($n = 4$).

Characterization of HVJ Envelope Vector

Electron microscopy of HVJ and HVJ envelope vector is shown in Fig. 2. Native HVJ was ~ 300 nm in diameter and contained viral RNA genome, which is visible as electron-dense material (Fig. 2A). When the HVJ envelope was isolated by inactivation of HVJ with UV or β-propiolactone and subsequent treatment with mild detergent (Fig. 2B), the HVJ particle-like structure was retained, but the electron-dense materials observed in native HVJ drastically diminished, suggesting that the HVJ genome was degraded or lost. However, when the HVJ envelope was constructed in the presence of plasmid DNA, electron-dense materials were observed again inside the HVJ envelope vector (Fig. 2C), indicating that plasmid DNA was incorporated into the HVJ envelope vector.

The protein composition of the HVJ envelope vector was analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE; Fig. 2D). Most of the native HVJ proteins were retained in the HVJ envelope vector. Fusion proteins HN and F1 (active form of F protein) were retained, and the molar ratio of these proteins in HVJ envelope vector was ~ 2–2.3, which is the same as that in native HVJ [26,27].

Gene Transfer to Cultured Cells

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 (Fig. 1B). Luciferase gene expression in all the cell lines tested was significantly augmented with protamine sulfate when compared with that in the absence of protamine sulfate. The increase in gene expression with protamine sulfate varied from 10- to 50-fold in different cell lines. A representative example is shown in Fig. 3. In human colon cancer HCT116 cells, luciferase gene expression was ~ 20 times higher with protamine sulfate in comparison with the level of expression in the absence of protamine sulfate. A 10-minute incubation was sufficient for high expression. The optimal conditions for *in vitro* gene transfer are summarized in Table 1. When the HVJ envelope vector containing green fluorescenct protein (GFP) expression plasmid was added to BHK-21 cells under the conditions shown in Table 1, GFP expression as determined by flow cytometry was ~ 80% (Fig. 4A). The gene transfer efficiency was dependent on the amount of vector and was not affected by 10% FCS. HVJ envelope vector did not appear to cause any observable cell damage due to cell–cell fusion. Nonadherent floating cells such as human erythroleukemia K562 cells and human T-cell leukemia NALM-6 cells have been resistant to current gene

TABLE 1: Results of *in vitro* gene transfer with HVJ envelope vector

Cell line	GFP (%)	RLU/mg protein	Incubation time (min)	PS conc. (μg/ml)	Centrifuge
Adherent cells					
HEK-293	40	2.64×10^6	60	300	-
BHK-21	80	9.35×10^7	10	10	-
B16-F1	50	1.13×10^7	60	50	-
M24	30	ND	10	10	+[a]
SAS	40	1.39×10^6	60	400	-
HeLa-S3	ND	5.79×10^5	60	100	-
Huh-7	ND	1.60×10^6	60	350	-
Nonadherent cells					
K562	50	2.10×10^5	10	100	+[b]
NALM-6	ND	1.90×10^5	10	100	+[b]
Primary cells					
HAEC	20	1.78×10^5	60	200	-
mDC	50	ND	90	0	+[c]

GFP (%), GFP expression rate determined by FACS analysis. ND. not done. RLU, luciferase activity (relative light units). Incubation time, time (minutes) required for transfection with HVJ envelope. PS conc., concentration of protamine sulfate (PS) in medium for transfection with HVJ envelope. Centrifuge, centrifugation of cells for transfection with HVJ envelope. HEK293, human embryonal kidney cells. BHK-21, baby hamster kidney cells. BL16-F1, mouse melanoma cells. M24, human melanoma cells. SAS, human tongue cancer cells. HeLa-S3, human uterocervical cancer cells. Huh-7, human hepatoma cells. K562, human erythroleukemia cells. NALM-6, human T cell leukemia cells. HAEC, human aortic endothelial cells. mDC, mouse dendritic cells.
[a] 1000*g* at 35°C.
[b] 18,500*g* at 35°C.
[c] 12,000*g* at 35°C.

doi:10.1006/mthe.2002.0647, available online at http://www.idealibrary.com on IDEAL

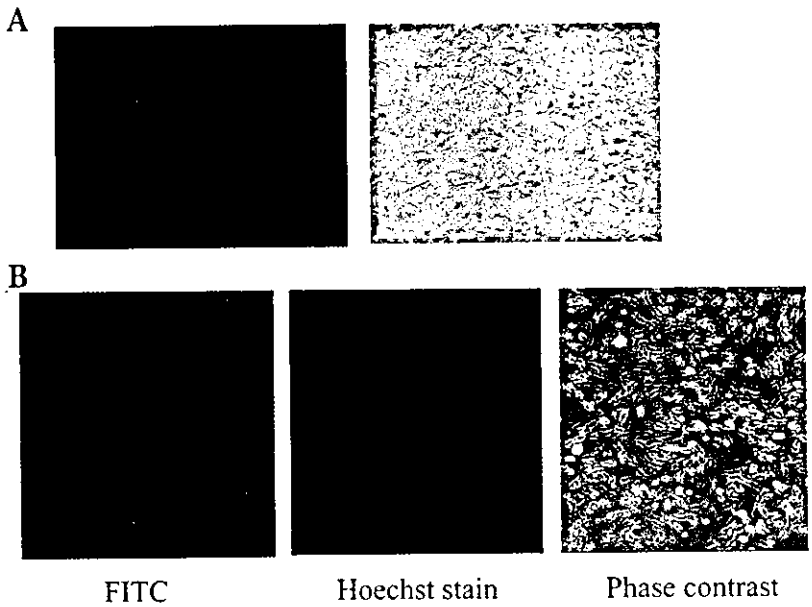

FIG. 4. GFP expression (A) and FITC-ODN transfer (B) in BHK-21 cells treated with HVJ envelope vector (3×10^9 particles). (A) HVJ envelope vector containing GFP plasmid was added to BHK-21 cells, and GFP (left) was observed by fluorescence microscopy (×200) 24 hours after transfer. At right is a phase-contrast image of the same field. (B) At 4 hours after transfer of FITC-ODN, FITC detection (left), nuclear staining with Hoechst 33258 (center), and phase-contrast image (right) of the same field were analyzed by fluorescence microscopy (×100).

transfer methods such as cationic liposome-mediated lipofection, electroporation, and HVJ-liposomes (Y.K., unpublished data). Luciferase gene expression in NALM-6 and K562 cells was increased with protamine sulfate treatment, but the level was still very low. However, when protamine sulfate was used in conjunction with centrifugation, as reported in retroviral vector transfection [28], luciferase gene expression in NALM-6 cells and K562 cells was enhanced 30- to 40-fold in comparison with that without centrifugation (data not shown). The optimal condition for gene transfer to such nonadherent cells was centrifugation of the cells and vector (6×10^9 particles) mixture at 18,500*g* for 10 minutes at 35°C in the presence of 100 μg/ml of protamine sulfate (Table 1). Primary cells such as human aortic endothelial cells and mouse dendritic cells were also effectively transfected with HVJ envelope vector without much cell damage. Approximately 50% of mouse dendritic cells and 20% of human aortic endothelial cells expressed GFP when assayed by fluorescence-activated cell sorting (FACS) 24 hours after transfection.

FITC-ODN were also transferred to the nuclei of BHK-21 cells at an efficiency of > 95% (Fig. 4B).

Gene Transfer to Animal Tissues

Another advantage of the present HVJ envelope vector system is possible *in vivo* application. Luciferase gene expression in mouse liver by HVJ envelope vector (3×10^9 particles) was two times higher than that by HVJ-liposomes prepared with inactivated HVJ (3×10^9 particles; Fig. 5A). In mouse uterus, we did not achieve effective gene transfer with HVJ-liposomes (3×10^9 particles), but high luciferase gene expression was obtained with HVJ envelope vector (3×10^9 particles; Fig. 5B). Transfer of the *lacZ* gene to uterus with HVJ envelope vector yielded gene expression mainly in the glandular epithelium of the endometrium (data not shown).

We next attempted to inject HVJ envelope vector intravenously. Luciferase gene-loaded HVJ envelope vector (6×10^9 particles) was injected into mouse tail vein, and luciferase activity in each organ was measured 24 hours after injection. Injection of HVJ envelope vector (6×10^9 particles) resulted in the highest luciferase activity and all mice lived, but 1 mouse out of 10 died 1 day after transfer of 9×10^9 particles of HVJ envelope vector. Luciferase activity was detected at significant levels in spleen and faintly in lung, but it was not observed in organs such as brain, kidney, bone marrow, intestine, liver, and testis (Fig. 6). Luciferase expression was detected in spleen for at least 1 week (data not shown). Similar results were obtained in three different experiments, one of which is shown in Fig. 6. Intravenous administration of naked plasmid (30 μg) containing luciferase gene resulted in expression in spleen and lung at much lower levels than that achieved with HVJ envelope. Compared with expression by naked plasmid DNA transfer, luciferase gene expression by HVJ envelope transfer was significant only in spleen, and not in other organs such as lung and liver (Fig. 6). FITC-ODN were also delivered to spleen by intravenous administration of HVJ envelope containing FITC-ODN (Fig. 7A). FITC-ODN were trapped by cells in the marginal zone of the spleen, and the ratio of the cells trapping FITC-ODN was estimated to be 6.3% of spleen cells (342/5411 cells counted). However, FITC-ODN were not detected in other organs such as lung and liver (data not shown). No specific fluorescence was observed in spleen when either empty HVJ envelope vector (Fig. 7B) or naked FITC-ODN (data not shown) were injected into mouse tail vein.

Discussion

We developed an HVJ envelope vector that traps exogenous DNA in inactivated HVJ particles, and we used this system to introduce DNA into various cultured cells and animal tissues.

HVJ envelope vector differs from reconstituted HVJ particles that are prepared by reassembling lipids and fusion proteins after solubilization of the virus particle. In the

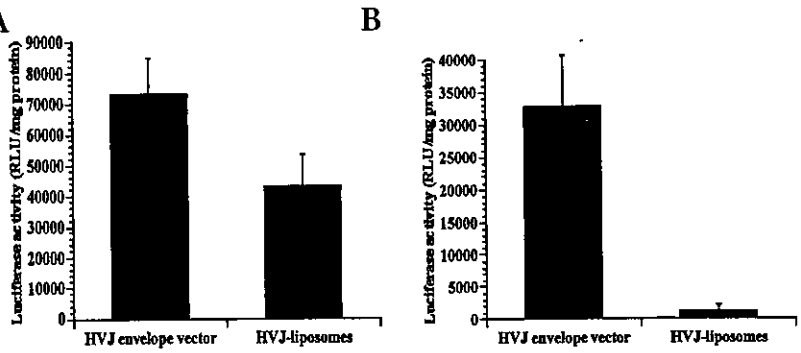

FIG. 5. Luciferase gene expression in mouse liver (A) and mouse uterus (B) by HVJ envelope vector (3×10^9 particles) and HVJ-liposomes. Results are shown as mean ± SD ($n = 5$).

preparation of HVJ envelope vector, plasmid DNA is incorporated into inactivated HVJ particles by treatment with mild detergent and without destruction of the virion. This procedure does not require dialysis, purification, or addition to the preparation of lipids or proteins, which are used for the preparation of reconstituted HVJ particles [21–25]. Therefore, the components of HVJ envelope vector are very close to those of native HVJ. The amounts of F1 and HN proteins in HVJ envelope vector are the same as those in native HVJ (Fig. 2). The ratio of these proteins is important for efficient fusion of the virus with the cell membrane [26,27], and in reconstituted HVJ particles, this ratio is altered [24]. Consequently, when hemagglutination of HVJ is assayed in chick red blood cells as described [27], the hemagglutinating activity derived from HN protein of HVJ envelope vector, which is one indicator of fusion activity, is similar to that of native HVJ and is ~ 40–50 times higher than that of HVJ-liposomes prepared from the same number of HVJ particles as HVJ envelope vector (data not shown). The disadvantage of the HVJ envelope vector is that the DNA-trapping efficiency is as low as that of anionic liposomes (15–20%) [17]. Without detergent treatment, no DNA was entrapped in the HVJ envelope. By detergent treatment without centrifugation, trapping efficiency was only 3–4%. Although the mechanism of DNA entrapment in the HVJ envelope is not clear, we speculate that small pores may be formed on the envelope by the detergent treatment and that DNA may be pushed into the particle by centrifugation. Alternatively, nucleic acid-binding proteins such as nucleocapsid protein (NP) [28] of HVJ may help to drag DNA into the particle. This possibility will be clarified when an NP-free viral particle is successfully formed.

Protamine sulfate was absolutely necessary for *in vitro* gene transfer with HVJ envelope vector. We hypothesized that the low transfection efficiency resulted from weak association of the vector with the cell membrane as a result of the negative charge on both the envelope and cell membrane. Therefore, we used protamine sulfate to augment attachment of the HVJ envelope vector to the cell surface by providing a cationic charge. Protamine sulfate has been used widely to enhance gene transfer by viral vectors and liposomes [29–34]. The optimal concentration of protamine sulfate for *in vitro* gene transfer differed among cells (Table 1). This is likely due to differences in sensitivity to HVJ and the net charge on the cell surface. HVJ envelope vector was useful for gene transfer to various cell lines, and a short incubation was sufficient for transfection. Under such conditions, little cell damage was observed. Thus, HVJ envelope vector should be useful for *ex vivo* gene therapies.

HVJ envelope vector transferred FITC-ODN to most cell nuclei (Fig. 4B), suggesting that the HVJ envelope vector system may be useful as a drug delivery system. Indeed, we have successfully used the HVJ envelope vector to accelerate radiation-induced apoptosis of cancer cells with an NF-κB decoy transfer (H.N., manuscript in preparation) and to contact dermatitis in mice by transfer of Stat6 decoy oligonucleotides (Hiroo Yokoseki, manuscript in preparation).

The HVJ envelope vector is more effective than HVJ-liposomes for *in vivo* gene transfer (Fig. 5). *LacZ* or luciferase gene transfer to lung, liver, uterus, eye, skin, muscle, and brain of animals such as mouse, rat, rabbit, and monkey was achieved by direct injection of the HVJ envelope vector. Among the organs we tested, HVJ envelope vector was more effective than HVJ-liposomes for gene transfer to liver, uterus, brain, eye, and lung. Similar levels of expression were detected in muscle and skin. Luciferase gene expression by HVJ envelope vector was more efficient than that by HVJ-liposomes in tumor

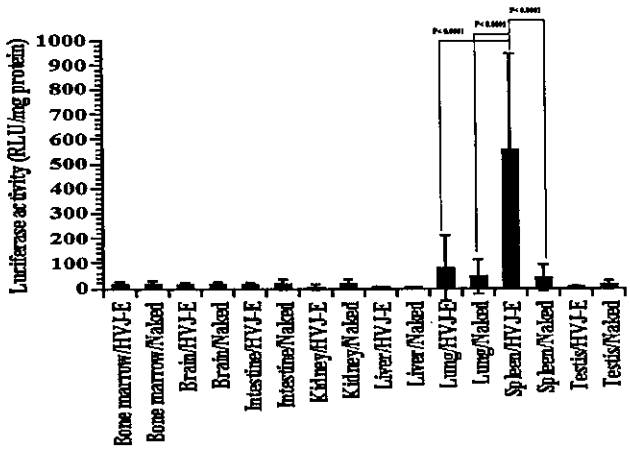

FIG. 6. Luciferase gene expression in various organs on day 1 after injection of HVJ envelope vector (HVJ-E) containing luciferase gene or naked plasmid with luciferase gene (naked) into mouse tail vein. $P < 0.0001$, significant difference between two groups. Results are shown as mean ± SD ($n = 7$).

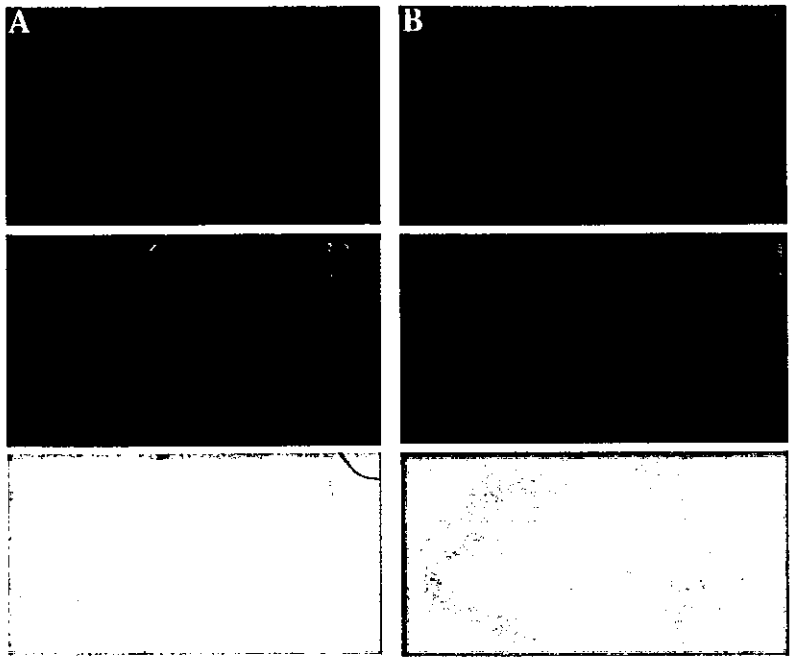

FIG. 7. FITC-ODN in spleen. Delivery of FITC-ODN to mouse spleen 2 hours after injection of 100 μl of HVJ envelope containing FITC-ODN into tail vein (A). In (B), empty HVJ envelope without FITC-ODN was injected in a similar manner. Upper, middle, and lower panels are FITC detection, Hoechst staining, and phase-contrast images, respectively.

masses derived from mouse melanoma cell (B16-F1), renal cancer cells (RENCA), and colon cancer cells (CT26) (S.Y., manuscripts in preparation). This predominance of HVJ envelope vector over HVJ-liposomes may be due to the stronger fusion activity of the HVJ envelope vector in comparison with that of HVJ-liposomes. The fusion activity of HVJ envelope vector, quantified as hemagglutinating activity, was ~ 50 times higher than that of HVJ-liposomes. The density of fusion proteins on HVJ envelope vector is greater than that of HVJ-liposomes because of the smaller particle size (~ 300 nm versus ~ 500 nm, respectively). This difference in density may influence fusion activity.

HVJ envelope vector targeted mainly spleen. Although colloidal particles are trapped in reticuloendothelial cells [35], predominant target tissues varied for the various 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 [25], because galactose residues of F protein are recognized by hepatocytes [25]. HVJ-liposomes containing both F and HN proteins target mainly liver, and faintly spleen and lung, when the vector is injected into the saphenous veins of monkeys [18], probably because phospholipids such as phosphatidylserine [35] present on the envelope are recognized by reticuloendothelial cells. LPD (liposome–protamine sulfate–plasmid DNA) vector targets lung, kidney, heart, liver, and spleen, with highest gene expression in the lung [14,15]. Analysis of the effects of mutations in the fusion glycoproteins of HVJ and alteration in the lipid profile of the envelope should clarify the mechanism underlying the spleen-specific targeting by HVJ envelope vector. Apart from the mechanism of tissue targeting, the spleen-targeting ability of HVJ envelope vector may be very effective for inducing immunity against infectious diseases and cancers. We reported earlier that strong antitumor immunity is generated when HVJ-liposomes containing melanoma-associated antigen gp100 mRNA were injected directly into mouse spleen [36]. Because direct injection into spleen is not practical for human gene therapy, intravenous administration of HVJ envelope vector containing tumor-associated antigen genes may yield an effective and practical strategy for cancer treatment.

In nonviral gene delivery systems, the gene expression period is dependent on the structure of the transferred plasmid. Although gene expression is detected for 2 weeks after transfer of GFP expression plasmid to rat brain and 2–4 weeks after transfer to mouse skeletal muscle, the duration of gene expression may be improved with an Epstein–Barr virus replicon plasmid [37,38].

Thus, HVJ envelope vector appears to be promising for *ex vivo* and *in vivo* gene transfer. Therefore, the HVJ envelope vector kit named GenomONE has been commercially available since April 1, 2002 (ISK, Osaka, Japan). The transfection efficiency using GenomONE was compared with other commercially available transfection kits. Consequently, luciferase gene expression in BHK-21 and primary human aortic endothelial cells (HAEC) transfected with GenomONE was 2–10 times higher than that with other gene transfer reagents such as LipofectAMINE 2000 Reagent (Life Technologies, Rockville, MD), Transfectam Reagent (Promega, Madison, WI), SuperFect Transfection (Qiagen, Hilden, Germany), and Effectene Transfection (Qiagen). HVJ envelope vector system also has advantages over current viral vector systems. Using HVJ envelope vector, gene expression was effectively detected in nondividing cells such as neurons and myotubes where gene transfer is not successful using retroviral vector [3]. Luciferase gene expression was not inhibited at all by three consecutive intramuscular injections of the DNA-loaded HVJ envelope vector (Y.K. and S.Y., unpublished data). The low immunogenicity of HVJ envelope vector is superior to highly immunogenic vectors such as adenoviral vector from the viewpoints of long-term gene expression and safety [3]. Moreover, HVJ envelope vector can deliver proteins, oligonucleotides, or anticancer drugs to cells both *in vitro* and *in vivo* as well as plasmid DNA (Y.K. and H.K., manuscript in preparation). Therefore, HVJ envelope vector can be used as not

only a DNA transfer vector system but also a drug delivery system. In this point, HVJ envelope vector obviously exceeds viral vectors in utility.

We have already evaluated the safety of the vector in various types of animals, and the clinical-grade HVJ envelope vector will be produced at Anges MG, Inc.

Materials and Methods

Virus production, purification, and inactivation. HVJ was amplified in chorioallantoic fluid of 10- to 14-day chick eggs as described [39]. Virus was inactivated with β-propiolactone (0.0075–0.001%) [40] and then purified by ion-exchange column chromatography. Alternatively, virus was purified by centrifugation and inactivated by UV irradiation (99 mJ/cm^2) as described [14]. In both cases, virus replication was lost completely, but hemagglutinating activity was not affected. Aliquots of the virus (3×10^{10} particles/1.5-ml tube) were centrifuged (18,500*g*, 15 minutes) at 4°C, and the viral pellet was stored at −20°C.

DNA preparation. Both luciferase expression vector (Promega) and GFP expression vector (Clontech Palo Alto, CA) were driven by cytomegalovirus (CMV) promoter. DNA was purified by Qiagen column (Hilden, Germany). FITC-ODN (random sequence) were purchased from Hokkaido System Science (Sapporo, Japan).

Cells. Primary human aortic endothelial cells (HAEC) were purchased from Sanko-Junyaku (Tokyo, Japan). Human melanoma cells (M24) were kindly provided by the John Wayne Cancer Institute (Santa Monica, CA). Mouse dendritic cells (mDC) were isolated from bone marrow of femur and tibia in our laboratory according to a method described [41], cultured for 4 days in the medium supplemented with 10 ng/ml recombinant granulocyte/macrophage colony-stimulating factor (GM-CSF; Genzyme-Techne, Minneapolis, MN) before transfection, and ~ 99% of the cells were identified as dendritic cells by FACS analysis after the staining with anti-CD11c antibody (PharMingen, Ontario, Canada). All other cell lines were purchased from American Type Culture Collection (ATCC, Rockville, MD). Adherent and nonadherent cells were cultured in Dulbecco's modified Eagle's medium and RPMI 1640, respectively, supplemented with 10% FBS.

Preparation of HVJ envelope vector. 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 or oligonucleotide (200 μg/50 μl) and 5 μl detergent. The mixture was centrifuged at 18,500*g* for 15 minutes 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 envelope vector was suspended in 300 μl of PBS. The vector was stored at 4°C until use. It was stable at least for 1 week at 4°C.

Trapping efficiency of DNA in the vector was examined by a method described earlier [42]. Briefly, HVJ envelope vector containing plasmid DNA was treated with DNase I (100 mg/ml) for 1 hour at 37°C, purified by centrifugation, and solubilized with 1% SDS. The DNA was then extracted with phenol and measured either by the absorbance at 260 nm or by intensity of the band after agarose gel electrophoresis.

For protein analysis, each sample (10 μg) was separated by SDS–polyacrylamide (10%) gel electrophoresis and stained with Coomassie blue.

HVJ, empty HVJ envelope vector, and HVJ envelope vector with plasmid DNA were analyzed by electron microscopy [42]. Ultrathin sections were stained with 3% uranylacetate, and the samples were examined with an electron microscope (H-7100, Hitachi).

Gene transfer in vitro and in vivo. For *in vitro* transfection, ~ 5×10^5 cells were prepared 1 day before transfection. HVJ envelope vector ($3–6 \times 10^9$ particles) was mixed with various concentrations of protamine sulfate (Table 1) and added to cells cultured in medium supplemented with 10% FBS. After 10–60 minutes of incubation at 37°C under 5% CO_2, the medium was replaced, and the cells were cultured overnight before examination of gene expression. In some cases, cells in a multiwell plate or in a plastic tube were centrifuged under the conditions shown in Table 1 after HVJ envelope vector was added.

GFP expression was observed under a fluorescent microscope without fixation, and the percentage of cells expressing GFP was determined by a flow cytometry method described earlier [43]. After transfer of FITC-ODN, cells were fixed with 4% paraformaldehyde, stained with 2 mg/ml Hoechst 33258, and observed by fluorescent microscopy.

For *in vivo* transfection, HVJ envelope vector ($3–9 \times 10^9$ particles) was injected directly into animal tissues without protamine sulfate. HVJ envelope vector (6×10^9 particles) containing luciferase gene (6 μg) or FITC-ODN (12 μg) suspended in 100 μl PBS was injected into the tail vein of BALB/c mice (8 weeks of age; $n = 7$ to 10). Naked luciferase plasmid (30 μg) was also injected into the tail vein in a similar manner. Fisher's test was used to determine the significance of differences. All animals were handled in a humane manner in accordance with the guidelines of the Animal Committee of Osaka University.

Luciferase activity was measured with a luciferase assay kit (Promega) [44], and the protein content of the samples was assayed by the Bradford method [45].

Acknowledgments

We thank Yoshio Okada (Senri Life Science Foundation) for evaluating our HVJ envelope vector system. This research was supported by Health Science Research Grants from the Ministry of Health and Welfare.

RECEIVED FOR PUBLICATION FEBRUARY 15; ACCEPTED MAY 22, 2002.

References

1. Marshall, E. (1995). Gene therapy's growing pains. *Science* 269: 1052–1055.
2. Ledley, F. D. (1995). Nonviral gene therapy: the promise of genes as pharmaceutical products. *Hum. Gene Ther.* 6: 1129–1144.
3. Mulligan, R. C. (1993). The basic science of gene therapy. *Science* 260: 926–932.
4. Morral, N., *et al.* (1999). Administration of helper-dependent adenoviral vectors and sequential delivery of different vector serotype for long-term liver-directed gene transfer in baboons. *Proc. Natl. Acad. Sci. USA* 96: 12816–12821.
5. Zou, L., Zhou, H., Pastore, L., and Yang, K. (2000). Prolonged transgene expression mediated by a helper-dependent adenoviral vector (hdAd) in the central nervous system. *Mol. Ther.* 2: 105–13.
6. Legendre, J. Y., and Szoka, F. C., Jr. (1992). Delivery of plasmid DNA into mammalian cell lines using pH-sensitive liposomes: comparison with cationic liposomes. *Pharm. Res.* 9: 1235–1242.
7. Wheeler, C. J., *et al.* (1996). A novel cationic lipid greatly enhances plasmid DNA delivery and expression in mouse lung. *Proc. Natl. Acad. Sci. USA* 93: 11454–11459.
8. Russell, D. W., and Miller, A. D. (1996). Foamy virus vectors. *J. Virol.* 70: 217–222.
9. Condreay, J. P., Witherspoon, S. M., Clay, W. C., and Kost, T. A. (1999). Transient and stable gene expression in mammalian cells transduced with a recombinant baculovirus vector. *Proc. Natl. Acad. Sci. USA* 96: 127–132.
10. Strayer, D. S. (1999). Gene therapy using SV40-derived vectors: what does the future hold? *J. Cell Physiol.* 181: 375–384.
11. Gebhart, C. L., Kabanov, A. V. (2001). Evaluation of polyplexes as gene transfer agents. *J. Control Release.* 73: 401–416.
12. Burns, J. C., Friedmann, T., Driever, W., Burrascano, M., and Yee, J. K. (1993). Vesicular stomatitis virus G glycoprotein pseudotyped retroviral vectors: concentration to very high titer and efficient gene transfer into mammalian and nonmammalian cells. *Proc. Natl. Acad. Sci. USA* 90: 8033–8037.
13. Naldini, L., *et al.* (1996). *In vivo* gene delivery and stable transduction of nondividing cells by a lentivirus vector. *Science* 272: 263–267.
14. Li, S., and Huang, L. (1997). *In vivo* gene transfer via intravenous administration of cationic lipid–protamine–DNA (LPD) complexes. *Gene Ther.* 4: 891–900.
15. Li, S., Rizzo, M. A., Bhattacharya, S., and Huang, L. (1998). Characterization of cationic lipid–protamine–DNA (LPD) complexes for intravenous gene delivery. *Gene Ther.* 5: 930–937.
16. Kaneda, Y., Iwai, K., and Uchida, T. (1989). Increased expression of DNA cointroduced with nuclear protein in adult rat liver. *Science* 243: 375–378.
17. Kato, K., Nakanishi, M., Kaneda, Y., Uchida, T., and Okada, Y. (1991). Expression of hepatitis B virus surface antigen in adult rat liver. *J. Biol. Chem.* 266: 3361–3364.
18. Tsuboniwa, N., *et al.* (2001). Safety evaluation of HVJ-AVE liposomes in nonhuman primates. *Hum. Gene Ther.* 12: 469–487.
19. Dzau, V. J., Mann, M., Morishita, R., and Kaneda, Y. (1996). Fusigenic viral liposome for gene therapy in cardiovascular diseases. *Proc. Natl. Acad. Sci. USA* 93: 11421–11425.
20. Kaneda, Y., Saeki, Y., and Morishita, R. (1999). Gene therapy using HVJ-liposomes: the best of both worlds? *Mol. Med. Today* 5: 298–303.
21. Uchida, T., Kim., J., Yamaizumi, M., Miyake, Y., and Okada, Y. (1979). Reconstitution of lipid vesicles associated with HVJ (Sendai virus) spikes. Purification and some properties

doi:10.1006/mthe.2002.0647, available online at http://www.idealibrary.com on IDEAL

of vesicles containing nontoxic fragment A of diphtheria toxin. *J. Cell Biol.* **80**: 10–20.
22. Suzuki, K., *et al.* (2000). Reconstituted fusion liposomes for gene transfer *in vitro* and *in vivo*. *Gene Ther. Regulat.* **1**: 65–77.
23. Bagai, S., and Sarkar, D. P. (1993). Targeted delivery of hygromycin B using reconstituted Sendai viral envelopes lacking hemagglutinin-neuraminidase. *FEBS Lett.* **326**: 183–188.
24. Ramani, K., Bora, R. S., Kumar, M., Tyagi, S. K., and Sarkar, D. P. (1997). Novel gene delivery to liver cells using engineered virosomes. *FEBS Lett.* **404**: 164–168.
25. Ramani, K., Hassan, O., Venkaiah, B., Hasnain, S. E., and Sarkar, D. P. (1998). Site-specific gene delivery *in vivo* through engineered Sendai virus envelopes. *Proc. Natl. Acad. Sci. USA* **95**: 11886–11890.
26. Nakanishi, M., Uchida, T., and Okada, Y. (1982). Glycoproteins of Sendai virus (HVJ) have a critical ratio for fusion between virus envelopes and cell membranes. *Exp. Cell Res.* **142**: 95–101.
27. Okada, Y. (1993). Sendai virus-induced cell fusion. *Methods Enzymol.* **221**: 18–41.
28. Myers, T. M., Smallwood, S., and Moyer, S. A. (1999). Identification of nucleocapsid protein residues required for Sendai virus nucleocapsid formation and genome replication. *J. Gen. Virol.* **80**: 1383–1391.
29. Swaney, W. P., Sorgi, F. L., Bahnson, A. B., and Barranger, J. A. (1997). The effect of cationic liposome pretreatment and centrifugation on retrovirus-mediated gene transfer. *Gene Ther.* **4**: 1379–1386.
30. Cornetta, K., and Anderson, F. (1989). Protamine sulfate as an effective alternative to polybrene in retroviral-mediated gene transfer: implications for human gene therapy. *J. Virol. Methods* **23**: 187–194.
31. Clark, P. R., *et al.* (1999). Polycations and cationic lipids enhance adenovirus transduction and transgene expression in tumor cells. *Cancer Gene Ther.* **6**: 437–446.
32. Lanuti, M., *et al.* (1999). Use of protamine to augment adenovirus-mediated cancer gene therapy. *Gene Ther.* **6**: 1600–1610.
33. Yang, Y.W., and Hsieh, Y.C. (2001). Protamine sulfate enhances the transduction efficiency of recombinant adeno-associated virus-mediated gene delivery. *Pharm. Res.* **18**: 922–927.
34. Sorgi, F. L., Bhattacharya, S., and Huang, L. (1997). Protamine sulfate enhances lipid-mediated gene transfer. *Gene Ther.* **4**: 961–968.
35. Allen, T. M., Williamson, P., and Schlegel, R. A. (1988). Phosphatidylserine as a determinant of reticuloendothelial recognition of liposome model of the erythrocyte surface. *Proc. Natl. Acad. Sci. USA* **85**: 8067–8071.
36. Zhou, W. Z., *et al.* (1999). RNA melanoma vaccine; induction of anti-tumor immunity by human gp100 mRNA immunization. *Hum. Gene Ther.* **10**: 2719–2724.
37. Calos, M. P. (1996). The potential of extrachromosomal replicating vectors for gene therapy. *Trends Genet.* **12**: 463–466.
38. Saeki, Y., Wataya-Kaneda M., Tanaka, K., and Kaneda, Y. (1998). Sustained transgene expression *in vitro* and *in vivo* using an Epstein–Barr virus replicon vector system combined with HVJ-liposomes. *Gene Ther.* **5**: 1031–1037.
39. Kaneda, Y. (1994). Virus (Sendai virus envelopes) mediated gene transfer. In *Cell Biology: A Laboratory Handbook* Vol. 3 (J.E. Celis, Ed.), pp. 50–57. Academic Press, Orlando, FL.
40. Race, E., *et al.* (1995). A multistep procedure for the chemical inactivation of human immunodeficiency virus for use as an experimental vaccine. *Vaccine* **13**: 1567–1575.
41. Inaba, K., *et al.* (1992). Generation of large numbers of dendritic cells from mouse bone marrow cultures supplemented with granulocyte/macrophage colony-stimulating factor. *J. Exp. Med.* **176**: 1693–1702.
42. Kaneda, Y., Uchida, T., Ishiura, M., Kim, J., and Okada, Y. (1987). The improved efficient method for introducing macromolecules into cells using HVJ (Sendai virus) liposomes with gangliosides. *Exp. Cell Res.* **173**: 56–69.
43. de Martin, R., Raidi, M., Hofer, E., and Binder, B. R. (1997). Adenovirus-mediated expression of green fluorescent protein. *Gene Ther.* **5**: 493–495.
44. Saeki, Y., *et al.* (1997). Development and characterization of cationic liposomes conjugated with HVJ (Sendai virus): reciprocal effect of cationic lipid for *in vitro* and *in vivo* gene transfer. *Hum. Gene Ther.* **8**: 1965–1972.
45. Guttenberger, M. (1998). Protein determination. In *Cell Biology: A Laboratory Handbook*, 2nd edition, Vol. 4 (J. E. Celis, Ed.), pp. 295–303. Academic Press, Orlando, FL.

Cancer Gene Therapy (2003) 10, 179–186

www.nature.com/cgt

A novel combination of suicide gene therapy and histone deacetylase inhibitor for treatment of malignant melanoma

Seiji Yamamoto,[1,2] Tomoki Yamano,[1,3] Maki Tanaka,[3] Dave SB Hoon,[3] Sonshin Takao,[2] Ryuichi Morishita,[1] Takashi Aikou,[2] and 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]First Department of Surgery, Faculty of Medicine, Kagoshima University, 8-35-1 Sakuragaoka, Kagoshima 890-8520, Japan; and [3]Department of Molecular Oncology, John Wayne Cancer Institute, Santa Monica, California 90404, USA.

One major problem associated with application of gene therapy to treatment of tumors is poor transgene expression. Although suicide gene therapy with the herpes simplex virus-thymidine kinase gene (HSV-*tk*) followed by administration of ganciclovir (GCV) was effective in the treatment of melanoma, it was still difficult to induce complete remission to cancer. A novel histone deacetylase inhibitor drug FR901229 was found to enhance transgene expression in tumor cells both *in vitro* and *in vivo*. Combination therapy with HSV-*tk*/GCV and FR901228 by direct injection into tumor enhanced antimelanoma effects. The number of apoptotic cells in melanoma tumors was increased significantly ($P<.05$) after combined suicide gene therapy and FR901228. Six times injection of HSV-*tk*/GCV and FR901228 prolonged mice survival compared to that of HSV-*tk*/GCV injection alone ($P=.021$). In total, 56% (10 of 18) of the mice survived 120 days after combined suicide gene therapy and FR901228 treatment, and no new tumors appeared in the surviving mice. However, only 19% (3 of 16) of the mice survived when treated with suicide gene therapy alone. This novel strategy may be applicable as a therapeutic regimen for the treatment of aggressive types of cancers.
Cancer Gene Therapy (2003) **10,** 179–186. doi:10.1038/sj.cgt.7700551

Keywords: suicide gene therapy; histone deacetylase inhibitor; melanoma; nonviral gene delivery

Since the early 1990s, gene therapy has been proposed as a strategy for the treatment of intractable human diseases. More than 500 clinical gene therapy protocols have been undertaken, and approximately 70% of these were for cancer (http://www.wiley.co.uk/genetherapy/). However, remarkable success in human gene therapy has not yet been obtained. These treatments may be hindered by gene-related problems such as low gene transfection efficiency and poor transgene expression. Among the many technical challenges associated with *in vivo* cancer gene therapy are the need to enhance gene expression and the need to develop more effective vectors. Numerous viral and nonviral (synthetic) methods of gene transfer have been developed.[1,2] Each delivery system has limitations as well as advantages, and further improvement of gene transfer vector systems will be obviously inevitable in the future. Regardless of vector development, it has been elucidated that silencing of transgene expression occurs in host cells, even though transgenes are retained in the host cell nucleus.[3] Similar silencing has also been reported in certain viral infections. For example, retroviral gene expression was inhibited after retrovirus infection of mouse embryos.[4,5] Although low or silenced transgene expression after transfection has been a major problem in human gene therapy,[6] the mechanism(s) by which transgene expression is regulated has not yet been reported. Recent studies on chromatin remodeling revealed that histone acetylase and deacetylase also regulate the level of transcription by modifying chromatin.[7,8] Agents, including trichostatin A (TSA), trapoxin (TPX), and *n*-butyrate, that inhibit histone deacetylation have been identified.[9,10] With the use of these agents, reactivation of virally transduced genes[11] and amplified expression of transgenes encoded by the recombinant adenovirus vector[12] have been reported. These data suggest that transgene expression can be regulated by chromatin modifications such as histone acetylation/deacetylation. Recently, amplification of transgene expression in cultured cells was also achieved by a novel histone deacetylase inhibitor, FR901228, which is derived from *Chromobacterium violaceum.* [13,14] This drug was first developed as an anticancer reagent because it induced growth arrest, differentiation, and/or apoptotic cell death.[15] Therefore, we attempted to evaluate the anticancer effect of the combination of cancer gene therapy and FR901228.

Among various strategies of cancer gene therapy, we chose suicide gene therapy using herpes simplex virus-thymidine kinase gene (HSV-*tk*)/ganciclovir (GCV)[16] because suicide gene therapy has multiple effects on

Received October 7, 2002.
Address correspondence and reprint requests to: Dr Y Kaneda, Division of Gene Therapy Science, Osaka University School of Medicine, 2-2 Yamada-oka, Suita, Osaka 565-0871, Japan. E-mail: kaneday@gts. med. osaka-u.ac.jp

killing cancer cells such as bystander effect[17] and induction of tumor immunity.[18] Here, we showed that this combination therapy could induce complete responses without recurrence in melanoma-bearing mice.

Methods

Construction of plasmid DNA

Luciferase (pcLuci: 7.4 kb) was constructed by cloning the luciferase gene from *pGEM-luci* (Promega, Madison, WI, USA) into *pcDNA3* (Invitrogen, San Diego, CA, USA) at the *Hind*III and *Bam*HI sites. The *EcoR*I–*Xho*I fragment of the HSV-*tk* gene was cloned into *pcDNA3* to generate *pc-tk* (7.14 kb). The pc-LacZ (9.2 kb) construct was generated by insertion of the *Hind*III–*BamH*I fragment of *pSV-β-galactosidase* (Promega) into *pcDNA3*.

Preparation of HVJ–liposomes

Cholesterol (Chol), egg yolk phosphatidylcholine (ePC), egg yolk sphingomyelin (eSph), dioleoylphosphatidylethanolamine (DOPE), and 3-beta {*N*-(*N'*,*N'*-dimethylaminoethane)-carbamoyl} cholesterol (DC-Chol) were purchased from Sigma Chemical Co. (St Louis, MO, USA). To prepare HVJ–liposomes, ePC, eSph, DOPE, Chol, and DC-Chol were mixed at a molar ratio of 16.7 : 16.7 : 16.7 : 40 : 10 as described previously.[19,20] For transfection of the *luciferase* gene or *LacZ* gene, 200 μg of plasmid (*pcLuci* and *pc-LacZ*) was incorporated into liposomes prepared from 10 mg of lipids, and the liposomes were fused with inactivated HVJ (hemagglutinating virus of Japan; Sendai virus). The HVJ–liposome complexes were then separated from free HVJ by sucrose density gradient centrifugation. For *in vivo* transfection of HSV-*tk* gene, 200 μg *pc-tk* and 151.2 μg *pcDNA3* plasmid (equal number of copies as *pc-tk*) were used. The final HVJ–liposome volume was approximately 200 μl.

Cell lines and drugs

Mouse melanoma B16F1 cells (American Type Culture Collection, Rockville, MD, USA) were seeded at 1×10^5 cells per well in six-well plates. Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), penicillin (50 unit/ml), and streptomycin (50 μg) and incubated at 37°C in a 5% CO_2–95% air atmosphere. Tissue culture medium and supplements were purchased from Nakarai Tesque (Kyoto, Japan). The following drugs were used in the study: GCV (Tanabe Seiyaku Co. Ltd, Osaka, Japan) and FR901228 (Exploratory Research Laboratories of Fujisawa Pharmaceutical Co. Ltd, Tsukuba, Japan). FR901228 was dissolved and diluted in phosphate-buffered saline (PBS, pH 7.4) to 250 μg/ml as described previously.[21] For *in vivo* experiments, FR901228 solution was mixed with HVJ–liposomes, and injected directly into tumors.

Animal experiments

All animal experimental protocols were approved in a humane fashion in accordance with the guidelines of the Animal Committee of Osaka University. Male C57BL/6 mice, 8 weeks of age, were obtained from the Japan SLC, Inc. (Hamamatsu, Japan). Mice were housed five per cage and allowed access to food and water *ad libitum*. In all experiments, mice were anesthetized by intraperitoneal injection of diluted pentobarbital (1 mg).

For tumor cell implantation, B16F1 cells were enzymatically detached from culture flasks and counted. Five million viable cells were resuspended in 100 μl of PBS and injected subcutaneously (s.c.) into the right flank of the mice.

X-gal staining of mouse melanoma cells in vitro

B16F1 cells were seeded at 1×10^5 cells per well in six-well plates on day 0. After replacing the culture medium with 2 ml of fresh medium, 20 μl of HVJ–liposomes containing 1.2 μg of *pc-LacZ* was added to each well with or without 100 nM FR901228 solution. One hour later, cell culture medium was changed to fresh medium. At 24 h after transfection, cells were stained with 2% X-gal staining solution (2 mM $MgCl_2$, 5 mM $K_3Fe(CN)_6$, 5 mM $K_4Fe(CN)_6$, and 1 mg/ml X-gal in PBS) at 37°C 20 overnight as described previously.[20]

In vivo *luciferase assay*

For luciferase assay, eight mice were used in each group. When tumors reached approximately 7–8 mm in diameter on day 7 after inoculation, 100 μl of HVJ–liposomes containing 20 μg of *pcLuci* mixed with various concentrations of FR901228 were injected directly into the tumors. Mice were killed and tumors were resected 24 h after injection of these HVJ–liposomes. Tumors were minced with scissors and homogenized completely in 5 × volume of cell culture Lysis 5 × Reagent (Promega). All steps were carried out on ice. After centrifugation at 12 000 rpm for 5 min, the supernatant was tested for luciferase activity. The luciferase activity of 20 μl of lysate was measured with a luminometer (Lumat LB 9501 luminophotometer, Belthold, Wildbad, Germany) as described previously.[20]

Suicide gene therapy for tumor-bearing mice

At 7 days after tumor inoculation, 100 μl of HVJ–liposome suspension containing *pc-tk* (20 μg) or *pcDNA3* control (15.1 μg, same number of copies as *pc-tk*) was injected into mouse melanoma tumors. In the combined treatment group, 1 μg of FR901228 was mixed with HVJ–liposomes and injected into the tumors directly. GCV (50 mg/kg/day) was administered i.p. once daily for days 8–12. After injection of HVJ–liposomes, tumor size was measured every 3 days, and the survival of the mice was monitored. Tumor volume was determined using the simplified formula of a rotational ellipse ($l \times w^2 \times 0.5$).[22]

For consecutive treatment, HVJ–liposomes or FR901228 or both were injected repeatedly at 2-day intervals, and each mouse received a total of six injections. GCV was given intraperitoneally (i.p.) for 20 days (days 8–27).

TUNEL assay

On day 4 after suicide gene therapy, tumors were removed and flash frozen in liquid nitrogen. Each tumor was cut into four pieces, and frozen sections from each piece were prepared as described previously.[20] Apoptotic cells were detected with the ApopTag Plus Fluorescein In Situ Apoptosis Detection Kit (Intergen, Purchase, New York, USA) and Hoechst 33258 (Nakarai Tesque) was used for counterstaining. The efficiency of the treatment was evaluated by counting cell numbers in four sections for each group (HSV-*tk*/GCV with FR901228, HSV-*tk*/GCV *pcDNA3*/GCV with FR901228).

Statistical methods

Bonferroni/Dunn test was used to determine the significance of differences, and $P<.05$ was considered significant. The survival data were analyzed via log rank test, and $P<.05$ was considered significant.

Results

LacZ gene expression in vitro

We used *LacZ* gene as a reporter to study the effect of FR901228, which enhances transgene expression. LacZ expression was enhanced ($P<.01$) significantly when *LacZ* gene was transferred to B16F1 cells in the presence of 100 nM FR901228. At 24 h after gene transfer with FR901228, 22% (167/770) of the cells expressed the LacZ (Fig 1a). In contrast, LacZ expression by cells not treated with FR901228 was observed in only 2% (20/855) of cells (Fig 1b).

Luciferase assay in vivo

A luciferase assay was used to study the effect of FR901228 *in vivo*. HVJ-liposomes containing a vector encoding *luciferase* gene were injected into the mouse melanoma tumors with various concentrations of FR901228. Firstly, we tested which route of FR901228 injection enhanced luciferase expression. We tested i.p. route, intravenous (i.v.) route and direct injection route. However, neither i.p. nor i.v. route increased transgene expression remarkably at less than 10 μg/ml (1 μg/body) of FR901228 dose (data not shown). On the other hand, the direct injection of FR901228 into the tumor enhanced gene expression significantly. For that reason we selected the direct injection route.

Luciferase activity was examined 24 h after transfer. Expression of luciferase was enhanced remarkably when transfection was combined with an FR901228 injection (Fig 2a). Luciferase activity increased in proportion to the dose of FR901228. Compared with control activity, luciferase activities of tumors treated with FR901228 were increased 8.7-fold at a dose of 10 μg/ml (1 μg/body) ($P<.01$) and 16.8-fold at a dose of 100 μg/ml (10 μg/body) ($P<.01$) on day 1 after transfection of the luciferase gene

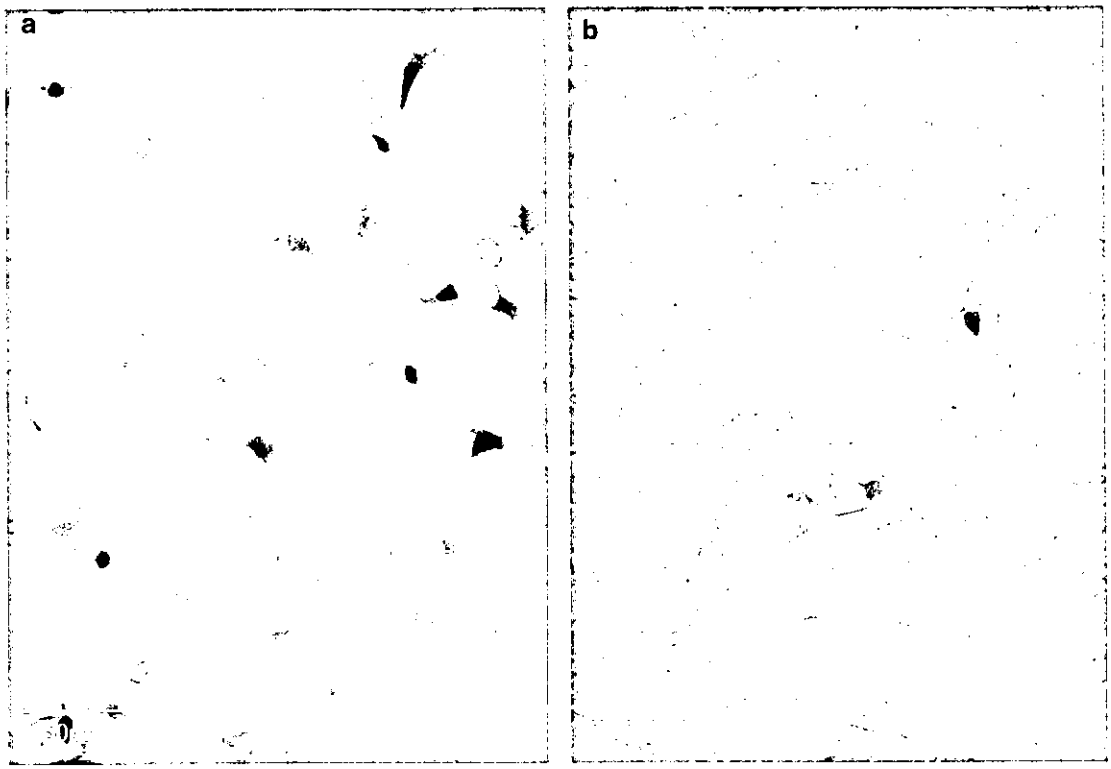

Figure 1 FR901228 enhanced *LacZ* gene expression. (**a**) *LacZ* expression was examined 24 h after the transfer of *pc-LacZ* with 100 nM FR901228, (**b**) *LacZ* gene expression without FR901228.

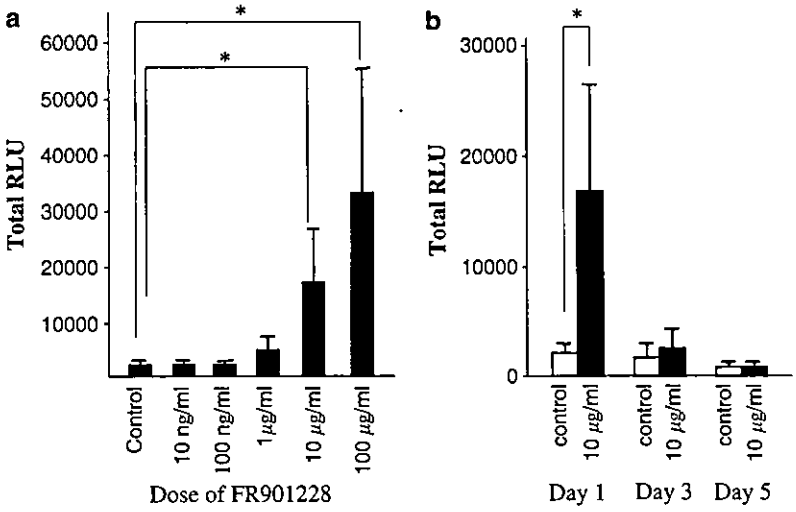

Figure 2 FR901228 enhanced luciferase gene expression in mouse melanoma tumor. **(a)** HVJ–liposomes containing 20 μg *pcLuci* were cointroduced with various concentrations of FR901228 into mice bearing melanoma tumors ($n=8$). Luciferase activity was measured 24 h after transfection. **(b)** *Luciferase* gene expression was measured at various time points after transfection with (■) or without (□) FR901228 (1 μg/body). Each group contains eight mice. Values are presented as mean ± SD. $^*P<.01$.

with FR901228. At concentrations of FR901228 less than 100 ng/ml, no significant gene expression was observed. On days 3 and 5, luciferase activity was not maintained (Fig 2b).

Apoptosis of melanoma cells

We then examined whether FR901228 could enhance the effectiveness of suicide gene therapy for cancer. HSV-*tk* gene was transfected via HVJ–liposomes into subcutaneous melanoma tumors on day 0. Tumors were injected with 10 μg/ml FR901228. GCV (50 mg/kg/day) was then administered i.p. daily for 3 days. On day 4, apoptosis was detected by both TUNEL assay and chromatin condensation with Hoechst 33258 (Fig 3). In tumors treated with HSV-*tk*/GCV and FR901228 (Fig 3a, d), 9% (53/565) of cells were apoptotic. However, 5% (29/639) of cells in tumors treated with HSV-*tk*/GCV alone (Fig 3b, e) and only 1% (9/645) of cells treated with *pcDNA*/GCV and FR901228 (Fig 3c, f) showed apoptotic characteristics. The number of apoptotic cells was significantly higher in tumors treated with HSV-*tk*/GCV and FR901228 than in those treated with *pcDNA*/GCV and FR901228 ($P<.01$) or those treated with HSV-*tk*/GCV alone ($P<.01$).

Combined treatment of suicide gene therapy and FR901228

To detect differences in efficacy of treatment with HSV-*tk*/GCV alone or in combination with FR901228, we developed tumor growth curves (Fig 4). HSV-*tk* or *pcDNA3* with 1 μg FR901228, diluted in 100 μl of PBS, were injected into the tumor directly and GCV was administrated i.p. for the following 5 days.

Animals treated with HSV-*tk*/GCV alone had a significant reduction in tumor size compared with control animals treated with *pcDNA*/GCV (median tumor volume ± SD on day 22 was 684 ± 430 mm^3 *versus* 1804 ± 809 mm^3; $P<.001$). Animals treated with *pcDNA*/GCV + FR901228 had a median tumor size of 1774 ± 650 mm^3. Animals receiving HSV-*tk*/GCV in combination with FR901228 had a median tumor volume of 269 ± 207 mm^3. Statistical analysis showed that mean tumor volume after combined treatment with HSV-*tk*/GCV and FR901228 was significantly smaller than that with HSV-*tk*/GCV ($P=.023$), *pcDNA*/GCV ($P<.001$), and *pcDNA*/GCV + FR901228 ($P<.001$). Tumors in animals treated with pcDNA/GCV + FR901228 were somewhat smaller than those in control mice treated with *pcDNA*/GCV alone. However, this difference was not statistically significant.

To assess treatment efficacy, we monitored the survival of tumor-bearing mice after treatment. As shown in Figure 5a, untreated animals (*pcDNA3*/GCV) had a median survival of 34 days. Animals treated with HSV-*tk*/GCV had significantly ($P<.01$) improved survival, with a median survival period of 47 days. A single injection of HSV-*tk*/GCV combined with FR901228 (1 μg/body) prolonged the survival period (median survival of 53 days) compared with that of mice treated with HSV-*tk*/GCV. However, the difference was not statistically significant ($P=.22$). Additionally, *pcDNA3*/GCV + FR901228 treatment did not improve the survival period any further (median survival of 35 days) compared with *pcDNA3*/GCV treatment.

Consequently, to evaluate the therapeutic effect of the combination treatment of HSV-*tk*/GCV and FR901228, we conducted consecutive gene therapy (Fig 5b). With repeated injections (2, 4, and 6 times) of HSV-*tk*/GCV, the survival rate of tumor-bearing mice was increased (data not shown). In mice injected six times with HSV-*tk*/GCV, 19% (3 of 16) showed complete remission. Consecutive treatment with HSV-*tk*/GCV + FR901228

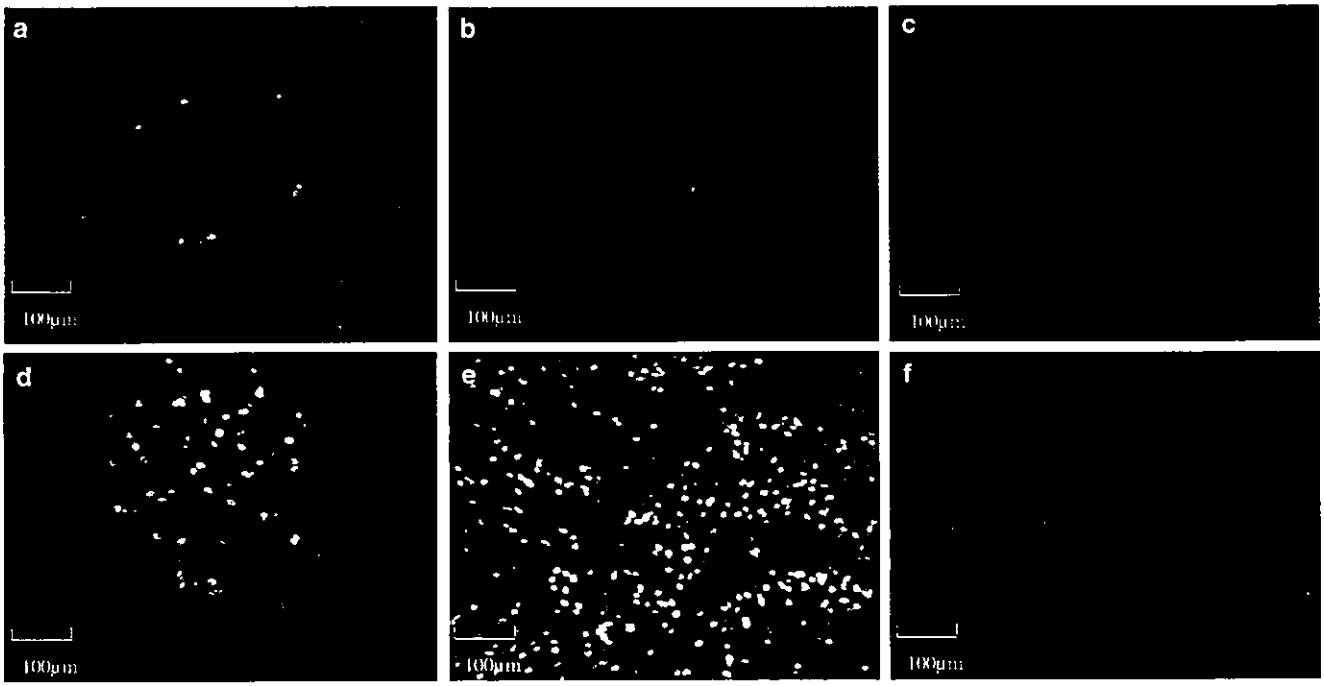

Figure 3 Apoptosis of melanoma cells *in vivo* detected by TUNEL staining. Apoptotic cells (green) in the frozen tumor sections were detected with an ApopTag Staining kit **(a–c)**. Sections were counterstained with Hoechst 33258 (blue) **(d–f)**. Mice were treated with HSV-*tk*/GCV and FR901228 (1 μg/body) **(a, d)**. Mice were treated with HSV-*tk*/GCV alone **(b, e)**. Mice were treated with *pcDNA3*/GCV and FR901228 (1 μg/body) **(c, f)**.

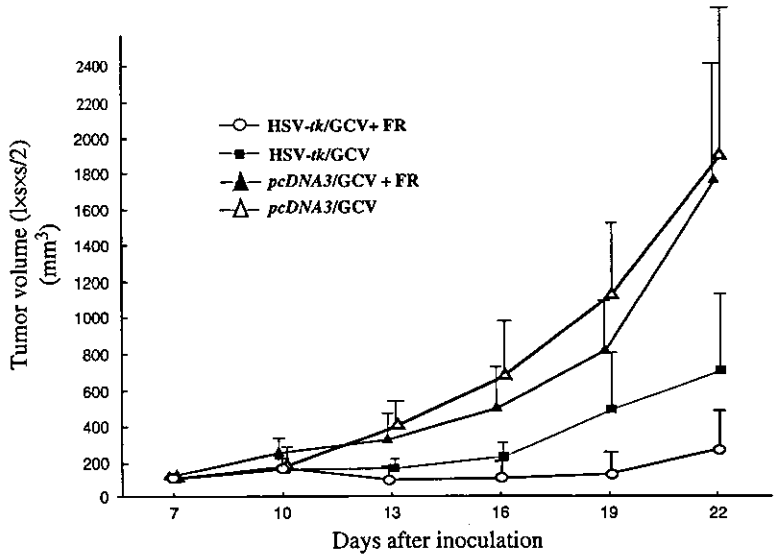

Figure 4 Treatment of established B16F1 tumors in mice with HSV-*tk*/GCV and in combination with FR901228 (1 μg/body). HVJ–liposomes containing HSV-*tk* or *pcDNA3* were injected directly into the tumor. FR901228 was mixed with HVJ–liposomes and injected. GCV was administered i.p. for the subsequent 5 days. Tumor volume was monitored after the treatment with HSV-*tk*/GCV+FR901228 (HSV-*tk*/GCV+FR, $n=25$), HSV-*tk*/GCV alone (HSV-*tk*/GCV, $n=25$), *pcDNA3*/GCV+FR901228 (*pcDNA3*/GCV+FR, $n=12$), and *pcDNA3*/GCV alone (*pcDNA3*/GCV, $n=15$). HSV-*tk*/GCV+FR *versus* HSV-*tk*/GCV: $P=.023$. HSV-*tk*/GCV *versus* *pcDNA3*/GCV: $P<.01$. HSV-*tk*/GCV+FR versus *pcDNA3*/GCV: $P<.01$. Results are shown as mean ± SD.

was more effective than consecutive HSV-*tk*/GCV injection alone ($P=.021$). When tumor-bearing mice were injected six times with HSV-*tk*/GCV + FR901228, 10 of 18 mice (56%) survived more than 120 days and experienced complete remission.

Discussion

We have described the *in vivo* amplification of transgenes by FR901228, resulting in a significant enhancement of the suicide gene therapy in melanoma-bearing mice.

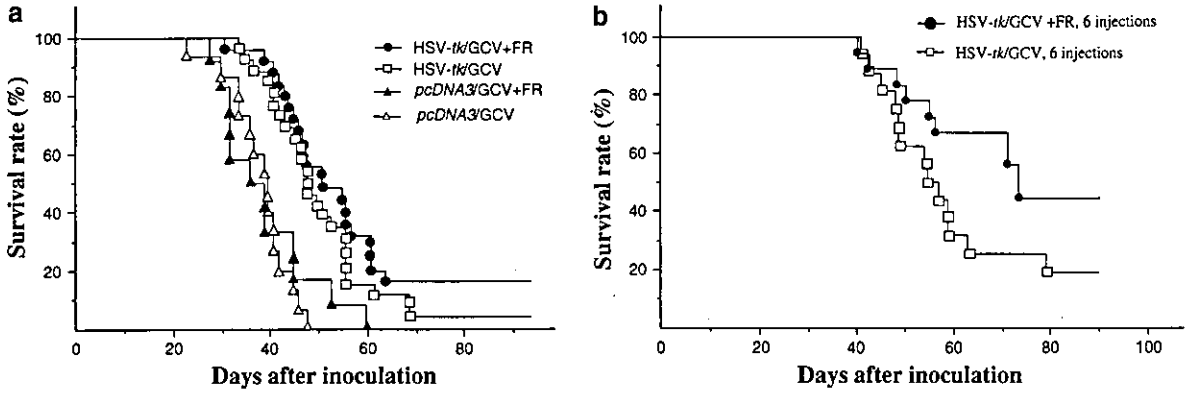

Figure 5 Kaplan–Meier survival analysis of tumor-bearing mice. **(a)** Survival of mice was monitored after single injection of HSV-*tk* gene combined with FR901228 (HSV-*tk*/GCV+FR; $n=25$), HSV-*tk* gene alone (HSV-*tk*/GCV, $n=25$), and *pcDNA3* with FR901228 (*pcDNA3*/GCV+FR; $n=12$) or *pcDNA3* alone (*pcDNA3*/GCV; $n=15$). FR901228 (1 μg/body) was mixed with HVJ–liposomes and used. All these animals were i.p. injected with GCV once daily for 5 days. **(b)** Survival of mice was monitored after six-times injection of HSV-*tk*/GCV+FR901228 (HSV-*tk*/GCV+FR, 6 injections, $n=18$) or HSV-*tk*/GCV alone (HSV-*tk*/GCV, 6 injections, $n=16$). The survival data were analyzed by log rank test. Consecutive treatment with HSV-*tk*/GCV+FR was more effective than consecutive injection of HSV-*tk*/GCV($P=.021$).

The observation that HDAC inhibitors such as FR901228 enhance transgene expression suggests that acetylation and deacetylation of cellular proteins are involved in transgene expression.[11,12,14] Acetylation and deacetylation of these proteins play important roles in the regulation of gene expression.[8,23–25] HDAC inhibitors regulate the level of transcription by modifying chromatin and activation of transcription factors.[26,27] Our previous report [14] showed that cellular histones were highly acetylated with FR901228. We analyzed mRNA enhanced by FR901228 using DNA microarray techniques. Only 1% of approximately 7000 genes were affected by the drug, and some genes were activated and others suppressed. However, the activation or suppression was less than 4 times as much as the basal transcription level of each gene without FR901228 (Yamamoto, unpublished data). Thus, activation of gene expression with FR901228 appears to be specific to exogenous genes, although the reason for such specific activation is presently unknown. One possible mechanism is that hyperacetylation of core histones by FR901228 may be sufficient to alter the chromatin structures of exogenous genes and activate expression, but it may not affect highly condensed chromatin structures involved in endogenous gene expression. Another mechanism involves the HDAC inhibitors activation of regulatory factors of the transgene by changing the chromatin structure of genes that encode the regulatory factors[26,27]. In this case, transgene expression may be indirectly regulated. The third possibility is direct acetylation of the transcription factors driving transgene expression by HDAC inhibitors[28–31]. Gene expression is indeed activated by TSA by acetylation of transcription factors such as p53[28] and GATA-1[30]. FR901228 may have a similar action on transcription factors.

FR901228 is able to cause both a p21-dependent G_1 and a p21-independent G_2 arrest, with the G_2 arrest appearing more cytotoxic than the G_1 arrest.[32–33] It has demonstrated potent cytotoxic activity against human tumor cell lines and *in vivo* efficacy against both human tumor xenografts and murine tumors.[34,35] On the basis of such findings, FR901228 was initially tested as an anticancer drug in the Phase I clinical trial.[36] We showed here that introduction of FR901228 into the tumor mass induced apoptosis. However, the direct injection of FR901228 into the tumor at this dose (less than 1 μg/body) was insufficient to control melanoma progression. Although the direct effect of HSV-*tk*/GCV induced apoptosis of cancer cells, HSV-*tk*/GCV alone was also insufficient (Fig 3b, e). Nevertheless, a combination of these therapies was efficacious, and the apoptosis ratio was increased (Fig 3a, d). FR901228 may have enhanced HSV-*tk* expression in transfected cancer cells and induced apoptosis in addition to its own effect.

One limitation of FR901228 as well as other HDAC inhibitors is the cytotoxicity at high doses. In our current *in vivo* luciferase experiments, a high dose (10 μg/body) of FR901228 was most effective for increasing the luciferase activity, but this high concentration of FR901228 induced inflammation and damaged the surrounding mouse skin. Repeated injection of FR901228 at this concentration (10 μg/body) also resulted in skin necrosis. However, the concentration of FR901228 used with gene therapy (1 μg/body) did not cause skin necrosis.

One other limitation of FR901228 is short half-life. The mean terminal half-life of FR901228 is 87 min,[21] and therefore the effect of the drug was only transient and high gene expression was not maintained. On days 3 and 5, luciferase activity in tumors transfected with *luciferase* gene combined with FR901228 was nearly the same as the activity in control tumors that received gene transfection alone (Fig 2b). Because of the short half-life of FR901228, we selected the direct injection route rather than the intravenous injection route. We tested several concentrations of FR901228 in intravenous and intraperitoneal injection. Using intravenous injection, approximately

5–10 times higher concentrations of FR901228 were needed to increase gene expression to the same level as the concentration in direct injection. In i.p. injection, more than 200 times concentration was needed. The long-term existence of lower concentration FR901228 in the target organ is most appropriate in terms of safety and efficacious therapy. Controlled release of FR901228 with a microcapsule may also result in more effective enhancement of therapeutic genes for cancer treatment.

Retention of a therapeutic gene by cancer cells is also necessary for an effective vector. One strategy to sustain gene expression is consecutive transfection. Gene transfer to the melanoma tumors has been achieved with HVJ–liposomes. Although a single injection of HSV-*tk*/GCV or FR901228 reduced tumor volume transiently (Fig 4), tumors began to grow again after day 13. Thus, a single injection of HSV–*tk*/GCV or FR901228 was insufficient for mice survival (Fig 5a). HVJ–liposomes containing HSV-*tk* gene can be injected consecutively because of their low immunogenicity.[37] Subsequently, we conducted the consecutive injection of HSV-*tk* gene-loaded HVJ–liposome combined with FR901228. In our present study, consecutive treatment with HSV–*tk*/GCV and FR901228 resulted in the significantly prolonged mouse survival compared with that obtained with HSV-*tk*/GCV alone (Fig 5b). Epstein-Barr virus (EBV) replicon plasmid will provide more effective sustained gene expression as described previously.[38] The expression of luciferase gene cloned in EBV plasmid also increased with FR901228 (Yamano, unpublished data).

In this experiment, we combined FR901228 with suicide gene therapy. Since FR901228 generally activates exogenous gene expression, combinations of FR901228 with other cancer gene therapy strategies may also be available for cancer treatment. Suicide gene therapy is known to have multiple anticancer effects. With suicide gene therapy, all cancer cells can be killed despite low transfection efficiency (bystander effect).[17] Furthermore, suicide gene therapy can induce tumor immunity.[18] In addition, it is reported that HDAC inhibitors mediate the expression of cell surface proteins such as CD86,[39] ICAMI,[39] and MAGE3.[40] These reports suggest the possibility of the use of HDAC inhibitors for increasing the expression of tumor-associated antigens. Moreover, in our experiment, mice cured from melanoma by suicide gene therapy showed strong delayed-type hypersensitivity (DTH) reaction after injection of recombinant tumor-associated antigens such as gp100 and TRP2 into the footpad (data not shown). Those mice also produced antibodies of very high titer (final liter: 6400–12 800) against gp100 and TRP2. Host immune responses against melanoma-associated antigens were significantly increased by the treatment of HSV-*tk*/GCV combined with FR901228.

Consequently, approximately two-thirds of those mice rejected melanoma when rechallenged with melanoma (Yamamoto, unpublished data). Therefore, it is expected that the combination of suicide gene therapy and FR901228 may efficiently prevent metastasis or regress metastatic foci of malignant melanoma.

Acknowledgments

We thank Rie Kosai for their excellent technical assistance. This work was supported by Grant 13218069 from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

References

1. Marshall E. Gene therapy's growing pains. *Science.* 1995;269:1052–1055.
2. Mulligan RC. The basic science of gene therapy. *Science.* 1993;260:926–932.
3. Palmer TD, Rosman GJ, Osborne WR, et al. Genetically modified skin fibroblasts persist long after transplantation but gradually inactivate introduced genes. *Proc Natl Acad Sci USA.* 1991;88:1330–1334.
4. Harbers K, Jahner D, Jaenisch R. Microinjection of cloned retroviral genomes into mouse zygotes:integration and expression in the animal. *Nature.* 1981;293:540–542.
5. Jahner D, Stuhlmann H, Stewart CL, et al. *De novo* methylation and expression of retroviral genomes during mouse embryogenesis. *Nature.* 1982;298:623–628.
6. Grossman M, Raper SE, Kozarsky K, et al. Successful *ex vivo* gene therapy directed to liver in a patient with familial hypercholesterolaemia. *Nat Genet.* 1994;6:335–341.
7. Roeder RG. The role of general transcription factors in transcription by polymerase II. *Trends Biochem Sci USA.* 1996;21:327–335.
8. Grunstein M. Histone acetylation and chromatin structure and transcription. *Nature.* 1997;389:349–352.
9. Yoshida M, Horinouchi S, Beppu T. Trichostatin A and trapoxin:novel chemical probes for the role of histone acetylation in chromatin structure and function. *Bioessays.* 1995;17:423–430.
10. Riggs MG, Whittaker RG, Neumann JR, et al. *n*-Butyrate causes histone modification in HeLa and Friend erythroleukaemia cells. *Nature.* 1997;268:462–464.
11. Chen WY, Bailey EC, McCune SL, et al. Reactivation of silenced, virally transduced genes by inhibitors of histone deacetylase. *Proc Natl Acad Sci USA.* 1997;94:5798–5803.
12. Dion LD, Goldsmith KT, Tang DC, et al. Amplification of recombinant adenoviral transgene products occurs by inhibition of histone deacetylase. *Virology.* 1997;231:201–209.
13. Nakajima H, Kim YB, Terano H, et al. FR901228, a potent antitumor antibiotic, is a novel histone deacetylase inhibitor. *Exp Cell Res.* 1998;241:126–133.
14. Yamano T, Ura K, Morishita R, et al. Amplification of transgene expression *in vitro* and *in vivo* using a novel inhibitor of histone deacetylase. *Molecular Ther.* 2000;1:425–431.
15. Marks PA, Richon VM, Rifkind RA. Histone deacetylase inhibitors:inducers of differentiation or apoptosis of transformed cells. *J Natl Cancer Inst.* 2000;92:1210–1216.
16. Oldfield EH, Ram Z, Culver KW, et al. Gene therapy for the treatment of brain tumors using intra-tumoral transduction with the thymidine kinase gene and intravenous gancyclovir. *Hum Gene Ther.* 1993;4:39–69.
17. Freeman SM, Abboud CN, Whartenby KA, et al. The "bystander effect": tumor regression when a fraction of the tumor mass is genetically modified. *Cancer Res.* 1993;53:5274–5283.