

A similar approach has been used to enhance the gene transfer efficiency of a receptor-mediated gene delivery system by combining fusion peptide derived from influenza virus hemagglutinin (Wagner *et al.*, 1992). A tissue-specific gene delivery system has been developed by binding tissue-specific molecules to a poly-L-lysine/DNA complex (Wu and Wu, 1988). Binding asialoglycoprotein and transferrin to a poly-L-lysine/DNA complex successfully targets DNA to hepatocytes and cancer cells, respectively (Wu and Wu, 1988; Zenke *et al.*, 1990). However, the limitation of this system is the degradation of the DNA in the lysosomes. To avoid such degradation, a fusion-mediated gene delivery system has been investigated using influenza fusion proteins. Influenza virus fuses with cell membranes at an acidic pH, and hemagglutinin (HA) protein on the viral envelope is involved in the fusion between viral envelope and endosomal membrane. It has also been elucidated that a mutant N-terminal peptide of influenza HA subunit, HA-2, can fuse with cell membranes at neutral pH. The transferrin/poly-L-lysine/DNA complex bound with the HA-2 peptide increases gene transfer efficiency in cultured cancer cells more than 1,000 fold compared with that in the absence of the peptide (Wagner *et al.*, 1992).

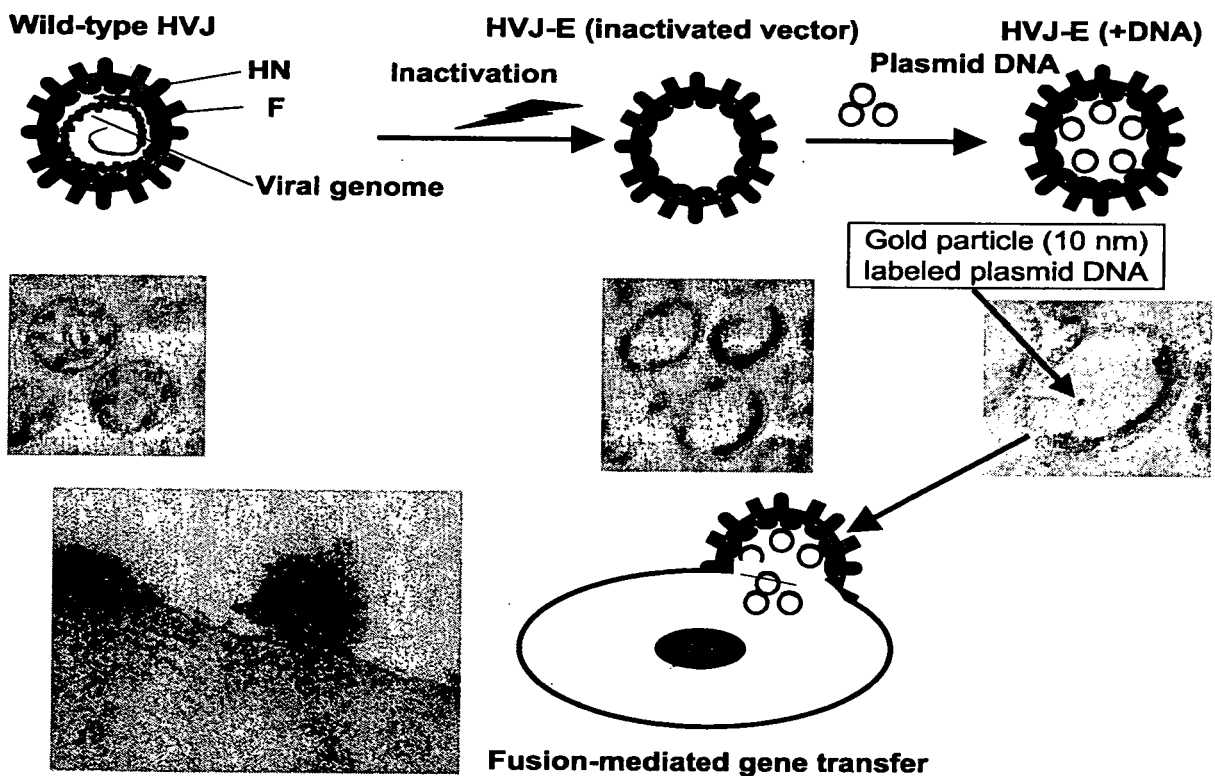
Reconstituted particles containing fusion proteins of HVJ have also been developed to promote fusion-mediated gene delivery (Bagai and Sarker, 1993; Ramani *et al.*, 1997). HVJ virion was completely lysed with detergent, and the lysates were mixed with DNA solution. In some cases, several lipids were added to the mixture. By removing the detergent with 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 lipid/DNA mixture in the presence or absence of detergent. Since F protein is recognized by the asialoglycoprotein receptor on hepatocytes, reconstituted HVJ particles containing only F protein have been constructed to specifically target hepatocytes *in vivo* (Ramani *et al.*, 1998). However, DNA trapping efficiency of the reconstituted particles was not so high. To improve the limitation, another approach was that liposomes containing fusion proteins of HVJ and DNA-loaded liposomes were prepared separately and then both liposomes were fused together (Suzuki *et al.*, 2000). These reconstituted fusion liposomes were as effective as conventional HVJ-liposomes, which contain the fully intact HVJ virion, in terms of the delivery of FITC-ODN and the luciferase gene to cultured cells. The LacZ gene was also transferred directly to mouse skeletal muscle *in vivo* using these reconstituted fusion particles.

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, vesicular stomatitis virus, and HVJ induce cell fusion. HVJ is the most abundantly produced in chick eggs. Therefore, we tried to construct an HVJ envelope vector system by incorporating plasmid DNA into inactivated HVJ particle (Kaneda

*et al.*, 2002a). In this review article, we explain the development of the new vector system and its application to gene therapy studies. Effective expression of a transgene is also a big issue for gene therapy, but we do not refer to this issue here.

## II. PREPARATION OF HVJ ENVELOPE VECTOR

There are some drawbacks to HVJ-liposomes, although they have been widely used for gene transfer both *in vitro* and *in vivo*. One disadvantage of HVJ-liposomes is the complicated procedure used to isolate and produce both inactivated HVJ and DNA-loaded liposomes. Additionally, the fusion activity of the HVJ-liposomes decreases to approximately 2% of that of native HVJ because of the reduced density of fusion proteins on the surface of HVJ-liposomes. To simplify the vector system and to develop a more effective gene delivery system, we attempted to incorporate plasmid DNA into inactivated HVJ particles without using liposomes (Fig. 12.1).



**Figure 12.1.** HVJ envelope vector system. For constructing HVJ envelope vector, gold-labeled plasmid DNA is mixed with inactivated HVJ particles purified through columns and the mixture is treated with mild detergent and centrifugation to incorporate DNA inside the particle. HVJ envelope vector can fuse with cell membrane to directly transfer DNA into cells.

HVJ is a mouse parainfluenza virus and is not a human pathogen (Okada, 1993). HVJ can fuse with cell membranes in both neutral and acidic conditions. Two distinct glycoproteins, HN and F, are required for cell fusion (Okada, 1993; Yeagle, 1993). HN is required for the binding of HVJ to cell surface sialic acid receptors and the subsequent degradation of the receptors by its sialidase activity. Then, F associates with lipids, such as cholesterol, in the cell membrane to induce cell fusion. The F glycoprotein is first synthesized as inactive F0 in cells infected with HVJ. F0 is then cleaved by host protease into the active F1 and F2 forms. F1 contains hydrophobic peptides with approximately 25 amino acids that induce cell fusion (Ghosh *et al.*, 2000; Yeagle, 1993). F protein produced in chick eggs is converted to the active F1 form by the protease in chorioallantoic fluid, while the virus produced from cultured cells contains inactive F0 that needs to be cleaved by a protease to form active F1. Cells in the rodent airway contain enzymes to convert inactive F0 to active F1. Therefore, HVJ induces severe pneumonia in rodents but not in humans. Large amounts of viral proteins are produced in infected cells. Viral nucleocapsid protein induces cytotoxic T cells (CTLs) against infected cells (Chen *et al.*, 1998). However, nucleocapsid protein is indispensable for virus production. Therefore, to develop highly efficient and minimally invasive vectors based on HVJ, our approach has been to use inactivated viral envelope in which the viral genome has been destroyed.

For this purpose, we have converted HVJ to a non-viral vector containing therapeutic genes instead of the viral genome (Kaneda *et al.*, 2002a). HVJ amplified in the chorioallantoic fluid of 10- to 14-day-old chick eggs was inactivated with  $\beta$ -propiolactone (0.0075% to 0.001%) (Race *et al.*, 1995) followed by UV irradiation (99 mJoule/cm<sup>2</sup>) (Kaneda, 2002b). Then, inactivated HVJ envelope was purified by ion-exchange column chromatography and gel filtration (Nakajima *et al.*, unpublished method). With this inactivation procedure, virus replication was completely destroyed, but hemagglutinating activity was not affected. HVJ particles in the chorioallantoic fluid were very heterogeneous with a diameter ranging from 150 to 600 nm. More homogeneous HVJ envelope was isolated with the improved purification method using the column procedure. The diameter of HVJ envelope obtained from the column procedure was 280 nm, and the zeta potential was approximately  $-5$  mV.

Aliquots of the inactivated virus ( $3 \times 10^{10}$  particles/1.5 ml tube) were centrifuged ( $10000 \times g$ , 5 min), and the viral pellet was mixed with exogenous plasmid DNA. Exogenous plasmid DNA was incorporated into inactivated HVJ by treatment with mild detergent and centrifugation. First, inactivated HVJ of  $3 \times 10^{10}$  particles was mixed with 0.24% to 0.80% Triton X-100 in the presence of plasmid DNA (200  $\mu$ g) in less than 100  $\mu$ l of Tris-EDTA buffer for 5 min on ice and the mixture was centrifuged at 10000 g for 5 min to move the DNA into the HVJ particle. The DNA trapping efficiency of HVJ envelope vector was approximately 15% to 20%. Without centrifugation, the DNA trapping efficiency was

approximately 3%. Different detergents were available for the preparation of the HVJ envelope vector including NP-40, CHAPS, octylglucoside, sodium cholate and dodecyl maltoside, although the optimal concentration of detergent for preparation of HVJ envelope vector is different. Without detergent treatment, DNA does not become incorporated into the viral particle. Electron microscopy confirms that DNA became incorporated into all of the particles of inactivated HVJ. The largest plasmid tested was 14 kb and its trapping efficiency was 18%.

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. To prepare the HVJ envelope vector, plasmid DNA is incorporated into inactivated HVJ particles by treatment with mild detergent. The virion is not destroyed and not subjected to the dialysis, purification, and addition of lipids or proteins that occurs during the preparation of reconstituted HVJ particles (Bagai and Sarker, 1993; Ramani *et al.*, 1997; Suzuki *et al.*, 2000). Protein analysis of HVJ envelope vector using SDS-polyacrylamide gel electrophoresis indicate that the composition of the HVJ envelope vector is very similar to that of native HVJ (Kaneda, *et al.*, 2002a). Most native HVJ proteins are retained in the HVJ envelope vector. Fusion proteins HN and F1 are retained, and the molar ratio of these proteins in HVJ envelope vector was approximately 2 to 2.3, which is the same as the ratio in native HVJ (Okada, 1993). This ratio of F and HN is very important for fusion activity. Therefore, the fusion activity of HVJ envelope vector is as robust as wild-type HVJ. Electron microscopic observation confirmed that the fusion between HVJ envelope vector and cell membrane occurs only 3 to 5 sec after the attachment of the plasmid-containing HVJ envelope vector to a cell surface (Fig. 12.1).

In contrast to recombinant HVJ viral vector (Yonemitsu *et al.*, 2000), the HVJ envelope is a non-viral vector system that consists of an envelope derived from wild type HVJ virus by inactivation and purification. Since the viral genome is inactivated in the HVJ envelope vector, the virus does not replicate and viral genes are not expressed in the cells that are transfected with the HVJ envelope vector. However, cells infected with recombinant HVJ viral vector produce viral proteins. The recombinant HVJ vector produces a large amount of therapeutic products, but it may cause cellular toxicity and be highly immunogenic, which makes it less desirable for repeated administration.

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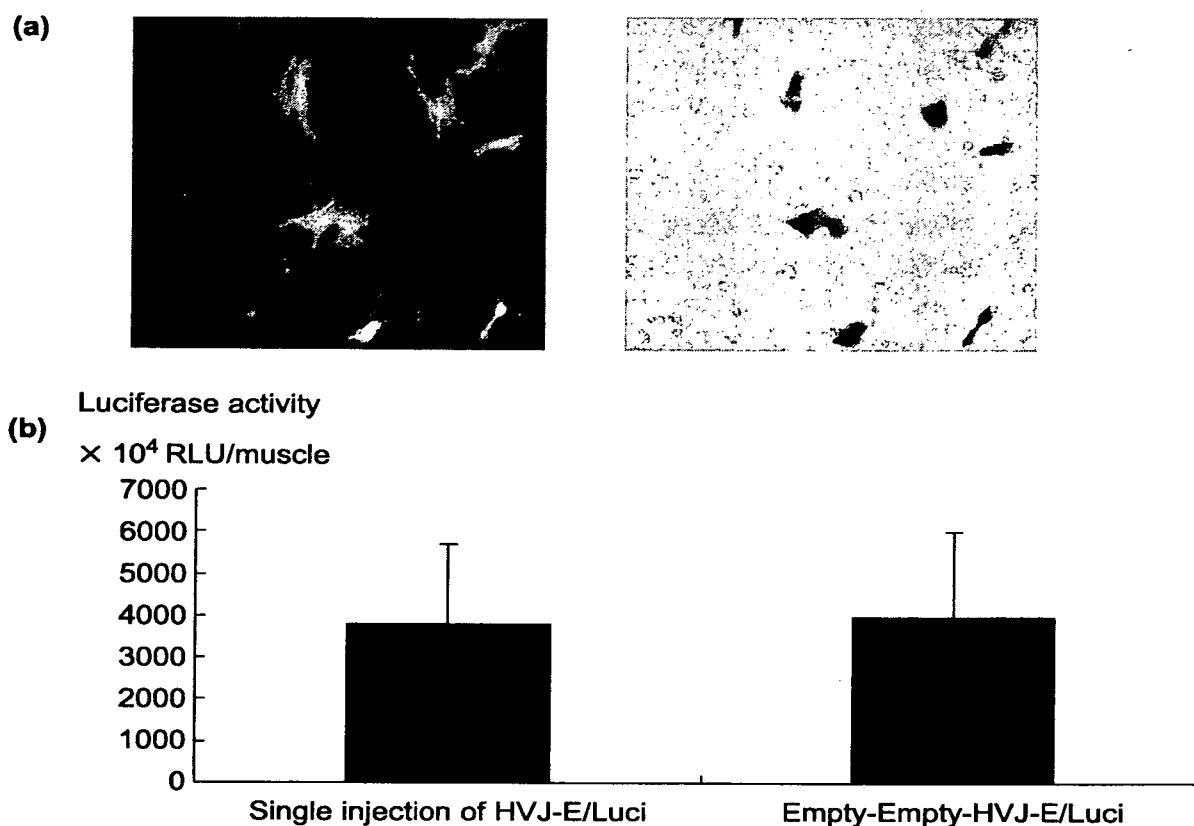
### **III. GENE TRANSFER TO CULTURED CELLS USING HVJ ENVELOPE VECTOR**

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 enhanced luciferase gene expression 10- to

50-fold in all cell lines tested. For example, in a mouse colon cancer cell line, CT26, luciferase gene expression was enhanced approximately 20-fold when compared to the expression level in the absence of protamine sulfate. A 10 min incubation period was sufficient for high levels of luciferase expression. The optimum conditions for *in vitro* gene transfer have been previously summarized (Kaneda *et al.*, 2002a). Mouse embryonal stem (ES) cells were also transfected using the mixture of the HVJ envelope vector and protamine sulfate. When HVJ envelope vector containing green fluorescent protein (GFP) expression plasmid was added to mouse ES cells, the efficiency of GFP expression (as determined by flow cytometry) was approximately 80%. The gene transfer efficiency was dependent on the amount of vector and was not inhibited by 10% fetal calf serum. Non-adherent floating cells are generally resistant to gene transfer methods, and the human T cell leukemia cell lines, NALM-6 and CCRF-CEM, have been particularly difficult targets. Luciferase gene expression in NALM-6 and CCRF-CEM was increased with protamine sulfate treatment, but the expression level was still very low. However, when protamine sulfate was used in conjunction with centrifugation, luciferase gene expression in NALM-6 cells and CCRF-CEM was enhanced 30- to 40-fold compared to the expression level without centrifugation. The optimal condition for gene transfer to these non-adherent cell lines was the centrifugation of the mixture of cells and vector ( $6 \times 10^9$  particles) at 10000 g for 30 min at 37 °C. Primary cells such as human aortic endothelial cells and rat neuronal cells were also effectively transfected with the HVJ envelope vector without significant cell damage.

Approximately 20 to 30 copies of plasmid DNA can be incorporated into one HVJ envelope vector particle when 200  $\mu$ g of 7 kb plasmid is mixed with  $3 \times 10^{10}$  particles of inactivated HVJ. When the DNA concentration is increased, more copies of plasmid DNA can be incorporated into the vector. Using HVJ envelope vector, two different plasmids can be delivered to the same cell. For example, HVJ envelope vector can be prepared when the LacZ gene and GFP gene are mixed with inactivated HVJ and the mixture is treated with mild detergent and centrifuged. When this HVJ envelope vector is added to BHK-21 cells, both proteins are clearly expressed in the same cells (Fig. 12.2a).

Small interfering RNA (siRNA) is an attractive and effective tool for suppressing target protein by specifically digesting its mRNA (Dorsett and Tuschl, 2004; Tijsterman and Plasterk, 2004). SiRNA is superior to antisense oligonucleotides and ribozyme in terms of efficiency and specificity (Miyagishi *et al.*, 2003; Yokota *et al.*, 2004), but finding a suitable delivery system for siRNA has been problematic (Sioud, 2004). Drugs, synthetic oligonucleotides, proteins and peptides, as well as siRNA, can be incorporated into the HVJ envelope



**Figure 12.2.** (a) Two different plasmids can be delivered to the same cell. HVJ envelope vector containing both GFP and LacZ gene is added to BHK-21 cells. At 24 h after gene transfer, both proteins clearly expressed in the same cells. (b) Gene expression is not inhibited after consecutive injection of HVJ envelope vector into mouse muscle. In both cases, luciferase gene expression in muscle is detected at 24 h after injection of HVJ envelope vector containing luciferase gene. In the mice shown by the blue bar, empty HVJ envelope vector has been injected into muscle twice in a 2-week interval before injection of luciferase gene-loaded vector. (See Color Insert.)

vector and delivered into cells. The HVJ envelope-mediated delivery efficiency of siRNA in cultured cells was 100%. Other researchers have also demonstrated that HVJ envelope vector efficiently delivers siRNA to islet cell lines (Itoh *et al.*, 2003) and Jurkat cells (Ishii *et al.*, 2003).

#### IV. GENE TRANSFER *IN VIVO* USING HVJ ENVELOPE VECTOR

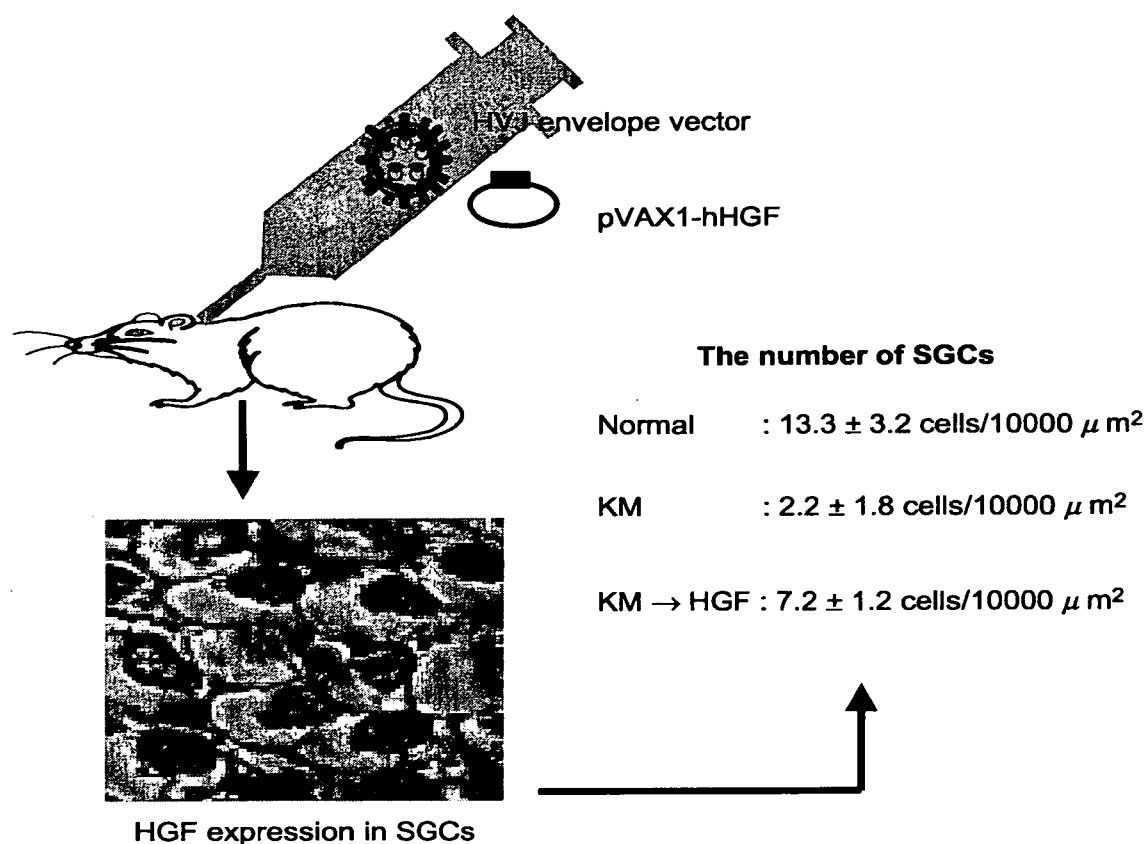
HVJ envelope vector system can be used for *in vivo* gene transfer. The HVJ envelope vector has mediated gene transfer to a variety of tissues (lung, liver, uterus, eye, skin, muscle, brain, heart and cancerous tumors) in animals

including mice, rats, rabbits and monkeys. In mouse liver, HVJ envelope vector-mediated luciferase gene expression was two times higher than the expression mediated by HVJ-liposomes prepared from inactivated HVJ (Kaneda *et al.*, 2002a).

In mouse skeletal muscle, consecutive injection of DNA-loaded HVJ envelope vector did not inhibit gene transfection. In this experiment, empty HVJ envelope vector was injected into mouse muscle tissue twice in a 2-week interval in one experimental group, while another group received saline injections. Two weeks later, HVJ envelope vector-containing luciferase gene was intramuscularly injected in all mice. As shown in Fig. 12.2b, similar luciferase gene expression was detected in the two groups. Thus, the HVJ envelope vector appears to be much less immunogenic than native HVJ which strongly induces CTLs against virus-infected cells.

We failed to get effective HVJ-liposome-mediated gene transfer in the mouse uterus. However, high levels of luciferase gene expression were obtained in the mouse uterus when the HVJ envelope vector was used (Nakamura *et al.*, 2003). Mice were anesthetized and subjected to laparotomy to expose the uterus. Twenty-five microliters of HVJ envelope vector ( $3 \times 10^9$  particles) containing plasmid DNA (16  $\mu\text{g}$ ) was slowly injected into the uterine cavity using a 30-gauge needle, and the cervix was clamped for 10 min. Then, the incision was closed to allow the mice to recover. After 24 h of transfection, the luciferase activity mediated by HVJ envelope vector was approximately 120 times higher than that obtained using Lipofectamine. A 5-fold increase in the amount of plasmid did not affect the level of luciferase activity when Lipofectamine was used. Luciferase activity was detected in the uterus for at least 3 days after transfection using the HVJ envelope. Transfer of the LacZ gene to the uterus with the HVJ envelope vector yielded gene expression mainly in the glandular epithelium of the endometrium. Few stroma cells were transfected with this procedure. Viral fusion proteins disappeared 3 days after gene transfer, but transferred DNA was detected in the uterus for 10 days. No transfer of injected DNA to mouse fetuses was detected in this experiment.

To safely transfer genes to brain tissue, we intrathecally injected HVJ envelope vector containing the LacZ gene into the cerebrospinal fluid (CSF) of male Wistar rats (270 to 300 g) (Fig. 12.3) (Shimamura *et al.*, 2003). The HVJ envelope vector was injected into the cisterna magna. Briefly, a stainless cannula (27 gauge) was introduced into the cisterna magna (subarachnoid space) of anesthetized rats. HVJ envelope vector (100  $\mu\text{l}$ ) containing the human hepatocyte growth factor (HGF) gene was infused at the rate of 50  $\mu\text{l}/\text{min}$  after removing 100  $\mu\text{l}$  of CSF. Then, the animals were placed head-down for 30 min. No behavioral changes, such as convulsions or abnormal movements, were observed. Cells that expressed  $\beta$ -galactosidase were present in the spiral ganglion



**Figure 12.3.** Gene therapy of hearing impairment by HGF gene. HVJ envelope vector containing human HGF gene is administered to the cerebrospinal fluid of a rat. Human HGF gene expression is immunohistochemically detected at SGCs in the inner ear. By HGF gene expression, the number of SGCs is rescued after KM insult. (See Color Insert.)

cells (SGCs), cerebral cortex, and medulla. Luciferase expression was also examined in the brain, cochlea, lung, spleen and liver of the rats that were intrathecally injected with HVJ envelope vector containing the luciferase gene. One day after injection, strong transgene expression levels were observed in the cochleae and various areas of the brain. The highest luciferase activity was observed in the medulla, which is the area of the brain closest to the injection point. In contrast, on Day 1 and Day 5 after transfection, luciferase activity was not detected in the lung, spleen, or liver of the luciferase-injected rats. Luciferase activity was also not detected in any of the tested organs of the control-injected rats. To determine the optimal amount of HVJ envelope vector, we intrathecally administered  $3 \times 10^{10}$ ,  $4.5 \times 10^{10}$ , and  $6 \times 10^{10}$  particles of HVJ envelope vector that contained 20  $\mu\text{g}$ , 30  $\mu\text{g}$  and 40  $\mu\text{g}$  of luciferase gene, respectively. The



highest expression level in the brain and cochlea resulted from the injection of  $4.5 \times 10^{10}$  particles of HVJ envelope vector (Oshima *et al.*, 2004).

Next, we intravenously injected HVJ envelope vector. Luciferase gene-loaded HVJ envelope vector ( $6 \times 10^9$  particles) was injected into the mouse tail vein, and luciferase activity in several organs was measured 24 h after injection. Luciferase expression was detected in the spleen for at least 1 week. Intravenously administered naked plasmid (30  $\mu$ g) that contained the luciferase gene resulted in luciferase expression in the liver, lung and spleen at much lower levels than those mediated by the HVJ envelope. When compared to the expression levels mediated by naked plasmid DNA transfer, luciferase gene expression mediated by HVJ envelope transfer was significant in the spleen, but not in the other organs (such as the lung and liver). When HVJ envelope vector containing FITC-ODNs was systemically injected, FITC-ODNs were observed in the marginal zone of the spleen. However, by intravenous injection of HVJ envelope vector, coagulation functions in the mouse blood is transiently suppressed, but the functions are recovered at 24 h after the injection. It is probably due to the hemagglutinating activity of HN protein. The protection of hemagglutinating activity of HN protein will be necessary for systemic injection of HVJ envelope vector to ensure the safety in gene therapy.

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## **V. GENE THERAPY FOR HEARING IMPAIRMENT USING HVJ ENVELOPE VECTOR**

By intrathecal injection of HVJ envelope vector, the vector reached the inner ear and gene expression was detected in SGCs which mediate auditory stimuli to the auditory nerve. Therefore, we tested treatment of hearing impairment with this approach (Oshima *et al.*, 2004). Hearing impairment is caused by the loss of hair cells and the degeneration of SGCs. To recover hearing impairment, some neurotrophic factors, such as neurotrophin-3 (Ernfors *et al.*, 1996), glial cell line-derived neurotrophic factor (Yagi *et al.*, 2000) and brain-derived neurotrophic factor (Staecker *et al.*, 1996) have been directly injected into inner ear tissue. However, we have reported that HGF also has pleiotropic effects on neural functions. HGF is a secretory protein that functions in an autocrine/paracrine manner on epithelial cells (Hayashi *et al.*, 2001) and also on the nervous system (Miyazawa *et al.*, 1998; Yoshimura *et al.*, 2002). By analyzing the molecular interactions of HGF, we found that HGF activates the transcription factor, Ets-1. Many factors can be activated by Ets-1 (Tomita *et al.*, 2003). HGF and its receptor, c-Met, are up-regulated by Ets-1, resulting in positive feedback of HGF activity. By the activation of VEGF and increases in metalloproteinases,

angiogenesis is indirectly enhanced. Ets-1 also up-regulates itself. Therefore, even a small amount of HGF can induce pleiotropic effects on various cells expressing its receptor and the activity of HGF can be sustained. Therefore, we used the HGF gene as a therapeutic molecule to treat hearing impairment induced by aminoglycoside administration.

To transfer the human HGF gene into the SGCs and central nervous system, we injected  $4.5 \times 10^{10}$  particles of HVJ envelope containing the HGF gene into the CSF in the cisterna magna. We then measured the protein level of HGF in the CSF by ELISA. Five days after transfection, human HGF was readily detected in the CSF. An increase of rat HGF was also observed in the CSF of the rats transfected with human HGF gene. The expression of human HGF was detected in rats for 2 weeks after transfection, and endogenous rat HGF concentration was approximately 10 times that of human HGF (mean value: approximately 3 ng/ml on Day 5). Immunohistochemical detection of human HGF expression in the cytoplasm of SGCs indicated that the gene transfer efficiency was greater than 70% (Fig. 12.3). Additionally, the expression of c-Met, which is the tyrosine kinase receptor of HGF, was greatly enhanced on SGCs, suggesting that the increased c-Met and HGF expression synergistically affects the survival of SGCs.

We examined whether HGF could rescue the loss of SGCs induced by kanamycin (KM) insult (Oshima *et al.*, 2004). A significant reduction of SGCs was induced by KM insult. The apoptosis was detected by TUNEL staining in SGCs. Pre-injection of HVJ envelope vector containing HGF gene completely prevented the apoptotic loss of SGCs. The apoptotic loss of hair cells in the inner ear was also rescued by HGF gene transfer using HVJ envelope vector.

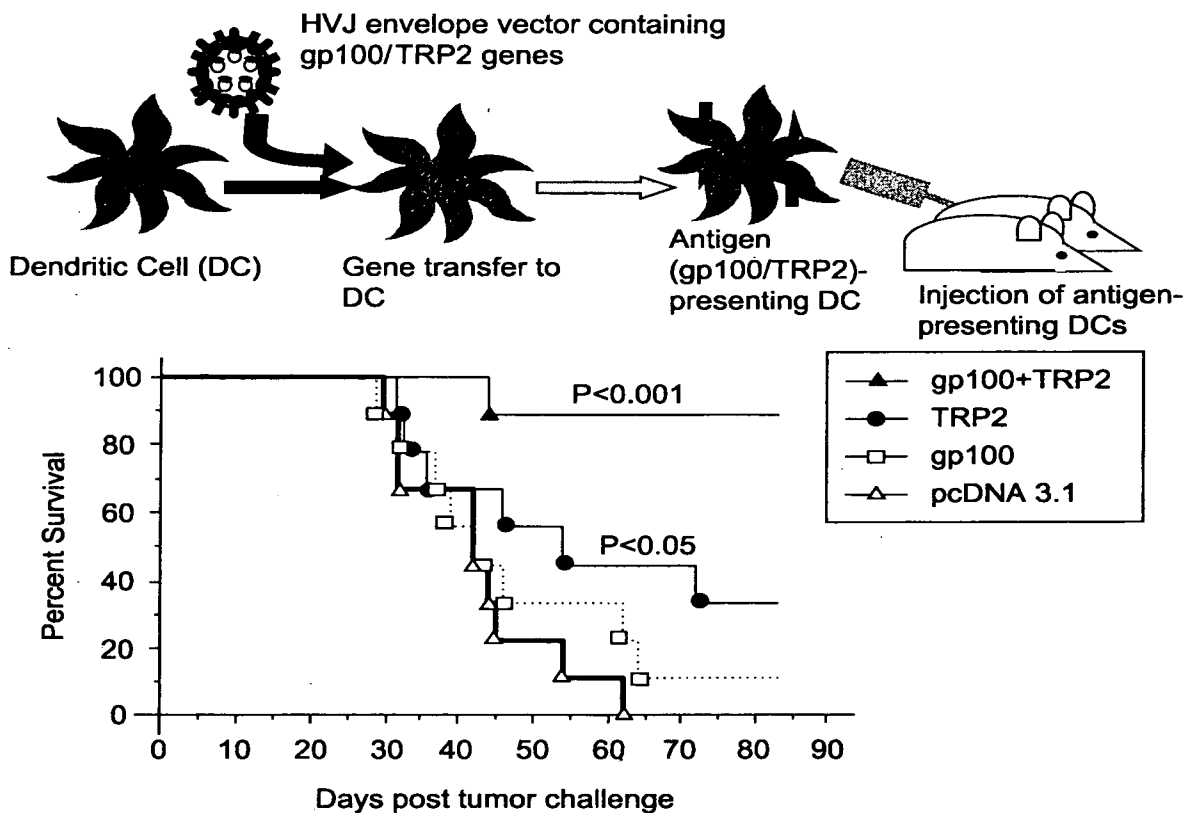
To examine the potential for human gene therapy, we transferred the HGF gene into rats after hearing impairment was observed. Transfection of the human HGF gene into the subarachnoid space of the hearing-impaired rats significantly reduced the threshold shift in hearing function detected by auditory brain stem response when compared to rats transfected with the control vector. We also measured the number of SGCs in mid-modiolar sections of the cochleae from rats transfected with human HGF gene after kanamycin treatment. As shown in Fig. 12.3, by KM insult, the number of SGCs decreased to an undetectable level, but with HGF gene transfer, the number of SGCs are recovered to almost half of the normal level before KM insult. These results suggest that SGCs can be recovered by HGF gene transfection even after KM insult and that the recovery of SGCs results in the improvement of hearing impairment.

Similarly, cerebral infarction induced by the obstruction of the mid-cerebral artery in rats was prevented by the intrathecal injection of HVJ envelope vector containing HGF (Shimamura *et al.*, 2004).

## VI. APPROACHES TO CANCER GENE THERAPY USING HVJ ENVELOPE VECTOR

### A. Transfection of dendritic cells (DCs) with melanoma-associated antigen (MAA) using HVJ envelope vector for immunotherapy of melanoma

Our first approach for developing cancer gene therapy was to construct cancer vaccines using the HVJ envelope vector system (Fig. 12.4). Polyvalent tumor antigen vaccines have been evaluated to increase the repertoire of anti-tumor T cells (Vilella *et al.*, 2003). In this study, we chose MAA genes, gp100 and TRP2, and transfected DCs with these two genes to evaluate *ex vivo* vaccination for prophylactic and therapeutic melanoma treatment.



**Figure 12.4.** Anti-tumor effect of DCs transfected with gp100 and/or TRP2 gene in a tumor-dissemination model. At 24h after intravenous administration of BL6 melanoma cells ( $10^6$ ), TAA-transfected DCs ( $10^6$ ) were intradermally injected. Control groups consist of DCs transfected with pcDNA3.1 ( $\Delta$ ) ( $n = 9$  mice). Experimental groups consist of vaccination with gp100-transfected DCs ( $\square$ ) ( $n = 9$  mice), TRP2-transduced DCs ( $\bullet$ ) ( $n = 9$  mice), and both gp100/TRP2-transfected DCs ( $\blacktriangle$ ) ( $n = 9$  mice).

DCs are the most potent antigen-presenting cells. DCs, are capable of highly effective presentation of antigens to naïve T cells and they can initiate immune responses (Figdor *et al.*, 2004). Several clinical studies have now been conducted in cancer patients with tumor-associated antigen (TAA)-loaded DCs (Nestle *et al.*, 1998; Thurner *et al.*, 1999). In several of these clinical studies the DCs were loaded by pulsing with protein antigens or with peptides derived from TAAs. Studies have demonstrated that *ex vivo* transfected DCs and adoptive therapy can be very effective in inducing antigen-specific immune responses. This type of strategy has been demonstrated in animals and pilot clinical studies for a variety of cancers (Gilboa *et al.*, 1998; Engleman, 1997).

Transfected DCs with vectors expressing TAA genes have shown promising results. Studies have demonstrated that xenogeneic TAAs are very strong immunogens capable of cross-priming to host syngeneic TAAs. Human TRP2 and gp100 proteins have a high amino acid sequence homology to their mouse counterparts. We used xenogeneic TAAs for several reasons: to break self-antigen tolerance in the host, to induce strong antitumor immunity, and to enhance individual TAA immunogenicity. The approach provides a large expression of TAAs and activates DC presentation. Therefore, a highly efficient transfection system is absolutely necessary for efficient induction of tumor immunity. In recent studies, adenoviral vector was the original vector of choice for efficiently transfecting DCs (Arthur *et al.*, 1997; Kaplan *et al.*, 1999). However, adenovirus does have serious disadvantages as a vector for engineering DCs because of its high toxicity. Some studies have demonstrated that plasmid DNA in liposomes (Pecher *et al.*, 2001), vaccinia virus (Yang *et al.*, 2000; Prabakaran *et al.*, 2002) and retroviruses (Akiyama *et al.*, 2002; Bello-Fernandez *et al.*, 1997) could be used to infect or transfect human DCs to present a variety of antigens. However, a highly efficient and minimally invasive vector system has not yet been achieved for transfection of DCs. To develop more efficient gene transfer to DCs that cause only minimal damage, we tested the potential of HVJ envelope vector for transfection of DCs with two different TAA genes (Fig. 12.4).

We used the luciferase gene to determine the optimal conditions for transfection of DCs. As previously reported, the optimum ratio of HVJ envelope vector to cultured cells is  $6 \times 10^3$  to  $1.2 \times 10^4$  (Kaneda *et al.*, 2002a). We identified  $1.2 \times 10^4$  as the optimal ratio and determined the most effective transfection conditions. When DCs were incubated with HVJ envelope vector containing luciferase gene, luciferase gene expression was not significant. We attempted to increase the luciferase gene expression by centrifuging the mixture of DCs and HVJ envelope vector. Centrifugation at 9000 g was much more effective for gene transfection than 3000 g and 13000 g. The viability of DCs after transfection was approximately 70%. The centrifugation time required for the most effective gene expression was 90 min. The highest luciferase gene

expression was obtained in DCs that were centrifuged for 90 min at 9000 g and 37 °C. Six days after DCs were isolated was the best time to transfect the DCs for optimal luciferase gene expression. Thus, the optimal conditions for the most effective transfection of DCs was at 9000 g for 90 min at 37 °C in DCs cultured for 6 days after isolation from bone marrow. Under these optimal conditions, we assessed the expression of yellow fluorescent protein (YFP) expression to determine the transfected DCs population. Almost all DCs that were recognized with phycoerythrin (PE)-conjugated CD11c antibody expressed YFP, as determined by fluorescence microscopy. Flow cytometry analysis revealed that approximately 99% of DCs expressed YFP.

Surface markers of mature DCs were studied by flow cytometry analysis to confirm that differentiation was not inhibited by gene transfection using HVJ envelope vector. The LPS-induced expression of CD40, CD80, CD86 and HLA-DR was equivalent on non-transfected DCs and transfected DCs. The non-specific phagocytotic activity of DCs (evaluated by uptake of FITC-dextran) was suppressed in transfected DCs as well as in non-transfected DCs. Thus, gene transfer with HVJ envelope vector did not inhibit LPS-induced maturation of DCs.

Next, we used the HVJ envelope vector to transfect the glycoprotein 100 (gp100) and tyrosine-related protein 2 (TRP2) genes into DCs. The expression of each transfected antigen was detected by flow cytometry analysis of cells stained with antigen-specific antibody. On Day 1, gp100 and TRP2 were detected in 36% and 63% of DCs, respectively. Approximately 80% of DCs expressed both antigens on Day 7.

DCs transfected with either the gp100 or TRP2 gene or both genes after one day were injected i.d. into C57BL/6 mice. The delayed-type hypersensitivity (DTH) response was assessed in mice immunized with MAA-transfected DCs at 24, 48 and 72 h after TAA protein challenge. Significant cutaneous DTH was detected in mice immunized with MAA gene-transfected DCs compared to the control mice that were injected with DCs without MAA. The DTH response to gp100 was significant in the groups immunized with gp100-transfected DCs or gp100/TRP2-transfected DCs. The level of response was equivalent in the two groups. A similar response to TRP2 was obtained in the groups immunized with TRP2-transfected DCs or gp100/TRP2-transfected DCs.

We examined CTLs against BL6 tumor cells on Day 7 after DC administration. TAA-specific CTLs were generated with variable response rates. Mice immunized with DCs expressing gp100 and TRP-2 had the highest level of CTL activity against <sup>51</sup>Cr-labeled BL6 target cells. Mice immunized with single TAA-transfected DCs generated significant CTL activity against BL6 cells when compared to the control groups that received HVJ envelope vector containing pcDNA-3.1. However, these responses were lower than those generated by the cotransfer of gp100 and TRP2. Vaccination of DCs transfected

with TAA genes induced TAA specific cellular immunity consisting of CTL activity and DTH-helper cell activity against melanoma cells.

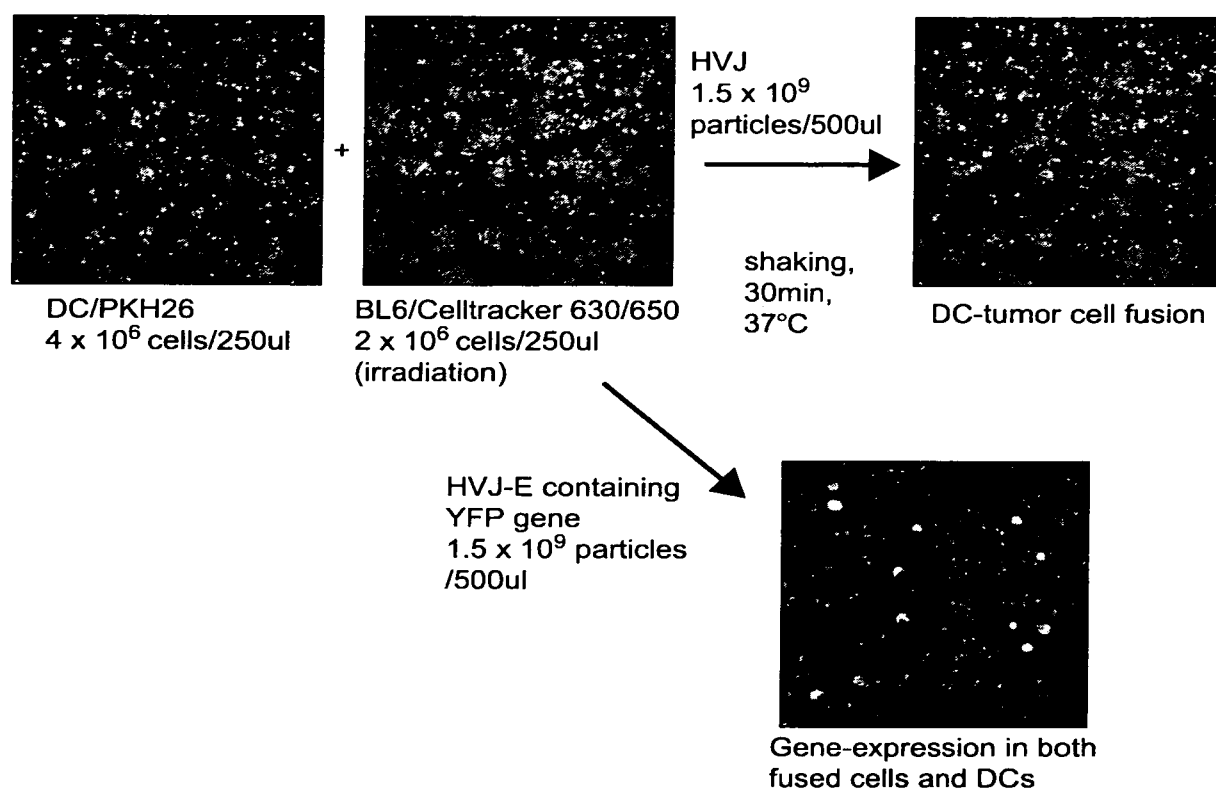
We tested the effect of vaccination with MAA-transfected DCs on the inhibition of tumor growth in mice. No tumor growth was observed in mice immunized with gp100/TRP2 transfected DCs, although significant tumor masses were palpable in all control mice injected with DCs without TAA. Immunization with DCs transfected with single TAA gene was not effective, as small tumor masses were detected. However the tumor growth rate was slower than that of the control group. In this prophylactic study, vaccination with DCs transfected with both gp100 and TRP2 was much more effective for inhibition of tumor growth than vaccination with DCs transfected with TRP2 alone.

To investigate the therapeutic effect of vaccination with TAA-transfected DCs, we vaccinated the mice with various DC vaccines 24 h after intravenous inoculation with  $10^4$  BL6 cells. All control mice injected with PBS-treated DCs and pcDNA3.1-transfected DCs died of lung metastasis in 45 days and 62 days after inoculation, respectively. Vaccination with DCs that were transfected with a single TAA gene improved likelihood of survival. Vaccination with TRP2 transfected DCs was more effective than vaccination with gp100-transfected DCs. Vaccination with gp100/TRP2-transfected DCs significantly prolonged the survival rate. Eight of nine mice vaccinated with gp100/TRP2-transfected DCs survived more than 80 days after tumor inoculation (Fig. 12.4). Vaccination with immature DCs that were not treated with LPS was not effective for improving survival, and all of these mice died in 60 days (including the mice that received gp100/TRP2-transfected DCs).

## **B. Fusion of DC-Tumor cells and simultaneous gene transfer to the hybrid cells using HVJ envelope for the prevention and treatment of cancers**

TAAAs have been identified in some cancers such as melanoma (Boon *et al.*, 1994; Kawakami and Rosenberg, 1997). However, TAAAs in many cancers have not been identified. The identification of TAAAs is required for the development of TAA-loaded DC vaccines.

To solve the problem, hybrid cell vaccines have been developed by fusing DCs with tumor cells (Gong *et al.*, 1997; Wang *et al.*, 1998). There is evidence that these DC-tumor-fused cells possess the properties of both tumor cells containing known and unknown TAAAs and DCs containing high levels of MHC class I and II molecules and co-stimulatory molecules for priming and activating naïve CD4+ and CD8+ T cells. Therefore, even though tumor cells lose MHC class I molecules, TAAAs can be presented on the surface of the fused cells by DC-derived MHC class I molecules. Polyethylene glycol and



**Figure 12.5.** Fusion of DCs and melanoma cells and simultaneous gene transfer to the fused cells. Mouse bone marrow-derived DCs stained with PKH26 and mouse melanoma BL6 labeled with Celltracker 630/650 are fused with inactivated HVJ ( $1.5 \times 10^8$  particles). When fused with HVJ envelope (HVJ-E) vector containing YFP gene, YFP gene expression is detected in both DCs and fused cells. (See Color Insert.)

electroporation have been used to induce tumor cell–DC fusion. HVJ has been identified as a powerful fusogen. We stained mouse DCs with red fluorescent reagent. Mouse melanoma cells were stained green and irradiated with gamma-rays. Both cells were mixed with inactivated HVJ. After 30 min, the fusion of DCs and tumor cells occurred with approximately 50% efficiency (Fig. 12.5).

We used HVJ envelope to generate powerful tumor-DC vaccines, because HVJ envelope can induce tumor cell–DC fusion and simultaneously transfer DNA or proteins to activate the immune response. To confirm simultaneous fusion and gene transfer mediated by HVJ envelope, DCs and tumor cells were mixed with HVJ envelope containing the GFP gene. As shown in Fig. 12.5, fusion between DCs and irradiated-tumor cells was observed, and YFP expression was detected in fused cells as well as in DCs.

Using this system, we attempted to generate anti-tumor immunity using HVJ envelope with or without the IL-12 gene. Ten days after the second immunization, spleen cells were isolated to assess cytolytic activity. The cytolytic

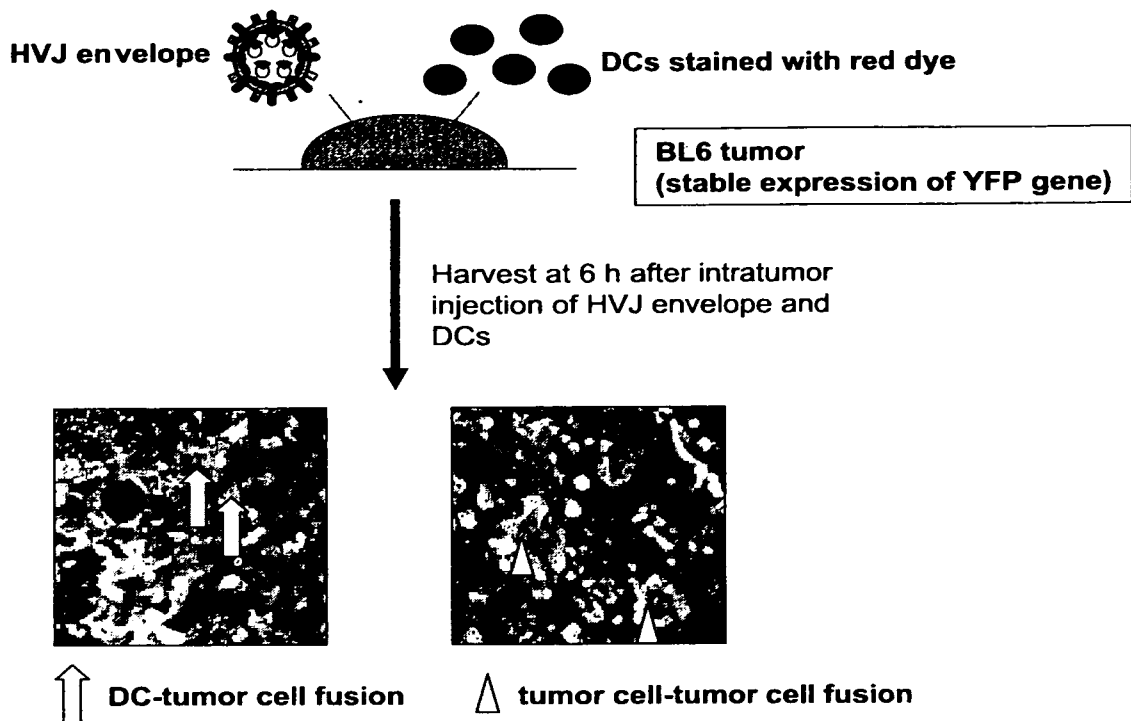
activity of the effector cells obtained from the mice immunized with fused cells was significantly higher than that obtained from other vaccination protocols such as PBS, IL-12, Mix (mixture of DCs and tumor cells without fusion) and mix plus IL-12. Higher cytolytic activity was observed in the mice that received fused cells plus IL-12 as compared with that in the mice that received fused cells alone.

First, we examined the effect of this hybrid cell vaccine on the prevention of tumor generation. After two vaccinations, melanoma cells or renal cancer cells were intradermally injected. The mice vaccinated with fused cells plus IL-12 had significantly increased survival; all of these mice were alive 60 days after tumor challenge in the B16BL6 tumor model. The survival rate was 20% in the mice vaccinated with fused cells without IL-12. All mice in the other groups died. The effect of fused cells and IL-12 on the enhancement of tumor-specific immunity in mice was also observed against renal cancer. Therefore, these findings indicate that immunization with fused cells plus IL-12 strongly induces Th1 cytokines and activates tumor-specific CTLs, resulting in significant protection from melanoma, which has known TAAs or renal cancers which has unknown TAAs.

Next, we treated solid tumors by *in vivo* fusion and gene expression using HVJ envelope vector. Mouse DCs and HVJ envelope vector were injected into solid tumors. The effect of the transfer of a therapeutic gene by HVJ envelope vector was also evaluated. To detect *in vivo* fusion of DCs with tumor cells, red-stained DCs were injected into melanoma masses expressing YFP with inactivated HVJ. Megakaryocytes, which indicate tumor cell-tumor cell fusion, were detected, as well as large orange-stained cells which indicate DC-tumor cell fusion. However, the fusion efficiency *in vivo* was much less than that of *in vitro* cell fusion (Fig. 12.6).

For therapeutic experiments, melanoma cells were intradermally injected. When the tumor diameter was greater than 5 mm, an injection of DCs and HVJ envelope vector was administered. Spleen cells were isolated and assayed for anti-melanoma CTL activity. No therapeutic genes were incorporated into the HVJ envelope vector. The co-injection of DCs and empty HVJ envelope vector resulted in significant anti-melanoma CTL activity. DCs or HVJ envelope vector alone did not induce CTL activity. When tumor growth was observed, the co-injection of DCs and empty HVJ envelope vector inhibited tumor growth, but the tumor volume still gradually increased. When DCs were co-injected with HVJ envelope vector that contained the IL-12 gene, a greater inhibitory effect was obtained. The co-injection of DCs and HVJ envelope vector effectively prolonged mouse survival. The most effective therapeutic effect was obtained when HVJ envelope vector that contained IL-12 gene was co-injected with DCs into the tumor mass; approximately 60% of these mice survived 35 days after treatment.



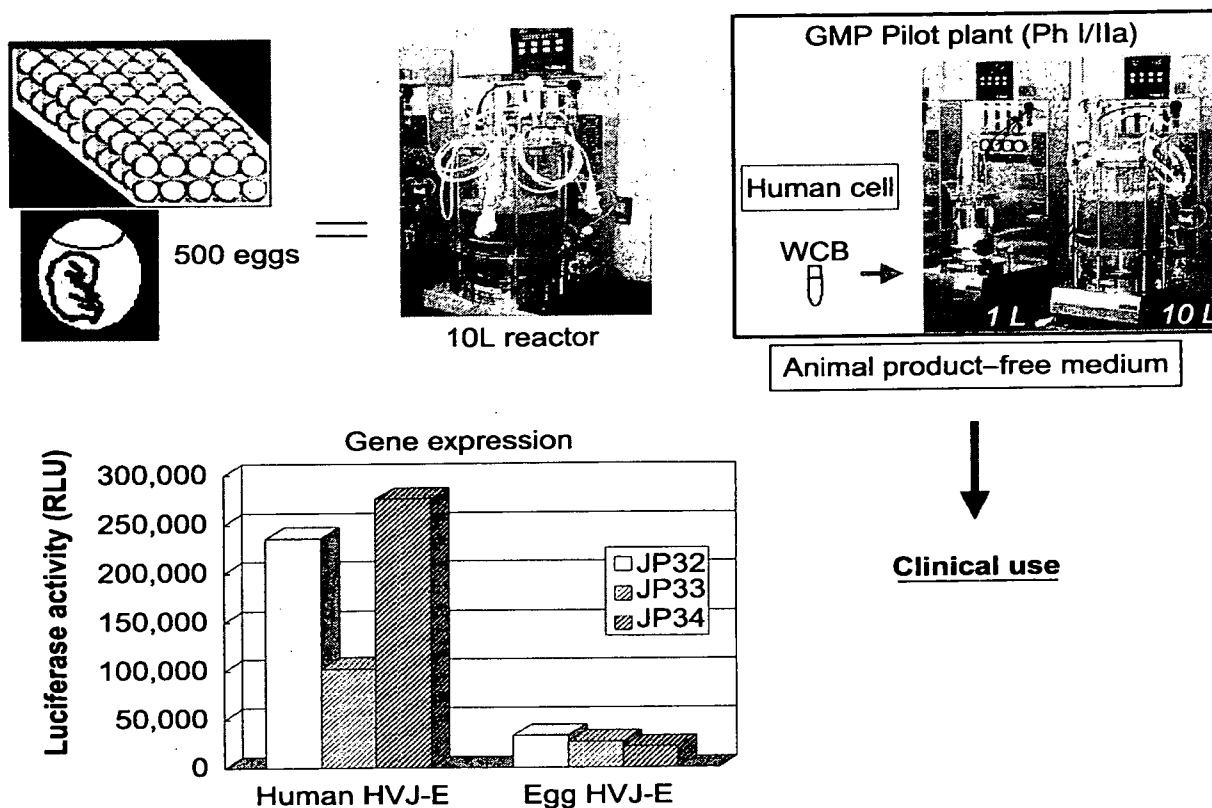


**Figure 12.6.** *In vivo* fusion of DCs and tumor cells. The mixture of DCs and inactivated HVJ is injected into intradermal tumor mass derived from BL6 expressing YFP gene. When DCs are labeled with red dye (PKH26), orange-colored fused cells are detected, suggesting DC-tumor fusion. Tumor-tumor cell fusion is also observed.

Thus, we developed a novel cancer vaccine using HVJ envelope-mediated DC-tumor cell fusion. The DC-tumor cell fusion was induced by HVJ envelope vector at a high efficiency. Both known and unknown TAAs were presented on fused cells by MHC class I molecules from DCs. Furthermore, IL-12 gene transfer was achieved in the fused cells. The expression of IL-12 stimulates the maturation of naïve T cells and promotes a Th1 response (Xu *et al.*, 2003). It is believed that the presentation of TAAs, and the promotion of a Th1 response, together induce an efficient anti-tumor CTL response.

## VII. TOWARD THE CLINICAL TRIAL

The HVJ envelope vector holds great promise for human gene therapy. We are improving the HVJ envelope vector system so that it can be used in clinical trials. So far, the virus has been produced in chick eggs (Okada, 1993), but egg-derived HVJ is difficult to use for clinical trials. It has been very difficult to produce large amounts of the virus in cultured cells. The production in cultured cells was less than 2% of that in chick eggs (Kaneda and Okada, unpublished



**Figure 12.7.** Development of clinical grade HVJ envelope vector. HVJ infects human cell from working cell bank (WCB), and human cell-derived HVJ is prepared using animal product-free medium in a pilot plant based on good manufacturing practice (GMP) standard. The envelope vector prepared from human cell-derived HVJ is more efficient in gene transfection compared with that from egg-derived HVJ. (See Color Insert.)

observation). We recently succeeded in producing HVJ in human cells. We cloned HEK 293 cells that produce a high titer of HVJ after infection, and determined the growth conditions for these cells in an animal product-free medium using a bioreactor. We are able to culture these cells in 10 liters of serum-free medium at a density of more than  $10^6$  cells/ml, and we can obtain HVJ at the efficiency of more than  $3 \times 10^9$  particles/ml (Fig. 12.7). The production in a 10 liter culture is comparable to that in 500 chick eggs. A pilot plant to commercially produce clinical grade HVJ envelope vector was established in a venture company named GenomIdea, Inc. (7-7-15 Saito-Asagi, Ibaragi, Osaka 567-0085, Japan; <http://www.anges-mg.com/news/030324.htm>). GenomIdea created a working cell bank, master cell bank and master virus bank for producing clinical grade HVJ for Phase I and IIa trials. They also set up an apparatus to purify inactivated HVJ using two different column systems. The human cell-derived HVJ envelope vector was more effective for gene transfection than

the egg-derived HVJ envelope vector (Fig. 12.7). Human cell-derived HVJ envelope vector is also available for drug delivery both *in vitro* and *in vivo*. This vector will soon be available for human gene therapy.

## VIII. MAIN ABBREVIATIONS

HVJ	hemagglutinating virus of Japan
HN	hemagglutinin-neuraminidase
F	fusion
FITC	fluorescein isothiocyanate
ODN	oligodeoxynucleotide
HA	hemagglutinin
CTLs	cytotoxic T cells
ES	embryonal stem
GFP	green fluorescent protein
siRNA	small interfering RNA
CSF	cerebrospinal fluid
HGF	hepatocyte growth factor
SGCs	spiral ganglion cells
KM	kanamycin
DCs	dendritic cells
MAA	melanoma-associated antigen
TAA	tumor-associated antigen
YFP	yellow fluorescent protein
PE	phycoerythrin
gp100	glycoprotein 100
TRP2	tyrosine-related protein 2
DTH	delayed-type hypersensitivity
WCB	working cell bank
GMP	good manufacturing practice
HVJ-E	HVJ envelope

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