BMB
reports

Mini Review

A non-replicating oncolytic vector as a novel therapeutic tool against cancer

*Yasufumi Kaneda**
Division of Gene Therapy Science, Graduate School of Medicine, Osaka University, Japan

Cancers are still difficult targets despite recent advances in cancer therapy. Due to the heterogeneity of cancer, a single-treatment modality is insufficient for the complete elimination of cancer cells. Therapeutic strategies from various aspects are needed. Gene therapy has been expected to bring a breakthrough to cancer therapy, but it has not yet been successful. Gene therapy also should be combined with other treatments to enhance multiple therapeutic pathways. In this view, gene delivery vector itself should be equipped with intrinsic anti-cancer activities. HVJ (hemagglutinating virus of Japan; Sendai virus) envelope vector (HVJ-E) was developed to deliver therapeutic molecules. HVJ-E itself possessed anti-tumor activities such as the generation of anti-tumor immunities and the induction of cancer-selective apoptosis. In addition to the intrinsic anti-tumor activities, therapeutic molecules incorporated into HVJ-E enabled to achieve multi-modal therapeutic strategies in cancer treatment. Tumor-targeting HVJ-E was also developed. Thus, HVJ-E will be a novel promising tool for cancer treatment. [BMB reports 2010; 43(12): 773-780]

INTRODUCTION

There are several barriers to be overcome in cancer gene therapy. One of the most important issues to be solved is gene delivery vectors. Numerous viral and non-viral methods for gene transfer have been developed for human gene therapy, but both viral and non-viral vectors have limitations as well as advantages. Whatever progress is achieved in gene therapy vectors, it would be impossible to achieve the delivery at 100% efficiency in cancer patients due to the heterogeneity of cancer cells and the complicated vasculature. For example, the most popular cancer gene therapy is the use of p53 gene which induces growth arrest and apoptosis. Although p53 gene therapy has been done in many cancer patients, anti-cancer effects are not sufficient by gene therapy alone (1). Combination with radiation or anti-cancer reagent is needed to augment p53 gene therapy (2). However, even by the combination therapy, complete elimination of cancer cells is impossible because of the heterogeneity of tumor-tissue. Actually, cancers frequently recur. It has been expected that immune system equipped with human body could eliminate residual cancer cells. Although the immune system greatly contributes to the treatment of infectious diseases, it is very complicated in cancers. Cancer immunotherapy is quite different from immunotherapy against infectious diseases (3). Numerous failures of cancer immunotherapy have indicated the difficulty. It has been gradually elucidated that cancer tissues produce factors to attenuate host immune defense system, which induces immunotolerance against cancers in tumor-bearing individuals (4). Both activation of effector lymphocytes against cancers and suppression of immunosuppressive factors are necessary for more effective immunotherapy (5). At the beginning of tumorigenesis, cancer cells can be eliminated by host immune system. Such battle may occur frequently in healthy individuals. When the activities of immune system are attenuated by several factors such as senescence, stress and immunosuppressive drugs, cancers begin to grow by escaping host immune surveillance. Finally, large tumor mass overwhelms immune system (6). At that time, immunotherapy has no more effect to eliminate cancer cells. Therefore, immunotherapy should be started at early stage of cancer. When large tumor mass is formed in patient, tumor mass should be removed as much as possible before immunotherapy. Therefore, to expect the effect of immunotherapy, combination of immunotherapy with any other treatment to remove tumor mass is recommended.

From those points of view on cancer therapy, multilateral therapeutic tools should be intensively committed to the battle. In the development of drug delivery system (DDS), safer vectors have been appreciated. Basically, the concept is correct, but, only for cancer treatment, DDS should have intrinsic anti-tumor activities. Therapeutic molecules incorporating into DDS with intrinsic anti-tumor activities can enhance anti-tumor activities. This might be an ideal cancer treatment and we can see such applications in armed type oncolytic viruses.

Oncolytic viruses have been used for cancer therapy. Current oncolytic viruses are live viruses and kill cancer cells by selective replication in cancer cells. Those are wild type vi-

*Corresponding author. Tel: 81-6-6879-3900; Fax: 81-6-6879-3909; E-mail: kaneday@gts.med.osaka-u.ac.jp
DOI 10.5483/BMBRep.2010.43.12.773

Received 15 November 2010

Keywords: Anti-tumor immunity, Apoptosis, Cancer, Gene therapy, HVJ

ruses or natural mutants such as E1B-55K deletion adenovirus, New Castle Disease virus and Edmonston strain of measles virus. Additionally, artificial mutants are developed by viral gene engineering such as telomerase-controlled adenovirus, ICP6/γ34.5-deleted herpes virus and thymidine kinase-deleted vaccinia virus (JX-594) (7, 8). To enhance anti-tumor effects, armed-type oncolytic viruses having therapeutic genes are produced. Cytosine deaminase/thymidine kinase gene-loaded E1B-55K deletion adenovirus and GM-CSF gene-loaded JX-594 have been reported (9, 10). These viral vectors will be promising therapeutic tools in future. However, as far as viral genome is intact, it is inevitable that live viruses remain in normal tissues of patients. That is not good from the view of safety. Furthermore, tumor-killing activity by those oncolytic viruses may be attenuated by anti-viral immunity raised in patients which can limit viral replication in cancer cells. The enhancement of tumor-immunity is desired for cancer therapy, but on the contrary, it may suppress the replication of oncolytic virus. The strategy may be caught in a dilemma.

Here, I am focusing on replication-incompetent virus with multiple anti-tumor activities. HVJ (hemagglutinating virus of Japan) envelope (HVJ-E) vector as developed using inactive Sendai virus particle as a drug delivery system (11). While repeating the treatment of tumor-bearing mice using HVJ-E containing therapeutic molecules, HVJ-E itself was found to have anti-tumor activities such as the generation of anti-tumor immunities and direct cancer killing activity (12).

Development of HVJ envelope vector

HVJ belongs to mouse parainfluenza virus and it is not a human pathogen. The virus is famous for inducing fusion with cell membrane at neutral pH, and HN and F-fusion proteins of the virus, contributes to the cell fusion (13). HN binds to acetyl type sialic acid and degrades the sugar chain with its neuraminidase activity. Then, F associates with lipids, such as cholesterol in the cell membrane to induce cell fusion. Using membrane fusion activity of HVJ, we have constructed fusion liposomes (HVJ-liposomes) by combining DNA-loaded liposomes with inactivated HVJ particle (14). However, HVJ-liposomes have been widely used for gene transfer both in vitro and *in vivo*, there are some drawbacks. One disadvantage of HVJ-liposome is the complicated procedure to prepare the vector, which has been a major obstacle for clinical use. Another is the weak fusion activity of the HVJ-liposomes, which is approximately 2% of native HVJ because of the reduction of density of fusion proteins on the surface of HVJ-liposomes. To simplify the vector system and to develop more effective gene delivery system, inactivated HVJ particle itself without liposomes was turned to a vector for drug delivery (11). As shown in Fig. 1, HVJ was inactivated with b-propiolactone (0.0075-0.001%) or by UV irradiation (99 mjoule/cm^2) as described previously, and then purified by ion-exchange column chromatography and gel filtration. The diameter of HVJ envelope (HVJ-E) was 220 nm and the zeta potential was approximately −5 mV. Exogenous plasmid DNA was incorporated into inactivated HVJ by treatment with mild detergent (for example; 0.02-0.05% Tween 80) and centrifugation (10,000 g, 5-10 min). Electronmicroscopy confirms that DNA is incorporated into all of the particles of inactivated HVJ. The largest DNA tested was a 14 kb plasmid DNA, with a resultant trapping efficiency of approximately 18%. Fusion between HVJ-E vector and cell membrane occurs within only 3-5 sec-

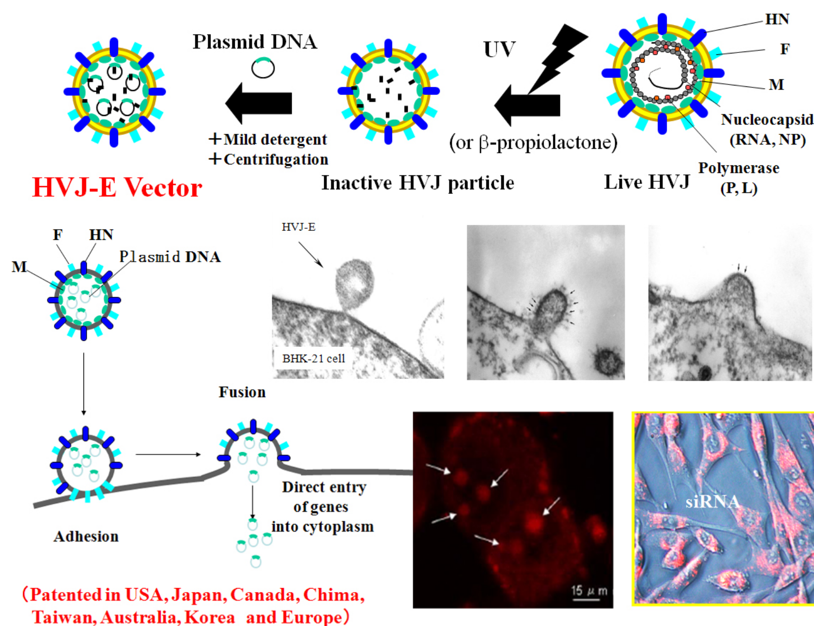

Fig. 1. Development of HVJ-E vector. HVJ-E vector derived from inactive Sendai virus particle incorporates DNA, siRNA or proteins by mild detergent solubilization and centrifugation. The vector directly introduces molecules inside to the cytoplasm via membrane fusion. A part of DNA rapidly migrates to the nucleolus. SiRNA is delivered to the cytoplasm at high efficiency. The patent of the vector has been already filed in USA, Europe, China, Canada, Korea, Taiwan and Japan.

onds immediately after the attachment of the plasmid-containing HVJ-E vector to a cell surface (Fig. 1). HVJ-E can produce cytokines and chemokines especially in dendritic cells and some cancer cells (12). However, the production of cytokines and chemokines in dendritic cells by HVJ-E vector was abolished by the treatment of HVJ-E with Triton-X100, but maintained by Tween 80-treated HVJ-E (15). Both detergents were available for incorporation of therapeutic molecules into HVJ-E vector. For cancer treatment, both immunological activities and drug delivery potency of HVJ-E are necessary, but, for the therapy of diseases other than cancers, induction of cytokine and chemokine may be harmful. Thus, using two different detergents at the incorporation step, it is possible to construct HVJ-E vector with or without anti-tumor immunity.

The applications of HVJ-E to drug delivery, gene therapy and gene screening are described elsewhere (15).

Since the viral genome is inactivated, there are no replication and viral gene expression in the cells infected with HVJ-E vector. To show the evidence of inability of replication, the amount of viral genome was analyzed using quantitative real-time RT-PCR. Renca cells were treated with UV-inactivated HVJ (HVJ-E) or live HVJ and RNA were isolated from the cells in 12 and 24 hours after the treatment. When cells were infected with HVJ-E, the level of viral RNA was much lower at 12 hr than that infected with live HVJ, and the RNA level decreased at 24 h compared with the level at 12 h. On the other hand, it increased with live HVJ. This result shows that UV-inactivated HVJ (HVJ-E) loses the ability to replicate its genome.

Gene transfer to mouse muscle by HVJ-E vector was not inhibited by repeated injections (16). This is probably because fusion occurs very rapidly so that fusion-mediated drug delivery finishes before sufficient binding the antibody to the vector.

Anti-tumor immunities by HVJ-E

When HVJ-E was injected into murine colon carcinoma (CT26) tumors growing in syngeneic Balb/c mice, tumors were eradicated in 60-80% of the mice and the tumor growth of the remainder was obviously inhibited (17). Induced adaptive anti-tumor immune responses were dominant in the tumor eradication process because the effect was abrogated in severe combined immunodeficient (SCID) mice. Actually, cytotoxic T lymphocytes (CTL) activities were much enhanced by HVJ-E injection. In murine renal cancer (Renca) mouse model, some inhibitory effects on tumor growth were seen even in SCID mice and the effect was abrogated by the coinjection of anti-GM1 antibody with HVJ-E which depletes natural killer (NK) cells (18). Therefore, the injection of HVJ-E into tumors induces strong anti-tumor immune responses including the activation of CTL and NK cells in tumor-bearing mice (Fig. 2). Furthermore, HVJ-E inhibited regulatory T cell (Treg)-mediated immunosuppression through interleukin (IL)-6 secretion from maturated dendritic cells (DCs) (17) (Fig. 2). IL-6 is known to inhibit Treg probably because of the increase of methylation of enhancer region of FoxP3 which is a key transcription factor in Treg (19, 20). We have been analyzing the mechanisms of induction of tumor-immunity by HVJ-E. One major effect was caused by the production of interferon-β (IFN-β). The component of HVJ-E responsible for production of IFN-β was degraded viral RNA fragment. Viral RNA can be recognized by cytoplasmic RNA receptors, retinoic acid-inducible gene-I

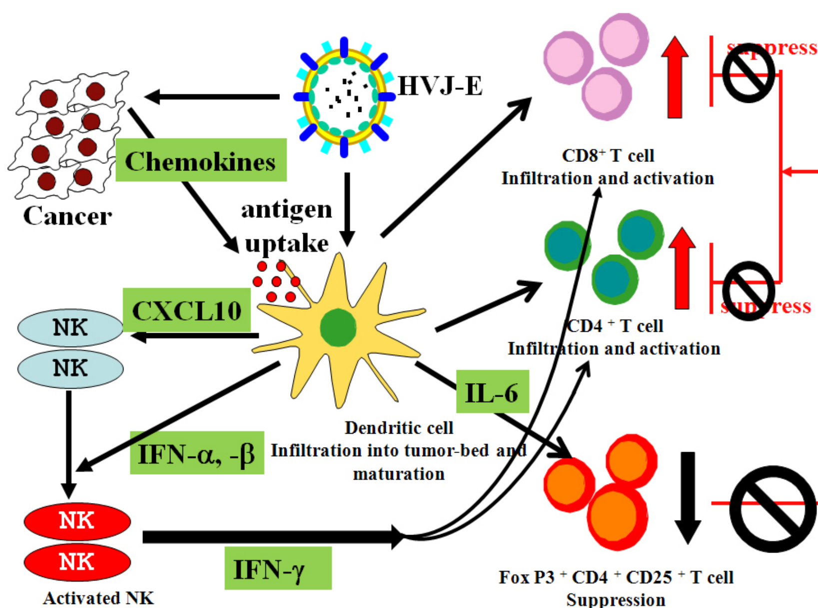

Fig. 2. Activation of CTL and NK cells and suppression of Treg by HVJ-E in tumor-bearing mice. HVJ-E induces the secretion of chemokines such as CCL2,3 and 5 from tumor tissue, which elicits the infiltration of $CD11c^+$ DCs, $CD4^+$ and $CD8^+$ lymphocytes to tumor-bed. DCs produce CXCL10 and IFN-β by HVJ-E, which activates recruited NK cells to secret IFN-γ. HVJ-E also induces maturation of DCs and tumor-antigens can be presented both by MHC class I and II, which are recognized by $CD4^+$ and $CD8^+$ lymphocytes to generate CTL. DCs also produce IL-6 by HVJ-E, which suppresses the function of $CD4^+$ $CD25^+$ $FoxP3^+$ Treg.

(RIG-I) and melanoma differentiation antigen-5 (MDA-5) (21). Sendai virus RNA is recognized by RIG-I and the RIG-I/RNA complex interacts with mitochondrial anti-viral signaling (MAVS) on mitochondria, which activates several transcription factors such as IRF3, IRF7 and NF-κB (22, 23). Those factors can activate the production of many cytokines and chemokines including IFN-β. Another effect, especially on the suppression of Treg, resulted from IL-6 which is produced from DCs. The component of HVJ-E for IL-6 production is F protein on HVJ envelope, not RNA inside the particle (24). Further analysis elucidated that the carbohydrates of F protein recognized by unknown receptor(s) on DCs induced the signal for IL-6 production via NF-κB activation. Thus, HVJ-E alone may be an effective treatment against cancer, and a clinical trial to test the safety and efficacy of HVJ-E in the treatment of malignant melanoma is underway. However, anti-tumor immune responses induced by HVJ-E may be suboptimal because its activation of effector T cells appears to be indirect, requiring several steps. First, HVJ-E induces the secretion of CXCL10 from DCs which recruits NK cells to the tumor-bed; then, IFN-k secretion from DCs induced by HVJ-E elicits activation of NK cells; and finally the activated NK cells produce IFN-c which promotes activation of effector T cells (18) (Fig. 2). Moreover, effector T cells should be activated efficiently to become CTL at the recognition of tumor antigens raised by MHC-class I molecule. Thus, HVJ-E alone does not directly activate effector T cells, and multiple injections of HVJ-E are needed for achieving effective immune responses (17, 18). More efficient activation of effector T cells by HVJ-E can be mediated by therapeutic molecules incorporated into HVJ-E. The trial is described in section 4.

Direct tumor killing by HVJ-E

When the castration-resistant human prostate cancer cells, PC3 and DU145, were treated with various amounts of HVJ-E, the viability of both cell lines was significantly suppressed by HVJ-E in a dose-dependent manner (25). Apoptosis of PC3 cells was clearly detected 24 hr after HVJ-E treatment by Tunel staining of fragmented DNA. Increased expression of caspase 3 and 8 was detected in PC3 cells after HVJ-E treatment, while the expression of caspase 9 remained unchanged. However, growth inhibition of normal prostate epithelium, PNT2, was not observed. Receptors for HVJ are ganglioside GD1a and sialyl paragloboside (SPG). Those gangliosides were highly produced in PC3 and DU145 cells. The amount of GD1a and SPG of those castration-resistant prostate cancer cells was 2-3 times more than that of PNT2. Sensitivity of HVJ-E to PC3 and DU145 is partly due to the high number of receptors for HVJ. However, PNT2 produces some amount of GD1a and SPG, which is sufficient for fusion with HVJ-E. Nevertheless, apoptosis was not caused in PNT2 by HVJ-E. What is the mechanism for tumor-selective killing by HVJ-E?

Microarray analysis of genes expressed in PC3 cells after HVJ-E treatment revealed seventeen IFN-induced genes (26) among the twenty most up-regulated genes (25). This result suggests that the secretion of type I IFN (IFN-α, -β) by tumor cells following HVJ-E treatment may stimulate IFN-related genes. It is known that live HVJ induces the production of type I IFN by RIG-I-mediated recognition of double-stranded viral RNA (21). HVJ-E also induces type I IFN secretion in murine dendritic cells in a fusion-dependent manner (24). Significant induction of IFN-α and -β was detected in the culture medium of PC3 cells following HVJ-E treatment, although IFN-α and -β were not secreted in PNT2 cells. In addition, IFN secretion was not detected in murine carcinoma cell lines, including CT26 and Renca (17, 18). RIG-I expression increased in both PC3 cells and DU145 cells after HVJ-E treatment, and this induction was blocked by antibody against the IFN receptor. Thus, HVJ-E augments production of the cytoplasmic receptor of its RNA genome through the production of type I IFN. Type

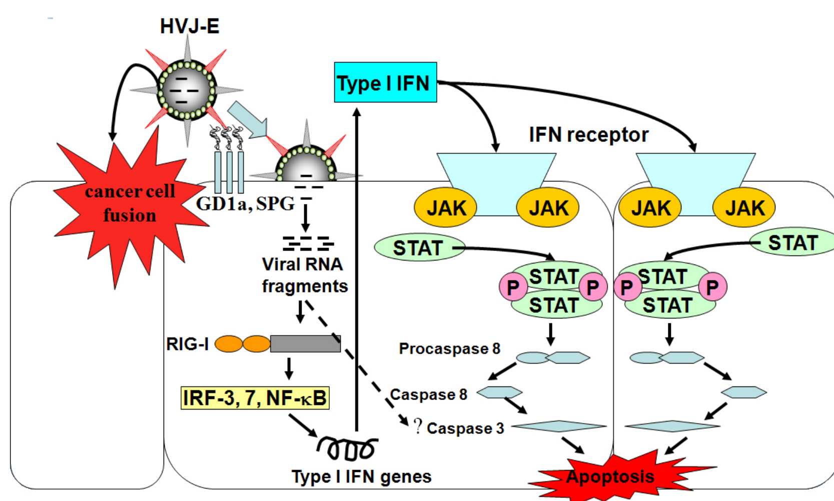

Fig. 3. A part of the mechanism of apoptosis of cancer cells by HVJ-E. Castration-resistant human prostate cancer cells produce high amount of gangliosides GD1a and SPG which are the receptors for HVJ. Then, cancer cell fusion frequently occurs by HVJ-E treatment. Those fused cells don't proliferate. By membrane fusion, viral RNA fragment is introduced into cancer cells. Cytoplasmic RNA receptor, RIG-I, recognizes the RNA, which associates with MAVS. Then, several transcription factors such as IRF3, IRF7 and NF-κB are activated. Those factors activate the expression of some genes including IFN-β. Secreted IFN-β binds to its receptor on cancer cell surface and activates Jak-Stat signaling pathway. Finally, caspases are activated to induce apoptosis off cancer cells. However, this pathway is one of the mechanisms of HVJ-E-mediated cancer cell killing. However, other pathways remain to be elucidated.

I IFN is known to induce apoptosis in some transformed cell lines via Jak-Stat pathway (27). By adding a Jak inhibitor to PC3 cells prior to HVJ-E treatment, pStat1 was reduced to an undetectable level even after HVJ-E treatment. Caspase 8 was also down-regulated in the presence of the inhibitor. When an antibody against the IFN receptor was added to cells 3 hr prior to HVJ-E treatment, Stat 1 phosphorylation and caspase 3 and 8 activation by HVJ-E were suppressed. Annexin V-positive cells increased with HVJ-E, but a significantly reduced number of apoptotic PC3 cells were observed following pre-treatment with the Jak inhibitor. From these results, IFN-β produced from PC3 cells by HVJ-E seems to be a trigger for apoptosis as shown in Fig. 3. However, with either IFN receptor antibody or Jak inhibitor, apoptosis of PC3 cells was not completely blocked. Moreover, many PC3 cells were resistant to IFN-β treatment. Therefore, some factors other than IFN-β may exist to induce apoptosis in PC3 cells by HVJ-E (28). Further analysis is needed for the complete elucidation of tumor-selective apoptosis by HVJ-E.

Apart from the mechanism, HVJ-E was very effective for the eradication of prostate cancers in mice. PC3 cells were intradermally inoculated into the backs of SCID mice, after which each tumor mass was directly injected with HVJ-E on days 10, 13 and 16 after inoculation. In the HVJ-E-treated group, the tumors were completely eradicated in all mice (25). It is known that HVJ-E eradicates mouse renal cancers by activating NK cells (18). Then, to test the effect of NK cells on tumor regression by HVJ-E treatment, an anti-asialo GM1 antibody was co-injected with HVJ-E three times into each PC3 tumor mass. Tumor regression by HVJ-E was slightly attenuated by the anti-asialo GM1 antibody, however, significant suppression of tumor growth was observed even in the presence of anti-asialo GM1 antibody. To further test the contribution of NK cells on tumor-regression, tumor-bearing NOD-SCID mice were treated with HVJ-E on days 15, 20 and 25 after tumor inoculation. In NOD-SCID mice, tumor growth was also suppressed by HVJ-E. However, tumor masses (50-60 mm^3) were palpable more frequently in NOD-SCD mice injected with HVJ-E than in SCID mice. Following injection of HVJ-E, the ratio of tumor-free mice on day 50 was 13/18 (72.2%) in NOD-SCID mice versus 11/13 (84.6%) in SCID mice. Therefore, in SCID mice, HVJ-E induced tumor regression partly due to the NK cell activation, but mainly by the induction of tumor-selective apoptosis.

Enhanced cancer treatment using armed-type HVJ-E

Cancer therapy using siRNA-loaded HVJ-E: HVJ-E vector is available for the delivery of proteins, anti-cancer small compounds and small interfering RNA (siRNA) (15). The efficiency of the delivery of siRNA to cultured cancer cells using HVJ-E is very high. It is generally 50-100%. Using HVJ-E, Rad51 siRNA was introduced to cancers both *in vitro* and *in vivo*. The siRNA enhanced anti-cancer effect of cis-diamminedichloroplatinum (II), one of the most widely used anti-cancer drugs, and inhibited cellular growth by inducing DNA double-strand breaks (29). For human glioma treatment, siRNA against Eg5 which is a motor protein involved in centriole separation and the formation of mitotic spindles (30) was also incorporated into HVJ-E. HVJ-E vector itself induced extensive apoptosis in some glioma cell lines such as U258MG and T98G. But, another glioma cells, U118 MG and A172, were not so sensitive to HVJ-E as glioma cells above. Then, Eg5 siRNA-loaded HVJ-E was constructed (31). The sRNA-incorporating HVJ-E showed much more tumor-killing activity than HVJ-E alone. The combination of Eg5siRNA and HVJ-E was very effective for inhibiting the growth of glioblastoma in both cultured cells and intracranial tumor model.

Cancer gene therapy using HVJ-E: To determine the best candidate cytokine for immunogene therapy with HVJ-E, granulocyte monocyte-colony stimulating factor (GM-CSF), IFN-β, IL-2, and IL-12 gene were selected to incorporate HVJ-E because those genes have been used for glioma treatment (32-39). The anti-tumor effect was examined in glioma-bearing mice by HVJ-E-mediated transfer of each cytokine gene (pVAX-mGM- CSF, pVAX-mIFN-β, pVAX-mIL-2, pVAX-mIL-12, or pVAX-Luc, a negative vector control) (40). Treatment with HVJ-E containing pVAX-mIL-2 showed the most dramatic inhibition of tumor growth among the groups (39). The percentage of mice exhibiting complete tumor eradication showed marked differences emerged between the treatment groups. Treatment with HVJ-E containing pVAX-mIL-2 resulted in eradication of 70% of all tumors, whereas no tumors were eradicated after the treatment with PBS or HVJ-E containing pVAX-Luc, and only 20%, 30%, or 40% of tumors were eradicated after the treatment with HVJ-E containing pVAX-mIFN-b, HVJ-E containing pVAX-mGM-CSF, or HVJ-E containing pVAX-mIL-12, respectively (n = 10 per treatment group). Therefore, IL-2 gene was further tested as the most attractive candidate for immunogene therapy using HVJ-E.

Then, the efficacy of IL-2 gene therapy using HVJ-E was assessed in an intracranial mouse glioma (RSV-M) model. RSV-M cells were stereotactically inoculated into the brains of mice; 7 days after inoculation, HVJ-E containing pVAX-mIL-2, HVJ-E containing pVAX-Luc, or PBS was injected into the tumor sites. There were no signs of toxicity such as weight loss. Seven days after treatment, the mice were euthanized and tumor volumes were evaluated. Tumors in the mice treated with HVJ-E containing pVAX-mIL-2 were significantly smaller than the tumors in the other treatment groups ($P < 0.05$). The survival period of mice treated with HVJ-E containing pVAX-mIL-2 was significantly longer than that of mice in the other treatment groups ($P < 0.05$).

To understand the potential mechanism of anti-tumor activity of IL-2 gene therapy using HVJ-E, mRNA expression of tumor-infiltrating immune cells such as $CD4^+$, $CD8^+$ and $Foxp3^+$ cells was measured in tumors from the three treatment groups, HVJ-E containing pVAX-mIL-2, HVJ-E containing pVAX-Luc, or PBS, 7 days after treatment. CD4 and CD8 expression was significantly increased in the tumors treated with

HVJ-E containing pVAX-mIL-2 relative to the expression in the other treatment groups ($P < 0.05$). Although it is reported that IL-2 also functions to expand and maintain Tregs in addition to stimulating proliferation and activation of effector T cells (41, 42), the expression of FoxP3, Treg marker, in the tumors treated with HVJ-E containing pVAX-mIL-2 was not significantly increased compared to the tumors with the other treatments. Thus, HVJ-E used for IL-2 gene therapy inhibited Treg expansion and, as a result, enhanced the proliferation of therapeutic effector T cells.

Development of tumor-targeting HVJ-E

In order to treat metastatic tumor foci, targeted drug delivery following systemic vector administration is desired. A new strategy was developed to construct tumor-targeting HVJ-E. In this strategy, first, a chimeric protein gene was constructed between F protein gene of HVJ and a tumor-targeting molecule such as transferrin and then, the chimeric gene was transferred to monkey kidney cells (LLCMK2 cells) in which HVJ effectively replicates. The construct was determined by evaluating the localization of chimeric protein on cell membrane (43). As a result, a signal sequence-depleted human transferrin gene was inserted into the F gene deletion mutant of HVJ, consisting of a signal sequence, transmembrane domain and cytoplasmic region (43, 44). Such chimeric protein on cell surface was incorporated into the progeny of HVJ produced from LLCMK2 cells after wild-type HVJ infection. Next step was the removal of HN protein from the progeny of HVJ produced from HVJ-infected LLCMK2 cells. HN binds to the viral receptor on cell membranes. This protein is also involved in the agglutination of red blood cells (13). To increase the targeting specificity and decrease hemagglutination, HN-depleted HVJ is needed. For this purpose, LLCMK2 cells were transformed with HN-specific siRNA. The HVJ progeny produced from those LLCMK2 cells lost HN, but retained F protein (45). No hemagglutination was detected by the HN-depleted HVJ. Finally, by combining the construction of chimera containing the F protein and a targeting molecule with the use of HN-specific siRNA to produce HN-depleted HVJ, tumor-targeting HVJ-E vector without HN was developed (44). In this case, transferrin was used as a tumor-targeting molecule. Transferrin receptor is very abundant in cancer cells, but the specificity is not so strict and other growing cells possess the receptor. Furthermore, endogenous transferrin in blood may competitively inhibit the targeting ability of transferrin-coated vectors. However, transferrin has been widely used for targeting to cancers (46).

To analyze the infectivity of tranferrin-F containing HN-depleted HVJ (chimera-F/ΔHN HVJ) constructed to target the transferrin receptor, HeLa cells were infected with chimera-F/ΔHN HVJ. Human embryonal kidney (HEK293) cells, which express lower levels of the transferrin receptor, were also infected. Expression of the transferrin receptor in human cervical cancer (HeLa) cells was 14-fold that of HEK293 cells. The infection efficiency was evaluated by fluorescence detection of F protein produced by infected cells. In the absence of excess transferrin, chimera-F/ΔHN HVJ demonstrated approximately three times greater infection efficiency of HeLa cells than wild-F/ΔHN HVJ. In HEK293 cells, both wild-F/ΔHN HVJ and chimera-F/ΔHN HVJ demonstrated lower rates of infection with no significant difference in rates of infection among both types of HVJ (44). In the presence of excess transferrin during infection, the infectivity of chimera-F/ΔHN HVJ was suppressed and not significantly different from that of wild-F/ΔHN HVJ in HeLa cells (44).

Next, the tumor-targeting efficiency of chimera-F/ΔHN HVJ-E was evaluated *in vivo*. For this purpose, chimera-F/ΔHN HVJ or wild-F/ΔHN HVJ were inactivated by UV-irradiation and Q-dots were incorporated into the inactivated viral particles. Q-dot-containing vectors were injected into the tail vein of nude mice bearing HeLa cell tumors on their back. At 48 hours, Red fluorescent Q-dots were detected in every tumor section when the Q-dots were introduced using chimera-F/ΔHN HVJ-E. However, fluorescence was barely detected after administration of wild-F/ΔHN HVJ-E containing Q-dots. Red fluorescent Q-dots were detected in the liver following administration of both HVJ-E vectors, but rarely seen in other organs, such as the lung, spleen and heart. By counting the number of cells containing red fluorescence within the tumor mass, a 32-fold enhancement of tumor-targeting efficiency was observed following administration of chimera-F/ΔHN HVJ-E, compared to wild-F HVJ-E (44).

Tumor-targeting molecules other than transferrin are also developed. For example, folate has been already clinically used for tumor-targeting (47). The anti-CD19 antibody targets B cell lymphoma (48), and immunotoxin therapy has been developed (49). Sigma receptors are known to be enriched in cancers (50). Its ligand is used for cancer-targeting drug delivery by conjugating with liposomes (51). In the future, it will be absolutely necessary to identify more specific cancer-targeting molecules. From the view to suppress recurrence of cancers, cancer stem cells should be targeted. Although several molecules have been reported as markers for cancer stem cells in glioma and mammary carcinoma, the concept is still controversial (52). No specific molecules for targeting have been identified to cancer stem cells. Although there are no data for the affinity of HVJ-E to cancer stem cells, cancer stem cell-targeting HVJ-E vector should be constructed in future.

CONCLUDING REMARKS

Here, I focused on HVJ-E vector. As a review article, similar vectors should be much more introduced. However, as I described in the Introduction, all other vectors with similar oncolytic function are replication-competent viruses. Until now, HVJ-E is only one replication-incompetent oncolytic virus. Furthermore, HVJ-E can induce multiple anti-tumor immunities and also can act as a gene delivery vector. From these points, HVJ-E is a unique vector which will become a promising ther-

apeutic tool against cancers. I hope that the concept of non-replicating oncolytic vector will be more appreciated for cancer therapy and many other vectors with similar functions will be found in future.

Acknowledgements
There is no financial conflict related to this article.

REFERENCES

1. Moon, C., Oh, Y. and Roth, J. A. (2003) Current status of gene therapy for lung cancer and head and neck cancer. *Clin. Cancer Res.* **9**, 5055-5067.
2. Fujiwara, T., Tanaka, N., Kanazawa, S., Ohtani, S., Saijo, Y., Nukiwa, T., Yoshimura, K., Sato, T., Eto, Y., Chada, S., Nakamura, H. and Kato, H. (2006) Multicenter phase I study of repeated intratumoral delivery of adenoviral p53 in patients with advanced non-small-cell lung cancer. *J. Clin. Oncol.* **24**, 1689-1699.
3. O'Neill, D. and Bhardwaj, N. (2005) Exploiting dendritic cells for active immunotherapy of cancer and chronic infection. *Methods Mol. Med.* **109**, 1-18.
4. Kim, R., Emi, M. and Tanabe, K. (2006) Cancer immunosuppression and autoimmune disease: beyond immunosuppressive network for tumor immunity. *Immunology* **119**, 254-264.
5. Dudley, M. E., Yang, J. C., Sherry, R., Hughes, M. S., Royal, R., Kammula, U., Robbins, P. F., Huang, J., Citrin, D. E., Leitman, S. F., Wunderlich, J., Restifo, N. P., Thomasian, A., Downey, S. G., Smith, F. O., Klapper, J., Morton, K., Laurencot, C., White, D. E. and Rosenberg, S. A. (2008) Adoptive cell therapy for patients with metastatic melanoma: evaluation of intensive myeloablative chemoradiation preparative regimens. *J. Clin. Oncol.* **26**, 5233-5239.
6. Kirkwood, J. M., Tarhini, A. A., Panelli, M. C., Moschos, S. J., Zarour, H. M., Butterfield, L. H. and Gogas, H. J. (2008) Next generation of immunotherapy for melanoma. *J. Clin. Oncol.* **20**, 3445-3455.
7. Samuel, M., Chow, P. K., Chan, Shih-Yen, E., Machin, D. and Soo, K. C. (2009) Neoadjuvant and adjuvant therapy for surgical resection of hepatocellular carcinoma. *Database Syst. Rev. Jan.* **21(1)**, CD001199.
8. Kelly, E. and Russell, S. J. (2007) History of oncolytic viruses: genesis to genetic engineering. *Mol. Ther.* **15**, 651-659.
9. Liu, T. C. and Kirn, D. (2008) Gene therapy progress and prospects cancer: oncolytic viruses. *Gene Ther.* **15**, 877-884.
10. Kirn, D. H. and Thorne, S. H. (2009) Targeted and armed oncolytic poxviruses: a novel multi-mechanistic therapeutic class for cancer. *Nat. Rev. Cancer* **9**, 64-71.
11. Kaneda, Y., Nakajima, T., Nishikawa, T., Yamamoto, S., Ikegami, H., Suzuki, N., Nakamura, H., Morishita, R. and Kotani, H. (2002) HVJ (hemagglutinating virus of Japan) envelope vector as a versatile gene delivery system. *Mol. Ther.* **6**, 219-226.
12. Kaneda, Y. (2010) Update on non-viral delivery methods for cancer therapy; possibilities of DDS with anti-cancer activities beyond delivery as a new therapeutic tool. *Expert Opinion Drug Delivery* **9**, 1079-1093
13. Okada, Y. (1993) Sendai virus-induced cell fusion; in *Methods in Enzymology volume 221*, Duzgunes N, (ed.). pp 18-41. Academic Press, Inc., San Diego, USA.
14. Kaneda, Y., Saeki, Y. and Morishita, R. (1999) Gene therapy using HVJ-liposomes; the best of both worlds. *Mol. Med. Today* **5**, 298-303.
15. Kaneda, Y. (2008) Applications on hemagglutinating virus of Japan in therapeutic delivery system. *Expert Opinion Drug Delivery* **5**, 221-233.
16. Kaneda, Y., Nakajima, T. and Yamamoto, S. (2005) Development of HVJ envelope vector and its application to gene therapy. *Adv. Genet.* **53PA**, 307-332.
17. Kurooka, M. and Kaneda, Y. (2007) Inactivated Sendai virus particles eradicate tumors by inducing immune responses through blocking regulatory T cells. *Cancer Res.* **67**, 227-236.
18. Fujihara, A., Kurooka, M., Miki, T. and Kaneda Y. (2008) Intratumoral injection of inactivated Sendai virus particles elicits strong antitumor activity by enhancing local CXCL10 expression and systemic NK cell activation. *Cancer Immunol. Immunother.* **57**, 73-84.
19. Pasare, C. and Medzhitov, R. (2003)Toll pathway-dependent blockade of $CD4^+CD25^+$ T cell-mediated suppression by dendritic cells. *Science* **299**, 1033-1036.
20. Lal, G., Zhang, N., van der Touw, W., Ding, Y., Ju, W., Bottinger, E. P., Reid, S. P., Levy, D. E. and Bromberg, J. S. (2009) Epigenetic regulation of Foxp3 expression in regulatory T cells by DNA methylation. *J. Immunol.* **182**, 259-273.
21. Kato, H., Takeuchi, O., Sato, S., Yoneyama, M., Yamamoto, M., Matsui, K., Uematsu, S., Jung, A., Kawai, T., Ishii, K. J., Yamaguchi, O., Otsu, K., Tsujimura, T., Koh, C. S., Sousa, C. R., Matsuura, Y., Fujita, T. and Akira, S. (2006) Differential roles of MDA5 and RIG-I helicases in the recognition of RNA viruses. *Nature* **441**, 101-105.
22. Seth, R. B., Sun, L., Ea, C. K. and Chen, Z. J. (2005) Identification and characterization of MAVS, a mitochondrial antiviral signaling protein that activates NF-kappaB and IRF 3. *Cell* **122**, 669-682.
23. Tang, E. D. and Wang, C. Y. (2010) TRAF5 is a downstream target of MAVS in antiviral innate immune signaling. *PLoS One* **5(2)**, e9172.
24. Suzuki, H., Kurooka, M., Hiroaki, Y., Fujiyoshi, Y. and Kaneda, Y. (2008) Sendai virus F glycoprotein induces IL-6 production in dendritic cells in a fusion-independent manner. *FEBS Letter* **582**, 1325-1329.
25. Kawaguchi, Y., Miyamoto, Y., Inoue, T. and Kaneda, Y. (2009) Efficient eradication of hormone-resistant human prostate cancers by inactivated Sendai virus particle. *Int. J. Cancer* **124**, 2478-2487.
26. Sadler, J. and Williams, B. R. G. (2008) Interferon-inducible antiviral effects. *Nat. Rev. Immunology.* **8**, 559-568.
27. Platanias, L. C. and Fish, E. N. (1999) Signaling pathways activated by interferons. *Exp. Hematol.* **11**, 1583-1592.
28. Tanaka, M., Shimbo, T., Kikuchi, Y., Matsuda, M. and Kaneda, Y. (2010) Sterile alpha motif containing domain 9 (SAMD9) is involved in death signaling of malignant glioma treated with inactivated Sendai virus particle (HVJ-E)

or type I interferon. *Int. J. Cancer* **126**, 1982-1991.
29. Ito, M., Yamamoto, S., Nimura, K., Hiraoka, K., Tamai, K. and Kaneda, Y. (2005) Rad51 siRNA delivered by HVJ envelope vector enhances the anticancer effect of cisplatin *J. Gene Med.* **7**, 1044-1052.
30. Blangy, A., Lane, H. A., d'Hérin, P., Harper, M., Kress, M. and Nigg, E. A. (1995) Phosphorylation by p34cdc2 regulates spindle association of human Eg5, a kinesin-related motor essential for bipolar spindle formation *in vivo*. *Cell* **83**, 1159-1169.
31. Matsuda, M., Yamamoto, T., Matsumura, A. and Kaneda, Y. (2009) Highly efficient eradication of intracranial glioblastoma using Eg5 siRNA combined with HVJ envelope. *Gene Ther.* **16**, 1465-1476.
32. Lefranc, F., Cool, V., Velu, T., Brotchi, J. and De Witte, O. (2002) Granulocyte macrophage-colony stimulating factor gene transfer to induce a protective anti-tumoral immune response against the 9 L rat gliosarcoma model. *Int. J. Oncol.* **20**, 1077-1085
33. Pan, D., Wei, X., Liu, M., Feng, S., Tian, X., Feng, X. and Zhang, X. (2009) Adenovirus mediated transfer of p53, GM-CSF and B7-1 suppresses growth and enhances immunogenicity of glioma cells. *Neurol. Res.* doi: 10.1179/174313209X455736.
34. Natsume, A., Mizuno, M., Ryuke, Y. and Yoshida, J. (1999) Antitumor effect and cellular immunity activation by murine interferon-beta gene transfer against intracerebral glioma in mouse. *Gene Ther.* **6**, 1626-1633.
35. Meijer, D. H., Maguire, C. A., LeRoy, S. G. and Sena-Esteves, M. (2009) Controlling brain tumor growth by intraventricular administration of an AAV vector encoding IFN-beta. *Cancer Gene Ther.* **16**, 664-671.
36. Glick, R. P., Lichtor, T., de Zoeten, E., Deshmukh, P. and Cohen, E. P (1999) Prolongation of survival of mice with glioma treated with semiallogeneic fibroblasts secreting interleukin-2. *Neurosurgery* **45**, 867-874.
37. Iwadate, Y., Inoue, M., Saegusa, T., Tokusumi, Y., Kinoh, H., Hasegawa, M., Tagawa, M., Yamaura, A., Shimada, H. (2005) Recombinant Sendai virus vector induces complete remission of established brain tumors through efficient interleukin-2 gene transfer in vaccinated rats. *Clin. Cancer Res.* **11**, 3821-3827.
38. Liu, Y., Ehtesham, M., Samoto, K., Wheeler, C. J., Thompson, R. C., Villarreal, L. P., Black, K. L. and Yu, J. S. (2002) In situ adenoviral interleukin 12 gene transfer confers potent and long-lasting cytotoxic immunity in glioma. *Cancer Gene Ther.* **9**, 9-15.
39. Hellums, E. K., Markert, J. M., Parker, J. N., He, B., Perbal, B., Roizman, B., Whitley, R. J., Langford, C. P., Bharara, S. and Gillespie, G. Y. (2005) Increased efficacy of an interleukin-12-secreting herpes simplex virus in a syngeneic intracranial murine glioma model. *Neuro. Oncol.* **7**, 213-224.
40. Matsuda, M., Nimura, K., Shimbo, T., Hamasaki, T., Yamamoto, T., Matsumura, A. and Kaneda, Y. (2010) Immunogene therapy using immunomodulating HVJ-E vector augments anti-tumor effects in murine malignant glioma. *J. Neurooncol.* In press.
41. Malek, T. R. and Bayer, A. L. (2004) Tolerance, not immunity, crucially depends on IL-2. *Nat. Rev. Immunol.* **4**, 665-674.
42. Furtado, G. C., Curotto de Lafaille, M. A., Kutchukhidze, N. and Lafaille, J. J. (2002) Interleukin 2 signaling is required for CD4(+) regulatory T cell function. *J. Exp. Med.* **196**, 851-857.
43. Kawachi, M., Tamai, K., Saga, K., Yamazaki, T., Fujita, H., Shimbo, T., Nimura, K., Nishifuji, K., Amagai, M., Uitto, J. and Kaneda, Y. (2007) Development of tissue-targeting HVJ envelope vector for successful delivery of therapeutic gene to mouse skin. *Hum. Gene Ther.* **18**, 881-894.
44. Shimbo, T., Kawachi, M., Saga, K., Fujita, H., Yamazaki, T., Tamai, K. and Kaneda, Y. (2007) Development of a transferrin receptor-targeting HVJ-E vector. *Biochem. Biophys. Res. Comm.* **364**, 423-428.
45. Saga, K., Tamai, K., Kawachi, M., Shimbo, T., Fujita, H., Yamazaki, T. and Kaneda, Y. (2008) Functional modification of Sendai virus by siRNA. *J. Biotechnol.* **133**, 386-394.
46. Kircheis, R. and Wagner, E. (2005) Transferrin receptor-targeted gene delivery systems; in *Polymeric Gene Delivery* Amiji, M. M. (ed.), pp 537-556. CRC Press LLC, Florida, USA.
47. Sudimack, J. and Lee, R. J. (2000) Targeted drug delivery via the folate receptor. *Adv. Drug Deliv. Rev.* **41**, 147-162.
48. Morris, J. C. and Waldmann, T. A. (2009) Antibody-based therapy of leukaemia. *Expert Rev. Mol. Med.* **11**, e29.
49. Vallera, D. A, Chen, H., Sicheneder, A. R., Panoskaltsis-Mortari, A. and Taras, E. P. (2009) Genetic alteration of a bispecific ligand-directed toxin targeting human CD19 and CD22 receptors resulting in improved efficacy against systemic B cell malignancy. *Leuk. Res.* **33**, 1233-1242.
50. van Waarde, A., Rybczynska, A. A, Ramakrishnan, N., Ishiwata, K., Elsinga, P. H. and Dierckx, R. A. (2010) Sigma receptors in oncology: therapeutic and diagnostic applications of sigma ligands. *Curr. Pharm. Des.* In press.
51. Chono, S., Li, S. D., Conwell, C. C. and Huang, L. (2008) An efficient and low immunostimulatory nanoparticle formulation for systemic siRNA delivery to the tumor. *J. Control Release* **131**, 64-69.
52. Clément, V., Dutoit, V., Marino, D., Dietrich, P. Y. and Radovanovic, I. (2009) Limits of CD133 as a marker of glioma self-renewing cells. *Int. J. Cancer* **125**, 244-248.