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Gene Therapy of Melanoma Using Inactivated Sendai Virus Envelope Vector (HVJ-E) with Intrinsic Anti-Tumor Activities

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1. Introduction

Despite the development of various cancer treatments, including surgical treatment, radiation, and anti-cancer reagents, cancer cells are not completely eliminated from the body in many cases, which allows the tumors to recur. Disease recurrence is the most difficult problem in cancer treatment. Much attention has been paid to cancer immunotherapy as a strategy to eliminate cancer cells from patients (1). However, numerous failures of cancer immunotherapy have indicated the difficulty of achieving anti-cancer immunity (1, 2). Cancer tissues produce factors that attenuate anti-tumor immunity and eventually induce immunotolerance against cancers in tumor-bearing individuals (3, 4). Therefore, to successfully eradicate cancer, first the tumor mass must be reduced as much as possible through surgery, radiation and chemotherapy; then, immunotherapy should be provided to increase the immune-activation signals and decrease the immune-suppression signals (5-7). Thus, multi-lateral strategies are needed in cancer treatment. Gene therapy has been anticipated to be a new tool for cancer treatment (8). Much attention has been also paid to immuno-gene therapy (9). However, it is still very difficult to achieve long-term remissions in cancer patients (10). Based on the concept of multi-lateral strategies, the control of multiple pathways of cancer growth is also necessary in gene therapy. We have developed a hemagglutinating virus of Japan envelope (HVJ-E) vector by using inactivated Sendai virus as a pseudovirion for gene and drug delivery (11). This vector can deliver siRNA, DNA, proteins, and anti-cancer drugs to cells *in vitro* and *in vivo* (12).

We previously reported that HVJ-E itself has a strong anti-tumor effect against mouse tumors, such as colon carcinoma and renal carcinoma, by the activation of cytotoxic T lymphocytes and natural killer cells and the suppression of regulatory T cells (13, 14). Recently, we also determined the direct tumor-killing activity of HVJ-E through the induction of type I interferon on hormone-resistant human prostate cancer cells and human glioblastoma cells (15, 16). Thus, HVJ-E is a versatile gene and drug delivery vector with

intrinsic anti-cancer activities. Therefore, it is expected that synergistic anti-tumor effects can be achieved by HVJ-E vector with incorporated therapeutic molecules.

Melanoma is one of the most aggressive tumors due to its strong ability to metastasize. In the United States, there were an estimated 62,480 new melanoma cases and 8,420 deaths caused by melanomas in 2008 (17). Although the 5-year survival rate of patients with localized melanoma at the early stage is greater than 90%, the survival rate drops to less than 20% once the melanoma has metastasized to distant sites (17).

Many chemotherapeutic agents have been used alone or in combination to treat melanoma (18). However, these therapies are insufficient for the eradication of melanoma cells, and recurrences are often observed. A recent report indicates that B-RAF inhibitor is a promising treatment for malignant melanoma patients (19). However, after the initial response, melanoma recurrence frequently occurred in the inhibitor-treated patients due to the up-regulation of other signaling molecules, such as RTK or N-RAS (20). Thus, when some signaling pathways are inhibited, cancer cells become resistant to the drug by the up-regulation of other pathways. Another report showed that the resistance to B-RAF inhibition in melanoma could be overcome by targeting both MEK and IGF-1receptor-mediated PI3 kinase (21). However, even when these pathways are inhibited, the elimination of all cancer cells is unlikely. Molecular-targeting drugs are very attractive because of their selectivity. However, this strategy may fall into a vicious cycle because cancers are heterogeneous and become adaptive to the drugs. Thus, a novel strategy is needed for the treatment of melanoma.

In the present study, we tested the possibility of HVJ-E with or without a therapeutic gene for the effective treatment of melanoma in a mouse model. We incorporated the IL12 gene into the HVJ-E vector (IL12/HVJ-E) and compared the tumor regression induced by IL12/HVJ-E and HVJ-E (without IL12 cDNA). We also performed a series of six HVJ-E injections into both mouse and human melanomas in mouse models to compare the suppression of tumor growth and survival time with three injections of HVJ-E.

2. Materials and methods

Cell lines and animals: Mouse melanoma F10 cells were purchased from the American Tissue Culture Collection, and human melanoma Mewo cells were purchased from the Japanese Collection of Research Bioresources. Mouse melanoma B16-BL6 cells expressing the luciferase gene were prepared by GenomIdea Inc. (Ikeda, Osaka, Japan). Cancer cells were grown in DMEM supplemented with 10% fetal bovine serum and 5% penicillin-streptomycin and maintained at 37°C in a humidified atmosphere with 5% CO₂. Murine bone marrow-derived DCs were generated as previously described. More than 90% of these DCs were positive for CD11c. Five- to six-week-old male C.B-17/IcrCrj-SCID mice and C57BL/6 mice were purchased from Charles River Inc. (Yokohama, Japan) and maintained in a temperature-controlled, pathogen-free room. All animals were handled according to the approved protocols and guidelines of the Animal Committee of Osaka University.

HVJ-E preparation and siRNA transfection: HVJ (VR-105 parainfluenza 1 Sendai/52, Z strain) propagated in HEK293 cells was collected and inactivated by UV irradiation (99 mJ/cm²) and β -propiolactone. The inactivated HVJ suspension (HVJ-E) was purified by GenomIdea Inc. by using four different columns. The titer of HVJ-E was examined by neuraminidase activity (mNAU). One mNAU corresponded to approximately 5×10^7

particles. Two hundred micrograms of plasmid DNA was mixed with 2000 mNAU HVJ-E in the presence of Tween 80 (final concentration, 0.05%).

Cytokine measurements: Mouse dendritic cells or F10 melanoma cells were seeded at 5×10^4 cells/well in 96-well plates. The following day, HVJ-E (MOI: 10^2 - 10^4) was added, and the cells were cultured for another 24 hr. After this, cytokines and chemokines were measured within the supernatant by ELISA performed with commercially available reagents (PBL Biomedical Laboratories, Piscataway, NJ, USA).

TUNEL assay: Twenty-four hours after three injections of HVJ-E or PBS, tissue sections were prepared by using a microtome. The sections were washed twice with PBS and fixed with 4% paraformaldehyde for 15 min at 4°C. The terminal deoxynucleotide transferase-mediated dUTP nick-end labeling (TUNEL) assay was performed according to the protocol of an *in situ* Apoptosis Detection Kit (Takara Bio Inc.).

Quantitative real-time RT-PCR: Isogen (Nippon Gene, Japan) was used to extract total RNA from tumors that had been resected and washed in PBS. A total of 2 µg of total RNA was reverse transcribed into cDNA and analyzed as *in vitro*. Probes and primer pairs specific for murine DX5, CD8, CD11c, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were purchased from Applied Biosystems. The concentration of target genes was determined by using the comparative CT method (threshold cycle number at the cross-point between amplification plot and threshold), and values were normalized to an internal GAPDH control.

ELISPOT assay: Mice were sacrificed 7 days after the fifth IP injection. Splenocytes were harvested from the spleen and cultured as previously described (15). A total of 1×10^4 lymphocytes were cultured with 0.2×10^4 mitomycin C-treated F10 cells at 37°C for 24 hr. The assay was performed by using a mouse IFN-gamma ELISPOT kit (R&D Systems). The number of spots was counted under a dissecting microscope (Leica, Cambridge, UK).

Tumor growth *in vivo*: Viable melanoma cells were re-suspended in 100 µl of PBS and intradermally injected into the backs of the mice. When each tumor had grown to approximately 4-6 mm in diameter, the mice were treated with intra-tumor injections of HVJ-E (in a total volume of 100 µl) or 100 µl of PBS. Tumor size was measured in a blinded manner with slide calipers, and tumor volume was calculated with the following formula: tumor volume (mm^3) = length \times (width) 2 / 2. In a spontaneous lung metastasis model, B16-BL6 cells (10^6 cells) were subcutaneously inoculated into a C57BL/6 mouse. The mice were injected with HVJ-E (300, 1000 and 2000 mNAU) or PBS three times on days 4, 8 and 12. On day 26, the mice were sacrificed. Lungs were isolated and fixed with 10% formalin solution, and the size and number of metastatic foci were examined.

Statistical analysis: *In vitro* results were analyzed with the Student's non-paired *t*-test. Comparisons of *in vivo* results were made by using Kaplan-Meier's method or Dunnett's test. Differences with *P* values < 0.05 were considered statistically significant.

3. Results

HVJ envelope (HVJ-E) was constructed by inactivation of HVJ (hemagglutinating virus of Japan; Sendai virus) with β -propiolactone (0.0075% - 0.001%) or UV irradiation (99 mJoule/cm 2), as described previously, and then purified by ion-exchange column chromatography and gel filtration. The diameter of 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 and centrifugation (10,000 g, 5-10 min). Various detergents, such as Triton X-100, NP-40, deoxycholate and Tween 80, were available for incorporating exogenous DNA into HVJ-E. The exogenous DNA incorporation rate was approximately 15% - 20% and did not vary significantly among the different detergents. However, when detergent-treated HVJ-E was added to dendritic cells or melanoma cells, the cytokine production from those cells was varied. IL-6, which plays a major role in the anti-tumor immunity of HVJ-E, was produced from dendritic cells treatment with Tween 80-treated HVJ-E as well as non-treated HVJ-E while it was dramatically suppressed by HVJ-E treated with other detergents (Fig. 1A). Tween 80-treated HVJ-E maintained the production of interferon- α and RANTES from dendritic cells, but Triton X-100-treated HVJ-E lost this ability (Fig. 1B and C). The optimum concentration of Tween 80 was 0.05%, which also enabled the incorporation of exogenous DNA into HVJ-E (data not shown). Mouse melanoma B16-F10 cells produced a small amount of interferon- β in response to HVJ-E. As shown in Fig. 1D, Tween 80-treated HVJ-E stimulated interferon- β secretion from melanoma cells in a dose-dependent manner. However, the interferon- β production induced by Triton X-100-treated HVJ-E was greatly suppressed. Therefore, we used 0.05% Tween 80-treated HVJ-E for cancer treatment in a mouse model.

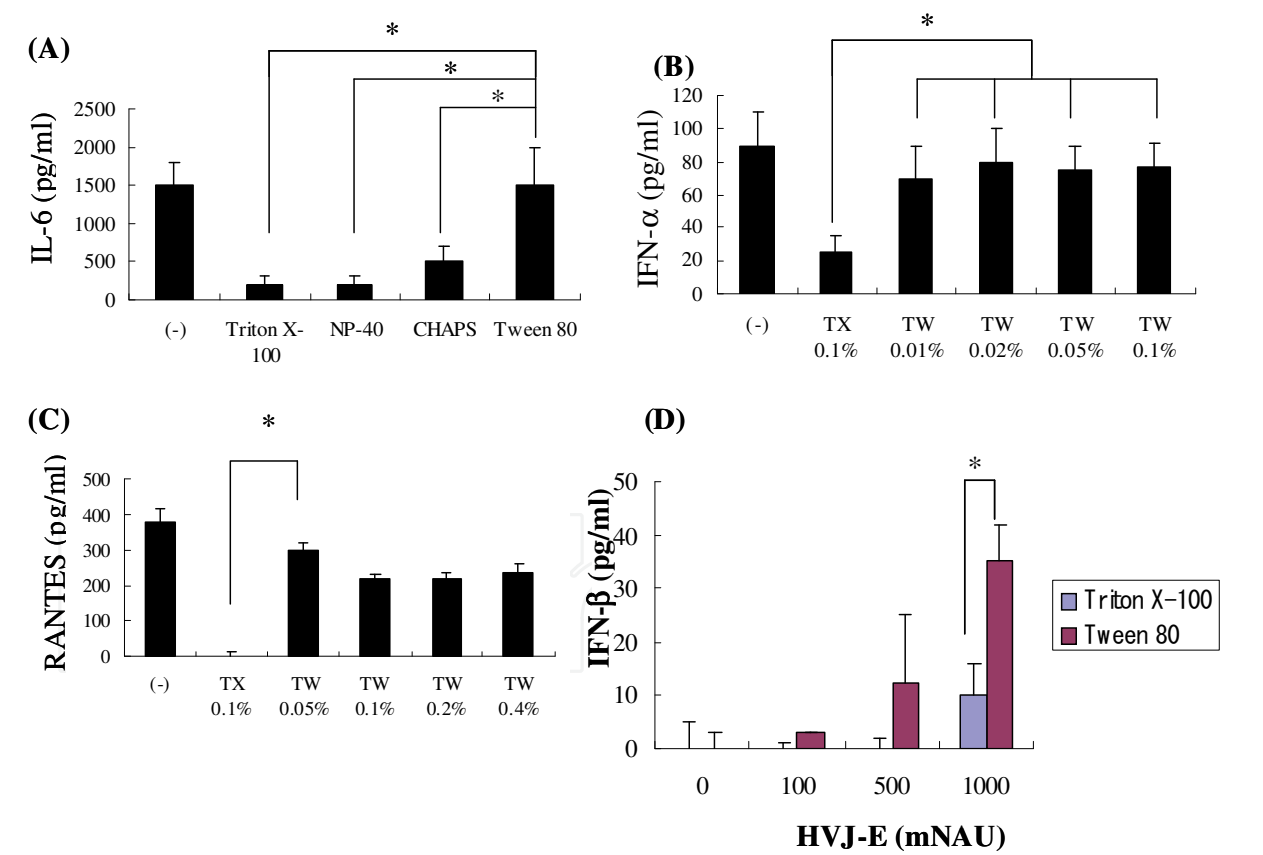


Fig. 1. The effect of detergent on the production of cytokine and chemokine from dendritic cells and tumor cells treated with HVJ-E.

IL-6 (A), IFN- α (B) and RANTES (C) in mouse dendritic cells were assessed by using ELISA kits 24 hours after HVJ-E treatment. In (A), HVJ-E was treated with detergent (0.2% Triton X-100, 0.1% NP-40, 1.25 CHAPS or 0.05% Tween 80). The concentration of each detergent

was required for incorporation of plasmid DNA into HVJ-E. In (B) and (C), 0.1% Triton X-100 (TX) and 0.01 – 0.4 % Tween 80 (TW) were used.

To test the anti-tumor effects of Tween 80-treated HVJ-E, mouse melanoma B16-F10 cells were intradermally inoculated into C57BL/6 mice. When the diameter of the tumor was approximately 5 mm, various amounts of HVJ-E were injected into tumors in a series of three injections with 4 days between injections (Fig. 2). Tumor regression was induced by HVJ-E. After three injections of HVJ-E (1000 mNAU), a TUNEL assay was performed on tumor sections to detect apoptosis. As shown in Fig. 3, TUNEL-positive cells were abundantly observed in the tumor tissues treated with HVJ-E but not in normal tissues.

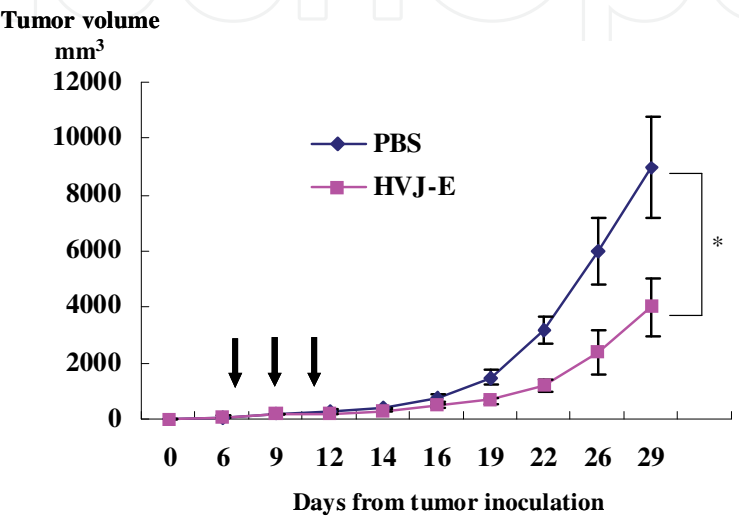


Fig. 2. Mouse melanoma treatment by three-times injection of HVJ-E

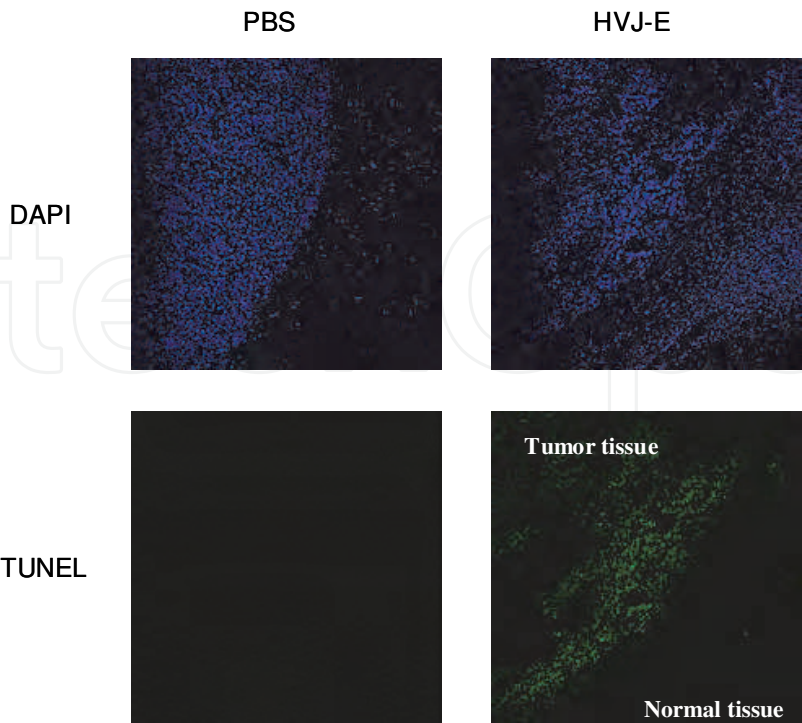


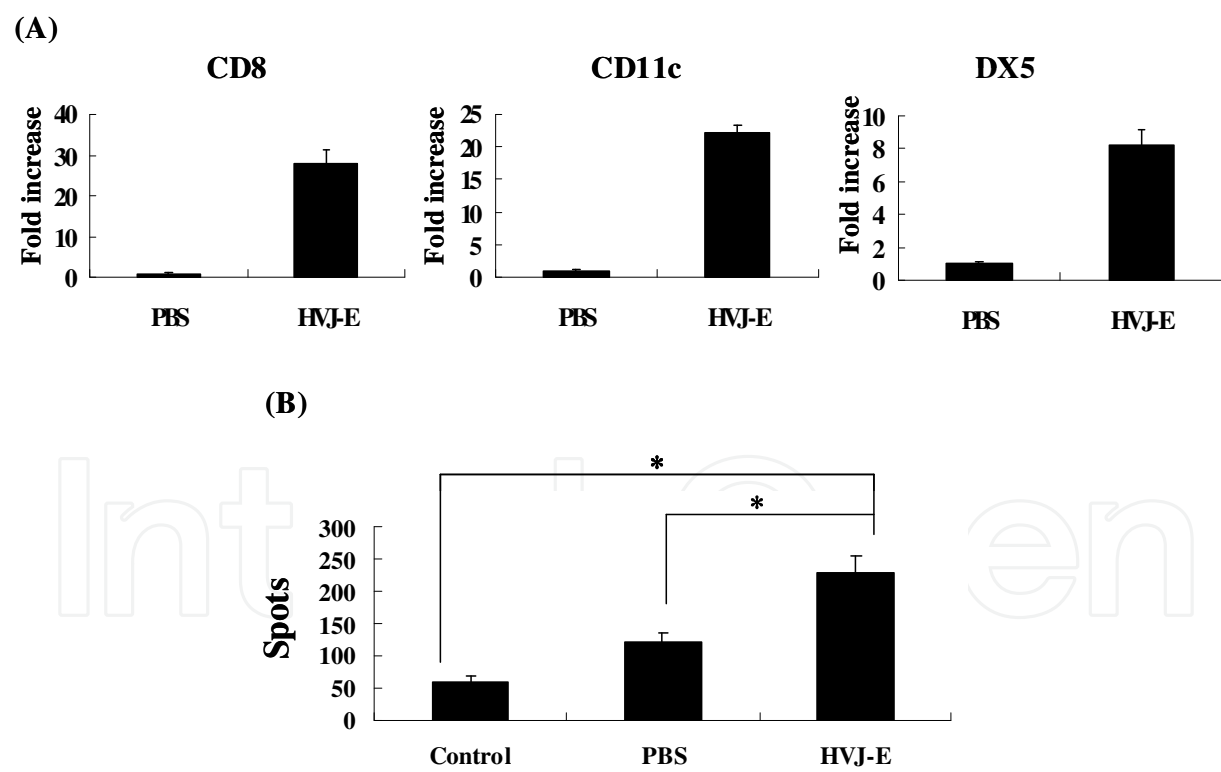
Fig. 3. Apoptotic cells in the tumor tissue by the injection of HVJ-E

C57BL/6 mice (each group; n=5) were challenged with 5×10⁵ F10 cells by intradermal injection on the dorsal surface. After tumors reached 4~6 mm in diameter, 1000 mNAU HVJ-E or PBS was injected into the tumors once every other day on days 6, 8 and 10. Tumor diameter was measured to calculate tumor volume. Bars, SD. *P <0.05.

Twenty-four hours after the third injection of HVJ-E (1000 mNAU) or PBS, tissue sections were prepared for a TUNEL assay. Successive sections were stained with DAPI to confirm the position of nuclei. The boundary between tumor and normal tissues was confirmed by H-E staining of successive sections and shown by a white dotted line.

As we have previously reported the generation of multiple anti-tumor immunities by HVJ-E, the infiltration of immune cells into the tumor bed was analyzed by RT-PCR at 48 hours after the last of three injections of HVJ-E (1000 mNAU). CD11c, CD8 and DX5 were highly expressed in melanoma tissues (Fig. 4A), indicating the infiltration of dendritic cells, CD8⁺ T cells and NK cells. Two weeks later, the cytotoxic T-cell response was evaluated by ELISPOT assay (Fig. 4B). A significant increase in interferon-γ spots was detected in spleen cells from mice injected with HVJ-E.

The subcutaneous mouse melanoma (B16-BL6) cells spontaneously metastasized to the lung. When the subcutaneous tumor mass was treated with HVJ-E, the ratio of mice with more than one melanoma focus in the lungs significantly decreased (Table 1). In particular, the number of large-sized metastatic foci (>0.7 mm in diameter) were dramatically reduced.



(A) The transcripts for CD8, CD11c, and DX5 isolated from melanoma masses were analyzed by real-time PCR. Values were normalized to an internal GAPDH control. The ratio of each transcript from HVJ-E-treated samples to that from PBS-injected samples is shown as the fold increase. Bars, SD. (B) ELISPOT assay of IFN-γ from lymphocytes collected from the spleen 7 days after the last injection of HVJ-E or PBS. Control samples were prepared from lymphocytes collected from untreated tumor-bearing mice. Bars, SD. *P <0.05.

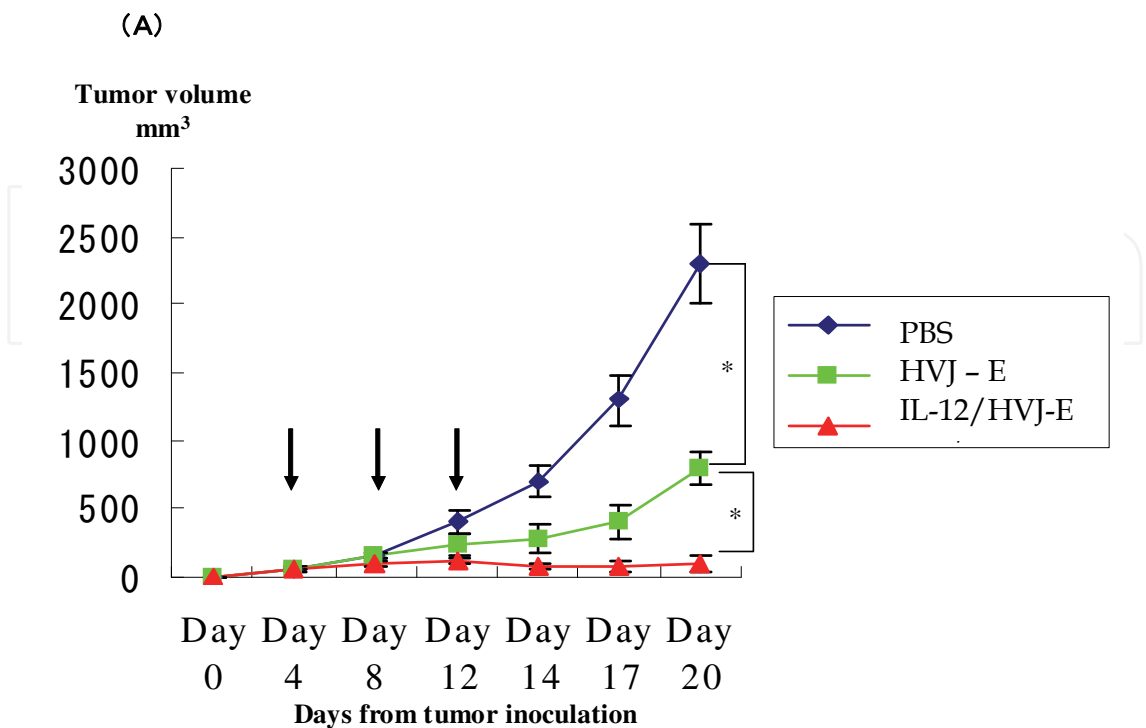
Fig. 4. Infiltration of immune cells into tumor tissue by intratumoral injection of HVJ-E

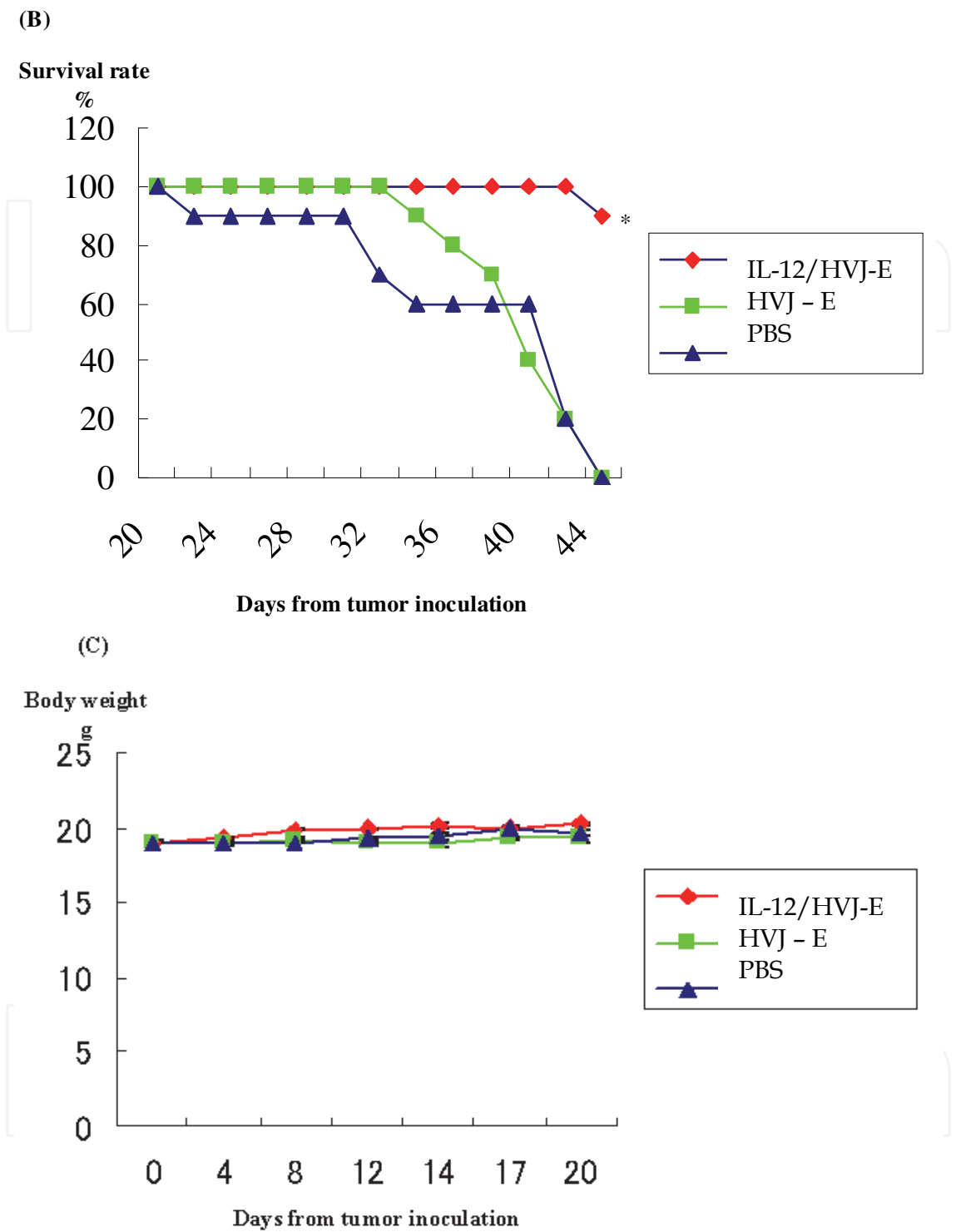
| Group | n | Lung (%) | | |
|-----------------|----|----------|----------------------|----------------------|
| | | Total | >0.4 mm in length | >0.7 mm in length |
| PBS | 10 | 100 | 80 | 70 |
| HVJ-E 300 mNAU | 11 | 82 | 18* | 9- |
| HVJ-E 1000 mNAU | 8 | 38* | 13* | 0- |
| HVJ-E 2000 mNAU | 9 | 44* | 22* | 0- |

*Statistical significance vs. PBS at $p<0.05$ by Steel's test
- Impossible for statistical analysis

Table 1. Ratio of mouse with more than one metastatic focus in lung

To increase the anti-tumor effects of HVJ-E, mouse IL-12 cDNA (IL-12/HVJ-E) was incorporated into HVJ-E, and the resulting vector was injected into the intradermal tumor mass of B16-BL6 cells. As shown in Fig. 5A, a series of three injections of IL-12/HVJ-E was more effective for melanoma regression than mock HVJ-E that contained vector plasmid without IL-12 cDNA. The survival and body weight change of tumor-bearing mice were analyzed. Fig 5B shows that 90% of mice injected with three times were alive on day 46, when all the mice injected with either mock HVJ or PBS had died. There was no decrease in the body weight of mice treated with IL-12/HVJ-E. No significant difference was observed in the growth rate of the PBS and IL-12/HVJ-E groups (Fig. 5C).

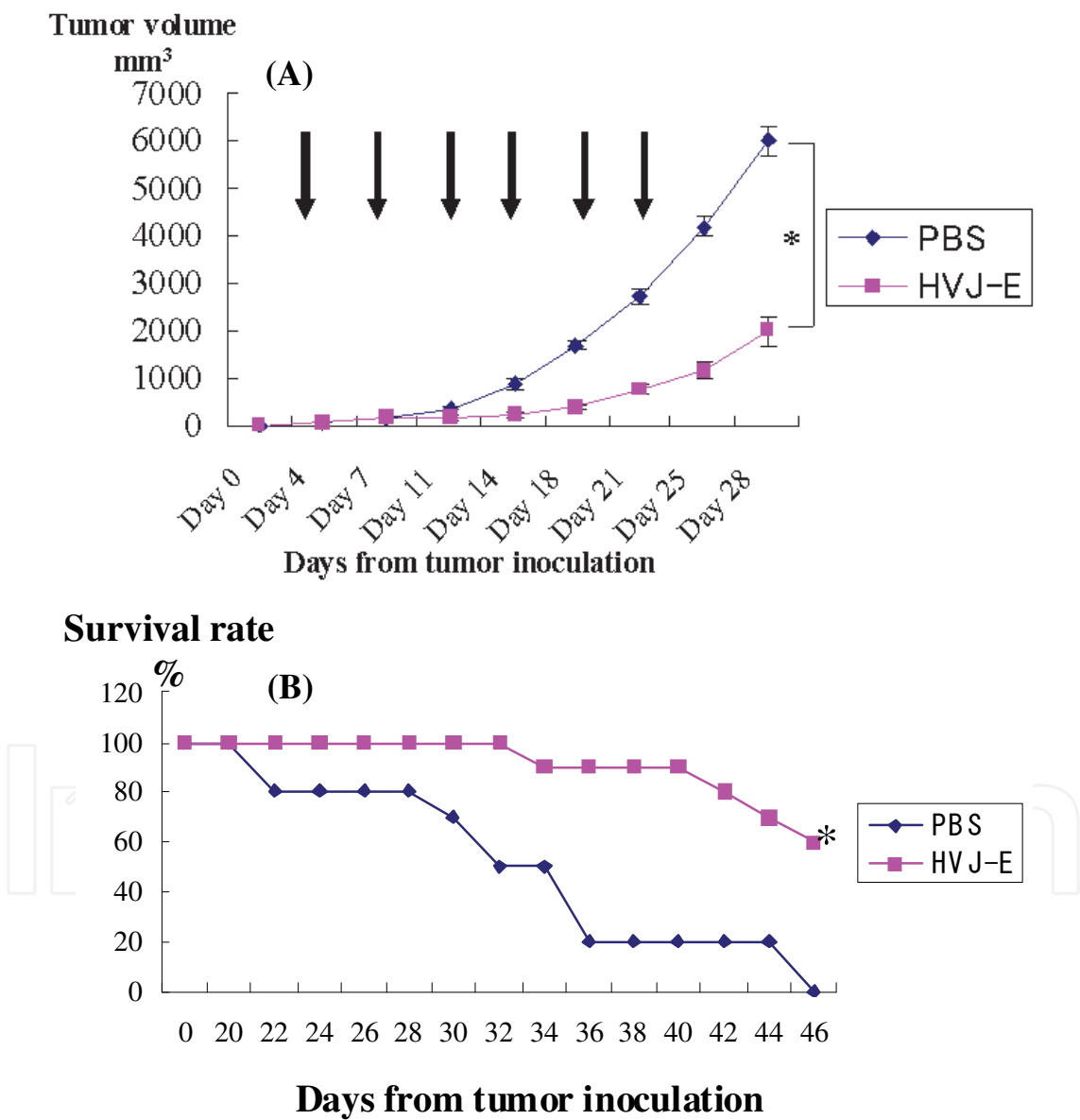




(A) C57BL/6 mice (each group; n=5) were challenged with 10^6 B16-BL6 cells by intradermal injection on the dorsal surface. After tumors reached 4~6 mm in diameter, HVJ-E (1000 mNAU), HVJ-E containing IL-12 cDNA or PBS were injected into the tumors on days 4, 8 and 12. Tumor diameter was measured to calculate tumor volume. Bars, SD. *P <0.05. (B) The survival of mice injected with HVJ-E (1000 mNAU) containing IL-12 cDNA (IL12/HVJ-E) or HVJ-E containing vector plasmid without IL12 (mock HVJ-E) or PBS (each group; n=10) is shown. *P <0.05. (C) Body weight of mice treated with IL12/HVJ-E, mock HVJ-E or PBS is shown (each group; n=10).

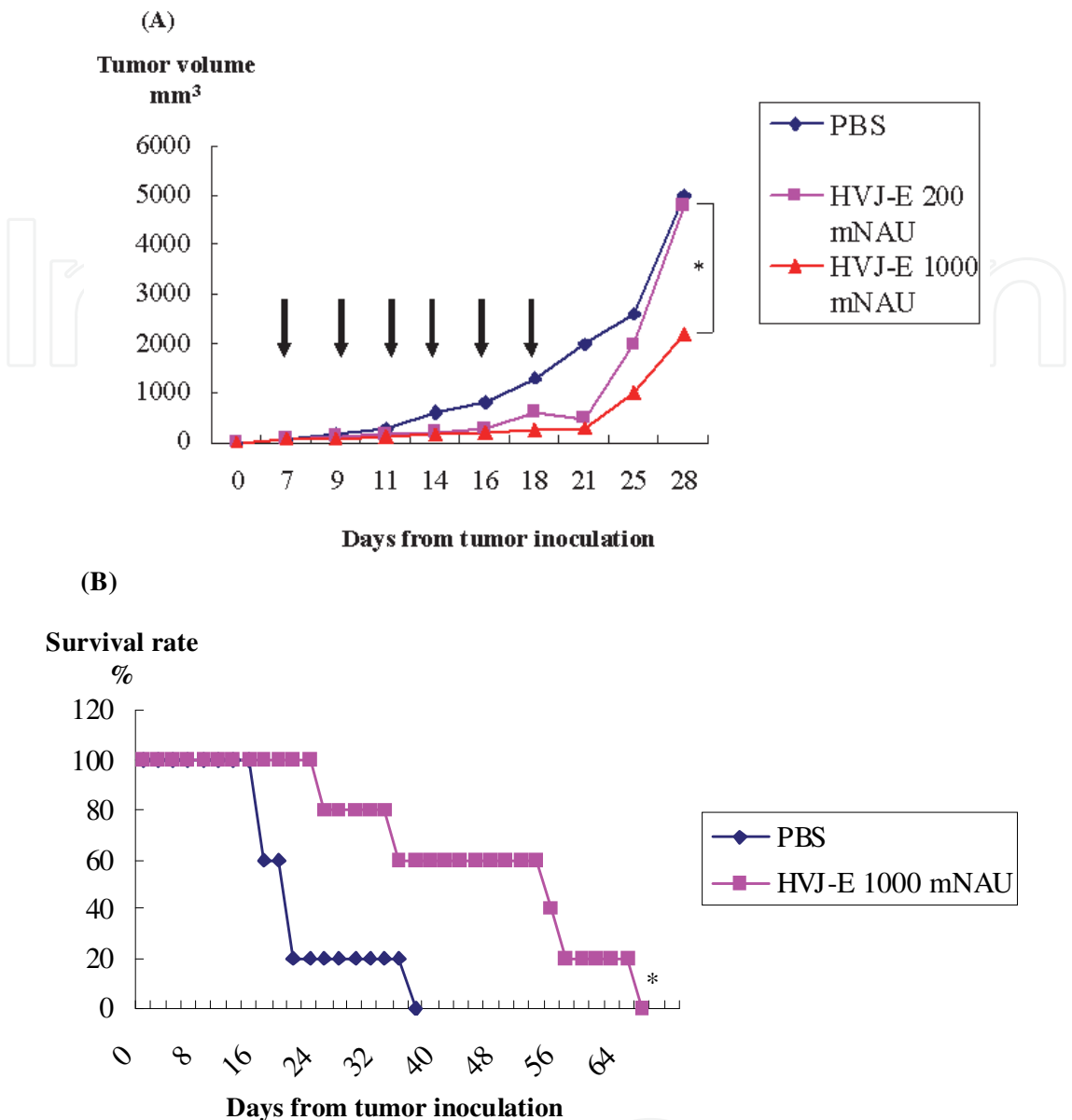
Fig. 5. Gene therapy of mouse melanoma by IL-12/HVJ-E

Three injections of HVJ-E without a therapeutic gene resulted in a significant regression of melanoma growth (Fig. 2), but the survival of tumor-bearing mice was not significantly prolonged as compared with the mice injected with PBS. Then, we tested whether a series of six injections of HVJ-E without therapeutic genes could prolong mouse survival. We found that the series of six injections inhibited melanoma (B16-BL6) growth and significantly prolonged the survival (Fig. 6 A and B). A similarly prolonged survival was also observed in B16-F10 melanoma model mice that received six HVJ-E injections (data not shown). Based on these results, we attempted to treat human melanoma (Mewo) in immunodeficient SCID mice. Six injections of HVJ-E suppressed the growth of human melanoma in a dose-dependent manner and significantly prolonged mouse survival (Fig. 7A and B).



(A) A series of six injections of HVJ-E (1000 mNAU) or PBS was administered to mice with intradermal tumor B16-BL6 melanoma on days 4, 8, 12, 16, 20 and 24 (each group; n=5). Bars, SD. *P <0.05. (B) The survival of mice injected with HVJ-E (1000 mNAU) or PBS (each group; n=10) is shown. *P <0.05.

Fig. 6. Mouse melanoma treatment by six times injection of HVJ-E



(A) Human melanoma Mewo cells (10^6 cells) were inoculated intradermally into SCID mice. Six injections of HVJ-E (200 mNAU, 1000 mNAU) or PBS were administered to the mice on days 7, 9, 11, 13, 15 and 17 (each group; $n=5$). Bars, SD. * $P < 0.05$. (B) The survival of mice injected with HVJ-E (1000 mNAU) or PBS (each group; $n=10$) is shown. * $P < 0.05$.

Fig. 7. Human melanoma treatment by six-times injection of HVJ-E

4. Discussion

HVJ-E was originally developed as a vector for the delivery of drugs and genes (11, 12). Then, it was found that vector itself has anti-tumor activities (13-16). For cancer treatment, a single modality of therapeutic strategy is not sufficient. By using HVJ-E, multi-lateral cancer treatment can be achieved by incorporating therapeutic molecules into HVJ-E with intrinsic anti-tumor activities. Here, we demonstrate that the anti-tumor activities of HVJ-E are enhanced by the incorporation of IL-12 cDNA. Our previous reports showed that HVJ-E induces the production of interferon- α , - β , IL-6 and CXCL10 in mouse dendritic cells

(13, 14). However, interferon- γ was not produced from dendritic cells treated with HVJ-E. Rather, interferon- γ was secreted from NK cells exposed to interferon- β produced from HVJ-E-treated dendritic cells (14). Since interferon- γ plays a key role in anti-tumor immunity (22), we selected IL-12, which induces interferon- γ in immune cells, for incorporation into HVJ-E. Local injection of the IL-12 gene into melanoma by using the canarypox virus or naked plasmid DNA has already been clinically tested and shows promising results (23, 24). In the present study, the IL-12 gene was coupled with HVJ-E, which has intrinsic anti-tumor activities; therefore, synergistic anti-tumor effects were expected. Indeed, in mouse tumor models of melanoma, IL-12/HVJ-E enhanced tumor regression and prolonged mouse survival more effectively as compared to mock HVJ-E without IL-12 cDNA. However, even when the therapeutic gene was not used, more frequent injections of HVJ-E increased the anti-tumor activities and prolonged the mouse survival. This result might be due to the accumulation of the anti-tumor effects of HVJ-E. A similar regression of human melanoma tumors in immunodeficient SCID mice was achieved by six injections of HVJ-E without therapeutic genes. In SCID mice, which lack T-cell immunity, NK cells activated by HVJ-E play a role in tumor suppression, as previously reported (14). Additionally, HVJ-E induces cancer cell killing in some human tumors, such as prostate cancer (15), glioblastoma (16), neuroblastoma and mammary carcinoma (YK; unpublished data). We found that HVJ-E itself induced apoptosis in Mewo cells in a dose-dependent manner, although the efficiency was not as high as the efficiency in glioblastoma and prostate cancers. This activity of HVJ-E also achieved significant regression of human melanoma in SCID mice and prolonged mouse survival. In SCID mice inoculated with Mewo cells, we compared the efficacy of HVJ-E to the efficacy of a clinically used anti-melanoma reagent, dacarbazine (DTIC). Three intratumoral injections of HVJ-E were sufficient for overcoming the tumor suppression effect by six intraperitoneal injections of DTIC.

The anti-tumor immunity induced by HVJ-E was shown to be caused by the production of cytokines and chemokines (14), which are produced in response to the recognition of viral RNA by RIG-I (25-27). HVJ-E contains viral RNA fragments (200 – 300 bases) that also have the potential to be recognized by RIG-I (28). Therefore, it is likely that the production of chemokines and cytokines induced by HVJ-E depends on the amount of viral RNA fragments introduced into the cytoplasm by membrane fusion. As shown in Figure 1, the production is dependent on the treatment of HVJ-E with detergent. We recently found that depletion of cholesterol from HVJ by methyl- β -cyclodextrin attenuated the infectivity of the virus due to the irreversible dysfunction of fusion protein (29). It is speculated that some detergents, such as Triton X-100 and NP-40, may damage fusion activity, while other detergents such as Tween 80 may maintain that activity. The difference might be due to the amount of envelope lipids depleted by the detergent treatment. Although the reason remains to be elucidated, Tween 80-treated HVJ-E is recommended for the treatment of cancer and infectious disease, and Triton X-100-treated HVJ-E is recommended for the treatment of other diseases, such as cardiovascular disease and inflammatory disease, which are exacerbated by cytokines and chemokines.

It has been determined that the selective cancer-killing activity of HVJ-E is mediated through the RIG-I/MAVS signaling pathway, which is activated by viral RNA fragments of HVJ-E (YK; manuscript in preparation). Therefore, Tween 80-treated HVJ-E can be equipped with both anti-tumor immunities and cancer-killing activities.

Recently, we also demonstrated the augmentation of the anti-tumor activities of HVJ-E by incorporating siRNA against motor protein Eg5 in glioblastoma treatment (30). The ability

of Eg5 siRNA to induce cell-cycle arrest compensated for HVJ-E in conducting apoptosis. Since the mechanism of apoptosis induced by Eg5 siRNA is different from that induced by HVJ-E, the combination of HVJ-E with Eg5 siRNA creates a synergistic anti-tumor effect. Thus, if a molecule to promote cancer progression after HVJ-E treatment is identified by comprehensive microarray analysis of transcripts in cancer cells, the combination of HVJ-E and siRNA against that molecule will be recommended as an effective cancer treatment.

5. Acknowledgment

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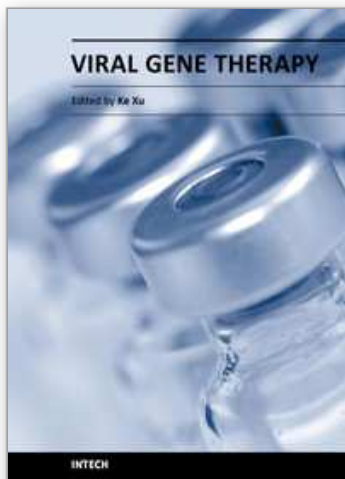
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The development of technologies that allow targeting of specific cells has progressed substantially in recent years for several types of vectors, particularly viral vectors, which have been used in 70% of gene therapy clinical trials. Particular viruses have been selected as gene delivery vehicles because of their capacities to carry foreign genes and their ability to efficiently deliver these genes associated with efficient gene expression. This book is designed to present the most recent advances in viral gene therapy

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