high gene expression, viral vectors such as retrovirus and adenovirus vectors have been utilized. However, in general, cancer gene therapy has not had satisfactory therapeutic effects. Therefore, to enhance the cancer-cell-killing effect, viruses that replicate mainly in cancer cells have been used for treatment [23]. Various types of oncolytic viruses have been developed by isolating viruses with inherent tumor selectivity [24, 25] and by engineering recombinant viruses [26, 27]. Furthermore, the combination of an oncolytic virus and gene therapy has been applied for cancer treatment, such as vaccinia virus including the GM-CSF gene [28]. Although these oncolytic viral treatments exhibited a strong therapeutic effect, safety might be a problem because the virus with an intact genome still exists in noncancerous cells [29].

An inactive virus that did not have the ability to amplify its progeny virus in host cells has also been used as a high-safety delivery vector for drugs and plasmids in cancer therapy. In particular, enveloped-virus-derived vectors have attracted attention because enveloped-vector-delivered molecules can escape endosomal degradation by direct introduction to the cytoplasm via membrane fusion [30]. A vector derived from an inactive enveloped virus is called a virosome, which is now an all-inclusive term for a reconstituted envelope that contains viral envelope proteins (Figure 1(a)) or viral envelope particles (Figure 1(b)) [31]. Several types of virosomes have been generated, for example, virosomes based on influenza virus [32], hepatitis B virus [33], human immunodeficiency virus [34], Newcastle disease virus [35], and Sendai virus [36, 37]. In many studies, virosomes have been used as vectors for drug delivery, with the inclusion of various therapeutic molecules, such as DNA, RNA, proteins, and drugs [38, 39]. Moreover, virosomes function as adjuvants to induce the activation of the immune system [40]; therefore, many groups are studying virosomes as tools for cancer therapy.

In this review, we introduce the previous research on virosomes, especially virosomes derived from the influenza (influenza virosome) and Sendai viruses (Sendai virosome) for the use in cancer therapy. The influenza virosome has been applied mainly as a delivery vector for TAAs and TAA-expressing plasmids. Sendai virosomes have been used as anti-cancer immune activators and apoptosis inducers.

2. Influenza Virosomes

Influenza virus is an Orthomyxovirus that has a nucleocapsid with a segmented single-stranded RNA genome and is covered with a viral envelope [41, 42]. Two types of membrane proteins, hemagglutinin (HA) and neuraminidase (NA), are present on the surface of the envelope. HA binds to sialic acid, which is its receptor, on the surface of host cells and is used for the adhesion of viral particles [43]. HA is responsible for membrane fusion of the viral envelope with the host cell membrane [44]. However, HA does not induce membrane fusion in neutral conditions, and it acquires its fusion activity through conformational change in acidic conditions [45, 46]. Viral particles are taken into the endosomes of host cells by endocytosis after HN-receptor binding, thereby exposing the particles to acidic conditions. Next, membrane fusion of the viral envelope with the endosomal membrane is induced by the conformational change of HA, and the viral genome is induced into the cytoplasm of host cells.

An influenza virosome is an artificial liposome that includes influenza membrane proteins [31] and is prepared by reconstituting influenza virus surface proteins and phospholipids [47]. The influenza viral envelope is first collapsed to phospholipids by the treatment with detergent, and the nucleocapsid is eliminated from the mixture. Then, the influenza virosome, including surface proteins and virus-derived phospholipids, is reconstituted from the mixture. An influenza virosome maintains its membrane fusion ability because it has HA on its surface [48]. Therefore, it works as a delivery vector to introduce macromolecules into the cytoplasm by including them in the virosome [38, 49]. Influenza virosomes have powerful immunogenicity. Vaccination with influenza virosomes induces protective levels of influenza-specific antibodies [50], and an influenza virosome is already licensed as an influenza vaccine [51]. Influenza virosomes also exhibit an adjuvant effect when they are coadministered with other antigens [52–54]; therefore, many groups have studied the application of influenza virosomes in the activation of antitumor immunity.

2.1. CTL Activation by Plasmid DNA Encapsulation in Influenza Virosomes. Correale et al. reported that TAA-specific CTLs were induced by the administration of an influenza virosome containing TAA plasmids in mice [55]. In this study, a plasmid expressing parathyroid hormone-related peptide (PTH-rP), which is a TAA expressed in prostate and spinocellular lung carcinomas, was included in an influenza virosome, which was administered intranasally. As a result, PTH-rP-specific CTL activity was significantly induced in mice, and this activity was also shown in human PBMCs activated by human DCs treated with the PTH-rP virosome. In addition, Cusi et al. demonstrated that TAA-specific CTLs were enhanced by the stimulation with an influenza virosome containing a CD40L-expressing plasmid [56]. CD40L binds to CD40 on APCs and upregulates the expression of its costimulatory molecules, B7.1 and B7.2, in the cells, which are important factors for the activation and amplification of naïve T cells [57, 58]. In this study, plasmids expressing carcinoembryonic antigen (CEA), which is a marker of colon cancer, and CD40L were encapsulated in influenza virosomes, and these virosomes were administered intranasally. Coadministration of CEA- and CD40L-virosomes resulted in a CEA-specific CTL response that was stronger than that in the CEA-virosome alone, by upregulating B7.1 and B7.2 expression on APCs.

2.2. CTL Activation by Peptide Encapsulation in Influenza Virosomes. Antigen presentation of TAAs by APCs is important for the activation of anti-cancer immunity. To activate CTLs, TAAs should be presented with MHC-I, which complexes with cytoplasmic antigens. Therefore, TAAs should be introduced to the cytoplasm for the effective activation

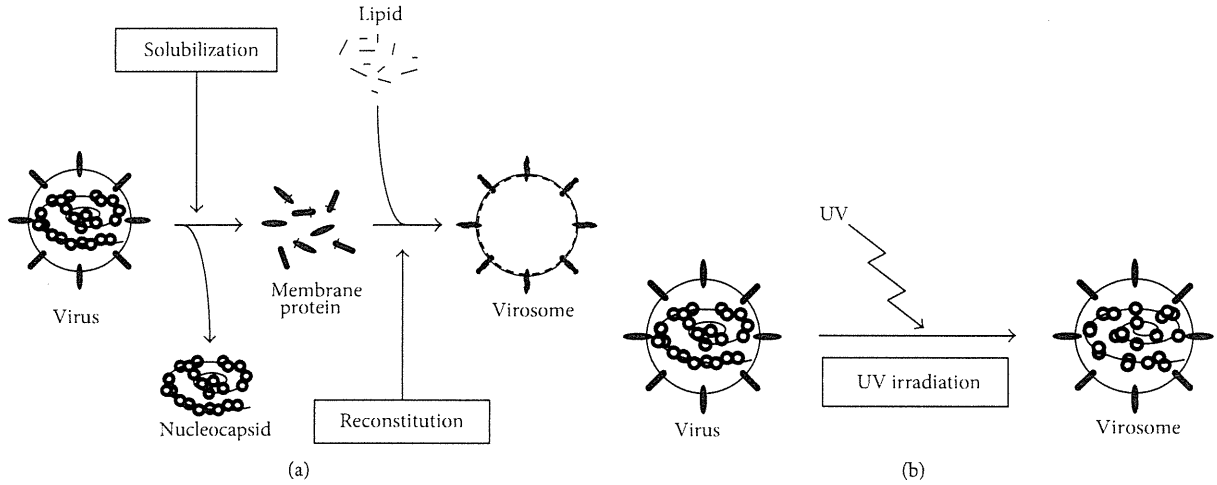

FIGURE 1: Concept of virosomes. (a) Reconstituted envelope containing viral envelope proteins. Viral membrane proteins are solubilized from viral particles, and artificial envelope is reconstituted with the viral proteins and exogenous lipids. (b) Viral envelope particles. Virus is inactivated with UV irradiation leading the fragmentation of viral genome.

of CTLs. Bungener et al. demonstrated influenza virosome-mediated OVA delivery to DCs [59] and that the delivery leads to OVA presentation on MHC-I and -II. Fusion-inactive virosomes presented OVA on MHC-II but not on -I. Therefore, it is suggested that influenza virosomes introduce encapsulated TAAs to the cytoplasm through membrane fusion and that TAA introduction is needed for the presentation of TAAs on MHC-I. Angel et al. reported influenza virosome-mediated delivery of TAAs to DCs [60]. The authors encapsulated the Melan-A peptide, which is a TAA from melanoma, in an influenza virosome and introduced the Melan-A peptide into plasmacytoid DCs (PDCs). Melan-A-containing, virosome-treated PDCs activated CD8 T cells more effectively than did free Melan-A peptide-pulsed PDCs. In addition, Correale et al. reported that PTH-rP-derived peptide (PTR)-4-encapsulated influenza virosomes significantly suppressed tumor growth [61]. In this study, PTR-4/virosome treatment effectively activated CTL activity, and the treatment inhibited the angiogenesis of tumors. The findings therefore suggest a new function of influenza virosomes in cancer therapy.

2.3. Modification of the Influenza Virus. To make influenza virosome-mediated cancer therapy more effective, modifications of the influenza virosome have been attempted. HA has an important function in influenza virosome-mediated delivery and immunostimulation. However, the HA receptor is ubiquitously expressed on nearly all cells. Therefore, the influenza virosome does not have affinity for specific cells. Mastrobattista et al. generated an influenza virosome that could target ovarian carcinoma (OVCAR-3) *in vitro* [62]. They coated influenza virosomes with polyethylene glycol (PEG) to inhibit HA-mediated binding, and then Fab' fragments of antiepithelial glycoprotein-2 (EGP-2) antibody (323/A3) were conjugated to the PEG on the virosomes. 323/A3-PEG-coated influenza virosomes exhibited low HA-mediated binding to sialic acid because of the PEG coating and gained specific binding for EGP-2-expressing ovarian cancer cells by 323/A3 conjugation. As a result, although the binding function of HA was depleted, the 323/A3-PEG virosomes were able to fuse with OVCAR-3 membranes. Because HA induced membrane fusion without binding to its receptor [63], it is thought that the 323/A3-PEG virosomes maintained their membrane fusion ability. Waelti et al. used the same strategy to demonstrate targeted delivery of doxorubicin (Doxo) to HER-2/neu-overexpressing breast cancer cells *in vivo* [64]. In this study, influenza virosomes were coated with anti-Neu mAb Fab' (7.16.4)-conjugated PEG (7.16.4/PEG), and Doxo was encapsulated in the 7.16.4/PEG-virosomes. Intravenous administration of Doxo-containing 7.16.4/PEG-virosomes significantly inhibited subcutaneous Neu+, but not Neu-, breast cancer. Jamali et al. recently reported the enhancement of the efficacy of influenza virosome-mediated delivery *in vitro* by reconstituting the virosome with cationic lipids [65].

As described above, influenza virosomes are useful for the cancer therapy. Recently, phase I clinical trial of influenza virosomes was carried out for the patients with metastatic breast cancer (MBC) [66]. In this trial, MBC patients were intramuscularly administrated influenza virosomes including three individual peptides of the extracellular domain of Her-2/neu protein. The trial tested the safety and Her-2/neu-specific immune responses. As a result, specific antibodies against naïve Her-2/neu protein were detected in serum. IL-2 production was significantly increased and Treg population was significantly decreased in PBMC. Although local erythema at the infection site has appeared in four patients, other serious side effects were not detected. Therefore, there is a possibility that influenza virosomes are used for future cancer therapy.

3. Sendai Virosomes

Sendai virus (hemagglutinating virus of Japan; HVJ) is a paramyxovirus that has a nucleocapsid with a single-stranded RNA genome and is covered with a viral envelope [67]. Two types of glycoproteins, hemagglutinin-neuraminidase (HN) and fusion protein (F), are present on the surface of the viral envelope [68]. HN enables the viral particle to adhere to the host-cell surface by binding to sialic acid [69], and then F induces membrane fusion of the viral envelope with the host-cell membrane [70]. F fuses these membranes under neutral conditions [71]; therefore, HVJ particles do not require uptake into the endosome for membrane fusion.

Previously, HVJ-liposomes were generated via reconstitution from HVJ surface proteins and phospholipids, similar to the influenza virosome [36]. Because HVJ-liposomes have membrane fusion ability, they have been used as a vector for DNA delivery [72]. However, because the membrane fusion efficiency of HVJ-liposomes is not high (approximately 2% of native HVJ) [73], an increase in the fusion activity of the vector is needed.

Kaneda et al. generated a new type of Sendai virosomes called HVJ-envelope (HVJ-E) [37]. HVJ-E is an inactivated HVJ particle that has been irradiated by UV light. The viral RNA genome is cleaved into many fragments; therefore, HVJ-E does not have the ability to produce progeny virus in infected cells. However, HVJ-E maintains its membrane fusion ability, which is dramatically higher than that of HVJ-liposomes [37]. HVJ-E has been used as a vector for plasmid DNA delivery to various cells and tissues [74–76]. In addition, plasmid DNA, anti-cancer drugs, and siRNAs have been delivered by HVJ-E, and there have been reports of cancer therapy using HVJ-E-mediated drug delivery [77, 78].

Cancer suppression by viral infection has also been reported [79]. Since that study, various viruses have been used for cancer therapy, and, in particular, the development of oncolytic viruses has attracted attention [80–83]. Oncolytic viruses function by inducing the lysis of cancer cells by infection [84]. Because the oncolytic activity is decreased by UV irradiation, it has been suggested that the viral amplification in cancer cells is responsible for oncolysis [85]. However, it is also possible that the virus's components contribute to the suppression of cancer. Recently, it was indicated that HVJ-E itself has an inhibitory effect against cancer growth [86, 87], and it was revealed that the viral components, in the absence of viral amplification, contribute to the anti-cancer effects. Since then, the HVJ-E-mediated anti-cancer effect has been studied.

3.1. HVJ-E for the Activation of Anticancer Immunity. Kurooka and Kaneda demonstrated that the intratumoral administration of HVJ-E dramatically eradicated intradermal cancer (Figure 2) [86]. They found that HVJ-E stimulated DCs to release various types of cytokines, such as interferon (IFN)-α, and -β, tumor-necrosis factor (TNF)-α and interleukin (IL)-6, and that IL-6 inhibited the proliferation of regulatory T cells (Tregs). Tregs negatively control effector T cells [88, 89] and interfere with the activation of anti-cancer immunity [90]. Therefore, HVJ-E-mediated eradication

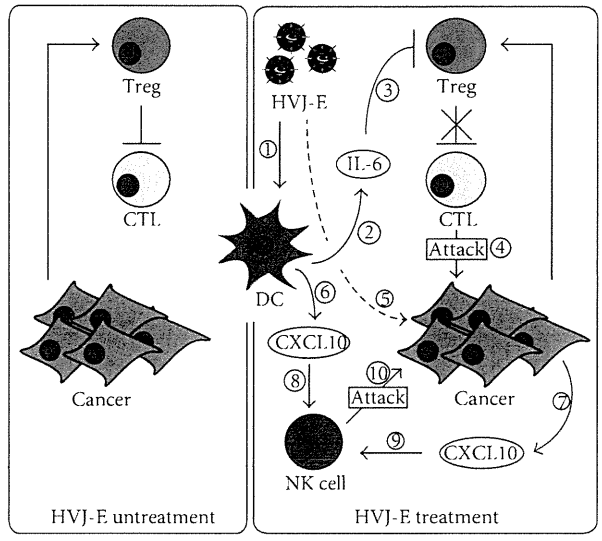

FIGURE 2: HVJ-E-mediated stimulation of anticancer immunity. Various modes of stimulations of the immune system that are induced by HVJ-E treatment. Black lines indicate the original reactions in cancer. Red lines indicate the reactions induced by HVJ-E treatment. *CTL Activation.* 1: HVJ-E stimulates dendritic cells (DCs). 2: DC secretes IL-6. 3: IL-6 suppresses regulatory T cell (Treg) function, which inhibits cytotoxic T cell (CTL) activity. 4: CTLs attack cancer cells. *NK Cell Activation.* 1, 5: HVJ-E stimulates DCs and cancer cells. 6, 7: DCs and cancer cells secrete CXCL10. 8, 9: CXCL10 attracts natural killer (NK) cells to cancer cells. 10: NK cells effectively attack the cancer cells.

of cancer results from the activation of anti-cancer immunity by IL-6-mediated suppression of Tregs. It is known that RNA viruses stimulate DCs via the recognition of the viral RNA genome by Toll-like receptor (TLR)-7 and -8 and Rig-I [91–93]. However, Suzuki et al. showed that the sugar chain of the F protein is important for HVJ-E-mediated, DC activation of IL-6 secretion [94]. Therefore, they suggested that DCs possess an unknown receptor for F that is involved in maturation.

In addition, HVJ-E suppressed tumor growth in the intradermal renal carcinoma SCID mouse model, in spite of their deficient T and B cells [95], which suggests that HVJ-E undergoes another stimulation mechanism that activates anti-cancer immunity. Fujihara et al. indicated that the anti-cancer activity of NK cells was led by the intratumoral administration of HVJ-E into the intradermal renal carcinoma SCID mouse model (Figure 1) [95]. In addition, HVJ-E directly stimulated cancer cells and induced their secretion of CXCL10. CXCL10 is a chemokine for monocytes/macrophages, T cells, NK cells, and DCs; therefore, it is suggested that active NK cells were attracted to the tumor by CXCL10.

Taken together, these reports demonstrate that HVJ-E is a powerful activator of anti-cancer immunity.

3.2. Direct Cancer Killing Activity of HVJ-E. Recently, it was reported that HVJ-E has a direct killing effect against cancer

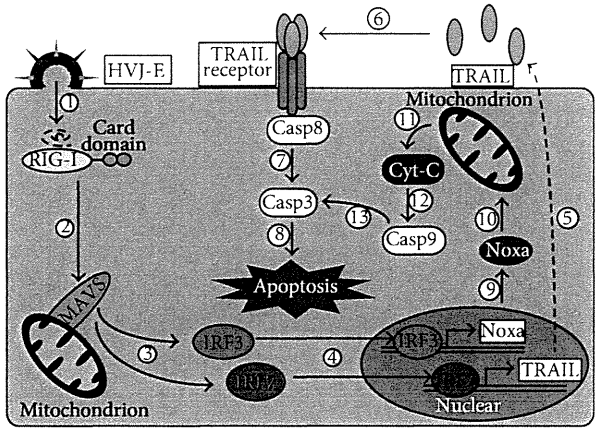

FIGURE 3: HVJ-E-mediated apoptosis pathway in cancer cells. HVJ-E-mediated signal transduction in cancer cells. 1: RNA fragments derived from the HVJ genome are introduced into the cytoplasm by membrane fusion, and RIG-I recognizes these RNAs. 2: RIG-I conveys the signal to the mitochondrial antiviral signaling adaptor (MAVS). 3: MAVS activates IRF-7 and -3. 4: activated IRF-7 and -3 localize to the nucleus. 5, 9: IRF-7 and -3 induce the expression of TRAIL and Noxa. 6: expressed TRAILs are recognized by the TRAIL receptor, and TRAIL receptors activate caspase-8 (Casp-8). 7: activated Casp-8 activates Casp-3. 8: activated Casp-3 induces apoptosis. 10, 11: Noxa induces the secretion of cytochrome-C (Cyt-C) into the cytoplasm from the mitochondria. 12: Cyt-C activates Casp-9. 13: Casp-9 activates Casp-3.

cells (Figure 3). Kawaguchi et al. showed that the viability of two castration-resistant human prostate cancer cell lines (PC3 and DU145) was remarkably decreased by the treatment with HVJ-E *in vitro* [96]. HVJ-E-treated PC3 cells exhibited some apoptotic phenotypes, namely, increases in the number of TUNEL-stained cells and in the expression levels of caspase-3 and caspase-8. However, HVJ-E-mediated inhibition of cell viability was not observed in normal prostate epithelium (PNT2), suggesting that HVJ-E-mediated apoptosis is specifically induced in cancer cells. HVJ-E contains many fragments of its RNA genome, and these RNA fragments are introduced to the cytoplasm by the fusion of HVJ-E and the cell membrane. Matsushima-Miyagi et al. revealed that the viability of prostate cancer cells (PC3 and DU145), but not normal prostate epithelium (PNT1 and PNT2), was significantly decreased by viral RNA introduction (Figure 2) [87]. The RNA fragments were recognized by RIG-I in the cytoplasm, and the signal was transduced to MAVS [97]. HVJ-E-mediated cell growth inhibition of PC3 was suppressed by the knockdown of RIG-I and MAVS, indicating that the RIG-I/MAVS signaling pathway is important for this process. Moreover, HVJ-E treatment induced the expression of TRAIL and Noxa (known as apoptosis inducers [98, 99]) in PC3 and DU145 cells, but not in PNT2 cells, via RIG-I/MAVS signaling. The fact that the knockdown of TRAIL and Noxa suppressed the HVJ-E sensitivity of PC3 and DU145, respectively, indicates that these apoptosis inducers are responsible for HVJ-E-induced cancer cell apoptosis. Furthermore, the knockdown of IRF7 and 3—transcription factors of TRAIL and Noxa, respectively [100, 101]—also suppressed the HVJ-E sensitivity of prostate cancer cells, suggesting that RIG-I/MAVS signaling regulates the expression of TRAIL and Noxa via IRF7 and 3 in cancer cells. Matsushima-Miyagi et al. [87] elucidates the mechanism of HVJ-E-induced cancer cell apoptosis. However, it is still unknown why the expression of these apoptosis inducers is induced in cancer cells by HVJ-E stimulation.

3.3. Combination Therapy with HVJ-E and Modification of HVJ-E. In attempts to enhance the strength and decrease the side effects of HVJ-E-mediated antitumor treatment, various combination therapies that include HVJ-E and modifications of HVJ-E have been used. Eg5 is an important factor in the early stages of mitosis [102] and its inhibition leads to mitotic arrest and results in apoptosis [103]. Matsuda et al. demonstrated that HVJ-E-mediated apoptosis in human glioblastoma cell lines (A-172, T98G and U-118MG) was effectively enhanced by the encapsulation of siRNAs against Eg5 in HVJ-E *in vitro* and *in vivo* [104]. The authors also observed that HVJ-E-mediated anti-cancer immunity was enhanced by the encapsulation of the IL-2 plasmid and that the astrocytoma cell line (RSV-M) was effectively eradicated when using this method *in vivo* [105].

HVJ-E adheres to the cell surface via HN binding to sialic acid (e.g., GD1a and SPG) [106]. Therefore, cancer cells with mild expression of these sialic acids exhibit low sensitivity to HVJ-E-mediated apoptosis because of their weak affinity for HVJ-E. To induce HVJ-E-mediated apoptosis in less sensitive cancer cells, Nomura et al. used the combination therapy of HVJ-E and 13-cis retinoic acid (13cRA) against human neuroblastoma cells (NB1), which are less sensitive to HVJ-E [107]. NB1 cells barely express GD1a and SPG and exhibit low sensitivity to HVJ-E-mediated apoptosis. 13cRA treatment induced the expression of GD1a in NB1 cells, and the HVJ-E sensitivity of NB1 cells was increased *in vitro*. Moreover, NB1 tumor volume in mice was significantly decreased and their survival rate was increased by the combination of HVJ-E and 13cRA *in vivo*.

Improvements to HVJ-E were made to enhance its performance. Sialic acids, such as GD1a and SPG, to which HN bind, are ubiquitously expressed in nearly all cells, and they are highly expressed in red blood cells. Therefore, HVJ-E does not have an affinity for a specific cell type, and it induces hemagglutination by intravenous administration. For the systemic administration of HVJ-E to treat cancer effectively, it must have high affinity for cancer cells and low affinity for sialic acids. Transferrin (Tf) is a protein in blood plasma that is responsible for ferric ion delivery, and the Tf receptor is highly expressed in various cancer cells. Shimbo et al. generated a cancer-targeting HVJ-E using Tf [108]. The HN on HVJ-E was depleted by siRNA [109], and Tf was presented on the surface of HVJ-E via the expression of a Tf/F recombinant fusion protein on HVJ-E. Tf-presented HVJ-E (Tf-HVJ-E) exhibited affinity for the human uterocervical cancer cell (Hela) line, which expressed the Tf receptor, and Tf-HVJ-E accumulated at tumor masses in mice after their systemic administration.

In addition, HVJ-E-mediated antitumor immunity was enhanced by HVJ-E modification. HVJ-E activates anti-cancer immunity; however, HVJ-E does not directly induce IFN-γ secretion. IFN-γ is an important factor for various anti-cancer activities [110], and IL-12 is a robust inducer of IFN-γ from immune cells [111, 112]. Saga et al. revealed that HVJ-E dramatically enhanced IL-12 activity for IFN-γ secretion from splenocytes; however, HVJ-E alone did not induce IFN-γ secretion [113]. The authors generated IL-12-conjugated- and HN-depleted HVJ-E (IL-12-HVJ-E) to enhance HVJ-E-mediated anti-cancer immunity. IL-12-HVJ-E induced secretion of IFN-γ from splenocytes *in vitro*. In addition, upon intratumoral injection, scIL12-HVJ-E activated antitumor immunity against mouse malignant melanomas (F10 melanoma) and suppressed tumor growth more effectively than the wild-type (wt) HVJ-E. Furthermore, upon intravenous injection, IL-12-HVJ-E, but not wt-HVJ-E, was especially localized to the lungs, where it induced IFN-γ expression and reduced the lung metastatic foci of F10 melanomas.

As described above, HVJ-E has the ability to induce anti-cancer effects in several types of cancers. Now, clinical trials of HVJ-E are ongoing to test its safety and anti-cancer immunity against melanoma and prostate cancer. Moreover, there is a possibility that the combination therapy of HVJ-E and other immune therapies, such as CTLA-4 antibody, exhibits a more effective activation of antitumor immunity, and it will be performed in the near future.

4. Conclusion

We have documented the utility of virosomes for cancer treatment. However, we believe that no omnipotent therapeutic technologies are currently available to completely eradicate various types of cancers. Cancers are heterogeneous and can transform themselves to be resistant to the treatment that they have received and to escape from the environment of cancer treatment [114]. In this scientific research field, it is absolutely necessary to identify the genes that direct tumorigenesis. However, in the clinical field, it is very important to prepare cancer treatments using a variety of therapeutic principles. Clinicians should provide cancer patients with the appropriate therapeutic tools according to the patient's condition. Thus, from a practical standpoint, virosome-mediated cancer therapy may have an important role in cancer treatment.

Conflict of Interests

The authors declare that there is no conflict of interests.

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The RIG-I/MAVS signaling pathway in cancer cell-selective apoptosis

Yasufumi Kaneda

Division of Gene Therapy Science; Department of Urology; Department of Pediatric Surgery; Graduate School of Medicine; Osaka University; Yamada-oka, Japan

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A replication-incompetent hemagglutinating virus of Japan (HVJ) envelope (HVJ-E) induces apoptosis selectively in cancer cells. Fragments of the viral RNA genome transported by HVJ-E are recognized by retinoic acid-inducible gene I (RIG-I) and mitochondrial antiviral signaling (MAVS). Specific pro-apoptotic factor are selectively upregulated in cancer cells downstream of the RIG-I/MAVS pathway.

Since the observation that some cancer patients underwent remission in response to viral infection, cancer virotherapy has attracted increasing attention.[1] Marked progress has been made in this field following the development of oncolytic viruses, i.e., viruses (including attenuated strains) that selectively replicate in cancer cells.[1,2] Of note, the oncolytic activity of these viruses is generally lost following irradiation.[3] Thus, replication appears to be required for oncolytic viruses, be they virulent or attenuated strains, to selectively kill cancer cells, although various cellular targets that may mediate this function have been identified.[2] Therefore, inactivated viral particles that fail to replicate and generate viral proteins so far have not been considered for the development of oncolytic viruses.

However, we have recently discovered that a replication-incompetent hemagglutinating virus of Japan (HVJ, also known as Sendai virus) envelope (HVJ-E) induces the apoptotic demise of human cancer cell lines, including prostate cancer PC3 and DU145 cells, mammary carcinoma MDA-MB-231 cells and lung cancer A549 cells, but not of non-transformed cells such as prostate epithelial PNT1 and PNT2 cells as well as primary human fibroblasts.[4] We have also found that fragments of the viral RNA genome selectively promote apoptosis in cancer cells via the upregulation of tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) and NOXA downstream of the retinoic acid-inducible gene I (RIG-I)/mitochondrial antiviral signaling (MAVS) pathway (**Fig. 1**).

The most striking finding of our recent research is that RIG-I/MAVS signaling can selectively apoptosis in cancer cells, suggesting this may constitute an ideal target for cancer therapy. The RIG-I/MAVS signaling pathway has been well investigated as a protection system that elicits innate immunity upon viral infection.[5] Indeed, when dendritic cells are treated with HVJ-E, Type I interferon (IFN) and CXCL10 are upregulated upon the activation of the RIG-I/MAVS signaling pathway by viral RNA fragments.[6,7] These factors promote in the activation of natural killer (NK) cells and, subsequently, cytotoxic T lymphocytes (CTLs) (**Fig. 1**). Based on these findings, we first reported that HVJ-E activates antitumor immunity.[6] Thus, in immune cells, the RIG-I/MAVS signaling pathway does not cause cell death, yet does so in cancer cells. Besch et al. have reported that synthetic RNA induces Type I IFN-independent apoptosis in human melanoma cells via the activation of RIG-I and melanoma-differentiation-associated gene 5 (MDA5).[8] According to their analysis, pro-apoptotic molecules including PUMA and NOXA are activated by polyinosinic:polycytidylic acid (polyI:C) or 5'-triphosphate-containing RNA transcribed in vitro in both melanoma cells and non-malignant skin cells, while the expression of the anti-apoptotic molecule BCL-X_L is induced in non-malignant cells only. Therefore, they conclude that melanoma-cell specific apoptosis as triggered by the activation of RIG-I and MDA5 occurs via a cytoplasmic pathway regulated by the balance between pro- and anti-apoptotic members of the BCL-2 protein family. In our experiments, the expression of anti-apoptotic proteins, including Bcl-X_L and pro-apoptotic molecules such as PUMA and BAX was unchanged upon the administration of HVJ-E to both PC3 and PNT2 cells. Instead, the expression of TRAIL and NOXA was selectively activated in prostate cancer cells, lung cancer cells and breast cancer cells downstream of RIG-I and MAVS. The factors that are responsible for cancer cell-specific apoptosis downstream of the RIG-I/MAVS pathway may therefore vary among cancers.

However, it is still unclear why pro-apoptotic genes are upregulated in cancer cells but not in normal cells downstream of the RIG-I/MAVS signaling pathway. Cancer cells differ from their normal counterparts relative to gene expression pattern, resulting from alterations in chromatin status as imposed by DNA methylation and histone modifications.[9] Our

Correspondence to: Yasufumi Kaneda; Email: kaneday@gts.med.osaka-u.ac.jp
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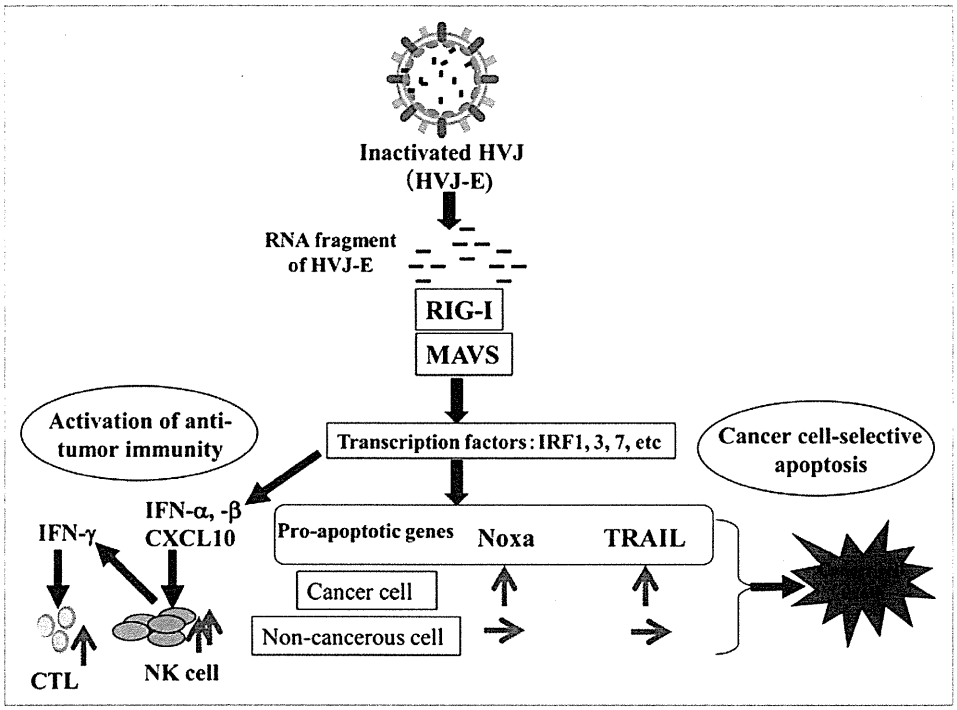

Figure 1. Signaling pathway for HVJ-E-mediated anti-tumor effects. Upon fusion with the plasma membrane, the hemagglutinating virus of Japan envelope (HVJ-E) introduces fragments of the viral RNA genome into the cytoplasm, which are recognized by retinoic acid-inducible gene I (RIG-I). The RIG-I/RNA complex associates with the mitochondrial antiviral signaling (MAVS) protein, which in turn promotes the activation of several transcription factors. In cells from the immune system, the RIG-I/MAVS pathway stimulate antitumor immunity via the production of some cytokines such as interferon (IFN)α, IFNβ and CXCL10. In cancer cells, this signaling pathway induces apoptosis upon the activation of pro-apoptotic factors, such as tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) and NOXA.

findings suggest that the epigenetic regulation of the loci coding for TRAIL and NOXA may vary between cancer cells and their normal counterparts. Based on preliminary experiments, we hypothesize that those loci may be silenced in cancer cells due to DNA methylation and/or histone deacetylation. Signaling via RIG-I/MAVS may therefore successfully relieve such a silencing in malignant cells, but not in normal cells. A detailed analysis of the transcriptional regulation of these (and other) genetic loci will allows us to better understand the mechanisms underlying tumorigenesis,.

Regardless of the molecular mechanism, HVJ-E can induce both anti-tumor immunity and cancer cell-selective apoptosis via the RIG-I/MAVS signaling pathway. As shown in **Figure 1**, when HVJ-E fuses with the membrane of cells from the immune system, such as dendritic cells and macrophages, antitumor immunity is induced. Conversely, when HVJ-E enters cancer cells, apoptotic cell death is induced. Although the effects of HVJ-E are limited in time due to its inability to replicate, antitumor immune response as induced by HVJ-E may eradicate the residual cancer cells that escape HVJ-E-induced apoptosis. In fact, we have demonstrate the antineoplastic potential of HVJ-E in various animal models.[4,7]

It is surprising that HVJ-E induces the apoptotic demise of cancer cells more effectively than live, replication-competent HVJ, with an increase in the multiplicity of infection (moi).[4] One of the reasons that underlie this observation may be that HVJ-infected, but not HVJ-E-treated, cells produce the viral C protein, which inhibits apoptosis in infected cells by modulating the activity of signal transducer and activator of transcription 1 and 2 (STAT1 and STAT2).[10] It is also possible that the inactive form of the fusion protein F0, which is expressed on the envelope of the viral progeny, may affect negatively their infectivity.[7] Indeed, HVJ infection cannot spread to other cells unless F0 is converted to F1 upon proteolytic cleavage.

Thus, HVJ-E may constitute an ideal tool for cancer therapy. Clinical-grade HVJ-E has already been generated and used in clinical trials enrolling melanoma and castration-resistant prostate cancer patients in Japan. Our recent findings provide solid evidence for the clinical application of HVJ-E.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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13-Cis retinoic acid can enhance the antitumor activity of non-replicating Sendai virus particle against neuroblastoma

Motonari Nomura,[1,2] Takashi Shimbo,[1,5] Yasuhide Miyamoto,[3] Masahiro Fukuzawa[2] and Yasufumi Kaneda[1,4]

[1]Division of Gene Therapy Science, Osaka University, [2]Department of Pediatric Surgery, Graduate School of Medicine, Osaka University, [3]Department of Immunology, Osaka Medical Center for Cancer and Cardiovascular Diseases, Osaka, Japan

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Hemagglutinating virus of Japan-envelope (HVJ-E) is a drug delivery vector based on inactivated Sendai virus. Recently, antitumor activities were found for HVJ-E itself and clinical trials of HVJ-E for some malignant tumors are now ongoing. We investigated the *in vitro* and *in vivo* antitumor effects of HVJ-E against neuroblastoma, which is one of the most common malignant solid tumors in childhood. The sensitivity of human neuroblastoma cell lines to HVJ-E correlated with the expression level of gangliosides, Sialylparagloboside (SPG) and GD1a, receptors for HVJ. Among the cell lines, SK-N-SH was the most sensitive to HVJ-E *in vitro* and total SPG and GD1a expression was the highest. Complete eradication of subcutaneous tumors derived from SK-N-SH cells was achieved by intratumoral injection of HVJ-E in SCID mice and no recurrence was observed for more than 300 days after HVJ-E inoculation. In contrast, NB1 cells expressed the lowest amount of GD1a and SPG and were resistant to HVJ-E *in vitro*. The expression of GD1a increased by 13-cis retinoic acid (13cRA), which is a therapeutic drug for high risk neuroblastoma, thus leading to an improved sensitivity to HVJ-E *in vitro*. Only growth inhibition of the subcutaneous tumors derived from NB1 cells was achieved by HVJ-E in the SCID mice, but the combination of 13cRA and HVJ-E could achieve partial eradication of the xenograft and also lead to an improved prognosis. In conclusion, HVJ-E is a promising therapeutic modality for neuroblastoma and 13cRA can be used as an adjuvant to HVJ-E. (*Cancer Sci* 2013; 104: 238–244)

Neuroblastoma is one of the most common malignant solid tumors in children and it is responsible for 12% of deaths associated with cancers in children.[1] Although the overall survival of neuroblastoma patients has improved using aggressive therapies in the past decades,[2,3] nearly 50% of high-risk neuroblastoma patients present with widespread dissemination of tumors and the long-term outcome is still very poor, even if intensive multimodal therapies are performed.[4] Some of these treatments are mutagenic, for example, radiotherapy causes somatic mutations in humans and germline mutations in animals.[5] Therefore, an alternative and less toxic cancer therapy is needed, especially for children.

It was reported that cancer cells could be killed after virus infection[6] and various viruses have therefore been used for cancer therapy.[6,7] Recently, we reported that UV-treated non-replicating Sendai virus (also known as hemagglutinating virus of Japan; HVJ) particle named HVJ-envelope (HVJ-E) can induce apoptosis in castration-resistant human prostate cancer cells and human glioblastoma cells [8–10] without toxicity in normal cells. Intratumoral injection of HVJ-E completely eradicated tumor masses of prostate cancer cells and glioblastoma cells in immunodeficient mice.[8,9] Furthermore, we discovered that HVJ-E also stimulated an antitumor immune response by activating cytotoxic T lymphocytes and natural killer (NK) cells and suppressing regulatory T cells.[11,12] Based on these direct and indirect antitumor activities, clinical trials to treat melanoma patients and castration-resistant prostate cancer patients with HVJ-E are now ongoing in Japan.

The mechanism underlying the direct antitumor activity of HVJ-E in neuroblastoma cells is being investigated to confirm whether a similar signaling pathway is involved in neuroblastoma cells, as has been suggested in prostate cancer and glioblastoma cells. Apart from the mechanism, the binding of HVJ-E to its receptor gangliosides is required for inducing cancer cell death. In the present study, we quantified the level of receptors for HVJ in various neuroblastoma cell lines and attempted to increase the expression of these receptors.

Among the recent therapies for advanced neuroblastoma patients, 13-cis retinoic acid (13cRA) is used for maintenance after high-dose chemotherapy combined with bone marrow transplantation.[2,3] It is well known that 13cRA can induce the differentiation of cancer cells from an immature form to a mature form,[13,14] but it is also known that 13cRA can alter the expression pattern of gangliosides.[15] Among the gangliosides, we focused on the possible changes in the synthesis of sialylparagloboside (SPG) and GD1a, receptors for HVJ,[16] because the expression of SPG or GD1a might be necessary for the interaction of HVJ with the target cells.

We concluded that many neuroblastoma cells were sensitive to HVJ-E, and this sensitivity correlated with the expression level of the HVJ receptor gangliosides. Furthermore, 13cRA enhanced the expression of HVJ receptors.

Materials and Methods

Cell lines and mice. Human neuroblastoma cell lines were obtained from the Health Science Research Resources Bank (Tokyo, Japan), European Collection of Animal Cell Cultures (Porton Down, UK) and the RIKEN Cell Bank (Ibaragi, Japan). The primary skin fibroblast cell NSF1227 derived from the forearm skin of a healthy man was established in our laboratory. NB1, NB19 and NB69 cells were maintained in RPMI-1640 (Nacalai Tesque Inc., Kyoto, Japan) supplemented with 15% fetal bovine serum (FBS), 100 IU/mL penicillin and 100 μg/mL streptomycin. SK-N-SH, SK-N-AS, SK-N-BE(2), IMR-32 and NSF1227 cells were maintained in Dulbecco's modified Eagle's medium (Nacalai Tesque Inc.) supplemented with 10% FBS, 100 IU/mL penicillin and 100 μg/mL streptomycin. Cells were incubated at 37°C in a humidified atmosphere of 95% air and 5% CO_2. Five-week-old female C.B-17

[4]To whom correspondence should be addressed.
E-mail: kaneday@gts.med.osaka-u.ac.jp
[5]Present address: Laboratory of Molecular Carcinogenesis, National Institute of Environmental Health Sciences, Research Triangle Park, North Carolina, USA.

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/Icr-scid/scidJcl mice were purchased from CLEA Japan Inc. (Tokyo, 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.

Preparation of HVJ-E. HVJ (VR-105 parainfluenza1 Sendai/52, Z strain from American Type Culture Collection) was amplified in chorioallantoic fluid of 10 14-day-old chicken eggs and purified by centrifugation and inactivated by UV irradiation (99 mJ/cm^2), as described previously.[17] The inactivated virus lost the ability of viral genome replication and viral protein synthesis, but retained activity for membrane fusion.[17]

Cell proliferation assay. A MTS assay using CellTiter 96 AQueous One Solution Cell Proliferation Assay Kit (Promega, Tokyo, Japan) was applied to evaluate cell viability. Cells were seeded in 24-well plates (5×10^4 cells/well in 500 μL of medium). Twenty-four hours later the cells were treated with HVJ-E (multiplicity of infection [MOI]: 100 10 000). Forty-eight hours after the treatment with HVJ-E, 100 μL of CellTiter 96 Aqueous One Solution Reagent was added to each well and the plates were incubated at 37°C. After transferring 100 μL of incubation medium from each well into a new 96-well plate, the absorbance at 490 nm was measured.

Pretreatment with 13-cis retinoic acid. Before HVJ-E treatment, NB1 or NSF1227 cells were treated with 10 μM 13-cis retinoic acid (Sigma, St Louis, MO, USA) for 24 h.

Interaction of PKH26-labeled HVJ-E with cells. The HVJ-E (3×10^{10} particles) was suspended in 1 mL Diluent C buffer and incubated with 1 mL of 4 mM PKH26 in Diluent C buffer at room temperature for 5 min (PKH26 Red Fluorescent Cell Linker Kit; Sigma). Labeling was stopped with 2 mL FBS. The labeled HVJ-E was washed with PBS and resuspended in 3 mL PBS. The day before HVJ-E treatment, cells were plated onto cover glasses in six-well plates (3×10^5 cells/well). The cells were incubated with PKH26-labeled HVJ-E (MOI of 10) at 37°C for 4 h, washed with PBS and fixed with 4% paraformaldehyde. Finally, the cells were stained with DAPI and observed using a confocal microscope.

Analysis of acidic glycosphingolipids from neuroblastoma cells. The majority of the experimental procedures have been reported previously.[8,18] Briefly, the acidic glycosphingolipids were extracted from each neuroblastoma cell line and the NSF1227 cell line (1×10^6 cells) and digested with recombinant endoglycoceramidase II from *Rhodococcus* sp. (Takara Bio, Shiga, Japan). The released oligosaccharides were labeled with 2-aminopyridine and separated using a high-performance liquid chromatography (HPLC) system equipped with a fluorescence detector. Normal-phase HPLC was performed on a TSK gel Amide-80 column (Tosoh, Tokyo, Japan). The molecular size of each PA-oligosaccharide is given in glucose units (Gu) based on the elution times of PA-isomaltooligosaccharides. Reversed-phase HPLC was performed on a TSK gel ODS-80Ts column (Tosoh). The retention time of each PA-oligosaccharide is given in glucose units based on the elution times of PA-isomaltooligosaccharides. Thus, a given compound on these two columns provides a unique set of Gu (amide) and Gu (ODS) values, which correspond to coordinates of the 2-D map. PA-oligosaccharides were analyzed using LC/ESI MS/MS. Standard PA-oligosaccharides, PA-GM1 and PA-GD1a, were purchased from Takara Bio and PA-LST-a and PA-SPG were obtained from our previous study.[19]

Real-time quantitative RT-PCR. RNA was extracted from cultured cells using the RNeasy Mini Kit (Qiagen Japan, Tokyo, Japan) and 1 μg total RNA was converted to cDNA with a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Tokyo, Japan). Human ST3GAL1, ST3GAL2, ST3GAL6 and β-actin were amplified using SYBR Premix Ex Taq (Takara Bio). All procedures were performed according to the manufacturer's instructions. The primers were as follows: human ST3GAL1-fp, 5′-GACTTGGAGTGGGTGGTGAG-3′; human ST3GAL1-rp, 5′-GGAACCGGGATGTAGGTGT-3′; human ST3GAL2-fp, 5′-GTCCAGAGGTGGTGGATGAT-3′; human ST3GAL2-rp, 5′-CAGCACCTCATTGGTGTTGT-3′; human ST3GAL6-fp, 5′-AGCCTGGTCCCTCTTTCG-3′; human ST3GAL6-rp, 5′-GGCCACAAGATACCCTCTCA-3′; human β-actin-fp, 5′-GAGCTACGAGCTGCCTGACG-3′; and human β-actin-rp, 5′-GTAGTTTCGTGGATGCCACAG-3′.

Tumor growth *in vivo*. Viable SK-N-SH cells (1×10^7 cells) were resuspended in 100 μL PBS and injected into the subcutaneous space on the back of SCID mice (day 0). When each tumor had grown to 90 110 mm^3, the mice were treated with intratumoral injections of HVJ-E (5000 HAU = 1.5×10^{10} particles in 100 μL PBS) or 100 μL PBS on days 6, 9 and 12. Tumor volume was measured in a blinded manner with slide calipers using the following formula: tumor volume (mm^3) = length × (width)^2/2.

Viable NB1 cells (1×10^7 cells) were resuspended in 100 μL PBS with or without 300 ng 13cRA and injected into the subcutaneous space on the back of the SCID mice. When each tumor had grown to 90 110 mm^3, the mice were treated with intratumoral injections of either HVJ-E (5000 HAU) or 100 μL PBS on days 6, 9, 12 and 15. A second intratumoral injection of 100 μL PBS with or without 300 ng 13cRA was performed on day 18 and HVJ-E treatment was performed again on days 21, 24, 27 and 30. Tumor volume was measured the same way as the SK-N-SH model.

Statistical analyses. Data are expressed as mean ± standard deviation. The two-tailed unpaired Student's *t*-test was used to determine the statistical significance of any differences between two groups. Probability values of $P < 0.05$ were considered to be statistically significant.

Results

Antitumor activity of HVJ-E against human neuroblastoma cell lines *in vitro*. A MTS assay was performed to evaluate cell viability and showed that the survival rates of SK-N-SH, SK-N-AS, SK-N-BE(2), NB19 and NB69 cells decreased by HVJ-E treatment in a dose-dependent manner, and SK-N-SH was the most sensitive to HVJ-E. In contrast, NB1 and IMR32 were not sensitive (Fig. 1a). As it has been reported that the malignancy of neuroblastoma correlates with MYCN amplification, we analyzed the expression of MYCN in each neuroblastoma cell line. A high level of MYCN expression was detected in SK-N-BE(2), IMR32, NB1 and NB19, but not in SK-N-SH, SK-N-AS and NB69 (Data S1, S2, Fig. S1). These results suggest that the sensitivity of neuroblastoma cells to HVJ-E is independent of MYCN. Using PKH26-labeled HVJ-E, SK-N-SH was found to have a stronger affinity for HVJ-E than NB1 and IMR32 (Fig. 1b), implying that the decreased survival of neuroblastoma cells might correlate with their affinity for HVJ-E.

Correlation of antitumor activity of HVJ-E with the expression of SPG or GD1a. To identify the limiting factor related to the sensitivity of cells to HVJ-E, we analyzed the expression levels of receptors for HVJ in each neuroblastoma cell line. It is known that gangliosides with sialylated terminal galactose residue can be receptors for HVJ. Among them, GD1a and SPG are thought to be representative receptors for HVJ. The expression pattern of acidic gangliosides in each cell line was analyzed using HPLC and each peak indicated the expression level of the corresponding ganglioside (Fig. 2a). The rate of each ganglioside's expression could therefore be estimated by calculating the area of each peak (Fig. 2b). An inverse correlation of

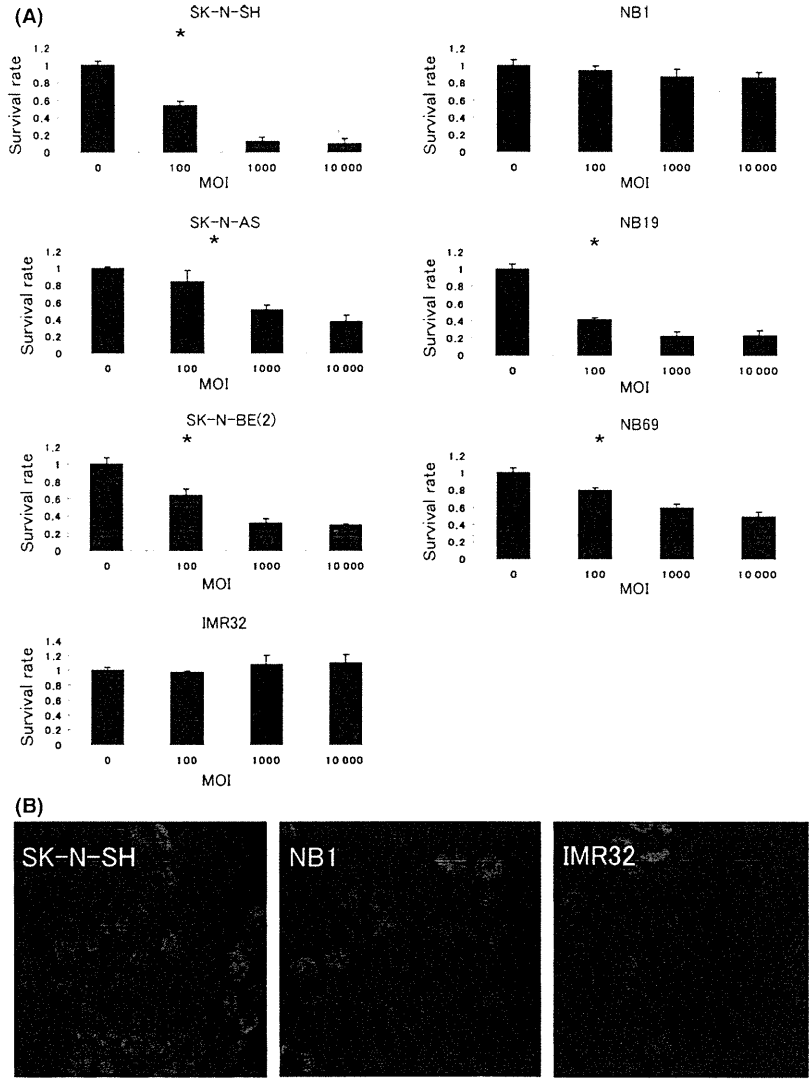

Fig. 1. Antitumor activity of hemagglutinating virus of Japan-envelope (HVJ-E) against neuroblastoma cell lines *in vitro*. (a) Each cell line was treated with a different multiplicity of infection (MOI) of HVJ-E for 48 h and then the survival rate was assessed using a MTS assay. Each value (mean ± standard deviation, $n = 4$) of survival was the ratio to the value without treatment. (b) The interaction of HVJ-E with the neuroblastoma cell lines SK-N-SH, NB1 and IMR32 was assessed. Each cell line was treated with 10 MOI of PKH26 (red)-labeled HVJ-E for 4 h. Nuclei were stained with DAPI (blue). Experiments were repeated three times and representative results are shown. $*P < 0.05$.

the cell survival rate with the expression rate of SPG, GD1a or SPG and GD1a was found (Fig. 2c).

Antitumor activity of HVJ-E against xenograft tumors derived from SK-N-SH cells. We first chose the SK-N-SH cell line, the most sensitive to HVJ-E, to show the antitumor effects of HVJ-E *in vivo*. Tumor volume significantly decreased by three intratumoral injections of HVJ-E (Fig. 3a) and all of the xenografted tumors were completely eradicated by HVJ-E treatment by day 49 after tumor inoculation (Fig. 3b). We also followed the prognosis of mice to prove the safety of HVJ-E. All mice in the PBS-treated group died within 100 days after tumor inoculation, but all mice in the HVJ-E-treated group survived more than 300 days without tumor recurrence or signs of toxicity (Fig. 3c). Thus, the first objective of the present study to show the antitumor effects of HVJ-E against human neuroblastoma *in vitro* and *in vivo* was successfully achieved.

Enhancement of antitumor activity of HVJ-E against the NB1 cell line by 13cRA *in vitro*. The next concern was how to kill the neuroblastoma cell lines that have a very low level of HVJ receptors. Since Hettmer *et al.*[15] reported that retinoic acid could alter the synthesis of the gangliosides of neuroblastoma cell lines, we hypothesized that the synthesis of GD1a might be altered by 13cRA. We chose the NB1 cell line as a model for this experiment, because the expression of SPG and GD1a was the lowest in this cell line out of the numerous cell lines used in the present study. Twenty-four hours after the treatment with 13cRA, the expression pattern of gangliosides was analyzed using HPLC. Each peak of the gangliosides was changed (Fig. 4a). The expression rate of GD1a increased by 13cRA, but the expression of SPG remained unchanged (Fig. 4b). Dimethylsulfoxide, which was used as the solvent for 13cRA, did not affect the synthesis of GD1a (data not shown).

To explain this facilitation of GD1a synthesis, we quantified the expression of sialyltransferases ST3GAL1 and ST3GAL2, which convert GM1 to GD1a,[20] using real-time PCR. The expression of ST3GAL2 was significantly enhanced by 13cRA (Fig. 4c). ST3GAL6, which synthesizes SPG,[20] was not detectable (data not shown). Next, we assessed the 13cRA-induced changes in the sensitivity to HVJ-E by MTS assay. The survival rate of NB1 cells significantly decreased using HVJ-E in the 13cRA-pretreatment group (Fig. 4d). The interaction of HVJ-E with NB1 cells was also strengthened by 13cRA, which was confirmed using PKH26-labeled HVJ-E (Fig. 4e). These data imply that 13cRA can enhance the antitumor activity of HVJ-E by altering the synthesis of ganglioside GD1a in NB1 cells. To ensure the safety of HVJ-E or 13cRA for normal cells, we also analyzed the expression of the gangliosides in the primary skin fibroblast cell line NSF1227.

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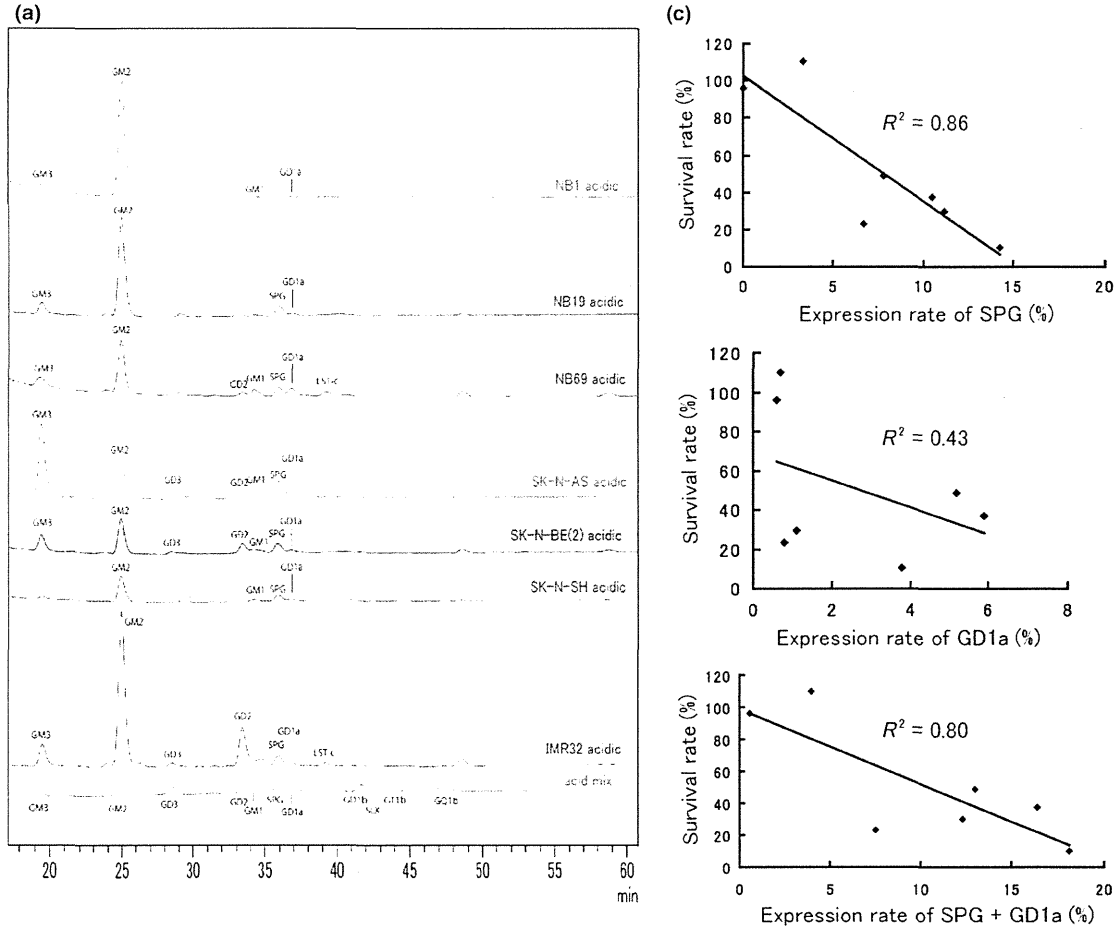

(b)

	Rate of expression (%)								
	GM3	GM2	GD3	GD2	GM1	SPG	GD1a	LST-c	total
NB1	2.5	94.9	1.3	–	0.8	–	0.6	–	100
NB19	7.2	83.9	1.5	–	–	6.7	0.8	–	100
NB69	10.3	65.2	2.3	2.2	3.9	7.8	5.2	3.0	100
SK-N-AS	44.7	28.9	4.7	3.5	3.8	10.5	5.9	–	100
SK-N-BE(2)	19.4	52.5	1.8	11.2	2.8	11.2	1.1	–	100
SK-N-SH	–	76.9	–	–	5.0	14.3	3.8	–	100
IMR32	8.7	69.5	1.1	15.5	–	3.3	0.7	1.1	100

Fig. 2. Relationship between the expression pattern of gangliosides and hemagglutinating virus of Japan-envelope (HVJ-E)-induced cell death. (a) Cells (1×10^6) of each cell line were collected and then the expression pattern of gangliosides was analyzed using HPLC; (b) the expression rate of each ganglioside was then calculated. (c) The XY scatter plots showed an inverse correlation between the cell survival rate and the expression of SPG or GD1a. R^2 is the Pearson's correlation coefficient index. $R^2 > 0.4$ indicates a relatively strong correlation.

SPG and GD1a were expressed in NSF1227, but the expression pattern was not changed with the 13cRA treatment (Fig. S2A,B). Moreover, NSF1227 cells were not killed by either HVJ-E alone or the combination of 13cRA and HVJ-E (Fig. S2C).

Enhancement of antitumor activity of HVJ-E against xenograft tumors derived from NB1 cells by 13cRA. Based on the findings from the *in vitro* experiments, we tested the antitumor effect of HVJ-E in the NB1-derived tumors xenografted into SCID mice. One treatment protocol comprising five injections was repeated twice. The first injection included 13cRA or PBS. For the other injections, either HVJ-E or PBS was used. More association of PKH26-labeled HVJ-E with cancer cells in the tumor mass was observed in mice treated with 13cRA compared with PBS (Data S3, Fig. S3A). The tumor volume of the 13cRA-treated groups (13cRA + PBS, 13cRA + HVJ-E) was significantly smaller in comparison with the 13cRA-untreated groups (PBS, HVJ-E). Moreover, the combination of 13cRA and HVJ-E almost completely eradicated the NB1-derived tumors. We also confirmed that the combination of 13cRA and HVJ-E induced both apoptosis and necrosis in the xenograft, although HVJ-E alone induced only apoptosis in the edge of the xenograft (Data S4, Fig. S3B).

Although these results were different from the findings obtained from *in vitro* experiments, the tumor volume of the HVJ-E group was significantly smaller than that of the PBS group (Fig. 5a,b). The expression of MICA/MICB, which is

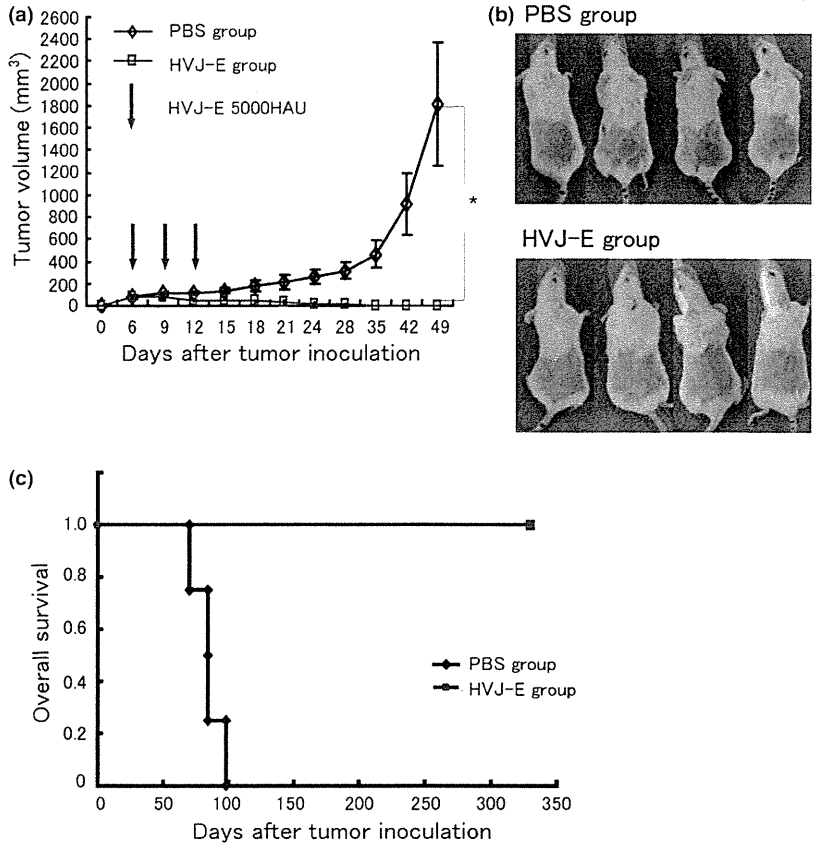

Fig. 3. Antitumor activity of hemagglutinating virus of Japan-envelope (HVJ-E) against xenograft SK-N-SH tumors in SCID mice. (a) Each value (mean ± standard deviation, $n = 4$) of the tumor volume was compared between the PBS and HVJ-E groups. (b) Complete eradication of tumors was achieved in the HVJ-E group by day 49 after tumor inoculation. (c) The overall survival was evaluated using Poisson's regression curve. No recurrence was observed in the HVJ-E group. *$P < 0.05$.

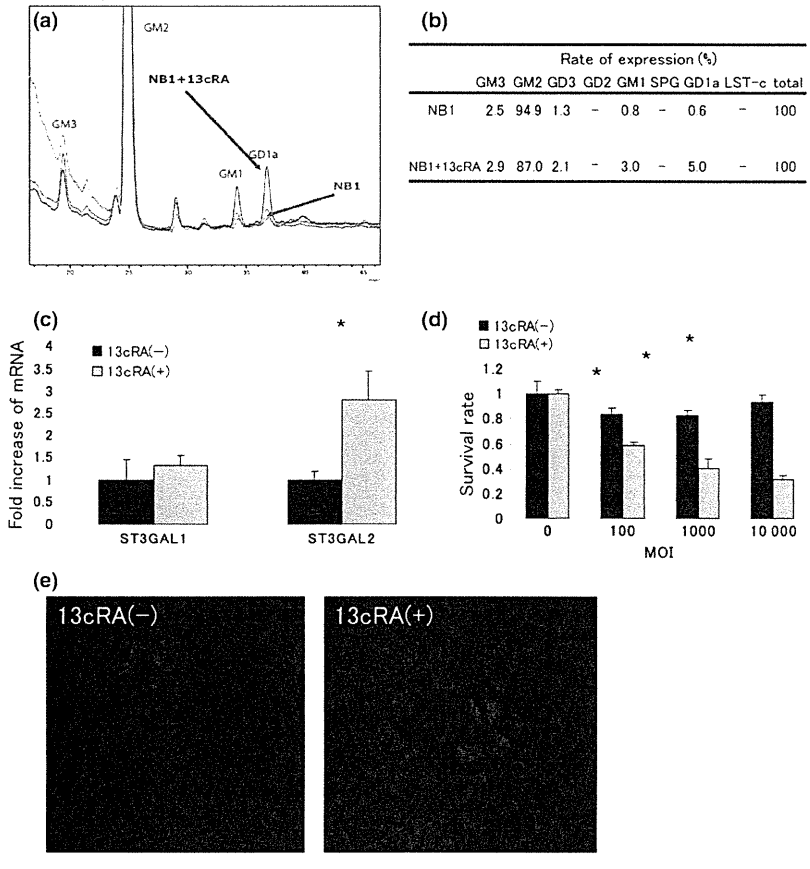

Fig. 4. Effects of 13cRA on the expression of gangliosides in NB1 cells and sensitivity to hemagglutinating virus of Japan-envelope (HVJ-E) *in vitro*. (a) NB1 cells with or without pretreatment using 10 μM 13cRA were collected and the expression pattern of gangliosides was analyzed using HPLC. (b) The expression rate of each ganglioside was calculated. Pretreatment with 13cRA enhanced the expression rate of GD1a. (c) The mRNA expression of ST3GAL1 and ST3GAL2 was analyzed using real-time PCR. Pretretament with 13cRA enhanced the mRNA expression of ST3GAL2. Each value (mean ± standard deviation, $n = 3$) of mRNA was the ratio to the value without treatment. (d) NB1 cells with or without 13cRA pretreatment were exposed to different multiplicity of infection (MOI) of HVJ-E for 48 h and the cell survival rate was then assessed using a MTS assay. Each value (mean ± standard deviation, $n = 4$) of survival was the ratio to the value without HVJ-E treatment. (e) The interaction of HVJ-E with NB1 cells with or without 13cRA pretreatment was assessed. NB1 cells were treated with 10 MOI of PKH26 (red)-labeled HVJ-E for 4 h. Nuclei were stained with DAPI (blue). Experiments were repeated three times and representative results are shown. *$P < 0.05$.

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the ligand for NKG2D on NK cells, was endogenously expressed in NB1 cells (Data S2, Fig. S4A), suggesting that NK cells in SCID mice can interact with NB1 cells. The mRNA expression of activated NK cell markers, CD69 and Interferon-gamma (IFN-γ), as well as CD49b (NK cell marker) in the xenograft of each group was also analyzed. HVJ-E alone could enhance the expression of CD49b, CD69 and IFN-γ in the xenografts (Data S1, Fig. S4B). It is likely that the activated NK cells infiltrated the tumor tissue and attacked the tumor cells *in vivo* without the direct interaction of HVJ-E and tumor cells. We performed only two cycles of each therapy because the first mouse in the PBS group died on day 33 after tumor inoculation. We also followed the prognosis of each group. The tumors of the HVJ-E-untreated groups (PBS, 13cRA + PBS) immediately re-grew and all mice in these groups soon died. Within a few weeks after the final treatments, the tumors of the mice in the HVJ-E-treated groups (HVJ-E, 13cRA + HVJ-E) also re-grew. The best prognosis of these mice was obtained in the 13cRA + HVJ-E group (Fig. 5c).

Therefore, the second objective of the present study, to enhance the sensitivity of a HVJ-E-resistant human neuroblastoma cell line to HVJ-E *in vitro* and *in vivo*, was also successfully achieved.

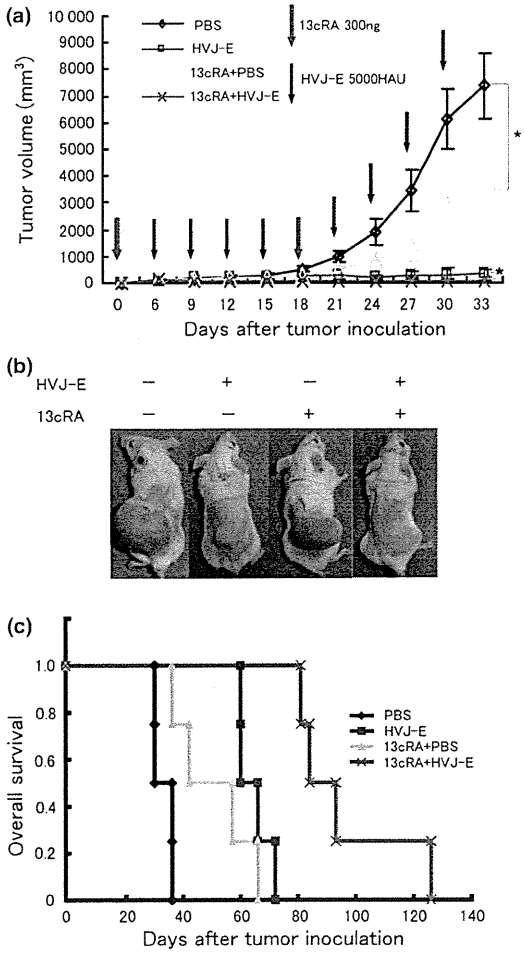

Fig. 5. Antitumor activity of hemagglutinating virus of Japan-envelope (HVJ-E) against xenograft NB1 tumors in SCID mice and the improvement of its activity using 13cRA. (a) Tumor volumes (mean ± standard deviation, $n = 4$) were compared among PBS, HVJ-E, 13cRA + PBS and 13cRA + HVJ-E groups. (b) The representative external appearance of xenografts from each group on day 33 after tumor inoculation showed the superiority of the combination of 13cRA and HVJ-E. (c) Overall survival was evaluated using a Poisson's regression curve. The best prognosis was obtained in the 13cRA + HVJ-E group. $^*P < 0.05$.

Discussion

In the past few decades many genetic features of neuroblastoma have been identified that correlate with the clinical outcome. Among them, MYCN amplification is one of the most critical risk factors in neuroblastoma patients and many patients classified into the high-risk group have MYCN amplification with a poor prognosis.[21] Herein, we demonstrated that HVJ-E could kill human neuroblastoma cells, even if MYCN was amplified.

Hemagglutinating virus of Japan is a mouse parainfluenza virus belonging to the paramixoviridae genus. Two glycoproteins, fusion (F) and hemagglutinin-neuraminidase (HN), are present on the viral envelope.[22] The first step of infection involves the binding of HN to its receptors. Hemagglutinin-neuraminidase has a neuraminidase activity and is thought to digest carbohydrate chains on the viral envelope. Following this, the hydrophobic region of the F protein, which is thought to function as a fusion peptide, invades the lipid bilayer through its association with lipid molecules such as cholesterol.[23] Therefore, the first step of infection requires expression of HN receptors SPG and GD1a. The results from the present study imply that the expression levels of SPG and GD1a can be used to predict the response to HVJ-E therapy. Therefore, neuroblastoma cells with a low expression of SPG and GD1a are likely to be resistant to HVJ-E and the increase of these gangliosides is necessary to overcome such resistance.

Therefore, we demonstrated that 13cRA might be a promising agent for the enhancement of the expression of SPG or GD1a. It has been known that 13cRA has the ability to induce cellular differentiation and to decrease the proliferation of neuroblastoma cells *in vitro*.[13] In the steady state, retinoic acid receptor (RAR) makes a heterodimer with retinoid X receptor (RXR) and this RAR RXR heterodimer binds to retinoic acid response elements (RARE) to repress the acetylation of histones, thereby silencing many genes.[24–26] However, once 13cRA binds to RAR, this repression becomes inhibited and the transcription of silenced genes becomes activated.[27] Therefore, many outcomes other than cellular differentiation can be obtained by the use of 13cRA. Enhancing the expression of ST3GAL2 and the synthesis of GD1a are other examples of 13cRA-induced outcomes.

In the standard protocol for high-risk neuroblastoma, the main therapy consists of surgery, radiotherapy and chemotherapy combined with bone marrow transplantation. The chemotherapy for high-risk patients is very intensive and is sometimes too toxic for children, but it must be administered repeatedly to achieve efficient eradication of tumors. The goal of the final phase of therapy is to eradicate any minimal residual disease (MRD), but these repeated chemotherapies often induce chemoresistance, therefore other agents for chemoresistant MRD are necessary. As a therapeutic modality for MRD, 13cRA is considered to be useful because of its ability to induce cellular differentiation. Considering the additional ability of 13cRA to enhance the antitumor activity of HVJ-E, a reduction in the cycles of chemotherapy and the earlier use of the combination of 13cRA and HVJ-E might be a novel strategy for high-risk neuroblastoma, especially when severe adverse drug reactions are observed during chemotherapy.

Herein, we demonstrated that 13cRA could enhance the antitumor activity of HVJ-E against NB1 cells, which were resistant to HVJ-E *in vitro*, but HVJ-E alone could also inhibit the tumor growth of NB1-derived xenografts to some degree *in vivo*. This is probably because HVJ-E activates NK cells in SCID mice. HVJ-E can activate NK cells without binding to tumor cells. This is because HVJ-E can directly induce the release of CXCL10 and interferon-β from dendritic cells to elicit the infiltration and activation of NK cells.[12] Our data also show that HVJ-E can activate NK cells without binding to NB1 cells. We used SCID rather than non-obese diabetic-SCID mice for the model of HVJ-E therapy, because the direct antitumor activity of HVJ-E *in vivo* has already been reported and we wanted to eradicate "human" neuroblastoma cell lines in a model closer to normal humans with some antitumor immunity.[8]

In conclusion, HVJ-E might therefore be a useful therapeutic modality for human neuroblastoma if SPG or GD1a are expressed in tumor cells and its anticancer effects can be enhanced by 13cRA. Therefore, combination therapy using HVJ-E and 13cRA might be an effective new method for the treatment of neuroblastoma.

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Disclosure Statement

The authors declare no conflicts of interest.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. MYCN expression in neuroblastoma cell lines.

Fig. S2. Antitumor activity of HVJ-E or 13cRA against a normal cell line.

Fig. S3. Interaction of HVJ-E with NB1 cells and the detection of apoptosis or necrosis *in vivo*.

Fig. S4. Expression of NK cell ligands in cancer cells and activation of NK cells in tumor tissue with HVJ-E.

Data S1–S4. Including: primers for real-time quantitative RT-PCR; antibodies and western blotting analysis; interaction of PKH26-labeled HVJ-E with NB1-derived xenografts; and detection of HVJ-E-induced apoptosis and necrosis in NB1-derived xenografts.

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Improvement of cancer immunotherapy by combining molecular targeted therapy

Yutaka Kawakami*, Tomonori Yaguchi, Hidetoshi Sumimoto, Chie Kudo-Saito, Tomoko Iwata-Kajihara, Shoko Nakamura, Takahiro Tsujikawa, Jeong Hoon Park, Boryana K. Popivanova, Junichiro Miyazaki and Naoshi Kawamura

Division of Cellular Signaling, Institute for Advanced Medical Research, Keio University School of Medicine, Tokyo, Japan

Edited by:
Antoni Ribas, University of California Los Angeles, USA

Reviewed by:
Howard L. Kaufman, Rush University Medical Center, USA
Graham R. Leggatt, University of Queensland, Australia

****Correspondence:***
Yutaka Kawakami, Division of Cellular Signaling, Institute for Advanced Medical Research, Keio University School of Medicine, 35 Shinanomachi, Shinjuku, Tokyo 160-8582, Japan.
e-mail: yutakawa@z5.keio.jp

In human cancer cells, a constitutive activation of MAPK, STAT3, β-catenin, and various other signaling pathways triggers multiple immunosuppressive cascades. These cascades result in the production of immunosuppressive molecules (e.g., TGF-β, IL-10, IL-6, VEGF, and CCL2) and induction of immunosuppressive immune cells (e.g., regulatory T cells, tolerogenic dendritic cells, and myeloid-derived suppressor cells). Consequently, immunosuppressive conditions are formed in tumor-associated microenvironments, including the tumor and sentinel lymph nodes. Some of these cancer-derived cytokines and chemokines impair immune cells and render them immunosuppressive via the activation of signaling molecules, such as STAT3, in the immune cells. Thus, administration of signal inhibitors may inhibit the multiple immunosuppressive cascades by acting simultaneously on both cancer and immune cells at the key regulatory points in the cancer-immune network. Since common signaling pathways are involved in manifestation of several hallmarks of cancer, including cancer cell proliferation/survival, invasion/metastasis, and immunosuppression, targeting these shared signaling pathways in combination with immunotherapy may be a promising strategy for cancer treatment.

Keywords: immunotherapy, immunosuppression, MAPK, STAT3, β-catenin

INTRODUCTION

By the time cancer cells are detected clinically, they have already evaded the immune-defense system (Robert et al., 2011). During their long development process, such cancer cells have lost highly immunogenic tumor antigens and acquired immunoresistant and immunosuppressive properties through various mechanisms (Yaguchi et al., 2011). Consequently, elimination of cancer cells by immunological strategies may not be easy. However, it has been revealed that the tumor antigens expressed by cancer cells are qualitatively or quantitatively different form the normal counterpart, and that cancer cells can be eliminated by T cells using various immune-interventions in some patients. We have previously identified human tumor antigens recognized by T cells (Kawakami et al., 1994a,b), and attempted to develop various antigen-specific immunotherapies (Rosenberg et al., 1998). For instance, the administration of gp100 melanoma antigen peptide vaccine along with IL-2 resulted in 16% objective response with 9% CR in the recent multicenter randomized trial (Schwartzentruber et al., 2011). Furthermore, adoptive immunotherapy using cultured melanoma-specific T cells following lymphomyeloablative treatment, which depletes various immunosuppressive cells and induces homeostatic proliferation of administered T cells, resulted in more than 70% objective response with about 20% durable CR in advanced melanoma patients with multiple metastases (Rosenberg et al., 2011). These observations indicate that active immunization may be further improved by various immune-interventions.

DEVELOPMENT OF EFFECTIVE IMMUNOTHERAPY BY COMPREHENSIVE REGULATION OF ANTI-TUMOR IMMUNE NETWORK

Analysis of mouse tumor models and human clinical trials using the identified tumor antigens revealed that following key points need to be addressed in order to regulate the anti-tumor immune network and develop effective immunotherapy (**Figure 1**) (Kawakami et al., 2004). (1) *Identification of appropriate tumor antigens for immunotherapy*: the ideal antigens should have tumor-specific expression and they should be involved in cancer cell proliferation/survival. They must also be expressed in cancer initiating cells. We have identified human glioma antigen SOX6, which is expressed in glioma stem-like cells. SOX6 is involved in cancer proliferation and is recognized by T cells (Ueda et al., 2004, 2010). Sox6-DNA vaccination was able to inhibit growth of murine glioma in a therapeutic setting (Ueda et al., 2008). (2) *Development of in situ tumor destruction methods to induce immunogenic cancer cell death:* break down of tumor releases endogenous tumor antigens and subsequently induces anti-tumor immune response (*Immunogenic cancer cell death*). This may be achieved possibly by using chemotherapy, molecular targeted drugs, anti-tumor antibody, irradiation, cryoablation, radiofrequency ablation, or oncolytic viruses. (3) *Development of methods to enhance dendritic cell (DC) functions:* the methods include augmentation of antigen uptake, cross presentation, and T cell stimulation by using adjuvants, cytokines, or agonistic antibodies. We have previously developed several protocols for combined immunotherapy of *in situ* tumor destruction and

subsequent DC activation. An example of this is the use of oncolytic HSV, which is capable of both direct tumor destruction and DC stimulation. Intratumoral administration of HSV not only inhibited the treated tumor but also suppressed untreated tumors at remote sites via induction of systemic anti-tumor T cells (Toda et al., 2002). Another protocol involves a combination of tumor cryoablation and subsequent intratumoral administration of DCs pretreated with TLR2-stimulating BCG-CWS (Mycobacterium bovis Bacillus Calmette-Guérin cell wall skeleton). This protocol induced T cell responses to multiple endogenous tumor antigens and suppressed growth of untreated remote tumors as well (Udagawa et al., 2006). (4) *Development of methods to activate and expand anti-tumor T cells in vivo:* this may be achieved possibly by immunization with tumor antigens, administration of cytokines, or agonistic antibodies against co-stimulatory molecules on T cells, or transfer of cultured anti-tumor T cells. We are currently attempting to use tumor-specific T cells cultured *in vitro* to treat patients with melanoma. (5) *Development of methods to reverse immunosuppression*: Various immunomodulating reagents are being studied to evaluate their efficacy in recovering immunosuppressive condition in cancer patients. These reagents include antibodies (e.g., anti-CTLA-4, anti-PD-1/PD-L1), chemotherapy, and molecular targeted drugs.

In this article, we will focus on the combined use of molecular targeted drugs with immunotherapy, that could possibly reverse immunosuppression and augment anti-tumor T cell responses.

MECHANISMS OF IMMUNOSUPPRESSION IN CANCER PATIENTS

Cancer cells, more specifically oncogene activation and subsequent signal activation in cancer cells, trigger multiple immunosuppressive cascades. These immunosuppressive cascades involve various immunosuppressive molecules such as TGF-β, IL-10, IL-6, VEGF, PD-L1, COX2, and IDO/TDO as well as immunosuppressive cells such as tolerogenic DCs, myeloid-derived suppressor cells (MDSCs), and regulatory T cells (Tregs). Ultimately, cancer cells generate immunosuppressive microenvironments in tumor and sentinel lymph nodes (Yaguchi et al., 2011). For example, an over production of TGF-β in tumor microenvironment resulted in accumulation of MDSCs, M2 macrophages and Tregs, and impairment of DC functions in tumor tissues and sentinel lymph nodes. We have shown that TGF-β-induced-Snail not only induces metastasis-causing epithelial-to-mesenchymal transition (EMT) of cancer cells but also enhances production of immunosuppressive cytokines and chemokines, including TGF-β, IL-10, CCL2, and TSP-1 (Kudo-Saito et al., 2009), which further promotes metastasis. These cytokines impair DC function, induce Tregs, and finally inhibit induction of anti-tumor T cells. CCL2 produced by cancer cells recruits MDSCs into tumor and CCL22 produced by M2 macrophages recruits $CCR4^+$ Tregs and Th2 cells into tumor and sentinel lymph nodes (Kudo-Saito et al., 2009, 2013; Tsujikawa et al., 2013). Therefore, TGF-β production in tumor microenvironment by either cancer cells or infiltrated immune cells triggers multiple immunosuppressive cascades involving various immunosuppressive cytokines, chemokines, and immune cells. It has been reported that inhibition of TGF-β signaling by injection of plasmid DNA containing TGF-β type II receptor cDNA near the tumor sites enhanced tumor antigen-specific T cells accompanied by decrease of Tregs through blockade of TGF-β signaling (Fujita et al., 2009). Therefore, blockade of the TGF-β dependent immunosuppressive cascade at either upstream signaling for TGF-β production, TGF-β itself, or its downstream events such as Treg induction may restore immunocompetence of cancer patients.

SIGNAL INHIBITORS MAY AUGMENT ANTI-TUMOR IMMUNE RESPONSES

To effectively reverse immunosuppressive condition in cancer patients, which molecules or cells should be targeted in the immunosuppressive cascades? Where should they be blocked, upstream, or downstream? Blockade of downstream immunosuppressive molecules, such as CTLA-4 and PD-1/PD-L1, was recently shown to be effective in augmenting anti-tumor immune responses in clinical trials (Hodi et al., 2010; Topalian et al., 2012). Targeting downstream immunosuppressive molecules (e.g., TGF-β, IL-10, IL-6, VEGF, CTLA-4, PD-1, PD-L1, IDO/TDO, Cox2) and cells (e.g., MDSCs and Treg) with antibodies or small molecule inhibitors may have specific and efficient inhibitory activity against immunosuppressive cascades. However, inhibition of one molecule or one cell type may not be sufficient to reverse caner immunosuppression in patients.

In order to reverse immunosuppression in tumor-bearing hosts, we have evaluated signal inhibition at upstream molecules, such as BRAF-MAPK, STAT3, and Wnt/β-catenin (Sumimoto et al., 2006; Iwata-Kajihara et al., 2011; Yaguchi et al., 2012) (**Figure 2**). Targeting a constitutively activated signaling in cancer cells will not only inhibit multiple downstream immunosuppressive events simultaneously but also suppress multiple intrinsic malignant features of cancer cells, such as proliferation, survival, and invasion. The destruction of cancer cells may result in release of various endogenous tumor antigens and contribute to induction of anti-tumor immune response, and subsequent decrease of tumor burden decreases total immunosuppressive activity. In developing molecular targeted therapy, the idea of personalized treatment strategy is crucial. This is because the contribution of target signaling molecules in immunosuppression may be different even among patients with same type of cancer. Another factor to consider is that signal inhibitors sometimes have direct effects on immune cells, including activation of immune cells (e.g., DC) and inhibition of various immunosuppressive cells (e.g., Treg, MDSC) (Iwata-Kajihara et al., 2011; Oosterhoff et al., 2012). A combination of both upstream and downstream blockade is also an attractive strategy. For instance, administration of signal inhibitors (e.g., BRAF inhibitor) and blockade of antibodies against major immuosuppressive molecules (e.g., TGF-β, PD-1/PD-L1, CTLA-4) may be effective. However, it should be noted that such upstream blockade may affect various normal cells and cause adverse effects, including suppression of anti-tumor immune response. Therefore, a careful evaluation of total *in vivo* activity of these signal inhibitors is needed in both animal tumor models and clinical trials.

MAPK SIGNALING INHIBITORS

A common mutation of BRAF (V600E), a molecule in MAPK signal pathway, was identified by systematic DNA sequencing of