Abstract

**Purpose:** The treatment of cancer with oncolytic viruses primarily depends on the selective viral replication in cancer cells. However, a replication-incompetent hemagglutinating virus of Japan (HVJ; Sendai virus) envelope (HVJ-E) suppresses the growth of human cancer cells as effectively as replication-competent live HVJ without producing toxic effects in nonmalignant cells. Here, we analyze the molecular mechanism of the oncolytic activity of HVJ-E.

**Experimental Design:** The molecules responsible for HVJ-E–induced cancer cell death were elucidated in prostate cancer cell lines, and the effect of HVJ-E on orthotopic prostate cancers was evaluated in nonobese diabetic–severe combined immunodeficient (NOD-SCID) mice.

**Results:** The liposome-mediated transfer of viral RNA genome fragments from HVJ-E suppressed the viability of prostate cancer cells but not the viability of the noncancerous prostate epithelium. Knockdown experiments using siRNAs showed that the cancer cell–selective killing induced by HVJ-E was mediated by retinoic acid–inducible gene I (RIG-I) and mitochondrial antiviral signaling protein (MAVS). Downstream of the RIG-I/MAVS pathway, both TNF-related apoptosis-inducing ligand (TRAIL) and Noxa were upregulated by HVJ-E in the castration-resistant prostate cancer cell line PC3 but not in the noncancerous prostate epithelial cell line PNT2. TRAIL siRNA but not Noxa siRNA significantly inhibited HVJ-E–induced cell death in PC3 cells. However, Noxa siRNA effectively suppressed HVJ-E–induced cell death in DU145 cells, another castration-resistant prostate cancer cell line, in which Noxa but not TRAIL was upregulated by HVJ-E. Furthermore, the orthotopic prostate cancers were dramatically eradicated in immunodeficient mice injected with HVJ-E.

**Conclusion:** The RIG-I/MAVS signaling pathway represents an attractive target for cancer therapy.

Clin Cancer Res; 18(22); 6271–83. ©2012 AACR.

Introduction

Cancer is an uncontrollable disease with high rates of morbidity and mortality. In many cases, cancer cells are not completely eliminated by surgery, thereby making disease recurrence a common and difficult problem associated with cancer treatment. To suppress the growth of residual or inoperable tumors, numerous therapeutic reagents have been developed to specifically eliminate neoplastic cells (1).

Prostate cancer is a disease that offers a second leading cause of cancer-associated death in males in the United States, and it represents a growing problem worldwide (2). Although most patients with prostate cancer initially respond to androgen ablation, castration-resistant prostate cancers develop within a couple of years in many cases (3), and such prostate cancers are difficult to treat.

Virotherapy has been receiving interest since the discovery of cancer remission in patients with cancer after virus infection (4, 5). Various viruses have been used for cancer treatment (5–7), but the efficacy is low due to the suppression of the virus by the host immune response (8). Remarkable progress has been made in cancer virotherapy following the development of oncolytic viruses, which enables viruses to replicate selectively in cancer cells (5, 9–14). Attenuated viral strains have also been used for cancer
Translational Relevance

Oncolytic viruses have been used in clinical trials to treat cancers. Cancer treatment involving the use of oncolytic viruses primarily depends on the selective replication of the viruses in cancer cells. However, replication-incompetent hemagglutinating virus of Japan (HVJ; Sendai virus) envelope (HVJ-E) obtained via UV irradiation was found to display effective oncolytic activity without producing toxic effects in nonmalignant human cells. The suppression of prostate cancer cell viability by HVJ-E is comparable with that induced by live HVJ particles. These results suggest that neither viral RNA replication nor viral protein synthesis is involved in the oncolytic activity of HVJ-E. However, the molecular mechanism of HVJ-E–induced apoptosis in cancer cells has not been fully investigated. Our data suggest that TNF-related apoptosis-inducing ligand (TRAIL) and Noxa represent promising target molecules for cancer cell–selective apoptosis when the retinoic acid–inducible gene I/mitochondrial antiviral signaling protein (RIG-I/MAVS) signaling pathway is activated. Clinical grade HVJ-E has already been applied in clinical trials for melanoma and castration-resistant prostate cancer in Japan. This article provides solid evidence for the clinical application of HVJ-E.

show that HVJ-E induces cancer cell–selective apoptosis by activating TNF-related apoptosis-inducing ligand (TRAIL) and Noxa in cancer cells via a signaling pathway involving a cytoplasmic helicase, retinoic-acid-inducible gene-I (RIG-1), and its adaptor, mitochondrial antiviral signaling protein (MAVS; also known as IPS-1, CARDIF, and VISA). The oncolytic activity of HVJ-E in vivo was clearly shown by the dramatic eradication of orthotopic prostate cancers in immunodeficient mice injected with HVJ-E.

Materials and Methods

Cells and mice

The hormone-resistant human prostate cancer PC-3 and DU145 cell lines, and the hormone-sensitive human prostate cancer cell line, LNCaP clone FGC cell line, the A549 human lung cancer cell line, and the MDA-MB-231 human breast cancer cell line were purchased from the American Type Culture Collection (ATCC). Human normal prostate epithelial cell lines, PNT1 and PNT2, were purchased from the European Collection of Animal Cell Cultures. The primary skin fibroblast cell, NSF1227, was established in our laboratory. PC3, A549, MDA-MB-231, and NSF1227 cells were maintained in Dulbecco’s modified Eagle’s medium (Nacalai Tesque Inc.), with 10% fetal bovine serum (FBS), and 100 μg/mL streptomycin (penicillin–streptomycin solution; Nacalai Tesque Inc.) in a humidified atmosphere of 95% air and 5% CO2. Nonobese diabetic–severe combined immunodeficient (NOD-SCID) mice, ages 6 weeks, were purchased from Japan Clea and were maintained in a temperature-controlled, pathogen-free room. All animals were handled according to the approved protocols and the guidelines of the Animal Committee of Osaka University (Osaka, Japan).

Preparation of HVJ-E

The HVJ (VR-105 parainfluenza 1 Sendai/52, Z strain from ATCC) was amplified in chorioallantoic fluid of 10- to 14-day-old chick eggs and was purified by centrifugation and inactivated by UV irradiation (99 ml/cm2) as described previously (26).

Evaluation of the viral RNA genome replication and viral protein synthesis of HVJ-E

To evaluate the viral RNA genome replication, cells in 6-well plates (6 × 10⁵ cells/well) were treated with live HVJ or HVJ-E (2 × 10⁵ particles), and the total RNA of cells was isolated after 12 and 24 hours. Using a primer set to detect the genomic RNA (nucleotide number 10786-10895) of HVJ (shown later), the amount of HVJ viral RNA was measured by real time-PCR (RT-PCR) and normalized to the amount of β-actin mRNA.

HVJ-genome forward primer (fp): 5'-GGCATAAGAGTTACTGCCCAGAA-3'
HVJ-genome reverse primer (rp): 5'-TGCACGCGCTATAGCTGTC-3'
To detect viral protein synthesis, cells in 12-well plates (2.4 × 10^3 cells/well) were treated with live HVJ or HVJ-E (8 × 10^8 particles), and the cells were harvested after 24 hours and lysed with sample buffer for a Western blot analysis as described later. To detect the F and HN proteins, a mouse monoclonal anti-F0 antibody F236 (a kind gift from Dr. Taira, Iwate University, Morioka, Japan; ref. 27) and a rabbit polyclonal anti-HN antiserum (isolated in our laboratory) were used, respectively.

Transfer of viral RNA or poly (I:C) to cells

The RNA from live HVJ or HVJ-E was isolated using a Gentra Purescript RNA Isolation Kit (Gentra Systems). Live HVJ or HVJ-E (∼10^13 particles) was lysed with Cell Lysis Solution, and the protein and DNA were removed with the Protein–DNA Precipitation Solution. Viral RNA was isolated using 100% isopropanol and the Gentra Glyogen Solution. Poly (I:C) was purchased from Sigma-Aldrich Japan, Inc. The RNA was transferred to cultured cells in 24-well cell culture plates by lipofection using Lipofectamine 2000 or Lipofectamine RNAiMAX (Invitrogen). After incubation for 4 hours, 100 µL of the cell culture medium was added into each well. These plates were thereafter incubated again at 37°C under 5% CO₂ for 24 or 48 hours.

SiRNAs (Noxa, TRAIL, IRF7, MAVS; Sigma-Aldrich Japan, Inc.) were transfected to cells using and the sequences were as follows:

- Human Noxa #1: 5'-AGCGCGUACACCUUGUGCGTT-3'
- Human Noxa #2: 5'-ACAGAGGCUCGUGAUGGUCAAGGUU-3'
- Human RIG-I #1: 5'-UGCAACAGGAGAGGCAUUCTT-3'
- Human RIG-I #2: 5'-UACAGCACCACCAGGAGUGTT-3'
- Human MAVS #1: 5'-ATGTCCATTGCCTGATTCTTTGTG-3'
- Human MAVS #2: 5'-GTAGTTTCGTGGATGCCACAG-3'
- Human IRF3: AACCUUGACCAUCACGAGCCUCUUG,
- Human TRAIL: 5'-CAUUCUUUGGGAUGAGAGATT-3'
- Human Noxa #1: 5'-CAUUCIUJIIUUGGGAAUGAGAGATT-3'
- Human Noxa #2: 5'-CGJHIJCIAUCAUJIIUGAGAGATT-3'
- Human RIG-I: 5'-GICUJICAAAGUGCAAGAuAIUJTT, 5'-AIAIUJCIGAUCJIIUGACACTT
- Human IRF3: 5'-ACCCJIIUGAGCAUCACCCJIIUG, CAAGAGGClJICUGAGUGGGCAACG
- Human IRF7: 5'-CGCCACAJIIUGCCJIIUCCJIIUGTT, 5'-AGCJCGJICJICAJIJUGGCJIIUGTT
- Human MAVS #1: 5'-GAUUGCJICJICAJIJUGUJUGCTT, 5'-UGCAACAJIGGACAGGGCAJIIUGTT
- Human MAVS #2: 5'-CACJICJICAJIJUGGCJIIUGJIIUJATT, 5'-LIAAGCJICJICAJIJUGGAGJIIUGTT

Human RIG-I #1: 5'-GAUUGCJICJICAJIJUGUJUGCTT, 5'-UGCAACAJIGGACAGGGCAJIIUGTT
- Human RIG-I #2: 5'-GAUUGCJICJICAJIJUGUJUGCTT, 5'-UGCAACAJIGGACAGGGCAJIIUGTT

Antibodies and Western blot analysis

- The anti-MAVS antibody (ab25084), anti-Noxa antibody (ab13654), and anti-CD11b antibody (ab8878) were purchased from Abcam Inc.
- Fluorescein isothiocyanate (FITC)-labeled anti-mouse CD49b/Pan NK cells antibody (DX5) was from BD Bioscience Pharmingen.
- The anti–Bcl-2 (50E3) antibody (#2870), anti–Bcl-xL (5H16) antibody (#2764), anti-Bax (D2E11) antibody (#5023), anti-Puma antibody (#4976), anti-TRAIL (C92B9) antibody (#3219), and anti–caspase-3 (8G10) antibody (#9665) were from Cell Signaling Japan Technology K.K.
- The anti-human p53 (DO-1) antibody, anti–IFN-regulatory factor 3 (IRF3; FL-425) antibody (sc-9082), anti-IRF7 (H-246) antibody (sc-9083), and anti–RIG-I (C-15) antibody (sc-48929) were from Santa Cruz Biotechnology, Inc.
- The anti–β-actin (AC-15) antibody (A5441) was from Sigma-Aldrich Japan, Inc.

The cell lysates were separated on polyacrylamide gels and transferred onto polyvinylidene difluoride (PVDF) membranes. Proteins were detected using the first antibodies described earlier followed by a horseradish peroxidase–linked secondary antibody (GE Healthcare Japan). Signals were detected with Chemi-Lumi one (Nacalai Tesque Inc.), according to the manufacturer’s instructions.

RNA extraction and quantification

RNA was extracted from cultured cells using an RNaseasy Mini Kit (Qiagen Japan), and 1 µg of total RNA was converted to cDNA with the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). The human TRAIL receptors (R1 and R2), TRAIL decoy receptors (DcR1 and DcR2), and β-actin were amplified by SYBR Premix Ex Taq (Takara Bio). All procedures were carried out according to the manufacturer’s instructions.

The primers were as follows:

- Human TRAILR1-fp: 5'-CAGAAACGTCCITGGAGCGCCGTAC-3'
- Human TRAILR1-rp: 5'-ATGTCCATITGGCAGCTTGTTTCTG-3'
- Human TRAILR2-fp: 5'-TGCTGACCGGTATGTCCTTGATTG-3'
- Human TRAILR2-rp: 5'-GCACCAAGTCTGCAAAGTCA-3'
- Human DcR1-fp: 5'-CCCTAAAGGTCTGCTGTATT-3'
- Human DcR1-rp: 5'-TTGGTGCCAGATATAGCTGGAGA-3'
- Human DcR2-fp: 5'-AAGTTGCTGCTTTCACTGTC-3'
- Human DcR2-rp: 5'-GATGGTGGAGAATTCAAC-3'
- Human β-actin-fp: 5'-GAGCTCAAGAGCTGCCAGAC-3'
- Human β-actin-rp: 5'-TATGGTTCGTGGATGCACACG-3'

MTS assay

A CellTiter 96 AQueous One Solution Cell Proliferation Assay Kit (Promega) was used to evaluate the cytotoxicity. After the transfection of siRNA or HVJ-E RNA, or after the treatment with HVJ-E, 100 µL of CellTiter 96 AQueous One Solution was added to each well, and the plates were incubated for 1 hour at 37°C. After the replacement of the incubation medium, the absorbance at 490 nm was measured in each well with a 96-well plate reader.
TUNEL assay and immunofluorescence of p53

Equal numbers of PC3 and PNT2 cells were mixed and were seeded at 5 x 10^4 cells per well on 8 chamber polystyrene vessel tissue culture–treated glass slides. The cells were incubated for 48 hours and then were treated with HVJ-E [multiplicity of infection (m.o.i.) 1,000] or PBS. Forty-eight hours after HVJ-E treatment, the apoptotic cells were evaluated. The cells were washed with PBS, and fixed with 4% paraformaldehyde for 15 minutes, followed by treatment with permeabilization buffer. The terminal deoxyribonucleotide transferase–mediated dUTP nick-end labeling (TUNEL) assay was carried out using an in situ apoptosis detection kit (Takara Bio) according to the manufacturer’s protocol. Next, immunohistochemical staining was carried out using the LSAB System-HRP (Dako), according to the manufacturer’s instructions. The cells were incubated overnight at 4°C with the anti-p53 antibody diluted in Dako REAL Antibody Diluent (Dako) at a 1:50 concentration. The cells were washed and labeled with a 1:500 dilution of Alexa Fluor 568 antibody (Invitrogen) at room temperature for 1 hour.

Treatment of prostate cancer in an orthotopic tumor model

NOD-SCID mice were anesthetized by an inhalation of isoflurane. Viable PC3 cells (8 x 10^4 cells) were resuspended in 20 µL of PBS and injected into the right dorsal lobe of the prostate of the mice as previously described (28). Ten days after tumor inoculation, when tumors were microscopically visualized, the mice were anesthetized, and PBS (40 µL) with or without HVJ-E (5 x 10^10 particles) was injected into the right dorsal lobe of the prostate. The mice were sacrificed on day 31 after tumor inoculation, and the tumors were harvested and fixed in 20% C2/C2 L of PBS and injected into the right dorsal lobe of the prostate of the mice as previously described (28).

Results

Neither viral genome replication nor viral protein synthesis was involved in cancer-selective apoptosis induced by UV-irradiated HVJ-E

To show the inactivation of UV-irradiated HVJ-E, viral genome replication and viral protein synthesis were examined in nonmalignant prostate epithelial cells (PNT2), and castration-resistant prostate cancer (PC3) cells, treated with either HVJ-E or live HVJ for 1 hour (Fig. 1A). Because both cell lines express the HVJ receptor gangliosides (23, 29), the viral RNA levels were increased in both cell lines at 12 and 24 hours following treatment with live HVJ. However, no increase in viral RNA levels between 12 and 24 hours were detected by RT-PCR in the HVJ-E–treated cells. Neither the viral F protein nor the HN protein was detected in either the PNT2 or PC3 cells treated with HVJ-E, whereas both proteins were clearly detected in both cell lines infected with live HVJ (Fig. 1B). Similar results were obtained in other cancer cell lines, such as the murine colon cancer cell line CT26 and the murine renal cancer cell line RENCA (data not shown). These results indicated that HVJ-E lacks the ability to induce viral genome replication and viral protein synthesis.

Next, the sensitivity of PC3 cells to HVJ-E was compared with that of live HVJ (Fig. 1C). The survival of PC3 cells was not affected by HVJ-E at a m.o.i. of 1 or 10, and the suppression of cell survival was first observed at a m.o.i. of 100. The suppression of PC3 viability induced by HVJ-E was lower than that induced by live HVJ; live HVJ decreased PC3 viability beginning at a m.o.i. of 1 and 10 at only 72 hours postinfection. However, the effects of HVJ-E were comparable with those of live HVJ at a m.o.i. of 100 or 1,000 at all of the time points tested. At a m.o.i. of 10,000, HVJ-E was slightly more effective with regard to cancer cell killing compared with live HVJ.

These results suggest that the mechanism for the oncolytic induction by HVJ-E does not involve viral genome replication or viral protein synthesis.

To clearly show the cancer cell–selective apoptosis induced by HVJ-E, HVJ-E was added to a mixed culture of PC3 and PNT2 cells. The expression of p53 was detected in the PNT2 cells but not in the PC3 cells (Fig. 1D), and dual p53-immunofluorescence and TUNEL staining showed that the TUNEL-positive apoptotic cells were exclusively p53-negative (Fig. 1E). From these results, HVJ-E induced apoptosis in the PC3 cells but not in the PNT2 cells. A higher magnification of the mixed culture revealed that the PNT2 cells were not apoptotic, although fusion did occur (arrowheads in Fig. 1F). In the PC3 culture, many unfused cells and a few fused cells were TUNEL-positive. This finding suggests that the formation of syncytia by cell–cell fusion via HVJ-E is not necessary for the induction of apoptosis. Apoptosis was also induced by HVJ-E in another human prostate cancer cell line, DU145, but not in NSF1227 human primary skin fibroblasts (Supplementary Fig. S1).

Viral RNA fragments of HVJ-E suppressed the viability of prostate cancer cells via the RIG-I/MAVS pathway

These findings prompted us to investigate which components of HVJ-E are required for the induction of apoptosis. As the intact HVJ genome is recognized by the cytoplasmic RNA receptor RIG-I (30–32), we hypothesized that RNA fragments from HVJ-E would also stimulate the RIG-I pathway. The size of the intact RNA genome of live HVJ is approximately 15 kb (33). Following UV irradiation, the RNA genome was broken, and no intact genome RNA was
Figure 1. Nonreplicating HVJ-E suppressed the survival of PC3 cells as efficiently as replicating live HVJ, and selective apoptosis was induced in PC3 cells by HVJ-E in a mixed culture of PC3 and PNT2 cells. A, PNT2 cells (top graph) and PC3 cells (bottom graph) were treated with either HVJ-E or live HVJ at a m.o.i. of 3,000 for 1 hour. The expression levels of genomic RNA corresponding to HVJ (nucleotide number 10786-10895) and cellular β-actin in these cells were measured via RT-PCR at 12 and 24 hours postinfection. A relative expression level of "1" indicates the ratio of viral RNA to β-actin RNA at 12 hours after HVJ-E treatment. The mean ± SD (n = 3) is shown. *, significance at P < 0.01. B, the expression of the HVJ proteins F (F0) and HN and cellular β-actin in both PNT2 cells (top blot) and PC3 cells (bottom blot) were assessed by Western blot analysis 24 hours postinfection. –, an uninfected sample. C, the survival of PC3 cells was assessed using the MTS assay at 24, 48 and 72 hours after treatment with either HVJ-E or live HVJ using 5 different m.o.i. values (1, 10, 100, 1,000, and 10,000). Each value (mean ± SD, n = 3) of survival is the ratio of the value without treatment at each time point. Three independent experiments were carried out, and similar results were obtained. *, significance at P < 0.05. D, p53 expression was assessed via Western blot analysis in PC3 and PNT2 cells. E, the immunofluorescence detection of p53 and apoptosis-mediated DNA fragmentation (TUNEL assay) in the mixed culture of PC3 and PNT2 cells 48 hours after HVJ-E treatment. "Control" indicates cells without HVJ-E treatment. The nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI). The phase contrast images of the same field as the immunofluorescence images are also shown (×200). F, a higher magnification (×400) of the mixed culture revealed that the PNT2 cells were TUNEL-negative although fusion had occurred (arrowheads). These results are representative of 2 independent experiments.
detected (Fig. 2A). When the intact RNA isolated from live HVJ was transferred to PC3 cells, their viability was suppressed in a dose-dependent manner; in contrast, the viability of PNT2 cells was not affected by this RNA transfer (Fig. 2B). Viral RNA fragments of HVJ-E were then transferred to the PC3 and LNCaP prostate cancer cell lines and the nonmalignant prostate epithelial cell lines PNT1 and PNT2 via lipofection (Fig. 2C). The viability of these cancer cell lines was reduced by the transfer of the HVJ-E RNA fragments in a dose-dependent manner, whereas the viability of the PNT1 and PNT2 cells was not affected. When poly (I:C), which is recognized by melanoma differentiation-associated gene 5 (MDA5; ref. 31) was transferred, the survival of both the PC3 and LNCaP cells was suppressed to a lesser degree than with HVJ-E RNA. Poly (I:C) also suppressed cell survival in the PNT1 and PNT2 cells (Supplemental Fig. S2).

To examine the involvement of the RIG-I/MAVS pathway in the recognition of exogenous RNA, RIG-I and MAVS siRNAs were transferred into PC3 cells, to suppress the expression of RIG-I and MAVS, respectively (Fig. 3A). When HVJ-E was added to these cells, the suppression of cell
viability was significantly attenuated in both the RIG-I siRNA- and MAVS-siRNA–transfected PC3 cells (Fig. 3B).

HVJ-E–mediated TRAIL and Noxa induction via the RIG-I/MAVS pathway decreased the viability of prostate cancer cells

We next examined the effector molecules responsible for apoptosis induced by HVJ-E. We extensively evaluated the expression levels of proapoptotic and antiapoptotic genes in HVJ-E-treated PC3 and PNT2 cells using cDNA microarrays, RT-PCR and Western blot analysis. As shown in Fig. 4A, TRAIL and Noxa were upregulated by HVJ-E in PC3 cells but not in PNT2 cells in a time- and dose-dependent manner. The expression of other proapoptotic and antiapoptotic genes, such as Bax, Puma, Bcl-xL, and Bcl-2 was not significantly affected by HVJ-E (Supplementary Fig. S3). The transfer of RNA fragments from HVJ-E into PC3 cells using cationic liposomes also upregulated the expression of both TRAIL and Noxa (Fig. 4B). Similar results were obtained by transferring RNA purified from live HVJ.

The effects of Noxa and TRAIL on HVJ-E–induced apoptosis in PC3 cells were examined via knockdown experiments using siRNA (Fig. 4C). TRAIL siRNA but not Noxa siRNA significantly inhibited HVJ-E–induced apoptosis in cancer cells. Because RIG-I and MAVS were involved in the observed HVJ-E–induced apoptosis (Fig. 4B), RIG-I siRNA and MAVS siRNA were transfected into PC3 cells, respectively, and the HVJ-E–induced expression of TRAIL was subsequently examined. As shown in Fig. 4D, the HVJ-E–induced expression of TRAIL was inhibited by the transfer of either siRNA into the cells. When another cytoplasmic helicase, MDA5, was suppressed using MDA5 siRNA, HVJ-E–induced TRAIL expression was not affected (data not shown).

Another advantage of inducing TRAIL expression for tumor suppression is that the expression of TRAILR1 (DR4) and TRAILR2 (DR5), which are involved in death-signal transduction (34, 35), was high in PC3 cells but not in PNT2 cells, whereas the TRAIL decoy receptors DcR1 and DcR2, which bind to TRAIL in the absence of signal transduction (34, 35), were highly activated in PNT2 cells but not in PC3 cells (Supplementary Fig. S4).

We next examined the molecular mechanisms of HVJ-E–induced apoptosis in other human cancer cell lines. These other human cancer cells, including the castration-resistant DU145 human prostate cancer cell line, A549 lung cancer cell line, and MDA-MB-231 mammary carcinoma cell line, were also sensitive to HVJ-E treatment (Fig. 5A). The induction of TRAIL and Noxa by HVJ-E was detected in the A549 and MDA-MB-231, but not in the DU145 cells, whereas Noxa was upregulated in all of the cancer cell lines tested (Fig. 5B). In the A549 and MDA-MB-231 cells, TRAIL siRNA significantly attenuated the suppression of cell viability induced by HVJ-E (data not shown). In the DU145 cells, Noxa siRNA significantly inhibited HVJ-E–induced cell death (Fig. 5C).

The transcription factors for Noxa and TRAIL are IRF3 and IRF7, respectively (36, 37), both of which are regulated by MAVS (38). HVJ-E–induced cell death was suppressed by IRF3 siRNA in DU145 cells and by IRF7 siRNA in PC3 cells (Supplementary Fig. S5). The IRF3 siRNA and IRF7 siRNA were not effective in suppressing HVJ-E–induced cell death.
in the PC3 and DU145 cells, respectively. These findings are consistent with our conclusion that the effector molecules for HVJ-E–induced apoptosis are Noxa in DU145 cells and TRAIL in PC3 cells (Figs. 4 and 5).

**Eradication of orthotopic prostate cancers by HVJ-E in immunodeficient mice**

Finally, to show the oncolytic activity of HVJ-E in vivo, we constructed an orthotopic prostate cancer model in immunodeficient NOD-SCID mice, and injected HVJ-E directly into the mouse prostate after tumor formation. As shown in Fig. 6A and B, tumor growth was extensively suppressed by a single injection of HVJ-E and the tumors disappeared in 7 of 10 mice, whereas a large tumor mass occupied the prostate in all of the tumor-bearing mice that did not receive HVJ-E treatment. Histologic analysis showed that the infiltration of CD11b-positive cells (Fig. 6D) but not CD49b-positive NK cells (Fig. 6E) was weakly detected in the tumor of the prostate injected with either HVJ-E or PBS. Although tubuloalveolar glands were histologically observed in the prostate injected with either HVJ-E or PBS (Fig. 6C), TUNEL-positive apoptotic cells were detected exclusively in the tumor treated with HVJ-E (Fig. 6F). Therefore, numerous small nuclei in the tumor of the prostate injected with HVJ-E (Fig. 6F) may be degraded nuclei of apoptotic cells. Similar results were observed in subcutaneous tumors treated with HVJ-E in NOD-SCID mice (Supplementary Fig. S6).

Thus, replication-incompetent HVJ-E effectively eradicated castration-resistant prostate cancers in vivo in an immunodeficient mouse model.

**Discussion**

In this study, we show that a nonreplicating Sendai virus particle called HVJ-E induces cancer-selective apoptosis via the upregulation of TRAIL and Noxa downstream of the RIG-I/MAVS pathway.
There are numerous reports describing oncolysis mediated by replication-competent viruses (5–20). Replication is required for cancer cell killing induced by both live and attenuated viruses, and the oncolytic ability of these viruses is lost following UV inactivation (18, 19). However, UV-irradiated HVJ-E displays oncolytic activity without viral genome replication and viral protein synthesis. One of the reasons that live HVJ fails to induce apoptosis more effectively than HVJ-E may be that C-protein is expressed in HVJ-infected cells but not in HVJ-E–treated cells (data not shown); C-protein is synthesized from the viral RNA genome to prevent the apoptosis of infected cells via the inhibition of Stat (39). It is also likely that the presence of the inactive form of the fusion protein, F0, expressed on the envelope of viral progeny produced from infected cells results in the inability of the progeny to infect cells (33). Therefore, HVJ infection is not spread to other cells unless F0 is converted to F1 via proteolytic cleavage as has been previously reported in rodent respiratory tissue (40).

The antitumor activities of HVJ-E have been reported in our previous publications (21–25). These activities include the activation of antitumor immunity and the induction of oncolysis. Although the activation mechanism of antitumor immunity has been elucidated (21–23), the precise mechanism of HVJ-E–induced oncolysis has not been extensively examined. In this article, we reveal that the activation of the RIG-I/MAVS pathway mediated by HVJ-E triggers

Figure 5. Effect of the HVJ-E–induced expression of Noxa and TRAIL on cell death in various cancer cell lines. A, the survival of castration-resistant DU145 prostate cancer cells, A549 lung cancer cells, and MDA-MB-231 mammary carcinoma cells was assessed via MTS assay 48 hours after treatment with HVJ-E at a m.o.i. of 0, 100, 1,000, or 10,000. The mean values ± SD (n = 3) are shown. B, the expression of TRAIL and Noxa was examined via Western blot analysis in DU145, A549, and MDA-MB-231 cells treated either with or without HVJ-E. The blots are representative of 2 independent experiments. C, using a method similar to that described in Fig. 4C, the effect of Noxa siRNA #1 was examined in DU145 cells. Noxa expression in siRNA-transfected DU145 cells was assessed via Western blot analysis following treatment either with or without HVJ-E. For the MTS assay, each value (mean ± SD, n = 4) of the survival rate is the ratio of the value without HVJ-E treatment. The blot and MTS assay results are representative of 2 independent experiments. *: significance at P < 0.05.
cancer cell–selective apoptosis. The activation of immune reactions following HVJ infection has been reported to be Toll-like receptor–independent (41). Instead, the cytoplasmic RNA receptor RIG-I recognizes the RNA of some viruses, such as HVJ, influenza virus, vesicular stomatitis virus (VSV), and the rabies virus, in addition to synthetic RNA and induces an immune response (30–32, 42, 43). Although the mechanism by which RNA fragments of the UV-irradiated HVJ genome are recognized in the cytoplasm has not been shown, our results suggest that the viral RNA fragments can be recognized by RIG-I. In addition to RIG-I, another cytoplasmic helicase, MDA5, also recognizes exogenous RNAs, such as poly (I:C) (30). However, HVJ-E–induced TRAIL expression was not affected by MDA5 siRNA (data not shown). These results suggest that the HVJ-E RNA fragments are likely recognized by RIG-I. Moreover, as shown in Supplementary Fig. S2, poly (I:C) was not significantly effective in cancer cell–selective killing as compared with HVJ-E–RNA.

The RIG-I/MAVS signaling pathway has been extensively investigated in immune cells (30–32) but not in cancer cells. Besch and colleagues reported that synthetic RNA induces type I IFN-independent apoptosis in human melanoma cells via RIG-I and MDA5 activation (44). According to their analysis, proapoptotic molecules, such as Puma and Noxa, were activated by poly (I:C) or 5'-triphosphate–conjugated RNA transcribed in vitro in both melanoma cells and nonmalignant skin cells, and the expression of the antiapoptotic molecule, Bcl-xL, was induced extensively in nonmalignant cells. Therefore, they concluded that melanoma-specific apoptosis occurs via a cytoplasmic RNA receptor pathway. However, in our experiments, the expression of antiapoptotic genes including Bcl-xL and proapoptotic molecules, such as Puma and Bax, was unchanged following HVJ-E treatment in both PC3 and PNT2 cells (Supplementary Fig. S3). Instead, we found that the expression of TRAIL and Noxa was activated selectively in prostate cancer cells, lung cancer cells, and breast cancer cells.

Figure 6. Extensive reduction of orthotopic prostate tumors following a single injection of HVJ-E into the prostate of immunodeficient mice. PBS (40 µL) with or without HVJ-E (5 × 10^10 particles) was injected into the prostate of NOD-SCID mice on day 10 after the inoculation of viable PC3 cells (8 × 10^5) into the prostate. Each prostate was prepared on day 31 after the inoculation of the PC3 cells. Arrowheads indicate the tumor mass, and the arrows indicate the site of the cancer cell injection. B, the tumor volume (mean ± SD) is shown for the HVJ-E–injected group (n = 10) and the control (without HVJ-E) group (n = 6). *, significance at P < 0.01. For histochemical analysis, either HVJ-E or PBS was injected into the prostate on day 29 after the inoculation of viable PC3 cells into the prostate. Two days after the injection of either HVJ-E or PBS, tumor sections were prepared for hematoxylin-eosin staining (C), immunologic staining using anti-CD11b (D), or anti-CD49b/Pan-NK cells antibody (E) and TUNEL staining (F).
downstream of RIG-I and MAVS. The genes responsible for the cancer cell–specific apoptosis downstream of the RIG-I/MAVS pathway may therefore vary among cancers. Another possibility is that stimulation by fusion-mediated RNA transfer, as with HVJ-E, may vary from liposome-mediated RNA transfer.

The RIG-I/MAVS pathway is known to induce type I IFN (30–32). In our experiments, IFN-β was secreted from the PC3 cells but not from the DU145, A549, MDA-MB-231, and PNT2 cells following HVJ-E treatment. IFN-α was not significantly produced in these cell lines by HVJ-E treatment. Therefore, we conclude that HVJ-E–induced apoptosis occurs independently of IFN production as suggested by Besch and colleagues (44) and our previous report (22), although IFN-β may augment HVJ-E–induced cancer-cell death via the upregulation of RIG-I (21). In both orthotopic and subcutaneous tumors, CD11b-positive cell infiltration was weakly detected following either HVJ-E or PBS. However, TUNEL-positive apoptotic cells were detected exclusively in tumors treated with HVJ-E (Fig. 6 and Supplementary Fig. S6). Therefore, we estimated that the eradication of tumors by HVJ-E in NOD-SCID mice likely resulted from the direct cancer-selective killing activity of HVJ-E.

When the expression of both TRAIL and Noxa was induced, cell death was suppressed by TRAIL siRNA but not Noxa siRNA (Fig. 4). This result suggests that a TRAIL–TRAIL receptor pathway may be more closely involved in inducing apoptosis than the Noxa pathway, because TRAIL activates both the extrinsic (via the death receptor) and intrinsic (via mitochondrial membrane permeability) apoptosis pathways (34, 35), whereas Noxa only affects the intrinsic apoptosis pathway (45, 46).

Our results and those reported in the previous study by Besch and colleagues (44) indicate that increased attention should focus downstream of the cytoplasmic helicase/MAVS pathway in terms of cancer cell–selective killing, which has long been considered to represent the ideal method for cancer therapy. Further studies should investigate the molecular mechanism of HVJ-E–induced proapoptotic gene expression that occurs selectively in cancer cells. Although it is impossible to clearly address this issue, our current hypothesis is that the promoter region of Noxa or TRAIL might be destabilized by the binding of HVJ-E–activated transcription factors, such as IRF3 and IRF7, so that it is released from the repressor complex as suggested elsewhere (47). The destabilization of the promoter region might occur more readily in cancer cells than in noncancerous cells due to the alteration of chromatin in cancer cells (48).

The induction of cancer cell apoptosis mediated by inactivated viral particles may not be restricted to HVJ-E. As cytoplasmic helicases and the adaptor MAVS seem to be essential for inducing cancer cell–selective apoptosis (ref. 44 and the present report), other RNA viruses, such as measles, mumps, vaccinia and NDV, should also induce apoptosis in cancer cells even if they are inactivated. However, UV-inactivated NDV has been reported to display reduced cytopathic effects in prostate cancer cell lines (18) and an UV-inactivated measles virus failed to suppress tumor growth in a Nalm-6 acute lymphoblastic leukemia xenograft model (19). The results shown in Fig. 1C suggest that the major differences between inactivated NDV and HVJ-E treatment are in the amount of viral particles used in each experiment. The concentration of inactivated HVJ-E particles used in this study was approximately 1,000 times higher than the inactivated NDV used in the previous report (18). If other RNA viruses were used in cell survival assays at a m.o.i. of 1,000 after inactivation, cancer cell–selective apoptosis may be induced as with HVJ-E.

Nevertheless, there is an advantage for the application of HVJ-E because high concentrations of HVJ are easily obtained from fertilized chicken eggs (~2 × 10^{14} particles per egg), and we have already succeeded in producing increased concentration of HVJ (~4 × 10^{14} particles from a 10 L culture) from a cultured human cell line, HEK293, and have prepared GMP grade HVJ-E (49). On the basis of the promising results of the preclinical toxicity tests using rodents and nonhuman primates, a clinical trial for the treatment of melanoma involving the direct injection of HVJ-E has commenced in Japan. The safety and efficacy of HVJ-E are being evaluated in patients with melanoma with the administration of 12 intratumoral injections of approximately 1.5 × 10^{11} or 5 × 10^{11} particles of HVJ-E.

Our present results suggest that the regulation of molecules downstream of the cytoplasmic helicase/MAVS pathway may vary between cancer cells and nonmalignant cells. About drug discovery, the cytoplasmic helicase/MAVS signaling pathway may be an effective target for generating novel cancer-selective therapies. Moreover, with regard to basic cancer research, further analysis of the chromatin state proximal to the genomic regions affected by cytoplasmic helicase/MAVS signaling may elucidate the differences between malignant and nonmalignant cells and provide novel insight about tumorigenesis.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

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Acknowledgments

The authors thank Dr. Yasutomo Nasu, Okayama University, for kindly teaching us the method to establish the orthotopic prostate cancer mouse model.
Grant Support

This study was supported by the Program for Promotion of Fundamental Studies in Health Sciences of the National Institute of Biomedical Innovation (Project ID: 10-03).

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Received May 16, 2012; revised September 12, 2012; accepted September 12, 2012; published OnlineFirst September 26, 2012.

References


Clinical Cancer Research

TRAIL and Noxa Are Selectively Upregulated in Prostate Cancer Cells Downstream of the RIG-I/MAVS Signaling Pathway by Nonreplicating Sendai Virus Particles


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