Accumulation of Cytosolic Calcium Induces Necroptotic Cell Death in Human Neuroblastoma

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Abstract
Necrosis has been studied extensively since the early days of medicine, with some patterns of necrosis found to be programmed like apoptotic cell death. However, mechanisms of programmed necrosis (necroptosis) are yet to be fully elucidated. In this study, we investigated how the hemorrhagglutinating virus of Japan-envelope (HVJ-E) induces necrosis in mouse xenografts of human neuroblastoma cells. HVJ-E–induced necrosis in this system was found to depend on phosphorylation of the death receptor kinase receptor interacting protein kinase 1 (RIP1) and on the production of reactive oxygen species. This process was interpreted as necroptosis, based on its suppression by the small molecule necrostatin-1, and it did not involve the TNF-α receptor pathway. We also demonstrated that increased concentrations of cytoplasmic calcium triggered necroptosis by activating calcium-calmodulin kinase (CaMK) II. Finally, we determined that RIP1 phosphorylation was mediated by CaMK II activation. Together, our results define an upstream pathway for the activation of necroptosis in neuroblastoma cells, with potential therapeutic implications. Cancer Res; 74(4); 1056–66. ©2013 AACR.

Introduction
The way in which a cell dies is decided according to the death stimuli and the endogenous expression level of death signaling effectors. Among the different mechanisms of cell death, there is much more information on apoptosis than necrosis, pyroptosis, or autophagy. Apoptosis is a well-known form of programmed cell death induced by the activation of caspase-8 or -9. Pyroptosis is also a form of caspase-dependent cell death like apoptosis, but the death stimuli are different from apoptosis. In contrast to apoptosis, caspase-1 is activated during the process of pyroptosis by the formation of inflammasome complex, which is induced by the recognition of Salmonella and Shigella species (1). In addition, a mechanism of cell death was recently identified that is morphologically necrotic but is induced by the same stimuli as apoptosis. This programmed necrotic cell death, which is referred to as necroptosis, is thought to be induced by apoptotic death stimuli, such as TNF-α and Fas ligand. The signal transduction for necroptosis is known to be caspase independent, and its mechanism is as follows: when a ligand binds to the death receptor in the cell, receptor interacting protein kinase 1 (RIP1) is deubiquitinated by CYLD and forms a complex with receptor interacting protein kinase 3 (RIP3), TNF receptor–associated death domain (TRADD), Fas-associated death domain (FADD), and caspase-8. Most human cells express caspase-8, which suppresses RIP1 or RIP3, but if the endogenous expression of caspase-8 is absent, RIP1 remains activated via phosphorylation of serine 161 (2–8). A necosome consisting of RIP1, RIP3, FADD, and TRADD induces mitochondrial complex I–mediated reactive oxygen species (ROS) production, which is an executor of necroptosis (2, 9). However, the upstream actions of the necrotic signaling pathway following death receptor stimulation, i.e., the mechanism by which RIP1 is phosphorylated, have not yet been clarified.

Neuroblastoma is one of the most common malignant solid tumors in children and is responsible for 12% of deaths associated with childhood cancer (10). Many genetic features and prognostic factors of neuroblastoma have been revealed. Moreover, most aggressive neuroblastoma cells reportedly do not express caspase-8 (11, 12), a key molecule in the extrinsic pathway of apoptosis. We recently reported a novel strategy for treating aggressive neuroblastoma with UV-treated, nonreplicating Sendai virus [also known as hemorrhagglutinating virus of Japan-envelope (HVJ-E)] particles (13). We also reported that HVJ-E induces necrosis in the xenografts derived from human neuroblastoma cells in severe combined immunodeficient (SCID) mice (13), but the mechanism of this necrotic cell death remains to be solved. Therefore, we reasoned that human neuroblastoma cell lines would be the ideal experimental materials for investigating the mechanism of necroptosis.

In this study, our first objective was to confirm whether the HVJ-E–induced cell death of human neuroblastoma cells is necroptosis, and our second objective was to investigate the
mechanism of RIP1 activation during necroptosis in cancer cells. We concluded that caspase-8-deficient cancer cells, like most neuroblastoma cells, are induced to undergo necroptosis by HVJ-E. The mechanism by which this occurs involves the phosphorylation of RIP1 by calcium-calmodulin kinase (CaMK) II, which is activated by membrane fusion with HVJ-E.

Materials and Methods

Cell lines

The human neuroblastoma cell lines SK-N-SH and SK-N-AS were obtained from the European Collection of Cell Cultures, and the monkey kidney cell line LLCMK2 and human prostate cancer cell line PC3 were from the American Type Culture Collection (ATCC). The SK-N-SH, SK-N-AS, and PC3 cells were maintained in Dulbecco's Modified Eagle Medium (DMEM; Nacalai Tesque Inc.), and the LLCMK2 cells were maintained in Minimum Essential Medium (Gibco-BRL). All media was supplemented with 10% FBS, 100 IU/mL penicillin, and 100 µg/mL streptomycin. The cells were incubated at 37°C in a humidified atmosphere of 95% air and 5% CO₂.

Preparation of HVJ-E

HVJ (VR-105 parainfluenza1 Sendai/52, Z strain from the ATCC) was amplified in the chorioallantoic fluid of 10- to 14-day-old chicken eggs and then purified by centrifugation and inactivated by UV irradiation (99 mJ/cm²), as previously described (14). The inactivated virus lost its ability to engage in genome replication and protein synthesis but retained its membrane fusion activity (14).

Generation of WT-HVJ-E, ΔHN–HVJ-E, and F₁/F₂-WT-HVJ-E

The majority of the experimental procedures have been previously reported (15). For wild-type (WT)–HVJ-E, the culture medium of HVJ-infected LLCMK2 cells was passed through a filter and then centrifuged at 100,000 × g for 2 hours at 4°C to precipitate the WT-HVJ particles. WT-HVJ was inactivated by UV irradiation. For Δ hemagglutinin neuraminidase (HN)–HVJ-E, the WT-HVJ particles were heated at 65°C for 30 minutes, thus denaturing the HN protein. F₁/F₂-WT-HVJ-E was prepared by treating WT-HVJ-E with 5 µg/mL trypsin for 30 minutes at 37°C.

Transmission electron microscopy

Cells were fixed with 2.5% glutaraldehyde in 0.1 mol/L phosphate buffer (pH 7.4) at 4°C and postfixed in 1% OsO₄ solution at 4°C for 1 hour. The samples were then dehydrated in a graded ethanol series and embedded in Quetol 812 epoxy resin (Nissin EM). Ultrathin sections (80 nm) were cut on a Reichert ultramicrotome (Ultracut E; Leica Microsystems), stained with uranyl acetate and lead citrate, and examined with a Hitachi H-7650 electron microscope (Hitachi).

Cell proliferation assay

A MTS assay was performed with the CellTiter 96 AQueous One Solution Cell Proliferation Assay Kit (Promega) to evaluate cell viability. Cells were seeded in 24-well plates (5 × 10⁴ cells per well in 500 µL of medium). Twenty-four hours later, the cells were treated with HVJ-E [multiplicity of infection (MOI) of 100–10,000], 0.1 to 10 ng/mL of human recombinant TNF-α (BD Biosciences), or 0.1 to 10 µmol/L of the calcium ionophore A23187 (Sigma-Aldrich Japan, Inc.). Twenty-four hours after this treatment, 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 the incubation medium from each well to a new 96-well plate, the absorbance was measured at 490 nm.

Western blotting

Anti-caspase-3, anti-caspase-8, anti-caspase-9, anti-PARP, anti-RIP1, anti-TNFFR1, anti-pan-CaMK II, and anti-phospho-CaMK II antibodies were purchased from Cell Signaling Japan Technology K.K., and the anti-β-actin antibody was purchased from Sigma-Aldrich Japan, Inc. The anti-RIP3 antibody was purchased from Santa Cruz Biotechnology, Inc. The anti-HN and F-antibodies were from Scrum Inc. Horseradish peroxidase–conjugated donkey anti-rabbit IgG (GE Healthcare) was used as the secondary antibody for the detection of caspase-3, caspase-9, PARP, RIP1, TNFFR1, pan-CaMK II, phospho-CaMK II, HN, and F. Horseradish peroxidase–conjugated sheep anti-mouse IgG (GE Healthcare) was used as the secondary antibody for the detection of caspase-8 and β-actin. Horseradish peroxidase–conjugated chicken anti-goat IgG (R&D Systems) was used as the secondary antibody for the detection of RIP3. Cell lysates were separated using polyacrylamide gels, and the proteins were transferred onto polyvinylidene difluoride membranes. The membranes were then blocked with blocking buffer (TBS containing 0.1% Tween 20 and 3% skim milk) for 1 hour and incubated with primary antibody in blocking buffer at 4°C overnight. The membranes were then washed with washing buffer (TBS containing 0.1% Tween 20) and incubated with the horseradish peroxidase–conjugated anti-mouse, anti-goat, or anti-rabbit secondary antibody (GE Healthcare) in blocking buffer for 1 hour. Signals were detected with ChemiLumi One (Nakalai Tesque Inc.) according to the manufacturer's instructions.

Measurement of ROS production

ROS generation was detected using a fluorescence probe called MitoSOX, which detects mitochondrial superoxide production. The cells were pretreated with 5 µmol/L MitoSOX at 37°C for 10 minutes according to the manufacturer's instructions.

Estimation of the intracellular Ca²⁺ concentration

The intracellular Ca²⁺ concentration was estimated with the Fura 2 Kit (DOJINDO) according to the manufacturer's protocol. Briefly, SK-N-SH cells (1 × 10⁶ cells) were seeded in a 96-well plate and incubated overnight. Then, the medium was removed, and the manufacturer's loading buffer was added to the cells. The cells were incubated at 37°C for 1 hour, and the buffer was replaced with recording buffer containing Fura 2-acetoxyethyl ester. Next, 1,000 MOI of HVJ-E with WT HN–inactivated F (WT.HN-F0) or WT HN-activated
F (WT.HN-F1/F2) were added to each well, and the resulting fluorescence intensity was estimated by recording the absorbance at the peak excitation wavelength of 340/380 nm and the peak emission wavelength of 510 nm.

**Detection of apoptosis and necrosis**

Following treatment with HVJ-E or A23187, SK-N-SH cells or the removed xenografts were fixed with 4% paraformaldehyde, and apoptosis or necrosis was detected with the GFP-Certified Apoptosis/Necrosis Detection System Kit (Enzo Life Science Inc.) according to the manufacturer’s protocol.

**Pretreatment of cells with inhibitors**

To inhibit the activity of pan-caspase, 20 μmol/L Z-VAD-FMK (Medical & Biological Laboratories) was added to the cells 1 hour before treatment with HVJ-E. To inhibit the activity of RIP1, 20 μmol/L necrostatin-1 (Enzo Life Science Inc.) was added to the cells 24 hours before exposure to HVJ-E. To inhibit the increase of cytoplasmic Ca2+, 10 μmol/L BAPTA-AM (Sigma-Aldrich Japan, Inc.) was added to the cells 30 minutes before exposure to HVJ-E or A23187. To inhibit the activity of CaMK II, 20 μmol/L CK59 (EMD Millipore) was added to the cells 1 hour before HVJ-E exposure.

**Cell transfection with siRNA**

SiGENOME SMARTpool Human RIP1 (M-004445-02-0005; Thermo Fisher Scientific), siGENOME SMARTpool Human RIP3 (M-005334-01-0005; Thermo Fisher Scientific), or ON-TARGET plus Human CAMK II α siRNA SMARTpool (L-004942-00-0005; Thermo Fisher Scientific) was used to transfect SK-N-SH cells. The siGENOME Non-Targeting siRNA Pool (D-001206-13-05; Thermo Fisher Scientific) was used as a control. The siRNA transfection was performed as follows: siRNA and Lipofectamine RNAimax (Invitrogen) were mixed in Opti-MEM and incubated for 20 minutes at room temperature, and then the mixture was added to cultured SK-N-SH cells in 6- or 24-well plates. The cells were incubated for 4 hours at 37°C, and the medium was exchanged with DMEM. The siRNA dosage was 100 or 20 pmol/well. The knockdown efficiency was confirmed by Western blotting at 72 hours after transfection.

**Labeling of cancer cells with 32P-orthophosphate and immunoprecipitation**

SK-N-SH cells [1 × 10⁶ cells per immunoprecipitation (IP) sample] were seeded 1 day before the labeling experiment, resuspended in phosphate-free medium, and incubated for 40 minutes at 37°C. The cells were centrifuged and resuspended in phosphate-free medium containing 0.1 μCi of 32P-orthophosphate (Perkin Elmer, Inc.) and incubated for 2 hours before stimulation with 1,000 MOI of HVJ-E for 2 hours. In some experiments, 20 μmol/L necrostatin-1 or 20 μmol/L CK-59 was added to the cells before HVJ-E stimulation. The cells were treated with HVJ-E for 2 hours before cell lysis.

For immunoprecipitation, the cell lysates were preclreated using 5 μg of the Normal Rabbit IgG–GFP Conjugate (Santa Cruz Biotechnology, Inc.) plus 30 μL of Protein A Agarose Fast Flow (Millipore) for 1 hour at 4°C. The agarose beads and nonspecifically associating proteins were removed by centrifugation at 12,000 × g for 10 minutes at 4°C. The supernatant was incubated with 5 μg of anti-RIP1 antibody or Rabbit Control IgG (ChIP grade; Abcam) plus 30 μL of Protein A Agarose Fast Flow for 12 hours at 4°C. The agarose beads were centrifuged at 3,000 × g for 2 minutes at 4°C, washed three times, and centrifuged as described above. The washed beads were boiled three times for 5 minutes at 95°C and resolved by SDS–PAGE. The gel was dried and subjected to autoradiography.

**Statistical analyses**

The data are expressed as the means ± SD. A two-tailed unpaired Student t test was used to determine the statistical significance of the difference between two groups. Probability values of P < 0.05 were considered to be statistically significant.

**Results**

**HVJ-E induces necrotic cell death in neuroblastoma cells**

We first analyzed the morphologic changes induced by HVJ-E in SK-N-SH cells. Twenty-four hours after the treatment of SK-N-SH cells with 1,000 MOI of HVJ-E, we observed swollen nuclei by transmission electron microscopy (Fig. 1A). Whether the whole cell body was swollen by HVJ-E could not be confirmed because the cells were fused together (Supplementary Fig. S1A). A MTS assay showed that the cell viability significantly decreased in a dose-dependent manner following treatment with different MOIs (100, 1,000, or 10,000) of HVJ-E in SK-N-SH and SK-N-AS cells (Fig. 1B). The HVJ-E–induced cell death was not significantly suppressed by the pretreatment with cytochalasin D, which binds to actin filaments and thus blocks actin polymerization, suggesting that cell fusion does not contribute to this cell death (Supplementary Fig. S1B).

**HVJ-E–induced cell death in neuroblastoma cells is not apoptosis but necroptosis**

Although we observed morphologic changes in necrotic SK-N-SH cells, we next needed to analyze the degree at which apoptosis contributes to this cell death. The cleavage of caspase-9, caspase-3, and PARP was detected by Western blot analysis at 12 hours after treatment with 1,000 MOI of HVJ-E, but caspase-8 was not endogenously expressed (Fig. 2A). However, the inhibition of PARP cleavage by Z-VAD-FMK could not significantly suppress HVJ-E–induced cell death (Fig. 2B and C). These results imply that this cell death is not caspase-dependent apoptosis. We could not narrow down the key factors responsible for HVJ-E–induced cell death. Therefore, we exhaustively investigated the changes in gene expression during this process by microarray analysis. However, no remarkable changes in the expression of individual genes were detected. Hence, we subjected the expressed genes to pathway analysis using MetaCore v6.5. The pathway analysis showed significant changes in some
pathways, including oxidative phosphorylation, ubiquinone metabolism, cytoskeleton remodeling, the immune response, and others (data not shown). Of these processes, we focused on the oxidative phosphorylation pathway because it produces ROS, which is one of the key executors of necrosis. Taking into consideration our findings on the

![Figure 1](image1.png)

Figure 1. Neuroblastoma cell death induced by HVJ-E. A, 24 hours after the treatment of SK-N-SH cells with 1,000 MOI of HVJ-E, the swollen nuclei (arrowhead) of the cells were observed by electron microscopy. Scale bar, 10 µm. B, the treatment of SK-N-SH and SK-N-AS cells (3 × 10^5 cells) with HVJ-E for 24 hours resulted in the cell death in a dose-dependent manner. Each survival value (mean ± SD; n = 4) was the ratio of the value with treatment to the value without treatment. *, P < 0.05.

![Figure 2](image2.png)

Figure 2. HVJ-E–induced cell death in SK-N-SH cells is not caspase-dependent apoptosis. A, SK-N-SH cells (3 × 10^5 cells) were treated with 1,000 MOI of HVJ-E for 6, 12, or 24 hours. The cleavage of caspase-9, caspase-3, and PARP was observed 12 hours after HVJ-E exposure, but caspase-8 was not endogenously expressed. B, the HVJ-E–induced cleavage of PARP was inhibited by pretreatment with Z-VAD-FMK. C, the HVJ-E–induced cell death was not significantly suppressed by pretreatment with Z-VAD-FMK. The experiments were performed in triplicate, and representative results are shown. Each survival value (mean ± SD; n = 4) was the ratio of the value with treatment to the value without treatment.
necrotic morphologic changes, nonapoptotic cell death and caspase-8 defects, we hypothesized that the HVJ-E–induced death of SK-N-SH cells is actually necroptosis. To confirm this hypothesis, we first used the RIP1 inhibitor necrostatin-1. The pretreatment of SK-N-SH cells with necrostatin-1 before HVJ-E exposure significantly suppressed cell death by inhibiting ROS production without inhibiting PARP cleavage (Fig. 3A–C). We confirmed that the HVJ-E–induced cell death was suppressed by pretreatment with necrostatin-1 also in SK-N-AS cells (Fig. 3C). To show that the lack of caspase-8 sensitizes the cells to necroptosis, caspase-8 cDNA was transferred to SK-N-SH cells. The restored caspase-8 in SK-N-SH cells resulted in the significant suppression of HVJ-E–induced cell death (Supplementary Fig. S2A and S2B). From these data, we suspected that HVJ-E–induced cell death might associate with the RIP1–RIP3 necrosome in caspase-8–deficient neuroblastoma cells. We used necrosulfonamide as the inhibitor of necroptosis, which blocks further downstream of necroptosis. The pretreatment of SK-N-SH cells with necrosulfonamide before HVJ-E exposure also significantly suppressed the cell death as well as necrostatin-1 (Supplementary Fig. S3). Knockdown of RIP3 using siRNA also suppressed the HVJ-E–induced cell death (Supplementary Fig. S4A and S4B). To further evaluate the involvement of RIP1 and RIP3 in HVJ-E–induced cell death, we demonstrated the HVJ-E–induced formation of RIP1–RIP3 necrosome by in situ proximity ligation assay, which can detect the protein–protein interactions using two antibodies derived from different species (Supplementary Fig. S5). These results imply that HVJ-E induces necroptosis in SK-N-SH cells.

**HVJ-E induces cell death in neuroblastoma cells by enhancing the cytoplasmic Ca2+ concentration**

We next attempted to unveil the upstream actions of HVJ-E–induced necroptosis in SK-N-SH cells. Necroptosis requires the phosphorylation of RIP1, which forms a complex with the TNF-α receptor in its ubiquitinated form before it is deubiquitinated by CYLD (9). By using 32P labeling, we demonstrated that RIP1 phosphorylation was induced by HVJ-E treatment for 2 hours, and this phosphorylation was suppressed by RIP1 knockdown (Fig. 3D). If this
SK-N-SH cell death is mediated by the TNF receptor, necroptosis should be induced by exposure to TNFα. However, TNFα was not able to induce cell death in SK-N-SH cells, and moreover, the TNF receptor was not endogenously expressed in SK-N-SH cells (Supplementary Fig. S6A and S6B), implying that this HVJ-E–induced cell death is not mediated by TNF receptor signaling. We confirmed that SK-N-SH cells could be killed only by HVJ-E with the WT HN protein (WT.HN) and HVJ-E with an activated fusion (F) protein (F1/F2) but not by HVJ-E with either denatured HN (ΔHN) or inactivated F (F0; Fig. 4A). Moreover, HVJ-E with F1/F2 significantly increased the cytoplasmic Ca2+ concentration compared with HVJ-E with F0 at 20 minutes after treatment of the SK-N-SH cells with 1,000 MOI of HVJ-E (Fig. 4B). This HVJ-E–induced cell death was significantly suppressed by pretreatment with the Ca2+–chelating agent BAPTA-AM (Fig. 4C), suggesting that a cytoplasmic Ca2+ increase induces cell death and that this increase in Ca2+ requires the fusion of HVJ-E to its target cells by the activated F protein.

**RIPl phosphorylation in necroptosis is mediated by CaMK II**

These results imply that a cytoplasmic Ca2+ increase upregulates HVJ-E–induced necroptosis. RIPI activation requires the phosphorylation of serine 161. Among the serine/threonine kinases, protein kinase C (PKC) and CaMK II are downstream of Ca2+ signaling and, therefore, they may be candidates for RIPI phosphorylation. Pretreatment with the PKC inhibitor bisindolylmaleimide before HVJ-E exposure did not significantly suppress HVJ-E–induced cell death, suggesting that PKC is not related to this type of cell death (Supplementary Fig. S7). Next, we detected the phosphorylation of CaMK II after treating SK-N-SH and SK-N-AS cells with HVJ-E (Fig. 5A). The HVJ-E–induced phosphorylation of CaMK II was inhibited by pretreatment with BAPTA-AM, implying that the increase in intracellular Ca2+ activates CaMK II (Fig. 5B). Knockdown of CaMK II resulted in the significant suppression of HVJ-E–induced cell death (Supplementary Fig. S8A and S8B). The endogenous expression of CaMK II was not detected (data not shown). Moreover, the inhibition

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**Figure 4. HVJ-E induces cell death in SK-N-SH cells by enhancing the intracellular Ca2+ concentration.**

HVJ particles produced by LLC-MK2 cells were either treated with trypsin for cleaving the F0 protein to form F1/F2 protein or heated to denature the HN protein. A, the expression of the HN, F0, or F1 proteins was detected by Western blot analysis. WT.HN, wild-type HN; ΔHN, denatured HN. Twenty-four hours after the treatment of SK-N-SH cells (3 × 10⁵ cells) with 1,000 or 10,000 MOI of the various types of HVJ-E, only the HVJ-E with WT.HN-F1/F2 protein could kill the cancer cells. B, SK-N-SH cells (1 × 10⁴ cells) were treated with 1,000 MOI of HVJ-E with WT.HN-F0 or WT.HN-F1/F2 protein. Only the HVJ-E with WT.HN-F1/F2 could enhance the intracellular Ca2+ concentration. C, HVJ-E–induced cell death was inhibited by pretreatment with BAPTA-AM. Each survival value (mean ± SD; n = 4) or intracellular Ca2+ concentration reflects the ratio of the value with HVJ-E treatment to the value without HVJ-E treatment. The experiments were performed in triplicate, and representative results are shown. *P < 0.05.
of CaMK II phosphorylation by pretreatment with the CaMK II inhibitor CK59 significantly suppressed HVJ-E–induced cell death in SK-N-SH and SK-N-AS cells (Fig. 5C and D), implying that CaMK II phosphorylation is related to this type of cell death.

The final objective of this study was to investigate the relationship between necroptosis and CaMK II phosphorylation. The HVJ-E–induced phosphorylation of RIP1, which was labeled using 32P, was significantly suppressed by pretreatment with necrostatin-1 and CK59 (Fig. 6A), suggesting that CaMK II phosphorylation is necessary for RIP1 phosphorylation. Moreover, using 7-Amino-Actinomycin D (AAD) staining, we confirmed that HVJ-E–induced necroptosis in SK-N-SH cells and showed that this necroptosis was inhibited by CK59 (Fig. 6B). The possibility that phosphorylated CaMK II is an upstream component of RIP1–dependent necroptosis was also supported by our results showing that CK59 pretreatment before HVJ-E exposure decreased ROS production and increased intracellular ATP (Supplementary Fig. S9A and S9B). This HVJ-E–induced necroptosis was also observed in xenograft tumors derived from SK-N-SH cells in SCID mice using AnnexinV-EnzoGold and 7-AAD (Supplementary Fig. S10).

In summary, we demonstrated that HVJ-E increases the cytoplasmic Ca\(^{2+}\) concentration followed by CaMK II phosphorylation, and that this activated CaMK II induces RIP1 phosphorylation and necroptosis (Fig. 7). To show that the increase of cytoplasmic Ca\(^{2+}\) induces necroptosis, we used Ca\(^{2+}\) ionophore A23187 instead of HVJ-E. As shown in Supplementary Fig. S11A, A23187 induced SK-N-SH cell death in a dose-dependent manner, and this cell death was significantly suppressed by pretreatment with the Ca\(^{2+}\) chelating agent BAPTA-AM. Using 7-AAD staining, we demonstrated that this A23187-induced cell death was necroptosis (Supplementary Fig. S11B).

**Discussion**

Necroptosis is inducible in many types of cells if apoptotic death signaling is inhibited by pretreatment with Z-
VAD-FMK and cycloheximide before being exposed to death ligands, such as TNF-α and the Fas ligand, and the resulting morphologic features are similar to those of necrosis (2, 3). In the ischemic brain, this type of cell death tends to be induced, and necrostatin-1 was first reported to be the agent that suppresses ischemic brain injury in mice through a mechanism that is distinct from apoptosis (16). Necrostatin-1 was later discovered to be a specific inhibitor of RIP1 (17). Therefore, cell death that is rescued by necrostatin-1 can be considered to be necroptosis, and RIP1 is believed to be the key necroptosis factor. It was recently reported that in addition to RIP1, RIP3 is essential for necroptosis because RIP3 is regulated by the caspase-8–FLIP complex (18) and mediates the embryonic lethality of caspase-8–deficient animals (19). Moreover, RIP3-knockout mice are very vulnerable to some viruses (6). Thus, necroptosis plays an important role in the inflammatory response or innate immune response to virus infection. The representative stimulator of necroptosis is TNF-α, and the mechanism of TNF-α–induced necroptosis has been well studied, but we could not study this factor here because its receptor is absent in SK-N-SH cells. Similar to HVJ-E in this study, other agents have been reported to stimulate necroptosis, such as kuguaglycoside C, a constituent of Momordica charantia (20). We chose RIP1 as the necroptosis marker for this study because a specific inhibitor of RIP3 was not available. The key executors of necroptosis are RIP1 and RIP3, and the downstream factors of these kinases are ROS and apoptosis-inducing factor (AIF). AIF is a Janus protein that exerts redox activity in the mitochondria and proapoptotic activity in the nucleus, but it can also regulate necroptosis (21). ROS also regulates apoptosis through other mechanisms involving AIF. Thus, the absolute markers of necroptosis that exist downstream of RIP1 and RIP3 have not yet been discovered. However, we chose ROS and ATP rather than AIF as markers of HVJ-E–induced necroptosis because a change in the oxidative phosphorylation pathway was detected in our pathway analysis. We have herein demonstrated that HVJ-E induces necroptosis in the neuroblastoma cell lines SK-N-SH and SK-N-AS.

We previously reported that HVJ-E induces apoptosis in castration-resistant human prostate cancer cells and human glioblastoma cells but does not cause toxicity in normal cells (22–24). Furthermore, we reported that HVJ-E stimulates an antitumor immune response by activating...
cytotoxic T lymphocytes and natural killer cells and suppressing regulatory T cells (25, 26). Among these direct and indirect HVJ-E antitumor activities, we recently discovered that HVJ-E induces apoptosis via activation of the retinoic acid–inducible gene-I (RIG-I)/mitochondrial antiviral-signaling protein pathway followed by activation of interferon regulatory transcription factor (IRF)3 or IRF7 in prostate cancer cells (27). HVJ is a mouse parainfluenza virus that belongs to the paramixoviridae genus. Two glycoproteins, HN and F, are present on the viral envelope (28). An infection occurs when HN binds to its receptor followed by the fusion of the hydrophobic region of the F protein to the lipid bilayer through its association with lipid molecules, such as cholesterol (29). After this first infection step, the HVJ virus genome is recognized by RIG-I, and death signaling effectors are activated (30, 31). However, no RIG-I expression was detected in the SK-N-SH cells in this study (data not shown). Therefore, we hypothesized that some mechanism that does not involve the innate immune system is associated with HVJ-E–induced necroptosis. It has been reported that HVJ-E enhances the cytoplasmic Ca²⁺ concentration of target cells upon fusion with the cell membrane, (28, 32–35) and we could confirm this increase in intracellular Ca²⁺.

Many intracellular Ca²⁺ effects are mediated by protein phosphorylation events that are catalyzed by a family of serine/threonine protein kinases called CaM kinases. Some CaM kinases phosphorylate gene regulatory proteins, such as CREB, and they regulate the transcription of specific genes. One of the best-studied CaM kinases is CaMK II, which is expressed in most animal cells and is especially abundant in the nervous system. It contributes up to 2% of the total protein of some regions in the brain and is highly concentrated in the synapses. CaMK II has a remarkable property in that it can function as a molecular memory device, switching to an activated state when exposed to Ca²⁺/calmodulin and then remaining activated (36, 37). This is because the kinase phosphorylates itself (autophosphorylation) and it also phosphorylates other proteins following activation by Ca²⁺/calmodulin. In this autophosphorylated state, the enzyme remains activated even in the absence of Ca²⁺, thereby prolonging the duration of its kinase activity. The enzyme maintains this activity until serine/threonine protein phosphatases suppress phosphorylated CaMK II and shut it off. CaMK II activation can thereby serve as a memory trace of a prior Ca²⁺ pulse, and it seems to have a role in some types of memory and learning in the vertebrate nervous system. We herein demonstrated that HVJ-E enhances the cytoplasmic Ca²⁺ concentration for a short time followed by the phosphorylation of CaMK II for a longer period. This finding suggests that the temporal increase in cytoplasmic Ca²⁺ can trigger CaMK II autophosphorylation, thereby causing necroptosis via RIP1 phosphorylation in caspase-8–deficient neuroblastoma cells. The first step in RIP1 activation is the deubiquitination of RIP1 by CYLD. RIP1 can then freely migrate to the cytoplasm and form a complex with RIP3. Although little is known about how this complex formation is regulated, it was recently reported that RIP1–RIP3 complex formation requires RIP1 deacetylation by NAD-dependent deacetylase SIRT2 (38). The final step of RIP1 activation requires the phosphorylation of serine 161 to produce ROS through the mitochondrial oxidative phosphorylation pathway, but the factor that phosphorylates RIP1 has not yet been discovered. Although it is not clear whether the regulation of RIP1 by CaMK II is direct or indirect, we are the first to discover one of the downstream Ca²⁺ signaling pathways associated with necroptotic cell death.

In conclusion, an increase in the cytoplasmic Ca²⁺ concentration induces necroptosis via CaMK II phosphorylation in caspase-8–deficient neuroblastoma cells.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions

Conception and design: M. Nomura, M. Fukuzawa, Y. Kaneda
Development of methodology: M. Nomura, Y. Kaneda
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): M. Nomura, A. Ueno
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): M. Nomura, Y. Kaneda

Figure 7. Schematic representation of death signaling stimulated by HVJ-E. HVJ-E enhances the cytoplasmic Ca²⁺ concentration by binding and fusing the target cells. Then, CaMK II is phosphorylated and thus activated. Once CaMK II is phosphorylated, it remains activated for a long time and exerts its kinase activity on its target proteins. Among the downstream effects of activated CaMK II, RIP1–dependent necroptosis is induced in the absence of endogenous expression.
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References


Accumulation of Cytosolic Calcium Induces Necroptotic Cell Death in Human Neuroblastoma

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