Supplementary data

Supplementary materials and methods

Mice. Five-week-old female C.B-17/lcr-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.

Tumor growth in vivo. Viable SK-N-SH cells (1×10^7 cells) were re-suspended in 100 µl of PBS and injected into the subcutaneous space 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 of PBS) or 100 µl of PBS on days 6 and 9. Three days after the last injection of HVJ-E, the mice were sacrificed and the xenograft tumor was extirpated.

Detection of the RIP1-RIP3 necrosome by in situ proximity ligation assay. Following 6 hours treatment with 1000 MOI of HVJ-E, SK-N-SH cells were fixed with 4% paraformaldehyde and the mixture of anti-RIP1 and anti-RIP3 antibody was added to
the samples. RIP1-RIP3 complex was detected with Duolink II Goat/Rabbit Red Starter Kit (Olink Bioscience. Uppsala, Sweden) according to the manufacturer’s protocol.

**Restore of caspase-8 expression.** Lipofectamine 2000 (Invitrogen) and pcDNA3-Casp8 (Addgene. MA, USA) were mixed in Opti-MEM and incubated for 5 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 pcDNA dosage was 25 or 5 μg/well. The restored caspase-8 expression was confirmed by Western blotting at 24 hours after transfection.

**Measurement of ATP.** The intracellular ATP levels in SK-N-SH cells were determined with the CellTiter-Glo Luminescent Cell Viability Assay Kit (Promega) according to the manufacturer's instructions. This assay generates a luminescent signal that is proportional to the amount of ATP in the cells.

**Pretreatment of cells with inhibitors.** To inhibit cell fusion, 0.025 μg/ml cytochalasin D (A.G. Scientific) was added to SK-N-SH cells 24 hours before exposure to HVJ-E, as previously described. To inhibit the effectors of downstream pathway of necroptosis,
1µM necrosulfonamide (EMD Millipore) was added to SK-N-SH cells 1 hour before exposure to HVJ-E. To inhibit the activity of PKC, increasing concentrations (up to 1 µM) of bisindolylmaleimide I (MERCK Millipore) was added to the cells 1 hour before treatment with HVJ-E.

Supplementary References


Supplementary Figure Legends

Figure S1. Cell fusion is not the main cause of HVJ-E-induced cell death. (A)

Twenty-four hours after the treatment of SK-N-SH cells (3×10^5 cells) with different MOIs (100, 1000 or 10000) of HVJ-E, cancer cell fusion was observed. (B) SK-N-SH cells (3×10^5 cells) were pretreated with 0.025 µg/ml of cytochalasin D for 24 hours and then treated with different MOIs (100, 1000 or 10000) of HVJ-E. Twenty-four hours after the HVJ-E treatment, the cell viability was not significantly increased by
pretreatment with cytochalasin D. Each survival value (mean ± standard deviation, n=4) was the ratio of the value with HVJ-E treatment to the value without HVJ-E treatment.

Figure S2. The expression level of caspase-8 regulates the HVJ-E-induced cell death in SK-N-SH cells. (A) The plasmid vector containing cDNA of caspase-8 (pcDNA3-Casp8) was transfected into SK-N-SH cells. The restored caspase-8 expression was confirmed by Western blot analysis. (B) SK-N-SH cells were treated with different MOIs of HVJ-E for 24 hours. The cells with restored caspase-8 expression got more resistant to HVJ-E than mock. Each survival value (mean ± standard deviation, n=4) was the ratio of the value with HVJ-E treatment to the value without HVJ-E treatment. * p < 0.05.

Figure S3. Downstream pathway of necroptosis is associated with HVJ-E-induced cell death in SK-N-SH cells. SK-N-SH cells (3×10^5 cells) with or without pretreatment with 1µM of Necrosulfonamide (NSA) were exposed to 1000 MOI of HVJ-E for 24 hours. The HVJ-E-induced cell death was significantly suppressed by the pretreatment with NSA. Each survival value (mean ± standard deviation, n=4) was the ratio of the value with HVJ-E treatment to the value without HVJ-E treatment. * p < 0.05.
Figure S4. The expression of RIP3 is necessary for the HVJ-E-induced cell death in SK-N-SH cells. (A) SK-N-SH cells (3×10^5 cells) were treated with 1000 MOI of HVJ-E. Knockdown of RIP3 by siRNA was confirmed by Western blot analysis. (B) The HVJ-E-induced SK-N-SH cell death was significantly suppressed by the knockdown of RIP3. Each survival value (mean ± standard deviation, n=4) was the ratio of the value with HVJ-E treatment to the value without HVJ-E treatment. * p < 0.05.

Figure S5. The formation of RIP1-RIP3 necrosome is facilitated by HVJ-E in SK-N-SH cells. SK-N-SH cells (3×10^5 cells) were exposed to 1000 MOI of HVJ-E for 6 hours. By in situ proximity ligation assay which can detect two kinds of proteins closed together, we demonstrated that the colocalization of RIP1 with RIP3 (red) was facilitated by HVJ-E treatment, The nuclei were stained with DAPI (blue).

Figure S6. HVJ-E-induced cell death in SK-N-SH cells is not mediated by the TNF receptor. (A) Twenty-four hours after the treatment of SK-N-SH cells (3×10^5 cells) with different doses (0, 0.1, 1 or 10 ng/ml) of TNF-α, the cell survival rate had not decreased. (B) The cells were then treated with or without HVJ-E, and the expression of
TNFR1 and β-actin was assessed by Western blot analysis. No TNFR1 expression was detected in the SK-N-SH cells. Each survival value (mean ± standard deviation, n=4) was the ratio of the value with HVJ-E treatment to the value without HVJ-E treatment.

**Figure S7. HVJ-E-induced cell death in SK-N-SH cells is not mediated by PKC.**

SK-N-SH cells (3×10^5 cells) were pretreated with different doses (0 nM, 1 nM, 10 nM, 100 nM or 1 µM) of the PKC inhibitor bisindolylmaleimide for 1 hour and then treated with 1000 MOI of HVJ-E. Twenty-four hours after the HVJ-E treatment, cell death was not significantly suppressed by the inhibition of PKC. Each survival value (mean ± standard deviation, n=4) was the ratio of the value with HVJ-E treatment to the value without HVJ-E treatment.

**Figure S8. The expression of CaMK IIα is necessary for the HVJ-E-induced cell death in SK-N-SH cells.** (A) SK-N-SH cells (3×10^5 cells) were treated with 1000 MOI of HVJ-E. Knockdown of CaMK IIα by siRNA was confirmed by Western blot analysis. CaMK IIβ was not expressed endogenously. (B) The HVJ-E-induced SK-N-SH cell death was significantly suppressed by the knockdown of CaMK IIα. Each survival
value (mean ± standard deviation, n=4) was the ratio of the value with HVJ-E treatment to the value without HVJ-E treatment. * p < 0.05.

**Figure S9. HVJ-E-induced necroptosis is inhibited by CK59.** SK-N-SH cells (3×10^5 cells) with or without Necrostatin-1 or CK59 pretreatment were exposed to 1000 MOI of HVJ-E for 24 hours. (A) The HVJ-E-induced production of ROS, which was detected by MitoSOX Red staining (red), was inhibited by pretreatment with Necrostatin-1 or CK59. The nuclei were stained with DAPI (blue) (B) Intracellular ATP production was also assessed. HVJ-E treatment decreased ATP production, which was inhibited by pretreatment with Necrostatin-1 or CK59. Each value (mean ± standard deviation, n=4) of intracellular ATP reflected the ratio of the value with HVJ-E treatment to the value without HVJ-E treatment. * p < 0.05.

**Figure S10. HVJ-E induces necroptosis in xenograft tumors derived from SK-N-SH cells.** Xenograft tumors were created in SCID mice using viable SK-N-SH cells. The mice were treated with two intratumoral injections of HVJ-E (5000 HAU). Three days after the last HVJ-E injection, the apoptotic cells were stained with
AnnexinV-EnzoGold (yellow), and the necrotic cells were stained with 7-AAD-Red (red). The nuclei were stained with DAPI (blue).

**Figure S11. Increased cytoplasmic Ca$^{2+}$ induces necroptosis in SK-N-SH cells.** (A) SK-N-SH cells (3×10$^5$ cells) with or without 10 µM BAPTA-AM pretreatment were exposed to 20 µM of A23187 for 24 hours, and the cell survival rate significantly decreased. The cell death was significantly suppressed by the BAPTA-AM pretreatment. (B) Necrotic cells were stained with 7-AAD-Red (red). The nuclei were stained with DAPI (blue). Each survival value (mean ± standard deviation, n=4) is the ratio of the value with HVJ-E treatment to the value without HVJ-E treatment. *p < 0.05.