ORIGINAL ARTICLE

Immunogenic cell death by oncolytic herpes simplex virus type 1 in squamous cell carcinoma cells

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Molecules essential for the induction of immunogenic cell death (ICD) are called damage-associated molecular patterns (DAMPs). The effects of oncolytic herpes simplex virus type 1 (HSV-1) on the production of DAMPs were examined in squamous cell carcinoma (SCC) cells. The cytopathic effects of HSV-1 RH2 were observed in mouse SCCVII cells infected at a high multiplicity of infection (MOI), and the amounts of viable cells were decreased. After being infected with RH2, ATP and high mobility group box 1 (HMGB1) were released extracellularly, while calreticulin (CRT) translocated to the cell membrane. A flow-cytometric analysis revealed an increase in the number of annexin-V and propidium iodide (PI)-stained cells; and the amount of cleaved poly (ADP-ribose) polymerase (PARP) was increased. The killing effect of RH2 was reduced by pan-caspase inhibitor z-VAD-fmk and the caspase-1 inhibitor z-VYAD-fmk, suggesting the involvement of apoptosis and pyroptosis. In C3H mice bearing synergic SCCVII tumors, the growth of tumors injected with the supernatant of RH2-infected cells was less than that of tumors injected with phosphate-buffered saline (PBS). These results indicate that oncolytic HSV-1 RH2 produces DAMPs from SCC cells to induce cell death. This may contribute to the enhancement of tumor immunity by oncolytic HSV-1.

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INTRODUCTION

Tumor cells exposed to chemotherapeutic agents and radiation may act as a tumor vaccine, indicating that dying cells release and expose to the cell surface molecules that promote tumor immunity. Unlike normal apoptosis caused by chemotherapy, which is mostly non-immunogenic or even tolerogenic, some cytostatic agents such as anthracyclines, oxaliplatin, bortezomib or radiotherapy and photodynamic therapy have been shown to cause cell death that enhances tumor immunity.^{1–3} This cell death is called immunogenic cell death (ICD).^{4–7} Molecules essential for the induction of ICD are called damage-associated molecular patterns (DAMPs),⁵ and include released molecules such as ATP and high mobility group box 1 (HMGB1) and/or those exposed to the cell surface such as HSP (heat-shock protein)70, HSP90 and calreticulin (CRT). Dendritic cells mature after being exposed to these molecules, process antigens and activate T cells to augment antitumor responses. Extracellular ATP and surface-exposed CRT act as ‘find me’ and ‘eat me’ signals, respectively, to immune cells.^{8–14}

Oncolytic virotherapy is a promising modality for many solid tumors because of its potential advantages over conventional cancer therapies. Herpes simplex virus (HSV), vaccinia virus and reovirus are the most extensively studied viruses at the clinical level.^{15–17} The clinical effects of HSV type 1 (HSV-1) were demonstrated in the treatment of skin melanoma and head and neck cancer.^{18–21} Now, oncolytic HSV-1, Imlygic (T-Vec), is commercially available for the treatment of surgically unresectable skin and lymph-node lesions in patients with advanced melanoma.²² These viruses exerted direct cytotoxic effects at the inoculated site of the tumors, whereas their effects on metastatic lymph nodes and organs were attributed to enhancing effects on tumor immunity.²³

Recent studies have shown that oncolytic viruses including adenovirus, parvovirus, reovirus, coxsackievirus, vaccinia virus, Newcastle disease virus and HSV induce immunogenic types of cell death, providing a danger signal and natural repertoire of tumor-associated antigens to dendritic cells, both of which are required to trigger adaptive immunity against cancer.^{7,24–28} HSV type 2 (HSV-2) produced DAMPs in mouse mammary gland tumor cells; however, information on HSV-1-induced DAMPs is limited.^{29–31}

We previously demonstrated that oncolytic HSV-1 RH2 deficient in the neurovirulent $\gamma_134.5$ gene inhibited the growth of mouse squamous cell carcinoma (SCC), even if tumors that were not directly injected with RH2, indicating its enhancing effects on tumor immunity.³² In the present study, using this model, we determined whether RH2 produced DAMPs from SCC cells to induce ICD.

MATERIALS AND METHODS

Cell culture

The mouse SCC cell line SCCVII was cultured in Eagle’s minimal essential medium (MEM) supplemented with 10% calf serum and 2 mM L-glutamine and grown in an incubator at 37 °C in a humidified atmosphere with 5% CO₂.³² MEM containing 5% calf serum and 2 mM L-glutamine was used for Vero monkey kidney cells. SAS cells derived from human oral SCC were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U ml^{−1} penicillin and 100 μg ml^{−1} streptomycin.³³

Viral infection

RH2 is an HSV-1 mutant deficient of the neurovirulent $\gamma_134.5$ gene and has mutations in gB, which is responsible for its fusogenic ability in human SCC cells.^{33,34} RH2 was grown in semi-confluent Vero cell monolayers; and

infectivity was determined by plaque formation on Vero cell monolayers covered with 0.3% methylcellulose.

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay

Cells grown in 96-well culture dishes were infected with RH2 at various multiplicity of infection (MOI), while controls were mock infected. After being incubated for various intervals, 10 μ l of a 5-mg ml⁻¹ MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) solution was added to each well with 100 μ l of medium. Cells were incubated for 4 h at 37 °C, and 100 μ l of 0.04 N HCl in isopropanol was then added. The sample was mixed thoroughly to dissolve the dark blue crystal. After standing overnight at room temperature, the plates were read on a Benchmark Plus microplate spectrophotometer (Bio-Rad Laboratories, Hercules, CA, USA) with a reference wavelength of 630 nm and a test wavelength of 570 nm. Background absorbance at 690 nm was subtracted from the 570-nm reading. Changes from controls (room air) were calculated.

ATP assay and HMGB1 enzyme-linked immunosorbent assay

The supernatants of RH2-infected SCCVII cells and uninfected control cells were collected; and cell debris was removed by centrifugation at 6200 r.p.m. for 5 min. Secreted extracellular ATP in the supernatants was measured with the ENLITEN ATP assay (Promega, Madison, WI, USA) according to the manufacturer's protocol, using a Turner Biosystems luminometer (TD-20/20; Promega). Supernatants were also used to detect HMGB1 with HMGB1 ELISA Kit II (Shino-Test, Kanagawa, Japan). Enzyme-linked immunosorbent assay was performed according to the manufacturer's protocol outlined for the normal sensitivity format of the assay; and the plates were read on a microplate spectrometer at a wavelength of 450 nm.

Confocal laser microscopic analysis

Cells were fixed in 4% paraformaldehyde phosphate buffer solution (WAKO, Osaka, Japan) and incubated with a mouse monoclonal antibody against CRT (Abcam, Cambridge, UK) diluted 1:250 in phosphate-buffered saline (PBS) with 0.3% bovine serum albumin for 30 min at room temperature. After washing, the cells were incubated with an FITC (fluorescein isothiocyanate)-conjugated goat polyclonal antibody diluted 1:100 and Alexa Fluor 633-conjugated wheat germ agglutinin (WGA) (Invitrogen, Carlsbad, CA, USA) for 30 min. After washing, coverslips were mounted onto microslides using a ProLong Gold Antifade Reagent with DAPI (4',6-diamidino-2-phenylindole) (CST Japan, Tokyo, Japan). The slides were analyzed under the confocal laser-scanning microscope Leica TCS SP8 (Leica Microsystems, Mannheim, Germany).

Flow-cytometric analysis

After the treatment, floating cells were harvested with medium and attached cells were dissociated with ethylenediaminetetraacetic acid-trypsin solution. Cells were collected by centrifugation at 1 000 r.p.m. for 5 min. FITC annexin-V and propidium iodide (PI) staining was performed using Vybrant Apoptosis Assay Kit#3 (Life Technologies, Carlsbad, CA, USA) following the manufacturer's directions. Cell pellets were suspended in 100 μ l binding buffer containing 10 mM HEPES, 140 mM NaCl and 2.5 mM CaCl₂ (pH 7.4) and incubated with 5 μ l of FITC annexin-V and 1 μ l of 100 μ g ml⁻¹ PI solution for 15 min at room temperature. Thereafter, 400 μ l of binding buffer was added, mixed gently and kept on ice. Stained cells were analyzed by a FACSCalibur flow cytometer using the CellQuest software (Becton Dickinson, San Jose, CA, USA).

Immunoblot analysis

For the detection of cleavage of poly (ADP-ribose) polymerase (PARP), cells were washed in PBS and lysed in a buffer containing 20 mM Tris-HCl (pH 7.4), 0.1% SDS, 1% Triton X-100, 1% sodium deoxycholate and protease inhibitor cocktail. After sonication on ice and subsequent centrifugation at 15 000 g for 10 min at 4 °C, the supernatant was collected and the protein concentration was determined using a Protein Assay Kit (Bio-Rad). Sample protein (20 μ g) was electrophoresed through a polyacrylamide gel and transferred onto a polyvinylidene fluoride (PVDF) membrane (Millipore, Bedford, MA, USA) by electroblotting. The membrane was probed with antibodies; and antibody binding was detected using an enhanced chemiluminescence (ECL) kit (Amersham Life Science, Arlington Heights, IL, USA) according to the manufacturer's instructions. Antibodies used were as follows: rabbit polyclonal antibodies against cleaved RAPR (#9544, Cell

Signaling Technology, Beverly, MA, USA) diluted 1:1000, mouse monoclonal antibodies against β -actin (Sigma, St. Louis, MO, USA) and horseradish peroxidase-conjugated secondary antibodies (Cell Signaling).

Lactate dehydrogenase release assay

The cytotoxicity of HSV-1 RH2 was measured using a lactate dehydrogenase (LDH) release assay. SCCVII cells were plated on 96-well plates at 2×10^3 per well and cultured for 24 h. After the treatment, the supernatants of cells were harvested and evaluated for the presence of the cytoplasmic enzyme LDH using MTX-LDH (Kyokuto, Tokyo, Japan) as directed by the manufacturer's instructions. In positive control wells, Triton X-100 was used at a final concentration of 0.8% to determine maximal LDH release. Percentage cytotoxicity was calculated as 100 $\times$ (MTX-LDH (Kyok – spontaneous LDH)/(maximum LDH release – spontaneous LDH)).^{30,32} To determine the effects of the inhibitor for cell death, necrostatin 1 (Sigma), the pan-caspase inhibitor z-VAD-fmk (R&D Systems, Minneapolis, MN, USA) and the caspase 1 inhibitor z-YVAD-fmk (BioVision, Milpitas, CA, USA), RH2-infected cells were incubated in the presence of these inhibitors for 24 h and the cytotoxicity of HSV-1 was determined by an LDH release assay. These inhibitors were dissolved in dimethyl sulfoxide and diluted in MEM. The final concentrations of necrostatin 1, z-VAD-fmk and z-YVAD-fmk were 20, 10 and 50 μ M, respectively. Dimethyl sulfoxide concentration was adjusted to lower than 0.1%.

Animal experiments with supernatants of infected cells

SCCVII cells were infected with RH2 at an MOI of 10. After being incubated for 24 h, the supernatant was harvested and concentrated 30-fold via centrifugation using Amicon Ultra-15 3 K Centrifugal Filter Devices (Merck, Darmstadt, Germany), according to the manufacturer's instructions. Infectious viruses in the concentrate were inactivated by ultraviolet irradiation for 60 min at an intensity of 0.15 mW cm⁻². Five-week-old C3H/HeJCl female mice were obtained from Clea Japan (Tokyo, Japan); and tumors were produced by a subcutaneous injection of 1×10^6 SCCVII cells into the backs of these mice. Once the tumor reached approximately 7 mm in diameter, animals were divided at random into two groups of six animals each. Tumors received an intratumoral injection of the concentrated supernatants of RH2-infected SCCVII cells in 50 μ l three times at an interval of 2 days. Animals in the control group received PBS instead of concentrated supernatants. Bidimensional tumor measurements were performed for 31 days with calipers, and tumor volumes were determined using the formula for a rotational ellipsoid ($L \times W^2 \times 0.52$). Experiments were performed with the approval of the Institute of Laboratory Animals, Osaka University Graduate School of Dentistry.

Statistical analysis

Statistical analyses were performed using the Student's *t*-test with Microsoft Excel (Microsoft, Redmond, WA, USA). Results were expressed as the mean $\pm$ s.d. Differences were considered significant at $P < 0.05$.

RESULTS

Cytopathic effects of RH2 on murine SCC cells

To determine the cytopathic effects of RH2 on SCCVII cells, the virus was inoculated at different MOI. Although cytopathic effects were not observed at a low MOI, cellular alterations appeared at an MOI of 10. Cell rounding appeared 12 h after infection, and became prominent at 24 h, with an increase in the number of floating cells (Figure 1a). When SCCVII cells were infected at an MOI of 10 or 100 and assayed by the MTT assay, cell viability decreased in time- and dose-dependent manners; it reached 57% of the control 24 h after infection with an MOI of 10 (Figure 1b).

Release of ATP and HMGB1 into the culture medium

Extracellular ATP is detectable as a correlate of apoptotic cell death induced by a wide range of anticancer agents including immunogenic chemotherapeutic compounds.³⁵ SCCVII cells were infected with an MOI of 10 to determine whether ATP was released extracellularly, and the amount of ATP in the supernatant was then measured. Extracellular ATP levels were low 12 h after

Figure 1. Cytopathic effects of RH2 on mouse squamous cell carcinoma (SCC) cells. **(a)** SCCVII cells were inoculated with herpes simplex virus type 1 (HSV-1) at a multiplicity of infection (MOI) of 10 or 100, incubated at 37 °C for 24 h, and observed under a phase-contrast microscope. **(b)** The viability of SCCVII cells infected with RH2 was measured by an MTT ((3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide)) assay at various intervals after infection, and the percentage to the control level was determined. * $P < 0.05$ versus uninfected control. $n = 5$. PBS, phosphate-buffered saline.

infection, increased from 24 h to a level that was maintained until 36 h (Figure 2a). This level increased further after HSV-1 infection at an MOI of 100. However, the ATP level in the supernatant of uninfected cells remained low during the incubation period for 36 h. Human SAS cells were also infected with RH2 at an MOI of 1, and the release of ATP was measured 24 and 48 h after infection. Although there was not apparent increase 24 h after infection, the amount of ATP in the supernatant was 9.6 pM, whereas that in untreated control was 2.9 pM ($n = 4$) 48 h after infection; there was a significant difference ($P < 0.05$) between the groups.

HMGB1 is an abundant nuclear non-histone chromatin-binding protein that is secreted by macrophages and monocytes activated by IL-1 β , tumor necrosis factor or lipopolysaccharide.^{5,10} In the untreated SCCVII cell culture, extracellular HMGB1 levels were markedly increased after a 24 h incubation, but decreased rapidly at 36 h. When cell cultures were infected with RH2 at an MOI of 10, a further increase in extracellular HMGB1 was observed 24 h after infection (Figure 2b). HMGB1 levels in the supernatants of RH2-infected cells decreased at 36 h.

Expression of CRT on the cell surface

CRT, a DAMP that is typically in the lumen of the endoplasmic reticulum, is translocated after the induction of immunogenic

apoptosis to the surface of dying cells, at which it functions as an eat-me signal for professional phagocytes.^{2,5,36} SCCVII cells were infected with RH2 at an MOI of 10 and were stained with an anti-CRT antibody. As a part of the outer leaflet of cellular plasma membranes, glycoproteins or glycolipids that contain sialic acid and *N*-acetylglucosamine residues are labeled with WGA.³⁷ SCCVII cells were stained with Alexa fluor 633-conjugated WGA and DAPI to indicate the cell membrane and nucleus, respectively. In untreated control cells, CRT was diffusely distributed in the cytoplasm and hardly detectable at the cell surface in most cells; however, its distribution changed 24 h after infection. CRT accumulated as puncture clusters at the plasma membrane (Figure 3). The cell membrane of RH-infected cells was partly, but intensively stained with Alexa Fluor WGA 633, indicating alterations in lectin binding sites on the cell surface.

Flow cytometry of infected cells

SCCVII cells were infected with RH2 at an MOI of 10, incubated for 24 h, and subjected to a flow-cytometric analysis to determine the proportion of apoptotic cells. Annexin-V(+) and PI(−) cells, suggesting early apoptotic cells, were present at populations of 2.4–0.5% 12 and 24 h after infection, respectively. The level of

early apoptotic cells was low during the experimental period for 36 h. In contrast, annexin-V(+) and PI(+) cells, suggesting late apoptotic or necrosis,^{38,39} were markedly increased 24 h after infection, reaching 33%, while the control level was 2.6% (Figure 4).

Figure 2. Release of ATP and high mobility group box 1 (HMGB1) into the culture medium. **(a)** SCCVII cells were infected at a multiplicity of infection (MOI) of 10 or 100, incubated for 24 h, and the supernatants were harvested. Uninfected cells were also incubated for 24 h, and the supernatants were harvested. The amounts of ATP in the supernatants were measured using an ATP assay. **(b)** HMGB1 released from SCCVII cells and RH2-infected SCCVII cells was measured using HMGB1 enzyme-linked immunosorbent assay (ELISA). * $P < 0.05$ as compared with uninfected control. $n = 4$. PBS, phosphate-buffered saline; SCC, squamous cell carcinoma.

Immunoblot analysis of PARP in RH2-infected cells

To determine the cleavage of PARP by caspases, an execution marker of apoptosis, SCCVII cells infected with RH2 were cultured for 24 h and then subjected to immunoblot analysis. As a positive control of drug-induced apoptosis, cells were treated with mitoxantrone (MTX) at a concentration of 2 μM for 24 h.² Cleaved PARP was increased in RH2-infected cells as well as in MTX-treated cells (Figure 5).

Effects of cell death inhibitors on RH2-induced cell death

Programmed necrosis, necroptosis, is a caspase-independent programmed cell death that results in necrosis via a regulated signal transduction pathway mediated by receptor-interacting protein kinases.^{40,41} When SCCVII cells were infected with RH2 at an MOI of 10 and incubated in the presence or absence of the necroptosis inhibitor necrostatin 1 for 24 h, cytotoxicity, as measured by an LDH release assay, was 12 and 13%, respectively, indicating that cell-killing effects were not altered by the inhibitor. However, in the presence of the pan-caspase inhibitor z-VAD-fmk, cell cytotoxicity 24 h after HSV-1 infection was markedly decreased and the percentage of LDH release by RH2 was 4% (Figure 6a). Pyroptosis is a type of cell death in which caspase-1 is activated with cellular enlargement and the effects of the caspase 1 inhibitor z-YVAD on the cytotoxicity of HSV-1 was also examined. In the presence or absence of z-YVAD-fmk, the percentages of LDH release by HSV-1 RH2 were 12 and 19%, respectively (Figure 6b), with a significant difference ($P < 0.05$) being observed between the two groups.

Effects of supernatants of RH2-infected cells on tumor growth

Tumors were produced by inoculating SCCVII cells. The concentrated supernatants of RH2-infected cells were injected into tumors three times at an interval of 2 days. PBS-treated tumors grew with the tumor volume reaching 2996 mm^3 at 25 days. In contrast, the growth of RH2-injected tumors was suppressed, and tumor volume was less than 1587 mm^3 . When tumor volumes were compared, a significant ($P < 0.05$) difference was observed between supernatant-injected tumors and PBS-injected tumors (Figure 7). No symptoms of neurological abnormalities or skin reactions at the injected sites were noted during the experiment.

DISCUSSION

The preapoptotic surface exposure of CRT as well as the release of ATP and HMGB1 are considered the optimal ICD combination for

Figure 3. Expression of calreticulin (CRT) on the cell surface. SCCVII cells were infected with RH2 at a multiplicity of infection (MOI) of 10, incubated for 24 h, and fixed in paraformaldehyde. Cells were stained with an anti-CRT antibody, Alexa Fluor 633-conjugated wheat germ agglutinin (WGA), and (4',6-diamidino-2-phenylindole) (DAPI) and then analyzed under a confocal laser-scanning microscope. A representative result was presented. PBS, phosphate-buffered saline; SCC, squamous cell carcinoma.

Figure 4. Flow-cytometric analysis. (a) SCCVII cells were infected with RH2 at a multiplicity of infection (MOI) of 10. After an incubation for 6, 12 or 24 h, cells were stained with annexin-V and propidium iodide (PI) and subjected to a flow-cytometric analysis. (b) The proportions of annexin-V (+) and PI (+) cells were determined. Experiments were performed three times. A representative result was presented. FITC, fluorescein isothiocyanate; PBS, phosphate-buffered saline; SCC, squamous cell carcinoma.

Figure 5. Immunoblot analysis of cleaved poly (ADP-ribose) polymerase (PARP). SCCVII cells were infected with RH2 at a multiplicity of infection (MOI) of 0.1, cultured for 24 h. Cellular proteins were subjected to immunoblot analysis to determine the expression of the cleaved PARP. MTX, Mitoxantrone. A representative result was presented. PBS, phosphate-buffered saline; SCC, squamous cell carcinoma.

dying tumor cells to enable the paracrine activation of dendritic cells and consequential priming of cytotoxic effectors.^{27,42}

Some viruses have been shown to induce ICD accompanying the release and surface exposure of DAMPs. Human non-small cell lung cancer cells treated with an oncolytic virus Coxsackievirus B3 induced ICD, and expressed abundant cell surface CRT and secreted ATP as well as translocated HMGB1.²⁶ Newcastle disease virus infection induced the *in vitro* characteristics of ICD in LG 261 glioma cells, including CRT and release of HMGB1, but not ATP, and also enhanced their antigenicity.²⁸ Workenhe *et al.*³¹ reported that an HSV-1 ICP 0 mutant and wild-type HSV-1 did not increase the release of HMGB1 under the *in vitro* conditions, while its injection into mouse tumors elevated serum HMGB1 levels and increased HSP70 and cleaved caspase 3 in tumor tissues. The origin of these DAMPs was not identified.

In the present study, mouse SCCVII cells were relatively resistant to infection with a low dose of HSV-1 and high amounts of the virus were required to initiate HSV-1 infection in the cells. At an MOI of 10 or 100, cell rounding and cell detachment were observed and cell viability was decreased, indicating the oncolytic effects of RH2 in these cells. When we examined the release of ATP and HMGB1, we found that it increased from 24 h after infection in accordance with the rounding and floating of cells. In this connection, Angelova *et al.*²⁷ reported that non-histone nuclear HMGB1 was released from non-dying cells or necrotic cells succumbing to programmed death pathways in pancreatic ductal adenocarcinoma cells infected with parvovirus. In consistent with

their results, the amount of HMGB1 in the medium was increased in uninfected SCCVII cells during incubations for 24 and 30 h (Figure 3a), indicating the active secretion of HMGB1 from living SCCVII cells under normal conditions. After RH2 infection, extracellular HMGB1 levels increased, which is consistent with the cell alterations due to the cytopathic effects of HSV-1. The increases observed in extracellular ATP and HMGB1 by HSV-1 RH2 indicate the passive release of these DAMPs due to the membrane damage. We also detected the accumulation of CRT in the plasma membrane by immunofluorescent antibody staining, while untreated cells showed the diffuse distribution of CRT. Thus, we concluded that RH2 infection produced DAMPs from SCC cells.

HSV-2 infection was previously shown to cause immunogenic apoptosis with the activation of caspase 7 and caspase 3 in mammary gland tumor cells.⁴³ However, Newcastle disease virus-mediated GL261 glioma cell death was not associated with the morphological traits of apoptosis. We observed marked increases in the numbers of annexin-V (+) and PI (+) cells, indicating the occurrence of late apoptosis or necrosis, whereas annexin-V (+) and PI (–) early apoptotic cells were not significantly increased.

Although final stage of cell death is necrosis, it was suspected that the annexin-V (+) and PI (+) cells at 24 h after infection were caused by programmed cell death, that is, apoptosis or programmed necrosis. Programmed necrosis, necroptosis, was previously reported in HSV-1-infected monocytes.⁴⁴ We examined this possibility using the specific inhibitor necrostatin 1; however, it did not affect the cytotoxicity of RH2. On the other hand, the pan-caspase inhibitor z-VAD-fmk inhibited cell death induced by RH2 and the increase of cleaved PARP was demonstrated. These findings suggested that RH2 infection caused apoptosis in SCC cells.

Another finding was that the caspase-1 inhibitor z-YVAD-fmk partly reduced the cytotoxicity of RH2. Pyroptosis is a caspase-1-dependent inflammatory type of cell death that involves the formation of an inflammasome complex, which was originally observed during the infection of macrophages. Specific features include the activation of caspase-1 or caspase-11, plasma membrane pore formation, cell swelling, osmotic lysis, nuclear condensation and the release of intracellular contents that promote the release of the inflammatory cytokines IL-18 and IL-1 β .⁴⁵ This type of cell death was previously reported in cytomegalovirus retinitis in mice, dengue virus-infected human monocytes and HSV-2 infection in melanoma cells.^{30,46,47}

Figure 6. Suppression of the cytotoxicity of RH2 by inhibitors of cell death. **(a)** SCCVII cells were infected with RH2 at a multiplicity of infection (MOI) of 10 and incubated in the absence or presence of the pan-caspase inhibitor z-VAD-fmk or necrostatin 1 for 24 h. Thereafter, the cytotoxicity of herpes simplex virus type 1 (HSV-1) was determined by a lactate dehydrogenase (LDH) release assay. **(b)** RH2-infected SCCVII cells were also incubated in the presence of the caspase 1 inhibitor z-YVAD-fmk for 24 h. * $P < 0.05$ versus control (dimethyl sulfoxide, DMSO). $n = 4$. SCC, squamous cell carcinoma.

Figure 7. Effects of supernatants of RH2-infected cells on the growth of tumors. Tumors were produced by a subcutaneous inoculation of SCCVII cells into C3H mice. The concentrated supernatants of RH2-infected SCCVII cells were injected into tumors three times at an interval of 2 days. Tumors in the control group received phosphate-buffered saline (PBS) instead of the supernatant. Tumor volumes were measured until 31 days after the initial injection. $n = 6$, * $P < 0.05$ versus control. SCC, squamous cell carcinoma.

Pyroptosis may also contribute to the RH2-induced cell death in SCCVII cells.

To elucidate the relationship between the production of DAMPs and the ability of oncolytic viruses to induce tumor immunity, previous studies demonstrated that the oncolytic virus enhanced antitumor ability and the production of DAMPs in their systems.^{26,28,31} In our model, in which two tumors were produced in C3H mouse synergic to SCCVII cells, the growth of untreated tumors as well as HSV-1-injected tumors was reduced. Spleen cells prepared from the mice that received an intratumoral injection of HSV-1 showed its specific cytotoxicity to SCCVII cells, but not to other murine tumor cells. CD8⁺T cells have been shown to enhance antitumor activity.³² To support these findings, we harvested the supernatants of RH2-infected cells, irradiated with UV to inactivate the infectious virus, and injected them into tumors of synergic mice. We found that tumor volume was significantly smaller than that in controls treated with PBS. This suggested that non-infectious molecules in the supernatants of RH2-infected cells suppressed the growth of tumors through tumor immunity.

In conclusion, oncolytic HSV-1 RH2 released ATP and HMGB1 and induced the translocation of CRT to the cell membrane, leading to cell death with the characteristic of apoptosis and pyroptosis. This may contribute to the enhancement of tumor immunity against SCCVII by RH2 in an *in vivo* SCC tumor model.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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