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Induction of necroptosis in multinucleated giant cells induced by conditionally replicating syncytial oHSV in co-cultures of cancer cells and non-cancerous cells

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Viral modifications enabling syncytium formation in infected cells can augment lysis by oncolytic herpes simplex viruses (oHSVs) which selectively kill cancer cells. In the case of receptor-retargeted oHSVs (RR-oHSVs) that exclusively enter and spread to cancer cells, anti-tumor effects can be enhanced in a magnitude of >100,000-fold by modifying the virus to a syncytial type (RRsyn-oHSV). However, when syncytia containing non-cancerous cells are induced by conditionally replicating syncytial oHSV (CRsyn-oHSV), syncytial death occurs at an early stage. This results in limited anti-tumor effects of the CRsyn-oHSV. Here, we investigated whether necroptosis is involved in death of the syncytia formed by the fusion of cancer cells and non-cancerous cells. Mixed-lineage kinase domain-like (MLKL), a molecule executing necroptosis, was expressed in all murine cancer cell lines examined, while receptor-interacting protein kinase 3 (RIPK3), which phosphorylates MLKL, was absent from most cell lines. In contrast, RIPK3 was expressed in non-cancerous murine fibroblast cell lines. When a CRsyn-oHSV-infected RIPK3-deficient cancer cell line was co-cultured with the fibroblast cell line, but not with the cancer cells themselves, MLKL was phosphorylated and syncytial death was induced. These results indicate that early necroptosis is induced in multinucleated giant cells formed by CRsyn-oHSV when they also contain non-cancerous cells.

INTRODUCTION

Oncolytic virotherapy is a promising new treatment for cancer. Of the viral candidates, oncolytic herpes simplex virus (oHSV) has been approved for clinical use in several countries, and many attempts are being made to develop new types of improved genetically modified oHSV.[1,2] Since cell surface receptors essential for HSV entry are expressed by most human cells, those viral genes necessary for efficient viral replication in normal cells are deleted to restrict oHSV replication to cancer cells. However, it has been reported that these conditionally replicating oHSVs (CR-oHSVs) often exhibit reduced efficiency for generating viral progeny in cancer cells.[3–6] Conversion of CR-oHSVs to induce direct cell fusion of infected cells with adjacent uninfected cells has shown promise for enhancing the anti-tumor effects of these viruses, which cause the formation of multinucleated giant cells termed syncytia. RP1, which employs the same genetic modifications in talimogene laherparepvec (Imlygic), expressing gibbon ape leukemia virus envelope fusogenic membrane glycoprotein (GALV-FMG), and HF10, which has a syncytia-inducing mutation (syn mutation), have been evaluated for safety and efficiency in clinical trials.[7,8]

Recently, we reported that syncytial CR-oHSV (CRsyn-oHSV), which has two syn mutations, causes fusion of cancer cells with normal cells and induces early syncytial death.[9] In contrast, receptor-retargeted syncytial oHSV (RRsyn-oHSV), which does not cause fusion of cancer cells with normal cells, does not induce syncytial death, resulting in long-lasting virus spread. Furthermore, RRsyn-oHSV exhibited the same level of infectivity as CRsyn-oHSV under *in vitro* conditions where only cancer cells were present, but nonetheless mediated markedly greater anti-tumor activity than CRsyn-oHSV (>1 million-fold higher in a mouse xenograft model of human glioblastoma U87) under *in vivo* conditions where cancer cells and non-cancerous cells are both present in the tumor. These findings suggested that the reduction of viral replication and spreading due to induction of early syncytial death is one of the factors that markedly reduces the therapeutic efficacy of CRsyn-oHSV. It has been reported that infection of human squamous cell carcinoma cell lines by RH2, one of the CRsyn-oHSVs, induced apoptosis, pyroptosis, and autophagic cell death, but data on the mechanism of syncytial death caused by such viruses is otherwise very limited.[10] In cancer cells in general, many signal transduction pathways, including cell death pathways, are disrupted. It is very possible that the factors that induce syncytial death under conditions where cancer cells and non-cancerous cells are both present are derived from the latter. Therefore, it is important to investigate the

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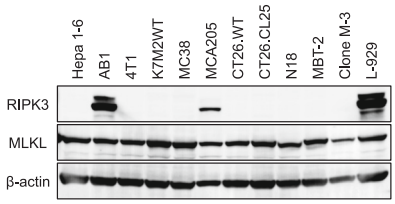

Figure 1. Expression of RIPK3 and MLKL in murine cancer cell lines
Detection of RIPK3, MLKL, and β-actin in murine cancer cell lines by immunoblotting. Cell lines are shown along at the top, and molecules detected on the left.

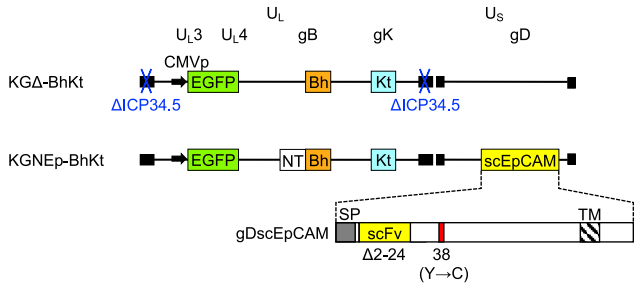

Figure 2. Schematic representation of CRsyn-oHSV and RRsyn-oHSV used in this study
Virus designations are shown on the left. UL, unique long segment; US, unique short segment; CMVp, human cytomegalovirus major immediate-early (HCMV-IE) promoter; EGFP, expression cassette for EGFP inserted in the intergenic region between the UL3 and UL4 genes; ΔICP34.5, deletion of ICP34.5 gene; Bh, R858H mutation in gB; Kt, A40T mutation in gK; NT, D285N/A549T mutations in gB that increase the rate of virus entry; scEpCAM, anti-hEpCAM scFv-fused gD; SP, signal peptide; TM, transmembrane region; Δ2-24, deletion of residues 2–24 of gD for ablation of HVEM binding; Red box, single amino acid substitution at residue 38 of gD for ablation of nectin-1 binding; Y, Tyrosine; C, Cysteine; Closed boxes, terminal and internal inverted repeats.

mechanisms responsible for the CRsyn-oHSV-induced syncytial death that occurs under conditions in which both cancer and non-cancerous cells are present, better mimicking the *in vivo* environment. However, the mechanisms of syncytial death induced by CRsyn-oHSV have not been investigated in co-cultures with the resulting mixed giant cell syncytia. An ability to intervene to suppress the induction of syncytial death following CRsyn-oHSV infection would facilitate the virus spread into a wider area of the tumor and enhance virotherapy applications. It will be challenging to develop improved CRsyn-oHSV agents that are less likely to induce syncytial death unless one has a detailed understanding of its mechanisms. To this end, it is essential to determine which cell death mechanisms are involved in the death of syncytia formed by the fusion of cancer cells and non-cancerous cells.

Necroptosis is a type of programmed cell death caused by specific phosphorylation of mixed-lineage kinase domain-like (MLKL) by receptor-interacting protein kinase 3 (RIPK3).[11–13] It is triggered following infection with wild-type HSV.[14] Necroptosis is characterized by the rupture of the cell membrane due to the formation of small pores by phosphorylated MLKL, and the outflow of intracellular molecules including ATP to the interstitium. Hence, necroptosis is likely to be involved in phenomena such as rupture of the cell membrane and loss of intracellular EGFP fluorescence observed during syncytial death induced by CRsyn-oHSV infection when cancer cells and non-cancerous cells are both present. Here, we evaluated whether the necroptosis pathway is involved in syncytial death induced by CRsyn-oHSV in an environment where cancer cells and non-cancerous cells coexist, as is the case in tumors.

RESULTS

RIPK3 is only expressed in some murine cancer cell lines, whereas MLKL is expressed in all

We first investigated the expression of RIPK3 and MLKL in 11 murine cancer cell lines by immunoblotting (Figure 1). Murine mesothelioma AB1 was the only cell line that expressed RIPK3 to a similar degree as in murine fibroblast L-929 cells, in which necroptosis can be efficiently induced.[15] Although RIPK3 was weakly expressed in murine fibrosarcoma MCA205 cells, it was not detected at all in the other nine cancer cell lines. On the other hand, MLKL was expressed in all of these cell lines. These results suggest that RIPK3 deficiency may render cancer cell lines less susceptible to necroptosis.

Phosphorylation of MLKL is enhanced when KGΔ-BhKt-infected Hepa 1-6-hEpCAM cells are co-cultured with non-cancerous 3T6 cells

We introduced human epithelial cell adhesion molecule (hEpCAM) into murine hepatocarcinoma Hepa 1–6 cells. Of the cancer cell lines shown in Figure 1, these cells are most tolerant of infection with the ICP34.5-deficient CRsyn-oHSV (KGΔ-BhKt) (Figure 2). We used hEpCAM-expressing Hepa 1–6 (Hepa 1-6-hEpCAM) as a model for cancer cells permissive not only for infection with KGΔ-BhKt but also hEpCAM-retargeted RRsyn-oHSV (KGNEp-BhKt) (Figure 2). KGNEp-BhKt expresses EpCAM-retargeted gD, which has a deletion that prevents HVEM binding, as well as a mutation preventing nectin-1 binding, and also includes an anti-hEpCAM single chain antibody (scEpCAM), which cannot bind to murine EpCAM, to retarget hEpCAM.[16,17] EpCAM-retargeted RR-oHSV has been shown to enter and spread between hEpCAM-positive cells in a specific manner.[17] As a model for non-cancerous cells, we used murine fibroblast 3T6 cells, which is frequently employed to evaluate the infectivity of CR-oHSV for normal cells.[18–20] Using these cell lines, we asked whether necroptosis was induced by syncytial oHSV infection in an environment where both cancer cells and non-cancerous cells were present.

First, we examined the expression of RIPK3 in the cells used in this experiment by immunoblotting. In a model of cancer cells lacking RIPK3, we confirmed that it was not expressed in Hepa 1–6 cells, but was detected in 3T6 cells, used as a model of non-cancerous cells (Figure 3A). These results emphasize that these cell lines are appropriate for the study described here, and also suggest that 3T6 cells could serve as a source of RIPK3 when fused with RIPK3-deficient cancer cells. To confirm that the results reported in our previous study were reproducible under the present experimental conditions using these cell lines, Hepa 1-6-hEpCAM cells infected with

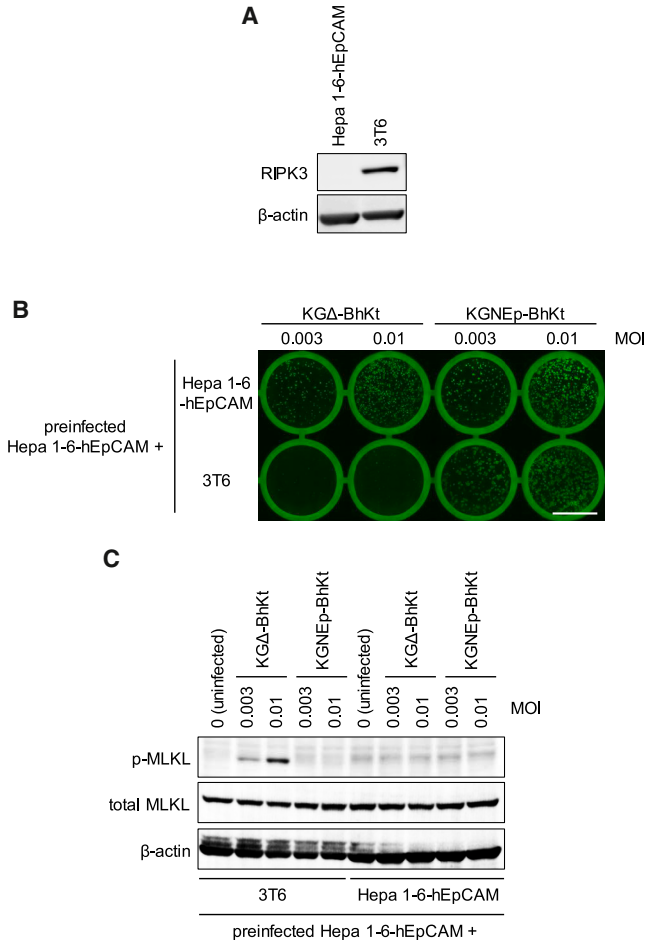

Figure 3. Induction of necroptosis in co-cultures of cancer and non-cancerous cells

(A) Detection of RIPK3 and β-actin in Hepa 1-6-hEpCAM and 3T6 cells by immunoblotting. Cell lines are shown at the top, the molecules detected are shown on the left.

(B and C) Hepa 1-6-hEpCAM cells were infected with the viruses shown at the top at MOIs of 0.003 or 0.01 for 2 h, seeded together with the same number of the cells shown on the left (B) or below (C), and cultured for 2 days. (B) EGFP signals in syncytial plaques are visible. Scale bar, 10 mm. (C) Detection of phosphorylated MLKL in lysates of infected cells by immunoblotting. Molecules detected are shown on the left.

KGΔ-BhKt or KGNEp-BhKt were co-cultured with the same number of uninfected Hepa 1-6-hEpCAM or 3T6 cells. EGFP signals were recorded 2 days post-infection (Figure 3B). Both viral constructs caused the formation of EGFP-expressing syncytial plaques by Hepa 1-6-hEpCAM cells alone. When Hepa 1-6-hEpCAM and 3T6 cells were co-cultured, KGNEp-BhKt infection resulted in the formation of EGFP-expressing plaques similar to cultures of Hepa 1-6-hEpCAM alone. In contrast, EGFP signals in the plaques observed after KGΔ-BhKt-infection were very weak. These results are consistent with our previous study in which syncytial death was accompanied by decreased EGFP signals in similar experiments where human cancer cells and non-cancerous cells were co-cultured. Thus, we considered it highly likely that, in accordance with the findings from the previous study, KGNEp-BhKt formed multinucleated giant cells consisting only of cancer cells (Hepa 1-6-hEpCAM cells), while KGΔ-BhKt formed multinucleated giant cells containing non-cancerous cells (3T6 cells). The implication was that early cell death was induced by increased membrane permeability in syncytia formed by KGΔ-BhKt.

In order to investigate whether necroptosis is involved in the death of multinucleated giant cells containing non-cancerous cells, the above-described infected cells were lysed and the phosphorylation of MLKL, the molecule responsible for necroptosis, was examined by immunoblotting (Figure 3C). Although MLKL was detected to similar degrees under all culture conditions, a viral load-dependent increase in phosphorylated MLKL was observed only when KGΔ-BhKt-infected Hepa 1-6-hEpCAM cells were co-cultured with 3T6 cells. These results suggest that necroptotic syncytial death was induced by phosphorylation of MLKL only when KGΔ-BhKt-infected Hepa 1-6-hEpCAM cells and 3T6 cells were both present. Interestingly, despite the absence of RIPK3 in Hepa 1–6 cells, necroptotic cell death still occurred in KGΔ-BhKt-infected Hepa 1-6-hEpCAM cells co-cultured with 3T6 cells. This finding suggests that early necroptosis might be induced when RIPK3-expressing cells are present in the syncytium.

Necroptosis associated with MLKL phosphorylation is induced when RIPK3-expressing cells are included in the syncytium

To assess whether RIPK3-expressing cells can serve as a source of RIPK3 to induce early necroptotic syncytial death upon infection with KGΔ-BhKt, Hepa 1–6 cells with or without enforced expression of RIPK3 (Hepa 1-6-RIPK3 and Hepa 1-6-mock, respectively) were compared. Hepa 1–6 cells infected with KGΔ-BhKt were co-cultured with Hepa 1-6-mock or Hepa 1-6-RIPK3 in the presence of dead cell stain SYTOX, which is impermeable to normal membranes and fluoresces strongly when bound to dsDNA. Fluorescence signals from EGFP and SYTOX were the compared (Figure 4A). When infected cells were co-cultured with Hepa 1-6-mock, SYTOX fluorescence signals were not observed in most plaques even 3 days after infection. In sharp contrast, when infected cells were co-cultured with Hepa 1-6-RIPK3, SYTOX fluorescence signals were observed in most plaques even only 2 days after infection. In addition, the fluorescence signals of SYTOX appeared earlier as the number of co-cultured Hepa 1-6-RIPK3 was titrated up. These results suggest that as a result of the fusion of RIPK3-expressing cells and KGΔ-BhKt-infected cancer cells deficient in RIPK3, the RIPK3 provided by the former induced early syncytial death. These results also suggest that even when it does not originate from non-cancerous cells, RIPK3 rapidly induces syncytial death in a dose-dependent manner. Interestingly, unlike the data shown in Figure 3B, relatively strong EGFP signals were observed in infectious foci formed by KGΔ-BhKt co-cultured with RIPK3-expressing cells, although SYTOX signals were also detected (see below for a discussion of this interesting observation).

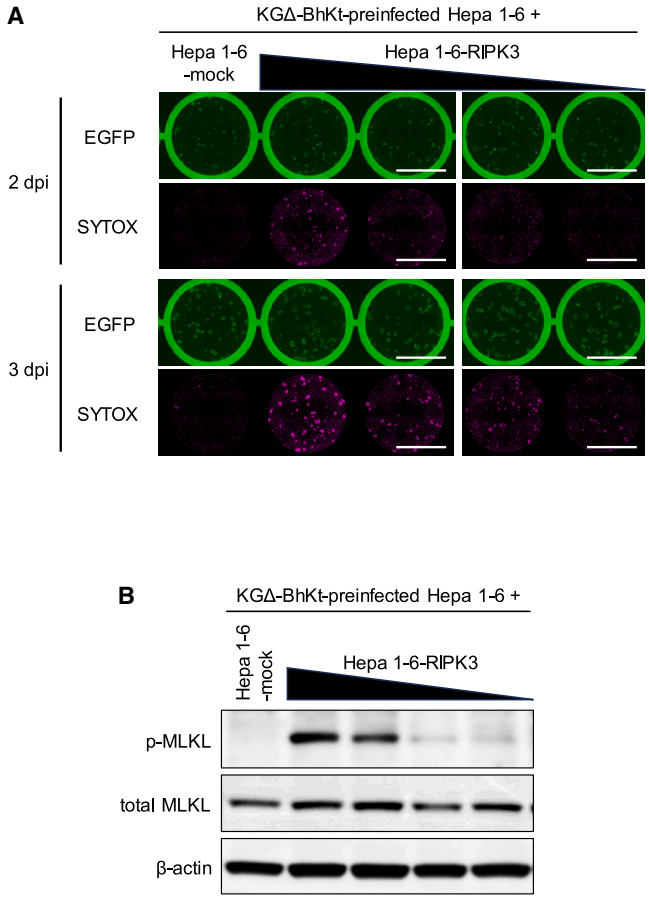

Figure 4. Induction of necroptotic syncytial death in co-cultures of CRsyn-oHSV infected cancer cells and RIPK3-expressing cancer cells

Hepa 1–6 cells were infected with KGΔ-BhKt at MOIs of 0.001 (A) or 0.01 (B) for 2 h, seeded together with the same number of cells shown at the top or with serially diluted Hepa 1-6-RIPK3 using Hepa 1-6-mock, and cultured in media containing SYTOX for 3 days (A) or 2 days (B).

(A) EGFP and SYTOX signals in syncytial plaques are seen. Scale bars, 10 mm.

(B) Detection of phosphorylated MLKL in infected-cell lysate by immunoblotting. Molecules detected are shown on the left.

In parallel experiments, cells cultured under similar conditions were lysed and MLKL phosphorylation was examined by immunoblotting (Figure 4B). Phosphorylated MLKL was not detected when infected cells were co-cultured with Hepa 1-6-mock cells. However, in the Hepa 1-6-RIPK3 co-cultures, phosphorylated MLKL was detected and increased as the number of co-cultured Hepa 1-6-RIPK3 cells increased. These results confirm that necroptotic syncytial death was induced when RIPK3-expressing cells, even if they are cancer cells, were included in the syncytium, also in a dose-dependent manner.

In summary, the phosphorylated MLKL-dependent necroptosis pathway is involved in syncytial death induced by KGΔ-BhKt under conditions where both cancer and non-cancerous cells are present. We conclude that RIPK3 supplied by RIPK3-expressing cells contained in syncytia is likely to be one of the inducers of early necroptotic syncytial cell death.

DISCUSSION

In the present study, we report that syncytia formation caused by CRsyn-oHSV infection of co-cultures of cancer cells and non-cancerous cells leads to necroptotic cell death of the syncytia. Furthermore, we also found that RIPK3 supplied by RIPK3-expressing cells contained in syncytia is likely to be one of the inducers of such necroptotic syncytial death. It remains unclear whether other types of antiviral responses such as apoptosis are also involved in syncytial cell death.

CRsyn-oHSVs that have been developed thus far can be classified into two types: (1) GALV-FMG type and (2) syn mutation type.[7,21–27] While human Pit-1 functions as a receptor for GALV-FMG, murine Pit-1 does not.[28–31] Therefore, when the GALV-FMG type virus is evaluated for anti-tumor activity in a human cancer xenograft model in mice (as commonly used to test the efficacy of almost all oHSVs reported so far), it does not induce a mixed syncytium of human cancer cells and mouse non-cancerous cells (expressing murine Pit-1), and virus spread can continue within the tumor. However, in the clinical environment where human cancer cells and human non-cancerous cells such as fibroblasts, vascular endothelial cells, and immune cells all coexist in the tumor, GALV-FMG type virus would induce syncytia of cancer cells and non-cancerous cells (expressing human Pit-1), resulting in early cell death. It is therefore highly likely that the therapeutic effects observed in the mouse models will not in fact translate to humans. Thus, the potential usefulness of GALV-FMG type CRsyn-oHSV may have been overestimated by the preclinical studies. In contrast, syn mutation type CRsyn-oHSV induces the formation of mixed syncytia of cancer cells and non-cancerous cells under both of the above conditions. In this respect, the evaluation of syn mutation type CRsyn-oHSV is considered to be more likely to translate to the human situation.

The results of the present study suggest that concomitant use of agents that inhibit the induction of necroptosis by CRsyn-oHSV may be an attractive way to augment its anti-tumor effects. However, as it has been reported that suppression of necroptosis-induced cell death leads to the induction of apoptosis,[32] it may not be sufficient to suppress only necroptosis. ICP34.5, which is often deleted in CR-oHSV, suppresses the activation of apoptosis via counteracting the inhibition of protein synthesis following detection of dsRNA resulting from viral infection.[33] In addition, in order to convert HSV to CR-oHSV, attempts have been made to delete ICP6, ICP27, and Us3 which are also involved in the suppression of apoptosis.[34–38] Thus, CR-oHSVs that lack these genes are expected to be susceptible to apoptosis-mediated cell death.

Furthermore, it is likely that the induction of cell death is not the only means by which non-cancerous cells suppress virus spread in tumors. Notably, in Figure 4A, the EGFP signals in infectious foci formed by KGΔ-BhKt co-cultured with RIPK3-expressing cancer cells that are

susceptible to CRsyn-oHSV infection were stronger than seen under otherwise the same conditions in Figure 3B where 3T6 cells that are resistant to CRsyn-oHSV infection were used. Antiviral responses that inhibit virus propagation are not limited to necroptosis (e.g., PKR response). Although necroptosis is induced by either of these two types of cells, the degree of inhibition of oHSV spread due to antiviral responses in 3T6 cell-containing syncytium could be greater than in Hepa 1-6-RIPK3 cell-containing syncytium. Therefore, it is also worth investigating the involvement of antiviral responses other than necroptosis (e.g., apoptosis and the PKR response) in future studies.

Based on the extreme complexity mentioned above, it is reasonable to propose that facilitating the formation of syncytia consisting only of cancer cells would be advantageous for efficient virus spread through the tumor. To achieve this, receptor-retargeting should be a rational approach, as it would help to prevent the formation of syncytia composed of both cancer and non-cancerous cells. However, for this tactic to succeed, highly cancer-specific molecules would need to be selected as targets for RRsyn-oHSV, but the availability of such antigens is very limited. In order to overcome this restriction, receptor-retargeted CRsyn-oHSV (RR-CRsyn-oHSV) which already has the ability to selectively replicate in cancer cells, would be very useful. Not only cancer-specific antigens, but also other molecules, provided they are not expressed in non-cancerous cells within tumors, would become selectable target candidates for RR-CRsyn-oHSV. In addition, an antibody-binding HSV (KGNc2) has recently been developed by our group which can latch on to antibodies suitable for application to RR-oHSV, such that pairs of target molecules and antibody can be efficiently screened for.[39] Moreover, even in the unlikely event that infection spreads to normal tissues, immediate induction of cell death by necroptosis would be valuable as the fourth safety mechanism following receptor-retargeting modification, conditionally replicating modification and anti-herpesvirus drugs. In order to maximize direct oncolytic activity, we suggest that it would be ideal to adopt cancer-specific antigens to develop RRsyn-oHSV without the necessity for such conditionally replicating modifications, thereby exploiting the original anti-cell death abilities of HSV.

In the future, *in vivo* experiments will be necessary to further evaluate the importance of necroptosis in syncytial cell death. However, because cells that have undergone necroptosis are thought to be rapidly eliminated by phagocytes *in vivo*, elaborate consideration of appropriate conditions will be necessary to determine optimal timing to detect phosphorylated MLKL. In the present study, we focused on the involvement of necroptosis in the death of multinucleated giant cells that contain non-cancerous cells. However, as mentioned above, necroptosis is closely related to other forms of cell death, so in the future, comprehensive investigations using cell death inhibitors should be performed in order to clarify these relationships. Clarification is also required as to whether antiviral responses other than cell death pathways, such as the PKR response, are involved in the death of multinucleated giant cells containing non-cancerous cells.

Although the conclusions from this study may not be generally applicable, we propose that it will be important to avoid necroptosis and other forms of cell death at the stage of premature oncolysis when developing a fusogenic oncolytic virus with strong anti-tumor activity. This may apply to syncytial oncolytic viruses derived from any virus species, and not only to syncytial oHSV. For example, in addition to RRsyn-oHSV, receptor-retargeted oncolytic measles virus (RR-oMV) and receptor-retargeted oncolytic vesicular stomatitis virus (RR-oVSV), which can infect cells solely via the target molecule, have also been developed. They are thought to be syncytial oncolytic viruses that, like RRsyn-oHSV, form multinucleated giant cells that do not contain non-cancerous cells.[40,41] Thus, in order to prevent the induction of early syncytial necroptosis causing premature oncolysis, MV and VSV can also be modified to receptor-retargeted versions. Because to the best of our knowledge, full receptor-retargeting technology has not been accomplished except for these three viruses, other syncytial oncolytic viruses derived from any virus species that do not harbor receptor-retargeting modifications are likely to form multinucleated giant cells that contain non-cancerous cells. This might result in early syncytial death causing premature oncolysis. In the future, however, strategies for augmenting the activity of non-retargeted syncytial oncolytic viruses by the achievement of receptor-retargeting technology, may become feasible. Last, in cancer treatment, while it is commonly believed that it is better to kill non-cancerous cells such as fibroblasts and vascular endothelial cells in the tumor to cause effective tumor regression, our conclusions from the present study suggest an interesting alternative scenario contrary to this paradigm.

MATERIALS AND METHODS

Cells

Murine fibroblast 3T6 (ECACC 86120801), murine hepatoma Hepa 1–6 (ATCC CRL-1830), murine osteosarcoma K7M2-WT (ATCC CRL-2836), murine colorectal adenocarcinoma MC38 (provided by Dr. Steven Rosenberg, National Cancer Institute, Bethesda, MD, USA), and murine neuroblastoma N18 (ECACC 88112301) cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM; Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS; Thermo Fisher Scientific). Murine bladder carcinoma MBT-2 (RIKEN BRC RCB0544) cells were cultured in Roswell Park Memorial Institute 1640 medium (RPMI 1640; Thermo Fisher Scientific) supplemented with 10% FBS. Murine breast cancer 4T1 (ATCC CRL-2539) and murine colon carcinoma CT26.WT (ATCC CRL-2638) cells were cultured in RPMI 1640 supplemented with 10% FBS and sodium pyruvate (Thermo Fisher Scientific). Murine mesothelioma AB1 (ECACC 10092305) cells were cultured in RPMI1640 supplemented with 5% FBS and HEPES (Thermo Fisher Scientific). Murine colon carcinoma CT26.CL25 (ATCC CRL-2639) cells were cultured in RPMI 1640 supplemented with 10% FBS, sodium pyruvate, non-essential amino acids (NEAA; Thermo Fisher Scientific), and 400 μg/mL G418 (Thermo Fisher Scientific). Murine fibrosarcoma MCA205 (provided by Dr. Steven Rosenberg) cells were cultured in RPMI1640 supplemented with 10% FBS, sodium pyruvate, NEAA, and 2-mercaptethanol (Wako,

Osaka, Japan). Murine melanoma Clone M-3 (ATCC CCL-53.1) cells were cultured in Ham's F-10 medium (Thermo Fisher Scientific) supplemented with 15% horse serum (HS, Thermo Fisher Scientific) and 2.5% FBS. Murine fibroblast L-929 (ATCC CCL-1) cells were cultured in Eagle's minimum essential medium (Thermo Fisher Scientific) supplemented with 10% HS. Hepa 1-6-RIPK3 and Hepa 1-6-mock cells were established by infection of Hepa 1–6 cells with murine RIPK3-expressing retroviral vector derived from pMXc-RIPK3 or negative control retroviral vector derived from pMXc-puro (provided by Toshio Kitamura, University of Tokyo, Tokyo, Japan) as described previously and selected for resistance to 4 μg/mL puromycin.[42] Plasmid pMXc-RIPK3 was constructed according to the following steps: Total RNA extracted from L-929 cells using RNeasy Mini Kits (QIAGEN, Hilden, Germany) was reverse-transcribed to cDNA using PrimeScript II 1st Strand cDNA Synthesis Kits (Takara Bio, Shiga, Japan). A murine RIPK3 gene-containing fragment was obtained by PCR using cDNA derived from L-929 cells as the amplification template with the primers 5′-aactcgagccaccatgtcttctgtcaagtta tggccta-3′ and 5′-aataagcggccgctacttgtggaagggctgccag-3'. The amplified fragment was inserted into multiple cloning sites of pMXc-puro. Plasmid constructs were confirmed by DNA sequencing. Hepa 1-6-mock, Hepa 1-6-RIPK3, and Hepa 1-6-hEpCAM established previously were cultured with DMEM supplemented with 10% FBS and 4 μg/mL puromycin.[9]

Viruses

EpCAM-retargeted RRsyn-oHSV (KGNEp-BhKt) and ICP34.5-deleted CRsyn-oHSV (KGΔ-BhKt) have been described previously.[9,42] These viruses contain an expression cassette for EGFP under the control of the human cytomegalovirus major immediate-early (HCMV-IE) promoter and possess two syncytial mutations (gB:R858H and gK:A40T) in viral glycoprotein B (gB) and glycoprotein K (gK). ICP34.5 genes of KGΔ-BhKt were deleted as described previously.[9] KGNEp-BhKt has entry-enhancing double mutations (gB:D285N/A549T) in gB and EpCAM-retargeted gD.[42,43] The propagation, purification, and titration of the viruses were essentially as described previously.[44,45]

Expression of RIPK3 and MLKL in murine cell lines

Cultured murine cancer cells were collected using trypsin-EDTA (Thermo Fisher Scientific) and resuspended in medium, washed twice with PBS (−) (Wako), and 3×10^6 cells per vial were frozen in liquid nitrogen. After thawing, cells were lysed in RIPA buffer (Wako) containing a protease inhibitor cocktail (cOmplete, EDTA-free protease inhibitor cocktail tablets; Roche, Basel, Switzerland) and phosphatase inhibitor cocktail solution I (Wako). The cell lysates were boiled in SDS sample buffer (Wako) containing dithiothreitol (DTT, Wako) and then separated using a 10% polyacrylamide gel (Wako) and transferred onto Immobilon-FL (Millipore, MA, USA). The membrane was washed using 1×Tris-buffered saline (TBS, Takara Bio), and incubated in PVDF Blocking Reagent for Can Get Signal (TOYOBO, Osaka, Japan) containing 5% skim milk (Wako) at 37°C for 2 h. After blocking, the membrane was washed three times with TBS containing 0.1% Tween 20 (Nacalai Tesque, Kyoto, Japan) (0.1% TBS-T). The membrane was then incubated in Can Get Signal Solution 1 (TOYOBO) containing primary antibodies at 4°C overnight. After washing three times with 0.1% TBS-T, the membrane was incubated in Can Get Signal Solution 2 (TOYOBO) containing secondary antibody at room temperature for 1 h. After washing another three times with 0.1% TBS-T, florescence signals were recorded using an Amersham Imager 600 (Cytiva, Tokyo, Japan). Murine anti-β-actin monoclonal antibody (1:2,000, A1978, Sigma-Aldrich, St Louis, MO, USA), rat anti-MLKL monoclonal antibody (1:2,000, ab243142, Abcam, Cambridge, MA, USA), and rabbit anti-RIPK3 monoclonal antibody (1:2,000, 95702, Cell Signaling Technology, Danvers, MA, USA) were used as primary antibodies. Cy2-labeled goat anti-murine IgG polyclonal antibody (1:2,000, ab6944, Abcam), Cy3-labeled goat anti-rat IgG polyclonal antibody (1:2,000, ab98416, Abcam) and Cy5-labeled goat anti-rabbit IgG polyclonal antibody (1:2,000, A10523, Thermo Fisher Scientific) were used as secondary antibodies.

Co-culture experiments and expression of phosphorylated MLKL

Single-cell suspensions of Hepa 1-6-hEpCAM cells were incubated with KGΔ-BhKt or KGNEp-BhKt at MOIs of 0 (no virus), 0.003, or 0.01 at 37°C for 2 h under constant rotation, co-seeded with an equal number of uninfected Hepa 1-6-hEpCAM or 3T6 cells, and incubated in culture medium in 24-well plates for 42 h at 37°C. EGFP signals were observed under a BZ X-800 fluorescence microscope (Keyence, Osaka, Japan). Thereafter, the cells were washed twice with ice-cold PBS and lysed by RIPA buffer containing protease inhibitor cocktail and phosphatase inhibitor cocktail solution I on iced water for 15 min. Preparation of running samples, SDS-PAGE, and transfer onto Immobilon-FL was as described above. Membranes were incubated in PVDF Blocking Reagent for Can Get Signal at 37°C for 2 h. After blocking, primary and secondary antibody staining was performed as described above. After florescence signal detection, enhanced chemiluminescence signals were recorded using an Amersham Imager 600. Rabbit anti-phosphorylated MLKL monoclonal antibody (1:1,000, 37333, Cell Signaling Technology), murine anti-β-actin monoclonal antibody, and rat anti-MLKL monoclonal antibody as described above were used as primary antibodies. Horseradish peroxidase (HRP)-labeled goat anti-rabbit IgG polyclonal antibody (1:3,000, 170–6515, Bio-Rad, Hercules, CA, USA), Cy2-labeled goat anti-murine IgG polyclonal antibody and Cy3-labeled goat anti-rat IgG polyclonal antibody as described above were used as secondary antibodies.

Cell death detection using dead cell stain SYTOX

Single-cell suspensions of Hepa 1–6 cells were incubated with KGΔ-BhKt at MOIs 0.001 or 0.01 at 37°C for 2 h under constant rotation, co-seeded with an equal number of uninfected Hepa 1-6-mock, Hepa 1-6-RIPK3 or mixed (Hepa 1-6-RIPK3:Hepa 1-6-mock = 1:1, 1:2, 1:4 or 1:8) cells, and incubated in 0.5 μM SYTOX Orange (Thermo Fisher Scientific) and 1% methylcellulose-containing medium (MOI 0.001) or 0.5 μM SYTOX Orange-containing medium (MOI 0.01) in 24-well plates for 48 h at 37°C. EGFP and SYTOX

Orange signals were captured under a BZ X-800 microscope 2 days or 3 days post-infection. Infected cell lysates were prepared 48 h post-infection as described above. Immunoblotting was performed as described above. Pairs of primary antibodies and secondary antibodies were as described above.

DATA AND CODE AVAILABILITY

The datasets of this article are available upon request.

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AUTHOR CONTRIBUTIONS

T.S. and H.U. contributed to the design of the work. T.S. contributed to the acquisition of the data. T.S. and H.U. contributed to the analysis and interpretation of the data. T.S. and H.U. drafted the work.

DECLARATION OF INTERESTS

H.U. is an inventor of intellectual property licensed to Oncorus (Cambridge, MA, USA) and Replay (San Diego, CA, USA). H.U. is a co-founder and employee of Good Hero Therapeutics, Inc. This work was supported in part by Grants-in-Aid from the Ministry of Education, Culture, Sports, Science and Technology of Japan; grant numbers 22K15553 (T.S.) and 22H02913 (H.U.).

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