Infection with Oncolytic Herpes Simplex Virus-1 Induces Apoptosis in Neighboring Human Cancer Cells: A Potential Target to Increase Anticancer Activity

Stephen F. Stanziale,[1] Henrik Petrowsky,[1] Prasad S. Adusumilli,[1] Leah Ben-Porat,[2] Mithat Gonen,[2] and Yuman Fong[1]

[1]Department of Surgery, Hepatobiliary Division and [2]Department of Epidemiology and Biostatistics, Memorial Sloan-Kettering Cancer Center, New York, New York

ABSTRACT

Purpose: **The antitumor efficacy of a herpes simplex virus (HSV)-1 oncolytic virus depends on the cytotoxic effect of the virus, but also on viral replication and spread within the tumor. Apoptosis is considered a defense mechanism of infected cells that minimizes the spread of viral progeny by limiting cellular production of virus. We sought to determine whether oncolytic HSV-1 infection induces apoptosis in neighboring, uninfected cells and whether manipulation of apoptosis can increase viral replication and cytotoxicity.**

Experimental Design: **NV1066 is an oncolytic HSV-1 mutant that contains the marker gene for enhanced green fluorescent protein. OCUM human gastric cancer cells were infected with NV1066 *in vitro* and inspected for apoptosis by Hoechst and terminal deoxynucleotidyltransferase-mediated nick end labeling staining and for infection by expression of green fluorescence.**

Results: **A significant increase in apoptosis was seen in cells infected by NV1066. More interestingly, a significant percentage (10%) of uninfected cells also proceeded to apoptosis. After NV1066 infection, cells were also treated with *N*-acetylcysteine (NAC), an inhibitor of apoptosis. By day 4 after infection, 2.7× more NV1066 was produced in cells exposed to NAC than in those not exposed to NV1066 ($P = 0.04$). NAC also increased tumor kill when administered with virus.**

Conclusions: **These data suggest that NV1066 induces apoptosis in uninfected cocultured cells, potentially hindering propagation of viral progeny and concomitant tumor kill. Inhibition of apoptosis may improve the efficacy of oncolytic HSV-1 therapy.**

INTRODUCTION

The antitumor activity of replication-competent, oncolytic herpes simplex virus (HSV)-1 depends on the cytotoxic effect of the virus and on the ability of the virus to replicate within the tumor to produce progeny for further tumor lysis. Factors that limit continued viral propagation are not completely understood. Latent HSV genomes can be detected up to 1 year after infection (1), but persistence of active HSV-1 virus or transgene expression is more ephemeral. Many groups have demonstrated that replication is terminated and virus is cleared within days of tumor infection (2–4). Increasing and sustaining viral production should enhance tumor killing.

Apoptosis is a host cell defense mechanism that limits viral infection by shutting down the cellular machinery necessary for viral production. The cellular response to HSV-1 infection includes phosphorylation of eukaryotic initiation factor-2α, which shuts off protein synthesis and terminates the lytic cycle of the virus. To counteract this apoptotic response, HSV-1 has evolved to include genes such as $\gamma_1 34.5$, which encodes the ICP45 protein (5). Intact viral ICP45 prevents phosphorylation of eukaryotic initiation factor-2α and thus precludes the host cell shut off of protein synthesis (6, 7). In fact, HSV-1 can prevent apoptosis induced by hyperthermia (8), chemically induced osmotic shock (9), or exposure to C2-ceramide, tumor necrosis factor α, and antibody to FAS (10). Several other HSV-1 genes and proteins are involved in preventing the host apoptosis response such as *ICP4* (8), *Us3* and *Us5* (11, 12), and *ICP27* (13), underscoring the importance of apoptosis to viral propagation.

Studies of HIV have demonstrated recently that HIV infection produces apoptosis in uninfected cells (14–18). HIV infection depletes circulating CD4+ T cells in part by the induction of apoptosis in these cells (14, 15, 17, 19–24) and kills lymphocytes within nodal tissue (25, 26) through varying mechanisms (16, 18, 24, 27–31). Our current experiments were conducted to determine whether a similar phenomenon is found with HSV-1 infection. Furthermore, we examined whether pharmacological manipulation of apoptosis can increase viral replication and oncolysis with the aim of a mechanistically based strategy to enhance viral efficacy.

MATERIALS AND METHODS

Cell Lines. OCUM-2MD3 human gastric cancer cells were a gift of Dr. Masakazu Yashiro (Osaka City University Medical School) and were maintained in DMEM with high glucose, 2 mM L-glutamine, and 0.5 mM sodium pyruvate. Vero cells were grown in MEM. All cell lines were maintained in 10% FCS, 100 μg/ml penicillin, and 100 μg/ml streptomycin.

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Requests for reprints: Yuman Fong, Department of Surgery, Hepatobiliary Division, Memorial Sloan-Kettering Cancer Center,1275 York Avenue, New York, NY 10021. Phone: (212) 639-2016; Fax: (212) 639-4031; E-mail: fongy@mskcc.org.

Virus. NV1066 is a replication-competent, attenuated herpes simplex-1 mutant virus derived from HSV-1 virus (F strain) as described previously (32). Briefly, the virus is deficient for *UL23* and the internal repeat region (there is only one copy of the immediate early genes *ICP0* and *ICP4* as well as the γ_1*34.5* gene). The enhanced green fluorescent protein gene (eGFP) under control of a cytomegalovirus promoter was inserted into the deleted internal repeat sequence region. NV1066 was propagated and titered on Vero cells (33).

Hoechst Staining. OCUM cells (1.0×10^4) were grown in 4-well slide chambers (Lab-Tek; Nalge Nunc) and treated with NV1066 at a multiplicity of infection [MOI (*i.e.*, virus: tumor ratio)] of 0.1. Separate populations were left untreated to serve as a negative control, and others were exposed to actinomycin D (Calbiochem), a known apoptosis-inducing agent, to serve as a positive control (34). Cells were incubated at 37°C for 48 h and then harvested, air dried, and fixed in 4% paraformaldehyde for 12 min. After two PBS washes, cells were stained with the DNA-specific fluorochrome Hoechst 33342 (1 μg/ml) for 10 min. Slides were washed in PBS twice and mounted. Several fields were examined under ×100 oil immersion using a Zeiss Axiophot-2 microscope until at least 200 cells were inspected for apoptotic nuclear morphology and expression of green fluorescence. Cells were first examined under a 4′,6-diamidino-2-phenylindole fluorescent filter, and nuclear morphology was assessed. Cells were determined to be apoptotic based on accepted criteria for nuclear morphology, which include nuclear pyknosis, chromatin condensation, nuclear blebbing and fragmentation, and formation of apoptotic bodies (35, 36). A partially blinded scientist examined the same microscopic field after changing the fluorescent filter to a green fluorescent protein (GFP) filter. Cell expression or nonexpression of enhanced green fluorescent protein (eGFP) was noted. A second completely blinded scientist confirmed the results. Any disagreement between investigators was resolved through discussion between the two. Photographs of representative microscopic fields were taken with a Zeiss LSM 510 confocal microscope at $63 \times 1.5 \times 10$ magnification under 4′,6-diamidino-2-phenylindole and GFP filters. Overlay images were created of the same field under the two filters within the system software.

Terminal Deoxynucleotidyltransferase-Mediated Nick End Labeling (TUNEL) Staining. OCUM cells (1.0×10^4) were grown, virally treated, harvested, and fixed in the same manner as described for the Hoechst staining. Cells were treated with 20 μg/ml proteinase K for 2 min and then fixed again in 4% paraformaldehyde for 2 min. After several PBS washes, endogenase peroxidase was quenched with 0.1% H_2O_2 for 15 min. After a rinse with distilled H_2O, slides were immersed in terminal deoxynucleotidyltransferase buffer [3 mM Tris (pH 7.2), 14 mM sodium cacodylate, and 1 mM cobalt chloride] for 10 min. Cells were then exposed to 30 units of terminal deoxynucleotidyltransferase (Roche) and 5 μM biotin-dUTP per slide at 37°C in a moist chamber for 1 h. The reaction was stopped with 2× SSC, and cells were blocked with 2% BSA in PBS to eliminate nonspecific reactivity. Next, slides were exposed to the secondary stain for 1 h, washed in PBS, and treated with 0.5% Triton X-100 for 2 min. Slides were stained with a filtered 3,3′-diaminobenzidine/0.0012% H_2O_2 solution, counterstained with Harris hematoxylin, washed in distilled H_2O, and mounted with fluorescent mounting media. Several fields were inspected under ×40 magnification in bright-field mode using a Zeiss Axiophot-2 for TUNEL positivity until at least 200 cells were examined. Without altering the microscopic field, the fluorescent filter was changed to a GFP filter. Cell expression or nonexpression of eGFP was noted. Photographs of representative microscopic fields were taken with a Zeiss Axiovert 200 microscope at 63×10 magnification under a GFP filter, using bright-field, phase-contrast microscopy with MetaMorph software. Overlay images were created of the same field by the two microscopy modes.

Expression of eGFP Corresponds to HSV Infection. OCUM cells (1×10^6) were grown and infected with NV1066 at MOIs of 0.01, 0.1, and 1. Cells were harvested at 6, 12, and 24 h after infection. eGFP-expressing cells were separated from non-eGFP-expressing cells by fluorescence-activated cell sorting (MoFlo; Dako Cytomation, Fort Colling, CO) and fixed on slides for immunohistochemistry. Uninfected cells served as negative controls. Slides were stained by the improved biotin-streptavidin amplified method (Biogenex Supersensitive Detection System) using a polyclonal antibody to HSV-1. Slides were examined using a Zeiss Axiovert 200 microscope for the presence of herpes and for eGFP expression. Overlay images were created of the same field viewed under routine light microscopy and eGFP microscopy modes. Each condition was performed in triplicate.

Flow Cytometry for eGFP. OCUM cells (4×10^4) were grown in 6-well plates, treated with NV1066 at various MOIs ranging from 0.01 to 0.1, and exposed to assorted concentrations of *N*-acetylcysteine (NAC). NAC is a known inhibitor of apoptosis (37–39). Cells were harvested on day 3 with 0.25% trypsin in 0.02% EDTA, washed in PBS, and brought up in 100 μl of PBS. Five μl of 7-amino-actinomycin (BD PharMingen) were added as an exclusion dye for cell viability. Data for eGFP expression from 10^4 cells were acquired on a FACSCalibur machine and analyzed with Cell Quest software. The experiment was performed more than three times, and a representative experiment is shown (Fig. 2).

Cytotoxicity Assay. OCUM cells (1.0×10^4) were grown in 24-well, flat-bottomed plates and subsequently infected with NV1066 at various MOIs ranging between 0 and 0.1. Cells were exposed *in vitro* to 10 mM NAC or not exposed and then incubated at 37°C for 1–7 days. Each day after infection, cells were washed in PBS and lysed with a 1.35% Triton X-100 solution (percentage volume/PBS) to release intracellular lactate dehydrogenase. The cytotoxic effect of viral infection on tumor cells was determined by release of cytoplasmic lactate dehydrogenase and comparison of these levels to levels in untreated control cells grown under identical conditions. Lactate dehydrogenase was quantified using a Cytotox 96 nonradioactive cytotoxicity assay (Promega, Madison, WI) that measures the conversion of a tetrazolium salt into a formazan product. Absorbance was measured at 450 nm using a microplate reader (EL 312e; Bio-Tek Instruments, Winooski, VT). Results are expressed as the surviving fraction, based on the percentage of the lactate dehydrogenase cells release compared with that of untreated control cells. All samples were tested in triplicate, and the experiment was repeated at least three times to ensure reproducibility.

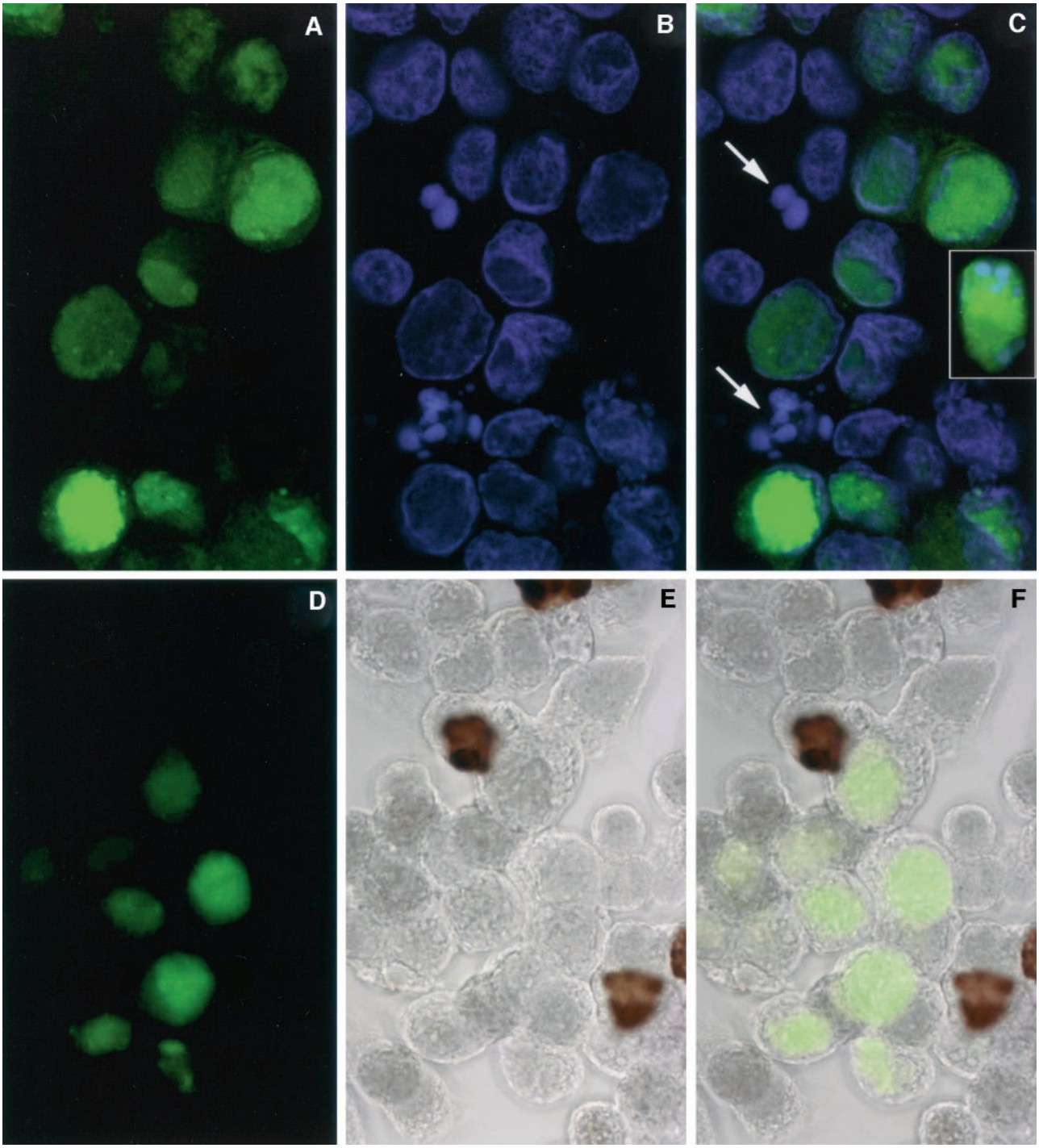

Fig. 1 NV1066 induces apoptosis in infected cells as demonstrated by nuclear staining (*A*−*C*) and terminal deoxynucleotidyl transferase-mediated nick end labeling (TUNEL; *D*−*F*). OCUM cells were infected with NV1066 (MOI = 0.1) and examined under separate filters for GFP expression and apoptosis. Representative fields are depicted here and quantitatively described in Table 1. *A,* infected cells stained with Hoechst DNA fluorochrome express enhanced green fluorescent protein (eGFP) as seen under a GFP filter at ×630 magnification. The eGFP protein can be found within nuclei and cytoplasm. *B,* under a 4′,6-diamidino-2-phenylindole filter, Hoechst staining illustrates high resolution of nuclear morphology to assess nuclear morphology. *C,* an overlay image is created by superimposing the GFP and 4′,6-diamidino-2-phenylindole filter images to concurrently evaluate nuclear morphology and eGFP expression. Characteristic morphology of apoptotic cells, including pyknosis, nuclear fragmentation, and apoptotic bodies, is seen in the cells at the *white arrows*. These cells do not express eGFP; that is, they are apoptotic but are not infected. The *inset* taken from another field illustrates that an apoptotic cell can express eGFP. Characteristic apoptotic bodies are shown within the eGFP-filled cytoplasm. *D,* infected cells are stained by TUNEL and examined under a GFP filter at ×945 magnification. Expression of eGFP represents viral infection. *E,* using phase-contrast, bright-field microscopy, TUNEL staining is evaluated. Several apoptotic cells are depicted by *brown-stained nuclei*. *F,* an overlay image is created to compare TUNEL positivity and eGFP expression. TUNEL-positive cells do not express eGFP, confirming that apoptosis occurs in noninfected cells.

Table 1 NV1066 induces apoptosis in uninfected and infected cells

Random microscopic fields of NV1066-treated cells were assessed for nuclear morphology and eGFP expression, counted, and averaged from three experiments. Cells are either apoptotic and do not express eGFP, not apoptotic and express eGFP, apoptotic and express eGFP, or not apoptotic and do not express eGFP. The last group is not represented in the table. There was a significantly higher rate of apoptosis in the infected cells compared with uninfected cells ($P < 0.005$, Fisher's exact test). Untreated samples represent the spontaneous rate of apoptosis in cultured cells. Actinomycin D was used as a positive control for the induction of apoptosis.

	Cell phenotype (%)		
	A^+/G^{-a}	A^-/G^+	A^+/G^+
Untreated	0.8 ± 0.1^b	0.1 ± 0.1	0.0 ± 0.0
MOI of 0.1	9.6 ± 2.8^b	12.8 ± 2.4	4.4 ± 1.9
Actinomycin D (1.2 μM)	18.4 ± 4.7	0.0 ± 0.0	0.0 ± 0.0

[a] A, apoptotic; G, enhanced green fluorescent protein; MOI, multiplicity of infection.
[b] $P = 0.009$.

Viral Titering. OCUM cells (1.0×10^4) were grown in 24-well, flat-bottomed plates and infected with NV1066 at various MOIs between 0 and 0.1 and then cultured with or without 10 mM NAC. Culture well supernatants were collected 4 days after infection. Serial dilutions were made of the supernatants, and viral plaques were counted on confluent Vero cells in a standard viral plaque assay. All samples were tested in triplicate, and the experiment was repeated at least three times to ensure reproducibility.

RESULTS

NV1066 Induces Apoptosis by Direct and Indirect Methods. NV1066 reliably infects OCUM cells at MOIs of 0.01 to 1 (data not shown). Expression of the viral marker gene eGFP is associated with NV1066 infection and is quantifiable. NV1066 also replicates within OCUM tumor cells, producing more virus and potentiating its oncolytic activity.

Exposure to NV1066 induces apoptosis in tumor cells. At MOI = 0.1, 14.0% of tumor cells are apoptotic 48 h after infection. Of these tumor cells, approximately one-third express eGFP and are considered directly infected by NV1066. Apoptosis also occurs in noninfected cells, constituting $9.6 \pm 2.8\%$ of the total cell population (Fig. 1 and Table 1).

Two assays confirmed this same finding. Hoechst nuclear staining was performed after exposure to NV1066 (Fig. 1, *A*−*C*) and counting at least 200 cells/sample (Table 1). This result was confirmed in seven additional experiments. Two *white arrows* in Fig. 1*C* mark apoptotic cells, which have no associated eGFP expression. These apoptotic cells have distinct morphology including pyknosis, nuclear fragmentation, and formation of apoptotic bodies. The overlay image shown in Fig. 1*C* demonstrates that these cells are not infected (*i.e.*, they do not express eGFP). There is significantly more apoptosis in cells exposed to NV1066 when compared with unexposed cells [$9.6 \pm 2.8\%$ (NV1066-exposed cells) *versus* $0.8 \pm 0.1\%$ (unexposed cells); $P = 0.009$, Fisher's exact test].

In total, 17.2% of cells express eGFP. Of these, $4.4 \pm 1.9\%$ are apoptotic and infected and are represented in the *inset* of Fig. 1*C*. These cells exhibit apoptotic morphology and expressed eGFP in overlay images.

A total of $12.8 \pm 2.4\%$ of cells express eGFP but are not apoptotic, constituting the largest population of infected cells. These cells have nuclear morphology consistent with viral infection (36). They are *green* in Fig. 1*A* but do not have apoptotic morphology in Fig. 1*B*. This corresponds with data derived from fluorescence-activated cell-sorting analysis for eGFP expression at day 2 (Fig. 2).

In Table 1, untreated cells grown under identical conditions did not have a significant proportion of apoptotic or eGFP-expressing cells. As a positive control, OCUM cells were treated with actinomycin D, a known inducer of apoptosis. In Table 1, $18.4 \pm 4.7\%$ of cells were apoptotic and uninfected (image not shown).

Although evaluation of nuclear morphology is regarded as a gold standard in the determination of apoptosis (35, 40), we confirmed our findings in each experiment with TUNEL staining (Fig. 1, *D*−*F*). Identification of apoptosis is better assured if more than a single assay is used (35). In the TUNEL assay, labeled uridine bases are attached to the DNA nicks characteristic of apoptotic cells. Infection with NV1066 induces TUNEL-positive apoptosis in uninfected neighboring cells, similar to the Hoechst staining experiments. Fig. 1*D* shows a cell culture

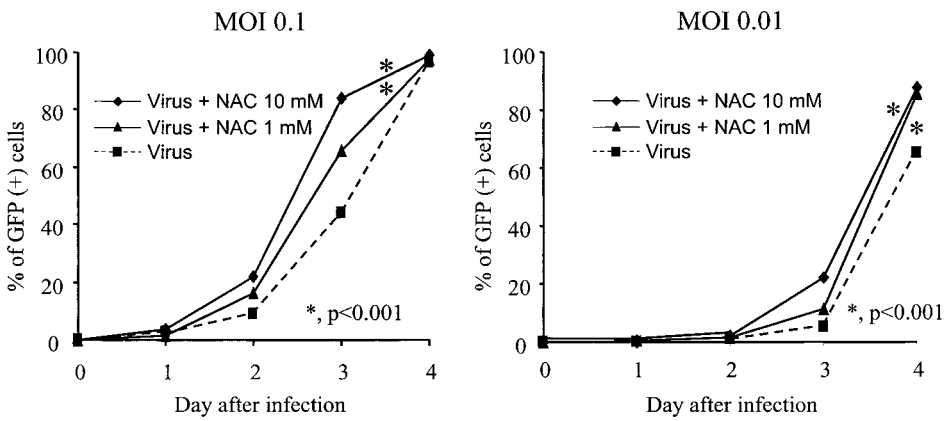

Fig. 2 Inhibition of apoptosis increases expression of the viral eGFP gene. Cells were infected with NV1066 at a multiplicity of infection (MOI) of 0.1 and a MOI of 0.01 and exposed to 10 mM *N*-acetylcysteine (NAC) or 1 mM NAC or left untreated. Cells were harvested and examined by flow cytometry for enhanced green fluorescent protein expression each day for 4 days after infection. Inhibition of apoptosis with NAC increases enhanced green fluorescent protein expression at both MOIs when compared with infection alone in this representative experiment ($P < 0.001$, *z*-test).

through a GFP filter exposed to NV1066. Fig. 1*E* shows the same field using phase-contrast, bright-field microscopy. Nuclei that take up 3,3′-diaminobenzidine stain in the TUNEL assay are *brown*, indicating apoptosis. The overlay image of the two microscopic modalities is shown in Fig. 1*F*. The percentage of apoptotic cells determined by TUNEL is approximately the same as the number of apoptotic cells determined by Hoechst nuclear staining (data not shown).

Mechanism of Induction of Apoptosis. The potential mechanisms by which uninfected cells are induced into apoptosis by infected ones are manifold (41, 42) but remain undetermined. We were unable to find a soluble mediator from infected cells that causes apoptosis. To determine whether a defective or incomplete viral particle shed into the culture media triggers apoptosis, we harvested "conditioned" media from infected cells, removed free virus by ultracentrifugation, and cultured naïve cells in this media. We also assayed supernatants of infected cells for tumor necrosis factor, a known mediator of apoptosis. Lastly, lysates from sonicated OCUM cells were incubated on naïve cells to simulate lysis and release of intracellular protoplasm. None of these approaches triggered apoptosis.

Another potential mechanism is that of abortive viral transcription or assembly resulting in nonproductive infection (41–43). It is plausible that a structural protein of the HSV-1 virion is responsible (44), just as the gp120 protein has been implicated in HIV-induced apoptosis (16, 18, 27, 28). In a study of human herpes virus-7, 25% of apoptotic cells were observed by electron microscopy to have empty virion capsids (43). However, in our studies, neither heat-inactivated nor UV-inactivated NV1066 induced apoptosis. We have data that suggest that greater cell density in culture correlates with greater occurrence of apoptosis. When compared with uninfected cells grown at similar confluence, there was a statistically significant increase in apoptosis at greater cell density ($P < 0.001$, Jonckheere-Terpstra test), suggesting that cell-to-cell contact plays a role in the induction of apoptosis.

Expression of eGFP Corresponds with NV1066 Infection. eGFP expression corresponds to immunohistochemistry-proven NV1066 infection. After cultured OCUM cells were infected with NV1066 at MOIs of 0.01, 0.1, and 1, they were fluorescence-activated cell-sorted into eGFP-expressing and non-eGFP-expressing populations. One hundred percent of eGFP-expressing cells were positive for HSV by immunohistochemistry. Of the fluorescence-activated cell sorted cells that did not express eGFP, none were positive for HSV by immunohistochemistry, except for <1% of cells infected at a MOI of 1 at 24 h. This finding supports the argument that eGFP is an accurate marker of HSV infection (45, 46). This was also confirmed in an *in vivo* model, in which we administered NV1066 i.p. to animals with i.p. carcinomatosis. Tissues that expressed eGFP contained NV1066 by quantitative PCR for an immediate early gene, and tissues that did not express eGFP did not contain the gene.[3]

Inhibition of Apoptosis Increases Expression of eGFP. OCUM cells infected with NV1066 *in vitro* reliably express eGFP in a MOI-dependent fashion that can be quantitated by flow cytometry (Fig. 2). Two, 3, and 4 days after treatment with NV1066 at a MOI of 0.01, 1%, 5%, and 65% of cells express eGFP. At a MOI of 0.1, 9%, 44%, and 96% of cells express eGFP. More important, however, is that NAC increases eGFP expression at both MOIs. Both 10 and 1 mM NAC increase eGFP expression compared with virus alone, with the higher concentration associated with greater eGFP expression than the lower concentration ($P < 0.05$ for all pairwise comparisons, *z* test). This effect did not extrapolate to concentrations of ≥20 mM NAC at either MOI (data not shown).

Inhibition of Apoptosis Increases Viral Infection and Tumor Kill. NV1066 is cytotoxic to OCUM cells in a dose-dependent fashion (Fig. 3*A*). Day 4 after infection was chosen as a representative day because it denotes the approximate halfway point of the experiments. By day 4, 40% of cells infected at MOI = 0.1 survived, compared with 88% of cells infected at a MOI of 0.01 ($P = 0.0003$, paired *t* test). Inhibition of apoptosis with 10 mM NAC increased tumor killing at both MOIs ($P < 0.05$ for MOI = 0.1 and $P = 0.20$ for MOI = 0.01). Importantly, exposure to 10 mM NAC without viral infection had no cytotoxic effect on cells during the 7 days of the experiment (data not shown).

Inhibition of apoptosis also increases NV1066 production by OCUM cells. Viral plaque-forming units were counted from supernatants of OCUM cells infected and then exposed or not exposed to 10 mM NAC. On each of the 7 days measured, NAC increased viral production. Fig. 3*B* shows the significant increase in viral production 4 days after infection in cells treated with NAC. At a MOI of 0.1, NAC is associated with 3× more viral production than untreated cells ($P = 0.05$, paired *t* test). At a MOI of 0.01, 19× more virus is produced ($P = 0.04$, paired *t* test). Others have demonstrated significant changes in viral production of a similar magnitude (3, 47–53).

DISCUSSION

Apoptosis is an active cellular process of self-destruction defined by morphological and biological criteria (54). Apoptosis protects an organism by ridding it of individual cells whose survival could be detrimental to the organism as a whole (41, 55). Viral infection often leads to an apoptotic response by a cell, thereby defending other cells from a similar fate (56) and limiting viral spread. Improving viral propagation in cancer cells is a potential approach to enhance efficacy of oncolytic viral therapy. This study confirms that a HSV-1 virus elicits apoptosis in infected cells (9, 11–13, 57, 58) but also demonstrates that the virus induces apoptosis in uninfected, cocultured cells. Chemical inhibition of this response improves viral production and tumor kill.

The observation that virally infected cells can induce apoptosis in uninfected, noncancerous cells has been described for other viruses including mouse hepatitis virus A59 (59), African swine fever virus (60), porcine reproductive and respiratory syndrome virus (61), human herpes virus-7 (43), human herpes

[3] S. F. Stanziale, B. M. Stiles, A. Bargava, S. A. Kerns, N. Kalakonda, and Y. Fong. Oncolytic herpes simplex virus-1 mutant expressing green fluorescent protein can detect and treat peritoneal cancer, submitted for publication.

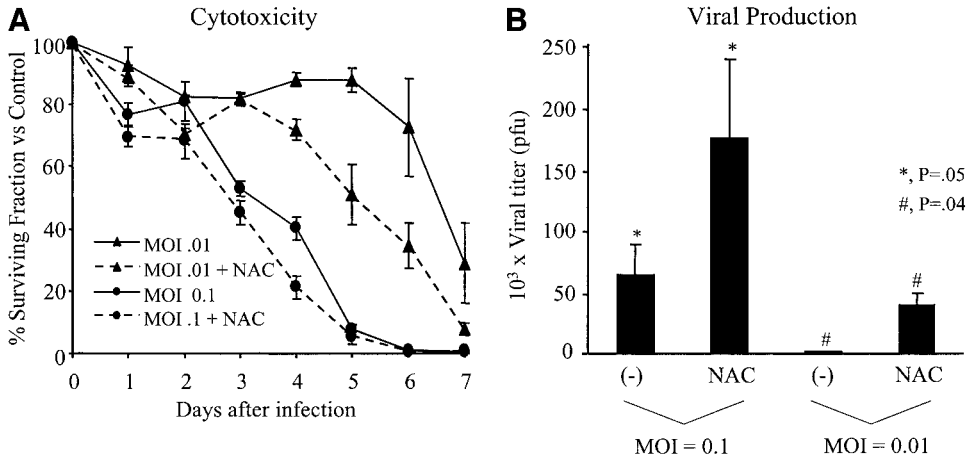

Fig. 3 Inhibition of apoptosis increases viral cytotoxicity and proliferation. OCUM cells are infected *in vitro* at multiplicities of infection of 0.1 and 0.01 and treated or not treated with 10 mM *N*-acetylcysteine (NAC). All samples were performed in triplicate and are presented as the mean ± SE. *A*, cells were harvested every day for a week, and cell survival was determined in a lactate dehydrogenase release assay. Cytotoxicity of viral treatment is enhanced in the presence of NAC at all time points until maximal lysis is achieved. *B*, supernatants from cell cultures were assayed in a standard viral plaque assay for viral count. By day 4, there is an increase of approximately 3× and 19× in viral production in the presence of NAC in the multiplicity of infection = 0.1 and 0.01 cultures, respectively.

virus-6 (62), and HIV (14–18, 63). In each, the uninfected cells induced into apoptosis were lymphoid cells. One group showed that HIV can induce apoptosis in uninfected cells in the lymph nodes of HIV-infected pediatric patients and simian immunodeficiency virus-infected macaques by undetermined "indirect mechanisms" (26). By labeling lymph nodes with *in situ* hybridization for viral transcripts and TUNEL for apoptosis, the group evaluated individual cells to show that apoptotic, uninfected cells were in close proximity to productively infected ones. In our study, we confirm that 10% of cells are apoptotic and are not infected. To ensure that this population is not infected, we demonstrate that eGFP expression corresponds to immunohistochemistry-proven HSV infection and confirm this in an *in vivo* model by quantitative PCR for NV1066. Secchiero *et al.* (43) infected lymphoblastoid cells with human herpes virus-7 and found two populations of cells: infected, necrotic multicellular syncytia; and uninfected, apoptotic single cells. Interestingly, empty viral capsids were visualized by electron microscopy in 25% of the apoptotic cells, potentially implicating nonproductive viral infection as a mechanism. Our study is novel in that it describes apoptosis in uninfected tumor cells by an oncolytic virus. Importantly, we establish that chemical inhibition of apoptosis increases viral replication and cytotoxicity. This implicates such apoptosis in limiting viral spread and favors killing by oncolytic viruses.

In our experiments, the expression of the viral eGFP protein is an appropriate marker of viral infection for several reasons. First, the gene construct was inserted into the viral genome with a constitutively expressed cytomegalovirus promoter, replacing two immediate early genes, *ICP0* and *ICP4*, in the viral genome. In our laboratory, we have detected eGFP as early as 6 h after infection at a MOI of 1. Additionally, we demonstrate that apoptotic cells retain the ability to express eGFP. In the *inset* of Fig. 1*C*, we demonstrate expression of eGFP within a cell of typical apoptotic nuclear morphology. This population, which accounted for 4.4 ± 1.9% of all cells, was expected; HSV-1 oncolytic viral mutants trigger apoptosis and lyse the tumor cells that they infect (64). Apoptosis without infection occurred in 9.6% of cultured cells, which, interestingly, is similar to the 10% of cells induced into apoptosis by HIV-GFP constructs in a previous study of HIV infection of lymphocytes (27).

NAC, an inhibitor of apoptosis, was used to confirm the hypothesis that such inhibition will alter viral efficacy. At day 2, there was a significant difference in apoptosis between NAC-treated and untreated cells that resulted in a significant difference in viral appearance (3, 47–53). This difference in viral appearance also parallels the difference in cytotoxicity and eGFP expression between the NAC-treated and untreated groups. Importantly, NAC at the treatment concentration of 10 mM has no independent cytotoxic effect on cultured cells in our cytotoxicity assay. NAC has been used clinically as a mucolytic for 30 years and is currently being used to decrease oxidative stress in HIV infection, cancer, and heart disease. In addition, NAC has had extensive use in the treatment of acetaminophen toxicity. Therefore, the application of NAC as an adjuvant in oncolytic therapy is plausible and merits *in vivo* testing.

In summary, these data show that the oncolytic HSV-1 mutant NV1066 induces apoptosis in uninfected, cocultured cells. Much like the induction of apoptosis in infected cells, this limits the lateral spread of viral progeny and likely protects the host against more widespread viral infection. Therefore, these data supply the impetus for further investigation into the combined use of inhibitors of apoptosis with HSV-1 oncolytic viral therapy. Improving propagation of viral progeny and concomitant tumor kill in such a way could potentially maximize the lateral spread of virus and minimize the required dose of oncolytic viral mutants for cancer therapy. Such a strategy could

have important implications on the efficacy of viral oncolytic therapy.

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REFERENCES

1. Ramakrishnan R, Fink DJ, Jiang G, et al. Competitive quantitative PCR analysis of herpes simplex virus type 1 DNA and latency-associated transcript RNA in latently infected cells of the rat brain. J Virol 1994;68:1864–73.

2. Jia WW, McDermott M, Goldie J, et al. Selective destruction of gliomas in immunocompetent rats by thymidine kinase-defective herpes simplex virus type 1 [see comments]. J Natl Cancer Inst (Bethesda) 1994;86:1209–15.

3. Andreansky S, Soroceanu L, Flotte ER, et al. Evaluation of genetically engineered herpes simplex viruses as oncolytic agents for human malignant brain tumors. Cancer Res 1997;57:1502–9.

4. Mineta T, Rabkin SD, Martuza RL. Treatment of malignant gliomas using ganciclovir-hypersensitive, ribonucleotide reductase-deficient herpes simplex viral mutant. Cancer Res 1994;54:3963–6.

5. Chou J, Roizman B. The γ1(34.5) gene of herpes simplex virus 1 precludes neuroblastoma cells from triggering total shutoff of protein synthesis characteristic of programmed cell death in neuronal cells. Proc Natl Acad Sci USA 1992;89:3266–70.

6. Chou J, Chen JJ, Gross M, Roizman B. Association of a M_r 90,000 phosphoprotein with protein kinase PKR in cells exhibiting enhanced phosphorylation of translation initiation factor eIF-2 α and premature shutoff of protein synthesis after infection with γ134.5- mutants of herpes simplex virus 1. Proc Natl Acad Sci USA 1995;92:10516–20.

7. He B, Chou J, Brandimarti R, et al. Suppression of the phenotype of γ(1)34.5- herpes simplex virus 1: failure of activated RNA-dependent protein kinase to shut off protein synthesis is associated with a deletion in the domain of the α47 gene. J Virol 1997;71:6049–54.

8. Leopardi R, Roizman B. The herpes simplex virus major regulatory protein ICP4 blocks apoptosis induced by the virus or by hyperthermia. Proc Natl Acad Sci USA 1996;93:9583–7.

9. Koyama AH, Miwa Y. Suppression of apoptotic DNA fragmentation in herpes simplex virus type 1-infected cells. J Virol 1997;71:2567–71.

10. Galvan V, Roizman B. Herpes simplex virus 1 induces and blocks apoptosis at multiple steps during infection and protects cells from exogenous inducers in a cell-type-dependent manner. Proc Natl Acad Sci USA 1998;95:3931–6.

11. Leopardi R, Van Sant C, Roizman B. The herpes simplex virus 1 protein kinase US3 is required for protection from apoptosis induced by the virus. Proc Natl Acad Sci USA 1997;94:7891–6.

12. Jerome KR, Tait JF, Koelle DM, Corey L. Herpes simplex virus type 1 renders infected cells resistant to cytotoxic T-lymphocyte-induced apoptosis. J Virol 1998;72:436–41.

13. Aubert M, Blaho JA. The herpes simplex virus type 1 regulatory protein ICP27 is required for the prevention of apoptosis in infected human cells. J Virol 1999;73:2803–13.

14. Meyaard L, Otto SA, Jonker RR, et al. Programmed death of T cells in HIV-1 infection. Science (Wash DC) 1992;257:217–9.

15. Groux H, Torpier G, Monte D, et al. Activation-induced death by apoptosis in CD4+ T cells from human immunodeficiency virus-infected asymptomatic individuals. J Exp Med 1992;175:331–40.

16. Oyaizu N, Adachi Y, Hashimoto F, et al. Monocytes express Fas ligand upon CD4 cross-linking and induce CD4+ T cells apoptosis: a possible mechanism of bystander cell death in HIV infection. J Immunol 1997;158:2456–63.

17. Lewis DE, Tang DS, Adu-Oppong A, Schober W, Rodgers JR. Anergy and apoptosis in CD8+ T cells from HIV-infected persons. J Immunol 1994;153:412–20.

18. Ullrich CK, Groopman JE, Ganju RK. HIV-1 gp120- and gp160-induced apoptosis in cultured endothelial cells is mediated by caspases. Blood 2000;96:1438–42.

19. Gougeon ML, Garcia S, Heeney J, et al. Programmed cell death in AIDS-related HIV and SIV infections. AIDS Res Hum Retroviruses 1993;9:553–63.

20. Meyaard L, Otto SA, Keet IP, Roos MT, Miedema F. Programmed death of T cells in human immunodeficiency virus infection. No correlation with progression to disease. J Clin Investig 1994;93:982–8.

21. Martin SJ, Matear PM, Vyakarnam A. HIV-1 infection of human CD4+ T cells in vitro. Differential induction of apoptosis in these cells. J Immunol 1994;152:330–42.

22. Terai C, Kornbluth RS, Pauza CD, Richman DD, Carson DA. Apoptosis as a mechanism of cell death in cultured T lymphoblasts acutely infected with HIV-1. J Clin Investig 1991;87:1710–5.

23. Sarin A, Clerici M, Blatt SP, et al. Inhibition of activation-induced programmed cell death and restoration of defective immune responses of HIV+ donors by cysteine protease inhibitors. J Immunol 1994;153:862–72.

24. Badley AD, McElhinny JA, Leibson PJ, et al. Upregulation of Fas ligand expression by human immunodeficiency virus in human macrophages mediates apoptosis of uninfected T lymphocytes. J Virol 1996;70:199–206.

25. Carbonari M, Pesce AM, Cibati M, et al. Death of bystander cells by a novel pathway involving early mitochondrial damage in human immunodeficiency virus-related lymphadenopathy. Blood 1997;90:209–16.

26. Finkel TH, Tudor-Williams G, Banda NK, et al. Apoptosis occurs predominantly in bystander cells and not in productively infected cells of HIV- and SIV-infected lymph nodes. Nat Med 1995;1:129–34.

27. Herbein G, Van Lint C, Lovett JL, Verdin E. Distinct mechanisms trigger apoptosis in human immunodeficiency virus type 1-infected and in uninfected bystander T lymphocytes. J Virol 1998;72:660–70.

28. Banda NK, Bernier J, Kurahara DK, et al. Crosslinking CD4 by human immunodeficiency virus gp120 primes T cells for activation-induced apoptosis. J Exp Med 1992;176:1099–106.

29. Li CJ, Friedman DJ, Wang C, Metelev V, Pardee AB. Induction of apoptosis in uninfected lymphocytes by HIV-1 Tat protein. Science (Wash DC) 1995;268:429–31.

30. Westendorp MO, Frank R, Ochsenbauer C, et al. Sensitization of T cells to CD95-mediated apoptosis by HIV-1 Tat and gp120. Nature (Lond.) 1995;375:497–500.

31. Parlato S, Santini SM, Lapenta C, et al. Primary HIV-1 infection of human CD4+ T cells passaged into SCID mice leads to selection of chronically infected cells through a massive fas-mediated autocrine suicide of uninfected cells. Cell Death Differ 2000;7:37–47.

32. Wong RJ, Joe JK, Kim SH, et al. Oncolytic herpesvirus effectively treats murine squamous cell carcinoma and spreads by natural lymphatics to treat sites of lymphatic metastases. Hum Gene Ther 2002;13:1213–23.

33. Shimamura T, Nakajima Y, Une Y, et al. Efficacy and safety of preoperative percutaneous transhepatic portal embolization with absolute ethanol: a clinical study. Surgery 1997;121:135–41.

34. Kleeff J, Kornmann M, Sawhney H, Korc M. Actinomycin D induces apoptosis and inhibits growth of pancreatic cancer cells. Int J Cancer 2000;86:399–407.

35. Darzynkiewicz Z, Traganos F. Measurement of apoptosis. Adv Biochem Eng Biotechnol 1998;62:33–73.

36. Monier K, Armas JC, Etteldorf S, Ghazal P, Sullivan KF. Annexation of the interchromosomal space during viral infection. Nat Cell Biol 2000;2:661–5.

37. Hockenbery DM, Oltvai ZN, Yin XM, Milliman CL, Korsmeyer SJ. Bcl-2 functions in an antioxidant pathway to prevent apoptosis. Cell 1993;75:241–51.

38. Kane DJ, Sarafian TA, Anton R, et al. Bcl-2 inhibition of neural death: decreased generation of reactive oxygen species. Science (Wash DC) 1993;262:1274–7.

39. Abello PA, Fidler SA, Bulkley GB, Buchman TG. Antioxidants modulate induction of programmed endothelial cell death (apoptosis) by endotoxin. Arch Surg 1994;129:134–40.

40. Darzynkiewicz Z, Bedner E, Traganos F, Murakami T. Critical aspects in the analysis of apoptosis and necrosis. Hum Cell 1998;11: 3–12.

41. Shen Y, Shenk TE. Viruses and apoptosis. Curr Opin Genet Dev 1995;5:105–11.

42. Razvi ES, Welsh RM. Apoptosis in viral infections. Adv Virus Res 1995;45:1–60.

43. Secchiero P, Flamand L, Gibellini D, et al. Human herpesvirus 7 induces CD4(+) T-cell death by two distinct mechanisms: necrotic lysis in productively infected cells and apoptosis in uninfected or nonproductively infected cells. Blood 1997;90:4502–12.

44. Koyama AH, Adachi A. Induction of apoptosis by herpes simplex virus type 1. J Gen Virol 1997;78:2909–12.

45. Jacobs A, Dubrovin M, Hewett J, et al. Functional coexpression of HSV-1 thymidine kinase and green fluorescent protein: implications for noninvasive imaging of transgene expression. Neoplasia 1999;1: 154–61.

46. Paquin A, Jaalouk DE, Galipeau J. Retrovector encoding a green fluorescent protein-herpes simplex virus thymidine kinase fusion protein serves as a versatile suicide/reporter for cell and gene therapy applications. Hum Gene Ther 2001;12:13–23.

47. Advani SJ, Chung SM, Yan SY, et al. Replication-competent, nonneuroinvasive genetically engineered herpes virus is highly effective in the treatment of therapy-resistant experimental human tumors. Cancer Res 1999;59:2055–8.

48. Carew JF, Kooby DA, Halterman MW, Federoff HJ, Fong Y. Selective infection and cytolysis of human head and neck squamous cell carcinoma with sparing of normal mucosa by a cytotoxic herpes simplex virus type 1 (G207). Hum Gene Ther 1999;10:1599–606.

49. Kooby DA, Carew JF, Halterman MW, et al. Oncolytic viral therapy for human colorectal cancer and liver metastases using a multimutated herpes simplex virus type-1 (G207). FASEB J 1999;6:499–504.

50. Oyama M, Ohigashi T, Hoshi M, et al. Treatment of human renal cell carcinoma by a conditionally replicating herpes vector G207. J Urol 2001;165:1274–8.

51. Petrowsky H, Roberts GD, Kooby DA, et al. Functional interaction between fluorodeoxyuridine-induced cellular alterations and replication of a ribonucleotide reductase-negative herpes simplex virus. J Virol 2001;75:7050–8.

52. Todo T, Rabkin SD, Chahlavi A, Martuza RL. Corticosteroid administration does not affect viral oncolytic activity, but inhibits antitumor immunity in replication-competent herpes simplex virus tumor therapy 2. Hum Gene Ther 1999;10:2869–78.

53. Yoon SS, Nakamura H, Carroll NM, et al. An oncolytic herpes simplex virus type 1 selectively destroys diffuse liver metastases from colon carcinoma. FASEB J 2000;14:301–11.

54. Kerr JF, Wyllie AH, Currie AR. Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. Br J Cancer 1972;26:239–57.

55. Williams GT. Programmed cell death: apoptosis and oncogenesis. Cell 1991;65:1097–8.

56. Teodoro JG, Branton PE. Regulation of apoptosis by viral gene products. J Virol 1997;71:1739–46.

57. Aubert M, Blaho JA. Modulation of apoptosis during herpes simplex virus infection in human cells. Microbes Infect 2001;3:859–66.

58. Ito M, Watanabe M, Kamiya H, Sakurai M. Herpes simplex virus type 1 induces apoptosis in peripheral blood T lymphocytes. J Infect Dis 1997;175:1220–4.

59. Godfraind C, Holmes KV, Coutelier JP. Thymus involution induced by mouse hepatitis virus A59 in BALB/c mice. J Virol 1995;69:6541–7.

60. Ramiro-Ibanez F, Ortega A, Brun A, Escribano JM, Alonso C. Apoptosis: a mechanism of cell killing and lymphoid organ impairment during acute African swine fever virus infection. J Gen Virol 1996;77: 2209–19.

61. Sirinarumitr T, Zhang Y, Kluge JP, Halbur PG, Paul PS. A pneumovirulent United States isolate of porcine reproductive and respiratory syndrome virus induces apoptosis in bystander cells both in vitro and in vivo. J Gen Virol 1998;79:2989–95.

62. Inoue Y, Yasukawa M, Fujita S. Induction of T-cell apoptosis by human herpesvirus 6. J Virol 1997;71:3751–9.

63. Meyaard L, Miedema F. Programmed death of T cells in the course of HIV infection. Adv Exp Med Biol 1995;374:115–20.

64. Spear MA, Sun F, Eling DJ, et al. Cytotoxicity, apoptosis, and viral replication in tumor cells treated with oncolytic ribonucleotide reductase-defective herpes simplex type 1 virus (hrR3) combined with ionizing radiation. Cancer Gene Ther 2000;7:1051–9.

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