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Herpes simplex virus thymidine kinase/ganciclovir–induced cell death is enhanced by co-expression of caspase-3 in ovarian carcinoma cells

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There is a need to enhance the efficacy of genetic prodrug activation therapy using herpes simplex virus thymidine kinase (tk) and ganciclovir (GCV) following disappointing results in early clinical trials. tk/GCV has been shown to lead to the activation of caspase-3, a potent executor of apoptosis. We demonstrate that co-expression of pro-caspase-3 with tk/GCV leads to enhanced cell death in ovarian carcinoma cells *in vitro*. Following transfection with recombinant adenoviral vectors encoding tk, GCV treatment leads to greater cell death in pro-caspase-3–expressing clones of SKOV3 and IGROV1 than control cells, as well as more rapid activation of caspase-3 and more rapid cleavage of PARP. Flow cytometry suggests that there is a greater degree of S-phase block in the pro-caspase-3–expressing clones than in control cells following treatment with tk/GCV. None of these effects is seen following transfection with a control adenovirus that does not encode tk. The increased cell death, early caspase-3 activation and PARP cleavage, and flow cytometric changes seen in pro-caspase-3–expressing cells can be partially inhibited by treatment with benzyloxycarbonyl–val–ala–asp fluoromethylketone, a synthetic caspase inhibitor. Our data suggest that co-expression of pro-caspase-3 may lead to a significant enhancement of the efficacy of tk/GCV therapy. **Cancer Gene Therapy (2001) 8, 308–319**

Key words: Thymidine kinase; ganciclovir; adenovirus; gene therapy; caspase-3; ovarian cancer.

Genetic prodrug activation therapy (GPAT) has great potential as a gene therapy strategy for cancer. Many different GPAT approaches have been described,[1–5] but the most frequently used method involves the thymidine kinase (tk) gene from herpes simplex virus (HSV) type 1, which catalyses the first step in the conversion of ganciclovir (GCV) into ganciclovir triphosphate,[6] a potent antimetabolite that interrupts DNA synthesis by erroneous incorporation as a false nucleotide. Despite many promising preclinical studies, the results of published clinical trials of tk/GCV have been disappointing, with little objective evidence of tumor responses in patients with recurrent gliomas[7–10] although one study has shown some reductions in prostate-specific antigen in men with prostate cancer.[11] There have, therefore, been many attempts to improve the efficacy of tk/GCV. These have included the use of the tk gene from other herpes viruses,[12,13] isolation of HSV1 tk mutants better able to activate GCV,[14] the use of alternative prodrugs,[15] combining tk/GCV with other GPAT strategies, such as cytosine deaminase/5-fluorocytosine,[16] and combined GPAT-immunotherapy strategies, *via* delivery of the genes for both tk and cytokines such as interleukins-2[17] and -4.[18]

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Recently, there has been some elucidation of the pathways by which activated GCV induces apoptosis in transfected cells. These include the accumulation of p53, CD95/Fas, and activation of both caspase-3 and caspase-8.[19,20] Caspase-3 is one of the family of cysteine proteases which have a central role in the induction and execution of apoptosis (reviewed in Ref.[21]). It is synthesized as a catalytically inert pre–pro-enzyme, with a large subunit, a small subunit, and a prodomain. The active enzyme is revealed only after the prodomain has been removed and the two subunits have been cleaved, the latter occurring in response to an apoptotic stimulus.

There has been considerable interest in caspase-3 as a potential tool for cancer gene therapy. Expression of pro-caspase-3 alone in tumors cells appears insufficient to induce apoptosis, but the addition of another stimulus, such as the simultaneous expression of Fas ligand, can induce extensive apoptosis of glioma cells *in vitro*.[22] Similarly, the combination of caspase-3 and exposure to the cytotoxic drug etoposide produces apoptosis *in vitro* and a significant reduction in tumor volumes in a rodent hepatoma model.[23] Another strategy has been to reverse the orientation of the two subunits of caspase-3, which produces a constitutively active enzyme.[24]

Here we report the first use of the combination of HSK-tk/GCV with caspase-3. We have previously demonstrated that the expression of pro-caspase-3 alone in ovarian carcinoma cells does not induce apoptosis.[25]

However, following adenoviral delivery of the tk gene, there is significantly greater death of ovarian carcinoma cells expressing pro-caspase-3 than of control cells upon exposure to GCV. Transfection of the pro-caspase-3–expressing cells with a control adenovirus that does not encode tk fails to enhance sensitivity to GCV. We also show that the increased tumor cell death is caused by caspase-3 activation and can be partially reversed on exposure to Benzyloxycarbonyl–val–ala–asp fluoromethylketone (zVAD-fmk), a synthetic caspase inhibitor.[26]

MATERIALS AND METHODS

Cell culture

SKOV3 and IGROV1 cells are both human ovarian carcinoma cell lines. SKOV3 cells were obtained from Imperial Cancer Research Fund (Clare Hall, UK) and IGROV1 cells were kindly donated by Dr. M. Ford (Glaxo-Wellcome, Stevenage, UK). Both cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal calf serum (FCS) and were incubated at 37°C with 10%

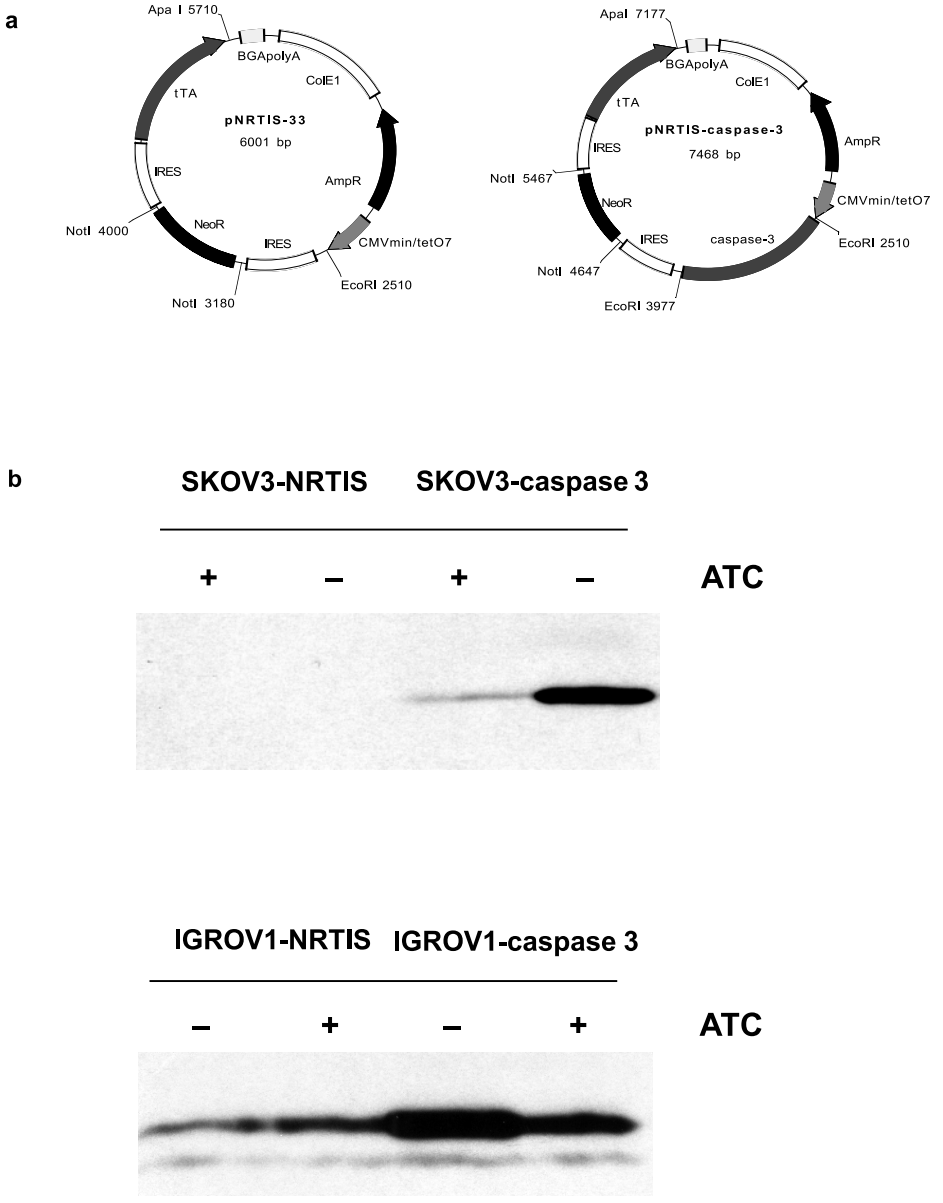

Figure 1. Tricistronic tetracycline-regulatable plasmids and the generation of stable cell lines expressing pro-caspase-3. Following transfection with either a tricistronic tetracycline-regulatable control vector, pNRTIS-33, or pNRTIS–caspase-3, cells were selected in medium containing 1 mg/mL G418. Protein was extracted from clones of SKOV3–NRTIS, SKOV3–caspase-3, IGROV1–NRTIS, and IGROV1–caspase-3 cells grown in the presence (+) and absence (–) of anhydrotetracycline (100 ng/mL for 72 hours), electrophoresed on an SDS-10% polyacrylamide gel and blotted with anti–caspase-3 antibody.

CO_2 in air. After transfection with the plasmids pNRTIS-33 or pNRTIS–caspase-3 (see below), cells were grown in medium supplemented additionally with 1 mg/mL G418.

Construction of plasmids

The construction of the tricistronic tetracycline-regulatable vector, pNRTIS-33, has been described previously.[27] Full-length human caspase-3 cDNA was generated by reverse transcriptase polymerase chain reaction (PCR) from OVCAR3 cells and cloned as an *Eco*RI fragment into pNRTIS-33.[25] Plasmid maps are presented in Figure 1a.

Adenoviral vectors and transfection of ovarian carcinoma cells

The construction, production, and purification of Ad *erb*B2-tk (also known as Ad5 1ETAS) have been described elsewhere.[28] Ad LM-tk was constructed as follows: a 2.6-kb *Xho*I–*Xba*I fragment containing the −1406/+33 MUC1 promoter and HSV tk gene was excised from pBS-LM-tk [29] and cloned into *Xho*I–*Xba*I digested pΔE1sp1A (Microbix, Toronto, Canada) to create pΔE1LM-tk. The control adenovirus, Ad LM-X, was constructed by ligating a 1.4-kb *Sal*I fragment containing the −1406/+33 MUC1 promoter alone from pBS-LM-tk into the *Sal*I site of pΔE1sp1A to create pΔE1LM-X. The recombinant viruses were generated after co-transfection of 10 μg each of one pΔE1 plasmid and pJM17 (Microbix) into low-passage 293 cells. This was followed by four rounds of limiting dilution and subsequent purification by double density CsCl gradient ultracentrifugation. Ad CMV-GFP was obtained from QBiogene (Harefield, UK). All four adenoviruses were shown to be replication-defective after infection of HeLa cells.

For cell viability assays, ovarian carcinoma cells were trypsinized on day 1 and 1×10^5 cells plated into each well of a 24-well plate. On day 2, medium was removed and adenovirus added in 200 μL of DMEM+5% FCS. After 90 minutes with gentle rocking, cells were refed with 1 mL DMEM+5% FCS. On day 3, cells were split, counted, and diluted to 10^4 cells/mL. Two-hundred microliters (i.e., 2000 cells) was added to each well of a flat-bottomed, 96-well plate. On day 4, medium was removed and GCV (Roche, Welwyn Garden City, UK) was added at concentrations 0, 0.01–100 μg/mL in quadruplicate in DMEM+10% FCS. Cell viability was assayed on day 11 by MTT assay.[30]

Reverse transcriptase PCR

On day 1, 1×10^6 cells were plated onto 6-cm dishes. Twenty-four hours later, they were infected with adenovirus at a multiplicity of infection (MOI) of up to 100 plaque-forming units/cell (total volume 1 mL in DMEM+5% FCS for 1 hour with gentle rocking). On day 4, total RNA was extracted (RNazol; Biogenesis, Poole, UK), incubated with 10 U RNase-free DNase I (Sigma, Poole, UK) for 1 hour at 37°C, and purified using RNeasy kits (Qiagen, Crawley, UK) according to manufacturer's instructions. An aliquot of 3 μg of total RNA was reverse-transcribed using First Strand cDNA Synthesis kit (Pharmacia, St. Albans, UK) with 0.2 μg random hexanucleotide primer. The PCR primers were as follows: HSV-tk forward 5′-AACAATGGGCATGCCTTATGC-3′, HSV-tk reverse 5′-TTATACAGCTCGCCGTTGGGG-3′ (expected band=540 bp), β-actin forward 5′-GAGCGGAATCGTGCGTGACATT-3′, β-actin reverse 5′-GATGGAGTTGAAGGTAGTTTCGTG-3′ (expected band 240 bp). PCR reaction constituents were as follows: 300 ng cDNA, 200 μM each deoxynucleotide, 20 pmol of each primer, 5 μL 10× buffer (15 mM $MgCl_2$, 250 mM KCl, 100 mM Tris–HCl, pH 8.8), and 5 U Taq DNA polymerase in a total reaction volume of 50 μL. Reaction conditions were as follows: 94°C for 5 minutes, 30 cycles of denaturation (94°C for 30 seconds), annealing (53°C for

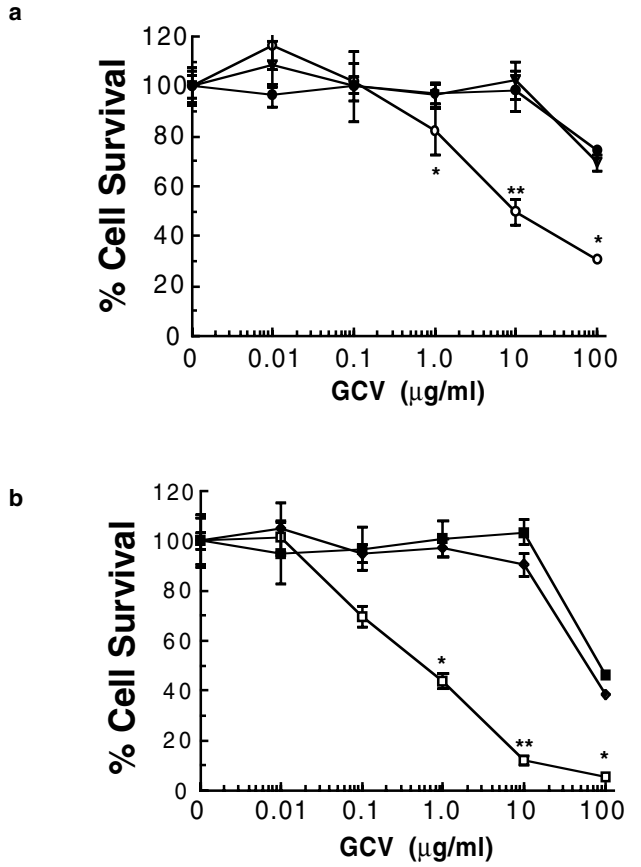

Figure 2. GCV dose–response curves for SKOV3–NRTIS and SKOV3–caspase-3 cells. SKOV3–NRTIS **(a)** and SKOV3–caspase-3 **(b)** cells were transfected with either control adenovirus (Ad LM-X, MOI 100), Ad *erb*B2-tk (MOI 100), or mock-infected. Forty-eight hours after transfection, cells were exposed to GCV (0–100 μg/mL) for 7 days when cell survival was estimated by MTT assay. Symbols are as follows: SKOV3–NRTIS mock-infected = ●; SKOV3–NRTIS+Ad LM-X = ▼; SKOV3–NRTIS+Ad *erb*B2-tk = ○; SKOV3–caspase-3 mock-infected = ■; SKOV3–caspase-3+Ad LM-X = ◆; SKOV3–caspase-3+Ad *erb*B2-tk = □. *$P<.001$; **$P<.0001$. In both graphs, values represent mean of four samples ± SD.

30 seconds for tk, 55°C for 30 seconds for β-actin), and extension (72°C for 30 seconds) followed by 10 minutes of extension at 72°C. PCR products were separated on a 1.5% agarose gel.

Western blot and caspase-3 enzymatic activity

Up to 1×10^6 cells were plated onto 6-cm dishes and transfected with adenovirus as described above. Forty-eight hours after transfection, they were treated with GCV (0 or 10 μg/mL). Cells were scraped into up to 100 μL lysis buffer (150 mm NaCl, 50 mM Tris pH 7.5, 0.05% sodium dodecyl sulfate, 1% Triton X-100) and sonicated on ice. Twenty micrograms of protein extract was electrophoresed on a 10% SDS-polyacrylamide gel and transferred onto nitrocellulose filter by semidry blotting. Primary antibodies used were a mouse monoclonal anti-PARP IgG (1:2000) or a rabbit polyclonal antihuman caspase-3 IgG (1:2000) (both Santa Cruz) and the secondary antibodies were, respectively, a rabbit antimouse or goat antirabbit IgG peroxidase conjugate (1:1500) (Dako). Antibody binding was visualized using ECL (Amersham).

Caspase-3 enzymatic activity was assayed using the acetyl-DEVD-AMC fluorogenic substrate assay (BD PharMingen Europe, Oxford, UK) according to the manufacturer's instructions. Briefly, cells were treated with GCV 48 hours after adenoviral transfection. Forty-eight hours thereafter, they were washed twice with cold phosphate-buffered saline and lysed on ice for 30 minutes with 400 μL enzymatic lysis buffer (10 mM Tris pH 7.5, 10 mM NaH_2PO_4/Na_2HPO_4, 130 mM NaCl, 1% Triton X-100, 10 mM sodium pyrophosphate). Fifty microliters of cell lysate was incubated for 1 hour at 37°C in the presence of 1 mL protease assay buffer (20 mM HEPES, 10% glycerol, and 2 mM DTT) and 20 μM acetyl-DEVD-AMC. Caspase-3 activity was assayed *via* measurement of liberated AMC from the acetyl-DEVD-AMC using a spectrofluorimeter (excitation wavelength, 380 nm; emission wavelength, 440 nm; scan duration, 20 seconds; emission measured at 10 seconds).

Flow cytometry

1×10^6 cells were plated onto 6-cm Petri dishes and infected with adenovirus. Forty-eight hours after transfection, GCV (10 μg/mL) was added. At various time points (0–96 hours), cells were trypsinized, washed twice with cold phosphate-buffered saline, and fixed in 70% ethanol at 4°C for at least 30 minutes. They were then washed twice with phosphate citrate buffer (192 μM Na_2HPO_4, 40 μM citric acid), treated with RNase A, and stained with propidium iodide. Cell cycle status was analysed using a FACScalibur flow cytometer (Becton Dickinson).

Lactate dehydrogenase (LDH) release assay

1×10^5 cells were plated on 24-well plates and infected with adenovirus. Twenty-four hours after transfection, cells were trypsinized and plated onto 96-well plates at a density of 10^4 cells/well and were treated, 24 hours thereafter, with GCV (0 or 10 μg/mL) in the presence and absence of 2 μM zVAD-fmk. After up to 96 hours of GCV treatment, LDH release from cells was assayed using an LDH cytotoxicity detection kit (Alexis Biochemicals, Nottingham, UK) according to the manufacturers' instructions.

RESULTS

Generation of pro-caspase-3–expressing ovarian carcinoma cells

Following transfection with the tricistronic tetracycline-regulatable plasmid, pNRTIS–caspase-3, or the control plasmid p-NRTIS-33 (Fig 1a), and selection by growth in medium containing the neomycin analogue, G418, clones of

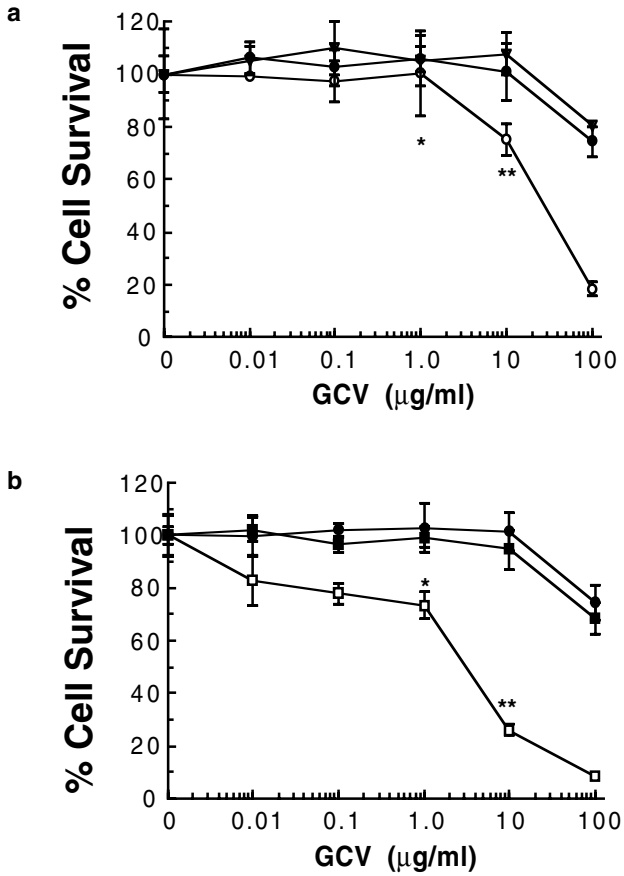

Figure 3. GCV dose–response curves for IGROV1–NRTIS and IGROV1–caspase-3 cells. IGROV1–NRTIS **(a)** and IGROV1–caspase-3 **(b)** cells were transfected with either control adenovirus (Ad LM-X, MOI 30), Ad LM-tk (MOI 30), or mock-infected. Forty-eight hours after transfection, cells were exposed to GCV (0–100 μg/mL) for 7 days when cell survival was estimated by MTT assay. Symbols are as follows: IGROV1–NRTIS mock-infected = ●; IGROV1–NRTIS + Ad LM-X = ▼; IGROV1–NRTIS + Ad LM-tk = ○; IGROV1–caspase-3 mock-infected = ■; IGROV1–caspase-3 + Ad LM-X = ◆; IGROV1–caspase-3 + Ad LM-tk = *P < .03; **P < .0001. In both graphs, values represent mean of four samples ± SD.

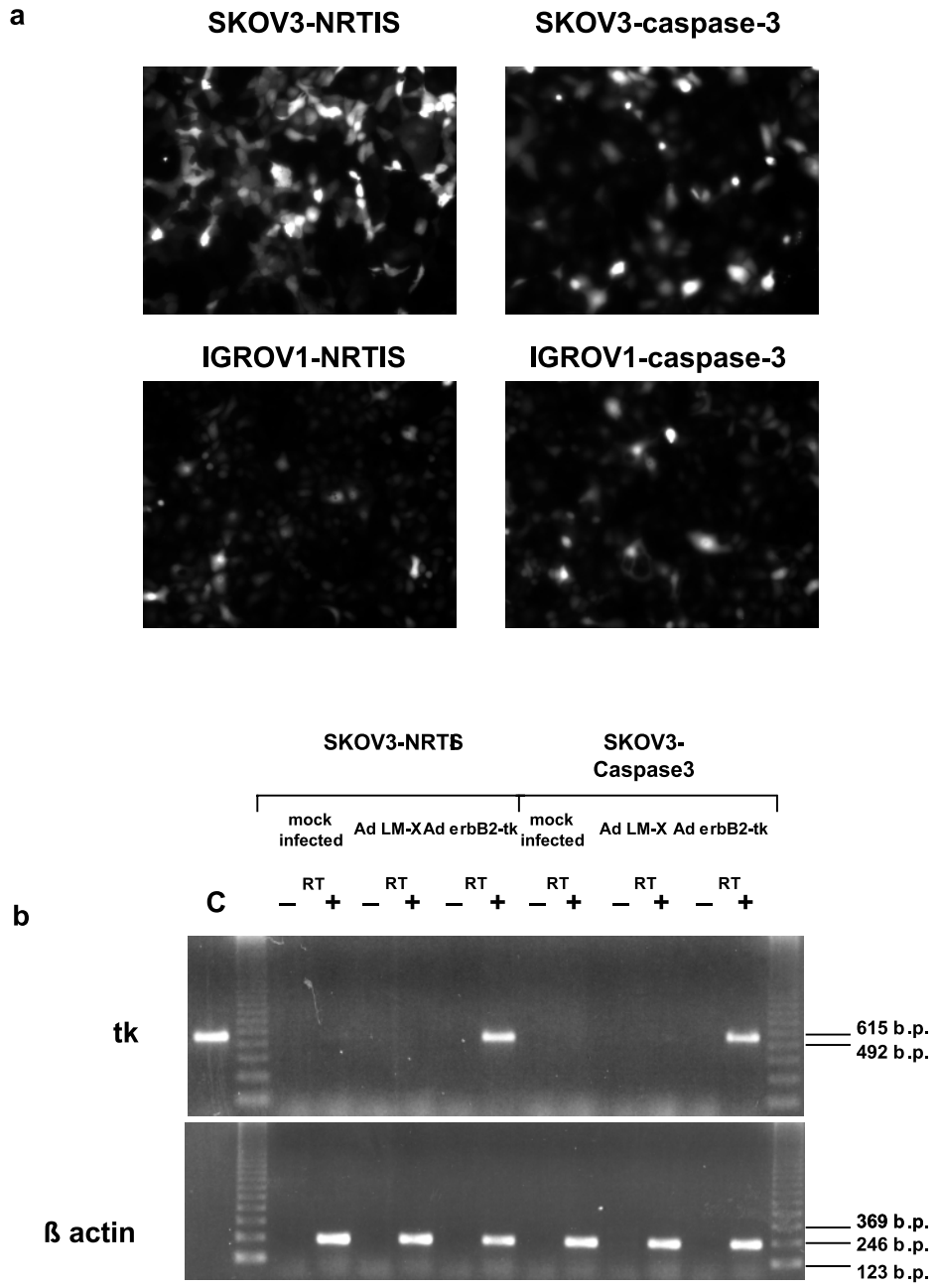

Figure 4. GFP expression and reverse-transcriptase PCR. **a:** 5×10^5 SKOV3–NRTIS, SKOV3–caspase-3, IGROV1–NRTIS, and IGROV1–caspase-3 cells were plated onto 6-cm Petri dishes. Twenty-four hours later, they were transfected with Ad CMV-GFP (MOI 30). Forty-eight hours thereafter, they were photographed under UV light. **b:** Total cellular RNA was extracted from SKOV3–NRTIS and SKOV3–caspase-3 cells following mock infection or transfection with either Ad LM-X (MOI 100) or Ad *erb*B2-tk (MOI 100) and reverse-transcribed as outlined in *Materials and Methods*. Following PCR using HSV tk or human β-actin–specific primers as outlined in *Materials and Methods*, PCR products were electrophoresed on a 1.5% agarose gel.

both SKOV3 and IGROV1 were isolated. Figure 1b shows the Western blots of SKOV3–NRTIS, SKOV3–caspase-3, IGROV1–NRTIS, and IGROV1–caspase-3 cells grown in the presence and absence of 100 ng/mL anhydrotetracycline. There is very little endogenous pro-caspase-3 expression in SKOV3–NRTIS cells. In the SKOV3–caspase-3 cells, there is some low-level expression in the presence of tetracycline, which increases dramatically on the removal of tetracycline suppression. In contrast, there is some detectable pro-caspase-3 expression in IGROV1–NRTIS cells that is not affected by the presence of tetracycline. Nonetheless, there is considerably greater pro-caspase-3 expression in the IGROV1–caspase-3 cells upon release from tetracycline suppression.

Following adenoviral delivery of the tk gene, exposure to GCV induces greater dell death in caspase-3–expressing cells

Two recombinant adenoviral vectors were employed to deliver the tk gene to the ovarian carcinoma cells. Ad *erb*B2-tk is a first-generation E1-deleted adenovirus, in which tk expression lies under the control of a 500-bp promoter element of the Her2/c-*erb*B2 gene. This virus was used to transfect SKOV3 cells, which are strongly positive for Her2/c-*erb*B2.[31] IGROV1 cells express MUC1 (I. McNeish, unpublished data) and were transfected with Ad LM-tk, another first-generation virus in which tk expression lies under the control of a 1.4-kb promoter fragment from the *MUC1* gene.[32]

Following infection of SKOV3–NRTIS cells with Ad *erb*B2-tk at a multiplicity of infection (MOI) of 10–100 plaque-forming units/cell, there was an increase in sensitivity to GCV (see Figure 2a), in accordance with previous results: IC_{50} for SKOV3–NRTIS cells >100 μg/mL, SKOV3–NRTIS+Ad *erb*B2-tk (MOI 10)≈20 μg/mL, SKOV3–NRTIS+Ad *erb*B2-tk, (MOI 100)≈10 μg/mL. There was no increase in sensitivity following infection with a control virus, Ad LM-X, at an MOI of 100.

In SKOV3–caspase-3 cells, infection with Ad *erb*B2-tk led to greater increases in sensitivity to GCV (see Figure 2b); the IC_{50} value for SKOV3–caspase-3+Ad *erb*B2-tk (MOI 100) was ~0.5 μg/mL. There was a statistically significant reduction in the proportion of cells surviving at given concentrations of GCV. Thus, for SKOV3–NRTIS+Ad *erb*B2-tk (MOI 100), cell survival at 10 μg/mL was 48.4±2.9% (mean±SD, n=4), whereas for SKOV3–caspase-3+Ad *erb*B2-tk (MOI 100), cell survival for the same drug concentration was only 7.1±2.1% (P<.001) (see Figure 2). Similar significant differences in cell survival were seen at lower doses of GCV (see Figure 2) and at the lower MOI of 10 (data not shown).

For IGROV1, similar results were seen. IC_{50} for IGROV1–NRTIS ~100 μg/mL, for IGROV1–NRTIS+Ad LM-tk (MOI 30) ~30 μg/mL, for IGROV1–caspase-3+Ad LM-tk (MOI 30) ~3 μg/mL (see Figure 3). Again, following infection with Ad LM-tk, the proportion of

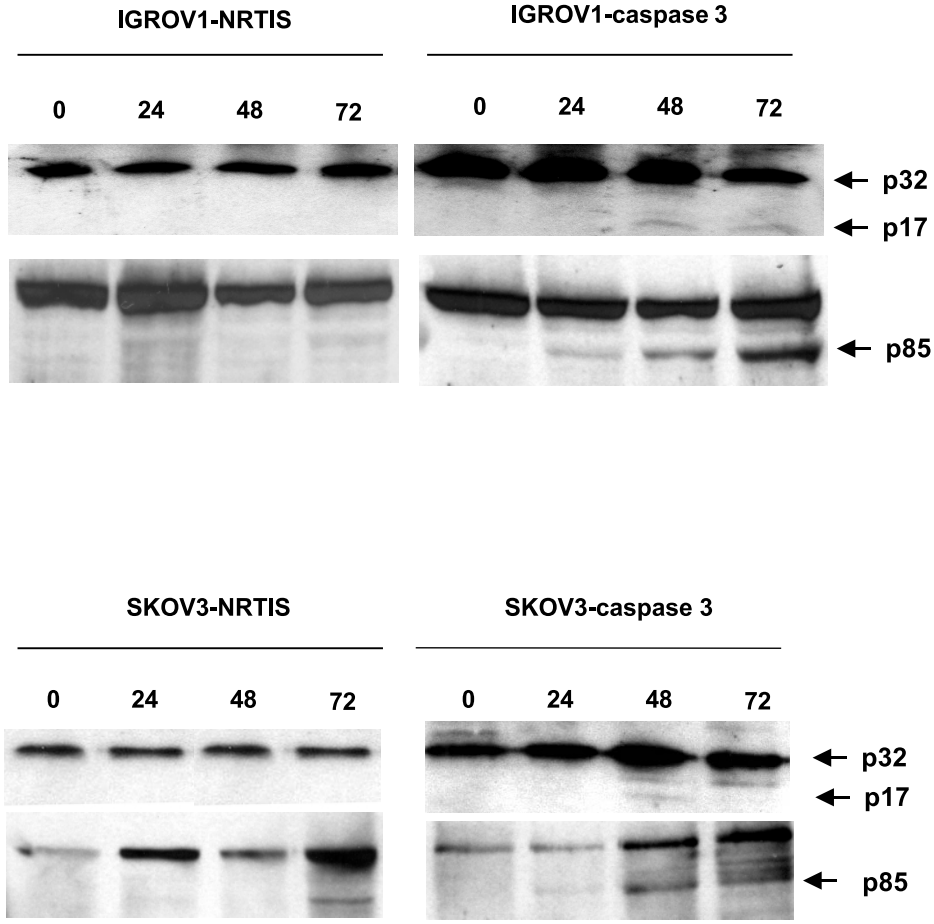

Figure 5. Detection of caspase-3 activation and PARP cleavage by Western blot 1×10^6 cells were transfected with either Ad LM-tk, MOI 30 (IGROV1–NRTIS, IGROV1–caspase-3) or Ad *erb*B2-tk, MOI 100 (SKOV3–NRTIS, SKOV3–caspase-3). Forty-eight hours later, 3 μg/mL GCV was added and cells were harvested 0, 24, 48, and 72 hours thereafter. Twenty micrograms of cellular protein was electrophoresed on an SDS-10% polyacrylamide gel, transferred to nitrocellulose membrane, probed with either a rabbit polyclonal anti–caspase-3 antibody or a monoclonal anti-PARP antibody and detected *via* ECL.

IGROV1–caspase-3 cells surviving at given concentrations of GCV is also significantly reduced compared to IGROV1–NRTIS cells.

To investigate whether pro-caspase-3 expression permitted greater transfection of cells by adenoviral vectors, SKOV3–NRTIS, SKOV3–caspase-3, IGROV1–NRTIS, and IGROV1–caspase-3 cells were transfected with the control adenovirus, Ad CMV-GFP (MOI 30). Figure 4a shows representative images of all four cells lines under UV light 48 hours after transfection. By reverse transcriptase PCR analysis, the amounts of tk mRNA expressed in the SKOV3–NRTIS and SKOV3–caspase-3 cells upon infection with Ad *erb*B2-tk are similar (Fig 4b). Thus, the difference in sensitivity to GCV of the caspase-expressing cells cannot be explained either by increased infectability with adenoviral vectors or increased expression of tk.

Activation of caspase-3 is seen in SKOV3–caspase-3 and IGROV1–caspase-3 cells in response to tk/GCV

In response to a pro-apoptotic stimulus, the 32-kDa pro-caspase-3 protein is cleaved into its p20 (large) and p12 (small) subunits. The large subunit is further reduced to the active p17 subunit.[21] Poly(ADP-ribose) polymerase (PARP) is cleaved into p85 and p25 proteins by activated caspase-3.[33] Following adenoviral delivery of the tk gene, cells were exposed to GCV for up to 72 hours. Western blotting demonstrates cleavage of both caspase-3 and PARP in both populations of pro-caspase-3–expressing cells after shorter incubations than in the respective control cells (see Figure 5). In IGROV1–NRTIS cells, there is no caspase-3 or PARP cleavage evident throughout the 72-hour time course, whereas in SKOV3–NRTIS cells, the p85 PARP cleavage band only becomes evident at 72 hours. By contrast, in both pro-caspase-3–expressing populations, there is evidence of PARP cleavage as early as 24 hours after the onset of GCV treatment.

Effect of zVAD-fmk

Cell viability assays and Western blots were performed in the presence and absence of 2 μM zVAD-fmk, a synthetic caspase inhibitor. Addition of zVAD-fmk had little effect on SKOV3–NRTIS cells, but reduced the cytopathic effect of tk/GCV in the SKOV3–caspase-3 cells significantly ($P<.002$; Figure 6a).

This reduction in cytotoxicity is mirrored by reduced caspase-3 activation and PARP cleavage as detected by Western blotting. SKOV3–NRTIS and SKOV3–caspase-3 cells were infected with Ad LM-X, Ad *erb*B2-tk, or mock-infected. They were then exposed to 10 μg/mL GCV in the presence or absence of 2 μM zVAD-fmk for 48 hours. In

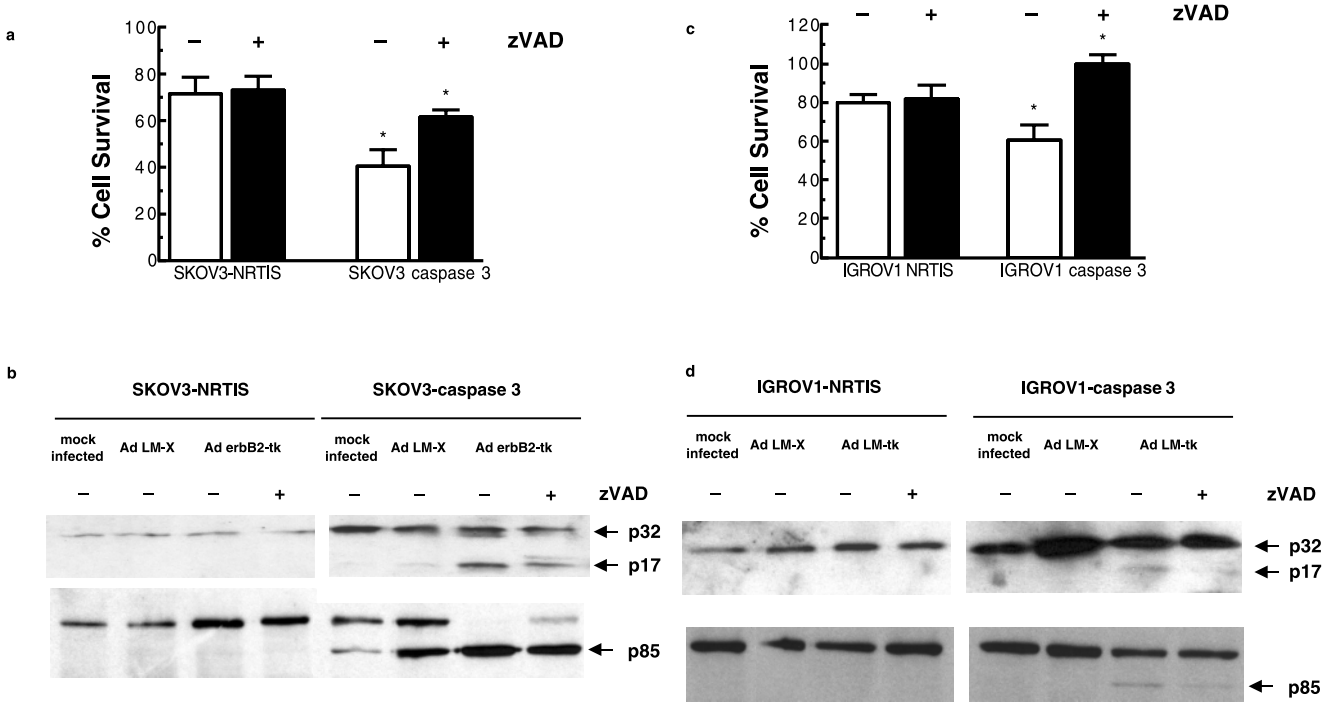

Figure 6. Effect of zVAD. **a:** following transfection with Ad *erb*B2-tk, MOI 100, SKOV3–NRTIS and SKOV3–caspase-3 cells were exposed to 10 μg/mL GCV for 7 days in the absence (white bars) or presence (black bars) of 2 μM zVAD. Cell survival was estimated by MTT assay. *$P<.001$. **b:** SKOV3–NRTIS and SKOV3–caspase-3 cells were mock-infected, infected with Ad LM-X, MOI 100 or Ad *erb*B2-tk, MOI 100. Forty-eight hours later, they were exposed to 10 μg/ml GCV for another 48 hours in the presence or absence of 2 μM zVAD. **c:** IGROV1–NRTIS and IGROV1–caspase-3 cells were infected with Ad LM-tk, MOI 30 and exposed to 10 μg/ml GCV in the absence (white bars) or presence (black bars) of 2 μM zVAD. Cell survival was estimated by MTT assay. *$P<.001$; **d:** IGROV1–NRTIS and IGROV1–caspase-3 cells were mock-infected, infected with Ad LM-X, MOI 30 or Ad LM-tk, MOI 30. Forty-eight hours later, they were exposed to 10 μg/mL GCV for another 48 hours in the presence or absence of 2 μM zVAD.

the SKOV3 cells, there is no caspase-3 activation or PARP cleavage in any of the samples. In the SKOV3–caspase-3 cells, there is some caspase-3 and PARP cleavage in the mock or Ad LM-X– infected cells following 48 hours GCV treatment. However, infection with Ad *erb*B2-tk leads to a dramatic increase of the cleavage of both proteins, which is partially reversed by exposure to zVAD-fmk (Fig 6b).

Similar results are seen with the IGROV1 populations. Thus, for IGROV1–NRTIS+Ad LM-tk (MOI 30), cell survival at 10 μg/mL GCV=79.9±4.3% (mean±SD; n=4) without zVAD, and 81.6±7.6% with zVAD (P=NS). For IGROV1–caspase-3 cells, the respective cell survival figures are 60.8±7.6% without zVAD-fmk and 100.1±4.5% with zVAD-fmk (P=.0001) (Fig 6c). Western blotting again shows no PARP cleavage in the IGROV1–NRTIS cells. However, there is demonstrable PARP cleavage in the IGROV1–caspase-3 cells after tk/GCV treatment that can partially be reversed by 2 μM zVAD-fmk (Fig 6d).

Caspase-3 enzymatic activity

Because PARP can be cleaved by multiple caspases and also because zVAD-fmk is a pan-caspase inhibitor, the specific enzymatic activity of caspase-3 was assayed by measuring the release of fluorogenic AMC, which is cleaved from acetyl-DEVD-AMC by the activated protease.[34] Treatment of both SKOV3–NRTIS and IGROV1–NRTIS with tk/GCV for 48 hours was associated with a very modest rise in caspase-3 activity, as measured by AMC fluorescence (Fig 7a and b). By contrast, there was a far greater increase in caspase-3 activity in both pro-caspase-3–expressing cells following tk/GCV treatment for the same duration and, once again, this activity was partially inhibited by treatment with 2 μM zVAD-fmk (Fig 7a and b).

Flow cytometry and LDH release

Following transfection with recombinant adenovirus and exposure to GCV, cells were harvested at various time points

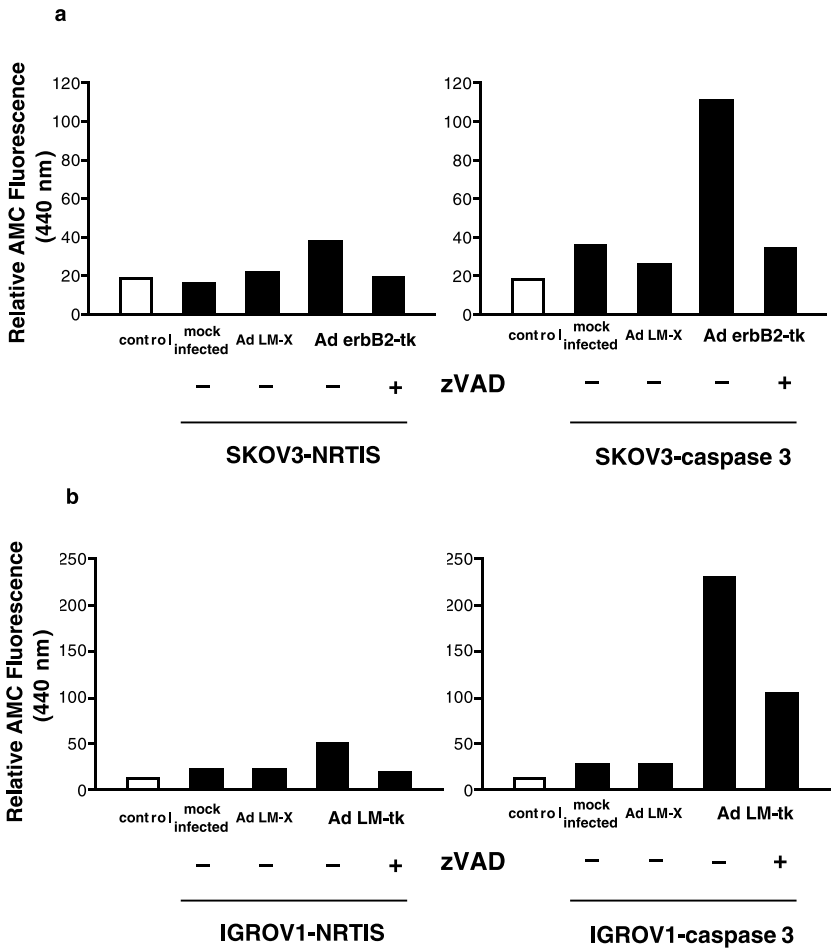

Figure 7. Caspase-3 enzymatic activity. **a:** SKOV3–NRTIS and SKOV3–caspase-3 cells were mock-infected, infected with Ad LM-X, MOI 100 or Ad *erb*B2-tk, MOI 100. Forty-eight hours later, they were exposed to 10 μg/mL GCV for 48 hours in the presence or absence of 2 μM zVAD, at which time they were lysed and caspase-3 enzymatic activity measured. **b:** IGROV1–NRTIS and IGROV1–caspase-3 cells were mock-infected, infected with Ad LM-X, MOI 30 or Ad LM-tk, MOI 30. Forty-eight hours later, they were exposed to 10 μg/mL GCV for 48 hours in the presence or absence of 2 μM zVAD, at which time they were lysed and caspase-3 enzymatic activity measured. In both experiments, control represents the background emission in the absence of any cell.

between 0 and 96 hours and cell cycle status analyzed by flow cytometry. For both SKOV3–NRTIS and SKOV3–caspase-3, tk/GCV treatment led to a gradual increase in the proportion of cells in S-phase, in keeping with the mechanism of action of activated GCV as a antimetabolite. At time 0, the percentage of cells in S-phase was similar in the two cell populations (SKOV3–caspase-3=11.58%, SKOV3–NRTIS=9.12%), but by 48 hours, the proportion of SKOV3–caspase-3 cells in S-phase was significantly greater than for the SKOV3–NRTIS cells (47.93% *vs.* 30.62%, $P<.01$). When cells were exposed to 3 μg/mL GCV for 48 hours in the presence of 2 μM zVAD-fmk, the proportion of cells in S-phase was very similar in SKOV3–NRTIS and SKOV3–caspase-3 cells (30.73 *vs.* 28.18%, respectively; Figure 8). Similar data were obtained with IGROV1–NRTIS and IGROV1–caspase-3 cells (data not shown).

To investigate further the possible mechanism of cell death, the release of LDH from treated cells was measured.[35] Loss of membrane integrity and consequent release of LDH are a feature of both necrotic and apoptotic cell death, but are seen early in necrosis and only in the very late stages of apoptosis.[35] Forty-eight hours following adenoviral transfection, cells were treated with GCV for up to 96 hours in the presence and absence of 2 μM zVAD. Figure 9 demonstrates the LDH release for both pairs of cells at 48 hours after the onset of GCV treatment. This time point was selected because both caspase-3 cleavage and enzymatic activity and PARP cleavage can be demonstrated in the pro-caspase-3–expressing cells at this time. For all four cell lines, there is no increase in the amount of LDH released from cells following tk/GCV treatment, regardless of caspase-3 expression, adenoviral transfection, or the presence/absence of zVAD. By contrast, treatment with the membrane-damaging agent Triton X-100 (1%) for 24 hours leads to large amounts of LDH release into the culture medium. The presence of GCV or zVAD alone has no effect on the LDH assay (data not shown). Therefore, the absence of significant LDH release after 48 hours of GCV treatment implies that the cells are undergoing predominantly apoptotic cell death. After 96 hours of tk/GCV, there was an increase in LDH release from both pro-caspase-3–expressing and –nonexpressing cells upon exposure to tk/GCV, in keeping with the observation that loss of membrane integrity can be seen in the very late stages of apoptosis[35] (data not shown).

DISCUSSION

GPAT, with HSV tk and GCV, has been shown to activate a wide variety of cellular pathways, including caspases-3 and -8, TRAIL, and p53 and leads to the up-regulation of death receptors such as CD95 and TNF-R1.[20] These data suggest that the efficacy of tk/GCV–mediated cell death could be enhanced by the manipulation of these pathways and we

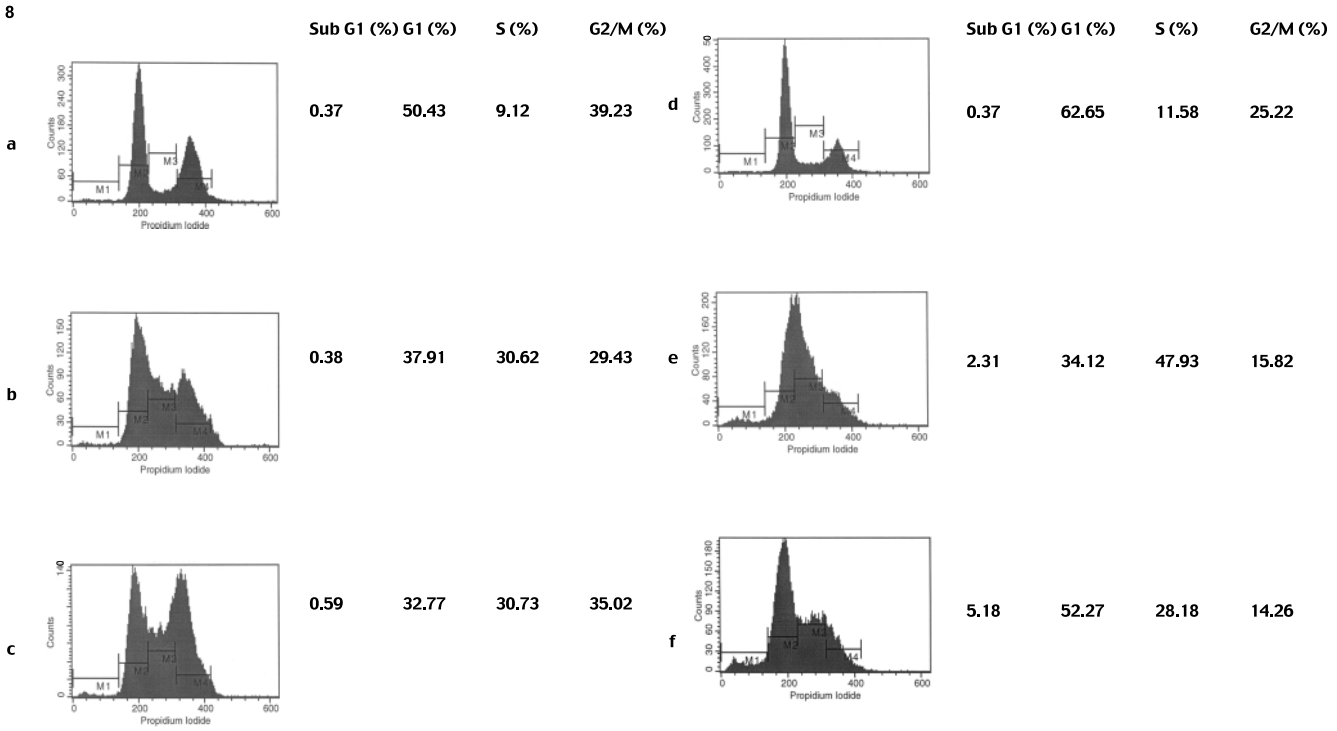

Figure 8. Flow cytometry. 1×10^6 SKOV3–NRTIS and SKOV3–caspase-3 cells were transfected with Ad *erb*B2-tk, MOI 100. Forty-eight hours later, they were exposed to GCV 10 μg/mL in the presence or absence of 2 μM zVAD. They were harvested at 0 and 48 hours, fixed in cold 70% ethanol, and cell cycle status was analyzed by FACS analysis following propidium iodide staining. **(a)** SKOV3–NRTIS+0 hours GCV; **(b)** +48 hours GCV; **(c)** +48 hours GCV+2 μM zVAD; **(d)** SKOV3–caspase-3+0 hours GCV; **(e)** +48 hours GCV; **(f)** +48 hours GCV+2 μM zVAD.

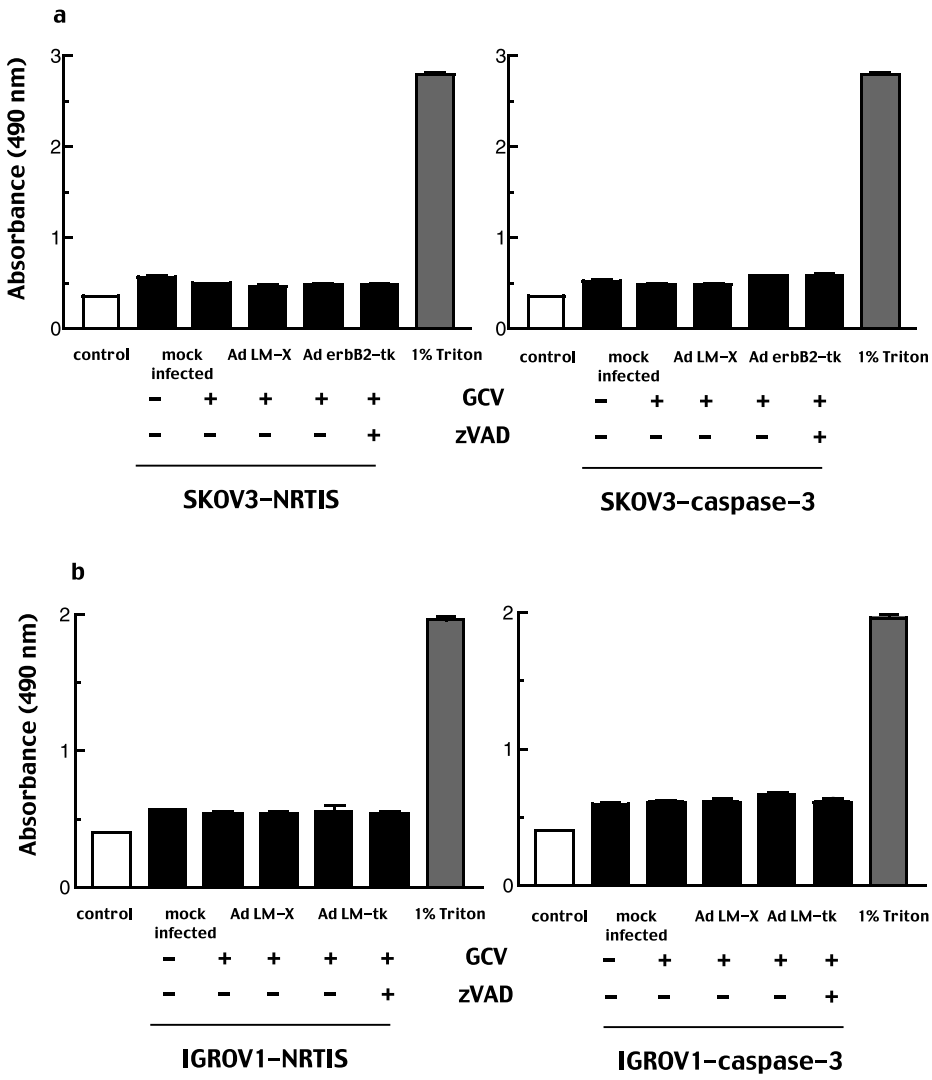

Figure 9. LDH release. **a:** SKOV3–NRTIS and SKOV3–caspase-3 cells were mock-infected, infected with Ad LM-X, MOI 100 or Ad *erb*B2-tk, MOI 100. Forty-eight hours later, they were exposed to 10 μg/mL GCV for 48 hours in the presence or absence of 2 μM zVAD; LDH activity in the culture medium was measured. **b:** IGROV1–NRTIS and IGROV1–caspase-3 cells were mock-infected, infected with Ad LM-X, MOI 30 or Ad LM-tk, MOI 30. Forty-eight hours later, they were exposed to 10 μg/mL GCV for 48 hours in the presence or absence of 2 μM zVAD, at which time LDH activity in the culture medium was measured. For both experiments, negative control was culture medium without cells or GCV (white bars). To assess maximal LDH release, cells were treated for 24 hours with 1% Triton X-100 (grey bars).

have demonstrated here that co-expression of pro-caspase-3 with tk/GCV does indeed lead to increased ovarian cancer cell death.

Other groups have demonstrated that expression of pro-caspase-3 alone in tumor cells does not induce apoptosis.[22,23] Previous work from our group has indicated that it is possible to derive ovarian carcinoma cell clones that stably express caspase-3,[25] following transfection with the tricistronic tetracycline-regulatable plasmid, pNRTIS–caspase-3, and selection by growth in medium containing the neomycin analogue, G418. The level of intrinsic pro-caspase-3 expression differs in the two cell populations investigated. Control SKOV3–NRTIS cells do not express any pro-caspase-3 as detected by Western blotting, which may explain why nontransfected SKOV3–caspase-3 cells are marginally more sensitive to GCV than control SKOV3–NRTIS cells. In contrast, control IGROV1–NRTIS cells do express some background pro-caspase-3 and, in the absence of tk, there is no difference in sensitivity to GCV between control and IGROV1–caspase-3 cells.

SKOV3 cells are Her2/c-*erb*B2–positive and they were transfected with Ad *erb*B2-tk, in which tk expression is controlled by a 500-bp element of the Her2/c-*erb*B2 promoter. SKOV3 cells are negative for MUC1 and there is minimal expression of tk when they are transfected with the second vector, Ad LM-tk, which contains a 1.4-kb element of the *MUC1* promoter (data not shown). By contrast, IGROV1 cells are strongly MUC1-positive and so

Ad LM-tk was used to sensitize these cells to GCV. When transfected with the relevant tk-encoding recombinant adenoviral vectors, there is a greater degree of sensitization of both pro-caspase-3–expressing cells lines to GCV than with their respective control cells. By contrast, there was no significant increase in GCV sensitivity when cell lines were exposed to a control adenovirus lacking a transgene. Coincidental to the increase in GCV sensitivity, there is both direct and indirect evidence of caspase-3 activation. The cleavage of caspase-3 into its active subunits can be demonstrated on Western blotting and the activity of the protease can be demonstrated by the cleavage of PARP and the liberation of fluorogenic AMC from the peptide acetyl-DEVD-AMC. Although PARP cleavage can occur in the absence of caspase-3 [MCF-7 breast carcinoma cells have a functional deletion of the caspase-3 gene but still demonstrate cleavage not only of PARP but also of the actin regulatory protein gelsolin and DNA-dependent protein kinase catalytic subunit ($DNA\text{-}PK_{cs}$) upon treatment with staurosporine and TNF[36]], its cleavage here in the presence of enzymatically active, cleaved caspase-3 provides further evidence that the caspase-3 is fully functional. The increase in sensitivity to GCV and also the activation of caspase-3 were both partially inhibited in the pro-caspase-3–expressing cells by exposure to the synthetic caspase inhibitor, zVAD-fmk, at a dose of 2 μM. By contrast, the presence of zVAD-fmk had no effect on the sensitivity of control cells to tk/GCV. The $t_{1/2}$ of caspase-3 in the presence of 1 μM zVAD-fmk is only 43 seconds,[26] and the $t_{1/2}$ values of caspases-1, -5, -7, -8, and -9 at the same dose of zVAD-fmk are even less, suggesting that this dose of zVAD-fmk is adequate to inhibit any activated caspase-3 in the pro-caspase-3–expressing cells. Therefore, the incomplete inhibition of tk/GCV effects upon exposure to zVAD-fmk indicates that other pathways, either fully or partially independent of caspase-3, may also be activated. This is corroborated by lack of effect of zVAD-fmk upon control cells.

The flow cytometric data from both populations of SKOV3 and IGROV1 cells following tk/GCV treatment do not demonstrate the classical sub-G1 population indicative of DNA cleavage that is a feature of apoptosis. Rather, there is an increase in the proportion of cells in S-phase and, even when GCV treatment extends to 96 hours, there is no noticeable sub-G1 population seen (data not shown). There are two possible explanations for this. Firstly, it has recently been shown that apoptosis can be induced in some adherent tumor cell lines that is not detectable by propidium iodide staining and flow cytometry.[37] Secondly, both SKOV3 and IGROV1 cells may undergo predominantly necrotic, rather than apoptotic, death upon treatment with tk/GCV. Previous work has indicated that the mode of cell death induced by tk/GCV may differ between cell lines, with B16 melanoma cells undergoing necrosis and CMT93 colorectal carcinoma cells undergoing apoptosis.[38] Similarly, it has been shown that other toxic stimuli, such as TNF, can induce both apoptosis and necrosis, depending on the cell line treated[39] or even within the same cell population.[40] Also, recent evidence indicates that, following Fas activation in L929 cells, inhibition of caspases can cause a switch from caspase-dependent apoptosis to caspase-independent necrosis,[41] which suggests strongly that this single stimulus can proceed *via* multiple pathways to produce simultaneous apoptosis and necrosis. Thus, the two modes of cell death can co-exist in many cells, although one may predominate over the other.

To elucidate whether there were significant degrees of necrosis occurring in these cells, the release of LDH from cells in response tk/GCV was measured. Loss of membrane integrity is seen early after cell injury in necrosis, but only in the very late stages of apoptosis.[35] At a time point (48 hours after onset of tk/GCV treatment) when it has already been demonstrated that caspase-3 is cleaved and is enzymatically active and also when PARP is cleaved, there is no difference in the amount of LDH released from pro-caspase-3–expressing cells compared to their respective control cells. This implies that SKOV3 and IGROV1 cells undergo predominantly apoptotic cell death in response to tk/GCV.

In summary, we have shown here that by utilising knowledge of the pathways stimulated by tk/GCV, it is possible to enhance the efficacy of this gene therapy strategy, which may have significant clinical potential in the treatment of ovarian cancer.

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