

Adenovirus-mediated expression of *HSV1-TK* or Fas ligand induces cell death in primary human glioma-derived cell cultures that are resistant to the chemotherapeutic agent CCNU

Tricia C. Maleniak,¹ John L. Darling,² Pedro R. Lowenstein,¹ and Maria G. Castro¹

¹Molecular Medicine and Gene Therapy Unit, School of Medicine, University of Manchester, Manchester M13 9PT, UK; and ²Department of Neurosurgery, Institute of Neurology, University College London, London WC1N 3BG, UK.

Due to minimal treatment success with surgery, radiotherapy, and chemotherapy, the aim of this study was to test the therapeutic potential of gene therapy for the treatment of glioblastoma multiforme (GBM). We have quantitatively analyzed two gene therapy approaches using short-term human glioma cell cultures derived from surgical biopsies (designated IN859, IN1612, IN2045, IN1760, and IN1265) and compared the results of gene therapy with the chemosensitivity of the same cells. All of the glioma cell cultures tested were susceptible to recombinant adenovirus (RAd)-mediated infection. Expression of herpes simplex virus type 1-thymidine kinase (RAd128), followed by ganciclovir treatment, induced apoptosis in all of the glioma cell cultures studied, including three that are resistant to the chemotherapeutic drug 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea (CCNU). Expression of murine Fas ligand (RAdhCMV-mFasL) also induced cell death in four of the five cell cultures studied. One cell culture that was resistant to CCNU was also resistant to apoptosis induced by mFasL expression. These results suggest that sensitivity to chemotherapeutic agents does not necessarily correlate with the sensitivity to gene therapy treatments. RAds expressing therapeutic gene products in human glioma cell cultures are able to induce apoptosis even in some cells that are resistant to a commonly used chemotherapeutic agent. Therefore, RAd-mediated gene transfer could be a good candidate to further develop gene therapy for the treatment of GBM. **Cancer Gene Therapy (2001) 8, 589–598**

Key words: Glioblastoma multiforme; herpes simplex virus type 1-thymidine kinase; murine Fas ligand; adenovirus; chemosensitivity.

Glioblastoma multiforme (GBM) is the most common and aggressive primary human brain tumor.¹ The diffuse and invasive nature of the tumor can lead to the infiltration and occupation of essential areas within the brain.² The first treatment option usually involves surgical resection to improve the symptoms associated with the tumor mass. Treatment after surgical resection can include the use of radiotherapy or chemotherapy. Total resection is difficult due to the lack of a defined tumor edge and nonapparent infiltration into normal brain tissue.³ The failure of chemotherapy at present is thought to be due to inadequate drug delivery and the development of drug-resistant tumor cells.⁴ Gene therapy has been proposed as another tool for the treatment of GBM and could prove to be a useful method of delivering therapeutic proteins to precise areas of the brain. Phase I/II clinical trials are now underway using gene therapy in combination with resection, radiotherapy, and chemotherapy.^{3,5–11}

Before the implementation of clinical trials in humans, new gene therapeutic strategies are tested in animal models of GBM. Animal models have proven to be reliable systems for evaluating efficacy, potential side effects, and for providing statistical survival data.¹² Syngeneic rat models of glioma (RG2/D74, BT₄A, F98, and CNS-1) have been characterized and are therefore most commonly used to determine treatment effectiveness.^{13–15}

Unfortunately, the invasive nature and diversity between human patients diagnosed with GBM is not well represented by any animal model. Thus far, *in vitro* studies using human glioma cell lines have provided evidence for cell death induced by two common experimental gene therapy treatments of GBM: expression of herpes simplex virus type 1-thymidine kinase (*HSV1-TK*), followed by ganciclovir (GCV) administration^{16,17} and the expression of murine Fas ligand (mFasL).^{18–20} These studies involve the use of immortalized cell lines (A-172, T98G, U-118MG, U-373MG, LN-18, U251). Therefore, it is possible that they have lost GBM characteristics by long-term passaging in the laboratory. We therefore decided to test these gene therapy strategies in short-term nonimmortalized human glioma cell cultures obtained from surgical biopsies that have been minimally passaged.^{21,22}

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Address correspondence and reprint requests to Maria G. Castro, Molecular Medicine and Gene Therapy Unit, School of Medicine, University of Manchester, Room 1.302 Stopford Building, Oxford Road, Manchester M13 9PT, UK. E-mail address: mcastro@fs1.scg.man.ac.uk



We investigated the efficacy of the *HSV1-TK*- and FasL-mediated suicide gene therapy strategies, using first-generation recombinant adenoviral (RA) vectors in five short-term human glioma cultures derived from surgical biopsies of different patients. We have determined that short-term human glioma cell cultures are susceptible to adenovirus-mediated transgene expression, and are therefore able to express potentially therapeutic gene products. Apoptosis was induced in all five cell cultures tested, following treatment of the cells with *HSV1-TK/GCV*; however, high levels of apoptosis were detected in only four of five cell cultures infected with a RA expressing mFasL. The sensitivity of the glioma cell cultures to the two gene therapy strategies was compared to the sensitivity to the chemotherapeutic drug, 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea (CCNU).²³ In this study, all of the cell cultures that were sensitive to CCNU were also sensitive to *HSV1-TK/GCV* and mFasL treatment. Of the three cell cultures that were resistant to CCNU, all were killed by *HSV1-TK/GCV*, but only two were killed by mFasL. These results suggest that *HSV1-TK/GCV* and mFasL gene therapy may markedly improve the prospects for successful treatment of GBM, even in cases in which cells are resistant to particular chemotherapeutic agents.

MATERIALS AND METHODS

Cell lines

Short-term glioma cell cultures designated IN859, IN1265, IN1612, IN1760, and IN2045, derived from surgical biopsies from patients with WHO grade IV astrocytoma (GM) were prepared as previously described.^{21,22} Cultures were maintained in Ham's F-10 medium supplemented with 10% (vol/vol) fetal calf serum (FCS), 100 U/mL penicillin, and 100 U/mL streptomycin. Cells were fed every 7–10 days or when the medium turned yellow. Cells were split 1:3–1:6 every 2–3 weeks and before use in experiments. Cell cultures were of similar passage number. Cervical carcinoma cell line HeLa (ATCC CCL-2) was maintained in DMEM medium supplemented with 10% (vol/vol) horse serum, 5% (vol/vol) newborn calf serum, 2 mM MEM nonessential amino acids, 2 mM L-glutamine, and 2 mM sodium pyruvate. Human glioma cell line LN18 (generously provided by Dr. A. Tassinato, University Hospital, Zurich, Switzerland) was maintained in DMEM medium supplemented with 5% (vol/vol) FCS, 2 mM MEM nonessential amino acids, and 2 mM L-glutamine. Human embryonic kidney cell line 293 (Microbix Biosystems, Toronto, Canada) was used for the propagation of RA stocks. 293 cells were grown in MEM medium supplemented with 10% (vol/vol) FCS, 2 mM MEM nonessential amino acids, and 2 mM L-glutamine.

Recombinant adenoviruses

The construction of RAs expressing *Escherichia coli* β -galactosidase (RA35),^{24,25} *HSV1-TK* (RA128),^{15,26} and mFasL (RAhCMV-mFasL),^{27,28} all under the control

of the major immediate early human cytomegalovirus promoter (mIE-hCMV), has been described in detail previously. The RAs were grown and purified as previously described.^{29,30} Double cesium chloride purified virus dialyzed in 10 mM Tris pH 7.5, 1 mM MgCl₂, 135 mM NaCl, and 10% glycerol were stored in small aliquots at -80°C until use in the experiments. All RAs were titrated by the 293 endpoint dilution assay and checked for the presence of replication competent adenovirus (RCA) by the supernatant rescue assay³¹ except RAhCMV-mFasL. RAhCMV-mFasL was verified to be free of RCA contamination by polymerase chain reaction, due to toxicity of mFasL expression on HeLa cells.³² Aliquots of vectors used were found to be negative for RCA at levels used in the experiments.

Short-term glioma cell culture growth curves

Six-well plates were seeded in triplicate with 5×10^4 cells/well. The following day, three wells of each glioma cell culture were trypsinized and cell counts were performed with an improved Neubauer hemacytometer. Counts were done every other day until day 10, approximately 2 days after cell growth reached confluency.

5-Bromo-4-chloro-3-indolyl β -D-galactopyranoside (X-Gal) expression and β -galactosidase activity

Six-well plates were seeded with 2×10^5 cells/well. Cultures were infected with RA35 at multiplicities of infection (MOIs) of 0, 1, 10, 30, 100, and 300. Forty-eight hours after the virus infection, the cultures were fixed for 30 minutes with 4% paraformaldehyde/0.24 M sucrose in 0.2 M phosphate buffer pH 7.4. X-Gal staining of the cultures was performed as previously described.²⁵ For each well, 10 fields were counted at $\times 10$ magnification for either positive (blue cells) or negative expression.

For β -galactosidase activity, duplicate wells at each MOI were harvested 48 hours after the virus infection as above. Cultures were scraped into the medium and the entire suspension was centrifuged at $200 \times g$ for 5 minutes. Pellets were resuspended in 100 μL PBS and freeze/thawed quickly three times. Cell lysates were stored at -80°C until assayed for β -galactosidase activity. Enzymatic activity was assayed as described previously.³³ Absorbance was read on a spectrophotometer at 420 nm. Enzyme units are defined as $380 \times A_{420 \text{ nm}}/\text{min}$.³⁴ Enzyme units were standardized against protein concentration performed with the BCA protein assay kit (Pierce, Rockford, IL).

GCV toxicity on short-term human glioma cell cultures

To test the toxicity of GCV administration, human glioma cell cultures were exposed to increasing levels of GCV. Six-well plates were seeded with 5×10^4 cells/well. Seventy-two hours later, GCV was added to the wells at concentrations of 0, 1, 10 and 100 μM in fresh media. GCV sodium salt lyophilized powder (Cymevene, Roche Discovery Welwyn, Hertfordshire, UK) was initially resuspended in sterile water (500 mg/10 mL), further

diluted in Dulbecco's PBS and stored at 4°C. Three days after the first GCV exposure, the medium was removed from all wells and GCV was administered again at the same concentrations in 2 mL of fresh medium. Seventy-two hours later, the wells were harvested and prepped for FACS analysis as described previously.³⁵ Fluorescence intensity of 5000 cells labeled with propidium iodide after cell permeabilization was measured using a FACScan (Becton Dickinson, Mountain View, CA). Apoptotic cells appear as a broad hypoploid DNA peak and are well distinguished from the normal diploid pattern of DNA associated within nonapoptotic cells.³⁶

Thymidine kinase expression and apoptosis induced by GCV administration

Six-well plates were seeded with 5×10^4 cells/well in duplicate. The following day, the cells were infected in duplicate with RAd128 at MOIs of 0, 1, 10, 30, 100, and 300. Cultures were monitored daily under the microscope for indication of cellular apoptosis (rounded cells detached from the plate). Before the first GCV dose, the infection medium was removed. Two and 5 days after infection with virus, fresh medium containing 10 μ M GCV was added to one set of plates for each cell culture. Eight days after virus infection, a final GCV administration was added to each

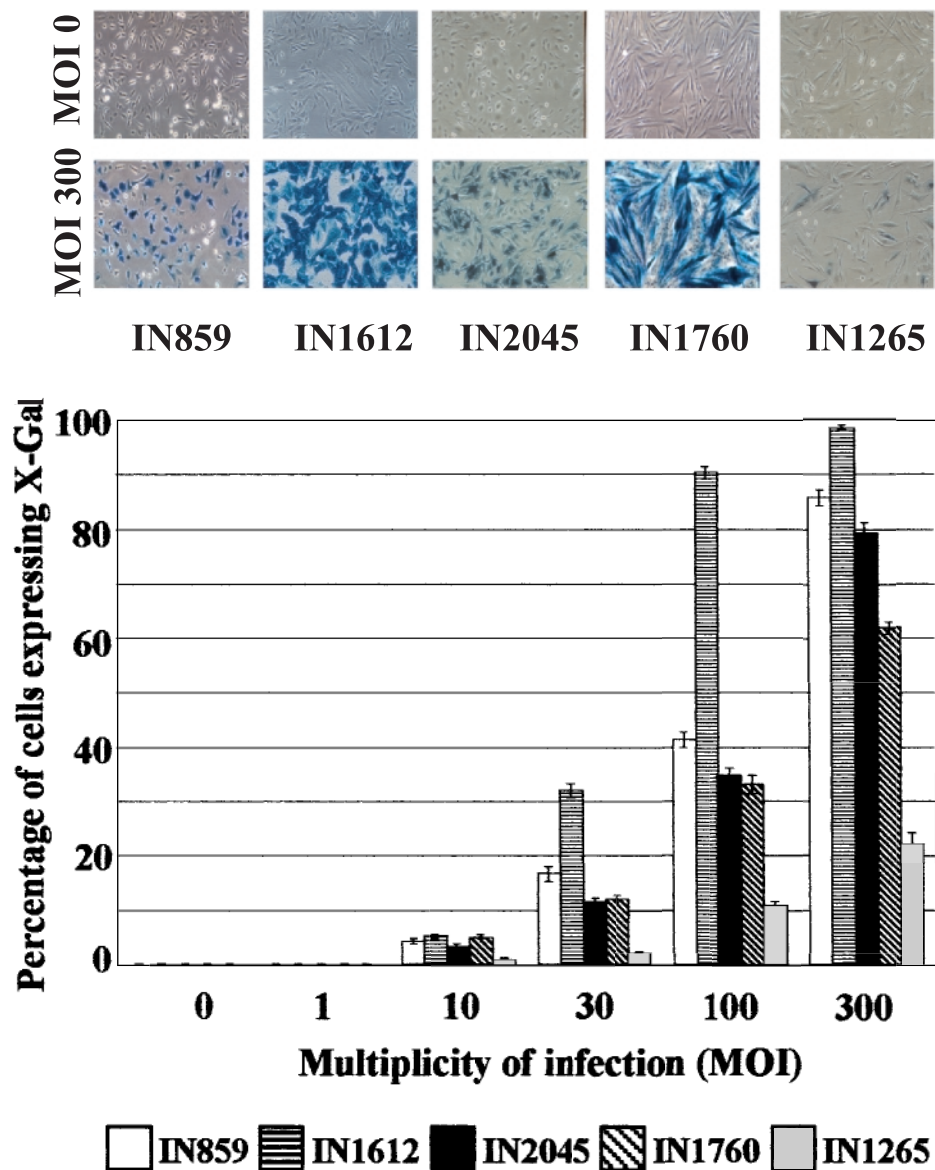


Figure 1. Expression of β -galactosidase expressed from a RAd vector in human glioma cell cultures. Cell cultures IN859, IN1612, IN2045, IN1760, and IN1265 were infected with RAd35, which expresses β -galactosidase under the control of the MIE-hCMV promoter, at MOIs of 0, 1, 10, 30, 100, and 300. Forty-eight hours after infection, the cells were fixed, stained using X-Gal histochemistry, and X-Gal positive cells (in blue) were counted. The top panels show the glioma cells not infected with RAd35. The bottom panels show the glioma cells infected with RAd35 at an MOI of 300. The graph depicts mean results of 10 fields \pm SEM. Magnification: $\times 10$.



well to bring the total well concentration to 100 μ M. The GCV-negative set of plates received fresh medium only. The following day, the supernatants and cells (after trypsinization) from each well were centrifuged and washed in PBS. Cell pellets were fixed in 4% paraformaldehyde/0.24 M sucrose in 0.2 M phosphate buffer pH 7.4 (diluted 1:4) and stored at 4°C until further preparation for FACS analysis as described previously.³⁵ To detect *HSV1-TK* expression, rabbit polyclonal thymidine kinase antibody (generously provided by M. Janicot, Rhone-Poulenc-Rorer, France) was used at a 1:1000 dilution and detected by FACScan after incubation with a swine antirabbit FITC-conjugated secondary antibody (DAKO #F0205, Cambridge, UK).

Fas ligand expression and induction of apoptosis

Cultures were seeded in six-well plates at 1×10^5 cells/well. The following day, the cultures were infected with either RAD35 or RAdhCMV-mFasL at MOIs of 0, 10, 100, 300, 500, and 1000. Three days after virus infection, the wells were harvested to measure the levels of apoptosis induced in the glioma cultures following FasL expression from the RAdS. Cells were prepared for FACS analysis as described previously.²⁸ Secretion of mFasL into the culture supernatants was also verified. To prepare the supernatants, the culture medium was centrifuged at $200 \times g$ for 10 minutes to remove any apoptotic cells. Five hundred microliters of clarified supernatant was applied to a confluent lawn of LN18 human glioma cells¹⁹ and incubated for 24 hours at 37°C. FACS analysis following propidium iodide incorporation was performed to assess cell death due to secreted mFasL in the supernatants.

In vitro sensitivity to CCNU

The short-term cell cultures were screened for sensitivity to CCNU using a modification of the 3-(4, 5 dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay. Technical details of the assay³⁷ and the ID_{50} values have been reported previously.³⁸

Statistical analysis

The experimental results were analyzed by the Student's *t* test, performed using GraphPad InStat[™] Version 2.00 software (GraphPad Software, San Diego, CA).

RESULTS

Expression of β -galactosidase driven by the MIE-hCMV promoter from RAdS in short-term cultured human glioma cells in culture

To assess whether the short-term human glioma cell cultures were susceptible to adenovirus infection and could express gene products encoded within RAdS, cells were infected with a RAd expressing β -galactosidase under the control of the MIE-hCMV promoter (RAD35).

Human glioma cell cultures infected with RAD35 expressed β -galactosidase at MOIs as low as 10, 48 hours after infection with virus (Fig 1). Increasing MOIs elicited increased expression in a dose-dependent manner, as

evidenced by increasing numbers of X-Gal positive cells with higher MOIs (Fig 1). The percentages of X-Gal positive cells at an MOI of 300, the highest MOI tested, were $86 \pm 1.4\%$ (IN859), $99 \pm 0.3\%$ (IN1612), $79 \pm 1.9\%$ (IN2045), $62 \pm 1.0\%$ (IN1760), and $22 \pm 2.1\%$ (IN1265).

β -Galactosidase activity was measured and standardized against protein concentration in cell lysates 48 hours after infection with MOIs of 0, 1, 10, 30, 100, and 300. Cell lines IN859 and IN2045 expressed 10- ($P < .0010$) and 14-fold ($P < .0011$) less β -galactosidase enzymatic activity, respectively, compared to cell line IN1760 at an MOI of 300 (Fig 2). The levels of β -galactosidase activity (enzymatic units per microgram protein) at an MOI of 300 were 0.8 ± 0.04 (IN859), 6.9 ± 0.50 (IN1612), 0.6 ± 0.10 (IN2045), 8.4 ± 0.24 (IN1760), and 3.4 ± 0.03 (IN1265).

Expression and cytotoxicity of HSV1-TK delivered by RAdS within human glioma cell cultures

Increasing *HSV1-TK* expression was observed in all cell cultures with increasing MOIs of RAD128 (Fig 3A). At the highest MOI tested (300), expression of immunoreactive TK protein levels, determined by MFC, were 90 ± 4.9 (IN859), 96 ± 1.5 (IN1612), 94 ± 1.4 (IN2045), 56 ± 18.9 (IN1760), and 76 ± 16.1 (IN1265).

Levels of apoptosis were assessed following GCV administration after infection with RAD128 (Fig 3). The best GCV treatment regime consisted of two 10- μ M GCV doses, followed by an addition of GCV to bring the final concentration to 100 μ M the day before harvesting the cultures. Treatment of the cultures with GCV alone at doses of up to 100 μ M did not induce significant levels of cell death compared to the untreated cells (Fig 4). Infection of all human glioma cell cultures with RAD128

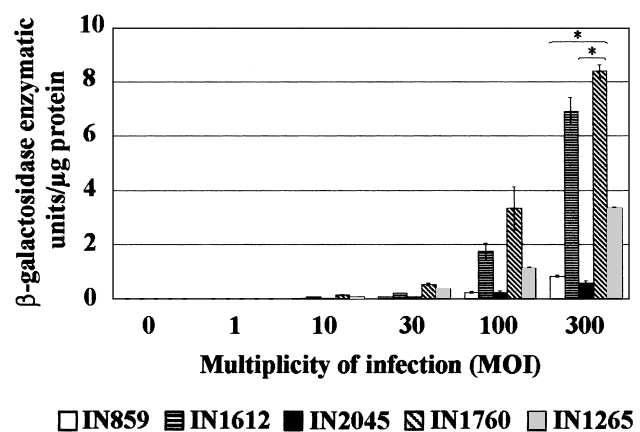


Figure 2. β -Galactosidase enzymatic activity levels expressed from a RAd vector in human glioma cell cultures. Cell cultures IN859, IN1612, IN2045, IN1760, and IN1265 were infected with RAD35 at MOIs of 0, 1, 10, 30, 100, and 300. Forty-eight hours after infection, the cell lysates were harvested and assayed for β -galactosidase activity and protein concentration. In cells IN859 and IN2045, enzymatic activity was considerably less compared to activity observed in cells IN1612, IN1760, and IN1265. The graph represents the mean enzymatic activity results \pm SEM. * $P < .01$.

in the presence of GCV induced apoptosis in a dose-dependent manner. Highly significant levels of apoptosis induced by GCV administration were observed in cell culture IN859 at an MOI of 30 ($P < .001$ vs RAD128-no

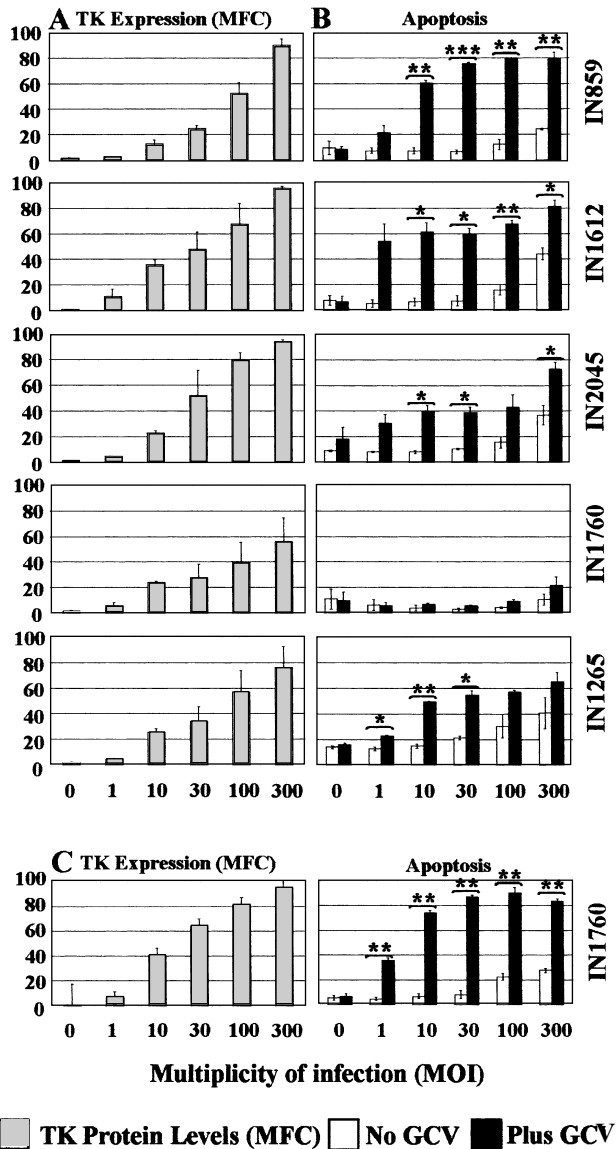


Figure 3. HSV1-TK protein levels and apoptosis induced by GCV administration in the human glioma cell cultures. **(A)** Cell cultures IN859, IN1612, IN2045, IN1760, and IN1265 were infected with RAD128, which expresses *HSV1-TK* under the control of the MIE-hCMV promoter, at MOIs of 0, 1, 10, 30, 100, and 300. TK protein levels (mean fluorescence channel, MFC) were detected by flow cytometry. **(B)** The percentage of apoptosis induced by GCV treatment in the cells was analyzed following infection with RAD128 at the same MOIs. Apoptosis was measured by FACS analysis after propidium iodide incorporation. **(C)** Cell culture IN1760 was infected with RAD128 at MOIs of 0, 1, 10, 30, 100, and 300 and harvested after a further 3-day incubation period. TK protein levels (MFC) and apoptosis were detected by flow cytometry as described above. White bars show apoptosis in cells infected with virus alone. Black bars show apoptosis with cells infected with virus, and then treated with GCV. * $P < .05$, ** $P < .01$, *** $P < .001$.

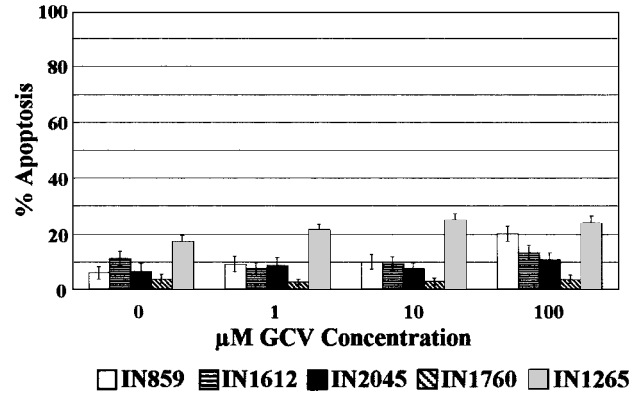


Figure 4. Effects of GCV on cell viability within human glioma cell cultures. Cell cultures were treated with two identical doses of GCV 3 and 6 days after plating the cells at concentrations of 0, 1, 10, and 100 μM . Three days after the last administration, cells were harvested and analyzed by flow cytometry for cell death. Cell culture IN859 was the only cell culture to display increased levels of cell death when GCV concentration increased. The graph depicts the percentage of cell death \pm SEM.

GCV cells), in addition to very significant levels at MOIs of 10, 100, and 300 ($P < .01$ vs RAD128-no GCV cells). Cell lines IN1612 and IN1265 also showed very significant levels of apoptosis at MOIs of 100 and 10, respectively ($P < .01$ vs RAD128-no GCV cells), and significant levels of apoptosis were observed in cell line IN1612 at MOIs of 10, 30, and 300; IN1265 at MOIs of 1 and 30; and IN2045 at MOIs 10, 30, and 300 ($P < .05$ vs RAD128-no GCV cells).

It is interesting to observe that in all of the glioma cells, MOIs as low as 1 are sufficient for the induction of apoptosis. In some cases, we saw higher levels of apoptosis than levels of *HSV1-TK* expression. For example, only 11% of IN1612 cells infected at an MOI of 1 were immunoreactive for *HSV1-TK*, yet greater than 54% cell death was observed by propidium iodide incorporation and FACS analysis. This is indicative of a strong bystander response, where cell death can be observed in cells not expressing the transgene. Moreover, *HSV1-TK* expression in the absence of GCV caused some cytotoxicity; when cultures were infected with RAD128 at an MOI of 300, $24 \pm 1.0\%$ (IN859), $44 \pm 4.7\%$ (IN1612), $37 \pm 7.5\%$ (IN2045), and $41 \pm 12.3\%$ (IN1265) of the cells underwent apoptosis.

At the first timepoint shown (Fig 3B), cell culture IN1760 displayed low levels of apoptosis. At an MOI of 300, cell death was observed at levels of $10 \pm 4.3\%$ without GCV and $21 \pm 6.7\%$ with GCV administration. Cell death induced by *HSV1-TK*/GCV treatment requires actively dividing cells. Therefore, growth rates of all cell cultures were assessed to determine whether there was a correlation between susceptibility to *HSV1-TK*/GCV-induced cell death and the rate of the proliferation in these cells. All cell cultures except IN1760 displayed similar growth rates and reached similar cell densities as the others, after 6 days in culture (Fig 5). Log phase growth occurred between 2 and 6 days, except for cell culture IN1760, which did not reach a

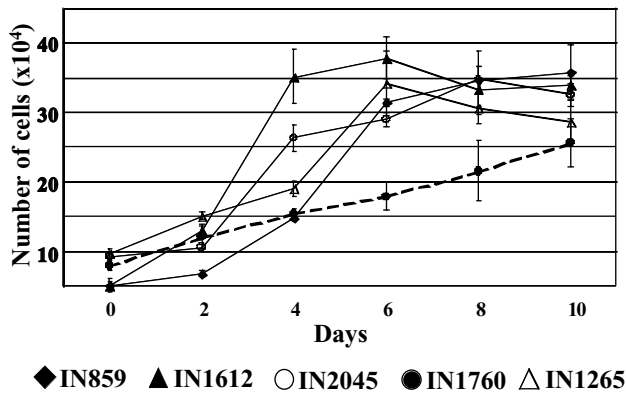


Figure 5. Cell proliferation of human glioma cell cultures. Cell cultures IN859, IN1612, IN2045, IN1760, and IN1265 were seeded in a six-well plate. Triplicate wells at each timepoint for each cell culture were counted every other day for 10 days. Cell culture IN1760, depicted with a dashed line, grew considerably slower than the other cell cultures and reached confluency at day 10. The other cell cultures grew quicker, approximately 3-fold from 2 to 6 days. After 6 days, the cells reached confluency and the growth curve plateaued. The graph depicts the mean cell counts \pm SEM.

similar cell density, and also grew steadily over the course of the 10-day experiment. At day 10, all wells displayed confluent monolayers.

Due to the slower growth rate of cell culture IN1760, we extended the harvest timepoint to observe apoptosis levels. Cell death was enhanced in cell culture IN1760 when the harvest timepoint was extended by a further 3 days (Fig 3C). Assessment of apoptosis levels in cell culture IN1760 at this timepoint at an MOI of 100 was $22.3 \pm 2.8\%$ without GCV and $90.5 \pm 4.2\%$ after GCV addition ($P < .01$ vs RAd35-no GCV cells).

Expression and cytotoxicity of mFasL from RAds within human glioma cell cultures

Cell cultures were infected with RAdhCMV-mFasL using MOIs ranging from 0 to 1000. Cells were harvested 3 days after infection, and levels of cell death were measured by flow cytometry (Fig 6). All cell cultures except IN1760 were susceptible to mFasL-mediated cytotoxicity. When the human glioma cell cultures were infected with RAd35, at the same MOIs of RAdhCMV-mFasL, no apoptosis was observed above background levels suggesting that cell death was due to mFasL expression and not cytotoxic viral infection.

Infection of all human glioma cell cultures with RAdhCMV-mFasL induced apoptosis in an MOI-dependent manner except for cell line IN1760. Cell line IN859 displayed the highest levels of cell death, even at low MOIs. At an MOI of 1000, cell death was 29.6-fold higher in RAdhCMV-mFasL-infected IN859 cells than RAd35-infected cells ($P < .01$). In cell culture IN1612 at an MOI of 1000, apoptosis levels were 9.5-fold higher than RAd35-infected cells ($P < .05$). Cell death in glioma cell culture IN2045 and IN1265 achieved 5.0-fold ($P < .05$ vs RAd35-infected cells) and 7.4-fold ($P < .001$

vs RAd35-infected cells) higher levels at an MOI of 1000 than RAd35-infected cells. In contrast, cell death in glioma cell culture IN1760 reached only $13.3 \pm 1.7\%$, not much higher than RAd35-induced cell death ($2.3 \pm 1.7\%$) yet still statistically significant ($P < .05$ vs RAd35-infected cells).

To further confirm that the cytotoxicity observed in the human glioma cell lines was due to mFasL expression from the RAds, supernatants were harvested from the glioma cell lines infected with RAdhCMV-mFasL and applied to cell line LN18, which is sensitive to mFasL cytotoxicity.¹⁶ Cell line LN18 was exposed to supernatants of the human glioma cell cultures infected with RAdhCMV-mFasL or RAd35 at an MOI of 1000. Cell death was measured by propidium iodide incorporation, followed by FACS analysis. Supernatants from RAdhCMV-mFasL-infected cells induced apoptosis in LN18 cells at levels of 34.9 ± 5.7 (IN859, $P < .05$ vs RAd35-infected cells); 23.9 ± 0.7 (IN1612, $P < .01$ vs RAd35-infected cells); 38.9 ± 4.1 (IN2045, $P < .05$ vs RAd35-infected cells); 19.4 ± 0.5

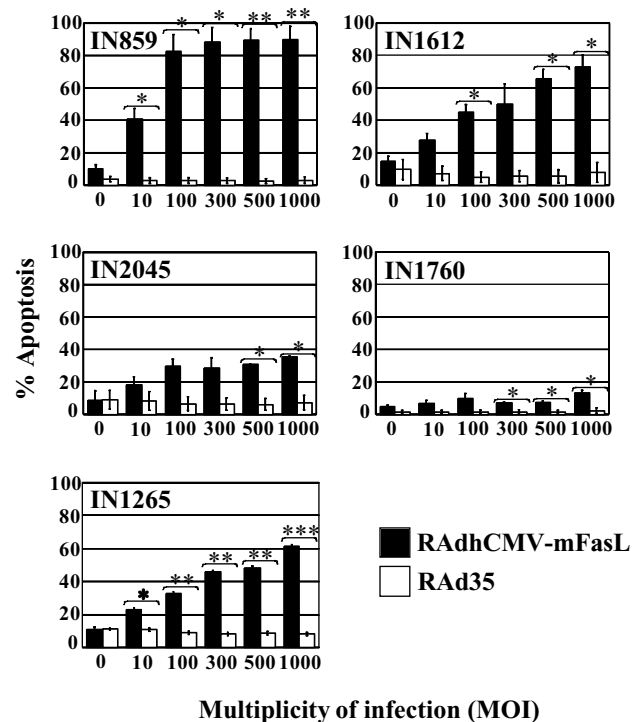


Figure 6. Induction of apoptosis by mFasL expression from a RAd vector within human glioma cell cultures. Cell cultures IN859, IN1612, IN2045, IN1760, and IN1265 were infected with RAdhCMV-mFasL, which expresses mFasL under the control of the MIE-hCMV promoter and RAd35, at MOIs of 0, 10, 100, 300, 500, and 1000. Three days after infection, the wells were harvested. Apoptosis was measured by FACS analysis after propidium iodide incorporation. Cell death is observed in cell cultures IN859, IN1612, IN2045, and IN1265 when infected with RAdhCMV-mFasL. Only very low levels of cell death are observed in cell culture IN1760 when infected with RAdhCMV-mFasL. Cell death does not appear to be induced by viral infection with high MOIs, because cells infected with RAd35 at the same MOIs did not undergo apoptosis. * $P < .05$, ** $P < .01$, *** $P < .001$.

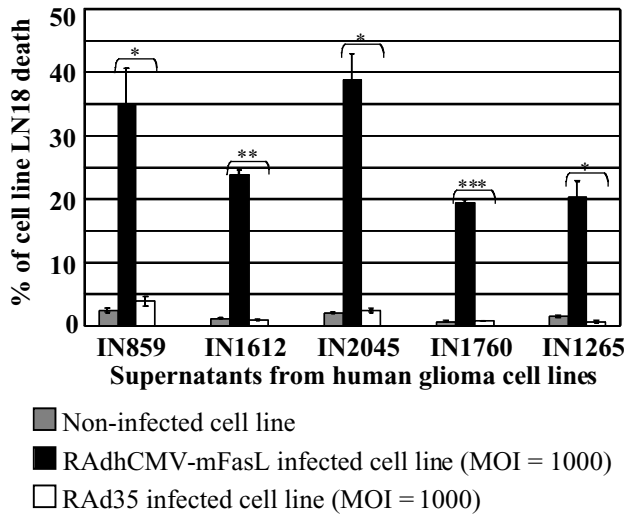


Figure 7. Secretion of mFasL in supernatants from human glioma cell cultures infected with RADhCMV-mFasL induces death in cell line LN18. Supernatants from human glioma cell cultures infected with RADhCMV-mFasL or RAD35 were harvested 3 days after infection. After clarification by centrifugation, the supernatants were applied to human glioma cell line LN18 (FasL-sensitive cell line). After an overnight incubation, cell death was observed by propidium iodide incorporation, followed by FACS analysis. Cell death is observed following exposure to supernatants from RADhCMV-mFasL-infected human glioma cells at a MOI of 1000, but not RAD35 at the same MOI. * $P < .05$, ** $P < .01$, *** $P < .001$.

(IN1760, $P < .001$ vs RAD35-infected cells); and 20.4 ± 2.4 (IN1265, $P < .05$ vs RAD35-infected cells) (Fig 7).

DISCUSSION

This study shows that short-term cultures of human glioma cells derived from surgical biopsies of patients with GBM with varying sensitivity to the chemotherapeutic drug CCNU were sensitive to cell death mediated from either conditionally cytotoxic (*HSV1-TK/GCV*) or directly cytotoxic (mFasL) gene therapy treatments. The cell cultures treated ranged in their *in vitro* sensitivity to the chemotherapeutic drug CCNU³⁷ (Fig 8). It has been suggested that CCNU achieves a concentration of about 5 $\mu\text{g}/\text{mL}$ in brain tumor tissue.³⁹ Therefore, cell cultures IN859 and IN1612 were considered to be sensitive to CCNU, whereas cell cultures IN2045, IN1760, and IN1265 were considered insensitive. Apoptosis was observed in all five cell cultures by *HSV1-TK/GCV* treatment, regardless of their sensitivity to CCNU. However, apoptosis was induced in four of the five cell cultures with expression of mFasL. Cell culture IN1760, appeared to be relatively resistant to the mFasL gene therapy approach. In contrast, cell cultures IN2045 and IN1265 that were also resistant to CCNU, were susceptible to both gene therapy modalities. Therefore, sensitivity to gene therapy treatment was not predicted by sensitivity to chemotherapy.

Even though we did not see 100% transduction efficiency by the adenovirus vector expressing β -galacto-

sidase, treatment of the human glioma cell cultures with *HSV1-TK/GCV* yielded high levels of cell death in all of the cell cultures tested. The best treatment modality occurred when the RAD128 infection was applied to the cell monolayer when the confluency reached no more than 20–30%. This appeared to be necessary to achieve enough cell proliferation following GCV administration to induce apoptosis. A strong bystander response was exhibited. In all of the cell lines at lower MOIs, *HSV1-TK* immunoreactive protein levels were lower than the observed level of cell death. For example, at an MOI of 30, *HSV1-TK* protein levels were approximately 25% in cell culture IN859, yet apoptosis levels reached over 75% following GCV administration. The cell death caused by the bystander effect could be due to the transfer of phosphorylated GCV through gap junctions⁴⁰ or the uptake of apoptotic bodies by nontransduced cells.⁴¹ This suggests that not all of the glioma cells need to express *HSV1-TK* to achieve efficient cell death within the culture.

Apoptosis was also observed in all of the human glioma cell cultures infected with RAD128 at the higher MOIs, even without the administration of GCV. This was due to expression of *HSV1-TK*, not viral infection, because apoptosis was not observed above background levels in human glioma cells infected with RAD35 at MOIs as high as 1000. Toxicity due to *HSV1-TK* expression has also been seen in the pituitary tumor cell line GH3³⁵ and in transgenic male mice expressing *HSV1-TK*.⁴²

The second gene therapy approach tested was the RAD delivery of mFasL. Four of the five cell cultures tested were killed by this treatment method. Glioma cell culture IN1760 was relatively resistant to this killing method. This suggests that these cells express very low or no Fas/APO-1 receptors on the cell surface, or are able to resist FasL-mediated cell death by inactivation of the downstream caspase cascade⁴³ or mitogen-activated protein kinase (MAPK) signaling pathway.⁴⁴ Increased expression levels of Fas/FasL in gliomas is thought to aid in the progression of the tumors by

Cell line	CCNU ID ₅₀ $\mu\text{g}/\text{ml}$	CCNU Sensitive?	HSV1-TK/ GCV Sensitive?	mFasL Sensitive?
IN859	1.65	Yes	Yes	Yes
IN1612	2.8	Yes	Yes	Yes
IN2045	5.62	No	Yes	Yes
IN1760	15.16	No	Yes	No
IN1265	15.8	No	Yes	Yes

Figure 8. Comparison of CCNU chemosensitivity to *HSV1-TK/GCV* treatment and mFasL-induced cell death in human glioma cell cultures. Short-term human glioma cell cultures were assayed for sensitivity to CCNU using a modified MTT assay.³⁷ The ID₅₀ results have been reported previously.³⁸ ID₅₀ values below 5 $\mu\text{g}/\text{mL}$ were classified as sensitive to CCNU.³⁹ Expression of *HSV1-TK* from a RAD, followed by GCV administration, induced apoptosis in all of the cell cultures tested. Expression of mFasL from a RAD induced apoptosis in all cell cultures except cell culture IN1760. *In vitro* sensitivity to chemotherapeutic agents (i.e., CCNU) does not indicate sensitivity to gene therapy strategies.



inducing extracellular signal-regulated kinase activation⁴⁴ and circumventing the host immune response mediated by Fas-positive lymphocytes.^{45,46} The use of mFasL as a treatment for GBM has been studied in the rat F98 glioma model, in which survival time was extended by 50% in rats treated with RAdhCMV-mFasL compared with the control animals.¹⁹

Clinical trials assessing the feasibility and adverse side effect of RAd delivering *HSV1-TK* for the treatment of GBM have started.^{3,8,11} Thirteen patients, nine of whom were diagnosed with GBM, were subjected to a single injection of RAd expressing *HSV1-TK* under the control of the Rous sarcoma virus promoter into an intratumoral site, followed by GCV treatment.⁸ The study found that the vector dose was safe when the dose did not exceed 2×10^{11} viral particles. Two of the patients diagnosed with GBM survived greater than 25 months, which was longer than expected. Severe and widespread inflammation was not observed in this study as was found in a rodent model of GBM.¹⁵ The inflammatory response to adenoviral vectors expressing *HSV1-TK* is still under scrutiny, as other clinical trial studies have seen widespread inflammation in the brain.¹¹ These results suggest the need for improving vector efficiency and expression and minimizing the host immune response against the vector, along with better vector and GCV delivery methods. Some of these objectives might be achieved with the use of stronger⁴⁷ or cell-type-specific promoters⁴⁸ within first-generation or high-capacity/“gut-less” RAd vectors.⁴⁹

Another adenovirus currently undergoing clinical trials is ONYX-015, which is an E1B-55K-deleted adenovirus vector that selectively replicates and lyses *p53*-deficient cancer cells. A high proportion of responding tumors has been seen in patients with recurrent squamous cell cancer of the head and neck when treated with a combination of cisplatin, 5-fluorouracil, and an intratumoral injection of ONYX-015.⁵⁰ This is in contrast to tumors treated with chemotherapy alone. The biopsies confirmed necrosis of the tumor cell population and that the vector displayed tumor selective replication with minimal toxic side effects for the patients.

Both of these studies show promising therapeutic benefit using adenovirus vectors for the treatment of cancer. These are encouraging results considering that the patients thus far used in the clinical trial cases usually are in the advanced stages of the disease and have already been treated by resection, radiation, and/or chemotherapy.

This study has demonstrated that the effectiveness of the treatment, whether chemotherapy or gene therapy, differs greatly between biopsies obtained from different patients. This is an important consideration when setting up clinical trials and establishing optimum treatment modalities. In addition, this study provides evidence that future screening of surgical biopsies would be advantageous to provide patients with GBM with the most beneficial strategy for individualized treatment. It also provides evidence for the use of a multifaceted approach using different gene therapy strategies in combination with currently used treatments, i.e., chemotherapy, radiotherapy, and/or surgery.

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REFERENCES

1. Kleihues P, Burger PC, Scheithauer BW. The new WHO classification of brain tumors. *Brain Pathol.* 1993;3:255–268.
2. Holland EC. Glioblastoma multiforme: the terminator. *Proc Natl Acad Sci USA.* 2000;97:6242–6244.
3. Eck SL, Alavi JB, Alavi A, et al. Treatment of advanced CNS malignancies with the recombinant adenovirus H5.010RSVTK: a phase I trial. *Hum Gene Ther.* 1996;7:1465–1482.
4. Bradford R, Koppel H, Pilkington GJ, et al. Heterogeneity of chemosensitivity in six clonal cell lines derived from a spontaneous murine astrocytoma and its relationship to genotypic and phenotypic characteristics. *J Neuro-Oncol.* 1997;34:247–261.
5. Klatzmann D, Valery CA, Bensimon G, et al. A phase I/II study of herpes simplex type 1 thymidine kinase ‘suicide’ gene therapy for recurrent glioblastoma. *Hum Gene Ther.* 1998;9:2595–2604.
6. Palu G, Cavaggioni A, Calvi P, et al. Gene therapy of glioblastoma multiforme via combined expression of suicide and cytokine genes: a pilot study in humans. *Gene Ther.* 1999;6:330–337.
7. Shand N, Weber F, Mariani L, et al. A phase 1–2 clinical trial of gene therapy for recurrent glioblastoma multiforme by tumor transduction with the herpes simplex thymidine kinase gene followed by ganciclovir. *Hum Gene Ther.* 1999;10:2325–2335.
8. Trask TW, Trask RP, Aguilar-Cordova E, et al. Phase I study of adenoviral delivery of the HSV-tk gene and ganciclovir administration in patients with recurrent malignant brain tumors. *Mol Ther.* 2000;1:195–203.
9. Market JM, Medlock MD, Rabkin SD, et al. Conditionally replicating herpes simplex virus mutant, G207 for the treatment of malignant glioma: results of a phase I trial. *Gene Ther.* 2000;7:867–874.
10. Rampling R, Cruickshank G, Papanastassiou V, et al. Toxicity evaluation of replication-competent herpes simplex virus (ICP 34.5 null mutant 1716) in patients with recurrent malignant glioma. *Gene Ther.* 2000;7:859–866.
11. Sandmair A-M, Loimas S, Puranen P, et al. Thymidine kinase gene therapy for human malignant glioma using replication-deficient retroviruses or adenoviruses. *Hum Gene Ther.* 2000;11:2197–2206.
12. Castro MG, Cowen R, Smith-Arica J, et al. Gene therapy strategies for intracranial tumours: glioma and pituitary adenomas. *Histol Histopathol.* 2000;15:1233–1252.
13. Chen S-H, Shine HD, Goodman J, et al. Gene therapy for brain tumors: regression of experimental gliomas by adenovirus-mediated gene transfer *in vivo*. *Proc Natl Acad Sci USA.* 1994;91:3054–3057.

14. Maron A, Gustin T, Le Roux A, et al. Gene therapy of rat C6 glioma using adenovirus-mediated transfer of the herpes simplex virus thymidine kinase gene: long-term follow-up by magnetic resonance imaging. *Gene Ther.* 1996;3:315–322.
15. Dewey RA, Morrissey G, Cowsill CM, et al. Chronic brain inflammation and persistent herpes simplex virus 1 thymidine kinase expression in survivors of syngeneic glioma treated by adenovirus-mediated gene therapy: implications for clinical trials. *Nat Med.* 1999;5:1256–1263.
16. Sturtz FG, Waddell K, Shulok J, et al. Variable efficiency of the thymidine kinase/ganciclovir system in human glioblastoma cell lines: implications for gene therapy. *Hum Gene Ther.* 1997;8:1945–1953.
17. Vandier D, Rixe O, Brenner M, et al. Selective killing of glioma cell lines using an astrocyte-specific expression of the herpes simplex virus-thymidine kinase gene. *Cancer Res.* 1998;58:4577–4580.
18. Shinoura N, Yoshida Y, Sadata A, et al. Apoptosis by retrovirus- and adenovirus-mediated gene transfer of fas ligand to glioma cells: implications for gene therapy. *Hum Gene Ther.* 1998;9:1983–1993.
19. Ambar BB, Frei K, Malipiero U, et al. Treatment of experimental glioma by administration of adenoviral vectors expressing fas ligand. *Hum Gene Ther.* 1999;10:1641–1648.
20. Shinoura N, Yoshida Y, Asai A, et al. Adenovirus-mediated transfer of p53 and fas ligand drastically enhances apoptosis in gliomas. *Cancer Gene Ther.* 2000;7:732–738.
21. Darling JL. The *in vitro* biology of human brain tumours. In: Thomas DGT, ed. *Neuro-Oncology: Primary Malignant Brain Tumours*. London: Edward Arnold; 1990:1–25.
22. Darling JL. Brain. In: Masters JRW, ed. *Human Cancer in Primary Culture: A Handbook*. Dordrecht: Kluwer; 1991: 231–251.
23. Darling JL, Lewandowicz GM, Thomas DGT. Chemosensitivity testing human malignant brain tumors. In: Kornblith PL, Walker MD, eds. *Advances in Neuro-Oncology II*. Armonk, NY: Futura Publishing Company; 1997:413–434.
24. Wilkinson GWG, Akrigg A. The cytomegalovirus major immediate early promoter and its use in eukaryotic expression systems. In: Greenaway PJ, ed. *Advances in Gene Technology*. Vol. 2. London: JAI Press; 1991:287–310.
25. Shering AF, Bain D, Stewart K, et al. Cell-type specific expression in brain cell cultures from a short human cytomegalovirus major immediate early promoter depends on whether it is inserted into herpesvirus or adenovirus vectors. *J Gen Virol.* 1997;78:445–459.
26. Cowsill C, Southgate TD, Morrissey G, et al. Central nervous system toxicity of two adenoviral vectors encoding variants of the herpes simplex virus type 1 thymidine kinase: reduced cytotoxicity of a truncated HSV1-TK. *Gene Ther.* 2000;7:679–685.
27. Larregina AT, Morelli AE, Dewey RA, et al. FasL induces fas/apo1-mediated apoptosis in human embryonic kidney 293 cells routinely used to generate E1-deleted adenoviral vectors. *Gene Ther.* 1998;5:563–568.
28. Morelli AE, Larregina AT, Smith-Arica J, et al. Neuronal and glial cell type-specific promoters within adenovirus recombinants restrict the expression of the apoptosis-inducing molecule fas ligand to predetermined brain cell types, and abolish peripheral liver toxicity. *J Gen Virol.* 1999; 80:571–583.
29. Lowenstein PR, Shering AF, Bain D, et al. The use of adenovirus vectors to transfer genes to identified target brain cells *in vitro*. In: Lowenstein PR, Enquist LW, eds. *Protocols for Gene Transfer in Neuroscience: Towards Gene Therapy of Neurological Disorders*. Chichester, UK: John Wiley and Sons, Ltd.; 1996:93–114.
30. Southgate TD, Kingston PA, Castro MG. Gene transfer into neural cells *in vitro* using adenoviral vectors. In: Crawley J, Rogawski M, Sibley D, Wray S, Skolnick P, eds. *Current Protocols in Neuroscience, Vol. 4: Gene Cloning, Expression, and Mutagenesis*. New York, NY: John Wiley and Sons Inc.; 2000:4.23.1–4.23.40.
31. Dion LD, Fang J, Garver RI Jr. Supernatant rescue assay vs. polymerase chain reaction for detection of wild type adenovirus-contaminating recombinant adenovirus stocks. *J Virol Methods.* 1996;56:99–107.
32. Rubinchik S, Ding R, Qiu AJ, et al. Adenoviral vector which delivers fasL-GFP fusion protein regulated by the tet-inducible expression system. *Gene Ther.* 2000;7:875–885.
33. Southgate TD, Windeatt S, Smith-Arica J, et al. Transcriptional targeting to anterior pituitary lactotrophic cells using recombinant adenovirus vector *in vitro* and *in vivo* in normal and estrogen/sulpiride-induced hyperplastic anterior pituitaries. *Endocrinology.* 2000;141:1–13.
34. Macgregor GR, Nolan GP, Fiering S, et al. Use of *E. coli lacZ* (β -galactosidase) as a reporter gene. In: Murray EJ, ed. *Methods in Molecular Biology, Vol. 7: Gene Transfer and Expression Protocols*. Clifton, NJ: The Humana Press Inc.; 1991:217–235.
35. Windeatt S, Southgate TD, Dewey RA, et al. Adenovirus-mediated herpes simplex virus type-1 thymidine kinase gene therapy suppresses oestrogen-induced pituitary prolactinomas. *J Clin Endocrinol Metab.* 2000;85:1296–1305.
36. Fraker PJ, King LE, Lill-Elghanian D, et al. Quantification of apoptotic events in pure and heterogeneous populations of cells using the flow cytometer. *Methods Cell Biol.* 1995;46:57–76.
37. Haselsberger K, Peterson DC, Thomas DGT, et al. Assay of anticancer drugs in tissue culture: comparison of a tetrazolium-based assay and a protein binding dye assay in short-term cultures derived from human malignant glioma. *Anti-Cancer Drugs.* 1996;7:331–338.
38. El-Dereby W, Ashmore SM, Branston NM, et al. Pretreatment prediction of the chemotherapeutic response of human glioma cell cultures using nuclear magnetic resonance spectroscopy and artificial neural networks. *Cancer Res.* 1997;57:4196–4199.
39. Levin VA. Clinical pharmacology of the nitrosoureas. In: Prestayko AW, Crooke ST, Baker LH, Carter SK, Schein PS, eds. *Nitrosoureas*. New York, NY: Academic Press; 1981:171–180.
40. Elshami AA, Saavedra A, Zhang H, et al. Gap junctions play a role in the bystander effect of the herpesvirus thymidine kinase ganciclovir system *in vitro*. *Gene Ther.* 1996;3:85–92.
41. Freeman SM, Abboud CN, Whartenby KA, et al. The 'bystander effect': tumor regression when a fraction of the tumor mass is genetically modified. *Cancer Res.* 1993;53: 5274–5283.
42. Salomon B, Maury S, Loubiere L, et al. A truncated herpes simplex virus thymidine kinase phosphorylates thymidine and nucleoside analogs and does not cause sterility in transgenic mice. *Mol Cell Biol.* 1995;15:5322–5328.
43. Nagata S. Apoptosis by death factor. *Cell.* 1997;88:355–365.
44. Shinohara H, Yagita H, Ikawa Y, et al. Fas drives cell cycle progression in glioma cells *via* extracellular signal-regulated kinase activation. *Cancer Res.* 2000;60:1766–1772.
45. O'Connell J, O'Sullivan GC, Collins JK, et al. The Fas counter-attack: Fas mediated T cell killing by colon cancer cells expressing Fas ligand. *J Exp Med.* 1996;184:1075–1082.



46. Hahne M, Rimoldi D, Schroter M, et al. Melanoma cell expression of Fas (Apo-1/CD95) ligand: implications for tumor immune escape. *Science*. 1996;274:1363–1366.
47. Gerdes CA, Castro MG, Lowenstein PR. Strong promoters are the key to highly efficient, noninflammatory and noncytotoxic adenoviral-mediated transgene delivery into the brain *in vivo*. *Mol Ther*. 2000;2:330–338.
48. Smith-Arica JR, Morelli AE, Larregina AT, et al. Cell-type specific and regulatable transgenesis in the adult brain: adenovirus-encoded combined transcriptional targeting and inducible transgene expression. *Mol Ther*. 2000;2:579–587.
49. Thomas CE, Schiedner G, Kochanek S, et al. Peripheral infection with adenovirus causes unexpected long-term brain inflammation in animals infected intracranially with first-generation, but not with high-capacity, adenovirus vectors: toward realistic long-term neurological gene therapy for chronic diseases. *Proc Natl Acad Sci USA*. 2000;97:7482–7487.
50. Khuri FR, Nemunaitis J, Ganly I, et al. A controlled trial of intratumoral ONYX-015, a selectively replicating adenovirus, in combination with cisplatin and 5-fluorouracil in patients with recurrent head and neck cancer. *Nat Med*. 2000;6:879–885.