Enhanced Tumor Cell Killing in the Presence of Ganciclovir by Herpes Simplex Virus Type 1 Vector-directed Coexpression of Human Tumor Necrosis Factor-α and Herpes Simplex Virus Thymidine Kinase

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ABSTRACT

Past studies have documented the promise of herpes simplex virus type 1 (HSV-1) thymidine kinase (TK) suicide gene therapy as a potential antitumor treatment. HSV-TK converts the pro-drug ganciclovir (GCV) into a toxic nucleotide analogue, the incorporation of which into cellular DNA blocks cell proliferation. In this report, we have examined the hypothesis that the effectiveness of HSV-TK suicide gene therapy can be enhanced by coexpression of the antitumor cytokine human tumor necrosis factor-α (TNF-α) from the same replication-defective HSV-1 vector. In vitro testing demonstrated that TNF-α expression from this vector potentiated the killing of both TNF-α-sensitive L929 tumor cells and TNF-α-resistant U-87 MG cells in the presence of GCV. Furthermore, treatment of established intracranial L929 tumors in vivo with the TNF-α/TK vector and GCV resulted in prolonged animal survival compared with treatment with parental HSV-TK vector in the presence or absence of GCV. Treatment of intracerebral U-87 MG tumors showed a clear benefit of TK therapy, but a significant further increase in survival using the TNF-α vector could not be demonstrated. We found that potentiation of cell killing in vitro required intracellular TNF-α because purified protein added to the culture medium of cells infected with HSV-TK vector failed to have the same effect. Accordingly, potentiation in vivo should depend on efficient infection, but immunohistochemical analysis indicated that virus administration by U-87 MG intratumoral injection was inadequate, resulting in an estimated <1% infection of all tumor cells. Moreover, the majority of infected tumor cells were localized at the tumor margin. Together, these results suggest that HSV-TK suicide gene therapy should provide a useful treatment for TNF-α-sensitive tumors and perhaps also for TNF-α-resistant tumors if vector delivery can be improved to increase the percentage of transduced tumor cells.

INTRODUCTION

Gene therapy is a relatively new technique with potential as a treatment for cancer and a wide variety of other diseases. One anticancer gene therapy approach that has already been applied in clinical settings is the direct transfer of genes that activate prodrugs locally or induce programmed tumor cell death (1–10). Although such “suicide” gene therapy approaches have been successful to varying degrees in both preclinical and clinical studies, it is possible that “cure” rates can be improved through the use of multiple therapeutic genes, the products or which destroy tumor cells by a combination of mechanisms. Experiments described in this report attempt to test this hypothesis by vector-mediated codelivery to the tumor mass of the antitumor cytokine TNF-α and the HSV-1 TK suicide gene followed by systemic treatment with GCV.

MATERIALS AND METHODS

Cell Culture and Virus Production. Vero African green monkey kidney cells (CCL81; ATCC, Rockville, MD), Vero-derived 7B cells (26), and mouse L929 fibrosarcoma cells (kindly provided by Dr. Leaf Huang, Department of Pharmacology, University of Pittsburgh) were maintained in MEM (Life Technologies, Inc., Gaithersburg, MD) supplemented with glutamine, antibiotics (PSN; Life Technologies, Inc.), and 10% fetal bovine serum (LTI. Pharmacology, University of Pittsburgh) were maintained in MEM supplemented with glutamine, antibodies, and 10% fetal bovine serum.

HSV-TK is a commonly used suicide gene in both experimental and clinical settings (1–10). Expression of HSV-TK in tumor cells results in tumor cell death when the cells are exposed to the antiviral prodrug GCV as the TK protein phosphorylates the prodrug into a toxic nucleotide analogue, which terminates strand elongation during DNA replication. A powerful feature of the TK/GCV approach is that the transduction of a small fraction of the targeted cells can result in significant antitumor activity (1–10). This has been ascribed to the killing of untransduced neighboring cells and is referred to as the bystander effect (1). It has been demonstrated that the cell-to-cell transfer of phosphorylated GCV via gap junctions between HSV-TK-transduced cells and neighboring uninfected cells is the primary mechanism for the bystander effect (11–15).

In an attempt to enhance the tumoricidal activity of TK/GCV suicide gene therapy, we have created a replication-defective HSV-1 vector expressing the human TNF-α gene in conjunction with HSV-TK. TNF-α has been demonstrated to possess an array of antitumor activities, including potent cytotoxicity exerted directly on tumor cells (16, 17), enhancement of the expression on tumor cell surfaces of HLA antigens (18) and intercellular adhesion molecule 1 (19) and on lymphocytes of interleukin-2 receptors (20), and promotion of the activation of such effector cells as NK cells, lymphokine-activated killer cells and CTLs (21–23). Yet, despite this promising antitumor profile, the clinical use of TNF-α has been constrained by the toxicity of systemic TNF-α administration (24, 25). The possibility exists, however, that this limitation can be overcome by the use of gene transfer methods in which vector-mediated local production of TNF-α at the site of tumor growth may allow for effective use of this cytokine as an antitumor agent without toxicity to other tissues.

In this study, we assessed the effectiveness of treating tumors with our HSV-1 vector expressing both TNF-α and HSV-TK. We present evidence from cell culture as well as in vivo experiments that the efficacy of HSV-TK suicide gene therapy can be increased by the simultaneous expression of TNF-α.
cycles of freeze-thawing (−80°C/37°C) and a single burst of sonication. The virus was then separated from cell debris by centrifugation and purified away from free protein and membrane particles by density gradient centrifugation (OptiPrep; Life Technologies, Inc.). Virus stocks were aliquoted into 2-ml cryogenic tubes (Corning Glass Works, Corning, NY) and stored at −80°C. Final titers averaged 5 × 10^8 to 5 × 10^9 pfu/ml.

**Construction of Plasmids and Recombinant Vectors.** Plasmids were isolated and grown in *Escherichia coli* strain DH5α (Life Technologies, Inc.). Plasmid-transformed bacteria were selected on YT agar plates (YT is 5 g of yeast extract, 5 g of NaCl, and 8 g of bacto-tryptone per liter distilled water, pH 7.0) containing 150 μg/ml ampicillin (Sigma Chemical Co., St. Louis, MO) and grown in 2× YT broth containing 150 μg/ml ampicillin.

Plasmid pBS-TNF contains a human TNF-α cDNA expression cassette flanked on both sides by HSV ICP22 sequences (a BamHI-PstI fragment, genomic position 131,398-133,372, and a HindIII-KpnI fragment, genomic position 133,465-134,787; Ref. 26). The TNF-α cDNA (ATCC) was under transcriptional control of the human CMV IE promoter (Fig. 1). pBS-TNF was used to recombine the TNF-α expression cassette into the ICP22 locus of viral vector THZ.1 using a procedure described previously (26). The insertion of the expression cassette was confirmed by Southern blotting, and the new vector (TH:TNF) was purified by three rounds of limiting dilution. Both TH:TNF and the parental THZ.1 are deficient for the HSV genes ICP4, ICP22, ICP27, and U24, with ICP22 of THZ.1

**Southern Blotting.** BamHI-digested viral DNAs were electrophoresed on 1% agarose gels in TAE buffer, blotted to Nytran membrane (Schleicher and Sciulli, Keene, NH), cross-linked in a UV Stratalinker 2400 (Stratagene, La Jolla, CA), and probed with [α-32P]CTP (DuPont NEN, Wilmington, DE) radiolabeled probes specific for the human TNF-α gene (a 0.6-kb XbaI-ClaI coding fragment) and the HSV-1 ICP22 gene (Accl-SalI fragment; HSV-1 genomic position 133,097-133,377).

**TNF-α Production by Infected Vero Cells.** Vero cells were infected with TH:TNF or THZ.1 at an MOI of 3.0. Supernatants from infected cultures were collected every 24 h for 7 days, and fresh medium was added each time. Samples were assayed for TNF-α using a commercial ELISA kit (Genzyme, Cambridge, MA). All assays were performed in triplicate.

**In Vitro Cytotoxicity Assays.** The cytotoxic effects of TH:TNF infection on cultured L929 mouse fibrosarcoma cells and U-87 MG human glioblastoma cells were examined using a standard MTT assay (28). Cells were infected in suspension for 1 h at MOIs of 0.1 and 3 and dispensed into 96-well, flat-bottomed microtiter plates (Becton Dickinson, Franklin Lakes, NJ) at a concentration of 1 × 10^5 cells (100 μl/well). The cells were incubated for 6 days with or without GCV (10 μg/ml) at 37°C in 5% CO2. On days 2, 4, 6, and 8 postinfection, 25 μl of MTT solution (5 mg/ml) was added to each well, and incubation at 37°C in 5% CO2 was continued for 30 min. Supernatants were removed, and 100 μl of DMSO were added to each well. After another 30-min incubation, absorbances were read by an EL312e plate reader using a 570-nm filter (Biotek Instruments, Winooski, VT).

**Western Blotting.** U-87 MG cells were infected with TH:TNF, THZ.1, or wild-type virus (KOS) at an MOI of 3.0 or 0.1 and incubated for 24 h at 37°C in 5% CO2. Cells were washed with PBS, lysed with lysis buffer (20 mM Tris (pH 7.2), 0.1% SDS, 0.1% Triton X-100, 10 mM phenylmethylsulfonyl fluoride, and 10 μg/ml leupeptin), and sonicated, and the protein concentrations of the lysates were determined. Forty μg of each lysate were resuspended in Laemmli buffer, and the samples were electrophoresed on a 10% SDS-polyacrylamide gels. Proteins were transferred to a nitrocellulose membrane and probed with a rabbit anti-HSV-TK polyclonal antibody (29).

**In Vivo Experiments.** All tumor experiments were carried out with female athymic nude mice (Hsd nu/nu), 7 weeks of age, purchased from Harlan Sprague (Indianapolis, IN).

**L929 Tumor Experiments.** Tumors were generated by intradermal implantation of 1 × 10^5 L929 mouse fibrosarcoma cells in the right flank. When the tumors reached 5 mm in diameter, treatment with TH:TNF or THZ.1 was started. Tumors were injected with 5 × 10^6 pfu intratumorally nine times at 48-h intervals, and 50 mg/kg GCV were administered i.p. every 24 h for 17 days (2, 8, 10). Tumor sizes were measured daily using a caliper, and animals were sacrificed when tumor area reached 2 cm^2. Each treatment group consisted of four animals assigned randomly.

**U-87 MG Tumor Experiments.** Intracerebral tumors were generated by stereotactic injection of 1 × 10^6 U-87 MG cells into the right frontal lobe (3 mm to the right of the midline, 1 mm caudal from bregma, 3 mm in depth from the dura; Ref. 30). Tumors were treated by injecting 1 × 10^6 pfu of vector in the same location 3 days after tumor implantation. Fifty mg/kg GCV were administered i.p. every 24 h for 10 days (2, 8, 10). Animals were monitored until death. Treatment groups consisted of 6–13 animals assigned randomly.

For the assessment of tumor size and vector transduction and to perform general histology, two mice per group (chosen at random) were killed, and cryostat sections (8 or 40 μm) of the brains were prepared and mounted on glass slides.

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![Fig. 1. A schematic representation of replication-defective recombinant HSV-1 vectors TH:TNF (ICP4-α, ICP27-α:CMVp-TNF-α, ICP22-α:CMVp-lacZ, ICP27-α, U24-α:ICP4p-α) and THZ.1 (ICP4-α, ICP22-α:CMVp-TNF-α, ICP27-α, U24-α:ICP4p-α). The top line illustrates the common backbone of the two viruses, which differed only in the ICP22 locus enlarged underneath. Repeat regions are illustrated at the top as open boxes, the tk locus as a checked box, and IE genes inactivated by deletion (ICP4 and ICP27) or insertion (ICP22) as boxes with a cross. U24 and U3 are the unique long and short regions, respectively, of HSV-1. In the illustrations underneath, the two transgenes (TNF-α cDNA and lacZ) are depicted as dark shaded boxes with the direction of transcription indicated by arrows, the human CMV promoter (CMVp) as a hatched box, the bovine growth hormone polyadenylation region as a light shaded box, and the interrupted ICP22 gene as an open box. B, BamHI map of the ICP22 region of TH:TNF and (C) Southern blot comparison of BamHI-digested THZ.1 and TH:TNF DNAs. Molecular size markers are indicated at the side of the Southern blot (in kb). The probes were a 380-bp AciI-SalI HSV-1 fragment (map position 133,097–133,377; ICP22 probe) and a 600-bp Clal-XbaI TNF-α cDNA fragment (TNF probe). The lacZ expression cassette of THZ.1 had no BamHI sites, and the fragment detected by the ICP22 probe is 4890 bp in size.

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Immunohistochemistry. Brains were fixed by perfusion with 4% paraformaldehyde and resected. Cryostat sections were prepared on poly-L-lysine slides, rinsed with 1% H2O2 in methanol and with PBS, and stained with a rabbit anti-HSV-1-antigen antibody (Accurate, Westbury, NY; 1:2000 dilution) in 5% normal goat or rabbit serum overnight at 4°C. The sections were washed three times with 0.3% Triton-X in PBS (PBS-T), incubated with biotin-conjugated goat anti-rabbit for 2 h at room temperature, and again washed three times with PBS-T. The sections were then incubated with avidin-biotin complex (ELITE kit; Vector, Burlingame, CA; 1:400 dilution) for 1 h at room temperature, washed with PBS-T three times, stained with 0.1% diaminobenzidine tetrahydrochloride (DAB substrate kit; Vector), washed with tap water, and counterstained with hematoxylin.

RESULTS

TH:TNF Vector Construction. A replication-defective genomic HSV-1 vector for coexpression of HSV-TK and the tumoricidal cytokine TNF-α was constructed by recombining a TNF-α expression cassette into the ICP22 locus of a previously described lacZ expression vector, THZ.1 (Fig. 1A, TH:TNF). The parental THZ.1 had the following features: (a) it was deleted for the two essential IE genes ICP4 and ICP27 and was therefore highly defective for early (E) and late (L) gene expression and incapable of productive infection; (b) it contained a lacZ expression cassette interrupting and thereby inactivating a third IE gene, ICP22; and (c) it had a modified tk locus in which the native E promoter (as well as the U24 gene encoded on the opposite DNA strand) had been replaced by the ICP4 IE promoter (ICP4p) to express TK as an IE gene product (27). It has been reported previously that combined IE gene deficiencies such as those of THZ.1 substantially reduce the undesirable cytotoxicity of wild-type HSV-1 and mutant vectors deleted for single IE genes (26), which results in extended transgene expression (26). The TNF-α expression cassette used for recombination consisted of a human TNF-α cDNA between the human CMV IE promoter (CMVp) and a polyadenylation region from the bovine growth hormone gene and was surrounded by ICP22 sequences in a plasmid background. The TNF-α cassette was introduced in place of the resident lacZ cassette of THZ.1 by homologous recombination using an efficient protocol described previously (26), and the structure of the final TH:TNF isolate was confirmed by Southern blot analysis (Fig. 1B).

The TH:TNF vector was tested for expression of TNF-α by infection of noncomplementing Vero cells and determination by ELISA of the amounts of TNF-α produced during successive 24-h periods over 7 days. As illustrated in Fig. 2A, medium (1 ml) collected after the first day contained 13.6 ng of TNF-α, but this was followed by a precipitous decline in production on day 2 and subsequent days (1–2 ng/24 h), and the protein was no longer detectable by day 7.

The bioactivity of TNF-α produced in this experiment was tested by exposure of cultured TNF-α-sensitive L929 fibrosarcoma cells to medium collected after the first day of infection. As measured by standard MTT viability assay, L929 cells treated with medium from either mock-infected or THZ.1 control-infected Vero cells showed no significant reduction in viability relative to untreated L929 cells (Fig. 2B). In contrast, a dramatic reduction was observed for cells treated with medium from TH:TNF-infected cells, and this reduction was essentially identical over time to that seen with uninfected medium supplemented with 10 ng of recombinant TNF-α protein (Fig. 2B). These results demonstrated that TNF-α produced by the TH:TNF vector was biologically active and comparable in specific activity to recombinant TNF-α.

Enhanced Killing of TNF-α-sensitive Cells in Vitro by TH:TNF and GCV. To determine whether vector-directed production of TNF-α could enhance HSV-TK/GCV-mediated cell killing of a TNF-α-sensitive cell line, L929 cells were infected at low multiplicity (MOI, 0.1) with TH:TNF or parental vector THZ.1, and the cells were maintained in the presence or absence of GCV. At 24-h intervals, cell viability was determined by MTT assay, and the results were plotted as the percentage of survival relative to mock-infected cells (Fig. 3A). THZ.1-infected cultures exposed to GCV displayed a 40% reduction in viability 3 days after infection, compared with <20% in the absence of GCV. Survival of TH:TNF-infected cultures was ~50% but was reduced to 35% in the presence of GCV. Hence, the combination of HSV-TK/GCV and TNF-α augmented the cytotoxic efficiency of each treatment alone.

The common observation that GCV treatment kills more cells than are actually infected with virus, producing activating TK protein, is referred to as the bystander effect. The bystander effect is thought to result from intercellular spread of activated GCV from infected cells to uninfected neighboring cells via gap junctions (11–15) and is therefore best observed at low MOI. For example, exposure of THZ.1-infected cells to GCV (Fig. 3A) reduced cell survival by nearly 25%, although <10% of the cells were infected with the nonreplicating vector. Likewise, the survival of TH:TNF-infected cultures was reduced by >15% in the presence of GCV, suggesting that the bystander effect was operational in these cultures as well and that cell killing by activated GCV was additive with the cytotoxic effect of TNF-α expression.

Fig. 3B shows the results of a second experiment performed at a 10-fold higher MOI, where ~70% of the cells are expected to be infected. A bystander effect was not obvious here for either THZ.1- or TH:TNF-infected cells. GCV reduced the viability of THZ.1-infected cells by 30%, an amount similar to that observed for the earlier MOI of 0.1. The TH:TNF-infected culture was obliterated in 2 days, but there was no evidence that GCV accelerated this process because the survival curves with and without GCV were essentially the same. These results suggested that combination treatment could be advantageous over single gene treatment at lower MOIs, although not at higher MOIs, which was encouraging because the level of infection attainable with nonreplicating vectors in vivo is generally low. Moreover, limited infection without viral spread should avoid the majority of undesirable side effects associated with sustained TNF-α expression at higher levels.

Enhanced Killing in Vitro by TH:TNF and GCV of a TNF-α-resistant Glioblastoma Cell Line. Unlike L929 cells, most human tumors are resistant to TNF-α-receptor-dependent cell killing, including those that arise in the central nervous system (31, 32). It was of
interest, therefore, to determine whether vector-directed TNF-α expression could enhance the killing by HSV-TK/GCV of U-87 MG cells, a well-defined human glioblastoma cell line which in our hands is resistant to TNF-α concentrations of as high as 100 ng/ml. Accordingly, U-87 MG cells were infected at low MOI, and viability with or without exposure to GCV was determined by MTT assay.

In the absence of GCV, cultures infected with TH:TNF at an MOI of 0.1 showed no significant reduction in viability compared with mock- or THZ.1-infected cells (Fig. 4A), which was consistent with the established TNF-α resistance of these cells. In the presence of GCV, the viability of THZ.1-infected cultures was reduced by 50% 6 days after infection, suggesting a robust bystander effect. Surprisingly, cultures infected with TH:TNF and exposed to GCV showed a substantially larger decrease in viability (90% by day 6; Fig. 4A; P < 0.01, Student’s t test). Although the mechanism of this enhancement is unclear, this observation indicated that the combination of vector-directed TNF-α expression and HSV-TK expression with GCV treatment could be beneficial not only against TNF-α-sensitive tumor cells like L929 but also against TNF-α-resistant tumors.

The preceding result could be a consequence of TK- and/or GCV-mediated sensitization of the cells to TNF-α or vice versa, TNF-α-mediated potentiation of the bystander effect. To distinguish between these alternatives, we tested whether TNF-α protein added to the growth medium would reduce the viability in the presence of GCV of U-87 MG cells infected with THZ.1 at an MOI of 0.1. The results showed that TNF-α added in this manner (10 ng/ml) had no significant effect (Fig. 4B), indicating that the increased cell killing by TK:TNF/GCV compared with THZ.1/GCV was dependent on intracellular production of TNF-α by the vector and was not mediated by secreted TNF-α or through sensitization of the cells to extracellular TNF-α by HSV-TK/GCV. The immunoblot of Fig. 4C demonstrates that there were no significant differences in the amounts of HSV-TK produced by U-87 MG cells infected with TK:TNF or THZ.1 at high or low MOI, which excluded the possibility that vector-produced TNF-α increased cell killing by raising the level of TK protein.

Interestingly, we observed that TH:TNF infection at high MOI caused a dramatic reduction in U-87 MG cell viability in the absence of GCV (Fig. 4D). Because these cells are resistant to high levels of extracellular TNF-α, this must also be due to intracellular TNF-α produced by the vector, suggesting that the two effects, increased killing at low MOI in the presence of GCV and at high MOI in the absence of GCV, may be manifestations of the same mechanism.

\[ \text{S. M., unpublished results.} \]
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TNF-α AND HSV-iK COMBINATION GENE THERAPY

FIG. 4. A, survival of TH:TNF- and THZ.1-infected U-87 MG cells in the presence or absence of GCV. Cells were infected at an MOI of 0.1, and survival was measured relative to untreated mock-infected cells, as before; bars, SD. B, TNF-α protein added to the medium of THZ.1-infected cells failed to reproduce the effect of TH:TNF infection on cell survival in the presence of GCV. U-87 MG cells were infected at an MOI of 0.1, and TNF-α was added at 10 ng/ml where indicated, with or without GCV. The graph shows survival 5 days after infection (averages of three determinations); bars, SD. C, immunoblot showing HSV-TK expression in infected U-87 MG cells. Cells were infected with TH:TNF (Lanes 1 and 4), THZ.1 (Lanes 2 and 5), or wild-type virus (KOS; Lanes 3 and 6) at MOIs of 3.0 or 0.1, and lysates were prepared 24 h later for electrophoresis of equal amounts of protein (40 μg/lane) on a 10% SDS-polyacrylamide gel, electroblotting, and reaction with a polyclonal HSV-TK-specific antibody (38). The lower level of HSV-TK expression in Lane 3 compared with Lanes 1 and 2 can be attributed to several causes, including the early HSV-rt promoter used for expression by KOS virus versus the immediate-early ICP4 promoter used by the two other viruses. D, TH:TNF infection of TNF-α-resistant U-87 MG cells at high MOI was cytotoxic in the absence of GCV. Cells were infected at an MOI of 3.0, and relative survival with or without exposure to GCV was determined as before. Unlike TH:TNF infection at low MOI (see A), infection at high MOI reduced cell survival dramatically; bars, SD.

HSV-positive cells were detected in tumors injected with medium (Fig. 7B). These observations would suggest that enhanced tumor control by TNF-α expression in the context of HSV-TK/GCV therapy may be demonstrable in this TNF-α-resistant brain tumor model only if a higher level of infection can be achieved. At the same time, it is noteworthy that HSV-TK/GCV therapy alone was beneficial in this model despite the limited spread of the virus, which can be attributed to the bystander effect.

DISCUSSION

It has been shown previously that suicide gene therapy can be an effective methodology for killing tumor cells in culture and in vivo (1–10). For example, the survival of rats implanted with tumor-generating 9 L gliosarcoma cells was prolonged 1.5 times or more by HSV-TK/GCV therapy, and some rats were cured completely (4, 7–10). However, the results of clinical trials indicate a need for greater efficacy. In a recent trial, 10 glioblastoma patients were treated by intratumoral delivery of the TNF-α and tk genes. We evaluated the ability of this vector to kill TNF-α-sensitive and TNF-α-resistant tumor cell lines in vitro and corresponding solid tumors in vivo in comparison with a vector that expressed only TK.

In experiments with the TNF-α-sensitive tumor cell line L929, we found that coexpression of HSV-TK with TNF-α resulted in greater cell killing in the presence of GCV than expression of TK alone. This trend was also observed in allograft experiments with athymic nude mice where established intradermal L929 tumors were treated by direct vector injections into the tumor mass. These experiments demonstrated that animals treated with the TH:TNF vector plus GCV had significantly longer survival times than animals in any of the control groups, including animals treated with parental HSV-tk vector plus GCV, and the results indicated that the combination of two tumor-killing mechanisms provided an additive effect. In this experimental tumor model, TNF-α could be released locally from transduced tumor cells and be taken up by neighboring tumor cells, providing an additional bystander effect to that of TK-activated GCV.

Because tumors are often resistant to exogenous TNF-α, it was of practical interest to examine the response of a representative TNF-α-resistant tumor cell line, U-87 MG, to infection with TH:TNF. In addition, these experiments would test the assumption that the effect of vector-derived TNF-α in the L929 experiments was mediated by interaction of secreted TNF-α with cell surface receptors. Surprisingly, we found that TH:TNF infection with exposure to GCV showed a significant increase in cell killing compared with THZ.1/GCV (90% versus 50% cell killing). We confirmed that this effect was independent
of cell surface receptor-mediated signaling by demonstrating that the addition of purified recombinant TNF-α to the culture medium of THZ.1-infected cells failed to increase cell killing. These results indicated that the intracellular production of TNF-α increased cell death due to activated GCV. We are presently exploring the underlying mechanism, which could involve, for example, increased formation of gap junctions between tumor cells, allowing for increased transmission of activated GCV, or inhibition of the activation of NF-κB, resulting in downregulation of the antipapoptotic pathway of the cell (33-35).

Unlike the earlier results with L929 cells, the additive effect of combination treatment observed with U-87 MG cells in vitro could not be duplicated in vivo. Independent inoculation of intracerebral U-87 MG glioblastomas with the TH:TNF and THZ.1 vectors accompanied by regular injections of GCV clearly prolonged the survival of the host animals significantly, but a meaningful difference between the two vectors was not observed. Because we had found that vector-directed TNF-α expression was short-lived in Vero cells, we determined whether repeated vector injections would differentiate between the TH:TNF and THZ.1 treatments. Regardless of whether the vectors were injected once or three times on alternate days, the differences in the results for the two vectors remained statistically insignificant. Whereas GCV treatment is able to reach uninfected cells that do not express the viral tk gene (bystander effect), the observation that TNF-α must be expressed intracellularly to enhance the killing of U-87 MG cells by activated GCV in vitro suggested that the benefits of TNF-α expression may be limited to infected tumor cells. We reasoned, therefore, that inefficient infection could explain the absence of a clear difference in vivo between TH:TNF and THZ.1. Indeed, we found by immunohistochemical staining of treated U-87 MG tumor sections that <1% of the tumor cells expressed HSV-1 antigens, which could be due in part to limited viral diffusion inside tumors. Hence, a more efficient procedure for vector delivery is required to establish whether TNF-α coexpression with HSV-TK is beneficial against TNF-α-resistant tumors. At the same time, eradication of solid tumors by any gene-therapy protocol alone is not a realistic goal at present, and the greater promise in this area may lie in postsurgical treatment to eliminate malignant cells left behind in the periphery; following surgery, the target cell population is much reduced in size and presumably more accessible to vector delivery. Accordingly, experiments are now in progress to compare our vectors under these conditions.

Local production of TNF-α is likely to have additional benefits for antitumor therapy by mechanisms other than direct tumor cell killing:

(a) Extracellular TNF-α damages endothelial cells and will cause thrombosis in the tumor vasculature when applied locally (36). This sensitizes the vasculature to radiation-induced collapse, and local production of TNF-α by virus-infected tumor cells therefore has the potential to enhance the efficiency of radiation treatment (37, 38). We have stained TH:TNF-injected U-87 MG tumor sections with humanspecific anti-TNF-α antibodies and observed a more widespread distribution of positive signals than with anti-HSV-1 antibodies; the specificity for human TNF-α was confirmed by the observation that the TNF-α antibodies failed to stain brain tumors injected with THZ.1 (data not shown). By indicating secretion and diffusion of vector-derived TNF-α, these results suggest that infection of even a fraction of the tumor could benefit the efficiency of radiation therapy. Moreover, vector-directed local TNF-α production should offer a clear advantage over systemic administration because it would prevent the severe side effects (e.g., hypotension, fever, diarrhea, and others) known to accompany systemic TNF-α delivery (24, 25).

(b) TNF-α-transduced cells can support the induction of antitumor immunity (39). For example, dendritic cells collected by leukapheresis from peripheral blood have been shown to efficiently mature in the presence of TNF-α and granulocyte/macrophage colony-stimulating factor (40), and TNF-α expression by autologous tumor cells can therefore increase the abundance of antigen-presenting cells required for sensitization of CTLs. Nonimmune antitumor responses involving activated NK and lymphokine-activated killer cells can also be induced by locally produced cytokines such as TNF-α (41),
and these activated cells have been shown to be effective in destruction of a variety of tumors in animal models (41). Whether NK cells played a role in our tumor models has not been determined, but inflammatory cells were observed at the site of virus injection using immunohistology. Thus, vector-directed TNF-α expression may be able to both increase local inflammation and to recruit and activate antigen-presenting cells that may induce antitumor immunity. Further preclinical studies using syngeneic tumor models where both direct tumor cell killing and the induction of specific immunity can be evaluated should be useful in determining whether the combination of TNF-α and tk gene therapy will provide enhanced effectiveness for the treatment of TNF-α-resistant tumors and by what mechanisms.

ACKNOWLEDGMENTS

We thank Richard Rouse and Kate Sullivan of the Department of Molecular Genetics and Biochemistry and Makiko Hartman of the Department of Neurology for technical assistance.

REFERENCES

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