Intratumoral 5-Fluorouracil Produced by Cytosine Deaminase/5-Fluorocytosine Gene Therapy Is Effective for Experimental Human Glioblastomas

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ABSTRACT

5-Fluorouracil (5-FU) is a potent antimetabolite used for chemotherapy of gastrointestinal (GI), breast, and head and neck malignancies. Although clinical trials have been conducted, the poor therapeutic index of 5-FU has precluded its clinical use for a number of other tumor types. It is unclear whether this lack of utility is due to problems with drug delivery or inherent insensitivity. Adenovirus (Ad) vector-mediated cytosine deaminase (CD)/5-fluorocytosine (5-FC) gene therapy has the potential to overcome pharmacokinetic issues associated with systemic 5-FU and is particularly well suited to use with tumors in which local control is paramount, such as recurrent, localized prostate cancer and malignant gliomas. In this study, the in vitro response by a panel of human tumor cell lines derived from both GI (colon, pancreas) and non-GI (prostate, glioma) tumors to 5-FU and to AdCMVCD (an Ad encoding Escherichia coli CD)/5-FC was examined. Whereas the sensitivity (IC50) of individual cell lines to these agents varied, no significant difference in median IC50 for either 5-FU or AdCMVCD/5-FC was evident for the four tumor types tested (P > 0.1). The relevant contributions of Ad gene transfer efficiency and inherent 5-FU sensitivity in determining response to AdCMVCD/5-FC were then assessed. Multiple linear regression analysis revealed that whereas both factors significantly contribute to the response, inherent 5-FU sensitivity was substantially more important (β = 0.78 versus 0.48; P < 0.001). Finally, the therapeutic efficacy of a single intratumoral injection of AdCMVCD followed by systemic 5-FC was assessed in three intracranial C6/B7 severe combined immunodeficient mouse models of human glioma. AdCMVCD/5-FC efficacy was specific, virus dose-dependent, and closely paralleled in vitro 5-FU and CD/5-FC sensitivity in two of three models tested. These results reveal that glioma cells are as sensitive as GI tumor cells to the antineoplastic effects of 5-FU, identify inherent 5-FU sensitivity as an important factor in determining CD/5-FC efficacy, and confirm previous findings in rat models that demonstrate the potential clinical utility of AdCMVCD/5-FC gene therapy for gliomas.

INTRODUCTION

5-FU, first described in 1957 (1), remains an essential component of chemotherapy for a number of solid tumors, particularly GI, breast, and head and neck malignancies. Overall, the single-agent response rate of 5-FU in GI tumors is 10–20% (2). 5-FU has also been actively investigated during the last 40 years for many non-GI tumors. However, the role of systemic 5-FU in therapy of these tumors has been limited by the fact that dose-limiting toxicities (myelosuppression and stomatitis) are usually reached before evidence of antitumor response. Potential explanations for this low therapeutic index include pharmacokinetic factors limiting accessibility of systemic 5-FU to tumor cells and inherent insensitivity of tumor cells to 5-FU.

Intratumoral delivery of genes encoding prodrug-activating enzymes, an approach termed GDEPT (3) or molecular chemotherapy (4), has the potential to become a powerful alternative method of drug delivery. In particular, the GDEPT combination of CD (EC 3.5.4.1) and 5-FC may be used to circumvent the pharmacokinetic limitations of systemic 5-FU. In this system, the nontoxic antifungal metabolite 5-FC is deaminated to the potent antitumor agent 5-FU within tumor cells expressing CD. Murine studies with CD-conjugated monoclonal antibodies (5), encapsulated CD protein (6), or tumor cells engineered to express the CD gene (7) have shown that high intratumoral concentrations of 5-FU, with minimal diffusion back into circulation, may be attained on conversion of systemically administered 5-FC.

The CD/5-FC GDEPT system likely to be used clinically involves intratumoral delivery of CD via viral vectors (VDEPT). We have demonstrated previously that intratumoral injection of an Ad encoding Escherichia coli CD (AdCMVCD) into mice bearing s.c. colon (8) and bile duct (4, 8) tumors followed by systemic 5-FC administration results in significant suppression of tumor growth. CD/5-FC VDEPT is logical because systemic 5-FU chemotherapy is standard of care for these tumors. However, GI tumors, particularly colorectal carcinomas, are frequently metastatic on initial presentation (9). With current vector technology, CD/5-FC VDEPT is best suited to local control of cancers that are easily accessible to intratumoral vector injection. Examples of such non-GI tumors include cutaneous SCCHN (10), recurrent and/or hormone-refractory localized prostate cancer (11), and gliomas (12). With the exception of SCCHN, 5-FU is not routinely used in the clinical management of these tumors. It is not clear whether this is due to an inherent lack of sensitivity or, alternatively, to poor drug delivery. In this report, we address both of these important issues in determining the potential clinical utility of interstitial 5-FU in such tumors. To address the former, the in vitro sensitivities of a panel of 14 human cell lines derived from both GI (colon, pancreas) and non-GI (prostate, glioma) tumors to 5-FU and to AdCMVCD/5-FC were quantified. To address the latter, the relative importance of Ad gene transfer efficiency versus inherent 5-FU sensitivity in determining AdCMVCD/5-FC toxicity in vitro was estimated using multiple linear regression analysis.

Finally, we chose gliomas as a representative non-GI tumor system for which (a) direct intratumoral vector injection is likely to be used clinically and (b) 5-FU is not routinely used. The CD/5-FC system has been previously investigated in preclinical models of glioma (6, 13–19). All of these studies used either rat C6 glioma cells injected intracranially in Wistar rats, an allogeneic model (20), or rat 9L gliosarcoma cells injected intracranially in syngeneic Fisher 344 rats or s.c. in xenogeneic nude mice. Whereas most of these studies used cell lines stably transfected to express CD, two investigated intratumoral injection of Ad vectors encoding CD (15, 18), and two used a fusion gene encoding CD and herpes simplex virus thymidine kinase (16, 18). Although comparisons of results with such diverse models

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3 The abbreviations used are: 5-FU, 5-fluorouracil; Ad, adenovirus; CAR, Coxsackie adenovirus receptor; CD, cytosine deaminase; CMV, cytomegalovirus; 5-FC, 5-fluorocytosine; GDEPT, gene-directed enzyme-prodrug therapy; GFP, green fluorescent protein; GI, gastrointestinal; ILS, increased length of survival; MOI, multiplicity of infection; pfu, plaque-forming unit; SCCHN, squamous cell carcinoma of the head and neck; SCID, severe combined immunodeficient; UPRT, uracil phosphoribosyltransferase; VDEPT, virus-directed enzyme-prodrug therapy; XRT, radiation therapy; GFP, green fluorescent protein; b.i.d., bis in die (twice a day); CNS, central nervous system; BCNU, carmustine; FDA, Food and Drug Administration.

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with different transgenes and produrg treatment regimens are difficult to make, CD/5-FC therapy, in general, produced significant tumor volume reduction (s.c.) or ILS (intracranial) in each model. Both C6 and 9L rat glioma models have been frequently used for experimental therapeutic studies. However, these models suffer from a number of problems that limit extrapolation to the clinical situation, most notably the lack of a syngeneic host (C6) and an intense antitumor immune response even in the absence of therapy [C6 and 9L (20)]. Orthotopic xenograft SCID mouse models of human glioma do not suffer from these limitations, permitting evaluation of experimental therapeutics in the absence of tumor immunogenicity. Thus, this is the first report to assess the therapeutic efficacy of CD/5-FC VDEPT in intracranial C.B17 SCID mouse models of human glioma using three human glioma cell lines displaying different genetic, morphological, and phenotypic profiles. Data presented provide the rationale for further evaluation of CD/5-FC-based VDEPT for human gliomas in clinical trials.

MATERIALS AND METHODS

**Tumor Cells, Animals, and Chemicals.** Human glioma cell lines D54MG, U87MG, U251MG (obtained from Darel Bigner; Duke University Medical Center, Durham, NC) and U118MG (American Type Culture Collection, Manassas, VA) were cultured in DMEM/F12 (Mediatech, Herndon, VA) containing 7% fetal bovine serum (Summit Biotechnology, Fort Collins, CO) and 2% glutamine. Human colon (LS174T and WiDr), pancreatic (AsPC-1, BxPC-3, and MIA PaCa-2), and prostate (DU145, LNCaP, and PC-3) carcinoma cell lines (American Type Culture Collection) were maintained in RPMI 1640 (Mediatech) containing 10% fetal bovine serum and 2% glutamine. All cells were cultured at 37°C in a 5% CO2 atmosphere without antibiotics and passed <12 times during the course of these experiments. C.B17 SCID mice were purchased from the Frederick Cancer Research Facility (Bethesda, MD) and housed under aseptic conditions in microisolator cages with sterile food and water ad libitum. 5-FC and 5-FU were purchased from Sigma Chemical Co. (St. Louis, MO) and SP Pharmaceuticals (Albuquerque, NM), respectively.

**Viruses and Monoclonal Antibodies.** AdCMV5GFP, a first-generation E1-, E3-deleted vector expressing GFP from the CMV immediate early promoter, was obtained from Corey Goldman (Cleveland Clinic, Cleveland, OH). The construction and characterization of AdCMVCD (4) and AdCMVhSSTR2 (21), an Ad encoding human somatostatin receptor subtype 2, have been described previously. All viruses were constructed by two-plasmid rescue in 293 cells using pACCMVpLPA shuttle vector and pJM17 rescue vector (4). Viruses were propagated and plaque titrated on permissive 293 cells and purified twice by centrifugation on CsCl gradients. All virus aliquots were maintained at ~80°C until use. Single lots of AdCMVCD, AdCMV5GFP, and AdCMVhSSTR2 were used throughout these experiments. The AdCMVCD lot proved free of contaminating wild-type Ad after infection of nonpermissive cells (A549 and HeLa; data not shown) and neurotoxicity after injection of up to 109 pfu into the brains of non-tumor-bearing C.B17 SCID mice as described below.

**5-FU and AdCMVCD/5-FC Toxicity.** Confuent cell monolayers were harvested with 0.25% trypsin/EDTA, plated (5000 cells/well in 100 µl of complete media) in 96-well tissue culture plates, and allowed to adhere overnight at 37°C. Ten serial half-log dilutions of 5-FU (six replicates/dilution) were made, and 100 µl of 5-FU-containing media were added directly to cells to achieve final concentrations of 0–200 µM per ml. Cells were incubated at 37°C for 5 days, and cellular respiration was determined using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Promega, Madison, WI) as per the manufacturer’s protocol. Fractional cell survival at each drug concentration was measured as the ratio of absorbance of 490 nm of cells incubated in the presence versus absence of drug, corrected for background absorbance of media alone. Fractional cell survival data were plotted against the logit of drug concentration and IC50 values extrapolated by piecewise linear regression as the concentration of drug producing a 50% reduction in corrected absorbance.

For AdCMVCD/5-FC toxicity experiments, cells were harvested from confluent monolayers and plated at 1.5–3 × 104 cells/well in T25 flasks (Conning, Corning, NY) overnight at 37°C. Cells were infected with varying amounts of AdCMVCD (0–300 pfu/cell) in 2 ml of OptiMEM (Life Technologies, Inc., Gaithersburg, MD) for 1 h at 37°C with continuous rocking. Twenty-four h after infection, cells were harvested with 0.25% trypsin/EDTA, plated into 96-well tissue culture plates at 5000 cells/well in 100 µl of complete media, and allowed to adhere overnight at 37°C. One hundred µl of media supplemented with serial dilutions of 5-FC were added, and 5-FC toxicity (IC50) was determined at 5 days as described above.

**Quantification of Ad Transducibility.** Ad gene transfer efficiency was quantified by monitoring expression of GFP in AdCMV5GFP-infected cells as described previously (22). Briefly, cells (0.5–1 × 105) were plated in 6-well plates (Corning), allowed to adhere overnight, and subsequently infected with AdCMV5GFP at various MOIs (0–500 pfu/cell) in 0.6 ml of OptiMEM for 1 h at 37°C with continuous rocking. Twenty-four h after infection, cells were harvested with 0.25% trypsin/EDTA (Mediatech), washed with buffer (PBS, 0.1% sodium azide, and 0.1% BSA), and resuspended at 1–105 cells/ml. Cells (104 cells/sample) were analyzed by flow cytometry at the University of Alabama at Birmingham Department of Rheumatology FACS Core Facility. MOI50 values, defined as the AdCMV5GFP MOI required to produce detectable GFP in 50% of cells, were determined for each cell line by a piecewise linear regression of data plotted as the logarithm of AdCMV5GFP MOI versus the percentage of GFP-positive cells.

**AdCMVCD/5-FC Treatment of C.B17 SCID Mice Bearing Intracranial Human Gliomas.** U87MG, D54MG, or U251MG tumors were established intracranially in C.B17 SCID mice as described previously (23–25). Briefly, mice were anesthetized with ketamine, and 0.5–1 × 106 cells (1 × 106 ml–1) were inoculated into the right frontal cerebral hemisphere via stereotactic injection. Tumors were allowed to grow for 5 days before stereotactic injection of 10 µl of saline, AdCMVCD, or AdCMVhSSTR2 (109 pfu). Two days after infection, mice were treated with either saline or 500 mg/kg 5-FC b.i.d. i.p. for 7 days and monitored daily for survival. When tumor-bearing mice displayed overt signs of neurological dysfunction, manifested primarily as a hunched appearance, lack of grooming, and lack of avoidance behavior when handled, they were sacrificed by lethal CO2 inhalation, and their brains were harvested for histopathological examination, confirming the presence of tumor in all sacrificed mice.

**Statistics.** Pairwise comparisons of mean 5-FU or 5-FC IC50 values calculated as described above were made using one-way ANOVA with Fisher’s modification using MINITAB v13.3 for Windows (Ministat, State College, PA). Median 5-FU and 5-FC IC50 values for each tumor type were calculated by grouping cell lines according to tissue of origin and compared by Kruskal-Wallis analysis using MINITAB. Pairwise comparisons of median IC50 values were performed with Dunn’s post-test using GraphPad InStat v3.05 (GraphPad, San Diego, CA). Multiple linear regression analysis on log-converted 5-FU IC50 and 5-FC IC50, and AdCMV5GFP MOI50 values was performed using GraphPad InStat v3.05. Assessment of the relative importance of each variable was made using the β standardized regression coefficient (26). Kaplan-Meier survival curves were analyzed by the log-rank test using GB-STAT v6.5 for Macintosh (Dynamic Microsystems, Silver Springs, MD), and specific pairwise comparisons were made. All comparisons were made using the 0.05 level of significance, unless otherwise stated.

**RESULTS**

**5-FU Sensitivity.** Sensitivity to continuous exposure of 5-FU was assessed for 14 cell lines derived from malignant gliomas and colon, pancreatic, and prostate carcinomas and was found to be variable, within a 2-log range (Fig. 1; IC50 range, 0.07–1.44 µg/ml). Cells could be grouped into three categories based on statistical analysis of the individual mean 5-FU IC50 for each cell line. Group I, consisting only of BxPC-3 pancreatic carcinoma cells, was most sensitive (IC50 = 0.07 ± 0.03 µg/ml). Group III, consisting of MIA PaCa-2 pancreatic carcinoma cells and U87MG and D54MG glioma cells, was the least sensitive (IC50 = 1.24–1.44 µg/ml). The largest group of cells, Group II, displayed intermediate 5-FU sensitivity. Only the difference in mean IC50 values for group I versus group III cells was statistically significant (P < 0.05). A notable exception was LS174T...
colon carcinoma cells, which were significantly more sensitive to 5-FU than were U87MG and D54MG glioma cells ($P < 0.05$).

**AdCMVCD/5-FC Sensitivity.** 5-FU sensitivity (IC$_{50}$ in $\mu g/ml$) was assessed with a subpanel of 11 cell lines after infection with AdCMVCD at 1, 10, 30, 100, and 300 MOI (pfu/cell). Dose-response curves from representative pancreatic, glioma, and colon cancer cell lines (AsPC-1, D54MG, and WiDR, respectively) are shown in Fig. 2. A significant linear relation ($P < 0.01$) between AdCMVCD MOI and 5-FU sensitivity (log-log scale) for each of the three cell lines was observed. Similar results were obtained with BxPC-3 and LS174T cells as described previously (27). The dose response with PC-3 and U251MG cells was not significantly linear ($P > 0.05$) despite sufficient data for analysis (three and four MOIs, respectively). Only two MOIs (100 and 300 MOI) were tested for four cell lines (DU145, LNCaP, U118MG, and U87MG), precluding accurate analysis of the linear relation with these cell lines (data not shown).

Like results with 5-FU, the 5-FC responses in the subpanel of cell lines infected with AdCMVCD at 100 pfu/cell were variable within a 2-log range (Fig. 3; IC$_{50}$ range, 1.0–196 $\mu g/ml$). Statistical analysis of mean 5-FC IC$_{50}$ for each individual cell line revealed two groups with similar responses. Group II was comprised of three cell lines that were significantly less sensitive than group I cells ($P < 0.05$): U118MG and U87MG glioma and AsPC-1 pancreatic cells (IC$_{50}$ = 83, 96, and 196 $\mu g/ml$, respectively). The remaining eight cell lines comprised group I (IC$_{50}$ range, 1.0–18.7 $\mu g/ml$).

**Tumor-type Response to 5-FU and AdCMVCD/5-FC.** Median 5-FU toxicity (IC$_{50}$) varied according to tumor type: 0.30, 0.37, 0.32, and 1.42 $\mu g/ml$ for colon, pancreatic, prostate, and glioma cell lines, respectively (Fig. 1). Similarly, response to 5-FC based on infection with AdCMVCD (100 pfu/cell) varied because the median 5-FC IC$_{50}$ under these infection conditions was 4.1, 99.8, 4.2, and 53.2 $\mu g/ml$, respectively (Fig. 3). Kruskal-Wallis analysis revealed no significant difference in either the median 5-FU IC$_{50}$ ($P = 0.10$) or the 5-FC IC$_{50}$ ($P = 0.24$) for the four cell types tested. These results were also independent of infection conditions because no difference in tumor-type response was evident at 10, 30, and 300 pfu/cell AdCMVCD ($P < 0.21$, 0.21, and 0.65, respectively; data not shown).

**Contribution of Inherent 5-FU Sensitivity and Ad Transducibility in Determining AdCMVCD/5-FC Response.** For AdCMVCD/5-FC GDEPT, two factors may influence the cellular response: (a) Ad gene transfer efficiency; and (b) inherent drug sensitivity. These variables, represented as MOI$_{50}$ and IC$_{50}$, respectively, were used to calculate 5-FC sensitivity (IC$_{50}$) after infection of 11 cell lines with AdCMVCD at 100 pfu/cell. Multiple regression analysis was performed on log-converted 5-FC IC$_{50}$ AdCMVGFP MOI$_{50}$ and 5-FC IC$_{50}$ values, and coefficients were determined for the equation shown below.

$$\text{Log}(5-\text{FC IC}_{50}) = b_0 + b_1 \times \text{Log}(5-\text{FU IC}_{50}) + b_2 \times \text{Log(AdCMVGFP MOI)}$$ (1)

This model described the data well (Table I; $P < 0.001$) and revealed that both AdCMVGFP gene transfer efficiency and inherent 5-FU sensitivity significantly contributed to overall 5-FC response after AdCMVCD infection. However, inherent 5-FU sensitivity was the more important factor of the two, as demonstrated by a $b_1$ $\beta$ weight of 0.78 compared with $b_2$, $\beta$ weight of 0.48 for Ad transducibility. Finally, as shown in Fig. 4, 5-FC IC$_{50}$ predicted by the above equation correlated strongly with measured 5-FC IC$_{50}$ ($P = 0.0001$).

**Intratumoral 5-FU Chemotherapy of Established Intracranial Gliomas in C.B17 SCID Mice.** The dose response of intratumoral 5-FU chemotherapy via AdCMVCD/5-FC was assessed in an estab-
two additional intracranial C.B17 SCID mouse models of human glioma (24). AdCMVCD/5-FC therapy significantly prolonged survival of mice bearing either intracranial D54MG tumors [Fig. 6B; median survival, 43 days versus 27 days (control); P = 0.002] or U251MG tumors [Fig. 6C; median survival, 35 days versus 22 days (control); P = 0.001]. This effect was dependent on AdCMVCD/5-FC because no prolongation of survival with control virus (AdCMVhSSTr2 ± 5-FC) and control drug (AdCMVGFP + saline) regimens was seen with either model (P > 0.05). Importantly, the magnitude of survival advantage in AdCMVCD/5-FC-treated animals differed among the three models. As shown in Table 2, the ILS for U87MG, D54MG, and U251MG tumors was 1.67, 2.25, and 1.59, respectively. With the exception of results with U251MG, the treatment response correlated well with the in vitro sensitivity of these cells to both 5-FU and AdCMVCD/5-FC (Figs. 1 and 3, U251MG > D54MG > U87MG).

**DISCUSSION**

This study addresses three important issues regarding CD5-FC VDEPT. First, data presented in Figs. 1 and 3 demonstrate that cell lines derived from both GI and non-GI tumors display similar in vitro sensitivity to both 5-FU and AdCMVCD/5-FC on continuous 5-day drug exposure using the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxy-methoxy-phenyl)-2-(4-sulfonyl)-2H-tetrazolium assay of cellular proliferation. Nonparametric analysis of median 5-FU (Fig. 1) and AdCMVCD/5-FC. As shown in Fig. 5, a single intratumoral injection of 10^9 pfu AdCMVCD followed by a twice daily, 7-day course of i.p. 5-FC at 500 mg/kg significantly prolonged median survival of mice bearing U87MG tumors (42 days) as compared with that of mice treated with intratumoral saline plus 5-FC (31 days; P < 0.05). Mice receiving 10^9 pfu AdCMVCD with systemic 5-FC lived significantly longer (61 days) than both control, saline/5-FC-treated mice (P < 0.01) and mice receiving 10^8 pfu AdCMVCD/5-FC (P < 0.05).

The specificity of the AdCMVCD/5-FC therapeutic effect was determined with mice bearing intracranial U87MG tumors and treated with either saline, AdCMVCD, or the control virus AdCMVhSSTr2 (10^8 pfu/mouse), followed by systemic saline or 5-FC as described above. As shown in Fig. 6A, a single intratumoral injection of 10^9 pfu AdCMVCD significantly prolonged the survival of C.B17 SCID mice bearing intracranial U87MG tumors compared with control, mock-infected animals treated with 5-FC (median survival, 67 days versus 49 days, respectively; P < 0.05). This effect was not due to AdCMVCD infection alone because survival of mice receiving 10^9 pfu AdCMVCD but no 5-FC was not significantly prolonged compared with that of control mice (median survival, 49.5 days; P > 0.22). The prolongation of survival was specific for AdCMVCD/5-FC because infection with AdCMVhSSTr2, which encodes the human SSTr2 (21) and does not induce CD activity in infected cells (data not shown), did not significantly prolong survival of mice in either the presence (53.5 days) or absence (46 days; data not shown) of 5-FC (P > 0.4).

The potential clinical efficacy of this approach was assessed in

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* ILS of animals receiving 10^9 pfu of AdCMVCD with systemic 5-FU (300 mg/kg b.i.d. i.p. for 7 days) versus animals receiving no AdCMVCD plus 5-FU. 5-FC regimen started 2 days after virus infection. ILS was determined from one to two separate experiments per tumor with 7–11 animals/group.

5-FC regimen started 3 days after virus infection.
MVC/D/5-FC (Fig. 3) sensitivity ($IC_{50}$) of 14 tumor cell lines (2 colon, 5 pancreatic, 4 glioma, and 3 prostatic cell lines) revealed no significant differences among the four tumor cell types tested ($P = 0.1$ and 0.24, respectively). A similar analysis was performed on publicly available 5-FU toxicity data from the National Cancer Institute Developmental Therapeutics Program Disease-oriented Anticancer Drug Screen (28). The National Cancer Institute screens approximately 10,000 compounds/year using a sulforhodamine B protein biomass assay to assess acute drug toxicity (2 days) with a panel of >60 human tumor cell lines from 9 different tissues (breast, CNS, colon, lung, leukemia, melanoma, ovarian, prostate, and renal). Analysis of data in the August 2000 database revealed no significant difference in median 5-FU GI50 (50% growth inhibition) values ($P = 0.17$; data not shown) for the nine different tissues. This analysis confirms that our results with 5-FU were not an artifact of the cell lines included in our panel, the toxicity assay used, or the time point analyzed. These results are consistent with the broad spectrum of activity demonstrated clinically with systemic 5-FU and suggest that intratumoral 5-FU may be active, with optimal drug delivery via CD/5-FC GDEPT, for both GI and non-GI tumors.

CD/5-FU VDEPT is a two-component system designed to produce high intratumoral 5-FU concentrations from systemically administered 5-FU. Both CD expression level within infected tumor cells and inherent 5-FU sensitivity of the tumor cells may contribute to its overall efficacy. Thus, the second focus of this study was to determine the relative importance of these two factors in vitro. We have previously shown that AdCMVCD MOI correlates directly with CD expression (27). To our knowledge, these data are the first to demonstrate a clear dose-response relationship between AdCMVCD dose and 5-FU toxicity.

Multiple linear regression analysis confirmed that both Ad gene transfer efficiency and inherent 5-FU sensitivity contributed significantly to AdCMVCD/5-FU toxicity in vitro (Table 1) and that quantification of these two factors could accurately predict 5-FU toxicity (Fig. 4). We have previously reported a facile method of quantifying Ad transduction efficiency by flow cytometry using AdCMVGFP that permits the statistical comparison of transduction efficiencies across different cell types, represented as a MOI50 value for each cell sample (22). We chose the method using AdCMVGFP for these studies, rather than AdCMVCD, due to its simplicity (direct autofluorescence) and its amenability to subsequent statistical analysis. However, differences in transcription and translation efficiencies of different transgenes within the same viral vector and driven from the same promoter may produce differing results. Preliminary experiments involving quantitation of AdCMVCD transduction efficiency using flow cytometric detection of CD by indirect immunofluorescence on a subset of the cell lines used in this study have demonstrated the concordance of these two methods (27, 29), suggesting that GFP is a suitable surrogate reporter gene for these studies.

These data also illustrate the importance of Ad gene transfer efficiency in determining CD/5-FU VDEPT efficacy. Ad gene transfer has been shown to depend on expression of the cellular receptors necessary for Ad entry, CAR (30, 31) and $\alpha_v$ integrins (32), on the surface of target cells. Lack of expression of CAR or $\alpha_v$ integrins on primary tumor cells from different tissues significantly affected Ad gene transfer (33–35). Moreover, in animal models, intratumoral Ad gene transfer is limited to the area around the needle track, presumably due to high interstitial pressure within the tumor that limits vector diffusion (36). Therefore, to maximize intratumoral CD expression and hence CD/5-FU efficacy, targeted Ad vectors capable of CAR-inde-
pended gene transfer (37), replicative Ad vectors capable of lateral infection throughout the tumor mass (38), or a combination of the two (39) may be used in future Ad-based CD vectors to overcome these problems.

Whereas both Ad gene transfer efficiency and inherent 5-FU sensitivity contributed significantly to CD/5-FC VDEPT efficacy in vitro, inherent 5-FU sensitivity was the more important factor ($\beta = 0.48$ versus 0.78, respectively; Table 1). There is, therefore, no irony in the fact that CD/5-FC GDEPT has primarily been investigated in preclinical models of both GI [bile duct (4), colon (8), and pancreas (40)] and non-GI [breast (41) and SCCHN (42)] malignancies for which systemic 5-FU has a clearly defined role in clinical management (2). However, these tumors, particularly colon and breast cancers, are frequently metastatic on initial presentation (9, 43). Whereas systemic CD/5-FC has been investigated for treatment of metastatic disease (44), current Ad-based VDEPT technology is most likely to be efficacious on direct injection into a tumor mass (45) or surgically voided tumor cavity (46). Thus, it is better suited to use in low-stage disease, where local control is possible, such as cutaneous SCCHN (10) or recurrent and/or hormone-refractory localized prostate cancer (11), or in high-grade tumors that display locally invasive growth and rarely metastasize, such as gliomas (12).

Based on the demonstrated efficacy of CD/5-FC VDEPT in animal models of GI tumors and the equivalent 5-FU in vitro sensitivity profile of both GI and non-GI tumor cells, the above-mentioned studies provided the rationale for further evaluation of CD/5-FC VDEPT in tumors not traditionally treated with systemic 5-FU. CD/5-FC VDEPT is particularly well suited to the treatment of gliomas because 90% of these tumors recur within 2 cm of the resection margin and rarely metastasize outside the CNS (47). Gliomas remain one of the more therapeutically intractable types of cancer; the median survival is <12 months even with surgery, XRT, and chemotherapy (48). Whereas the roles of cytoreductive surgery and external beam XRT are established in the clinical management of newly diagnosed and recurrent gliomas, the value of adjunctive systemic chemotherapy is less well defined. BCNU, a lipophilic, non-phase-specific alkylating agent, is the most commonly used systemic chemotherapeutic and, until 1999, was the only FDA-approved drug for malignant gliomas (49). Prolongation of patient survival by nitrosourea-based mono- and poly-chemotherapy has been modest, primarily due to the emergence of drug-resistant cells. FDA approval of temozolomide in 1999 for recurrent anaplastic astrocytoma marked the biggest change in neuro-oncology in 20 years (50), but its role in the management of malignant gliomas is far from clear (51). Combination systemic chemotherapy and chemoradiotherapy with BCNU and non-cross-resistant, phase-specific drugs such as 5-FU, 5-bromodeoxyuridine, 5-iododeoxyuridine, hydroxyurea, and other antimetabolites has been investigated in various clinical trials for gliomas over the past 40 years (see review, Refs. 52 and 53). Whereas no clear role for these drugs has been established, antimetabolites remain attractive due to their capacities to potentiate the effects of XRT (54) and to overcome or circumvent BCNU resistance (49). Narrow therapeutic indices, the intact blood-brain barrier at the actively proliferating tumor margin, and the relatively low growth fraction of gliomas have limited the utility of antimetabolites for gliomas and precluded clinical evaluation of their direct antineoplastic effect (49).

Alternative drug delivery approaches may overcome the obstacles associated with systemic 5-FU chemotherapy. Intratumoral chemotherapy has been previously investigated in gliomas, and sustained release, biodegradable BCNU polymers (Gliadel) have recently been approved by the FDA for use in recurrent gliomas (55). This approach has also been attempted for antimetabolites such as 5-FU. Menei et al. (56) recently reported results of a Phase I trial investigating the combination of interstitial 5-FU with concurrent external beam XRT. High levels of 5-FU in the CSF, but not blood, were seen up to 30 days after surgical implantation of biodegradable microspheres containing 5-FU, with minimal neurological side effects. Although only eight patients were studied, there was a distinct trend toward improved local control and prolonged survival in patients receiving high-dose interstitial 5-FU with concurrent XRT.

CD/5-FC GDEPT is an attractive alternative to polymer-based interstitial 5-FU. Unlike 5-FU, 5-FC is amenable to oral administration due to its efficient GI absorption, readily penetrates the blood-brain barrier after systemic administration, and is FDA-approved for CNS fungal chemotherapy (57). Thus, the third aim of this study was to investigate the in vivo efficacy of intratumoral 5-FU/CD/5-FC VDEPT in experimental human gliomas. In all three intracranial C.B17 SCID mouse models, combined intratumoral injection of AdCMVCD with systemic 5-FU significantly prolonged survival ($P < 0.05$). This effect was virus dose dependent (Fig. 5) and specific for the CD/5-FC combination (Fig. 6) because treatment with neither AdCMVhSSTr2 ± 5-FU nor AdCMVCD without 5-FU significantly prolonged survival ($P > 0.05$). Interestingly, the mean ILS in two of three models tested (U87MG and D54MG; Table 2) closely mirrored the in vitro response of these cells to both 5-FU (Fig. 1) and AdCMVCD/5-FC (Fig. 3). Results with U251MG cells were the exception, displaying an intermediate ILS but superior sensitivity to both 5-FU and AdCMVCD/5-FC. Perhaps additional in vivo experiments with this model or modification of the treatment regimen would yield results that correlate more closely with in vitro sensitivity profiles. Alternatively, there may be undetected differences in the growth profiles of U251MG cells in vitro and in vivo that may explain the differences in sensitivity. Future testing of AdCMVCD/5-FC therapy in multiple different tumor models, such as pancreatic and prostate cancer, should permit a more definitive evaluation of this correlation.

A recent study by Lambin et al. (58) quantified the therapeutic gain that may be obtained with CD/5-FC GDEPT and concurrent fractionated XRT. Based on published pharmacokinetic data for 5-FC, these authors suggested that, assuming only 1–3% local CD conversion efficiency, maintenance of serum 5-FU at 25–100 $\mu$g/ml (standard antifungal treatment values) may permit intratumoral 5-FU production of 0.6–0.9 $\mu$g/ml, a level previously shown to achieve radiosensitization values of 1.1–1.2 in vitro (59). This level of sensitization, when combined with 60 Gy of fractionated XRT (2 Gy/fraction), might increase local tumor control 20–40%. This level of increased local control may be detected in a randomized, two-arm clinical trial involving 260 or 60 total patients, respectively (60). Data in Fig. 1 demonstrate that the mean 5-FU IC$_{50}$ values for three of four human glioma cell lines tested were in this range (1.17–1.44 $\mu$g/ml), and the mean 5-FU IC$_{50}$ for the other cell line, U251MG, was even lower (0.35 $\mu$g/ml). 19F magnetic resonance spectroscopy studies are currently under way to assess intratumoral CD conversion efficiency on intratumoral AdCMVCD infection. In addition, concurrent CD/5-FC VDEPT and fractionated XRT studies with these cell lines and tumor xenografts are currently ongoing to directly assess the potential gain of this approach for experimental malignant gliomas.

GDEPT is also potentially more flexible than polymer delivery systems because additional genes may be included to potentiate the GDEPT effect. For example, we and others (61–63) have conducted preclinical GDEPT studies to investigate the combination of CD and UPRT, an enzyme that catalyzes production of (5-fluoro)UMP in the first step of the (fluoro)uracil salvage pathway and is down-regulated in most 5-FU-resistant fungi (57). Coexpression of CD and UPRT has been shown to increase the toxic effect of 5-FC by 1–3 orders of magnitude over treatment with CD/5-FC alone (61–63). Preclinical studies with CD/UPRT-based
VDEPT and combination chemotherapy (BCNU, hydroxyurea, irinotecan, and leucovorin) and chemoradiotherapy are currently ongoing to maximize the potential of CD/5-FC GDEPT for malignant gliomas. Such manipulation of downstream 5-FU metabolism and modulation of its cytotoxic effects via adjunctive therapies should improve efficacy because inherent 5-FU sensitivity was shown to be the more important factor in Ad-mediated CD/5-FC GDEPT (Table 1).

This study represents the first study to use CD/5-FC VDEPT in experimental human gliomas and demonstrates the efficacy of intratumoral 5-FU produced via a single intratumoral injection of AdCMVCD and a 7-day b.i.d. regimen of systemic 5-FC (500 mg/kg). These efficacy results compare favorably with other reports of CD/5-FC GDEPT and suggest that future protocol optimization of viral injection (fractionated dosing), 5-FC regimen (prolonged administration), and concurrent fractionated external beam XRT may be even more efficacious, providing the rationale for further investigation of this approach via clinical trials. This study is also the first to systematically evaluate VDEPT in multiple murine models of the same tumor type using cell lines displaying distinct genetic, morphological, and phenotypic properties. Results demonstrate that response to CD/5-FC VDEPT is variable and that both Ad gene transfer efficiency and inherent 5-FU sensitivity are critical factors in determining tumor response. Most importantly, these findings are likely to reflect the variable responses to CD/5-FC VDEPT that may be encountered clinically in human tumors that are even more heterogeneous.

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Intratumoral 5-Fluorouracil Produced by Cytosine Deaminase/5-Fluorocytosine Gene Therapy Is Effective for Experimental Human Glioblastomas

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