Diffusible Cytotoxic Metabolites Contribute to the in Vitro Bystander Effect Associated with the Cyclophosphamide/Cytochrome P450 2B1 Cancer Gene Therapy Paradigm

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ABSTRACT

Tumor cells become sensitive to the inert prodrug cyclophosphamide (CPA) after transfer of the gene encoding cytochrome P450 2B1. This enzyme activates CPA into 4-hydroxycyclophosphamide, which ultimately degrades into acrolein and phosphoramidomustard, the antitumor and DNA-alkylating metabolite. It is imperative that any prodrug-activating gene therapy strategy designed to prevent cancer possess the capacity to affect the proliferation of tumor cells even when they do not express the transgene (bystander effect), because current methodologies cannot achieve gene transduction in all tumor cells. Prodrug-activating gene therapy schemes designed to date exhibit a bystander effect that is not mediated by conditioned medium in culture and may depend on cell contact. In contrast, we find that CPA sensitized, P450-expressing C6 glioma cells (C6-P450) transfer cytotoxicity to nonexpressing cells by releasing diffusible metabolites through the medium. A 3-h exposure to the prodrug is necessary and sufficient to achieve killing of the transfected cells, and medium conditioned by these cells can kill untransfected cells with similar potency. This bystander effect occurs in the presence of CPA even when only 10% of cells in culture express the P450 2B1 gene, and it is not reproduced by cells that have been irradiated. In an animal model of intracerebral brain tumors, expression of the P450 2B1 gene within the neoplastic cells enhanced significantly the antitumor effect of CPA, even when it was administered systemically. This study shows that CPA/P450 2B1 gene therapy represents a novel tumor-killing strategy that diffuses cytotoxicity to neighboring C6 glioma cells that do not express the CPA-activating P450 2B1 gene product, and that this bystander effect results from diffusible metabolites that are not expressed by the CPA-activating gene.

INTRODUCTION

One major goal of oncological chemotherapeutics is to maximize toxic effects on tumor cells and minimize them on normal cells. A strategy developed recently uses the selective introduction of a chemosensitivity gene into neoplastic cells (1–5). This approach provides a wider therapeutic window for existing antitumor drugs and allows the use of novel drugs not originally designed for cancer therapy.

We have recently shown that C6 glioma cells, genetically engineered to express the hepatic-specific enzyme cytochrome P450 2B1, acquire sensitivity to the prodrug CPA (5). This prodrug normally requires bioactivation by liver-specific enzymes of the cytochrome P450 family (such as cytochrome P450 2B1) to exert its antitumor function (6). The primary cytochrome P450 metabolite of CPA, 4-HCPA, is unstable and spontaneously decomposes into two hydrophilic metabolites, PM and acrolein. PM produces DNA alkylation, resulting in strand breaks during DNA replication. Acrolein promotes the formation of covalent links in cellular proteins, but it is not a major factor in the cytotoxicity of CPA toward tumor cells in vitro (7). Unlike antimetabolites, the cytotoxicity of PM and acrolein is not restricted to a particular phase of the cell cycle, rendering CPA and other alkylating agents particularly suited for the treatment of solid tumors (8), in which a large proportion of the neoplastic cells are not in the S-phase (9).

The antitumor effects of prodrug-activating gene therapies, such as the ones that use ganciclovir/HSV-TK, 5-fluorocytosine/Escherichia coli cytosome deaminase (3), and 6-thioxanthine/E. coli gpt (4), are amplified by the bystander effect, which sensitizes neighboring tumor cells that do not themselves express the prodrug-activating gene (1, 10–18). The most widely studied bystander effect is that associated with ganciclovir/HSV-TK, and, at least in vitro, this mechanism appears to involve the transfer of toxic metabolites through cell-to-cell contacts, perhaps across gap junctions (14, 16). Experimental evidence shows that the medium conditioned by prodrug-activating cells does not participate in the bystander effect associated with the ganciclovir/HSV-TK gene therapy strategy (13). On the contrary, because CPA usually is activated by hepatic cytochrome P450 enzymes into metabolites that can diffuse for relatively long distances, one would expect that a gene therapy strategy that uses the CPA-activating P450 2B1 gene would exhibit a bystander effect that is qualitatively different from that associated with ganciclovir/HSV-TK. In fact, in this study, we show that cytotoxicity occurs in neighboring C6 glioma cells that do not express the CPA-activating P450 2B1 gene product, and that this bystander effect results from diffusible metabolites that are...
transmitted through the medium from P450-expressing to P450-nonexpressing tumor cells. We also show, in an intracerebral tumor model, that expression of the P450 gene leads to a significant enhancement of the antitumor effect of CPA, even when the prodrug is administered systemically. These findings show that the intratumoral generation of the cytotoxic metabolites of CPA and their ability to diffuse into the extracellular environment can provide an enhanced anticancer effect in the setting of a prodrug-activating gene therapy strategy.

MATERIALS AND METHODS

Chemicals and Cell Lines. CPA was purchased from Sigma Chemical Co. The cell lines C6-Neo and C6-P450 were generated by transfection of rat C6 glioma cells (19) with plasmids bearing the neomycin phosphotransferase gene and the cDNA for rat cytochrome P450 2B1, as described (5). The cell line 9L-P450 was generated by transfecting rat 9L gliosarcoma cells with the same plasmids. The cytochrome P450 used is the most active in metabolizing CPA of the 12 rat liver P450 enzymes tested (6). Cells were grown in DMEM with high glucose (catalogue no. 10–013-LM; CELLGRO) supplemented with 10% FCS, 100,000 units/liter penicillin, and 100 mg/liter streptomycin (Sigma) in a 5% CO2 incubator.

Colony Formation Assay. For colony formation assays, cells were plated at a density of 1000 cells/10-cm dish in triplicate. The cloning efficiency was approximately 25% for control cultures. The next day, 0.5 mm CPA was added, and incubations were carried out for 6 days. Cells were then rinsed once with Hank’s buffered saline (GIBCO-BRL) and stained with Giemsa (Fisher Diagnostics), and colonies larger than 1 mm in diameter were counted.

Cell Proliferation Assays. For cell proliferation assays, cells (2 × 105/dish, unless otherwise noted) were plated onto 10-cm dishes. After 24 h, CPA was added to a final concentration of 0.5 mm. Incubations were continued for 4 days, and cell numbers were assayed using a Coulter counter (Coulter Electronics, Inc.), after harvesting in trypsin-EDTA.

CPA Pulse and Washout Assay. To determine the temporal kinetics of CPA-mediated cytotoxicity, 2 × 106 cells were plated onto each 10-cm dish. After 24 h, 0.5 mm CPA was added, and cells were incubated for the times indicated in Figs. 1–4. Cells were then trypsinized and replated at a density of 2 × 105 cells/10-cm dish. Nine days later, cell numbers were evaluated by Coulter counting.

Coculture Assay. Cocultures of C6-P450 and C6 cells were incubated in triplicate at various ratios (where 0, 10, 50, 90, and 100% of the cells were C6-P450 cells) to achieve a total number of 2 × 106 cells/10-cm dish. After 24 h, 0.5 mm CPA was added, and 4 days later, cells were counted as described previously. To determine the specificity of the cell-mediated killing effect from the coculture assays, C6 cells were incubated overnight with C6 cells that expressed the neomycin phosphotransferase gene (C6-Neo), with irradiated C6 cells, with irradiated C6-Neo, and with irradiated C6-P450 cells. γ-Irradiation was performed by exposing cells to a total of 6000 rads emitted by a 51Cr source. Cells exposed to this level of radiation did not proliferate but, instead, remained attached to tissue culture dishes for 7 days before detaching and losing viability.

Conditioned Medium Assay. To assess the effect of conditioned medium, cells were cocultured in dishes, as indicated in Fig. 2, using the Falcon insert system (Fisher Scientific). This system involves the use of tissue culture dishes (diameter, 3.5 cm) that contain micropore membranes (pore diameter, 0.45 μm) that physically separate cell populations plated into the upper and lower chambers but permit the exchange of components in the medium. C6 cells (3.4 × 105 cells) were plated into the bottom chamber, and C6-P450 (3.4 × 105 cells) or C6-Neo cells (3.4 × 105 cells) were plated into the upper chamber (on top of the filter) in a total volume of 5 ml medium. After an overnight incubation, 0.5 mm CPA was added to this medium. Four days later, the membrane on which the C6-P450 or C6-Neo cells were growing was removed, and then the number of C6 glioma cells in the lower chamber was determined by counting with a Coulter apparatus. Surviving C6 cells were then replated at a density of 2 × 105 cells/10-cm dish without CPA. Nine days later, these cells were trypsinized and counted using the Coulter apparatus.

Animal Studies. All animal studies were conducted according to the institutional guidelines promulgated by the Massachusetts General Hospital Committee on Animal Care (Charlestown, MA). 9L or 9L-P450 cells were trypsinized, counted, washed in HBSS, and resuspended at a density of 20,000 cells/μl in DMEM. Cells (2 μl) were inoculated stereotactically into the frontal lobes of Fischer 344 rats (weight, 200 g; three rats/group), as described (18). Three days later, CPA (100 mg/kg) was administered either as an i.p. injection (dissolved in 0.5 ml saline) or as an i.v. injection (dissolved in 0.5 ml saline). At the 21-day time point (19 days after CPA administration), rats were killed as described (18). Brain tumors were cryosectioned into 50-μm slices (every sixth slice was saved) and stained using cresyl violet staining, as described (18). Tumor volumes were calculated by multiplying the following formula: average tumor area (in mm2) × number of slices in which tumor is present × 0.3 (in mm).

RESULTS

Effect of CPA on the Proliferation of C6 Cells Expressing the Cytochrome P450 2B1 Gene. The generation of cell lines C6-P450 (designated as C450–8 previously) and C6-Neo (designated as CNeo-1 previously) has been described (5). These cell lines were derived from C6 glioma cells stably transfected with the rat cytochrome P450 2B1 gene and the neomycin phosphotransferase gene, respectively. Fig. 1A shows that C6, C6-P450, and C6-Neo glioma cells proliferate at similar rates in the absence of CPA. However, in the presence of 0.5 mm CPA, there was selective and complete growth inhibition of C6-P450 cells over the course of 10 days.

Death of Cells Expressing the Cytochrome P450 2B1 Gene Occurs within Hours of Exposure to CPA. To investigate the time course of the killing action of CPA, a pulse and washout assay with CPA was performed: 2 × 106 cells were exposed for various time periods to 0.5 mm CPA (pulse period). The cells were then washed and trypsinized to remove excess...
Fig. 1 Cell proliferation assays of C6, C6-Neo, and C6-P450. A, the proliferation rates of C6, C6-Neo, and C6-P450 cells are shown in the absence of CPA over a 10-day time course; B, the same experiment was performed in the presence of CPA (0.5 mM). ■ C6-P450 cells; □ C6 cells; ●, C6-Neo cells. A total of $2 \times 10^6$ C6 or C450-8 cells were plated onto 10-cm dishes in triplicate. The next day, 0.5 mM CPA or medium was added to all dishes. At each time indicated, cells were trypsinized and counted. Points, means; bars, SE.

Table 1 CPA pulse and washout assay

<table>
<thead>
<tr>
<th>CPA pulse and washout assay</th>
<th>Time after addition of CPA (h)</th>
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<tr>
<td></td>
<td>0.5</td>
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<tr>
<td>C6</td>
<td>11.6 ± 0.4</td>
</tr>
<tr>
<td>C6-P450</td>
<td>12 ± 1</td>
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* C6 or C6-P450 cells ($2 \times 10^6$) were plated onto 10-cm dishes in duplicate. After 24 h, 0.5 mM CPA was added to each dish. At the indicated time, cells were trypsinized and replated at a density of $2 \times 10^5$ cells/10-cm dish in fresh medium in triplicate. After 9 days, cells in each dish were trypsinized and counted. 

prodrug and/or metabolites (washout) and replated at a density of $2 \times 10^6$ cells/10-cm dish. Cells were counted 9 days later. Table 1 shows that a 3-h pulse of CPA was sufficient to completely inhibit the growth of the replated C6-P450 cells. Proliferation of the replated parental C6 glioma cells was unaffected by CPA, even after a 96-h period of drug exposure. This demonstration that the killing effect of CPA on cells is complete within 3 h indicates that CPA is converted rapidly within the C6-P450 cells into its cytotoxic metabolite(s).

**Bystander Effect.** We sought to determine the presence of a bystander effect in the CPA/cytochrome P450 2B1 gene therapy paradigm. Fig. 2A shows that, when C6 and C6-P450 cells were cocultured so that 10% of cells on a dish expressed the P450 gene, there was a 75% decrease in the proliferation of C6 cells (from $2 \times 10^6$ to $8 \times 10^5$ cells) in response to CPA over 4 days. Because C6-P450 cells do not proliferate in the presence of CPA, cells that are counted in the coculture assay consist of C6 cells. When the percentage of cocultured cells containing the P450 gene was increased to 50%, there was a proportional decrease in C6 cell proliferation in response to CPA, to approximately 85% of control. When the percentage of C6-P450 cells was increased further so that these cells represented 90% of cells on a dish, there was complete inhibition in the proliferation of the remaining C6 cells over a 4-day period of exposure to CPA. We conclude that the process of growing the two cell populations in close proximity to one another provides powerful transcellular toxicity in the CPA/cytochrome P450 2B1 gene therapy paradigm.

In a control study, CPA was added to cocultures in which C6 cells represented 90% of cells in the dish, and the remaining 10% of the cells consisted of the following: (a) C6-Neo cells; (b) irradiated C6 cells; (c) irradiated C6-Neo cells; and (d) irradiated C6-P450 cells (Fig. 2B). It was evident that coculture with C6-Neo cells did not affect the proliferation of C6 cells (Fig. 2B, column 2). It also was evident that killing C6 cells by irradiation did not mediate toxicity on the remaining naive C6 cells (Fig. 2B, column 3). There was a small, but not statistically significant ($P > 0.1$, as determined by Student’s t test), decrease in the proliferation of C6 cells cocultured with irradiated C6-Neo cells (Fig. 2B, column 4). However, there was a statistically significant decrease (30%) in the proliferation of C6 cells cocultured with irradiated C6-P450 cells (Fig. 2B, column 5; $P < 0.05$).
Fig. 2  Bystander effect. A, the proliferation of C6 cells was assayed in the presence of 0.5 mM CPA when 0, 10, 50, 90, and 100% of the cells contained the P450 2B1 gene. The total number of cells/dish at the start of the experiment was 2 × 10^6 cells. Cells from each dish were counted 5 days later. Final values, the average from three plates. Columns, means; bars, SE. B, C6 cells were cultured alone (2 × 10^6 cells; column 1) and in combination with C6 cells that expressed the Neo gene (column 2), irradiated C6 cells (column 3), irradiated C6-Neo cells (column 4), and irradiated C6 cells that expressed the P450 gene (column 5). In columns 2–5, the total number of cells/dish was 2 × 10^6, and C6 cells accounted for 90% of cells in the dish at the start of the experiment. All cells were grown in the presence of 0.5 mM CPA for 4 days. Only the decrease seen in column 5 achieved statistical significance (P < 0.05, as determined by Student’s t test).

Table 2  Diffusion of cytotoxic metabolites

<table>
<thead>
<tr>
<th>C6 exposed to conditioned medium from</th>
<th>Time after addition of CPA (h)</th>
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<tr>
<td></td>
<td>0.5</td>
</tr>
<tr>
<td>C6</td>
<td>247 ± 3^a</td>
</tr>
<tr>
<td>C6-P450</td>
<td>263 ± 6</td>
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</table>

^a Conditioned medium from C6 or C6-P450 cells exposed to 0.5 mM CPA for the indicated times (0.5, 1.5, 3, 6, 10, 24, 32, 48, 72, and 96 h) was harvested and added to plates containing C6 cells plated overnight (1000 cells/dish with clonogenic efficiency of approximately 25%). Colony numbers were determined 6 days later. Colonies greater than 1 mm in diameter were counted. Values represent means ± SE.

^b Number of colonies.

0.05, as determined by Student’s t test) in the presence of CPA. This finding suggested that irradiated C6-P450 cells may be able to activate CPA at levels sufficient to confer toxicity onto neighboring C6 cells.

Transfer of Cytotoxic Metabolites to Naive C6 Cells through Medium Harvested from C6-P450 Cells. The generation of toxic metabolites produced a cytotoxic bystander effect to neighboring cells. We sought to determine whether conditioned medium could transfer the cytotoxic effect of CPA metabolized by C6-P450 cells onto parental C6 cells. Conditioned medium was harvested from C6 or C6-P450 cells exposed to CPA for the time periods shown in Table 2 and was then added to C6 glioma cells to evaluate their colony-forming ability. The results in Table 2 show that a 3-h exposure of C6-P450 cells to CPA was sufficient to generate conditioned medium, which, when added to C6 cells, decreased their cloning efficiency by approximately 45%. The inhibitory activity of this conditioned medium increased with the length of exposure of the C6-P450 cells to CPA. Complete inhibition of C6 cell colony formation was achieved through incubation with medium harvested from C6-P450 cells exposed to CPA for 24 h (Table 2). This finding indicates that CPA-treated C6-P450 cells release soluble toxic metabolites that accumulate in the medium.

To further demonstrate the presence of secreted cytotoxic metabolite(s), C6 and C6-P450 cells (1:1 ratio) were cultured using Falcon coculture inserts, which provide for a physical separation of the two cell types through the use of a micropore filter, yet allow the free diffusion of their supernatants. As a control, C6 and C6-Neo cells (1:1 ratio) were also plated in this configuration. CPA was added to the culture medium, and the number of C6 cells in the lower culture compartment was determined 4 days later. Under these culture conditions, there was an approximate decrease of 50% in the number of C6 cells cocultured with C6-P450 cells in the presence of CPA (Fig. 3A) compared with C6 cells cocultured with C6-P450 cells in the absence of CPA or with C6 cells cocultured with C6-Neo cells. When these C6 cells were stimulated to proliferate by trypsinization and replating at lower density, the toxic effects of
Fig. 3  Mediation of bystander effect by conditioned medium. A, C6 (3.5 × 10⁵ cells) were cocultured with C6 (3.5 × 10⁵ cells; column 1), C6-Neo (3.5 × 10⁵ cells; column 2), or C6-P450 (3.5 × 10⁵ cells; column 3) on a dish separated by a 0.45-μm filter (Falcon insert system) in the presence of 0.5 mM CPA. After 5 days, the number of C6 cells was determined by using a Coulter counter. B, the surviving C6 cells from the previous experiment were trypsinized and replated at a density of 2 × 10⁵ cells/dish. C6 cell numbers were then counted 9 days later. Columns, means; bars, SE.

CPA metabolites generated by C6-P450 cells were accentuated, resulting in an approximate decrease of 90% in C6 cell number (Fig. 3B). Therefore, diffusible cytotoxic factor(s) or metabolites are transferred through the culture medium from P450-positive to P450-negative tumor cells.

**In Vivo Experiments.** To ascertain the in vivo anticancer effect of a CPA/P450 2B1 gene therapy strategy, 9L or 9L-P450 tumors were established in the frontal lobes of Fischer 344 rats. Three days later, saline or CPA was administered by i.p. or i.v. injection. Rats were killed at the 21-day time point, and tumor volumes were determined. Fig. 4 shows that, after CPA administration, there was a reduction in tumor volume in all groups compared with the tumor volumes measured after saline administration. Although the percentage of decrease of 9L-P450 tumor volumes was more pronounced than that of 9L tumor volumes, only i.p. administration of the produg produced a statistically significant difference. These results indicated that the intratumoral enzymatic activation of CPA enhanced significantly the anticancer effect of the produg, even when it was administered systemically.

**DISCUSSION**

**CPA/P450 2B1 Gene Therapy for Cancer.** The insertion of the rat cytochrome P450 2B1 transgene into tumor cells to render them sensitive to the antitumor action of CPA holds promise as a novel therapeutic strategy against tumors (5). We have shown previously that fibroblasts genetically engineered to produce a retrovirus vector that bears the above-mentioned gene will induce tumor regression in animal models of peripheral and brain tumors (5). The major objective of this study were: (a) to evaluate whether the expression of the P450 2B1 gene in tumor cells would also sensitize P450 2B1-negative tumor cells to CPA (bystander effect); (b) to characterize the cellular mechanism(s) that contributed to this bystander sensitization; and (c) to evaluate therapeutic effectiveness in an animal model of intracerebral tumors. The relevance of providing evidence for a bystander effect in a prodrug-activating gene therapy strategy is that current gene therapy techniques do not permit the transfer of a therapeutic gene into all tumor cells. Our findings show the
presence of a bystander effect in the CPA/P450 2B1 gene therapy strategy, the mechanism of which appears to depend primarily on transfer of cytotoxic metabolites from CPA-expressing to CPA-nonexpressing tumor cells through the extracellular environment. Because the CPA metabolite PM does not diffuse efficiently across cell membranes (20), it is likely that the active metabolites in this conditioned medium are the dif fusible ones, i.e., 4-HCPA and its tautometer aldophosphamide.

**Bystander Effect.** The presence of a diffusible bystander effect differentiates the CPA/cytochrome P450 2B1 gene therapy paradigm from the ganciclovir/HSV-TK gene therapy strategy (1, 10–18). In the case of the latter strategy, conditioned medium from ganciclovir-treated tumor cells containing the HSV-TK gene is not cytotoxic to untreated, naive tumor cells (13), although toxic metabolites are thought to be transferred across cell contacts (14, 16). We hypothesize that the formation of diffusible cytotoxic metabolites in CPA/cytochrome P450 2B1 gene therapy will provide a significant therapeutic boost against tumors. Although these metabolites may lead to some undesirable toxicity to normal cells, their exclusive generation within the tumor by directed gene delivery should maximize neoplastic cell killing and minimize deleterious effects on normal cells.

Although the cellular mechanisms that characterize the bystander effect in vitro appear to be relatively straightforward, other mechanisms may be operative in vivo. For instance, immune responses against the tumor that expresses the antigens derived from the producer cells, the viral vector, and/or the foreign gene that is expressed by the virus vector have been implicated in the in vivo bystander effect (13, 21–24). Disruptions in the neovascularity of tumors also have been shown to contribute to in vivo bystander sensitization (25). Ultimately, it is likely that an interplay of these factors will contribute to in vivo tumor regression after viral-mediated therapeutic gene delivery.

It is of interest to note that a small percentage of tumor cells remained attached to the dishes at the end of our in vitro assays. This may reflect the fact that these presumably viable cells may not have been dividing during the assay and, therefore, may have escaped the cytotoxic action of PM. It is probable that, given enough time, these cells would have died as well. Alternatively, this may reflect the development of resistance to the action of PM and/or down-regulation of cytochrome P450 2B1 gene expression.

**In Vivo Significance.** The in vivo findings provide evidence for a statistically significant therapeutic reduction in tumor volume, even when CPA is administered by a systemic route. Because the liver’s ability to metabolize CPA would be far greater than that of a 9L tumor that expresses the P450 2B1 gene, this result might be unexpected. Several hypotheses could be contemplated to explain this finding: (a) 4-HCPA, the activated transport form of CPA, when generated intratumorally, has more time to diffuse into neoplastic cells before decompartmentalization into PM (the hydrophilic anticancer metabolite) compared with 4-HCPA that was generated by the liver; (b) CPA metabolites generated by the liver are not readily available for brain tumor cell uptake and toxicity; and (c) acrolein generated within the tumor may synergize the anticancer effect of PM, whereas acrolein generated in the liver may not. Assays of activated metabolites in 9L-P450 versus 9L tumors might provide insight in the overall contribution of the hepatic versus the neoplastic system in prodrug conversion and aid in explaining the above-described result. Nevertheless, our experimental results clearly indicate that a gene therapy approach might enhance the anti-tumor effect of CPA. We have demonstrated previously a similar benefit for CPA/P450 2B1 gene therapy in a s.c. rat C6 glioma model in athymic mice (5), and, more recently, these results were replicated in a s.c. 9L gliosarcoma model in rats (26). To further maximize an intratumoral therapeutic effect and minimize the hepatic contribution to prodrug metabolism, alternative delivery approaches (carotid artery injection, intratumoral polymer delivery, and intrathecal infusion) will be tested in the future.

Ultimately, the ability to enhance onco logically chemotherapy through the delivery of genes that would allow the intracellular conversion of prodrugs into active drugs should achieve the objective of maximum cytotoxicity for tumor cells with minimal effects on normal cells. The combination of several prod drug gene therapy systems that have different modes of action (e.g., ganciclovir/HSV-TK targets cells in the S-phase, whereas CPA/cytochrome P450 2B1 targets cells in all phases), as well as the expression of cytokines that expand the immune response, such as interleukin 4 (27, 28), granulocyte-macrophage colony-stimulating factor (29), and antisense RNAs that alter tumor cell metabolism, such as insulin-like growth factor 1 (30), should expand the antitumor effectiveness of cancer gene therapy.

**ACKNOWLEDGMENTS**

The excellent technical assistance of M. Chase is acknowledged. We thank Drs. N. W. Kowall and D. F. Siwek (Edith N. Rogers Memorial Veterans Administration Hospital, Bedford, MA) for assistance with tumor sectioning and computerized tumor volume measurements.

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