Intratumoral Activation and Enhanced Chemotherapeutic Effect of Oxazaphosphorines following Cytochrome P-450 Gene Transfer: Development of a Combined Chemotherapy/Cancer Gene Therapy Strategy

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ABSTRACT

Cyclophosphamide and its isomer ifosfamide are cell cycle-nonspecific alkylating agents that undergo bioactivation catalyzed by liver cytochrome P-450 enzymes. The therapeutic efficacy of these oxazaphosphorine anticancer drugs is limited by host toxicity resulting from the systemic distribution of activated drug metabolites formed in the liver. Since tumor cells ordinarily do not have the capacity to activate oxazaphosphorines, we examined whether introduction into tumor cells of a cDNA encoding CYP2B1, a major catalyst of oxazaphosphorine activation, sensitizes the cells to the cytotoxic effects of cyclophosphamide and ifosfamide. Here we show that 9L gliosarcoma cells stably transfected with a cDNA encoding rat CYP2B1 are highly sensitive to cyclophosphamide and ifosfamide cytotoxicity as compared to parental 9L cells or 9L cells transfected with an Escherichia coli β-galactosidase gene. The CYP2B1 enzyme inhibitor metyrapone protects the CYP2B1-expressing 9L cells from oxazaphosphorine cytotoxicity, demonstrating that the chemosensitivity of these cells is a direct consequence of intracellular prodrug activation. Moreover, CYP2B1-expressing 9L cells potentiate the cytotoxic effects of cyclophosphamide and ifosfamide toward cocultured CYP2B1-negative 9L tumor cells. This "bystander effect" does not require cell-cell contact, and therefore may have the therapeutic advantage of distributing cytotoxic drug metabolites to a wide area within a solid tumor mass. In vivo experiments using Fischer 344 rats implanted s.c. with CYP2B1-expressing 9L tumor cells demonstrated that intratumoral expression of the CYP2B1 gene provides a substantial therapeutic advantage over that provided by liver cytochrome P-450-dependent drug activation alone; cyclophosphamide treatment resulted in complete growth inhibition of CYP2B1-positive tumors, whereas only a modest growth delay effect was obtained with CYP2B1-negative tumors. These studies establish that drug-activating CYP genes may be useful for the development of novel combined chemotherapy/gene therapy strategies for cancer treatment utilizing established cancer chemotherapeutic agents.

INTRODUCTION

Conventional chemotherapy aims to kill malignant cells without major toxicity to normal host cells and tissues. Although some notable successes in the treatment of specific tumor types (e.g., childhood leukemias) have been achieved using this approach, more limited success has been obtained in the treatment of solid tumors. This failure is primarily due to the low therapeutic index of many anticancer drugs, as well as to the intrinsic or acquired drug resistance that often characterizes tumor cells in the clinic. To circumvent these difficulties, novel approaches based on the application of the principles of gene therapy to cancer therapeutics have gained wide attention. To overcome these difficulties, novel approaches based on the application of the principles of gene therapy to cancer therapeutics have gained wide attention (1, 2). One therapeutically attractive strategy is based on the direct transfer to the tumor of a drug susceptibility gene, or "suicide gene," which encodes an enzyme that can activate a prodrug intratumorally, thereby rendering the tumor cells sensitive to chemicals which are otherwise nontoxic. One widely studied example is the HSV-TK4 gene, the protein product of which activates the antiviral drug and nucleoside analogue ganciclovir (3, 4). Tumor cells transduced with the HSV-TK gene using retroviral or adenoviral vectors are killed by ganciclovir, while normal host cells, which do not express HSV-TK, are unaffected (5-7). A second example is the bacterial cytosine deaminase gene, which confers chemosensitivity to the relatively nontoxic 5-fluorouracil precursor 5-fluorocytosine (8-10). Although both HSV-TK/ganciclovir and cytosine deaminase/5-fluorocytosine may potentially be useful for cancer gene therapy, ganciclovir was originally introduced into the clinic for treatment of herpes viral infection (11-13), while 5-fluorocytosine is an antifungal drug (14). There are, therefore, few detailed biochemical or pharmacological studies on the application of these drugs in cancer treatment.

The oxazaphosphorines, cyclophosphamide and ifosfamide (Fig. 1), are therapeutically inactive cancer chemotherapeutic prodrugs that must first be activated by liver CYP metabolism to achieve therapeutic effects in cancer patients. Conceivably, CYP gene transfer in combination with cyclophosphamide or ifosfamide treatment could correspond to a useful therapeutic strategy to increase the activity and improve the therapeutic index of this class of chemotherapeutic agents. CYP gene transfer could provide an opportunity to minimize the systemic toxicity associated with conventional oxazaphosphorine treatment, which derives from drug activation in the liver by a CYP-catalyzed 4-hydroxylation reaction (Fig. 1), followed by release into the circulation of activated drug metabolites. Although these activated metabolites can effect tumor regression, their formation is accompanied by inevitable cytotoxic side effects toward critical host tissues such as bone marrow and kidney. Since tumor cells do not normally express significant levels of CYP enzymes, introduction of these enzymes into tumor cells by gene transfer may sensitize the cells to oxazaphosphorines as a consequence of the resultant direct, intratumoral prodrug activation. This could provide a basis for improved anticancer effects using oxazaphosphorine-based chemotherapy.

Recently, the specific liver cytochrome P-450 enzyme catalysts of cyclophosphamide and ifosfamide activation were identified in both rat (15, 16) and human (17) liver. Three specific rat liver cytochrome P-450 enzymes, CYP2B1 (phenobarbital inducible), CYP2C6 (constitutively expressed), and CYP2C11 (constitutively expressed and present only in adult male rats), are major catalysts of cyclophosphamide activation in adult rat liver (15), while a CYP3A enzyme

4 The abbreviations used are: HSV-TK, herpes simplex virus thymidine kinase; CYP, cytochrome P-450; CYP2B1, CYP form 2B1; 9L-Z, 9L cells that stably express E. coli β-galactosidase; 9L-ZP, 9L cells that stably express E. coli β-galactosidase and rat CYP2B1; 9L-F3, 9L-P13 and L450-Z, 9L cells that stably express rat CYP2B1; X-Gal, 5-bromo-4-chloro-3-indolyl-β-D-galactoside.

5 Individual cytochrome P-450 enzymes are designated according to the systematic nomenclature for CYPs (46).
Furthermore, it is not known whether tumor cells can be similarly sensitized to oxazaphosphorine cytotoxicity. Moreover, the extent to which this drug sensitivity can be transferred to adjacent, non-CYP-expressing tumor cells via a bystander effect is unclear. Finally, the therapeutic benefits of this strategy in vivo are uncertain given the very high level of endogenous CYP expression in liver, coupled with the high activity of several liver CYP enzymes with oxazaphosphorine substrates (15–17). The present paper evaluates these important questions through a series of in vitro and in vivo studies based on ex vivo gene transfer using the rat 9L gliosarcoma model. Together, these studies establish the feasibility of this approach and suggest that substantial therapeutic benefits may be achieved by using cyclophosphamide and CYP2B1 in a novel combined chemotherapy/cancer gene therapy strategy.

MATERIALS AND METHODS

**Chemicals.** Cyclophosphamide and ifosfamide were obtained from the Drug Synthesis and Chemistry Branch of the National Cancer Institute (Bethesda, MD). 4-Hydroperoxy cyclophosphamide was obtained from Nova Pharmaceutical Corporation (Baltimore, MD). Metyrapone was purchased from Aldrich Chemical Co. (Milwaukee, WI).

**Stable Transfection of 9L Cells.** Rat 9L gliosarcoma cells (20) were grown in α-MEM (GIBCO-BRL) containing 10% fetal bovine serum, 10 units/ml penicillin, and 10 mg/ml streptomycin. Cells were maintained in a humidified atmosphere of 5% CO2/95% air. 9L cells were co-transfected with a rat CYP2B1 expression plasmid (pMT2-CYP2B1; kindly provided by Drs. Milton Adesnik and Allison Reiss, New York University Medical School) and pCMV-ßgal.Neo (a generous gift from Dr. H. Li, Dana-Farber Cancer Institute) to a molar ratio of 10:1 using Lipofectin (GIBCO-BRL) according to the instructions of the manufacturer. The plasmid pCMV-ßgal.Neo contains a neomycin phosphotransferase gene, which confers resistance to G418, and also the lacZ (β-galactosidase) gene of *Escherichia coli*, which serves as a control and provides a convenient cell marker. Stable transfectants were cloned under selection in 1 mg/ml G418 (GIBCO-BRL). Cell lines resistant to G418 were selected and cloned. Western blot analysis showed a single protein band of approximately 52 kilodaltons corresponding to the molecular mass of purified CYP2B2 in samples prepared from the parental 9L cell line designated 9L-ZP. No CYP2B1 protein was detected in parental 9L cells or in 9L-Z cells, which were shown to express β-galactosidase (X-Gal staining) but not CYP2B1 (Fig. 2; data not shown). The clonal cell lines 9L, 9L-Z, and 9L-ZP were used for further studies. Analysis of CYP2B1-dependent enzyme activities (see "Materials and Methods") confirmed that the CYP2B1 transformant 9L-ZP expresses CYP2B1 protein in an enzymatically active form and at a level corresponding to ~1–2% of that of phenobarbital-induced adult male rat liver, while CYP2B1 activity (testosterone 16β-hydroxylation) was not detectable in parental 9L cells or in 9L-Z cells (data not shown). A similar level of CYP2B1 expression was obtained in several independent 9L/CYP2B1/ß-galactosidase transfectants (9L-ZP, 9L-ZP, 9L-ZP, and 9L-ZP), as well as in several transformants that do not express β-galactosidase (clonal cell lines designated 9L-P3, 9L-P13, and L450-2) (data not shown).

**RESULTS**

**Stable Expression of CYP2B1 Gene in 9L Gliosarcoma Cells.** 9L cells were cotransfected with an expression plasmid encoding rat CYP2B1 and a β-galactosidase expression plasmid containing a neomycin resistance gene in a 10:1 molar ratio. Cell lines resistant to G418 were selected and cloned. Western blot analysis of isolated 9L cell microsomes using a rabbit polyclonal antibody specific to CYP2B showed a single polypeptide band of approximately 52 kilodaltons corresponding to the molecular mass of purified CYP2B1 in samples prepared from the parental 9L cell line designated 9L-ZP. No CYP2B1 protein was detected in parental 9L cells or in 9L-Z cells, which were shown to express β-galactosidase (X-Gal staining) but not CYP2B1 (Fig. 2; data not shown). The clonal cell lines 9L, 9L-Z, and 9L-ZP were used for further studies. Analysis of CYP2B1-dependent enzyme activities (see "Materials and Methods") confirmed that the CYP2B1 transformant 9L-ZP expresses CYP2B1 protein in an enzymatically active form and at a level corresponding to ~1–2% of that of phenobarbital-induced adult male rat liver, while CYP2B1 activity (testosterone 16β-hydroxylation) was not detectable in parental 9L cells or in 9L-Z cells (data not shown). A similar level of CYP2B1 expression was obtained in several independent 9L/CYP2B1/β-galactosidase transfectants (9L-ZP, 9L-ZP, 9L-ZP, and 9L-ZP), as well as in several transformants that do not express β-galactosidase (clonal cell lines designated 9L-P3, 9L-P13, and L450-2) (data not shown).

**Effects of Oxazaphosphorines on Cultured 9L and 9L/ZP Cells.** We first tested whether 9L cells which express CYP2B1 are sensitive to the cytotoxic effects of cyclophosphamide and ifosfamide. CYP2B1-positive (9L-ZP and L450-2) and CYP2B1-negative (parental 9L and 9L-Z) cells were cultured with various concentrations of cyclophosphamide or ifosfamide. The number of viable cells present 5 days after drug treatment was then determined. As shown in Fig. 3A, cyclophosphamide inhibited the growth of CYP2B1-positive cells in a concentration-dependent manner (IC50 = ~70 μM). Growth of...
Western blot analysis of CYP2B1 in parental 9L cells and in 9L cells that stably express CYP2B1. Microsomal proteins prepared from cultured cells (20 μg protein/lane) were electrophoresed on 10% SDS/polyacrylamide gels, transferred to nitrocellulose, and probed with polyclonal rabbit anti-CYP2B1 antibodies as described in “Materials and Methods.” 9L-ZP1 (Lane 2) corresponds to a second clone derived from the same selection as 9L-ZP, expresses CYP2B1, and exhibits an oxazaphosphorine sensitivity very similar to that of 9L-ZP (data not shown). Phenobarbital-induced rat liver microsomes (0.5 or 1 μg; Lanes 5 and 6, respectively) were used as a standard for CYP2B1 (2B1, lower band of doublets in Lanes 5 and 6).

CYP2B1-positive cells was also inhibited by ifosfamide, but this required a somewhat higher drug concentration (IC₅₀ = ~145 μM) (Fig. 3B). These findings are consistent with our earlier observation that CYP2B1 activates ifosfamide with a 3-4-fold lower catalytic efficiency (Vₘₐₓ/Kₘ) than cyclophosphamide (16). In contrast, parental 9L cells and 9L-Z cells manifested no adverse effects when grown in the presence of millimolar concentrations of cyclophosphamide or ifosfamide. In control experiments, we established that CYP2B1-positive and CYP2B1-negative cells are both inherently sensitive to activated cyclophosphamide, which we presented to the cells in the form of 4-hydroperoxycyclophosphamide (Fig. 3C).

Effects of P-450 Enzyme Inhibition on Oxazaphosphorine Sensitivity of CYP2B1-positive 9L Cells. To verify that the expression of CYP2B1 per se is responsible for the chemosensitivity of the CYP2B1-positive cells to cyclophosphamide and ifosfamide, we used a CYP2B1-selective enzyme inhibitor, metyrapone (24), to inhibit cellular CYP2B1 activity. In the presence of 10 μM metyrapone, the cytotoxic effects of cyclophosphamide and ifosfamide toward 9L-ZP cells were nearly eliminated (Fig. 4). By contrast, metyrapone did not block the cytotoxic effect of the chemically activated derivative,
We subsequently monitored the effect of cyclophosphamide treatment on both CYP2B1-negative and CYP2B1-positive cells within the mixed cell population. Cells marked with the lacZ gene (β-galactosidase), which can be identified as blue cells after staining the cultures with the β-galactosidase substrate X-Gal, were used to distinguish the two types of cells in culture. Equal numbers of parental 9L cells were mixed with lacZ-marked CYP2B1-positive cells (9L-ZP). Following cyclophosphamide treatment, cells were fixed and stained with X-Gal to reveal the cytotoxicity of cyclophosphamide to the two-cell populations. As illustrated in Fig. 6, cyclophosphamide dramatically inhibited growth of the CYP2B1-positive cells (blue-stained cells). A substantial, albeit somewhat lower inhibition of the growth of the CYP2B1-negative cells (unstained cells) was observed. The few remaining cells showed marked morphological abnormalities and may no longer be viable. The CYP2B1 inhibitor metyrapone protected both cell types from cyclophosphamide killing; however, microscopic evaluation revealed morphological distortions in some of the CYP2B1-positive cells but not in the CYP2B1-negative cells. These findings indicate that 9L cells that express CYP2B1 are more susceptible to cyclophosphamide cytotoxicity as a consequence of prodrug activation that occurs within the tumor cell, but that substantial cytotoxicity toward adjacent CYP2B1-negative cells also occurs.

We next assessed whether this bystander killing of CYP2B1-negative cells by the adjacent CYP2B1-positive cells requires direct cell-cell contact by analogy to the case of HSV-TK-positive and HSV-TK-negative tumor cells and ganciclovir treatment (6, 25–27).

4-hydroperoxy cyclophosphamide (Fig. 4), a finding that is consistent with metyrapone protection via inhibition of CYP2B1-catalyzed oxazaphosphorine activation. Therefore, the chemosensitivity of CYP2B1-expressing cells to cyclophosphamide and ifosfamide is dependent on the presence of a functional CYP2B1 enzyme within these cells.

Analysis of Bystander Cytotoxicity Effect. We next examined whether CYP2B1-negative 9L cells can be rendered susceptible to cyclophosphamide cytotoxicity when cocultured with CYP2B1-expressing tumor cells. Parental 9L cells and CYP2B1-positive 9L-ZP cells were used for these experiments since they have similar doubling times in culture. Equal numbers of 9L and 9L-ZP cells were mixed, and the mixed culture was then treated with cyclophosphamide. We anticipated that if cyclophosphamide cytotoxicity was restricted to the CYP2B1-positive cells, then the total cell number would be decreased by approximately 50% compared to drug-free controls, as predicted on the basis of the selective (>90%), cytotoxicity of cyclophosphamide toward 9L-ZP cells, which comprise one-half of the mixed cell population. On the other hand, if the CYP2B1-positive cells chemosensitize the adjacent CYP2B1-negative cells, then both cell types should be eliminated following treatment of the coculture with cyclophosphamide. As shown in Fig. 5, more than 80% of the total cell population was eradicated when the mixed culture was treated with cyclophosphamide. Moreover, the CYP2B1 enzyme inhibitor, metyrapone, could largely abrogate this effect. The cells in the mixed culture showed a similar pattern of sensitivity to ifosfamide, albeit at a somewhat higher drug concentration. In contrast, there was no killing of either cell population when 9L-Z cells were mixed with parental 9L cells (data not shown). These studies demonstrate that CYP2B1-positive cells confer a bystander killing effect on adjacent CYP2B1-negative cells by a mechanism that involves CYP2B1 enzyme activity.

Fig. 4. CYP2B1 enzyme inhibitor metyrapone (MTP) blocks the cytotoxic effects of cyclophosphamide (CPA) and ifosfamide (IFA) but not 4-hydroperoxy cyclophosphamide (4HC) on CYP2B1-expressing cells. 9L-Z and 9L-ZP cells (1 × 10^5) were treated with cyclophosphamide (CPA) and ifosfamide (IFA) but not 4-hydroperoxy cyclophosphamide (4HC) on CYP2B1-expressing cells. 9L-Z and 9L-ZP cells (1 X 10^5) were treated with cyclophosphamide. As shown in Fig. 5, more than 80% of the growth ratio (percentage) relative to drug-free controls, as predicted

Fig. 5. Cytotoxicity of oxazaphosphorines toward mixed cultures of 9L and 9L-ZP cells. Equal numbers of parental 9L cells were mixed with 9L-ZP cells (total initial cell number = 1 × 10^6/30-mm tissue culture dish). Cells were untreated or were treated with either 1 mM cyclophosphamide (CPA) or 2 mM ifosfamide (IFA) in the absence or presence of 10 μM metyrapone (MTP), as indicated. Cell numbers were determined 5 days after beginning drug treatment. Data [mean ± range (bars) for duplicate determinations] are expressed as growth ratio (percentage) relative to drug-free controls.
For these experiments, parental 9L cells were seeded in the bottom chamber of Falcon coculture inserts, and either CYP2B1-positive (9L-ZP) or CYP2B1-negative (9L-Z) cells were placed in the top chamber of the coculture inserts. The two cell populations are physically separated in this coculture system but share the same culture medium. Cyclophosphamide treatment for 5 days killed not only the CYP2B1-positive 9L cells in the top chamber (data not shown) but also the parental 9L cells cultured in the bottom chamber (Fig. 7). The killing of both cell populations could be effectively blocked by the CYP2B1 inhibitor metyrapone. In contrast, there was no killing of either cell population when 9L-Z cells were cocultured with parental 9L cells. To assess whether the bystander killing of cocultured CYP2B1-negative cells is dependent on the number of cocultured CYP2B1-positive cells, a variable number of 9L-ZP cells (ranging from $10^4$ to $10^6$ cells) was placed in Falcon culture inserts and cocultured with $10^5$ parental 9L cells. Fig. 7B demonstrates that the cytotoxicity of cyclophosphamide toward the 9L cells in the bottom culture chamber (abscissa) is directly correlated with the initial number of 9L-ZP cells in the top chamber (ordinate). Thus, in the case of CYP2B1/cyclophosphamide, the bystander killing effect is at least partly due to the transfer to the non-CYP-expressing cells of soluble cytotoxic metabolite(s) formed via cytochrome P-450-catalyzed drug metabolism. This bystander effect is therefore distinct from that of the HSV-TK/ganciclovir system, where intimate cell-cell contact is necessary for bystander cytotoxicity to occur (26, 27).

Effects of CYP2B1 Expression on Cyclophosphamide Sensitivity of 9L Tumors in Vivo. The studies described above establish that CYP2B1-negative 9L gliosarcoma cells that are stably transfected to express CYP2B1 become highly sensitive to cyclophosphamide and ifosfamide cytotoxicity. We next used these cells as an ex vivo gene transfer model to evaluate in vivo the feasibility of using the CYP2B1/oxazaphosphorine system for cancer gene therapy. An in vivo tumor growth delay study was carried out to compare the cyclophosphamide sensitivity of CYP2B1-negative 9L tumors to that of CYP2B1-expressing 9L tumors. CYP2B1-negative cells (9L and 9L-Z) and CYP2B1-expressing cells (9L-ZP and L450-2) were grown s.c. as solid tumors in female Fischer 344 rats. Female rats were used for these experiments since liver microsomal cyclophosphamide activation in this animal model is catalyzed primarily by a single P-450 enzyme (CYP2C6) (15) and at a rate (15, 16) that is comparable to that observed in human liver microsomes (17). Cyclophosphamide treatment of CYP2B1-expressing tumors led to complete inhibition of tumor growth (Table 1; Fig. 8). The CYP2B1-negative 9L tumors showed some growth delay following cyclophosphamide treatment, but this antitumor effect was short-term, with aggressive tumor growth eventually returning. The temporary growth delay of the parental 9L tumors results from the activation of cyclophosphamide by cytochrome P-450 present in the liver, which in the case of adult female rats is catalyzed primarily by cytochrome P-450 form 2C6 (15). These in vivo tumor model studies establish that intratumoral expression of the CYP2B1 gene and the associated intratumoral prodrg activation can render solid tumors highly susceptible to oxazaphosphorine treatment in vivo.

**DISCUSSION**

Rat 9L gliosarcoma cells were used as a model to assess the utility of CYP gene transfer as a paradigm for chemosensitization of tumors by introduction of genes for drug-metabolizing enzymes that activate known, established cancer chemotherapeutic agents. 9L cells, originated from a rat brain tumor (20), can be grown in culture or can be implanted either s.c. or intracranially in Fischer 344 rats. 9L cells express cytochrome P-450 reductase, which transfers electrons required for all microsomal CYP-dependent enzyme reactions, but contain little or no endogenous CYP enzyme activity, making them well suited as a recipient cell line for experiments involving cytochrome P-450 gene transfer. The primary goals of the present studies were: (a) to evaluate whether expression of CYP2B1 in this gliosarcoma line sensitizes the cells to oxazaphosphorines; (b) to establish
whether adjacent, non-CYP-containing cells become drug sensitive via a bystander effect; and (c) to ascertain whether this chemosensitization in vitro translates into a therapeutic advantage in vivo in the context of an intact liver system, which has the capacity to catalyze oxazaphosphorine activation at an overall rate that greatly exceeds that of the tumor itself. Our findings establish that transfer of oxazaphosphorine-activating CYP2B1 gene into 9L tumor cells does indeed render these cells preferentially susceptible to cyclophosphamide and ifosfamide, both in vitro and in vivo, and therefore, it may be useful for application to cancer therapy.

In vitro and in vivo studies of the HSV-TK/ganciclovir system have indicated that HSV-TK-transduced cells treated with ganciclovir exert a bystander killing of non-HSV-TK-transduced cells which they contact (6, 25–27). The precise mechanistic basis for the bystander killing effect remains unclear, but it appears to involve transfer of activated ganciclovir metabolites or other toxic substances through cell–cell contact. This bystander effect can be of great therapeutic significance because it indicates that eradication of the tumor can, in principle, be achieved even if only a subset of a tumor cell population is effectively transduced with the drug sensitivity gene. Consequently, we carried out experiments to model whether CYP gene transfer is associated with a bystander effect, i.e., whether CYP-expressing cells can sensitize adjacent tumor cells to cyclophosphamide. We observed that CYP2B1-positive cells do confer a bystander killing of CYP2B1-negative cells by a mechanism that requires enzymatically active CYP2B1. This bystander killing effect involves, at least in part, intercellular transfer of soluble cytotoxic metabolite(s), as indicated by the chemosensitivity conferred by 9L-ZP cells to parental 9L cells, even when contact between the two cell populations is prevented. Conceivably, the bystander killing that we observed could additionally involve cell–cell contact mechanisms as well. 4-Hydroxycyclophosphamide formed by CYP2B1 (Fig. 1) is believed to be readily diffusible across cell membranes (28), and it is likely that the release of this primary metabolite, or perhaps its cytotoxic decomposition products phosphoramide mustard and acrolein, contribute to the lethal effect of cyclophosphamide on neighboring CYP2B1-negative cells. Other mechanisms such as the transfer of apoptotic signals from dying 9L-ZP cells to 9L cells could also play a role. Further investigations will be required to elucidate in detail the mechanisms of cell death in this ex vivo model of CYP gene transfer.

The lack of a requirement for cell–cell contact to achieve CYP2B1/cyclophosphamide bystander cytotoxicity may represent an important therapeutic advantage of cytochrome P-450 gene therapy over the HSV-TK/ganciclovir system by providing for more extensive distribution of activated drug within a tumor mass. In addition, unlike HSV-TK/ganciclovir, which produces activated metabolites the cytotoxicity of which is limited to cells in the DNA synthesis (S phase) of the cell cycle, the CYP2B1/oxyazaphosphorine system generates metabolites that are effective in killing tumor cells in a cell cycle-independent manner. Thus, the toxicity to tumor cells of phosphoramide mustard-derived interstrand DNA cross-links becomes manifest at whichever point the tumor cells begin to replicate, resulting in complete tumor growth inhibition in the cyclophosphamide-treated 9L-ZP and L450-2 tumors was assessed by palpation or by anatomical examination 7–8 weeks after cyclophosphamide treatment. Results were combined from three independent experiments. Representative tumor growth curves for an experiment involving 9L-Z and 9L-ZP tumors are shown in Fig. 8.

Table 1 Effect of cyclophosphamide on CYP2B1-negative and CYP2B1-positive 9L tumors grown s.c. in Fischer 344 rats

<table>
<thead>
<tr>
<th>Tumor</th>
<th>Saline</th>
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<tr>
<td>9L</td>
<td>0/11</td>
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<td>9L-Z</td>
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<td>9L-ZP</td>
<td>0/9</td>
<td>0/9</td>
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<tr>
<td>L450-2</td>
<td>0/11</td>
<td>11/11</td>
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a Rats were injected with 2 × 10⁶ CYP2B1-negative tumor cells (9L or 9L-Z) or CYP2B1-positive tumor cells (9L-ZP or L450-2). Cyclophosphamide was administered as a single i.p. injection at 100 mg/kg body weight 7 days after tumor implantation. The completeness of tumor growth inhibition in the cyclophosphamide-treated 9L-ZP and L450-2 tumors was assayed by palpation or by anatomical examination 7–8 weeks after cyclophosphamide treatment. Results were combined from three independent experiments. Representative tumor growth curves for an experiment involving 9L-Z and 9L-ZP tumors are shown in Fig. 8.
Fig. 8. Growth inhibitory effects of cyclophosphamide toward 9L-Z and 9L-ZP tumors grown in vivo. Female Fisher 344 rats (n = 5) were each inoculated with 9L-Z cells and with 9L-ZP cells by s.c. injection into the outer thighs (2 × 10⁶ 9L-Z cells on the right thigh and 2 × 10⁶ 9L-ZP cells on the left thigh of each rat). Seven days after tumor implantation, each rat received a single i.p. injection of cyclophosphamide (100 mg/kg body weight) or saline as control. Tumor areas were measured until the rats were sacrificed. Data shown are mean ± SEM (bars) for n = 5 tumors/group.

Complete tumor regression following cyclophosphamide treatment was observed in both of these 9L/CYP2B1 tumors but not in the CYP2B1-negative 9L and 9L-Z tumors. Apparently, there is a very substantial “proximity effect” in the case of intratumoral cyclophosphamide activation. This may indicate that the primary metabolite 4-hydroxycyclophosphamide/aldophosphamide (Fig. 1) or perhaps its plasma protein-stabilized sulfhydro adduct (31) has limited access to the tumor vasculature or a lower degree of cell membrane permeability when formed in the liver than would be anticipated on the basis of earlier studies (28, 32). Alternatively, given the short intrinsic half-life of 4-hydroxycyclophosphamide (t½ = 5.2 and 3.3 min in rat and human plasma, respectively; (32)), significant decomposition of this primary metabolite may occur before it reaches the tumor from its site of generation in the liver. Thus, the effective intratumoral concentration of alkylating metabolites may be substantially higher in the case of CYP2B1-expressing 9L tumors as compared to non-CYP2B1-expressing 9L tumors, despite the much higher inherent metabolic capacity of liver in these rats.

An additional possibility is that the substantially enhanced cytotoxicity of cyclophosphamide toward CYP2B1-expressing 9L tumors results from sensitization of the tumor cells to phosphoramide mustard, the primary DNA-alkylating metabolite, by the protein-alkylating metabolite acrolein. Acrolein, derived from 4-hydroxycyclophosphamide by chemical decomposition, is formed in equimolar amounts with phosphoramide mustard (Fig. 1), and is an important contributing factor to cyclophosphamide-associated cardio-toxicity (33) and to the endocrine toxicities that cyclophosphamide can have, as indicated by its importance for the depletion of serum testosterone levels and the modulation of liver CYP and steroid metabolizing enzyme profiles following cyclophosphamide treatment (34). Although acrolein derived from liver cyclophosphamide activation does not mediate the antitumor activity of the parent drug (28), it is conceivable that in our CYP gene transfer/intratumoral cyclophosphamide activation model, acrolein formed locally potentiates the cytotoxic effects of phosphoramide mustard, perhaps by a glutathione depletion mechanism (compare Refs. 33 and 35). Further studies will be required to evaluate these possibilities.

In a recent study, the expression of foreign genes within 9L cells was shown to have a potential negative influence on tumor growth in vivo, even in the case of 9L tumors expressing control genes, such as neomycin phosphotransferase (36). While the mechanism underlying this effect is still unclear, one possibility is that the protein products of these foreign genes may act as strong immunogens, thereby decreasing the growth of genetically modified 9L tumors, even in the absence of cytotoxic drug treatment (36). In our studies, however, genetically modified 9L cells (CYP2B1 or β-galactosidase) only regressed in the case of the CYP2B1 gene, and then again, only following cyclophosphamide treatment (Fig. 8). In addition, since CYP2B1 is an endogenous rat liver protein, the expression with 9L tumor cells of CYP2B1 seems less likely than a foreign gene to provide a significant immunogenic stimulus. In this context, the use of mammalian genes for cancer gene therapy, i.e., rat CYP2B1 for model studies using rat tumors or its human counterpart, CYP2B6 [which also activates cyclophosphamide (17)] for human tumors, has the potential advantage of avoiding complications due to possible immunological responses associated with the expression of HSV-TK or other foreign genes.

Taken together, the present studies establish a model system for further investigation of the therapeutic utility of transferring oxazaphosphorine-activating cytochrome P-450 genes into tumor cells. The killing of tumor cells by oxazaphosphorines is shown to proceed in an efficient manner even if only a subset of a tumor cell population is efficiently transfected with the drug-activating cytochrome P-450.
gene. A substantial improvement in the therapeutic activity of cyclophosphamide or ifosfamide may thus be anticipated when these drugs are combined with CYP2B1 gene transfer, even if the efficiency of CYP gene transfer that can be achieved using viral vectors or other gene transfer approaches is less than 100% with respect to gene transduction into tumor cells. This approach also has the potential for being superior to alternative pharmacological strategies, e.g., intrathecal injection of 4-hydroxypropyclophosphamide in the case of brain tumors (37, 38), as a consequence of the sustained, intracellular generation of activated oxazaphosphorine metabolites that occurs when CYP2B1 is expressed within tumor cells. In addition, the chemotherapy/gene therapy concepts and strategies developed in this study may potentially be extended to other established cancer chemotherapeutic agents (39) and other cytochrome P-450 genes (40) using any one of a number of approaches to achieve selective, targeted gene delivery to tumor cells. Recombinant retrovirus (6, 25) and recombinant adenovirus (41) have been used as gene transfer vehicles to deliver the HSV-TK gene into brain tumors in situ and to sensitize brain tumor cells to the prodrug ganciclovir. Recent studies have suggested that the CYP2B1/oxazaphosphorine system may also be applicable to the chemotherapeutic treatment of brain tumors using similar approaches, i.e., local injection of retrovirus carrying the CYP2B1 gene, followed by treatment with cyclophosphamide (42). In the more general case of systemic tumors, selective delivery of the CYP2B1 gene to tumor cells might best be achieved using tumor tissue-specific DNA-regulatory sequences to direct the expression of the CYP prodrug-activating enzyme within tumor cells. DNA-regulatory sequences from several tumor-expressed genes, including α-fetoprotein (hepatoma) (43), tyrosinase (melanoma) (44, 45), the onco- gene ERBB2 (pancreatic cancer) (46), carinoembryonic antigen (lung cancer) (47), and DF3/MUC1 (breast cancer) (48) have been shown to direct selective expression of a prodrug activating enzyme in each of the indicated tumor cell types. These or other DNA elements might also prove useful in achieving targeted gene therapy in the case of CYP2B1/cyclophosphamide or CYP2B1/ifosfamide. The goal of future studies will be to optimize drug efficacy by increasing the specificity and selectivity of anticancer oxazaphosphorines, while minimizing the dose-limiting systemic toxicities traditionally associated with the use of these agents.

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