Gene-directed Enzyme Prodrug Therapy with a Mustard Prodrug/Carboxypeptidase G2 Combination

Richard Marais, Robert A. Spooner, Yvonne Light, Janet Martin, and Caroline J. Springer

ABSTRACT

The gene for the bacterial enzyme carboxypeptidase G2 (CPG2) was expressed internally in mammalian cells. Mammalian-expressed CPG2 had kinetic properties indistinguishable from bacterially expressed CPG2. Human tumor cell lines A2780, SK-OV-3 (ovarian adenocarcinomas), LS174T, and WiDr (colon carcinomas) were engineered to express constitutively either CPG2 or bacterial β-galactosidase. These lines were subjected to a gene-directed enzyme prodrug therapy regime, using the prodrug 4-

INTRODUCTION

One of the major problems with current cancer therapies is the lack of specificity of treatment, which leads to harmful side effects in normal tissues, especially the gut lining and bone marrow (1). Current research is thus focused on the development of more selective methods for the delivery of toxic compounds to cancer cells. Gene therapy may be broadly defined as a genetic technology aimed at modifying cells for therapeutic gain that has been proposed as one such method to achieve greater selectivity (2).

In cancer gene therapy, both malignant and nonmalignant cells may be targeted for therapeutic benefit. The possibility to render cancer cells more sensitive to chemotherapy or toxins, either by suppressing the expression of resistance genes (e.g., multidrug resistance gene) or by introducing “suicide genes” has been considered (2). The latter includes two approaches: (a) toxin gene therapy, whereby transfected genes are able to generate toxins; and (b) enzyme-activating prodrug therapy whereby transfected genes express foreign enzymes that can activate prodrugs inside the cancer cells. This latter approach is termed GDEPT (for virally directed enzyme prodrug therapy; Ref. 3), or GDEPT3 (4, 5).

GDEPT is a two-step approach to targeted chemotherapy of human cancer. In the first step, the gene for a foreign enzyme is delivered to the tumor in a form that directs tumor-specific expression of the foreign protein. In the second step, a nontoxic prodrug is administered that is converted to a cytotoxic drug by the action of the expressed enzyme. Since the foreign enzyme is expressed only in the tumor, the cytotoxic drug will be restricted to the tumor. Theoretically, such an approach should enhance the therapeutic index of chemotherapeutic agents by minimizing systemic toxicity (3, 5).

A variety of different methods for gene delivery has been considered. These include retrovirus (6), naked DNA (7), liposomes (8), and adenovirus (9) delivery. Tissue-specific promoters such as those of tyrosinase (10, 11), α-fetoprotein (12), and carcinoembryonic antigen genes (13, 14) have been used to effect selectivity of expression in target tissues. Since expression of the foreign enzyme with these methods is unlikely to occur in all cells of a targeted tumor in vivo, a bystander cytotoxic effect is required whereby the prodrug is cleaved to an active drug that kills not only tumor cells expressing the foreign enzyme but also neighboring nonexpressing tumor cells (3). In animal models, when as few as 2% of the tumor cells express foreign enzyme after subsequent treatment with a suitable prodrug, long-term survivors can be obtained (3). Therefore, an expression efficiency of 10–20% should be enough to achieve 100% cell kill in tumors, and efficiencies of 1–5% are considered sufficient for a therapeutic response (3).

A number of different enzyme/prodrug systems has been designed for GDEPT. These include purine nucleoside prodrugs, which are activated by viral thymidine kinase (6, 7, 15–24) or thymidine phosphorylase (11, 25, 26), 5-fluorocytosine activated by bacterial cytosine deaminase (3, 27–31), cyclophosphamide and ifosfamide activated by bacterial nitroreductase (4).

In all the prodrug/enzyme examples cited above, the expressed enzyme converts the prodrug to an intermediate metabolite, which requires further catalysis by cellular enzymes within the tumor before the active drug is formed. If the cellular enzymes responsible for this second phase of activation become defective or deficient in the tumor cells, this would lead to resistance of the tumor to the prodrug (5). Thus, it is preferable that the active moiety be released directly from the prodrug cleavage by the expressed enzyme.

For maximal benefit, the released drug should be effective against both cycling and noncycling cells. Most drugs are active only against cycling cells, whereas mustard alkylating drugs are also cytotoxic to noncycling cells (33, 34). They have the added advantage that their cytotoxicity is dose related, and their use is less prone than other classes of drugs to induce resistance (33, 34). We have previously used the bacterial enzyme CPG2 (which has no mammalian homologue) to activate a glutamic acid prodrug derivative of a benzoic acid mustard in an ADEPT context (35, 36). We have demonstrated the efficacy of an antibody-CPG2 conjugate that effected CMDA prodrug activation in tumor xenograft models in nude mice, such as choriocarcinoma (37) and ovarian (38), colorectal (39, 40), and breast carcinomas (41). The CPG2 enzyme removes the glutamic acid moiety from the prodrug releasing the active mustard drug (Fig. 1; Ref. 42). No further enzymatic processing is required to activate the drug (43, 44).

These properties suggested that the CPG2 enzyme could be a good candidate for GDEPT if the corresponding gene could be expressed in mammalian cells. The present study was designed to investigate the
feasibility of this proposal within COS cells (monkey kidney) and in four human tumor epithelial cell lines. Kinetic parameters were measured for all of the modified lines. Cytotoxicity assays in combination with prodrug and the bystander effects were studied for GDEPT protocols.

MATERIALS AND METHODS

Plasmid Construction. All cloning procedures were performed using standard molecular biology techniques (45). The cpg2 gene was manipulated to delete the first 22 amino acids that code for the signal peptide. This was achieved by PCR, in which the PCR primers were designed to change codons 21 and 22 to a BamHI site and the stop codon to an EcoRI site. The 5' cpg2 primer (no. 1, see below) was used in conjunction with the 3' cpg2 primer (no. 2, see below) to amplify the cpg2 gene using the plasmid pNM830 (46). The PCR product was digested with the restriction endonucleases BamHI and EcoRI and cloned into those sites in the polylinker of the mammalian expression vector pEFPlink.2 (47). An initiator ATG is supplied by the vector, which is in frame with the cpg2 reading frame and is in a highly conserved Kozak motif to ensure efficient translation (48). This plasmid is referred to as pEFcpg2* and encodes a protein, the predicted structure of which is MAGS motif to ensure efficient translation (48). This plasmid is referred to as pMCEFcpg2*. The lacZ gene was cloned into pMCEF— to create the plasmid pMCEF—lacz. The vector pEFlacZ, which codes for bacterial β-galactosidase, was constructed by cloning the NcoI/XhoI fragment from the plasmid pMLVlacZ(50) into those sites in the plasmid pEFPlink2. The plasmid pMCEF— was created by cloning the end-repaired HindIII fragment from the plasmid pEFPlink* into the end-repaired XhoI site of a modified version of pMClNeo Poly(A) (Stratagene). The pEFPlink fragment contains the elongation factor 1α promoter (51) to direct efficient expression of the foreign protein and a multiple cloning site flanked by the 5' and 3' untranslated regions from the human β-globin gene, which are provided to give efficient mRNA processing, polyadenylation, and translation. The pMCEF— vector, therefore, contains the elongation factor 1α promoter, which directs expression of the foreign gene, and a polyoma enhancer which directs expression of the neo* gene. These two promoters are juxtaposed on opposite strands of the plasmid, giving divergent expression in opposite directions. The cpg2* coding sequence was cloned from pEFcpg2* as an NcoI/XhoI fragment into the NcoI/SpeI sites of the plasmid pMCEF— to create the plasmid pMCEFcpg2*. The lacZ gene was cloned into pMCEF— from the plasmid pEFPlacZ, using the same strategy, to create the vector pMCEFlacZ.

To express CPG2* in S9 insect cells, cpg2* was cloned into the insect cell vector pVLH6, a derivative of pVL941 (PharMingen). The EcoRI site in pVL941 was destroyed by digesting the plasmid with EcoRI, end-repairing the overhangs and then religating the plasmid. The EcoRV/BamHI fragment from pAcYl (52) was then cloned into those sites in pVL941 to create the plasmid pVLH6. This plasmid contains the multicloning site (4080) CCATG CCG CAT CAT CAT CAT GCG CCGG TAC CAG

Oligonucleotides. The following oligonucleotides were used. For oligonucleotides nos. 1 and 2, differences from the CPG2 sequence are italicized and contain the engineered restriction sites; sequences are 5'→3': no. 1, CGC GGA TCC GTT GCA CAC TTG GTT GCT C; no. 2, CGC GGA TTC GAC CGT GAT GAC AAG GCC; no. 3, C ATG CCG CAT CAC CAT CAT CAC GC; and no. 4, CAT GGC GTG ATG ATG GTG ATG ATG CGG.

Prodrug Synthesis. The CMDA prodrug was synthesized and characterized as described previously (53). CPG2 Specific Antisera. Rabbit antisera were raised to H6CPO2* expressed in S99 insect cells by the methods described previously by Marais et al. (54). For purification of H6CPO2*, 1.5 X 10⁸ cells were infected with virus particles, incubated for 48 h, and extracted as reported previously (54), except the extraction and dilution buffers contained only 0.5 mM EDTA and 0.06% (w/v) 2-mercaptoethanol. The H6CPO2* protein was purified by Ni²⁺-NTA agarose affinity chromatography (Qiagen Ltd.) according to the manufacturer's instructions, eluting the protein with 90 mM imidazole. The eluted protein was judged to be pure by silver-stained SDS polyacrylamide gels (data not shown). Rabbits were inoculated with —150 µg of purified protein, and subsequent bleeds were examined for production of antibodies by immunoprotein blotting using crude insect cell extracts expressing H6CPO2*.

Cell Culture and Transfection. The human carcinoma tumor cell lines A2780 and SK-OV-3 (ovarian adenocarcinomas), LS174T and WiDr (colon carcinomas), and the COS-7 cell lines were obtained from American Type Culture Collection. All cell lines were maintained in DMEM supplemented with 10% FCS (DMEM/FCS; 37°C, 10% CO₂). Transient transfection of COS-7 cells was performed as described previously for NIH3T3 cells using LipofectAMINE (47). For stable line construction, the human tumor cell lines were transfected with either pMCEFcpg2* or pMCEFlacZ using LipofectAMINE. Forty eight h following transfection, the cells were recultured into medium supplemented with 2 mg/ml neomycin (G418, Geneticin; Life Technologies, Inc.). Individual G418-resistant colonies were cloned by limiting dilution, and all clones that could degrade MTX were selected for further characterization.

Mammalian Cell Extraction and Kinetic Analysis of CPG2. Cells were grown to confluence, and extracts were prepared by washing twice with 5 ml of PBSA (137 mM NaCl, 3.4 mM KCl, 10 mM Na₂HPO₄, 1.8 mM KH₂PO₄, pH 7.4), followed by the addition of 200 µl of extraction buffer (250 mM Tris-HCl, 10% v/v glycerol, and 1% v/v Triton X-100, pH 7.5). The cells were lysed in situ (5 min at room temperature), and the extracts were collected and clarified by centrifugation in a microfuge (5 min at 14,000 rpm); the supernatant fractions were stored at —70°C. Cell extracts were subjected to kinetic analysis for CPG2 activity by a modification of the method described previously (37). Briefly, cell extract containing —200 ng of CPG2* was added to 1 ml of CPG2 assay buffer (100 mM Tris-HCl, and 260 µM ZnCl₂, pH 7.3) containing MTX at concentrations from 0.5–100 µM, and the rate of change of absorbance at 320 nm was measured. Km₅ were calculated from standard regression analysis.

Cytotoxicity Assays. To determine the sensitivity of the cell lines to the CMDA prodrug in vitro, cells were seeded into 6-well tissue culture plates at 3 x 10⁵ cells/well (SK-OV-3, A2780, and WiDr) or 2 x 10⁶ cells/well (LS174T) and allowed to grow to confluence. CMDA was prepared in DMSO immediately prior to use and diluted in DMEM/FCS; then 225 µl were used to replace the medium in wells at 6–4000 µM concentrations. After incubation (1 h), the medium was replaced with 1 ml of medium containing CMDA at the same concentration and incubated for an additional 18 h. The cells were washed and trypsinized; then ~3% of the cells were seeded into fresh dishes. After 4 days of further growth, cell viability was assessed by incorporation of [3H]thymidine (0.4 µCi/ml for 6 h). The cells were washed twice with PBSA, fixed in 5% trichloroacetic acid (4°C for 20 min), washed twice with methanol, and air dried. The fixed cells were solubilized (1 ml, 1% SDS/0.2 M NaOH)}
Fig. 2. Expression of CPG2* in COS-7 cells. A, immunoprotein blot analysis. COS-7 cells were transfected with either pEFlacZ (Lane 1) or pEFCpg2* (Lane 2), and detergent extracts were prepared. For each cell line, 5 μg of soluble proteins were analyzed on an 8% polyacrylamide gel and probed with the rabbit CPG2-specific polyclonal antiserum. The position of standard molecular mass marker proteins is indicated (× 10^5), and the CPG2* band is indicated by the arrowhead. B, CPG2 enzymatic activity in transfected cells. Detergent extracts from cells transfected with either pEF-FlacZ (Lane 1) or pEF-Cpg2* (Lane 2) were tested for CPG2 activity, using 5 μg of soluble protein and CMDA as a substrate. The degradation of CMDA was monitored at 305 nm, and the results are expressed in the form of amount CMDA degraded. C, kinetic analysis. Lineweaver-Burk plots, where the X-axis intercept of a linear regression equals −1/Keq, are shown for CPG2* expressed in mammalian cells (○) and for bacterially expressed CPG2 (O).

and the soluble fraction was added to 6 ml of scintillation fluid to determine the thymidine incorporation; the results are expressed as percentage growth of control cells that were treated with vehicle alone. For bystander cytotoxicity assays, mixtures of CPG2*-expressing cell lines and their appropriate lacZ cell line were seeded as above and treated with either 1 mM (A2780 and LS174T) or 2 mM (SK-OV-3 and WiDr) CMDA.

RESULTS

Expression of CPG2 in COS Cells. Our initial experiments were designed to address whether the bacterial enzyme (CPG2) could be expressed in mammalian cells in an active form. Wild-type CPG2 is a secreted enzyme found in the bacterial periplasm (46). The mature protein is a homodimer consisting of subunits, the molecular weight of which is ~42,000, and the monomers are inactive. We were concerned that if CPG2 were secreted from tumor cells, it could escape from the tumor, giving rise to nonspecific toxicity owing to production of active drug at distant sites. Therefore, we expressed CPG2 internally in a form that could not be secreted from mammalian cells. We also wished to establish whether the CPG2 in mammalian cells was active, since problems due to incorrect folding of the protein, lack of formation of dimers, or to sequestration into a hostile cell subcompartment might render this enzyme inactive. To this end, we chose to use a transient transfection system based on COS-7 cells to examine whether CPG2 could be expressed in mammalian cells.

Our first priority was to create a protein that would not be secreted from mammalian cells. The first 22 codons of the cpg2 gene encode a signal peptide that is responsible for targeting the protein to the periplasm of bacteria and which is removed by proteolysis following membrane translocation (57). We used PCR-directed mutagenesis to remove the sequences encoding the signal peptide and cloned the altered gene into the mammalian expression vector pEFlink.2, which uses the promoter from the elongation factor 1α gene to direct expression of foreign proteins in mammalian cells (47). This promoter was chosen because it is active in a wide variety of cell types (51); the expression construct is referred to as pEF-Cpg2*.

We used immunoprotein blotting to determine whether CPG2* protein was expressed in COS-7 cells. COS-7 cells were transfected with either pEF-Cpg2* or pEFlacZ (which codes for the bacterial β-galactosidase enzyme), and detergent extracts were prepared. These extracts were examined using a CPG2-specific rabbit polyclonal antiserum. The results show that an immunoreactive band with an apparent molecular weight of ~42,000 was detected in cells transfected with pEF-Cpg2*, which was absent in the cells transfected with pEFlacZ (Fig. 2A). We also found that when CPG2* was analyzed in nondenaturing gels, it migrated with a mobility that is consistent with the protein being a dimer (data not shown). The detergent extracts were then analyzed for CPG2 activity, using CMDA as a substrate. The extract from the cells transfected with pEF-Cpg2* was able to degrade CMDA, whereas there was no such activity in the control β-galactosidase extract (Fig. 2B). Conversely, using O-nitrophenyl β-D-galactopyranoside as a substrate, we could detect β-galactosidase activity in the extract from the cells transfected with pEFlacZ but not in the extract from the cells transfected with pEF-Cpg2* (data not shown).

Using MTX as a substrate, the mammalian-expressed CPG2* was found to have a Keq of 7 μM, which is in close agreement with our own findings for the bacterially expressed wild-type protein (Fig. 2C) and with those published previously for CPG2 purified from the periplasm of Escherichia coli and Pseudomonas sp. strain RS16 (46). To determine whether CPG2* expressed in COS-7 cells was secreted, we examined the tissue culture supernatant from the pEF-Cpg2*-transfected cells for CPG2 activity, and none was detectable (data not shown). These data, taken together with the lack of the signal peptide and the fact that CPG2* can be released from the transfected cells with the detergent Triton X-100, suggest that CPG2* is not secreted by mammalian cells.

Establishment of Cell Lines Constitutively Expressing CPG2*. The results presented above show that when CPG2* is expressed internally in mammalian cells, it is soluble and fully active, indicating that neither the alterations that have been introduced into the coding sequence nor the intracellular location affect the enzymic properties of CPG2. Because we wished to create a model system to examine the potential of CPG2* in a GDEPT approach, we established mammalian tumor cell lines that constitutively expressed CPG2*. This was achieved by cloning the gene encoding CPG2* into the mammalian expression vector pMCEF– to create the plasmid pMCEF-Cpg2*. The vector pMCEF– uses the elongation factor 1α promoter to direct foreign gene expression, but it also contains the NeoR gene; therefore, the vector can be used to select cells with the cytotoxic drug G418. We chose the human colon carcinoma cell lines LS174T (58) and
WiDr (59) and the human ovarian adenocarcinoma cell lines SK-OV-3 (60) and A2780 (61) for this analysis.

For each tumor line, cells were transfected with either pMCEF-cpg2* or the plasmid pMCEF LacZ (which directs expression of bacterial β-galactosidase), and G418-resistant colonies were selected. G418-resistant colonies were examined for CPG2* expression by enzymic assay, using MTX as a substrate, and for β-galactosidase activity using O-nitrophenyl β-d-galactopyranoside as a substrate (data not shown). All the cell lines were found to express >0.4 IU CPG2*/mg soluble protein (data not shown). We then examined the susceptibility of the cell lines to prodrug treatment in tissue culture by incubating each line with increasing concentrations of the prodrug CMDA. For these experiments, the cells were treated with CMDA twice, and then the medium was replaced with fresh prodrug-free medium. Thus, the cells were incubated with the prodrug for only 19 h. The rate of survival of the cells was determined by [3H]thymidine incorporation.

The results show that the cell lines fall into two different categories (Table 1): those that are highly susceptible to CMDA, with IC50 in the 5–50 μM range (LS174T and A2780); and those that are less sensitive to CMDA, with IC50 in the range 250–600 μM (WiDr and SK-OV-3). For each tumor cell line, one clone was selected to express either CPG2* or β-galactosidase for further study. The CPG2*-expressing lines were selected to express similar amounts of CPG2 activity (~1 unit/mg of detergent-soluble proteins; Fig. 3A and Table 2). Each selected line was then subjected to immunoprotein blotting analysis. An immunoreactive band with Mr ~42,000 was detected in the cell lines that contained CPG2 activity but not in those that contained β-galactosidase activity, confirming the presence of the cpg2* gene (Fig. 3B).

Following G418 selection, the selected cell lines were transferred into medium lacking G418 to determine the stability of expression and to assess whether CPG2* was toxic to mammalian cells. No differences in the levels of CPG2* enzyme activity were detected, even when the cells were maintained in the absence of G418 selection for 4 months, and no significant differences were detected in the rate of cell growth of the CPG2*-expressing cells compared to control β-galactosidase-expressing cells of the same lineage (data not shown). Because we did not observe any reduction in the expression of the protein with time or major differences in the rate of growth rate of cells, we conclude that the expression of CPG2* in these cell lines is stable, and CPG2* is not toxic to mammalian cells.

The IC50 for the selected CPG2*-expressing cell lines were compared to their corresponding β-galactosidase lines. The IC50 for the selected WiDr-CPG2* line was found to be >11 fold lower than the IC50 for the WiDr-lacZ line (Fig. 4; Table 1); the IC50 of the selected A2780-CPG2* line was >92-fold lower than the IC50 for the A2780-lacZ line, and the IC50 of the selected SK-OV-3-CPG2* line was >16 fold lower than the IC50 for the SK-OV-3-lacZ line; the IC50 for the selected A2780-CPG2* line was >92-fold lower than the IC50 for the A2780-lacZ line, and the IC50 of the selected SK-OV-3-CPG2* line was >16 fold lower than the IC50 for the SK-OV-3-lacZ line. These data show that for each tumor cell line, expression of CPG2* significantly increases the sensitivity of the cells to the CMDA prodrug.

Table 1 Expression of CPG2* in human tumor cell lines

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Clone</th>
<th>IC50 (μM CMDA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A2780</td>
<td>A 1°</td>
<td>23.2 ± 2.9</td>
</tr>
<tr>
<td></td>
<td>A 2</td>
<td>44.4 ± 8.1</td>
</tr>
<tr>
<td>LS174T</td>
<td>L 1</td>
<td>7.6 ± 0.36</td>
</tr>
<tr>
<td></td>
<td>L 2</td>
<td>25.8 ± 1.6</td>
</tr>
<tr>
<td></td>
<td>L 3</td>
<td>271.1 ± 4.4</td>
</tr>
<tr>
<td></td>
<td>L 4</td>
<td>291.1 ± 8.9</td>
</tr>
<tr>
<td></td>
<td>L 5</td>
<td>39.7 ± 0.8</td>
</tr>
<tr>
<td>SK-OV-3</td>
<td>S 1°</td>
<td>258 ± 14</td>
</tr>
<tr>
<td></td>
<td>S 2</td>
<td>459 ± 36</td>
</tr>
<tr>
<td></td>
<td>S 3</td>
<td>535 ± 12</td>
</tr>
<tr>
<td></td>
<td>S 4</td>
<td>546 ± 17</td>
</tr>
<tr>
<td>WiDr</td>
<td>W 1°</td>
<td>257 ± 15</td>
</tr>
<tr>
<td></td>
<td>W 2</td>
<td>237 ± 38</td>
</tr>
</tbody>
</table>

*a The clone from each cell line type picked for further study.

IC50 for the WiDr-lacZ line (Fig. 4; Table 1); the IC50 of the selected SK-OV-3-CGP2* line was >16 fold lower than the IC50 for the SK-OV-3-lacZ line; the IC50 for the selected A2780-CGP2* line was >92-fold lower than the IC50 for the A2780-lacZ line, and the IC50 of the selected LS174T-CGP2* line was >92-fold lower than the IC50 for the LS174T-lacZ line. These data show that for each tumor cell line, expression of CPG2* significantly increases the sensitivity of the cells to the CMDA prodrug.
The cells with varying concentrations of prodrug. The IC50s are shown and are defined as the concentration of prodrug required to kill 50% of the cells, compared to vehicle-treated controls.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Clone</th>
<th>CPG2 activity (units/mg)</th>
<th>IC50 (μM CMDA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A2780</td>
<td>lacZ</td>
<td>0</td>
<td>2150.0 ± 180</td>
</tr>
<tr>
<td></td>
<td>CPG2*</td>
<td>0.964</td>
<td>23.2 ± 2.9</td>
</tr>
<tr>
<td>LS174T</td>
<td>lacZ</td>
<td>0</td>
<td>2770.0 ± 260</td>
</tr>
<tr>
<td></td>
<td>CPG2*</td>
<td>1.256</td>
<td>29.1 ± 8.9</td>
</tr>
<tr>
<td>SK-OV-3</td>
<td>lacZ</td>
<td>0</td>
<td>4180.0 ± 452</td>
</tr>
<tr>
<td></td>
<td>CPG2*</td>
<td>1.013</td>
<td>258.0 ± 14</td>
</tr>
<tr>
<td>WiDr</td>
<td>lacZ</td>
<td>0</td>
<td>3230.0 ± 120</td>
</tr>
<tr>
<td></td>
<td>CPG2*</td>
<td>0.787</td>
<td>277.0 ± 38</td>
</tr>
</tbody>
</table>

For each cell line, the levels of CPG2 activity have been determined by standard kinetic analysis and are expressed as a function of unitsof activity/mg of cell protein in the cell extracts (units/mg). The sensitivity of the cell lines to CMDA was determined by treating the cells with varying concentrations of prodrug. The IC50s are shown and are defined as the concentration of prodrug required to kill 50% of the cells, compared to vehicle-treated controls.

The susceptibility of the cell lines to CMDA was determined by treating the cells with the CMDA prodrug. The sensitivity of the cell lines to CMDA was determined by [3H]thymidine incorporation. The results are expressed as the proportion of surviving cells relative to the vehicle-treated controls. For each cell line, the control cell lines expressing β-galactosidase (0) are shown with their corresponding CPG2* expressing lines (●) and are expressed as a function of units of activity/mg of cell protein in the cell extracts (units/mg). The sensitivity of the cell lines to CMDA was determined by the sensitivity of the CPG2* expressing lines to CMDA (1 mM for A2780 and LS174T; 2 mM for SK-OV-3 and WiDr). The proportion of surviving cells relative to the vehicle-treated controls is shown in Fig. 5 and expressed in Table 3. They indicate that all of the clones expressing CPG2* were able to direct a bystander effect. The results with the SK-OV-3 and the WiDr clones were similar, 50% of the cells in the culture mixture could be killed when only 1–2% of the cells were expressing CPG2*. 90% of the cells were killed when ~8% of the cells expressing CPG2*, and 100% cell kill was achieved when about 12% of the cells expressed CPG2* (Fig. 5; Table 3). The A2780 and LS174T clones were able to direct an even greater bystander effect; 50% cell kill occurred when only 0.1 and 1.6%, respectively, of the cells were CPG2* expressors; 90% of the cells were killed when 2.0 and 3.1%, respectively, of the cells were CPG2* expressors. Total cell kill occurred when only an estimated 5 and 3.7%, respectively, of the cells were CPG2* expressors (Fig. 5 and Table 3).

DISCUSSION

We have analyzed the potential use of the enzyme CPG2 together with the prodrug CMDA as a mammalian GDEPT model. To prevent CPG2 secretion, we removed the signal peptide from the cpg2 gene to make CPG2* and cloned the altered gene into mammalian expression vectors. The altered protein had the same kinetic properties as wild-type CPG2 purified from bacterial cells but was located intracellularly. The altered gene was cloned into a variety of mammalian tumor cells for constitutive expression. The stable cell lines expressing CPG2* were subjected to GDEPT protocols, using the CMDA prodrug. The sensitivity of the CPG2* expressing lines fell into two categories: the more susceptible A2780 and LS174T lines; and the less sensitive WiDr and SK-OV-3 lines. Clearly, this is not lineage dependent because A2780 and SK-OV-3 are derived from ovarian adenocarcinomas, whereas WiDr and SK-OV-3 are derived from colon carcinomas.

We found that in each case, the selected cell lines expressing CPG2* were significantly more sensitive to the prodrg than the β-galactosidase controls, with IC50 differentials between 11- and 95-fold. Bystander assays of mixtures of the CPG2* and control lines expressing β-galactosidase and the cell line expressing CPG2* were mixed in various proportions in tissue culture and treated with the appropriate concentration of CMDA (1 mM for A2780 and LS174T; 2 mM for SK-OV-3 and WiDr). The proportion of cells surviving was determined by [3H]thymidine incorporation and is expressed as the proportion of surviving cells relative to the cells expressing 100% β-galactosidase treated with the prodrug. The dashed line predicts cell survival if there were no bystander effect. Bars, SD.
demonstrated that total cell kill could be obtained when only 3.7 to 12% of the cells were expressing CPG2*.

To obtain selective toxicity in GDEPT, there should be no endogenous enzyme capable of catalyzing the prodrug to the cytotoxic moiety. In previous ADEPT clinical trials, when the CMDA prodrug was administered to patients without prior injection of an antibody-CPG2 conjugate, there was no CMDA related toxicity (62), nor was there any conversion of the prodrug to the active drug, as monitored in plasma by high-performance liquid chromatography and the more sensitive technique of liquid chromatography-mass spectrometry (63). Taken together, these data demonstrate that there is no human enzyme capable of converting CMDA to the active drug when it is administered systemically. Thus, the required selectivity of GDEPT can be attained by the CPG2 enzyme.

In clinical trials with CMDA in ADEPT, doses of 2.2–5.5 mmols/m²/days of prodrug were administered to patients (62), and concentrations in excess of 3 mmol have been measured in the plasma of patients. The concentrations of 1–2 mmol CMDA used in the in vitro bystander cytotoxicity studies described herein, therefore, fall within the range of concentrations that could be achieved in patients. At these concentrations, the CMDA was not cytotoxic to the cells in vitro that were not expressing CPG2. However, in GDEPT this level of prodrug concentration would be effective to kill those cells that express the enzyme and would be sufficient to direct a substantial bystander response.

Activated CMDA exerts its cytotoxicity by cross-linking DNA; thus, it is able to kill both cycling and noncycling cells. This contrasts with the most commonly used GDEPT enzyme/prodrug system, thymidine kinase/ganciclovir, which is cytotoxic only during the S phase of the cell cycle (64). It has been proposed that resistance to ganciclovir in GDEPT is due to the proportion of cells in G0 at the time of ganciclovir administration (65). Resistant tumor outgrowth occurred, despite up to 30 days of continuous ganciclovir administration, indicating that some tumor cells can remain in G0 for long periods. Tumors that grew out remained sensitive to ganciclovir on additional administrations of the prodrug, showing that acquired resistance was not the cause for the lack of response. In contrast, mustard alkylating agents are not cell cycle dependent and are able to exert their cytotoxic effects in a cell cycle independent manner (33, 66). The alkylating agent prodrug CMDA can be converted directly by CPG2 to the mustard drug, without intermediate metabolites (42). We found that cells expressing CPG2* intracellularly were between 10- and 100-fold more sensitive to CMDA than cells of the same lineage expressing bacterial β-galactosidase. Thus, CMDA used in combination with CPG2* should provide advantages over the existing prodrug enzyme systems used in GDEPT.

It has been shown that gene delivery to tumors in vivo does not lead to modification of 100% of the cells was determined by logarithmic regression of the data presented in Fig. 5.

**Table 3 The bystander effect in vitro**

<table>
<thead>
<tr>
<th>Cell line</th>
<th>CMDA conc.* (mm)</th>
<th>50% cell death</th>
<th>90% cell death</th>
<th>100% cell death</th>
</tr>
</thead>
<tbody>
<tr>
<td>A2780</td>
<td>1</td>
<td>0.1%</td>
<td>2.0%</td>
<td>5.0%</td>
</tr>
<tr>
<td>LS174T</td>
<td>1</td>
<td>1.6%</td>
<td>3.1%</td>
<td>3.7%</td>
</tr>
<tr>
<td>SK-OV-3</td>
<td>2</td>
<td>1.3%</td>
<td>7.7%</td>
<td>12.0%</td>
</tr>
<tr>
<td>WiDr</td>
<td>2</td>
<td>2.2%</td>
<td>8.3%</td>
<td>11.5%</td>
</tr>
</tbody>
</table>

*conc., concentration.*

**REFERENCES**


Gene-directed Enzyme Prodrug Therapy with a Mustard Prodrug/Carboxypeptidase G2 Combination

Richard Marais, Robert A. Spooner, Yvonne Light, et al.


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/56/20/4735

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.