Sensitization of Human Tumor Cells to CPT-11 via Adenoviral-mediated Delivery of a Rabbit Liver Carboxylesterase

Monika Wierdl, Christopher L. Morton, James K. Weeks, Mary K. Danks, Linda C. Harris, and Philip M. Potter

Department of Molecular Pharmacology, St. Jude Children’s Research Hospital, Memphis, Tennessee 38105

ABSTRACT

Irinotecan, 7-ethyl-10-[4-(1-piperidino)-1-piperidino]carbonyloxycamptothecin (CPT-11) is activated by carboxylesterases (CE) to yield the potent topoisomerase I inhibitor, SN-38. We have demonstrated previously that a rabbit liver CE is approximately 100-1000-fold more efficient at drug activation than a highly homologous human CE. In an attempt to use rabbit CE expression in combination with CPT-11 for gene therapy approaches for the treatment of cancer, we have developed an adenoviral vector expressing this intracellular CE. After transduction, this virus produces very high levels of CE activity in a panel of human tumor cell lines and results in marked sensitization to CPT-11 of all of the transduced cells. Reductions in IC50 values for this drug ranged from 11–127-fold. Additionally, comparison with an adenovirus expressing a secreted form of the rabbit CE indicated that a collateral effect could be achieved with reductions in the IC50 values ranging from 4–19-fold. These data suggest that the described reagents may be suitable for use in vivo in a viral-directed enzyme prodrug therapy approach using CPT-11.

INTRODUCTION

CPT-11 is an anticancer camptothecin prodrug that has demonstrated good antitumor activity both in animal xenograft models of human tumors and in patients undergoing therapy with this drug (1–7). This prodrug is metabolized by esterases to yield SN-38, a potent topoisomerase I inhibitor, which is cytotoxic (8–11). Activation of CPT-11 by human esterases is poor, resulting in very low plasma SN-38 levels after administration to patients (7–11). In contrast, very high levels of SN-38 are observed in mice and rats after treatment with CPT-11, indicating that the enzymes involved in drug catalysis are either much more efficient or much more abundant (8, 12, 13).

We have demonstrated recently (9, 14) that, similar to rodent enzymes, a rCE is highly proficient at drug activation, being greater than 100-fold more efficient than a homologous human enzyme. Because low level drug metabolism is observed in patients treated with CPT-11, selective activation of the drug at the tumor site may be a viable approach to increase antitumor activity. As a prelude to these types of gene therapy experiments, we describe here the construction and characterization of adenoviral vectors designed to express either an intracellularly localized or a secreted form of the rabbit CE and determine the ability of these vectors to sensitize human tumor cell lines to CPT-11.

MATERIALS AND METHODS

CPT-11. CPT-11, kindly provided by Dr. J. Patrick McGovren (Pharmacia Upjohn, Kalamazoo, MI), was dissolved in methanol and stored at −20°C. For growth inhibition assays, the drug was diluted in media immediately before use.

Cell Lines and Plasmids. The rhabdomyosarcoma cell line Rh30 was provided by Dr. Peter Houghton (St. Jude Children’s Research Hospital, Memphis, TN), and the neuroblastoma cell line, NB-1691, was obtained from the Pediatric Oncology Group. The lung carcinoma line A549, the colon adenocarcinoma line HT29, the neuroblastoma line SK-N-AS, the glioblastoma line U373MG, and the embryonal kidney cell line 293 were all purchased from the American Type Culture Collection (Rockville, MD). All of the cell lines were cultured in DMEM containing 10% FCS at 37°C under an atmosphere of 10% CO2/90% air. For studies with adenovirus, FCS was heat inactivated at 56°C for 30 min. U373MG cell lines transfected with either the full-length rCE cDNA (U373MGpIRESRABC) or the truncated cDNA (U373MGpIRESRABFL) were generated by electroporation as described previously (9, 15, 16). The adenoviral vector pAV56a was obtained from Genetic Therapy Inc. (Gaithersburg, MD), and AdRl327 was kindly provided by Dr. Elio Vanin (St. Jude Children’s Research Hospital).

Construction of Recombinant Adenovirus. Adenoviral vectors were generated by homologous recombination of pAV56a with adenoviral DNA (AdRl327; Ref. 17) after transformation into 293 cells. An agarose overlay was applied to the cells, and plaques were allowed to form (typically 10–14 days). Individual plaques were isolated, DNA was prepared, and the presence of the rabbit CE cDNA was determined by PCR. Additionally, the absence of wild-type adenoviral DNA was monitored by PCR analysis of the E1a gene (18). Plaques that did not contain wild-type sequences were subjected to two more rounds of plaque purification before large-scale amplification and preparation by cesium chloride ultracentrifugation. Adenoviral titers were determined using 293 cells, and aliquots of virus were stored at −80°C. moi is defined as the number of plaques produced in 1 × 106 293 cells in a total volume of 1 ml of media after incubation with virus for 1 h.

CE Assays. CE activity was determined in sonicated cell extracts or culture media by a spectrophotometric assay using o-nitrophenyl acetate as a substrate (9, 19). Briefly, samples were incubated with 3 mM o-nitrophenyl acetate in 50 mM HEPES (pH 7.4), and the change in absorbance at 405 nm was measured using a Jasco 920 fluorescence detector, and the sensitivity of this system was 20 pg/920 fluorescence detector, and the sensitivity of this system was 20 pg/μl. Data were expressed as μmol o-nitrophenol produced/min of protein or μl of culture media.

CPT-11 Conversion Assays. Conversion of CPT-11 to SN-38 was monitored by incubating either cell extracts or media samples with 5 or 25 μM CPT-11 for 18 h in 50 mM HEPES (pH 7.4) at 37°C. Reactions were terminated by the addition of an equal volume of acidified methanol, and particulate matter was removed by centrifugation at 100,000 × g for 30 min at 4°C. Concentrations of both drugs in the supernatant were then determined by high performance liquid chromatography.

Detection of CPT-11 and SN-38. CPT-11 and SN-38 concentrations in biological samples were determined by high performance liquid chromatography as described previously (13, 20). Detection was achieved using a Jasco 920 fluorescence detector, and the sensitivity of this system was 20 pg/μl and 1.5 pg/μl for CPT-11 and SN-38, respectively.

Western Analysis. Whole cell extracts were separated in 4–20% precast SDS-PAGE gels (Invitrogen, Carlsbad, CA), proteins were transferred to Immobilon-P membrane by electroblotting, (21) and Western analysis was performed as described previously (22). CEs were detected using a horseradish peroxidase-conjugated rabbit antiperoxidase esterase antibody (Research Diag...
nistics, Inc., Flanders, NJ) and enhanced chemiluminescence (Amersham Life Sciences, Arlington Heights, IL). As a control for loading, an anti-TFIID antibody was used to reprobe the membrane (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Prestained molecular weight protein markers (Invitrogen) were used to visualize the molecular mass of the immunoreactive protein species.

Growth Inhibition Assays. Growth inhibition assays were performed in triplicate in 6-well multiwell plates. Cells were plated at the following densities: A549, 5 × 10^4 cells/well; HT29, 6 × 10^4 cells/well; NB-1691, 1 × 10^5 cells/well; Rh30, 5 × 10^4 cells/well; SK-N-AS, 5 × 10^4 cells/well; and U373MG, 7 × 10^4 cells/well. Then, the cells were transduced with adenovirus for 24 h. The following day, the medium was removed, and the cells were allowed to grow until the CE activity no longer increased, typically 3–4 days. At this time, the cells were exposed to CPT-11 for 2 h, and after replacing with fresh media, the cells were allowed to grow for an additional three cell doublings (determined from a nondrug-treated well) or a maximum of 6 days. Cell number was measured using a Coulter Z2 (Coulter Electronics, Luton, England), and IC_{50} values were calculated from data plots using Prism software (Graphpad Software, San Diego, CA).

Collateral Effect Assays. To distinguish the activation of CPT-11 by an extracellular enzyme as opposed to intracellular activation and transfer of SN-38 to neighboring cells, i.e., a bystander effect, we have termed the former mechanism a collateral effect. To assess a collateral effect from secreted CES, ~1 × 10^5 cells were transduced with adenovirus at a moi of 5, and CE expression in the media was monitored. After 4 days, the medium was harvested and applied to untransduced cells in 6-well multiwell plates. CPT-11 was added for 2 h, and after replacing with fresh media, the cells were allowed to grow for three cell doublings of untreated control cells or a maximum of 6 days. Cell number and IC_{50} values were then determined as described above.

RESULTS

Construction of an Adenoviral Vector Expressing rCE. To assess the ability of viral vectors to deliver the rabbit CE cDNA to human tumor cells, we constructed an adenovirus designed to express this enzyme. To generate this vector, we ligated the 1.7-kb EcoRI fragment (9) into pSP65Cla (23), allowing the removal of the cDNA with Clal overhangs to facilitate ligation into pAVS6a (Fig. 1). This plasmid was cotransfected into 293 cells with Clal-restricted Ad5327 DNA (17). After analysis of plaque DNA for the presence of the cDNA and the absence of the Ela gene (18), large-scale preparations of Ad5RSvrCE were performed. Titers of 3 × 10^{10} plaque-forming units/ml were achieved for Ad5RSvrCE. A control virus containing no cDNA (AdVC) was also generated and demonstrated a similar titer (2.2 × 10^{10}) to Ad5RSvrCE.

Adenoviral-mediated Expression of the rCE in Rh30 Cells. To confirm that expression of the rCE could be achieved from adenoviral vectors, Rh30 cells were transduced with Ad5RSvrCE at an moi of 5, and cells were harvested at 24-h intervals. Extracts were prepared for CE assays and CPT-11 conversion analyses. As can be seen in Table 1, high levels of CE activity (up to ~1100 μmol/min/mg) were present in cell extracts after viral transduction that peaked at 72 h. Similarly, the yields of SN-38 produced from these extracts increased and peaked at the same time point. After 72 h, the levels of CE activity dropped and the conversion of CPT-11 decreased in parallel. This reduction may be attributable to viral-mediated or CE-mediated cytotoxicity. SDS-PAGE analysis of these cell extracts confirmed the above data, with the presence of the expected Mr 65,000 immunoreactive band in all of the samples exhibiting CE activity (Fig. 2). Approximate equal loading of the Western analysis was confirmed by analysis with an anti-TFIID antibody.

Adenoviral-mediated Sensitization of Human Tumor Cell Lines to CPT-11. Having determined that adenoviral-mediated delivery of the rCE generated high levels of enzyme activity in Rh30 cell extracts, we assessed the ability of these reagents to induce cytotoxicity in cells in combination with CPT-11. Cells were transduced at a moi of 5, and sensitivity to CPT-11 was assessed after 4 days. An example of a typical growth inhibition curve is demonstrated in Fig. 3, in which Rh30 cells expressing the rCE were sensitized >60-fold to CPT-11. Table 2 demonstrates the change in IC_{50} for a panel of human tumor cell lines after treatment with Ad5RSvrCE and CPT-11. A minimum of an 11-fold reduction in the IC_{50} was observed, consistent with the hypothesis that adenoviral-mediated delivery and expression of the rCE can sensitize cells to CPT-11. Indeed, in some cell lines such as Rh30 and U373MG, very high levels of CE activity were detected in cell extracts, and this resulted in 64–127-fold changes in the IC_{50} values, respectively (Table 2).

rCES Activates CPT-11. Having demonstrated that sensitization to CPT-11 for a panel of human tumor cells lines could be achieved by adenoviral-mediated delivery of the rabbit CE cDNA, we at...
between the levels of SN-38 produced and the amount of CE activity. CPT-11 decreased concomitantly. Additionally, there is a correlation cause we observed a collateral effect from media containing rCES, we induce growth inhibition in untransfected U373MG cells.

To determine whether a collateral effect could be achieved with AdRSVrCES, medium was harvested from cells transduced with this virus at an moi of 5 and applied to untransduced cells in combination with CPT-11. Because only basal levels of intracellular CE were present, the toxicity of CPT-11 was attributable to the extracellular conversion to SN-38 by the secreted rabbit liver enzyme. This approach was adopted as opposed to mixing of transduced cells because adenosine gene expression might affect growth rates and, hence, apparent cytotoxicity to CPT-11. Table 5 indicates the IC$_{50}$ values for a panel of cell lines after incubation with media derived from AdRSVrCES-transduced cells and CPT-11. Significant differences in the levels of CE activity in the media paralleled a corresponding reduction in the IC$_{50}$ values for CPT-11, and sensitization to the drug was observed in A549, HT29, Rh30, and U373MG cells. In contrast, although AdRSVrCE allowed expression of intracellular CE and sensitized the two neuroblastoma lines NB-1691 and SK-N-AS to CPT-11, no CE activity was detected in the media after transduction with AdRSVrCES. The possibility that the protein secretory pathway is disrupted in neuroblastoma cells is currently being investigated.

**DISCUSSION**

This study demonstrates the sensitization of a panel of cell lines to CPT-11 after adenosine-mediated delivery of a rCE cDNA. We have reported previously (9, 14, 15, 24) that cells transfected with plasmids expressing the rCE are sensitized cells to the drug. In this manuscript and the accompanying study, we have expanded these studies by using adenosine to deliver the prodruk-activating CE to potentiate the toxicity of CPT-11 for proposed gene therapy experiments. We have demonstrated in six of six cell lines that very high intracellular levels...
of CE activity can be achieved after adenoviral transduction at modest mois (≤5) and result in significant sensitization to CPT-11. Our results are in contrast to data presented by Kojima et al. (25) who used adenoaviruses to transfer and express a human CE in tumor cell lines. Of 11 cell lines tested, only three demonstrated more than a 5-fold decrease in the IC₅₀ values for CPT-11. The results of Kojima et al. (25) were achieved using a moi of 50.

In our study, experiments performed at moi of 50 with AdRSVrCE resulted in exceedingly high levels of CE activity (>30,000 μmol/min/mg) and led to growth arrest of the cells (data not shown). In the two cell lines common to both studies, A549 and HT29, Kojima et al. (25) achieved changes in the IC₅₀ values of ~17-fold and zero, respectively. In our studies with the rabbit CE, we observed reductions in the IC₅₀ of 46- and 11-fold using a moi of 5. These differences may be attributable to the ability of the rabbit CE to preferentially activate CPT-11 in comparison with the human CE. We have reported previously (14) that the rabbit enzyme is at least 100-fold more efficient at drug activation as compared with a highly homologous human CE.

Data in Tables 2 and 5 indicate that although all of the cell lines expressing or exposed to medium containing the rabbit CE were sensitized to CPT-11, there was no direct comparison in the levels of CE activity and the fold change in the IC₅₀ value. This is probably attributable to inherent differences in the sensitivity of the cells to SN-38, because we have observed a 200-fold range in the IC₅₀ values attributable to inherent differences in the sensitivity of the cells to CPT-11 before or after transduction with CE activity and the fold change in the IC₅₀ value. This is probably expressing or exposed to medium containing the rabbit CE were efficient at drug activation as compared with a highly homologous previously (14) that the rabbit enzyme is at least 100-fold more activate CPT-11 in comparison with the human CE. We have reported may be attributable to the ability of the rabbit CE to preferentially activate CPT-11 compared with this drug with several cell lines. However, as indicated in Table 4, there is a direct correlation between the level of CE activity and the amount of SN-38 produced. Hence, we cannot a priori predict the IC₅₀ values of cells to CPT-11 before or after transduction with AdRSVrCE or AdRSVrCES, but it is probable that exposure to these adenoaviral vectors will significantly increase the toxicity to cells by this drug.

This manuscript describes the construction of two different adenoaviruses for use in VDEPT with CPT-11. AdRSVrCE, which encodes an intracellular CE, would be suitable for the elimination of tumor cells from bone marrow (26). Cytotoxicity would be limited to cells transduced with the virus. However, for the treatment of solid tumors, AdRSVrCES would be the reagent of choice. This virus encodes the secreted form of the rabbit CE and should induce a collateral effect by producing SN-38 in the extracellular fluid. Hence, untransduced cells would be subjected to the cytotoxic effects of the drug. To what extent this occurs would depend upon many factors including the level of CE expression, the elimination of CE from the extracellular fluid, binding of SN-38 to extracellular proteins, and exogenous metabolism of CPT-11.

We envisage that each of the described vectors may be best applied for specific applications. The use of an intracellular enzyme might be most appropriate for ex vivo purging of tumor cells before autologous stem cell rescue (26). In contrast, for the treatment of solid tumors in situ, a collateral effect may be beneficial. Because of the characteristics of currently available adenoaviruses, it is unlikely that 100% of cells in a tumor will be transduced after local application of virus. Therefore, approaches that increase the effectiveness of extracellular CPT-11 activation should result in increased antitumor activity. Hence, in addition to the adenovirus encoding the intracellular CE, we generated a second adenoavirus expressing a secreted form of the rCE (15). Studies with this virus demonstrated that in four of six cell lines, sufficient CE was secreted into the media to sensitize untransduced cells to CPT-11. Because cultured cells are incubated in considerably more media than would be present in the extracellular fluid of a solid tumor, these in vitro studies may underestimate the effectiveness of the described approach to induce a collateral effect in vivo. We are currently performing human tumor xenograft experiments to assess the efficacy of both AdRSVrCE and AdRSVrCES in inducing tumor regressions in combination with CPT-11.

A second consideration for the use of VDEPT in cancer therapy concerns the biological characteristics of the solid tumor. As mentioned previously, the neuroblastoma cell lines transduced with AdRSVrCES did not secrete the CE, although high levels of enzyme were present after transduction with AdRSVrCE, and these cells were sensitized to CPT-11. Additional experiments have indicated that less protein is synthesized in NB1691 cells after transduction with AdRSVrCES, although this is partially dependent upon the density at which the cells were plated and transduced (data not shown). We are currently characterizing other neuroblastoma cell lines to determine whether this result is unique to this cell type. If so, the production of a collateral effect using the secretion of a drug-activating enzyme may be difficult to achieve in neuroblastoma tumors.

In summary, there are several potential uses of replication-deficient viral vectors in VDEPT cancer therapy. These include the direct injection of solid tumors with virus, the elimination of residual tumor cells after surgical resection or chemotherapy, and the ex vivo purging of tumor cells from bone marrow preparation before autologous stem cell rescue. It is our opinion that direct administration of virus to large tumors either to induce apoptosis or to sensitize cells to drugs is probably not the most effective use of these reagents for cancer treatment. Although such studies may be applicable in very defined

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### Table 4 Collateral effect in U373MG cells from media containing rCE

<table>
<thead>
<tr>
<th>Media Percentage (CE+)</th>
<th>CE Activity (μmol/min/ml)</th>
<th>SN-38 Produced (pmol/μl/ml)</th>
<th>IC₅₀ (μl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>100:0</td>
<td>27.7 ± 2.7</td>
<td>0.24</td>
<td>62.5</td>
</tr>
<tr>
<td>90:10</td>
<td>29.4 ± 1.1</td>
<td>0.54</td>
<td>43.3</td>
</tr>
<tr>
<td>50:50</td>
<td>41.0 ± 1.5</td>
<td>2.70</td>
<td>26.8</td>
</tr>
<tr>
<td>0:100</td>
<td>54.8 ± 2.8</td>
<td>5.12</td>
<td>14.8</td>
</tr>
</tbody>
</table>

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### Table 5 IC₅₀ values after treatment with media derived from AdRSVrCES transduced cells and CPT-11

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Virus</th>
<th>Media CE activity after 4 days (μmol/min/ml)</th>
<th>CPT-11 IC₅₀ (μl)</th>
<th>Fold change in IC₅₀</th>
</tr>
</thead>
<tbody>
<tr>
<td>A549</td>
<td>AdVC</td>
<td>5.3 ± 0.1</td>
<td>41.4</td>
<td></td>
</tr>
<tr>
<td></td>
<td>AdRSVrCES</td>
<td>119.4 ± 7.0</td>
<td>5.4</td>
<td>8</td>
</tr>
<tr>
<td>HT29</td>
<td>AdVC</td>
<td>7.2 ± 0.2</td>
<td>26.1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>AdRSVrCES</td>
<td>34.4 ± 0.8</td>
<td>6.8</td>
<td>4</td>
</tr>
<tr>
<td>NB-1691</td>
<td>AdVC</td>
<td>6.1 ± 0.2</td>
<td>4.6</td>
<td></td>
</tr>
<tr>
<td></td>
<td>AdRSVrCES</td>
<td>10.1 ± 0.2</td>
<td>3.9</td>
<td>1</td>
</tr>
<tr>
<td>RH30</td>
<td>AdVC</td>
<td>4.5 ± 0.3</td>
<td>18.4</td>
<td></td>
</tr>
<tr>
<td></td>
<td>AdRSVrCES</td>
<td>162.3 ± 5.9</td>
<td>0.28</td>
<td>66</td>
</tr>
<tr>
<td>SK-N-AS</td>
<td>AdVC</td>
<td>7.5 ± 0.3</td>
<td>19.2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>AdRSVrCES</td>
<td>11.9 ± 0.4</td>
<td>19.9</td>
<td>1</td>
</tr>
<tr>
<td>U373MG</td>
<td>AdVC</td>
<td>14.2 ± 0.4</td>
<td>7.7</td>
<td></td>
</tr>
<tr>
<td></td>
<td>AdRSVrCES</td>
<td>122.5 ± 7.1</td>
<td>0.4</td>
<td>19</td>
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</table>
clinical situations such as nonmetastatic brain tumors where surgery is not possible, it is unlikely that this approach would be applicable to solid malignancies in general. Hence, we are pursuing alternative strategies to maximize the efficacy of these gene therapy approaches.

We believe that the greatest clinical utility for replication-deficient vectors will be for the treatment of residual disease at the primary site after resection of solid tumors and for the purging of tumor cells from bone marrow used for autologous transplant (26). This latter procedure uses replication-deficient adeno virus expressing the intracellular form of the rabbit CE (AdRSVrCE, described in this study) to sensitize tumor cells to CPT-11 in vivo, and preclinical studies describing such an approach are found in Meck et al. (26). In the case of purging, selectivity of gene expression is afforded by the ability of the viral vectors to transduce tumor cells preferentially to CD34+ cells. In the case of residual disease, however, it will be necessary to design tumor-specific expression vectors to effect tumor-selective cytotoxicity.

In conclusion, we have constructed and monitored the efficacy of adenoviral vectors designed to express either an intracellular localized or a secreted rCE and demonstrated that both can sensitize cells to CPT-11. Additionally, a collateral effect has been observed with some type of tumor cells with the latter virus indicating that this reagent may be suitable for gene therapy approaches for the treatment of solid tumors in combination with CPT-11.

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5082
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