Suicide Gene Therapy of Human Colon Carcinoma Xenografts Using an Armed Oncolytic Adenovirus Expressing Carboxypeptidase G2

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Abstract

We have designed a targeted systemic suicide gene therapy that combines the advantages of tumor-selective gene expression, using the human telomerase promoter (hTERT), with the beneficial effects of an oncolytic adenovirus to deliver the gene for the prodrug-activating enzyme carboxypeptidase G2 (CPG2) to tumors. Following delivery of the vector (AdV.hTERT-CPG2) and expression of CPG2 in cancer cells, the prodrug ZD2767P was administered for conversion by CPG2 to a cytotoxic drug. This system is sometimes termed gene-directed enzyme prodrug therapy (GDEPT). Here, we have shown that it is applicable to 10 human colorectal carcinoma cell lines with a direct correlation between viral toxicity and CPG2 production. SW620 xenografts were selected for analysis and were significantly reduced or eradicated after a single administration of AdV.hTERT-CPG2 followed by a prodrug course. The oncolytic effect of adenovirus alone did not result in DNA cross-links or apoptosis, whereas DNA cross-links and apoptosis occurred following prodrug administration, showing the combined beneficial effects of the GDEPT system. The apoptotic regions extended beyond the areas of CPG2 expression in the tumors, indicative of significant bystander effects in vivo. Higher concentrations of vector particles and CPG2 were found in the AdV.hTERT-CPG2 plus prodrug–treated tumors compared with the virus alone, showing an unexpected beneficial and cooperative effect between the vector and GDEPT. This is the first time that a tumor-selective GDEPT vector has been shown to be effective in colorectal carcinoma and that apoptosis and significant bystander effects have been identified as the mechanisms of cytotoxicity within the tumor. [Cancer Res 2007;67(10):4949–55]

Introduction

Despite recent advances in the treatment of colorectal cancer, the death rate remains high. In 2002, more than 56,000 people died from the disease in the U.S.4 Agents with novel mechanisms of action are needed both in second- and third-line metastatic disease when other options have been exhausted, along with improvements in the treatment for earlier clinical settings. Oncolytic adenovirus therapy has emerged as a promising candidate (1, 2). It has been shown to produce clinical benefits in patients with colon cancer (3) and has recently been approved for cancer treatment in China (4). Oncolytic adenoviruses are particularly efficient in combination with chemotherapy (5–7). They are also efficient gene delivery vectors (8) and can be administered to patients via the hepatic artery (3, 6), which makes oncolytic adenoviruses attractive for systemic gene therapy of metastasized colorectal cancer.

We have constructed the oncolytic adenovirus AdV.hTERT-CPG2 for gene-directed enzyme-prodrug therapy (GDEPT; refs. 8, 9). GDEPT is a suicide gene therapy approach that directs chemotherapeutic agents to tumors selectively (10). AdV.hTERT-CPG2 replicates in, and kills, telomerase-positive tumor cells, and at the same time targets expression of the GDEPT enzyme carboxypeptidase G2 (CPG2) to the cancer cells (8) for conversion of the alkylating agent mustard prodrug ZD2767P (11, 12) to a cytotoxic drug. It is unlikely that all cells of a tumor (particularly non–cancer stromal cells) will synthesize CPG2 following the administration of AdV.hTERT-CPG2. Therefore, a so-called “bystander effect” is required, i.e., the ability of CPG2-expressing, prodrug-activating cells to kill adjacent, nonexpressing cells (13).

Here, we describe the use of an oncolytic adenoviral vector that can target tumors systemically and selectively for activation of a potent prodrug. We show significant long-term antitumor efficacy following a single systemic administration of AdV.hTERT-CPG2 followed by a course of prodrug delivery in a xenograft model of human colorectal cancer. We identify the mechanisms for this efficacy and show that the oncolytic effect of the virus alone is enhanced significantly by the effects of the activated prodrug ZD2767P, resulting in DNA cross-linking, apoptosis, and bystander killing. Thus, combining the replicating virus with our GDEPT system has significant advantages over the oncolytic vector or GDEPT alone.

Note: The basic oncolytic virus program of Genetic Therapy, Inc. was acquired by Cell Genesys, Inc., South San Francisco, California.

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Materials and Methods

Cells and adenoviruses. The replicating adenoviruses AdV.hTERT-CPG2 and AdV.hTERT containing have been previously described (8). Cell lines were maintained in DMEM containing 10% fetal bovine serum. Cell survival was determined using MTS assays (CellTiter96 Aqueous Nonradioactive Cell Proliferation Assay, Promega). Synthesis of ZD2767P (11), CPG2 assays (10), GDEPT, and bystander experiments (8) were done as described previously. Floating and adherent cells were pooled and analyzed by flow cytometry (14) for cell cycle studies or Annexin V FITC binding (Annexin V FITC Apoptosis Detection Kit, Calbiochem).

Western immunoblotting. We used anti-poly(ADP-ribose) polymerase (PARP; BD Biosciences PharMingen), anti-cyclin A (Ab-6, clone 6E6; Labvision Corporation), anti-cyclin E (M20), anti-cyclin D1 (M20, sc-718), anti-cyclin B1 (GNS1, sc-245), or anti-ERK2 antibodies (C14, all from Santa Cruz Biotechnology).

Reference

4 http://www.cdc.gov/cancer/colorectal/statistics
Comet assays. Assays were done in duplicate. Four hours after treatment, half of the cells were incubated in 100 µmol/L of H₂O₂ (Sigma) to induce DNA fragmentation. Olive tail moments (ref. 15, 100 for each sample) were analyzed using the CometAssay kit (Trevigen, AMS Biotechnology) and CometScore software.5

Animals. SW620 xenografts were established in female mice (CrlCD1-Fox nu/nu, 7 × 10⁶ cells s.c., eight animals per group; Charles River). After 6 days, PBS or adenovirus was administered into the tail vein (10¹¹ vp/kg), following allocation to treatment groups by stratified distribution on tumor size. ZD2767P (300 mg/kg) was administered as described (16) 5 days after adenovirus therapy. Additional courses of prodrug (six doses in total) were injected at weekly intervals. Tumor volumes were calculated relative to the mean of the first measurement and plotted while at least five animals survived. Animals were culled when tumors reached 1.7 cm in any dimension or a mean of 1.5 cm in two perpendicular dimensions. P values were calculated using GraphPad Prism version 4.02 for Windows (GraphPad Software). CPG2 assays were as described (17).

Immunofluorescence. Xenografts were frozen in isopentane 5 days after the second prodrug dose (10¹¹ vp/kg). Cryosections (5 µm) were exposed to rabbit polyclonal antibody against cleaved caspase-3 (Cell Signaling) and rat monoclonal anti-CPG2 antibody (ICR hybridoma unit), followed by Alexa-Fluor 488–conjugated anti-rabbit and Alexa-Fluor 555–conjugated anti-rat secondary antibodies (Molecular Probes, Invitrogen). Nuclei were counterstained with TO-PRO-3 (Molecular Probes, Invitrogen). Samples were imaged on a Leica confocal microscope at 20× magnification using the same intensity parameters.

Real-time PCR. Adenoviral copy numbers were synthesized as described (18). For coxackievirus B3 and adenovirus receptor (CAR) detection, cDNA was synthesized from total RNA (Trizol, Invitrogen) using random nonamers forward (mouse + human) 5′TTTGTCTGCCAGAATATAAAATCACTTGA, reverse (human) 5′TTTGTCTCCAGAATATAAAATACCTTGA, probe (mouse + human) 5′-FAM-AGTCGGAGACCGAGGACC[TAMRA]-6-FAM). Glycer- aldehyde-3-phosphate dehydrogenase forward (mouse + human) 5′TTCT- ATTGAACCTACA, reverse (mouse) 5′TGACTCCAGACATCT, reverse (human) 5′TGGACTCCAGACT, probe (mouse + human) 5′-FAM-CCATACACATCTTCCA[TAMRA]-6-FAM). Tumor cDNA was analyzed using human primers, murine primers were used for all other tissues.

Results and Discussion

The telomerase reverse transcriptase gene is active in a wide range of human cancers, including colorectal carcinoma (18). Therefore, we hypothesized that the oncolytic vector AdV.hTERT-CPG2 would target colorectal carcinoma cells. The viral cytotoxicity was measured by determining the EC₅₀ value for each cell line (Table 1). In order to assess the suitability of the virus as a GDEPT gene delivery vector, we also measured the CPG2 enzyme activity in the cells following infection. The adenovirus was potent in all cell lines (EC₅₀ values, 0.02–511 pfu/cell; CPG2 levels, 0.3–4.2 units/mg). There was a significant inverse correlation between EC₅₀ values and CPG2 levels [log EC₅₀ = -0.77 × (CPG2); P = 0.024] (Table 1). These data show that AdV.hTERT-CPG2 is an effective oncolytic vector for delivering CPG2 to colon cancer.

We selected a colon cancer cell line, SW620, which grows reproducibly as xenografts, for further study to determine the mechanism of cell death of our therapy. Cell cycle analyses showed that the adenovirus arrested SW620 cells in the S-G₂-M phase (Fig. 1A). Infection with AdV.hTERT-CPG2 altered the expression of the cell cycle regulators cyclin D1 and cyclin E, but not that of cyclin A or B1 (Fig. 1B), providing further evidence that adeno- viruses trap the infected cells in S-G₂-M phase, as reported by Bernt et al. (19). Next, we examined the survival of SW620 cells after GDEPT treatment. In combination with the prodrug ZD2767P, AdV.hTERT-CPG2 sensitized SW620 cells 160-fold to the prodrug, showing the antitumor cell activity of our therapy (Fig. 1C).

For systemic cancer gene therapy in vivo, tumor-selective gene expression is crucial. Therefore, we tested adenoviral targeting in nude mice bearing SW620 xenografts. We examined CAR and found that it is expressed in a range of mouse tissues (Fig. 2A). We also found that livers, guts, and lungs have higher CAR expression levels than tumors (Fig. 2A). Murine CAR is known to function as a receptor for adenoviruses (20). However, when we administered AdV.hTERT-CPG2 via a single tail vein injection and measured the CPG2 activity in the tissues, we showed that the adenovirus selectively targeted CPG2 to the tumors (20.6 units/g; Fig. 2B), despite the ubiquitous presence of the CAR receptor (Fig. 2A). We detected little or no CPG2 activity (<0.07 units/g) in the brains, bone marrow, kidneys, spleens, ovaries, guts, or lungs from the animals. The two nonmalignant tissues with the highest CPG2 activity levels were muscle (0.2 units/g, 102-fold less than tumors) and livers (0.7 units/g, 29-fold less than tumors). These results indicate selective targeting of AdV.hTERT-CPG2 for colon carcinoma cells in our in vivo model.

To test the efficacy of the GDEPT therapy in vivo, we administered the AdV.hTERT-CPG2 adenovirus systemically, followed by the prodrug ZD2767P, to nude mice bearing SW620 xenografts. In order to select the lowest optimum dose of AdV.hTERT-CPG2 for these experiments, we initially assessed three different viral dosages (10¹¹, 3.3 × 10¹¹, and 6.7 × 10¹¹ vp/kg). We examined the concentration of CPG2 expressed in the tumors, livers, and plasmas at 4, 8, and 14 days after virus administration (Table 2). Based on these results, we selected a dose of 10¹¹ vp/kg, which gave excellent tumor/liver and tumor/plasma ratios (803:1 and 398:1, respectively, 8 days after virus administration). Neither AdV.hTERT-CPG2 nor the prodrug alone affected the growth of the xenografts, whereas this GDEPT in combination induced a significant tumor growth delay (Fig. 2C). The average body weight

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5 http://www.autocomet.com/main_home.php

<table>
<thead>
<tr>
<th>Cell line</th>
<th>EC₅₀ (pfu/cell)</th>
<th>CPG2 activity (units/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SW480</td>
<td>0.02 (2 × 10⁻³–0.2)</td>
<td>4.17</td>
</tr>
<tr>
<td>HCT 116</td>
<td>0.06 (0.02–0.19)</td>
<td>1.58</td>
</tr>
<tr>
<td>SW620</td>
<td>0.07 (0.02–0.18)</td>
<td>2.9</td>
</tr>
<tr>
<td>LS 174T</td>
<td>0.18 (0.11–0.26)</td>
<td>2.05</td>
</tr>
<tr>
<td>LoVo</td>
<td>0.33 (0.26–0.47)</td>
<td>0.79</td>
</tr>
<tr>
<td>DLD-1</td>
<td>0.48 (0.39–0.6)</td>
<td>3.31</td>
</tr>
<tr>
<td>HT-29</td>
<td>0.76 (0.65–0.84)</td>
<td>2.67</td>
</tr>
<tr>
<td>Caco-2</td>
<td>0.77 (0.57–1.04)</td>
<td>2.12</td>
</tr>
<tr>
<td>BE</td>
<td>1.19 (0.05–2.88)</td>
<td>0.26</td>
</tr>
<tr>
<td>Colo 205</td>
<td>511 (457–572.3)</td>
<td>0.3</td>
</tr>
</tbody>
</table>

NOTE: EC₅₀ effective viral dose required for 50% cell kill; the values in parentheses indicate the 95% confidence intervals. Cell survival was determined 7 d after infection. For activity assays, cells were infected for 3 d using 0.1 pfu/cell.
losses (% of initial weight ± SE) while at least five animals remained were not more than 6.2 ± 3.3 for GDEPT, 3 ± 2.1 for the adenovirus, and 5.1 ± 2.1 for the prodrug alone, compared with 4.7 ± 1.1 in the control group, indicating that the treated groups had limited or no toxicity compared with the controls. The median survival of the controls or the animals that received only the adenovirus was 52 days (Fig. 2D). ZD2767P without AdV.hTERT-CPG2 did not increase median survival (Fig. 2D). In contrast, GDEPT more than doubled the control survival to 111 days (Fig. 2D). This showed that in combination with ZD2767P, AdV.hTERT-CPG2 is effective even when virus-mediated tumor cell killing is low (Fig. 2C and D). Our data show that arming an oncolytic adenovirus with CPG2 improves its efficacy, thus allowing the use of lower viral doses and improving the safety of adenoviruses.

A consideration when engineering an oncolytic adenovirus for GDEPT is that the activated drug may inhibit viral replication. To investigate the effect of GDEPT on the replication of the adenovirus in vivo, we used quantitative real-time PCR and measured the adenoviral copy numbers in SW620 xenografts of mice that had received a single tail vein injection of AdV.hTERT-CPG2 or AdV.hTERT-CPG2 in combination with prodrug (Fig. 3A). We detected ~10 times more adenoviral particles per gram of tumor in the GDEPT-treated animals compared with the animals that had only been injected with the adenovirus (Fig. 3A). There was a correlation between the adenoviral load and CPG2 enzyme activity in the tumors; CPG2 activity levels were 3.6-fold higher in the tumors after AdV.hTERT-CPG2 plus ZD2767P treatment, compared with the adenovirus alone (Fig. 3B). Following a single injection of ~2.5 × 10^11 viral particles per mouse, we detected a total viral load in the tumors of 1.7 × 10^11 (GDEPT) and 1.1 × 10^11 particles (virus only) 8 days later, a 4-fold increase over the inoculum, proving that AdV.hTERT-CPG2 had replicated. Thus, CPG2-activated ZD2767P does not inhibit the replication of AdV.hTERT-CPG2 in vivo. Moreover, GDEPT facilitates the accumulation of the adenovirus in colon cancer xenografts. Activation of ZD2767P results in higher CPG2 expression levels from the adenovirus, which in turn results in the conversion of a larger number of prodrug molecules, providing an amplification effect. By contrast, it was reported that the vaccinia/purine nucleoside phosphorylase system abolished viral replication in the presence of the prodrug (21). Another study described the antiviral effect of the activated prodrug generated by a vaccinia vector expressing cytosine deaminase (22). Also, thymidine kinase–activated prodrug inhibited the replication of herpes simplex virus 1 vectors (23, 24), and the prodrug CIB1954 was shown to block the replication of a nitroreductase-expressing oncolytic adenovirus (25). Of note, here we find that the CPG2-activated prodrug ZD2767P actually enhances the viral particle concentration in AdV.hTERT-CPG2–infected tumors with a concomitant increase in CPG2 concentration. This positive feedback can therefore increase both GDEPT and viral antitumor efficacy.

To our knowledge, this advantageous cooperation between an oncolytic vector and activated prodrug has not been observed in any other GDEPT system. Thus, AdV.hTERT-CPG2 is an excellent vector for CPG2-mediated GDEPT in vivo.

We examined the mechanism by which the GDEPT kills tumor cells and investigated the consequences of prodrug activation on SW620 cells using single cell gel electrophoresis (“comet”) assays. In untreated cells, hydrogen peroxide treatment induced DNA fragmentation, resulting in comet tails (ref. 15; Fig. 3C). The DNA cross-linking agents nitrogen mustard and cisplatin prevented the migration of the DNA from the nuclei, retarding the electrophoretic mobility and reducing the comet tails (Fig. 3C). ZD2767P alone did not induce substantial DNA...
cross-links in uninfected cells or cells that were infected with the nonexpressing adenovirus AdV.hTERT (Fig. 3C). Similarly, the adenoviruses alone did not affect comet tail formation (Fig. 3C). However, AdV.hTERT-CPG2 plus ZD2767P significantly reduced the comet tails, showing that the activated prodrug cross-linked the DNA (Fig. 3C). Cross-linking prevents DNA from being separated for synthesis and cell division, killing the cancer cells in S-G2-M phase, which is also when AdV.hTERT-CPG2 arrests infected cells (Fig. 1A), suggesting that both components of our therapy may cooperate (Fig. 1C).

### Table 2. CPG2 biodistribution after systemic delivery of AdV.hTERT-CPG2

<table>
<thead>
<tr>
<th>Viral dose (vp/kg)</th>
<th>Days after virus administration</th>
<th>Tumor (units/g)</th>
<th>Liver (units/g)</th>
<th>Plasma (units/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(10^{11})</td>
<td>4</td>
<td>0.76 ± 0.62</td>
<td>0.08 ± 0.12</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>5.46 ± 11.1</td>
<td>0.07 ± 0.02</td>
<td>0.01 ± 0.03</td>
</tr>
<tr>
<td></td>
<td>14</td>
<td>4.52 ± 1.7</td>
<td>0.07 ± 0.06</td>
<td>0.01 ± 0.01</td>
</tr>
<tr>
<td>(3.3 \times 10^{11})</td>
<td>4</td>
<td>11.92 ± 10.22</td>
<td>0.01 ± 0.01</td>
<td>0.01 ± 0.01</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>3.22 ± 1.7</td>
<td>0</td>
<td>0.01 ± 0.01</td>
</tr>
<tr>
<td></td>
<td>14</td>
<td>12.59 ± 8.42</td>
<td>0.02 ± 0.04</td>
<td>0.03 ± 0.04</td>
</tr>
<tr>
<td>(6.7 \times 10^{11})</td>
<td>4</td>
<td>53.63 ± 97.36</td>
<td>0.06 ± 0.04</td>
<td>0.03 ± 0.03</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>15.75 ± 8.36</td>
<td>0.03 ± 0.03</td>
<td>0.02 ± 0.01</td>
</tr>
<tr>
<td></td>
<td>14</td>
<td>8.49 ± 5.04</td>
<td>0.03 ± 0.02</td>
<td>0.02 ± 0.01</td>
</tr>
</tbody>
</table>

NOTE: CPG2 activity data are the means from five animals ± SD.
We then tested whether GDEPT induces apoptosis in SW620 cells. We did two different apoptosis assays: loss of membrane phospholipid asymmetry and selective permeability were studied using the flow cytometric Annexin V-propidium iodide assay, whereas PARP cleavage was assessed by Western blotting. Both assays showed the induction of apoptosis following GDEPT treatment (Fig. 3D). By contrast, no evidence of apoptosis was seen in untreated cells or in cells that received either the
AdV.hTERT-CPG2 adenovirus or the prodrug alone (Fig. 3D). Together, these data show that CPG2-activated ZD2767P kills human colorectal cancer cells by forming DNA cross-links and inducing apoptosis. They also indicate that the GDEPT cooperates with the replicating adenovirus to kill the tumor cells.

We next assessed the bystander effect of our AdV.hTERT-CPG2/ZD2767P therapy using mixed cultures of WM-266-4 cells, which are poorly infected by adenovirus and therefore do not express CPG2 following treatment with AdV.hTERT-CPG2 and SW620 cells, which are ~800-fold more receptive to adenovirus-mediated killing and express 2.9 units/g of CPG2 following infection (Table 1). Initially, we tested whether the adenovirus alone could mediate a bystander effect. We used a viral dose that selectively lysed only the SW620 cells in the cocultures (0.2 pfu/cell). Fifty percent cell death was achieved when only 8.8% of the cells were SW620, indicating that AdV.hTERT-CPG2 could induce bystander killing on its own in the absence of prodrug (Fig. 4A).

Next, we examined the GDEPT-mediated bystander effect. We treated the WM-266-4/SW620 cocultures with low levels of AdV.hTERT-CPG2 (0.002 pfu/cell) that do not mediate a substantial viral-mediated bystander effect. Under these conditions, in the presence of ZD2767P, the GDEPT mounted a bystander effect with 50% of the cells in the culture being killed when only 9% of the cells were CPG2-expressing SW620 cells (Fig. 4B). The data suggests that the AdV.hTERT/ZD2767P therapy mounts two types of bystander effects: prodrug-mediated and virus-mediated.

We then examined the in vivo effect of our therapy on established tumors (Fig. 4C). We treated mice bearing SW620 xenografts systemically with AdV.hTERT-CPG2, followed by two courses of ZD2767P, and examined the expression of CPG2 and the apoptosis marker cleaved caspase-3 in the tumors by immunofluorescence. CPG2 was detected only in the tumors of animals that had received the adenovirus, but not in the controls or in the prodrug alone group (Fig. 4C). AdV.hTERT-CPG2 without prodrug did not activate caspase-3. By contrast, substantial apoptosis was seen in the tumors after GDEPT treatment (Fig. 4C). These findings confirmed the in vitro data.

Figure 4. In vivo bystander effect and apoptosis. A, virus-mediated bystander effect. SW620 and WM-266-4 cells were mixed at various proportions and infected using AdV.hTERT-CPG2 at a dose that only lysed SW620 cells (0.2 pfu/cell). Cell survival was expressed as the percentage of uninfected cells. Dashed line, prediction of cell survival in the absence of a bystander effect. Points, combined results from three experiments; bars, SE. B, GDEPT-mediated bystander effect. SW620/WM-266-4 cocultures were treated with AdV.hTERT-CPG2 (0.002 pfu/cell), followed by 50 μmol/L of ZD2767P. Under the experimental conditions used, only the SW620 cells expressed CPG2. Cell survival was determined as the percentage of infected cells that did not receive any prodrug. C, apoptosis and bystander effect in vivo. AdV.hTERT-CPG2 was administered via a single tail vein injection into mice bearing SW620 xenografts, followed by systemic ZD2767P. Detection of CPG2 (red), cleaved caspase-3 (a marker for apoptosis; green), and colocalization (yellow) in the tumors by immunofluorescent labeling.
(Fig. 3D) and showed that the oncolytic adenovirus and GDEPT kill tumor cells via different mechanisms, making it less likely for resistance to the combined treatment to develop. Notably, in GDEPT-treated tumors, we detected apoptosis in uninfected cells that did not express CPG2, showing a GDEPT-mediated bystander effect of the therapy in vivo (Fig. 4C). We have thus shown an effective GDEPT system which can selectively and systemically target CPG2 to tumors for the activation of a prodrug capable of killing cells by apoptosis and of providing a bystander effect.

References


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