Systemic Gene-Directed Enzyme Prodrug Therapy of Hepatocellular Carcinoma Using a Targeted Adenovirus Armed with Carboxypeptidase G2

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Abstract

Hepatocellular carcinoma is the fifth most common cancer worldwide, and there is no effective therapy for unresectable disease. We have developed a targeted systemic therapy for hepatocellular carcinoma. The gene for a foreign enzyme is selectively expressed in the tumor cells and a nontoxic prodrug is then given, which is activated to a potent cytotoxic drug by the tumor-localized enzyme. This approach is termed gene-directed enzyme prodrug therapy (GDEPT). Adenoviruses have been used to target cancer cells, have an intrinsic tropism for liver, and are efficient gene vectors. Oncolytic adenoviruses produce clinical benefits, particularly in combination with conventional anticancer agents and are well tolerated. We rationalized that such adenoviruses, if their expression were restricted to telomerase-positive cancer cells, would make excellent gene vectors for GDEPT therapy of hepatocellular carcinoma. Here we use an oncolytic adenovirus to deliver the prodrug-activating enzyme carboxypeptidase G2 (CPG2) to tumors in a single systemic administration. The adenovirus replicated and produced high levels of CPG2 in two different hepatocellular carcinoma xenografts (Hep3B and HepG2) but not other tissues. GDEPT enhanced the adenovirus-alone therapy to elicit tumor regressions in the hepatocellular carcinoma models. This is the first time that CPG2 has been targeted and expressed intracellularly to effect significant therapy, showing that the combined approach holds enormous potential as a tumor-selective therapy for the systemic treatment of hepatocellular carcinoma. (Cancer Res 2005; 65(12): 5003-8)

Introduction

Systemic cancer treatments often lack specificity, leading to unwanted toxicity in healthy tissues. Gene-directed enzyme prodrug therapy (GDEPT) is a suicide gene therapy approach that has been developed to target tumor cells selectively (1). The GDEPT enzyme carboxypeptidase G2 (CPG2) converts alkylation agent mustard prodrugs, such as ZD2767P (2, 3), into potent cytotoxic drugs that induce apoptosis. The use of CPG2 when transfected and expressed as a surface-tethered enzyme in model nontargeted GDEPT protocols has been shown (3, 4). Oncolytic adenoviruses target all cancer cells but have an intrinsic tropism for liver; they are also efficient gene vectors (5). They are well tolerated and produce clinical benefits, particularly in combination with conventional anticancer agents (6–8). Adenoviral vectors have been used with other GDEPT systems where one of the two components has been given systemically. A replication-selective adenovirus that expressed rabbit carboxylesterase was given i.v., which activates the intratumorally (i.t.) given prodrug CPT-11 (9). Another system used an i.t. injected replication-selective adenovirus expressing the Escherichia coli nitroreductase enzyme, which activates the i.p. given prodrug CB1954 (10). To our knowledge, our GDEPT system is the only one that has been used where both vector and prodrug components are systemic, which is ultimately desirable for clinical application. The virus replicates in and kills tumor cells selectively both in vitro and in vivo, while also delivering CPG2 and rendering tumor cells sensitive to ZD2767P. Our results show that GDEPT synergizes with viral oncolysis, significantly enhancing the efficacy of the adenovirus.

Materials and Methods

Cell lines and adenoviruses. HepG2, Hep3B, and Wi38.VA13/2RA cells were obtained from American Type Culture Collection (LGC Promochem, London, United Kingdom). Replication-deficient adenoviruses were generated using the AdEasy vector system and 293 cells (Qbiogene, Promochem, London, United Kingdom). AE1-2a cells were used to produce the oncolytic adenoviruses (11). The ratio of viral particles to plaque-forming units (pfu) was 1:50 for AdV.hTERT-CPG2 and 1:6.4 for AdV.hTERT. Both viruses contain a 245-bp fragment of the hTERT promoter containing 167 bp of downstream sequence that is upstream of the transcription start site and 78 bp of down- stream sequence. cpg2 has been described (1) and was cloned into AdV.hTERT-CPG2 using published protocols (12).

Western immunoblot analysis. Unless stated otherwise, cells were infected with 100 (AdV.CMV-GFP) or 2.5 × 10⁻⁴ (AdV.hTERT-CPG2; multiplicity of infection, MOI of 5 × 10⁻⁴) particles per cell (ppc) and analyzed. Protein detection was performed using CPG2-specific antiserum (1), anti-Ad5 capsid protein (ab6982, Abcam, Cambridge, United Kingdom), anti-GFP antiserum (a kind gift of Dr. Doreen Cantrell, Dundee, United Kingdom), anti-ERK2 (C-14, Santa Cruz Biotechnology, Santa Cruz, CA), and anti-E1A antibody (MS8, Neomarkers, Strachet Scientific, Sobbam, United Kingdom).

Activity assays. For time course studies (Fig. 1D), cells were infected with oncolytic adenoviruses using 2.5 × 10⁻⁴ ppc (MOI = 5 × 10⁻⁴) or 5 ppc for AdV.CMV-CPG2. In Figs. 1F and 2C, cells were infected using 5 ppc (MOI = 0.1). CPG2 assays were as described (1, 13). Prodrug synthesis and cytotoxicity assays. ZD2767P was synthesized as described (2). For GDEPT, cells were infected in 24-well plates using 0.5 ppc; 100-fold stocks of ZD2767P were prepared in DMSO 72 hours after infection and added to the cells. After 24 hours, 2% of the cells were replated...
Figure 1. AdV.hTERT-CPG2 is a tumor-selective oncolytic adenovirus that expresses CPG2 in cancer cells. A, schematic representations of the adenoviruses used in this study. AdV.hTERT-CPG2 and AdV.hTERT are replicating adenoviruses with the E1a gene under the control of the hTERT promoter. Additionally, AdV.hTERT-CPG2 encodes for CPG2. AdV.CMV-CPG2 and AdV.CMV-GFP are replication-deficient adenoviruses that express CPG2 and GFP, respectively, under the control of a CMV promoter. Dose-dependent expression of CPG2 from AdV.hTERT-CPG2 (B) and AdV.CMV-CPG2 (C). Hep3B cells were infected and lysates were analyzed 3 days later for CPG2 and adenoviral hexon protein by Western blotting. D, CPG2 activity was measured in triplicate at various times following adenoviral infection of Hep3B cells. AdV.hTERT-CPG2 and AdV.hTERT, 2.5 × 10^−2 ppc; AdV.CMV-CPG2, 5 ppc. Columns, means; bars, ± SE. E1a expression following infection of Hep3B and Wi38.VA13 cells with AdV.hTERT-CPG2 at 2.5 × 10^−2 ppc. E, CPG2 enzyme activity in the 10-day lysate from (E). G, AdV.hTERT-CPG2 selectively kills cancer cell lines. Phase-contrast photograph of Hep3B and Wi38.VA13 5 days after infection with AdV.hTERT-CPG2 (2.5 ppc). F, GFP expression in Hep3B and Wi38.VA13 cells three days after infection with AdV.CMV-GFP by (H) Western blot (100 ppc) and (I) fluorescence (1,000 ppc). The transduction efficiency was ~80%. Equal protein loading was confirmed by probing the blots for ERK2 (C, E, and H).
into 96-well plates and after a further 6 days, cell survival was determined using the CellTiter 96 Aqueous Nonradioactive Cell Proliferation Assay (Promega, Southampton, United Kingdom). Results are expressed relative to untreated controls and IC_{50} values are defined as the prodrug concentration or viral particle number per cell required to kill half of the cells. For photographs of cytopathic effects (Fig. 1C, 20× magnification), cells were infected for 5 days using 2.5 ppc (AdV.hTERT-CPG2) or 1.000 ppc (AdV.CMV-GFP).

**Viral/prodrug interactions.** AdV.hTERT was incubated with 50 μM/L ZD2767P and 10 milliunits/mL purified CPG2 for 1.5 hours at 37°C and subsequently used to infect Hep3B cells for cell survival determination. The potency of this mixture was confirmed by adding it to Hep3B cells, which resulted in total cell death (data not shown). To determine viral burst, Hep3B cells were infected with AdV.hTERT-CPG2 (0.5 pcc, MOI = 0.01) and 50 μM/L ZD2767P were added after 72 hours. After 3 days, cell lysates were prepared by five rounds of freeze/thawing and used to infect 293 cells. The cells were overlaid with agarose and plaques were counted 17 days later.

**Real-time PCR.** Animals were injected with 10^{12} viral particles/kg (2 × 10^{10} pfu/kg) i.v. DNA from different tissues was isolated using the DNeasy tissue kit (Qiagen, Crawley, United Kingdom). AdV.hTERT-CPG2 was added to untreated tissue homogenates for standard curves. Primers were derived from the cpg2 gene (1): 5′ primer, 5′-CGAGGAGGAGAAAGGTTCTTCT-3′; 3′ primer, 5′-TCGCCACCTGGTGGCT-3′; probe, 5′-[6-FAM]TGCAGCCGACCTGGATCTCAGAA[TAMRA-6-FAM]-3′ (Qiagen). Samples were derived in triplicate using the ABI PRISM 7700 sequence Detection System (Applied Biosystems, Cheshire, United Kingdom) and the Quantitect Probe PCR kit (Qiagen). The sensitivity limit was 10^7 viral particles per gram tissue.

**Animals.** Xenografts were established in female athymic mice (Crl:Cd1-Fox nu/nu, 9 weeks old; Charles River, Kent, United Kingdom) by injecting 10^5 cells s.c. into the right flank (eight animals per group). After 14 days, PBS or adenovirus was given into the tail vein (10^{12} viral particles/kg, which equals 2 × 10^{10} pfu/kg and 2.5 × 10^{10} viral particles per mouse with an average body weight of 25 g) following allocation to treatment groups by stratified distribution on tumor size. For targeting studies (PCR, activity assays), tumors and livers were collected at times after viral infection as stated. For adenovirus/prodrug therapy, ZD2767P (300 mg/kg) was given as described (3) 7 days after adenovirus. Additional courses of prodrug (six doses in total) were injected at weekly intervals for Hep3B or every 7 to 12 days (HepG2 xenografts). Tumor volumes were calculated relative to the mean of the first two measurements and plotted, in the treatment groups, while at least five animals survived. The plots for Hep3B xenografts show the combined analyses of two therapies. Doubling times were generated from nonlinear exponential fitting using GraphPad Prism version 4.02 for Windows (GraphPad Software, San Diego, CA). Animals were culled when tumors reached 1.7 cm in any dimension or a mean of 1.5 cm in two perpendicular dimensions. Alanine aminotransferase (ALT) serum concentrations (Beckman Coulter, Kent, United Kingdom) were compared by unpaired, two-tailed t test.

**Figure 2.** AdV.hTERT-CPG2 is an effective vector for GDEPT. A, AdV.hTERT-CPG2 increases the sensitivity of HepG2 cells to the prodrug ZD2767P. Uninfected cells (IC_{50} = 10.8 μM/L) or cells infected with 0.5 ppc AdV.hTERT-CPG2 (IC_{50} = 0.6 μM/L) were treated with prodrug and cell viability was determined. The 95% confidence intervals did not overlap (uninfected cells: 4.9-23.7 μM/L and infected cells: 0.08-4.2 μM/L). AdV.hTERT-CPG2 mediated (B) cytotoxicity and (C) CPG2 expression in Hep3B and HepG2 cells. Columns, means; bars, ±SE. Each experiment was done in triplicate. D, the activated form of ZD2767P does not destroy the cytotoxicity of the adenoviruses. Hep3B cells were infected in triplicate with AdV.hTERT (IC_{50} = 0.02 ppc) or with AdV.hTERT-CPG2 (IC_{50} = 0.05 ppc). No significant difference in cytotoxicity was observed (95% confidence intervals, 6.7 × 10^{-3} to 3.3 × 10^{-2} and 1.7 × 10^{-2} to 1.2 × 10^{-1} ppc, respectively). E, viral burst of AdV.hTERT-CPG2 infected Hep3B cells in the absence or presence of prodrug. Differences between prodrug-treated and untreated samples were not significant. Columns, means from three experiments; bars, ±SE.
The normal telomerase reverse transcriptase gene (hTERT) is silent in normal mouse cells but is reactivated in >85% of human cancers (14). We placed the adenoviral E1a gene under the control of the hTERT promoter, creating the oncolytic adenovirus AdV.hTERT (Fig. 1A). Subsequently, we replaced the viral E3gp 19-kDa gene (15) with the *Pseudomonas* gene cpg2 (AdV.hTERT-CPG2), placing cpg2 under the control of the viral E3 promoter (Fig. 1A).

CPG2 was not expressed when we infected the hepatocellular carcinoma cell line Hep3B with AdV.hTERT but was present in cells infected with AdV.hTERT-CPG2 or a nonreplicating adenovirus expressing CPG2 from the cytomegalovirus (CMV) promoter (AdV.CMV-CPG2; Fig. 1A), the amount of protein corresponding to the viral dose applied (Fig. 1B-C). We did not detect CPG2 enzyme activity in Hep3B cells infected with AdV.hTERT, and as expected for a replicating adenovirus, the levels of CPG2 enzyme activity increased with time following infection with AdV.hTERT-CPG2 (Fig. 1D). By contrast, CPG2 activity in the cells infected with AdV.CMV-CPG2 was extremely low at this level of infectivity and did not increase with time (Fig. 1D). We did not detect adenoviral E1a protein or CPG2 expression in AdV.hTERT-CPG2-infected WI-38VA13 cells, a telomerase-negative cell line (ref. 16; Fig. 1E-F) and AdV.hTERT-CPG2 killed Hep3B but not WI-38VA13 cells (Fig. 1G). However, we detected GFP in WI-38VA13 cells infected with the nonreplicating adenovirus AdV.CMV-GFP (Fig. 1A and H-I), showing that these cells are susceptible to adenoviral infection. Together, these results show that replicating adenoviruses are more efficient than nonreplicating adenoviruses at delivering CPG2 to hepatocellular carcinoma, but that AdV.hTERT-CPG2 does not deliver CPG2 to cells in which the telomerase promoter is silent.

We hypothesized that the components of our system would synergize because adenoviruses trap cells in the S phase of the cell cycle (17), which is when activated ZD2767P kills cells. Indeed, AdV.hTERT-CPG2 sensitized the hepatocellular carcinoma cell line HepG2 to ZD2767P ~20-fold (Fig. 2A-D), despite the fact that HepG2 cells were 100-fold less receptive to adenoviral-mediated killing than Hep3B cells (Fig. 2B) and expressed 4.5 times less CPG2 (Fig. 2C). The reasons for the apparent lower susceptibility of HepG2 cells in vitro are not known. Both cell lines express the CAR receptor and high levels of hTERT mRNA, but Hep3B cells internalize adenoviral particles more efficiently than HepG2 cells (18–20). However, both cell lines are comparably efficient at transferring adenoviral DNA into the nucleus and replicating the viral genome (20). Exogenous CPG2 plus prodrug did not affect the cytotoxic effect of AdV.hTERT viral particles (Fig. 2D and beneficially, ZD2767P did not affect the viral burst size of AdV.hTERT-CPG2 (Fig. 2F). Thus, our adenovirus is an excellent vector for GDEPT, even in adenovirus-resistant hepatocellular carcinoma cell lines, effecting cell death with ZD2767P.

We tested adenoviral targeting by injecting mice bearing Hep3B xenografts with AdV.hTERT-CPG2 via a single tail vein injection and characterizing the tumors. CPG2 enzyme activity in the tumors of these animals peaked at ~4 units/g 15 days after infection but was not found in their livers or lungs (Fig. 3A). We used quantitative real-time PCR to measure viral copy numbers in the mice (Fig. 3B). Following a single injection of 2.5 × 10^10 viral particles per mouse, we detected a total tumor load of 9.6 × 10^10 particles 15 days later, an increase of ~4-fold over the inoculum, proving that viral replication had occurred in the tumors. Note that there was a close correlation between viral load and CPG2 activity in the tumors (Fig. 3A-B).

We then treated mice bearing palpable Hep3B or HepG2 hepatocellular carcinoma xenografts systemically with the adenovirus followed by ZD2767P. As shown previously with other cell lines (3), ZD2767P alone does not significantly affect the growth of Hep3B or HepG2 tumor xenografts (data not shown). In two independent experiments with Hep3B xenografts (plotted combined), we found that AdV.hTERT-CPG2 alone caused a growth delay (Fig. 3C; tumor volume doubling times: controls, 7.6 days; AdV.hTERT-CPG2, 15.4 days), whereas GDEPT induced a substantial growth delay (Fig. 3C; 39.9 days). In HepG2 xenografts, we observed a similar growth delay with AdV.hTERT-CPG2 alone (Fig. 3D; controls, 6 days; AdV.hTERT-CPG2, 13.2 days), but importantly, HepG2 tumor growth was significantly reduced by GDEPT (Fig. 3D; 28.2 days). In the Hep3B xenografts, AdV.hTERT-CPG2 alone only increased the 75% quartile survival from 33.8 to 44.8 days, whereas GDEPT almost doubled the control survival to 64 days (Fig. 3E). Similarly, the 75% quartile survival of the HepG2 xenografts treated with AdV.hTERT-CPG2 alone only increased from 36.8 to 39 days, whereas GDEPT more than doubled the control survival to 74.3 days (Fig. 3F). Median survivals are not quoted because of the high survival at 90 days in the GDEPT group. Two animals in the HepG2 GDEPT group underwent total tumor regression. These results show that GDEPT synergizes with AdV.hTERT-CPG2 to inhibit tumor growth and is even effective in tumors where *in vitro* viral-mediated cell killing is low (Fig. 2B).

In these studies, we have shown that replicating adenoviruses can deliver CPG2 to tumors for GDEPT protocols. This is the first time that cytotoxic CPG2 has been shown effective in GDEPT protocols. CPG2 has several advantages as a prodrug-activating enzyme. There is no equivalent human enzyme; thus, endogenous prodrug activation does not occur; it does not require cofactors whose availability could be limited or absent in tumors; it converts prodrugs directly to cytotoxic drugs without requiring intermediate host enzymes that could be deficient/defective in tumor cells. Furthermore, activated ZD2767P kills cycling and noncycling cells; thus, quiescent tumor cells are unlikely to survive and regrow. GDEPT mounts a robust bystander effect, which is important, as although the adenovirus is engineered to target only cancer cells and thus spare normal tissue, it is unlikely that all cells of a tumor (particularly noncancer stromal cells) will become infected. Using GDEPT, those cells not targeted can still be killed through the bystander effect. Thus, we have shown that CPG2 plus ZD2767P cooperate with replicating adenoviral vectors to induce hepatocellular carcinoma cell killing.

The mean bodyweights of the animals were not significantly affected by the GDEPT protocols (data not shown) and serum ALT levels were not significantly elevated (untreated animals: 72 ± 28 IU/L, GDEPT: 53 ± 20 IU/L; *P > 0.05*), indicating that there was no hepatotoxicity, which can be a problem with adenoviral vectors (11). In this study, we have used 10^13 particles/kg systemically. A similar total dose has been safely used in man (21), suggesting that AdV.hTERT-CPG2 would be well tolerated in patients. We could detect no CPG2 in gut, brain, spleen, kidney, or muscle from mice bearing either Hep3B or HepG2 xenografts and given virus, despite the fact that adenoviruses will infect murine cells and that the hTERT promoter is active in mouse cells (14, 22). In livers and lungs from these mice, average CPG2 concentrations were at least 270 times lower than in tumors [except in two cases when tumor levels were very high (tumors: 24.1 and 56.2 units/g; livers: 2.1 and
1.3 units/g, respectively)]. This shows selectivity of the AdV.hTERT-CPG2 for hepatocellular carcinoma cells. ZD2767P toxicology and pharmacokinetics have been examined in patients in non-GDEPT studies (23). The prodrug is well tolerated and rapidly cleared from the circulation in patients. The cytotoxic agent formed from ZD2767P is the bifunctional DNA interstrand cross-linking alkylating agent 4-(bis(2-iodoethyl)amino)phenol and the short half-life of the active drug prevents it from diffusing from the tumors into the circulation (2). Moreover, quiescent normal hepatocytes will be intrinsically more resistant than the rapidly dividing tumor cells. Normal liver toxicity is not therefore expected in this system.

We have previously shown that 0.5 units/g CPG2 in tumors was sufficient to activate prodrug in patients when antibodies were used to target CPG2 (24). Here we show that tumoral CPG2 concentrations were extremely high following a single systemic adenovirus administration. In the combined adenovirus-treated groups from both Hep3B therapy experiments, the mean CPG2 concentration at death was $11.7 \pm 3.1$ units/g SE. This indicates that the AdV.hTERT-CPG2 replicated in the tumors. In the HepG2 tumors, we measured slightly lower mean CPG2 levels at death ($3.2 \pm 1.7$ units/g), which is consistent with our in vitro data (Fig. 2C). The CPG2 concentrations achieved here far exceed those obtained when using an antibody-CPG2 fusion protein to target the enzyme to tumors (peak $1 \pm 1$ units/g, 8 hours after injection; ref. 25).

We have shown that the adenovirus synergized with GDEPT in vitro to induce cancer cell killing and in vivo, it cooperated with
ZD2767P to inhibit tumor growth, and extended the life span of the mice. Importantly, five animals from the GDEPT group were culled in accordance with humane practice under the UK Home Office Licence regulations because although their tumors were small, they developed lesions. Four of these animals (25% of the total treatment group) showed clear responses to the therapy. In contrast, all of the control mice and the 13 mice culled following adenovirus treatment alone (81% of the treatment group) were culled because their tumors reached size limits. To our knowledge, this is the first time that a single systematic administration of a replicating viral vector has shown significant efficacy against human tumor xenografts in GDEPT protocols.

Hepatocellular carcinoma is the third most common cause of cancer-related deaths worldwide (26). Oncolytic adenoviruses are attractive delivery vectors for liver gene therapy because of their natural tropism for hepatocytes and because they can be given by hepatic artery infusion, generally with only mild to moderate adverse events (21, 27). Unarmed oncolytic adenoviruses have only achieved partial tumor responses; thus, approaches that improve their potency are urgently required. Our data shows that arming AdVhtERT with CPG2 provides a promising strategy to improve the efficacy and safety of oncolytic adenoviruses. The hTERT gene is active in a wide range of human cancers, which should enable broad application of AdVhtERT-CPG2 to different cancer types.

Our approach has a multifaceted mode of attacking tumors. First, there are successive rounds of adenovirus-mediated cancer cell killing. Second, the adenovirus delivers long-lived expression of CPG2, which is able to activate a large number of prodruk molecules, providing an amplification effect. Third, the bystander effect leads to killing of uninfected stromal cells (e.g., vasculature), which would otherwise escape the effects of the oncolytic adenovirus. Finally, it should be emphasized that both adenoviruses and the prodruk can be given systemically and that both have already been assessed separately in clinical trials. We show here that we have developed a targeted, tractable GDEPT therapy designed for clinical applications.

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References

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