

Adenovirus vector-mediated delivery of the prodrug-converting enzyme carboxypeptidase G2 in a secreted or GPI-anchored form: High-level expression of this active conditional cytotoxic enzyme at the plasma membrane

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Carboxypeptidase G2 (CPG2) is a powerful prodrug-converting enzyme. Without a requirement for endogenous enzymes or cofactors, it can directly activate mustard alkylating prodrugs to cytotoxic species, killing both quiescent and dividing cells. This paper provides the first report of its use in the context of a clinically relevant delivery vehicle using adenovirus vectors. To strengthen the efficacy of the prodrug-activating system, the enzyme has been engineered to be secreted or glycosylphosphatidylinositol (GPI) anchored to the extracellular membrane of tumor cells, resulting in an enhanced bystander effect by facilitating diffusion of the active drug through extracellular, rather than intracellular, activation. Using the vectors, we have achieved expression of functional secreted or GPI-anchored CPG2 in a panel of tumor cell lines demonstrating no loss in efficacy as a result of GPI anchor retention. Despite variable transduction efficiencies inherent to these vectors, greater than 50% cell kill was achievable in all of the cell lines tested following only a single exposure to the prodrug ZD2767P. Even in cell lines refractive to infection with the vectors, substantial cell death was recorded, indicative of the enhanced bystander effect generated following extracellular prodrug activation. A direct evaluation of the efficacy of our system has been made against adenoviral delivery of herpes simplex virus thymidine kinase plus ganciclovir (GCV), a suicide gene therapy approach already in the clinic. In a short-term human glioma culture (IN1760) resistant to the clinical chemotherapeutic drug CCNU (1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea), thymidine kinase/GCV effected no cell killing compared to 70% cell killing with our system.

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Existing treatments for cancers using localized therapies such as surgery and radiotherapy only succeed in about one-third of patients, with the majority of cases requiring additional systemic therapy using anticancer drugs designed to target particular aspects of the cellular replication of tumor cells. The toxicity of these anticancer agents to normal cells is their major limitation; therefore, the current challenge in cancer chemotherapy is to create therapeutics with a larger

differential toxicity to normal and tumor cells and/or improved tumor cell killing.

To meet this challenge, great efforts have been made in the development of enzyme prodrug therapies.¹ Enzyme-activated prodrugs are chemical entities that are relatively inert in comparison to their active drugs, but which can be converted *in vivo* to pharmacologically active, toxic molecules by a specific activating enzyme. The enzyme can be targeted to the tumor cells by linking it chemically or genetically to an antibody recognizing a tumor-specific antigen,² or the tumor can be transduced with the gene encoding for the enzyme. By restricting enzyme expression to the tumor, when prodrug is administered systemically, its activation and subsequent cytotoxicity will be confined to the tumor mass. To achieve the levels of enzyme expression required for an effective therapy, viral vectors have been used to deliver the enzyme encoding genes to the tumor cells.

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Previously, first-generation adenoviruses have been used to deliver herpes simplex virus thymidine kinase (HSV-TK) and cytosine deaminase in clinical gene therapy trials.^{3–7}

In this paper, we have focused on the bacterial enzyme carboxypeptidase G2 (CPG2). CPG2 was isolated from the *Pseudomonas R16* strain⁸ from which the CPG2 gene has also been cloned.⁹ Its crystal structure has been determined, showing it to function as a zinc-dependent dimeric enzyme.¹⁰ CPG2 can activate mustard alkylating prodrugs to cytotoxic species by hydrolytic cleavage of the glutamate moiety, and as no mammalian homologue for CPG2 exists, this rules out the possibility of nonspecific prodrug conversion by endogenous enzymes. In combination with the prodrug CMDA (4-[(2-chloroethyl)(2-mesyloxyethyl)amino] benzoyl-L-glutamic acid), CPG2 is the only prodrug-converting enzyme to have progressed into phase I/II clinical trials using an antibody-directed enzyme prodrug therapy (ADEPT) approach.⁷ Subsequently, other prodrugs possessing optimal biological and chemical half-lives have been developed to achieve maximum bystander effects with minimum systemic toxicity. The active species of one of these drugs, ZD2767P or [4-[bis(2-iodoethyl)-amino]phenyl oxy carbonyl]-L-glutamic acid, has been shown to be 300-fold more potent than CMDA.¹¹

Marais *et al*¹² highlighted the potential of using CPG2 in a gene therapy approach by stably expressing the enzyme in human tumor cell lines. They achieved CPG2 expression within the extracellular space by linking the transmembrane region of the human tyrosine kinase receptor c-erb B2 to the C-terminus of CPG2. Cells expressing membrane-bound CPG2 were able to extracellularly activate the hydrophilic prodrug CMDA into its nonpolar cytotoxic metabolite (which can thus cross the plasma membrane) to elicit an important cytotoxic effect and also achieve an important bystander cell killing on neighboring cells.^{12–14}

Alternatively, naturally secreted proteins can be engineered for posttranslational glycosylphosphatidylinositol (GPI) anchoring into the extracellular membrane by the addition of the last exon of Thy1.^{15,16} Such GPI anchors are ubiquitous in eukaryotes and are employed by a wide variety of cell surface glycoproteins. The GPI enzyme hybrid will be secreted but retained on the outer lipid leaflet of the plasma membrane by its C-terminal GPI membrane anchor, thus achieving display of the active enzyme on the cell surface. We have previously demonstrated that HSV1-derived viral vectors can be used to target transgenes as GPI-anchored proteins to the outside leaflet of plasma membranes, without disrupting the targeting machinery of host epithelial cells or neurons.¹⁷

Within this report, we demonstrate that GPI anchoring of CPG2 to the extracellular membrane of tumor cells retains enzyme activity presumably by allowing it to fold and dimerize to form its natural enzymatic structure. Further, we have placed both the secreted and GPI-anchored CPG2 cassettes within a clinically relevant vector context to achieve enhanced tumor cell transduction. Adenoviral-mediated delivery enabled the achievement of sufficient expression levels of GPI-anchored CPG2 to sensitize tumor cells *in vitro* to the prodrug ZD2767P. Because CPG2 is anchored to the plasma membrane, it will avoid systemic

release *in vivo*, but still allow the extracellular conversion of prodrug to its cytotoxic counterpart, which is then able to diffuse throughout the tumor milieu to kill adjacent, uninfected tumor cells.

Methods

Cell culture

All cell culture reagents were purchased from Sigma Aldrich (Poole, Dorset, UK) for Gibco-BRL (Paisley, Glasgow, UK). All tissue culture plastic ware were purchased from Greiner (Stonehouse, Gloucestershire, UK). GL261 cells were kindly provided by Dr P Walker (Laboratory of Tumor Immunology, University Hospital Geneva, Switzerland) and the MT539MG cells from Dr YG Gillespie (Brain Tumor Research Laboratories, University of Alabama at Birmingham, Birmingham, AL). The two human glioma short-term cultures IN859 and IN1760 were generated by one of us (JL Darling; Institute of Neurology, Department of Neurology, University College-London, London, UK). LoVo cells were purchased from the American Tissue Culture Collection (Manassas, VA, USA) (ATCC no. CCL229). All cell lines were grown in culture medium composed of Dulbecco's modified Eagle's medium supplemented with sodium pyruvate (1 mM), glutamine (2 mM), nonessential amino acids (0.2 mM), 10% (vol/vol) horse serum, and 5% (vol/vol) newborn calf serum at 37°C, 5% CO₂.

Reagents and antibodies

CPG2 protein was detected using the primary antibodies, mouse monoclonal α CPG2#627 (diluted 1/1000) or rabbit polyclonal α CPG2#440 (diluted 1/2500), both provided by AstraZeneca (Cheshire, UK). The secondary antibody FITC-labeled goat α rabbit immunoglobulin (Ig) G (DAKO F0205, Carpinteria, CA, USA, diluted 1/50) was used for flow cytometry and immunocytochemical analysis and goat α mouse horseradish peroxidase-labeled antibody (DAKO PO448, diluted 1/200) for Western blot analysis. Phosphatidylinositol-specific phospholipase C (PI-PLC) was purchased from Oxford Glycosystems (Abingdon, Oxfordshire, UK). A total of 10⁶ cells were treated with 1 U/mL PI-PLC for 3 hours at 37°C. Tunicamycin was purchased from Sigma Aldrich and used at 10 μ g/mL.

The CPG2 enzyme

All cloning procedures were performed using standard molecular biology techniques. The CPG2 structural gene encoding amino acid residue Q26-K415 inclusive, which had been synthesized from the published sequence,⁸ was cloned into the AstraZeneca plasmid pNG3-VKss-HuCK (NCIMB deposit no. 40798) that contained the human cytomegalovirus (hCMV) promoter and SV40pA. The CPG2 gene was isolated by restriction digestion with *Sac*II and *Eco*RI and inserted downstream of the 5' kappa antibody light chain signal sequence (VKss) derived from the murine anti-CEA monoclonal antibody A5B7. This plasmid, pNG3/RC/CPG2, was then used in all mammalian cell expression and secretion studies using transfected cells.

CPG2 gene mutagenesis

Three putative amino acid glycosylation recognition sites within the *CPG2* gene, at positions 222–224 (N-I-T), 264–266 (N-W-T), and 272–274 (N-V-S), were negated by a mutation strategy using polymerase chain reaction (PCR), in which the asparagine residue (N) of each site was mutated to glutamine (Q), generating the *CPG2(Q3)* gene. The vector pNG3/RC/CPG2 was used as a template for three initial PCR reactions. Reaction 1 used synthetic oligonucleotide primers A4 and B2, reaction 2 used synthetic oligonucleotide primers A4 and A3, and reaction 3 used synthetic oligonucleotide primers B3 and B5. The PCR reaction products 1 and 2 contained the mutated 222 and 264+272 glycosylation sites, respectively, with product 3 being a copy of the C-terminal segment of the *CPG2* gene. Products 2 and 3 were joined in reaction 4 using the synthetic oligonucleotide primers A4 and B5. Product 4 was used as a template in reaction 5 using the synthetic oligonucleotide primers B5 and B2. The resulting product 5 and product R1 were used in a final joining reaction 6 using the synthetic oligonucleotide primers A4 and B5. This final product now contained all three mutated glycosylation sites and could be cloned between *SacII* and *EcoRI* sites in pNG3-VKss-HuCK to generate plasmid pNG2/RC/CPG2(Q3), which was used alongside pNG3/RC/CPG2 in mammalian cell expression and secretion studies (primer sequences: A2-5'TAGCGG-ATGCACGTCCAGTTGTAGTGGCCGTTCCGGAGCG5'; A3-5'CGCTTCTTGGACGCGAAGTTGACCTGGTAGC-GGTTCCGGCCGTTGCAGAGCTTGTAGTAGGG5'; A4-5'AAGCTTCCGCGGGCAGAAGCGCGACAACGTG'; B2-5'ATCACCGCAAGGCCTCG3'; B3-5'GTCTCGAA-CATCATCCCC3'; B5-3'GACCCGCGGCCGTTCACTAT-TCTTAAGTTTCGCTT5').

Thy1 addition to CPG2 and CPG2(Q3)

The last exon of the rat brain antigen *Thy1* gene¹⁵ was linked in frame through a proline codon to the C-terminus of both CPG2 and CPG2(Q3) using a two-step cassette mutagenesis strategy. Firstly, two synthetic oligonucleotides Thy1A upper and Thy1A lower were annealed together to produce a double-stranded DNA cassette, Thy1A. Thy1A encodes the CPG2/CPG2(Q3) 3' end sequence, separated from the 5' Thy1 sequence by a single proline codon, and also introduced a unique *NheI* restriction site. The Thy1A cassette was inserted into pNG3/RC/CPG2 or pNG3/RC/CPG2(Q3) between *BsrGI* and *EcoRI* sites.

A second pair of synthetic oligonucleotides, Thy1B upper and Thy1A lower, was annealed together to produce the DNA cassette, Thy1B. Thy1B encodes the residual 3' Thy1 sequence terminated by two-stop codons. This cassette, which carried intrinsic 5' *NheI* and 3' *EcoRI* cohesive ends, was inserted into the intermediate plasmids to construct the plasmid pNG3/RC/CPG2-Thy1 and pNG3/RC/CPG2(Q3)-Thy (oligonucleotide sequences: Thy1A upper, 5'GT-ACATGGCTGCGCGCCTGATCATGGATCTGGGCGCC-GGCAAGCCCGACAACTGGTCAAGTGTGAGGGCA-TCAGCCTGCTAGCTGATAAG3'; Thy1A lower, 3'TACC-GACGCGCGGACTAGTACCTAGACCCGCGGCCGTT-CGGGCTGTTTGACCAAGTTCACACTCCCGTAGTCGG-

ACGATCGACTATTCTTAA5'; Thy1B upper, 5'CTAGCT-CAGAACACNTCGTGGCTGCTGCTGCTCCTGCTCTC-CCTCTCCCTCCTCCAGGCCACGGATTTTCATGTCCCT-GTGATAAG3'; Thy1B lower, 3'GAGTCTTTGTGNAGCA-CCGACGACGACGAGGACGAGAGGGAGAGGGAGG-AGGTCCGGTGCCTAAAGTACAGGGACACTATTCTT-AA5').

Recombinant adenovirus (rAd) generation

The cDNA cassettes within pNG3/RC/CPG2(Q3) and pNG3/RC/CPG2(Q3)-Thy were isolated by *BglIII* and *XbaI* digestion and each cloned into the multiple cloning site of p Δ E1sp1A (Microbix Biosystems, Toronto, Canada) to generate four shuttle vectors: p Δ E1sp1A/CPG2, p Δ E1sp1A/CPG2(Q3), p Δ E1sp1A/CPG2-Thy1, and p Δ E1sp1A/CPG2(Q3)-Thy. rAds were generated by cotransfection using the calcium phosphate coprecipitation method¹⁸ of each shuttle vector and pJM17 (Microbix Biosystems) into 293 cells to obtain replication-defective adenoviruses by homologous recombination. Adenovirus clones were harvested and analysed by Southern blotting to confirm the presence of the correct cDNA cassette. Positive clones were grown up and purified using double cesium chloride gradient as previously described.¹⁷ All rAd stocks were titrated using the 293 end point dilution assay and shown to be free from endotoxin (lipopolysaccharide) as previously described¹⁹ and negative for the presence of replication-competent adenovirus (at 10⁹ plaque-forming units) using the supernatant rescue assay.²⁰

Immunocytochemical studies

Cells were grown on poly-L-lysine-coated coverslips and infected with either rAd/CPG2(Q3) or rAd/CPG2(Q3)-Thy1 at a multiplicity of infection (MOI) of 100. Forty-eight hours postinfection, the cells were fixed. The subcellular distribution of the CPG2 protein was determined by staining of both permeabilized and unpermeabilized cells using a rabbit polyclonal antibody specific for CPG2 followed by a secondary antibody against rabbit IgG conjugated to the fluorochrome FITC and analysed by fluorescence microscopy. Alternatively, to quantify the percentage of CPG2-positive cells, they were detached prior to fixing. The cells were then either immunostained for CPG2 as for attached cells or incubated in PI-PLC using 1 U/mL for 3 hours at 37°C, followed by immunostaining for CPG2. The percentage of cells expressing CPG2 was determined by measuring the fluorescence intensity of 10,000 cells using a FACScan (Becton Dickinson, Mount View, CA), comparing both pre and post PI-PLC treated cells and using uninfected cells as a negative control.

Cytotoxicity studies

Six well plates were seeded at 10⁵ cells/well and 24 hrs later infected at increasing MOI (0, 1, 10, 30, 100, and 300) with rAd/CPG2(Q3) or rAd/CPG2(Q3)-Thy1. After 48 hrs infection, the cells were fed with either culture medium alone or culture medium containing 20 μ M prodrug ZD2767P (supplied by AstraZeneca) and incubated for a further

72 hrs. Cells were harvested, permeabilized and then processed for CPG2 immunoreactivity and DNA content. CPG2 immunoreactivity was performed as already described with the addition of propidium iodide prior to flow cytometry analysis to detect the level of DNA fragmentation in the cells.²¹ The fluorescence intensity of 10,000 cells was analysed using a FACScan (Becton Dickinson, Mount View, CA) and cells with sub-G₀/G₁ DNA stainability were identified as being apoptotic. Alternatively, the sulfurhodamine (SRB) dye-based cytotoxicity assay was used.²² The colorectal cell line LoVo was seeded in 96-well plates at a density of 2×10^3 cells/well and infected at increasing MOI with rAd/CPG2(Q3) or rAd/CPG2(Q3)-Thy1. Forty-eight hours postinfection, the cells were thoroughly washed with culture medium alone and then incubated with culture medium containing the produg ZD2767P (0.3–10 μ M) for 1 hour at 37°C. Four days later, trichloroacetic acid was added, and the amount of cellular protein adhering to the plate was assessed by the addition of SRB dye.

Results

Generation of adenoviral vectors expressing secreted and GPI-anchored CPG2(Q3) enzyme

Endogenous CPG2 enzyme is localized in the periplasmic space of *Pseudomonas* sp. bacteria.⁸ To target CPG2 to the

mammalian secretory pathway, we used the mammalian kappa antibody light chain secretory signal sequence (amino acid sequence KLAATMDFQVQIFSFLISASVIMSR), with enzyme expression driven by a hCMV promoter.

The three putative amino acid glycosylation recognition sites within the CPG2 gene, at positions 222–224 (N-I-T), 264–266 (N-W-T), and 272–274 (N-V-S), were mutated by changing the asparagine residue (N) of each site to glutamine (Q) generating the CPG2(Q3) gene (Fig 1) because this is reported to improve enzyme activity in mammalian cells.¹² Also, we anchored the enzyme to the extracellular membrane through a GPI anchor. This was achieved by the addition of the last exon of Thy1 to the C-terminus of CPG2(Q3) linked through a proline codon (Fig 1), an approach already proven to achieve GPI anchor display of secreted tissue inhibitor of metalloproteinases (TIMP).^{15–17}

Western blot analysis upon cell supernatants from COS 7 cells, transfected with plasmid DNA encoding for either CPG2 or CPG2(Q3), confirmed that eukaryotic expression of CPG2 results in the secretion of two protein products of greater molecular mass than that of bacterial CPG2. These bands represent two glycosylated CPG2 protein species, the production of which can be blocked by the glycosylation inhibitor tunicamycin (Fig 2, Lanes 1 and 2). This indicates that in COS 7 cells, glycosylation of native CPG2 is not 100% efficient, thus allowing a mix of glycosylated and nonglycosylated CPG2 protein (the third and smallest

RAAd E1/E3 vector constructs

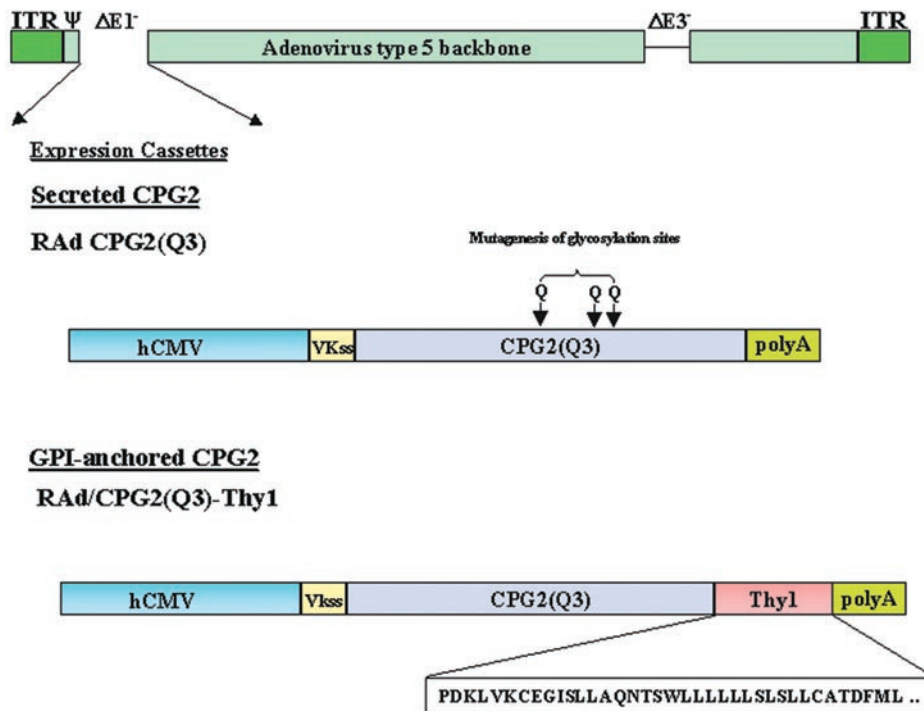


Figure 1 Diagrams showing the construction of the secreted and GPI-anchored CPG2 encoding adenoviral vectors. In each case, the constitutive hCMV promoter drives expression with each CPG2 gene flanked at its 3' end by a SV40polyA. CPG2 protein is targeted for secretion from the cell by the 5' kappa antibody light chain secretory leader sequence (VKss). The first expression cassette encodes for the secreted CPG2(Q3) variant in which three potential glycosylation sites [asparagine (N) residues 222, 264, 272] within the wild-type CPG2 have been mutated to glutamine residues (Q). Expression cassette 2 carries the CPG2(Q3)-Thy1 gene for the expression of GPI-anchored CPG2 protein. The Thy1 sequence has been placed at the 3' end of the CPG2(Q3) gene linked through a proline codon (amino acid sequence is shown).

protein product of identical mass to bacterial CPG2 protein) to be secreted from the cells. This heterogeneous mix of glycosylated and nonglycosylated CPG2 protein had a combined specific activity of 74 ± 13 U/mg, being 7-fold lower than that of bacterial CPG2 (which has a specific activity of 500 U/mg). In contrast, CPG2(Q3) expression produces a single nonglycosylated protein product of identical 42 kDa molecular weight as bacterial CPG2 (Fig 2, Lanes 4 and 5) with tunicamycin addition having no effect on its production, thus confirming the removal of all potential glycosylation sites from within the wild-type *CPG2* gene (Fig 2, Lane 3). The specific activity of CPG2(Q3) was recorded at 107 ± 17 U/mg and although this is lower than that of bacterial CPG2, it is still a highly active enzyme.

E1⁻/E3⁻ adenovirus vectors were generated by homologous recombination between the plasmid pJM17, which encodes for the adenoviral genome, and the plasmid pΔE1sp1A, containing either the expression cassette for the transgenes *CPG2(Q3)* or *CPG2(Q3)-Thy1*. The expression cassettes were inserted into the E1 region of the adenoviral genome, generating two rAd vectors, rAd/CPG2(Q3) for the expression of secreted CPG2 enzyme and rAd/CPG2(Q3)-Thy1 to deliver the cassette for the expression of GPI-anchored CPG2 enzyme, both of which were confirmed by Southern blot analysis (data not shown).

CPG2(Q3)-Thy1 expression results in the attachment of CPG2(Q3) enzyme into the extracellular plasma membrane through a GPI anchor

Madin Darby canine kidney (MDCK) cells, which are polarized epithelial cells showing strict apical membrane sorting of both endogenous and exogenous GPI-anchored proteins, were infected with either rAd/CPG2(Q3) or rAd/CPG2(Q3)-Thy1 at a MOI of 100. Forty-eight hours postinfection, the cells were incubated with PI-PLC to cleave off any GPI-anchored proteins from the extracellular membrane. Following thorough washing of the cells and fixation, the percentage of cells expressing CPG2 was determined using flow cytometry both pre- and post-PI-PLC treatment. MDCK cells infected with rAd/CPG2(Q3) only stained positive for CPG2 when permeabilized, showing that 21% of the cells was secreting CPG2 protein. PI-PLC treatment had no effect on this result (Fig 3b). Both permeabilized and unpermeabilized cells stained positively for CPG2 when infected with rAd/CPG2(Q3)-Thy1. Fifty-two percent of the cells showed CPG2 expression when the cells were permeabilized. In contrast to cells expressing secreted CPG2, this signal was not lost if unpermeabilized cells were analysed, showing that 30% of the unpermeabilized cells was displaying CPG2 extracellularly (Fig 3c). The mechanism of extracellular CPG2 protein attachment was confirmed to be through a GPI anchor by the ability of PI-PLC to cleave it off the membrane. Uninfected cells showed no CPG2 staining with or without permeabilization (Fig 3a).

Further confirmation that CPG2(Q3)-Thy1 was extracellularly displayed came from immunocytochemical staining for CPG2 protein upon MDCK cells infected with either rAd/CPG2(Q3) or rAd/CPG2(Q3)-Thy1. Again MDCK

cells were infected with either rAd/CPG2(Q3) or rAd/CPG2(Q3)-Thy1 at a MOI of 100. Forty-eight hours postinfection, the cells then fixed. When unpermeabilized cells were stained for CPG2; rAd/CPG2(Q3) infected cells did not stain at all. In contrast, rAd/CPG2(Q3)-Thy1-infected cells displayed punctate membrane staining indicative of a GPI-anchored protein (Fig 3e). rAd/CPG2(Q3)-infected MDCK cells only stained for CPG2 if permeabilized when the endoplasmic reticulum of these cells stained positively for CPG2 (Fig 3d). This targeting of CPG2(Q3)-Thy1 for GPI anchor addition into the extracellular membrane of rAd/CPG2(Q3)-Thy1-infected cells was not cell type-specific. When the experiment was repeated in two short-term human glioma lines IN859 and IN1760, the human colorectal adenocarcinoma cell line (LoVo), a rat glioma cell line (CNS1), and the murine glioma cell lines MT539MG and GL261, the same pattern of staining was seen in all six different cell types, identical to that observed in MDCK cells (data not shown).

GPI anchoring of CPG2(Q3) into the extracellular membrane does not negate the functionality of this dimeric enzyme

Bacterial CPG2's native form is homodimeric, consisting of two identical, antiparallel, associated subunits. Therefore, it was considered essential that enough flexibility be provided to allow the C-terminally GPI-tethered subunits to form the native CPG2 dimerization. Endogenous GPI-anchored enzymes that require dimerization to become functional exist.²³ We speculated that by using this GPI mode of membrane attachment for CPG2, we would achieve extracellular

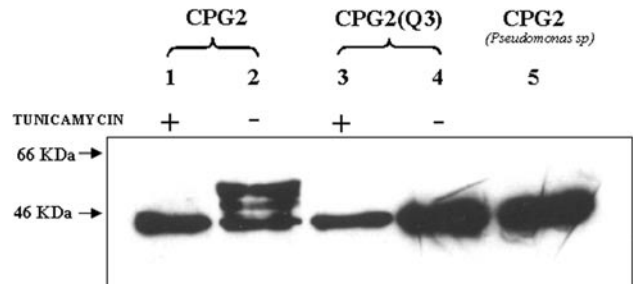


Figure 2 Western blot analysis to detect secreted CPG2 protein performed upon crude cell supernatants, taken from COS7 cells transiently transfected with either plasmid pNG3/RC/CPG2 or pNG3/RC/CPG2(Q3) and expressed in the presence or absence of 10 μg/mL tunicamycin. The blot shows that expression of the secreted form of CPG2 gives a distinct pattern of three different protein products. The smallest band has the same mobility as bacterial CPG2 (43 kDa). This is most likely unglycosylated CPG2, indicating that the glycosylation process within COS cells is not 100% efficient. The two larger bands probably represent glycosylated CPG2 forms. Tunicamycin addition completely blocks glycosylation of CPG2, with Western blot analysis showing the expression of the single 43-kDa band only that represents unglycosylated CPG2. Supernatant analysed from CPG2(Q3) expression gives a single band of 43 kDa, the mobility of which is unaffected by tunicamycin, proving that the mutagenesis of the three asparagine residues to glutamine residues has resulted in the expression of a uniform Q3 variant of the CPG2 protein.

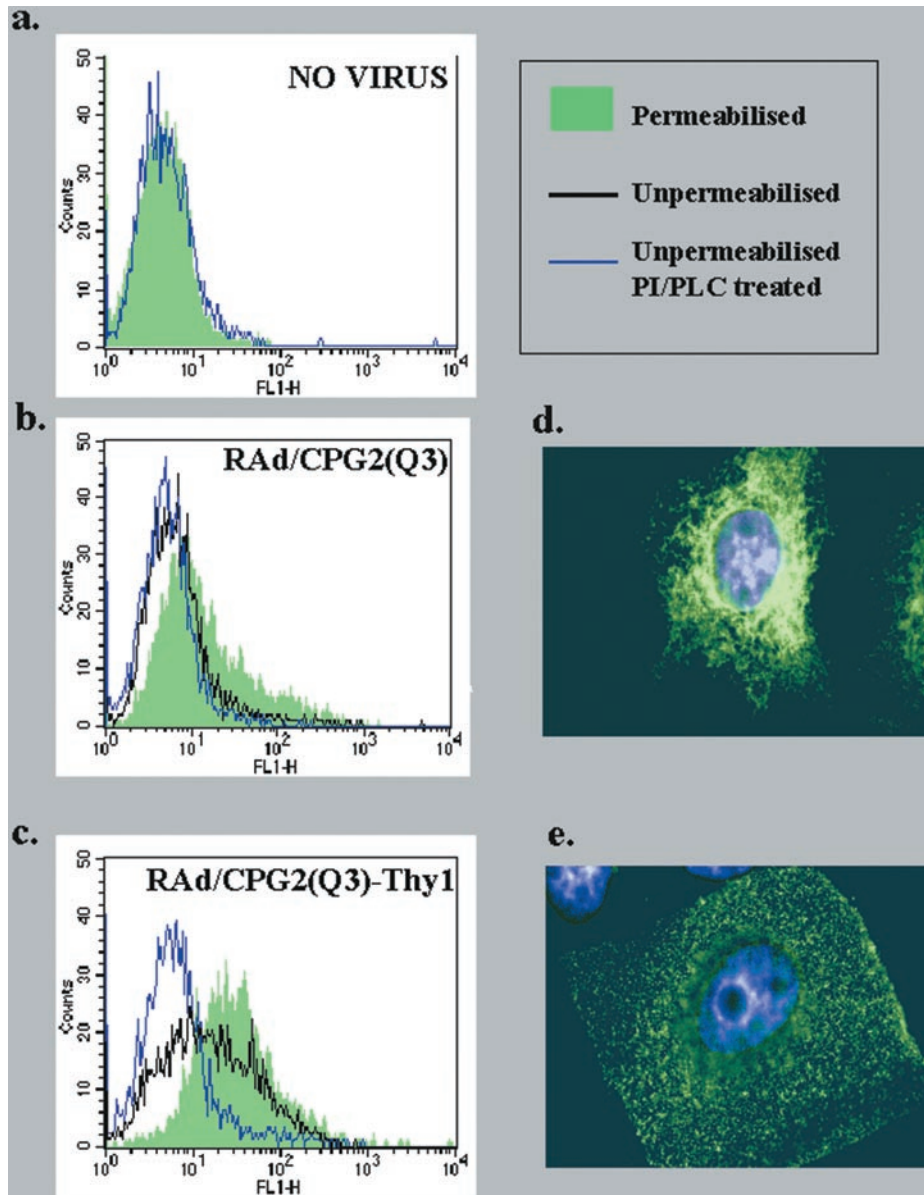


Figure 3 Confirmation that the addition of the last exon of Thy1 to the C-terminus of the *CPG2(Q3)* gene has resulted in the posttranslational GPI anchoring of CPG2 enzyme to the extracellular membrane. MDCK cells were infected with either rAd/CPG2(Q3) or rAd/CPG2(Q3)-Thy1 (MOI of 100) and the percentage of cells expressing CPG2 was determined using flow cytometry. Only permeabilized cells stained positive for CPG2 (21%) when infected with rAd/CPG2(Q3) and phospholipase C treatment had no effect (**Panels a and b**). Unpermeabilized cells stained positive for CPG2 when infected with rAd/CPG2(Q3)-Thy1 (30%) as well as permeabilized cells (52%), and this extracellular CPG2 was shown to be GPI-anchored to the membrane by cleavage with phospholipase C (**Panel c**). Immunocytochemical staining for CPG2 protein in the MDCK cells demonstrated that cells infected with rAd/CPG2(Q3) only stained for CPG2 when permeabilized, showing positive CPG2 staining within their endoplasmic reticulum (**Panel d**). However, unpermeabilized cells infected with rAd/CPG2(Q3)-Thy1 produced a punctate staining pattern upon their extracellular membrane indicative of GPI-anchored proteins (**Panel e**).

surface tethering of active CPG2 enzyme. The ability of GPI-anchored CPG2(Q3) enzyme expression and prodrug to inhibit cell proliferation was measured using the SRB dye-based cell proliferation assay.²² Two days postinfection with increasing MOI of either rAd/CPG2(Q3) or rAd/CPG2(Q3)-Thy1, the LoVo cells were thoroughly washed, then exposed to a single dose of between 0.3 and 10 μ M prodrug. Three days later, the total protein per well was assessed. In each case, the cells had been washed prior to

prodrug addition to remove any enzyme secreted into the supernatant (and not bound to cells), whereas the hydrophilic ZD2767P prodrug cannot permeate the cells and thus be converted to active drug by enzyme that remains within the secretory pathway. Thus, without surface-bound enzyme, one would expect little or no active drug to be generated from the prodrug and hence extremely poor levels of cell killing to occur. Neither rAd/CPG2(Q3) or rAd/CPG2(Q3)-Thy1 infection alone produced any cytotoxic

effects even at MOI of 200. As expected, cells expressing secreted CPG2(Q3) were viable irrespective of virus infection or prodrug dose (Fig 4B). In comparison, LoVo cells displaying CPG2(Q3) on their membranes showed dose-dependent cell death the level of which rose with increasing prodrug concentration. A maximum loss of cell survival (80%) was observed when the LoVo cells were infected at MOI of 200 with rAd/CPG2(Q3)-Thy1 (Fig 4A) followed by a single prodrug dosing of 10 μ M. However, a 50% loss in cell survival was achievable when the cells were infected with as low as 50 infectious particles per cell followed by a single prodrug dosing of 10 μ M. This clearly proves that GPI-anchored CPG2(Q3) enzyme anchored into the extracellular membrane of LoVo cells is functional.

Adenoviral delivery and expression of secreted and GPI-anchored CPG2(Q3) in six tumor cell lines

The transduction efficiency and ability to express both secreted CPG2(Q3) and GPI-anchored CPG2(Q3)-Thy1

using rAd vector delivery were assessed in two human glioma short-term cultures (IN859 and IN1760), a human colorectal cell line (LoVo), two murine gliomas (MT539MG and GL261), and a rat glioma cell line (CNS1). The tumor cells were seeded in six-well plates at a density of 10^5 cells/well then infected using increasing viral doses of either rAd/CPG2(Q3) or rAd/CPG2(Q3)-Thy1. After 3 days, the percentage of cells expressing CPG2 was determined quantitatively using flow cytometry as previously described²³ (Fig 5). The cells were permeabilized prior to staining for CPG2 immunoreactivity to detect secreted CPG2 protein within the secretory pathway of infected cells but unpermeabilized to detect extracellular GPI-anchored CPG2. At the highest MOI (MOI of 300 for IN859, IN1760, LoVo, and CNS1; MOI of 1000 for MT539MG and GL261 cells), all of the cell lines, with the exemption of the murine glioma MT539MG cells, showed detectable levels of GPI-anchored CPG2 protein [IN859 (68%), IN1760 (85%), LoVo (37%), CNS1 (34%), MT539MG (6%), and GL261(13%)] and secreted CPG2 expression [IN859 (75%), IN1760 (77%),

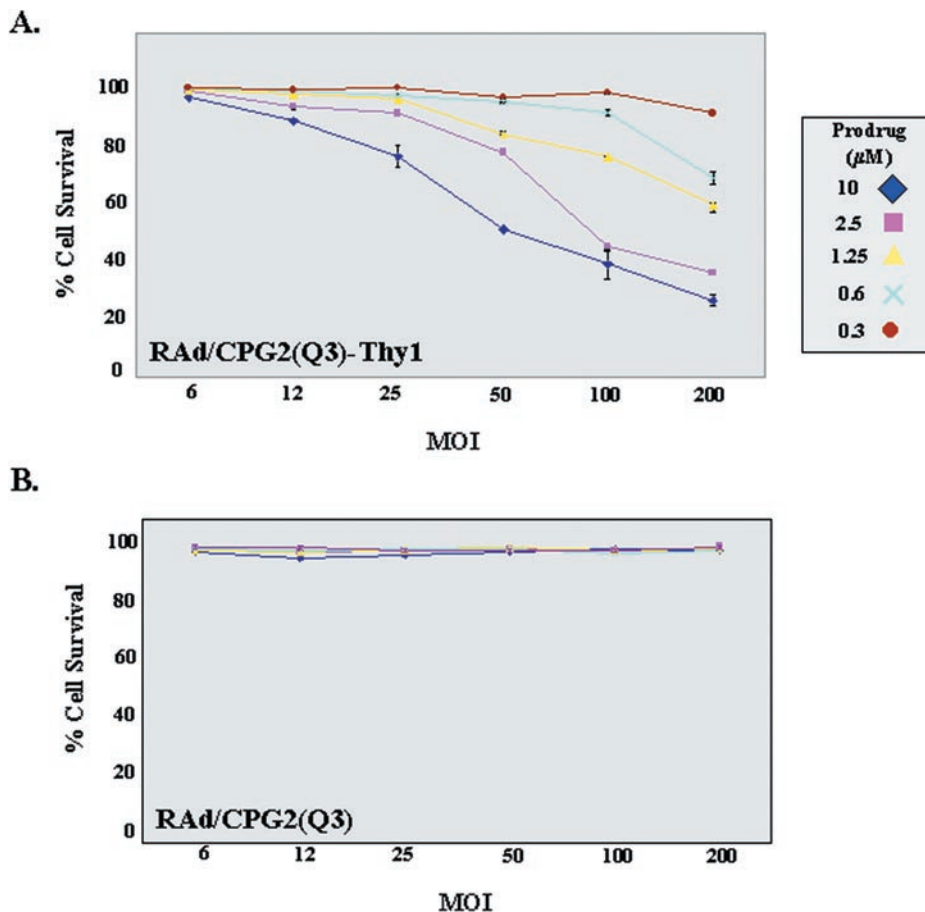


Figure 4 *In vitro* cytotoxicity testing of rAd/CPG2(Q3) or rAd/CPG2(Q3)-Thy1 with the prodrug ZD2767P in LoVo human colorectal adenocarcinoma cells. LoVo cells were infected with increasing MOI of virus (MOI of 6, 12, 25, 50, 100, and 200). After 48 hours, the cells were thoroughly washed, then exposed to a single dose of between 0.3 and 10 μ M prodrug. Cytotoxicity was assessed 72 hours later by measuring the total protein per well using the SRB dye-based cytotoxicity assay. Cells infected with rAd/CPG2(Q3), therefore expressing secreted CPG2 protein, were unaffected following prodrug exposure as active CPG2 enzyme was removed by washing. However, cells infected with rAd/CPG2(Q3)-Thy1 were sensitized to the prodrug even after washing, confirming that the GPI-anchored CPG2 enzyme was retained on the extracellular membrane from where it could activate the prodrug.

LoVo (8%), CNS1 (20%), MT539MG (0%), and GL261 (17%)]. In general, the cell lines expressed both membrane-bound and secreted CPG2 in a dose-dependent manner. The differences between the level of GPI-anchored CPG2 expression compared to that of secreted CPG2 were not significant in any of the cell lines except for the LoVo cells in which a greater percentage of cells expressing GPI-anchored CPG2 was detected. The human glioma cell lines, IN859 and IN1760, were the easiest to transduce, with enzyme expression detectable even after infection with MOI of 1. The human LoVo and rat CNS1 cells required infection with at least 10 plaque-forming units/cell before enzyme expression was detectable and the two murine glioma cell lines, MT539MG and GL261, proved the most difficult to transduce. Even after infection at MOI of 1000, secreted CPG2 expression from MT539MG cells was undetectable.

Tumor cell cytotoxicity of prodrug ZD2767P following adenoviral vector-mediated delivery of secreted and GPI-anchored CPG2(Q3)

To determine the prodrug sensitivity of the different cell lines, following adenoviral vector-mediated delivery of

either secreted or GPI-anchored CPG2, the tumor cells were seeded into six-well plates at 10^5 cells/well then infected at increasing MOIs with either rAd/CPG2(Q3) or rAd/CPG2(Q3)-Thy1. After 3 days, prodrug was administered to the cells, without prior washing, at a dose of $20 \mu\text{M}$. Cell death was observed microscopically and the level of propidium iodide incorporation, as a measure of the extent of apoptosis, was determined using flow cytometry after a further 3 days as previously described²¹ (Fig 6). Virus infection or prodrug dosing alone at $20 \mu\text{M}$ had no significant effect on cell viability beyond that observed in untreated controls. In all of the cell lines, irrespective of their susceptibility to adenovirus infection, at least 50% cell death could be induced following only a single prodrug dose, and consistent with the CPG2 enzyme expression analysis, maximum levels of cell death were induced using the highest MOI. Using the adenovirus vector expressing GPI-anchored CPG2, efficient cell killing was recorded when cells were infected at MOI of 300 (or MOI of 1000 for the murine glioma cell lines), prior to exposure to the prodrug ZD2767P [IN1760 ($67 \pm 4.2\%$), IN859 ($70 \pm 5.9\%$), CNS1 ($66 \pm 16.3\%$), MT539MG ($65 \pm 2.9\%$), and GL261 ($67 \pm 1.4\%$)]. The only

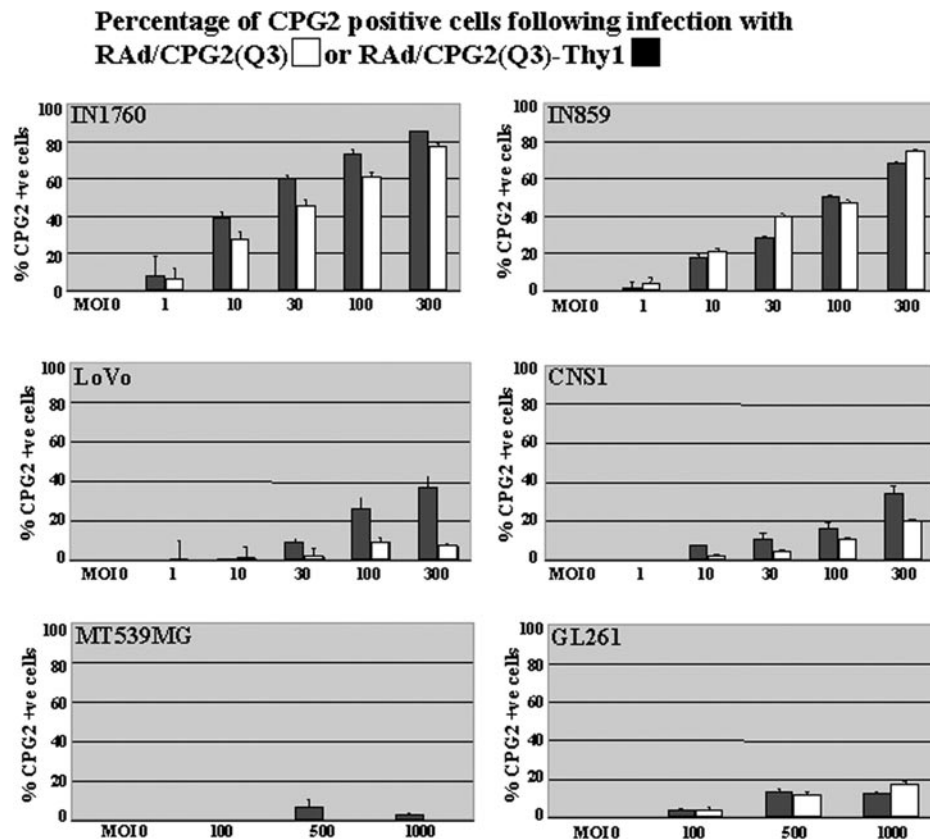


Figure 5 Determining the levels of expression of both secreted and GPI-anchored CPG2 achieved using adenovirus vector gene delivery in six different tumor cell lines. A quantitative analysis of CPG2 expression was carried out 3 days postinfection with rAd/CPG2(Q3) or rAd/CPG2(Q3)-Thy1. The short-term human glioma cultures IN859 and IN1760, the human colorectal cell line LoVo, and rat glioma CNS1 cells were dosed with MOI of 0, 1, 10, 30, 100, and 300. The murine glioma cell lines, MT539MG and GL261, were dosed at the higher range of 100, 500, and 1000. The cells were fixed, and the percentage of cells expressing CPG2 was determined by flow cytometry upon permeabilized cells expressing secreted CPG2 (white bars) and unpermeabilized cells expressing GPI-anchored CPG2 (black bars).

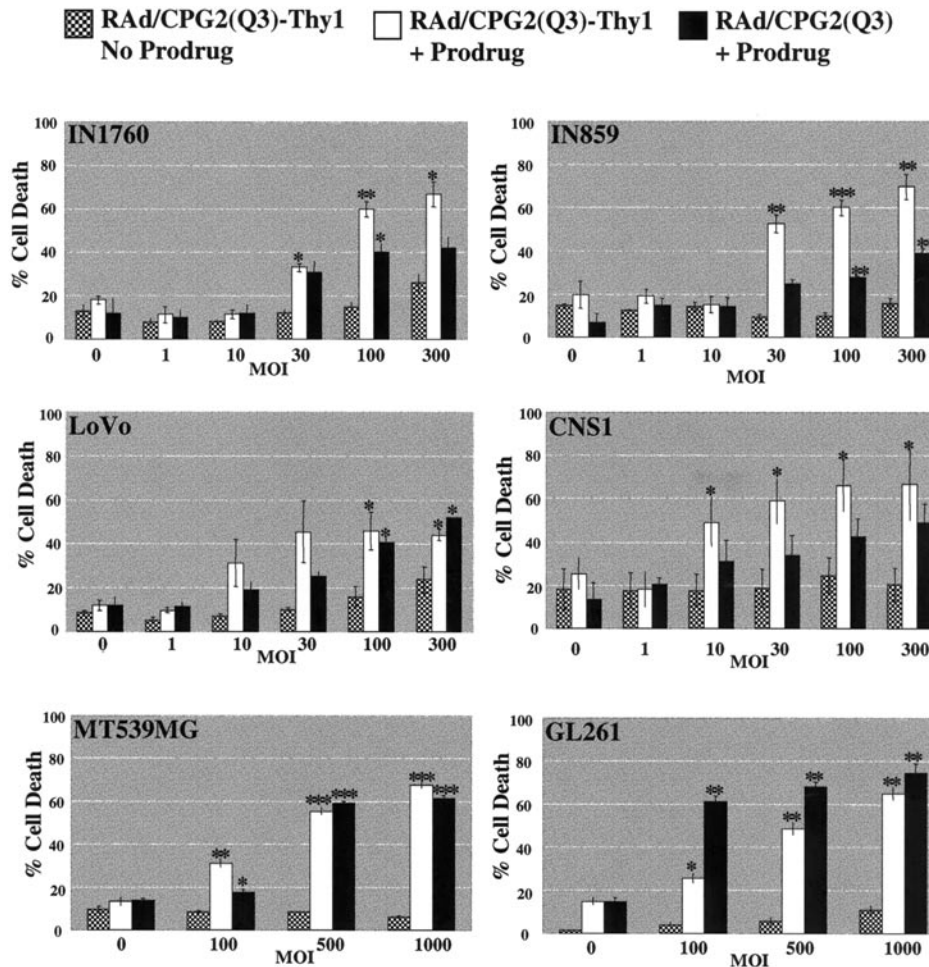


Figure 6 Comparison of the cell killing ability of adenoviral vector delivered, secreted, and GPI-anchored CPG2(Q3)-Thy1 and a single dose of prodrug ZD2767P in six tumor cells. The cells were infected at increasing MOI (IN859 and IN1760, LoVo, and CNS1 cells were dosed with MOI of 0, 1, 10, 30, 100, and 300 and MT539MG and GL261 with MOI of 100, 500, and 1000) with either rAd/CPG2(Q3) or rAd/CPG2(Q3)-Thy1. After 3 days, prodrug was administered to the cells, without prior washing, at a dose of 20 μ M. Cell death was observed microscopically and the level of propidium iodide incorporation, indicative of the extent of apoptosis, was determined using flow cytometry after a further 3 days.

exception was in the LoVo cells where the highest induction of cell death was observed at MOI of 100 (46 ± 8.8), which may be due to transgene-mediated toxicity of GPI-anchored CPG2(Q3) within this cell type at higher viral doses. Efficient cell killing was also recorded when each of the cell lines was infected at the highest viral dose with the adenovirus vector encoding for secreted CPG2 prior to prodrug exposure [IN1760 ($42 \pm 4.8\%$), IN859 ($39 \pm 1.8\%$), CNS1 ($49 \pm 9.0\%$), LoVo ($52 \pm 0.3\%$), MT539MG ($61 \pm 1.1\%$), and GL261 ($74 \pm 4.6\%$)].

This clearly demonstrates that adenoviral-mediated gene delivery of secreted or GPI-anchored CPG2(Q3) can sensitize a panel of different tumor cell lines to the prodrug ZD2767P. By comparing the CPG2 enzyme expression data in Figure 5 and the cytotoxicity data in Figure 6, we can ascertain the level of the bystander effect. In all cell lines at low MOIs, CPG2 immunoreactive protein levels were significantly lower than the observed level of cell death, indicating a strong bystander response. In particular in the murine glioma cell lines, MT539MG and GL261, where less

than 17% of the cells was immunoreactive for CPG2, up to 74% cell killing was achievable.

Discussion

In this paper, we have developed an efficient CPG2 enzyme/prodrug gene therapy using first-generation adenovirus vector delivery and expression of the enzyme in a secreted or extracellular GPI-anchored form. We used a mutated, nonglycosylatable form of the CPG2 gene, CPG2(Q3), to ensure the expression and secretion of a homogeneous, nonglycosylated CPG2 protein within mammalian cells. Although the substitution of three amino acids within the dimerization domain of the enzyme results in an approximately 5-fold reduction in the specific activity of CPG2(Q3) compared to bacterial CPG2, the enzyme is still highly active. In addition, attempts to restore activity by substituting alternative amino acids other than glutamine at each glycosylation site have met with limited success.¹³ To target the gene to the extracellular leaflet of the plasma

membrane, we used the GPI attachment signal encoded within the last exon of the rat brain antigen Thy1, which was cloned in frame but separated from the C-terminus of the *CPG2(Q3)* gene through a proline-encoding codon. The GPI membrane anchor allowed the necessary flexibility for the *CPG2(Q3)* enzyme to maintain its enzymatic function, thus suggesting that the enzyme subunits were still able to dimerize while anchored into the outer lipid leaflet to form an active enzyme conformation. Expression cassettes for the expression of secreted or GPI-anchored enzyme were then inserted into the E1 region of an adenovirus vector. The recombinant adenoviral material produced, which expressed either secreted or GPI-anchored *CPG2*, was used to investigate whether a bystander effect could be determined. A bystander effect in this case is thought to occur due to the extracellular activation of prodrug and diffusion of the active drug throughout the tumor mass. GPI-anchored *CPG2* should avoid systemic release of the enzyme into the extracellular space *in vivo*, but will still allow this extracellular conversion of prodrug to its cytotoxic product, thereby reducing the percentage of tumor cells that have to be transduced to generate an effective antitumor treatment.

We chose to use *CPG2* in combination with the hydrophilic and relatively membrane-impermeable optimized prodrug *ZD2767P* that is cleaved by *CPG2* to release a very potent and highly reactive nitrogen mustard drug. These mustard alkylating agents have the advantage of exerting dose-dependent cytotoxicity by cross-linking DNA in both proliferating and quiescent cells. In comparative studies with other *CPG2* prodrugs, *ZD2767P* has shown improved antitumor activity in a colorectal tumor xenograft model. In addition, the activated metabolite of the prodrug *ZD2767P* has a short chemical half-life of approximately 2 minutes in plasma.¹¹ It has been predicted that a diffusion range of 100–200 μm for a cytotoxin in tumor tissue would provide efficient localized cell killing with limited systemic leakage and that a tissue half-life of ~ 1 minute for an activated cytotoxin would be sufficient to provide an effective but localized bystander effect with minimal diffusion into peripheral nontumor tissue.²⁴

We tested the potential effectiveness of these vectors in two human glioma short-term cultures IN1760 and IN859 and the human colorectal tumor cell line LoVo. We also tested the cell killing ability of our system in cell lines from two other species, the rat glioma CNS1 cell line and two murine glioma cell lines MT539MG and GL261 cells, which exhibited different adenoviral transfection efficiencies. The two human glioma cell lines, IN1760 and IN859, were transduced with high efficiency, expressing both secreted and GPI-anchored *CPG2* when infected with only one virus particle per cell. The LoVo and CNS1 cells required infection with MOI of 10 or greater, and the murine glioma cell lines MT539MG and GL261 required a 10-fold greater viral dose of MOI of 1000. Despite these differences in transfection efficiency, in all of these cell lines, at least 50% of the cells was killed when infected at the highest MOI followed by a single administration of prodrug *ZD2767P* (20 μM). The sensitivity of different cell lines used in this study to *CPG2(Q3)* versus *CPG2(Q3)*-Thy1 differs. The reasons for this are, at present, unclear; it could be due to different

cellular physiology with respect to their efficacy to anchor the *CPG2(Q3)*-Thy1 form to the plasma membrane, or to fold and dimerize the enzyme.

A direct comparison of our data to assess the efficacy of our system against the most widely used suicide gene therapy approach HSV-TK/ganciclovir (GCV) has been made.²⁵ Three administrations of GCV (two doses of 10 μM followed by a single dose of 100 μM over a 9-day period) were required to effect equivalent cell killing in the IN859 short-term human glioma, but in the IN1760 short-term human glioma, which exhibits a longer doubling time, no cell killing was observed with TK/GCV, compared to 70% killing with *CPG2/ZD2767P*. In addition, two murine cell lines that were transduced by the adenovirus vectors at extremely low efficiency were killed on a single exposure to prodrug even when infected at a MOI at which *CPG2* immunoreactive cells could not be detected, demonstrating the clear bystander effect achievable with this system.

In summary, we have shown the efficacy of using adenovirus vectors to deliver *CPG2*. The vectors are able to transduce a panel of tumor cell lines in which both secreted and GPI-anchored *CPG2(Q3)* enzymes were correctly processed and targeted, resulting in active enzyme release out of the cell or retention on the extracellular plasma membrane. In addition, despite the inherent refractivity of a tumor cell to adenovirus infection, transfection of only a small fraction of the cell population was required to achieve significant cell death upon administration of prodrug. These results suggest that recombinant adenoviral vector delivery of *CPG2* may significantly increase the percentage of tumor cells that can be sensitized to hydrophilic prodrugs such as *ZD2767P* and that extracellular activation of the prodrug could potentially enhance the bystander effect without necessitating the transduction of every tumor cell.

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