Relative Efficiency of Tumor Cell Killing \textit{in Vitro} by Two Enzyme-Prodrug Systems Delivered by Identical Adenovirus Vectors$^1$

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ABSTRACT

Enzyme-prodrug therapy for the treatment of cancer is an experimental procedure that is under intensive investigation. However, the relative merits of the various systems for use under specific conditions are still being determined. We have compared the efficacy of cell killing by the herpesvirus thymidine kinase (HSVTK)/ganciclovir and the purine nucleoside phosphorylase (PNP)/9-(3-D-2-deoxy-erythropentofuranosyl)6-methylpurine enzyme/prodrug systems. These were chosen because of their differential dependence on DNA replication for their mechanism of action. The HSVTK and PNP genes, expressed from the identical prostate-specific antigen promoter, were transduced into human prostate and breast cancers cells using the same human adenovirus vector. The kinetics of cell killing in the presence of the respective prodrugs was monitored using a nondestructive assay that measured total cell bioactivity. The PNP/9-(3-D-2-deoxy-erythropentofuranosyl)6-methylpurine system was clearly superior in its ability to cause cell death \textit{in vitro}. Cells were killed in about half the time and at a 5–10-fold lower input of virus relative to the HSVTK/ganciclovir system. The PNP system may offer advantages for the treatment of slow-growing tumors in which the daily proliferative rate is low or in situations in which gene delivery or expression is inefficient.

INTRODUCTION

New experimental approaches for the treatment of cancer are being developed using gene therapy. Some strategies being investigated include the introduction of multidrug resistance genes into bone marrow cells for improved chemotherapy, replacement of defective tumor suppressor genes, the delivery of cytokine genes and/or antigens to stimulate the immune system, and the use of virally directed EPTs$^3$ to achieve cell killing (strategies are summarized in Ref. 1). Several cell-killing systems are under investigation (2). In principle, neither the enzyme nor the prodrug is toxic to the target cells, but the combination of the two results in the production of a lethal metabolite. Delivery of the gene encoding the enzyme is most efficiently achieved by a viral vector, although not all of the target cells need be infected, because a “bystander effect” is also observed (3–5). Additional cell killing may occur due to diffusion of the toxic metabolite into cells not expressing the gene (6–8), but a component of the immune system also appears to be involved because a reduction in the number of experimental metastases at remote sites was also observed in intact but not in athymic mice (9). Eventually, specific cell killing may be achieved by using a tissue-specific promoter to control gene expression (9, 10).

Our strategy to develop new treatments for late-stage prostate cancer has been to use an adenovirus vector to deliver EPT genes for cell killing. The most commonly used cell killing system uses the HSVTK enzyme together with GCV (11). In HSVTK-expressing cells the prodrug is phosphorylated and becomes incorporated into DNA as replication proceeds (12). In resting and slowly dividing cells, however, its efficacy may be limited. GCV, a phosphorylated compound, is also unable to diffuse freely between cells (2). Because human prostate PC-3 cells were more resistant to HSVTK/GCV-mediated destruction than other prostate tumor lines (13) and because there may be a correlation between cell killing \textit{in vitro} and tumor elimination \textit{in vivo} (14), it was decided to investigate the use of an alternative killing system. The \textit{Escherichia coli} \textit{DeoD} gene product, PNP, differs from the mammalian enzyme in its use of adenine-based nucleosides as substrates (15, 16) and can metabolize such compounds into toxic, nonphosphorylated molecules that are capable of diffusion between cells (17). One metabolite, MeP, significantly reduced cellular protein, RNA, and DNA synthesis and caused a major reduction in cell number even when growth was arrested in 1% serum (17). The improved bystander effect may be beneficial in eliminating tumors \textit{in vivo} (10), particularly for conditions such as late-stage prostate cancer, in which it has been determined that the percentage of proliferating cells is very low (18). Here, we investigate killing of a prostate cancer cell

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\footnotesize{The abbreviations used are: EPT, enzyme prodrug therapy; PSA, prostate-specific antigen; TK, thymidine kinase; HSVTK, herpes-virus thymidine kinase; GCV, ganciclovir; MeP, 6-methylpuprine; PNP, purine nucleoside phosphorylase; 6MPDR, 9-(3-D-2-deoxy-erythropentofuranosyl)MeP; RSV, Rous sarcoma virus; Ad5, adenovirus type 5; MOI, multiplicity of infection; pi, postinfection; pfu, plaque-forming unit(s).}
line in vitro by the PNP and HSVTK EPT systems controlled by the same PSA promoter and delivered by the same viral vector. Killing of certain breast cancer cell lines was also investigated because some of these also produce PSA mRNA (19).

MATERIALS AND METHODS

Recombinant Adenoviruses. The recombinant Ad5/RSV/ lacZ, in which the lacZ gene was expressed from the RSV promoter, was obtained from Dr. M. Perricaudet (Institut Gustave Roussy, Centre National de la Recherche Scientifique, Villejuif, France). Replication-deficient recombinant Ad5 viruses carrying the HSVTK or E. coli DeoD genes linked to a PSA promoter were constructed. A 630-bp PSA promoter fragment containing an Xbal sequence near its 5' end was isolated from WBC DNA by PCR amplification and subcloned into pGem-T vector (Promega Corp. Madison, WI). Plasmid pTK1 (20) containing the HSVTK gene (including the endogenous polyadenylation signal) was cut with BglII/PvuII, end-filled with Klenow, and subcloned into BamHI-cut, end-filled pSP64 (Promega, Madison, WI). The gene was then released by digestion with EcoRI (end-filled) and XbaI and subcloned into the SpeI and NotI (end-filled) sites of pGem/PSA. Similarly, the E. coli DeoD gene (GenBank accession no. M60917) was amplified from genomic DNA using PCR primers that introduced SpeI and BamHI sites at bases 105 and 849, respectively, of the GenBank sequence and digested with those enzymes. A 513-bp fragment containing a SV40 polyadenylation signal was prepared by digestion of pJC119 (21) with BamHI and SalI. This fragment was cloned in a three-way ligation with the DeoD PCR fragment into pGem/PSA vector cut with SpeI and SalI. The PSA/TK and PSA/PNP cassettes were then excised with XbaI and SalI and subcloned into an adenovirus shuttle vector, pXCX3, which was cut with SpeI and SalI. pXCX3-derived plasmids containing the PSA/gene cassettes were transfected into human 293 cells together with plasmid pJM17 (22) to rescue recombinant viruses Ad5/PSA/TK and Ad5/PSA/PNP (Fig. 1). Plaques were picked, virus stocks were grown (22), and their genomes were confirmed as correct by restriction enzyme digestion. Viruses were propagated in 293 cells and purified by banding on CsCl density gradients, followed by dialysis (22).

Cell Lines and Virus Infection. Human cell lines, including the prostate PC-3 and breast cancer lines MCF-7 and T47-D2, were obtained from American Type Culture Collection and grown in RPMI plus 10% FCS. Cells were infected as described previously (23) using various MOIs between 10 and 200 pfu/cell. Cells infected with the Ad5/RSV/lacZ recombinant were stained for β-galactosidase expression.

Prodrugs. GCV was a gift of Syntex Inc. (Palo Alto, CA). The deoxyadenosine analogue 6MPDR was synthesized as described previously (24) and purified by high-performance liquid chromatography. A 5 mM stock was prepared in 0.01 M Tris, pH 7.2, and diluted as appropriate for addition to cell cultures. Prodrugs were added to cell cultures, when appropriate, immediately after infection and again whenever the medium was changed (usually every 3–4 days).

Alamar Blue Assay of Metabolic Activity. Cell metabolic activity was determined quantitatively at various times pi using the Alamar Blue assay (Alamar, Sacramento, CA). The system incorporates an oxidation-reduction indicator that both fluoresces and changes color in response to chemical reduction of the growth medium resulting from cell growth. Alamar Blue was diluted 1:10 in cell growth medium, and after incubation for 2 h at 37°C absorbance at 600 nm (oxidized form) was subtracted from absorbance at 570 nm (reduced form). Differential absorbance was plotted against virus input.

RESULTS

Infection of Cells with Ad5/RSV/lacZ. The ability of Ad5 vectors to infect prostate and breast cancer cell lines was monitored using an Ad5/RSV/lacZ recombinant. Cells were infected at multiplicities ranging between 1 and 100 pfu/cell and stained after 24 h. The number of infected PC-3 cells changed little with increasing MOI, although the intensity of staining suggested that lacZ expression was higher in susceptible cells when the MOI was increased. At a MOI of 100, less than 5% of the cells stained blue (Fig. 2). However, at a MOI of 100, the same stock of Ad5/RSV/lacZ infected a much higher proportion of MRC-5 human lung fibroblasts (Fig. 2; i.e., the PC-3 cells were more recalcitrant to infection). MCF-7 cells also showed a definite increase in the number of infected cells as the MOI increased from 1 to 50. However, at a MOI of 10, less than 10% of MCF-7 cells were blue (data not shown). For the experiments described below, a low level of infection was used because of...
the potential to emphasise any bystander effect, which might contribute to cell killing.

**Titration of Prodrugs.** To determine the level of prodrug that could be tolerated without ill effect, cell lines were exposed continuously to various concentrations of GCV or 6MPDR for 1–3 weeks. Periodically, metabolic activity was measured using the Alamar Blue assay. PC-3, MCF-7, and T47-D2 cells showed no visible effects when maintained in 50 μM GCV or 20 μM 6MPDR for the term of these experiments (data not shown).

**Killing of Prostate PC-3 Cells *in Vitro*.** The ability of Ad5/PSA/Tk to kill prostate PC-3 cells *in vitro* was investigated. Cells were left uninfected or were infected with Ad5/PSA/Tk at a range of MOIs (1–100 pfu/cell). At a MOI of 100, a reduction of ~50% in PC-3 cell metabolism was observed at 14 (data not shown) and at 23 days pi compared with control cells incubated without virus or without GCV (Fig. 3, A and B). A greater degree of cell killing was observed if PC-3 cells were reinfected once or twice during the course of the experiment (data not shown).

Similarly, cell killing by the PNP/6MPDR EPT system was determined using the same Ad5 vector and PSA promoter. PC-3 cells were infected with Ad5/PSA/PNP at MOIs of 0–200 pfu/cell and maintained in the presence of 20 μM 6MPDR. Compared with the HSVTK/GCV system, the effects on PC-3 cell metabolism were far more dramatic. After 7 days in the presence of virus, but without prodrug, there was no detectable effect on the cells (Fig. 4A). However, when virus was used at MOIs of 10–200 in the presence of prodrug, most of the cells had rounded up and lifted off after 7 days. Metabolic activity was therefore reduced by 66–80% compared with uninfected, prodrug-treated control cells (Fig. 4A). At 14 days pi, there was little detectable effect by the virus in the absence of prodrug, but in the presence of 20 μM 6MPDR, metabolic activity was undetectable (Fig. 4B) because all of the cells were lost. By this time, the drug itself was also beginning to affect cell metabolism. Nevertheless, it is clear that when using the same promoter and the same vector for gene delivery, the PNP/6MPDR system was significantly more efficient at killing PC-3 cells *in vitro* than the HSVTK/GCV system. The PNP/6MPDR system also killed PC-3 cells more efficiently at a virus input that was 2–5-fold lower than that required for an effect with HSVTK/GCV.

**Killing of Breast Cancer Cells.** Because certain breast cancer tumor lines also produce PSA mRNA (19), the PSA promoter fragment should be active in such cells. Therefore, MCF-7 or T47-D2 cell monolayers were left uninfected or were infected at a MOI of 10 with Ad5/PSA/Tk in the presence of 20 μM GCV. However, by 13 days pi there was no visible decline in cell metabolism (data not shown). Next, the ability of Ad5/PSA/PNP to kill MCF-7 and T47-D2 cells in the presence of prodrug was investigated. Monolayers of T47-D2 cells were

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**Fig. 2** Infection of human PC-3 or MRC-5 cells with an Ad5/RSV/lacZ recombinant. Cells left uninfected or infected at the MOI indicated were fixed and stained for β-galactosidase activity at 24 h pi.
infected with Ad5/PSA/PNP at MOIs of 10–50 in the presence of 20 \( \mu M \) 6MPDR, and cells were assayed for bioactivity. At 9 days pi, there was no discernable effect of the drug on control T47-D2 cells, but no metabolic activity was detectable in virus-infected cells, even at a MOI of 10 (Fig. 5A). Similarly, MCF-7 cells were infected with Ad5/PSA/PNP at MOIs of 0–200 in the presence of 20 \( \mu M \) 6MPDR. After 7 days, MCF-7 cells were not affected by the presence of the virus or the drug alone, but the combination of virus and prodrug was lethal, even at the lowest MOI of 10 (Fig. 5B).

**DISCUSSION**

Several EPT systems for the experimental therapy of cancers are being assessed (2). The HSVTK/GCV system (11), which is frequently used, is thought to exert its effect by phosphorylating GCV so that it becomes a substrate for cellular DNA synthesis. However, systems that depend on DNA replication may have limited efficacy against slowly growing tumors. Certain prostate tumors in which the number of proliferating cells is low (<3%) are included in this group (18). We therefore decided to compare the cell-killing ability of the PNP/6MPDR system, based on earlier observations with colon and melanoma cells, in which the toxic purines produced generated an excellent bystander effect (10, 17). Our strategy was to control the respective suicide genes using the same promoter, deliver them with an identical adenovirus vector, and assess the efficacy of cell killing in vitro to generate data from which to design subsequent in vivo experiments. The prostate cancer line PC-3 was chosen as one target cell because it has several characteristics of late-stage, androgen-insensitive prostate cancers and because it forms tumors when xenografted s.c. into nude mice.

Although a low level of PC-3 cell infection was obtained in these experiments, this was advantageous because it increased our ability to detect the contribution of bystander effects to cell killing by these EPT systems. When <5% of the cells were infected, there was little effect of GCV on PC-3 cells in the presence of Ad5/PSA/TK (Fig. 3), showing that the bystander effect obtained was not extensive. Phosphorylated GCV is unable to diffuse across membranes into other cells but can apparently spread between cells via gap junctions when cells are in contact (6, 8). Cell killing may result when bystander cells take up apoptotic vesicles released from dying cells (3), but, in thyrocytes at least, it was shown that GCV can induce apoptosis directly via a pathway that is independent of p53 (25). GCV-mediated cell killing can also be observed when as few as 3% of cells are expressing TK (26), as long as the level of TK expression is adequate (27). The small degree of cell killing we observed may therefore have been due to an insufficient level of PSA promoter activity. In separate experiments, the activity of this promoter fragment has been monitored by transfection of...
plasmids into a range of cell types. These data confirm that in vivo certain tumors to killing in vitro, aed cell killing can vary significantly, and there appears to be RNA levels and stability, translation efficiency, and enzyme, RNA, and DNA synthesis in cells (17). However, other free to diffuse across membranes and can reduce the level of obtained compared with HSVTK/GCV. This was presumably pi (Fig. 4), indicating that a much greater bystander effect was observed with the PNP/6MPDR combination. Although PSA expression apparently decreases in late-stage compared with early-stage breast cancers (19), according to our results, only a low level of promoter activity may be required to achieve EPT-mediated cell death.

The situation of toxicity due to the production of MeP and similar prodrug metabolites has been raised previously and is an issue relevant to in vivo experiments (10, 17). Because the prostate is a nonessential tissue, some collateral damage due to local diffusion might be tolerable. However, for the future application of EPT to prostate cancer metastases, the use of a tissue-specific promoter (10) will be important. The identification of prostate-specific promoters therefore remains a priority. In addition, there is evidence that HSVTK/GCV activity is enhanced by immune components (9) involving cytokines (29). If immune complementation also occurs with the PNP/prodrug system, combination therapy may ultimately be the most effective, and this will be tested in future experiments in vivo.

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