Gene-directed enzyme prodrug therapy for prostate cancer in a mouse model that imitates the development of human disease

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Abstract

Background Gene-directed enzyme prodrug therapy (GDEPT) based on the E. coli enzyme purine nucleoside phosphorylase (PNP) represents a new approach for treating slow growing tumours like prostate cancer (PCa). Expressed enzyme converts a systemically administered prodrug, fludarabine phosphate, to a toxic metabolite, 2-fluoroadenine. Infected and neighbouring cells are killed by a bystander effect that results from the inhibition of DNA and RNA synthesis.

Methods These studies were carried out using the transgenic adenocarcinoma of the prostate (TRAMP) model that mimics human PCa development and progression. Control TRAMP mice were injected intraprostatically with vector vehicle and thereafter intraperitoneally with saline or fludarabine phosphate (≈600 mg/m2/day) once daily for 5 consecutive days. Treated mice received a single intraprostatic injection containing 1010 particles of OAdV220, an ovine adenovirus which expresses the E. coli PNP gene under the control of the Rous sarcoma virus promoter, followed by systemic fludarabine treatment. The weight of the genitourinary tract, seminal vesicles and the prostate as well as animal survival were monitored. Tumours were also analysed histologically.

Results Preliminary studies showed that fludarabine alone caused no significant change in genitourinary (GU) tract weight in TRAMP mice. Animals injected with vector and prodrug showed a significant reduction (36–47%) in GU tract weight (ANOVA p = 0.0002) and a 35–50% reduction in seminal vesicle weight (ANOVA p = 0.0007). In particular, the target organ showed a significant 57% reduction in prostate weight (ANOVA p = 0.0007). PNP-GDEPT mice also showed a survival advantage over control mice. Histological analysis suggested that the cancer progression was slowed in GDEPT-treated animals.

Conclusion A single course of GDEPT based on OAdV-delivered PNP and fludarabine produced highly significant suppression of PCa progression in immune-competent TRAMP mice. Copyright © 2004 John Wiley & Sons, Ltd.

Keywords prostate; TRAMP model; preclinical studies; GDEPT; PNP; fludarabine phosphate; ovine adenovirus

Introduction

Prostate cancer (PCa) can be a difficult and intractable disease. The prognosis of patients with PCa that has metastasised is poor despite initial responses
to androgen ablation therapy. For patients with organ-confined disease, treatments such as radical prostatectomy and radiation therapy may produce complications such as impotence, incontinence or rectal or bladder injury, and metastatic disease frequently develops after surgery [1–3]. Our goal and that of others [4–7] has been to investigate gene therapy strategies to provide new, less invasive approaches for treating early as well as late-stage PCa.

Strategies for prostate cancer gene therapy have been based on: (1) corrective gene replacement to restore suppressor gene activity, or to abrogate oncogene activity; (2) immunotherapy to augment the body's immune response against prostate cancer; or (3) cytotoxic reduction of PCa by induction of apoptosis, antiangiogenesis, gene-directed radioisotopic therapy or by gene-directed enzyme prodrug therapy (GDEPT; reviewed in [6–8]). Tissue ablation by one of several methods is permissible since the prostate gland is a non-vital organ. GDEPT most often involves the use of a viral vector to deliver a gene (usually viral or bacterial) not found in mammalian cells and one whose product can convert a relatively non-toxic prodrug to an active drug [9]. An advantage of GDEPT is derived from a local bystander effect through which comprehensive cell killing is achieved without the need to express the gene in all cells [10,11]. This is provided by the ability of the toxic metabolite 2-fluoroadenine (2FA) to diffuse freely across cell membranes or via gap junctions. This is important given that in vivo gene transfer efficiencies do not approach 100% with current gene delivery vectors.

Of several GDEPT systems under investigation for PCa we have focused on the E. coli enzyme purine nucleoside phosphorylase (PNP), that converts the prodrug fludarabine phosphate into the highly toxic metabolite 2-fluoroaradene (2FA) [4,5,12]. 2FA is highly toxic due to its ability to inhibit RNA, DNA and protein synthesis. Unlike other GDEPT metabolites such as ganciclovir triphosphate, 2FA is a non-phosphorylated metabolite allowing it to freely diffuse across cell membranes to induce a potent bystander activity [13–15]. By permeating the pathway for RNA synthesis, 2FA is also toxic to both dividing and non-dividing cells, an important requirement for slow-growing cancers such as PCa [16,17].

We and others have been assessing the therapeutic potential of PNP-based GDEPT for treating cancer in vivo. An attenuated vaccinia vector was used to deliver PNP intratumourally with the prodrug 6-methylpurine-2-deoxyriboside (6MPDR) in a murine hepatic metastasis model [18]. Vector (10⁶ infectious units) delivered intraperitoneally (ip) into athymic nude mice bearing hepatic metastases, followed by administration of the prodrug (10mg/kg) every other day for 14 days, produced a 30% cure rate and significant prolongation of animal survival [18]. Mohr et al. [19] used a human adenoviral vector to deliver PNP under the control of a human cytomegalovirus early promoter (HCMV) and the prodrug fludarabine in a mouse model of hepatocellular carcinoma (HCC). Subcutaneous (sc) and intrahepatic tumour formation in nude mice produced by HCC lines transduced with PNP was abolished if mice were treated with fludarabine. Established sc HCC tumours in nude mice that were injected intratumourally with 5 x 10⁶ infectious units of vector followed by ip treatment with 250 µg fludarabine injected three times per day for 5 days showed 70% survival for 4 weeks when all control animals died during this period. Although 50% of established tumours became undetectable week after prodrug treatment, they finally progressed in all animals. In addition, we previously constructed a vector based on ovine atadenovirus; the prototype of a new adenovirus genus [20]. The vector, OAdV220, carrying the PNP gene under the control of the constitutive Rous sarcoma virus (RSV) promoter, was used together with the prodrug fludarabine phosphate. This PNP-GDEPT system retarded the growth of androgen-independent, syngeneic RM1 tumours and human PC3 prostate tumours grown sc in immune-competent and immune-deficient mice, respectively [5]. Transduction of RM1 cells with OAdV220 before sc implantation dramatically inhibited tumour growth when fludarabine phosphate was administered systemically and increased mouse survival in a dose-dependent manner, but lesser efficacy was observed in established tumours [5]. While these mouse models have been extremely useful for demonstrating proof of concept for PNP-GDEPT treatment of androgen-independent PCa, they do not recapitulate the development and progression of human disease.

The TRAMP model shows prostate cancer progression that more closely mimics the development of human disease [21,22]. In this transgenic model, the expression of Tag is regulated by the −426 to +28 region of the rat probasin promoter. This regulatory element is androgen-regulated and mice express Tag when approaching puberty at 8 weeks of age. One hundred percent of TRAMP mice spontaneously develop PCa regardless of their genetic background [22]. However, some variability in development of metastases in TRAMP mice that appears to be dependent on the genetic background of the animals has been observed. TRAMP mice on a pure C57BL/6 background showed some PCa metastasis to the periaortic lymph nodes [23]; however, we found this only in those mice with poorly differentiated PCa [24]. Other groups have observed that these mice also develop metastases in the lungs [25]. However, we did not observe any lung metastases in pure C57BL/6 TRAMP mice up to the age of 33 weeks [24]. In contrast, C57BL/6 TRAMP x FVB)F1 mice developed metastases in the lymph nodes and lungs as well as in the liver and to a small extent in the bone [26,27]. Recent studies have established the utility of TRAMP mice for assessing various forms of PCa therapy involving chemoprevention [25,27–29], immunotherapy [30,31], ionising radiation and/or oncolytic virus treatment [32]. We showed in principle that pure C57BL/6 TRAMP mice with well to moderately differentiated PCa could be utilised within a 25–33 weeks of age treatment to harvest window for...
preclinical GDEPT studies [24]. In the current study, we demonstrate that PNP-GDEPT delivered by OAdV caused significant suppression of PCa progression in and increased survival of TRAMP mice.

Methods

TRAMP breeding and care

Animals were bred at the University of New South Wales Biological Resources Centre (BRC) under specific pathogen free (SPF) conditions. Female TRAMP mice heterozygous for the Tag transgene were mated with male C57BL/6 inbred mice to provide heterozygous (C57BL/6 TRAMP x C57BL/6)F1 offspring. Pups were routinely tail clipped (<5 mm) between 2–3 weeks of age. Tail tips were stored at −20 °C in sterile Eppendorf tubes until processed for genotyping by PCR as described previously [24]. Following genotyping, groups of 40 TRAMP+ males and approximately 10 non-transgenic (TRAMP−) male littermates (age-matched controls) were supplied at age ∼20 weeks. These mice were housed in filter-capped cages with access to mouse chow and acidified water ad libitum within a PC2 animal laboratory under SPF conditions. Animal monitoring and experimentation were approved by and conducted under the guidance of The University of New South Wales (UNSW)/Prince of Wales Hospital Animal Care and Ethics Committee, and their housing was consistent with the guidelines of the Australian Office of the Gene Technology Regulator and the UNSW Institutional Biosafety Committee.

Recombinant vector

The ovine adenovirus vector OAdV220 containing a 522-bp portion of the Rous sarcoma virus 3’ LTR linked to the E. coli PNP gene and bovine growth hormone (BGH) polyadenylation signal (RSV/PNP/BGH expression cassette) was constructed, rescued, purified and characterized as previously described [5]. The ovine atadenovirus vector OAdV220 containing a 522-bp portion of the Rous sarcoma virus 3’ LTR linked to the E. coli PNP gene and bovine growth hormone (BGH) polyadenylation signal (RSV/PNP/BGH expression cassette) was constructed, rescued, purified and characterized as previously described [5]. The ovine atadenovirus vector OAdV220 containing a 522-bp portion of the Rous sarcoma virus 3’ LTR linked to the E. coli PNP gene and bovine growth hormone (BGH) polyadenylation signal (RSV/PNP/BGH expression cassette) was constructed, rescued, purified and characterized as previously described [5].

PNP assay

To determine the in vivo transduction efficiency of OAdV220 in TRAMP prostates, mice were treated intraprostatically with 1010 vector particles (vp). Control mice received 1010 vp of OAdVwt. Two days after treatment, the prostate and seminal vesicles were harvested and snap frozen at necropsy. Tissues were then homogenised in 0.4–1.2 mL of 50 mM potassium phosphate buffer, pH 7.4, depending on the amount of tissue, followed by three rounds of snap freezing on dry ice and thawing in a 37 °C water bath to complete cell lysis. Cell debris was removed by centrifugation for 5 min at 15,000 g and supernatants transferred to fresh tubes on ice. The protein concentration was measured using a BCA test kit (Pierce, Rockford, IL, USA). PNP activity in tissue homogenates (500 µg protein) was determined by an assay that monitored the conversion of a substrate 6MPDR to 6MP by reverse-phase high-performance liquid chromatography (HPLC) as previously described [5]. 6MPDR was synthesised and purified by HPLC as previously described [33]. Briefly, lysate was incubated in a 1.2 mL reaction volume with 500 nmol of 6MPDR for 2 h before stopping the reaction by heating to 100 °C. Activity was expressed as conversion units (nmol 6MPDR converted/mg protein/h). Fludarabine phosphate was purchased from Schering AG (Germany), and administered ip daily at ∼600 mg/m2/mouse/day in a 100 µL volume for 5 days as previously described [5].
were sacrificed within the age window of cancer-related death (~33 weeks of age). Mice included in survival studies were sacrificed when a 20% weight loss was observed or if the animal showed any form of severe distress.

RM1 prostate cancer cells were kindly provided by Dr T. Thompson (Baylor College, Texas, USA) and grown in DMEM medium (Invitrogen, Grand Island, NY, USA) containing 2 mM L-glutamine (Invitrogen), 0.2 M HEPES buffer solution (Invitrogen), sodium bicarbonate (0.85 g/l; Sigma Chemical Co., St Louis, MO, USA), penicillin G (2 units/mL) and streptomycin sulfate (100 units/mL) (Invitrogen) and 10% fetal calf serum (FCS; Invitrogen). The cells were free of mycoplasma and used in the log phase of growth. Male C57BL/6 mice were bred and maintained at the BRC and were used at 6–8 weeks of age. Orthotopic RM1 tumours in C57BL/6 mice were developed by implanting 5 × 10^3 RM1 tumour cells into the dorsal prostatic lobe following a low abdominal transverse incision. On day 4, mice were anaesthetised and the previous incision reopened for intraprostatic treatment with OAdV220 or control as described above. The mice were monitored twice weekly as described above and sacrificed on day 18.

Genitourinary tract harvest and measurement

At the time of sacrifice, the lower genitourinary (GU) tract including the testes, emptied bladder, prostate and seminal vesicles was removed and the wet weight (wt) was recorded to the nearest 0.0000 g. The testes, bladder and prostate were then dissected individually, weighed and recorded. As the seminal fluid was quickly lost post-dissection, seminal vesicle weight was determined by subtraction of the testes, bladder and prostate weights from the GU tract weight. Prostate volume (V) was calculated from prostate diameters (d) measured at right angles using a vernier caliper, V = π/6(d1 × d2)^3/2 [34].

GDEPT treatment was compared with the control group. Suppression in GU tract development was calculated as follows:

Percentage reduction = \[
\frac{\text{mean wt}_{\text{control}} - \text{mean wt}_{\text{treatment}}}{\text{mean wt}_{\text{control}} \times 100}
\]

Microscopic analysis of the PCa histopathology in these mice was also performed.

Preparation and histological analysis of tissues

Tissues collected at necropsy were processed and analysed as previously described [24]. Histology sections were evaluated blindly for the presence of primary PCa and metastasis in the lymph nodes and lungs using light microscopy. Mitotic figures were scored in 10 high-power fields. PCa progression was classified as previously described by Gingrich et al. [26]. Briefly, in well-differentiated PCa the glandular architecture with open lumina is maintained. Epithelial papillary projections with cell proliferation and increased mitotic activity occur and the basal cell layer is no longer detectable. In moderately differentiated PCa, distinct glandular units are maintained. However, epithelial proliferations form cribriform structures and solid sheaths with abundant atypical mitoses. These proliferations obliterate some of the gland lumina. Poorly differentiated PCa showed a total loss of glandular architecture. Extreme cellular atypia with atypical mitotic figures and areas of necrosis were present.

Data analysis

Measurements for each mouse were entered and analysed using the GraphPad PRISM V2.01 software for Windows 95. A one-way analysis of variance (ANOVA) was performed if the data in multiple groups were normally distributed. A Tukey's post-test was performed if the ANOVA indicated a significant difference between treatments on the GU tract development of ~33-week-old TRAMP+ mice.

Results

Histological characterisation of animals

The progression of disease in 27 TRAMP+ mice was previously monitored by histological analysis to identify those most suitable for GDEPT treatment [24]. One case of PIN (4%) was observed, but, in agreement with others [26], most (C57BL/6 TRAMP x C57BL/6)F1 transgenic male offspring aged 25–33 weeks exhibited a range of PCa progression from well- (26%) to moderate- (37%) or poorly differentiated (33%) adenocarcinoma (Figure 1).

PNP expression delivered by OAdV220 in vivo

We previously demonstrated that injection of 10^{10} vp of OAdV220 into sc RM1 tumours in C57BL/6 mice produced PNP activity that persisted in tumour homogenates for at least 6 days post vector injection [5]. In this study groups of three TRAMP+ mice received one iprost injection of 10^{10} vp of OAdVwt or test vector OAdV220 (Figures 2A and 2B). Similarly, RM1 tumours in C57BL/6 mice received one injection of 5 × 10^9 vp OAdV220 or vector buffer (Figure 2C). Prostate tumours and adjacent seminal vesicles were harvested 2–3 days post vector injection, homogenised and assayed for PNP activity. As expected vector buffer or OAdVwt treatment
Figure 1. Genitourinary (GU) tracts isolated from (C57BL/6 TRAMP × C57BL/6)F1 TRAMP+ and TRAMP− mice. Macroscopic photographs were taken of 25–28-week-old TRAMP− and TRAMP+ lower GU tracts including the bladder (b), prostate (p) and seminal vesicles (sv). The typical histopathology seen for the prostate in these mice is illustrated at 10× (A) and 40× (B) magnification. (A) The TRAMP− prostate uniformly showed glands lined by a single layer of tall columnar epithelial cells with round basally placed nuclei. The glandular units are surrounded by a thin layer of smooth muscle (2–3 layers) and loose connective tissue. The glands show large open lumens. (B) At higher magnification the normal small round to oval basally placed nuclei can be seen and the tall columnar epithelium is surrounded by a layer of flattened basal cells. The TRAMP+ prostate predominantly shows well to moderately differentiated PCa (A). Generally, there is an increase in the size of each gland unit. All glands show some areas of perforated epithelium (cribriform or basket-like) structures. Glands showing well-differentiated PCa have open lumens (lower left corner) whereas those showing moderately differentiated PCa display almost completely filled lumens. At 40× magnification (B), there are areas with recognisable glandular architecture; however, the epithelial cells are atypical with atypical mitoses. Other areas (left) have a disorganised growth pattern with sheet formation and an increased number of mitotic figures. It is difficult to identify the basal layer surrounding the epithelium, suggesting invasion.

produced no detectable PNP activity in the prostate or seminal vesicles of these mice. On average (±SD), OAdV220 treatment resulted in the prostate homogenate conversion of 324 ± 39 and 195 ± 120 nmol 6MPDR/mg protein/h from TRAMP+ and RM1 tumour-bearing mice. The TRAMP+ seminal vesicle homogenates showed a conversion of 68 ± 36 nmol 6MPDR/mg protein/h. These data confirmed that OAdV220 effectively delivered the
PNP gene and expressed it similarly in RM1 as well as TRAMP+ mouse prostate tumours in vivo. Some distribution of virus to the adjacent seminal vesicles also occurred in the TRAMP+ mice.

**Impact of OAdV220 alone on prostate tumour growth**

We previously demonstrated that transducing RM1 cells ex vivo with high levels of OAdV220 ranging from $10^2$–$10^4$ vp per cell followed by sc implantation in C57BL/6 mice showed no significant impact on tumour growth [5]. For example, all mice treated with the vector alone were dead due to excessive tumour burden by day 45 ($\geq 15 \times 15 \text{ mm diameter or } 1767 \text{ mm}^3$, at which time the mice were sacrificed). At day 45, 100% of mice treated with $10^4$ vp OAdV220 + fludarabine phosphate were still alive. A mock transduction was also performed and cells were implanted in mice followed by treatment with saline or fludarabine. These mice were also all dead by day 45 and did not show a difference in tumour growth rate. This study showed that OAdV220 alone or fludarabine alone did not impact sc RM1 tumour growth. The effect of OAdV220 alone on PCA growth was further explored in the RM1 model herein. RM1 tumour-bearing mice were treated iprost with vector buffer or $10^{10}$ vp OAdV220 and both groups were given saline ip for the following 5 days (Figure 3). The $10^{10}$ vp OAdV220 treatment showed a mean tumour volume ± SEM of $1228 \pm 219 \text{ mm}^3$ and

![Figure 2. OAdV220-delivered PNP expression in vivo. TRAMP+ mice (n = 3) received one iprost injection of $10^{10}$ vp wild-type OAdV or OAdV220. C57BL/6 mice bearing RM1 tumours (n = 3) received one injection of $5 \times 10^8$ vp OAdV220 or vector buffer. TRAMP+ prostate tumours (A) and their adjacent seminal vesicles (B) or RM1 tumours (C) were harvested 2–3 days post vector injection. These tissues were individually homogenised and soluble protein (500 µg) was assayed for PNP activity as described in the Methods. Homogenate PNP activity was presented as 6MPDR conversion units (nmol 6MPDR converted/mg protein/h).](image)

![Figure 3. Impact of OAdV2220 used alone on prostate tumour growth. Groups of 7–8 RM1 tumour-bearing C57BL/6 mice were treated iprost with vector buffer or $10^{10}$ vp OAdV220 and both groups were given fludarabine phosphate (∼$600 \text{ mg/m}^2$/day/mouse, A) or saline ip (B) once daily for 5 days thereafter. Mice were sacrificed 18 days following RM1 cell implantation and the tumour volume was determined from the prostate tumour diameters measured at right angles. Data are presented as mean tumour volume ± SEM for each treatment group.](image)
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did not impact on tumour growth when compared with the control mice (1007 ± 220 mm³). When the same experiment was repeated with fludarabine treatment ip, 10¹⁰ vp OAdV220 GDEPT (1054 ± 292 mm³) showed a 25% reduction in RM1 prostate tumour growth (similar observation to Voeks et al. [5]) when compared with the fludarabine alone control (1404 ± 558 mm³). These data show that OAdV220 alone does not impact on mouse PCa growth.

Impact of fludarabine phosphate on GU tract development of TRAMP+ mice

Preliminary experiments were performed to determine whether, at the systemic dose administered (~600 mg/m²/day/mouse), fludarabine phosphate treatment alone had any effect on GU tract and prostate tumour development. Groups of ~13 TRAMP+ mice were treated at ~25 weeks of age with nil vector:nil pro-drug (group 1), vector buffer A iprost and saline ip (group 2) or vector buffer A iprost and fludarabine phosphate ip (group 3). All mice were sacrificed at ~32 weeks of age (Figure 4). GU tract weight which ranged from 2.25 ± 0.11g (mean ± SE) to 4.11 ± 1.47g did not differ significantly among these three groups (ANOVA p = 0.3047). A Tukey’s multiple comparison post-test showed there was no difference in GU tract development when the control groups were compared with the fludarabine phosphate treated group. These results were also reflected in the prostate weight and volume measurements. In addition, assessment of prostates by microscopy showed that 71–80% of mice across all groups had well to moderately differentiated PCa and 20–29% had poorly differentiated PCa (Table 1). Thus, treatment with fludarabine phosphate alone did not impact on TRAMP+ mouse GU tract development.

Impact of PNP-GDEPT on GU tract development in TRAMP+ mice

Efficacy

We next examined the therapeutic efficacy of OAdV220 plus fludarabine phosphate treatment on prostate tumour development in vivo. Our strategy was to treat the TRAMP+ prostate with one injection of vector at ~25 weeks of age followed by one systemic course of fludarabine phosphate administered for 5 consecutive days thereafter. Mice were sacrificed at ~33 weeks of age and the impact of GDEPT on the GU tract development in these mice was assessed. Animals with a large tumour burden and poorly differentiated tumours were excluded from the study, as explained in the Methods. Groups of 9 TRAMP+ mice received: vector buffer A iprost and saline ip for 5 days (group 1); OAdV220 iprost (10¹⁰ vp in vector buffer A per prostate) and fludarabine phosphate ip (~600mg/m²/day/mouse) for 5 days (group 2); OAdV220 iprost (10¹⁰ vp in vector buffer B per prostate) and fludarabine ip (group 3). The impact of GDEPT on development of the lower GU tract is

Figure 4. Impact of fludarabine phosphate treatment alone on the lower GU tract development of TRAMP+ mice. Groups of 13 TRAMP+ mice ~25 weeks of age were treated iprost with nil (group 1) or vector buffer A (groups 2 and 3). These mice then received nil (group 1), saline (group 2) or fludarabine phosphate (~600 mg/m²/day/mouse, group 3) ip once daily for 5 days thereafter. TRAMP−mice were included to provide age-matched controls (group −). Following treatment, mice were sacrificed at ~32 weeks of age. The GU tract wet weight that included the testes, bladder, prostate and seminal vesicles was recorded. These tissues were further dissected and the prostate weight was measured. The prostate diameters were measured at right angles using a vernier caliper from which the tumour volume was determined.
Table 1. Impact of fludarabine phosphate treatment alone on TRAMP+ mouse prostate histopathology

<table>
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<th>Treatment Group</th>
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<th>PCa (%)</th>
<th>WD (%)</th>
<th>MD (%)</th>
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Abbreviations: PIN, prostatic intraepithelial neoplasia; PCa, prostate cancer; WD, well-differentiated PCa; MD, moderately differentiated PCa; PD, poorly differentiated PCa.

Treatments: TRAMP+ mice were treated iprost with nil (group 1) or vector buffer (groups 2 and 3). These mice then received nil (group 1), saline (group 2) or fludarabine phosphate (~600 mg/m²/day/mouse, group 3) ip once daily for 5 days thereafter.

Figure 5. Impact of PNP-based GDEPT on the lower GU tract development of TRAMP+ mice. Groups of 9 TRAMP+ mice ~25 weeks of age were treated iprost with vector buffer A (group 1) or 1 x 10¹⁰ vp OAdV220 in vector buffer A (group 2) and vector buffer B (group 3) on day 0. These mice then received saline (group 1) or fludarabine phosphate (~600 mg/m²/day/mouse, groups 2 and 3) ip once daily for 5 days thereafter. Tissues of 5 mice from each group were macroscopically photographed prior to further tissue processing. TRAMP−mice were included to provide age-matched controls for the experiment.

The GU tract, prostate and seminal vesicle weight and prostate volume were determined for each animal (Figure 6). Mean (±SE) GU tract weights of 2.73 ± 0.22, 1.44 ± 0.14 and 1.73 ± 0.21 g was observed for groups 1, 2 and 3, respectively. Similar trends in mean prostate weight of 0.35 ± 0.18, 0.15 ± 0.01 and 0.15 ± 0.02 g were also observed for groups 1, 2 and 3, respectively. The seminal vesicles made up the majority of the GU tract with mean weights of 2.12 ± 0.18, 1.06 ± 0.13 and 1.38 ± 0.02 g. When compared with the buffer control (group 1), GDEPT (groups 2 and 3) significantly reduced (ANOVA p = 0.0002) the GU tract weight by 36–47% (Tukey’s post-test: group 1 vs. group 2, p < 0.001; group 1 vs. group 3, p < 0.01; group 2 vs. group 3, p > 0.05). A similar reduction (35–50%) in seminal vesicle weight (ANOVA p = 0.0007) was also observed (Tukey’s post-test: group 1 vs. group 2, p < 0.001; group 1 vs. group 3, p < 0.05; group 2 vs. group 3, p > 0.05). The prostate weight was reduced even further (57% ANOVA p = 0.0007, Tukey’s post-test: group...
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Figure 6. GU tract weight, seminal vesicle weight, prostate weight and volume in PNP-based GDEPT-treated TRAMP+ mice. Following treatment (see Figure 5) mice were sacrificed at ~33 weeks of age. The GU tract wet weight including the testes, bladder, prostate and seminal vesicles was recorded (g). These tissues were further dissected and the testes, bladder, prostate weights were measured. The seminal vesicle weight was determined by subtraction of the testes, bladder and prostate from the GU tract weight. The prostate diameters were measured at right angles using a vernier caliper from which the tumour volume was determined. TRAMP−mice were included to provide age-matched controls for the experiment.

Table 1

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</tr>
<tr>
<td>Group 2</td>
<td>0.50</td>
<td>0.50</td>
</tr>
<tr>
<td>Group 3</td>
<td>0.25</td>
<td>0.25</td>
</tr>
<tr>
<td>Group (-)</td>
<td>0.00</td>
<td>0.00</td>
</tr>
</tbody>
</table>

1 vs. group 2, p < 0.01; group 1 vs. group 3, p < 0.01; group 2 vs. group 3, p > 0.05). This experiment was repeated with similar results.

Histology

Microscopic assessment of prostates harvested from the two experiments showed that control mice had moderately differentiated PCa (50%) and well-differentiated PCa (38%) with one animal (12%) presenting with PIN (Table 2). In contrast, PNP-GDEPT mice showed mostly well-differentiated PCa (71%) and PIN (24%); very few animals displayed moderately differentiated PCa (6%). On average, the control mice had 29.4 ± 6.8 mitotic bodies per 10 high-powered fields, whereas PNP-GDEPT mice showed a 41% reduction in observed mitotic bodies (17.4 ± 3.4). Taken together, these results suggest that PNP-GDEPT suppressed TRAMP+ PCa development and may preferentially kill rapidly dividing cells.

In control mice with moderately differentiated tumours many glands were completely filled with proliferating epithelium that obliterated the lumen. Although there were areas of recognisable glandular architecture, the epithelial cells were atypical showing cribriform structures and many mitotic figures (>60 in 10 fields). Well-differentiated tumours showed some glands that were partly filled with proliferating epithelium; however, the lumina remained open. These glandular units lacked basal cells. Invasive glands showing a loss of the basal layer surrounding the acini epithelial cells were also seen. Epithelial cribriform structures and many mitotic figures (~40 in 10 fields) were observed. The one control mouse with PIN showed normal glandular architecture with some epithelial proliferation forming papillary structures. There was a slight increase in mitotic activity; however, the basal cell layer remained intact. In contrast, PNP-GDEPT-treated mice showed predominantly well-differentiated tumours and most glands displayed only a single epithelium cell layer with a reduction in the number of cribriform structures. The PNP-GDEPT mice presenting with PIN showed no invasion. Overall, the histopathology in the prostate of TRAMP+ PNP-GDEPT mice further supported the ability of the treatment to suppress PCa development.

Impact of PNP-GDEPT on the survival of TRAMP+ mice

In a separate experiment the survival of PNP-GDEPT TRAMP+ mice was also extended (Figure 7). Groups of 9 TRAMP+ mice were treated iprost at ~25 weeks of age with vector buffer A (group 1) or 1010 vp OAdV220 in vector buffer A (group 2) on day 0. These mice then received saline (group 1) or fludarabine phosphate (~600 mg/m2/day/mouse, group 2) ip once daily for 5 days. Mice were routinely weighed and palpated in the lower abdominal region thereafter. Only 30% of control TRAMP+ mice persisted beyond 115 days post iprost treatment when 67% of PNP-GDEPT animals were still alive. This study was terminated 128 days post vector injection.
Table 2. Impact of PNP-GDEPT on TRAMP+ mouse prostate histopathology

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>n</th>
<th>Negative (%)</th>
<th>PIN (%)</th>
<th>PCa (%)</th>
<th>WD (%)</th>
<th>MD (%)</th>
<th>PD (%)</th>
<th>Mitosis Mean ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>(1)</td>
<td>8</td>
<td>nil</td>
<td>1 (12.5%)</td>
<td>7 (87.5%)</td>
<td>3 (37.5%)</td>
<td>4 (50%)</td>
<td>nil</td>
<td>29.38 ± 6.76</td>
</tr>
<tr>
<td>(2) and (3)</td>
<td>17</td>
<td>nil</td>
<td>4 (23.5%)</td>
<td>13 (76.4%)</td>
<td>12 (70.6%)</td>
<td>1 (5.9%)</td>
<td>nil</td>
<td>17.38 ± 3.4</td>
</tr>
</tbody>
</table>

Abbreviations: PIN, prostatic intraepithelial neoplasia; PCa, prostate cancer; WD, well-differentiated PCa; MD, moderately differentiated PCa; PD, poorly differentiated PCa.

Discussion

This study on the efficacy of PNP-based GDEPT has been carried out in a mouse model that more closely mimics the development and progression of PCa in humans [21,22]. Until recently, preclinical therapeutic studies were performed in animal models implanted with PCa tumour cells, a method that does not display the natural kinetics of tumour induction within the anatomical site. In addition, we found that the TRAMP+ model can be used successfully for preclinical experimentation provided that mice with poorly differentiated PCa and a large tumour burden are excluded prior to treatment at ∼25 weeks of age. This exclusion was justified on the grounds that these mice recovered slowly from the prostate treatment surgery and often do not survive to the study endpoint [24]. By largely excluding such animals, a treatment window between 25 and 33 weeks of age became accessible to orthotopic treatment involving GDEPT. This window allows time to recover from the intensive surgery performed during the experiment and, more importantly, allows for a statistically significant progression in GU tract weight, seminal vesicle weight and prostate weight or volume in the mice. We chose to assess the GDEPT system based on *E coli* PNP and fludarabine [13,14,16]. This GDEPT system induces a powerful bystander effect but its overall potency is still influenced by the number of cells in which the PNP gene is expressed.

Recombinant human adenoviruses (hAd) have been widely used for gene delivery since they efficiently enter many types of mammalian cells and express their transgene. However, the successful application of hAd in human clinical trials may be limited in certain circumstances by their immunogenicity, safety and effectiveness in the face of preexisting natural immunity [35–37]. To address these concerns, we are developing a strategy for gene delivery based on the use of OAdV, a non-human ovine atadenovirus vector that infects but replicates abortively in non-ovine cell lines [38–40]. Methods to produce recombinant OAdV vectors have been developed [41,42]. Importantly, OAdV was able to express a transgene *in vivo* in the presence of immunity to a common human adenovirus [43]. The vector also has a favourable biosafety profile in that it lacks apparent transforming activity [44] and it is not complemented by a replicating human adenovirus [39]. Although OAdV uses a receptor that is unidentified but distinct from CAR, an important AdV5 receptor, it nevertheless transduces a wide range of human and non-human cell types (reviewed in [45]). We have shown here and in previous animal studies [5] that
Preclinical GDEPT Studies using the TRAMP Model

OAdV220 efficiently delivers the PNP transgene to mouse prostate cells. As PNP was controlled by the RSV promoter that is active in many cell types, the only specificity in the orthotopic GDEPT procedure used here was provided by physical delivery of the vector to the prostate. In mice, the vector is fairly evenly distributed between major organs after intravenous delivery and its tropism is not liver-dominant [43]. If vector diffused to other organs from the prostate (and this was not monitored here) there was no observable impact on animal welfare or survival in this study, even following treatment with fludarabine. There was, however, some distribution of the vector to the nearby seminal vesicles as judged by the levels of PNP expression (Figure 2). The seminal vesicles are by far the larger organ by weight, compared with the prostate to which the vector was delivered, thus substantial virus may have initially been present in the total organ.

PNP-GDEPT-treated mice also showed a survival advantage over control mice (Figure 7). Interestingly, microscopic assessment of the prostates harvested showed that PCa development was slowed in those animals receiving PNP-GDEPT (Table 2). Whereas control TRAMP+ mice had predominantly moderately (50%) or well-differentiated PCa (37%) and one animal presented with PIN (13%), the PNP-GDEPT mice mostly showed well-differentiated PCa (71%) and PIN (24%). Very few mice had moderately differentiated PCa (6%). Furthermore, PNP-GDEPT-treated mice showed a substantial (41%) reduction in observed mitotic bodies when compared with the controls. As the number of mitoses seen in the treated mice was lower than in controls, it is likely that the GDEPT was preferentially effective in rapidly dividing cells where both DNA as well as RNA synthesis is likely to be affected. In contrast, in non-dividing cells, RNA synthesis is the principle target of toxic purines such as ZPA.

This study has demonstrated the efficacy of the PNP-GDEPT delivered by an ovine adenoavirus in a mouse model that mimics PCa development and progression in humans. Recent human trials involving adenoviral gene delivery have renewed the emphasis on safety, above all, in the development of gene therapy strategies [46,47]. The use of a transcriptionally targeted vector in conjunction with physical delivery directly to the prostate would provide an even greater safety margin. We are currently constructing OAdV containing the prostate targeting promoter rat probasin with an SV40 enhancer [4] to deliver the PNP-based GDEPT to human advanced stage PCa in xenograft mouse models. However, this prostate-targeting promoter that drives PNP expression effectively in human cells does not express in murine cells. We anticipate that the use of GDEPT in combination with other strategies, such as those which deplete androgen-sensitive cells or enhance antitumour immunity, may also provide greater efficacy in future studies.

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References


