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Adenovirally delivered enzyme prodrug therapy with herpes simplex virus-thymidine kinase in composite tissue free flaps shows therapeutic efficacy in rat models of glioma

Structured Abstract

Introduction—Free flap gene therapy exploits a novel therapeutic window when viral vectors can be delivered to the flap *ex vivo*. We investigated the therapeutic potential of the thymidine kinase (TK)/ganciclovir pro-drug system in treating residual disease when delivered into a free flap by intra-arterial injection of an adenoviral vector (Ad.TK).

Methods—We demonstrated direct *in vitro* efficacy of the Ad.TK/ganciclovir system by treating a panel of malignant cell lines with Ad.TK/ganciclovir to show significant cell kill proportional to the multiplicity of infection (MOI) of Ad.TK. Indirect (bystander) cytotoxicity was demonstrated by transferring conditioned medium from Ad.TK-infected malignant, or non-malignant, producer cells to uninfected tumour cells. We investigated the effect of Ad.TK/ganciclovir therapy *in vivo*, using models of microscopic (MiRD) and macroscopic (MaRD) residual disease in a rodent superficial inferior epigastric artery flap model.

Results—We observed retardation of tumour volume growth in both MiRD and MaRD models ($p < 0.05$) and improvements in animal survival (MiRD median survival: MOI10 = 28 days, MOI 50 = 25 days, control = 18.5 days, $p = 0.0004$; MaRD median survival: MOI 50 = 30 days, control = 18 days, $p = 0.0005$). Gene expression studies demonstrated that viral genomic material was found predominantly in flap tissues but declined over time.

Conclusion—In summary, we describe the utility of virally-delivered enzyme/pro-drug therapy (VDEPT), using a free flap as a vehicle for delivery. We discuss the merits and limitations of this approach and the unique role of therapeutic free flaps in the reconstructive armamentarium.

Introduction

Surgical excision remains the mainstay of management for loco-regional disease across a variety of tumour types and offers the best chance of achieving cure. Whilst radical approaches to tumour excision, in the context of primary disease, are more selectively utilised than historically practised (1–3), they still retain a major role in the management of locally advanced and recurrent disease (4, 5). When such resections take place, the most popular technique for the reconstruction of extirpative defects is free tissue transfer (6–8), however, free flaps currently do not deliver any therapy to the underlying disease process.

Ex vivo gene therapy in free flaps has been described previously (9–12) and a variety of gene therapy strategies may be applied to deliver targeted cancer therapies to microscopic residual disease at the tumour bed (12). We have previously shown that the intra-vascular administration of viral vectors into flaps achieves greater transfection efficiencies than non-viral and percutaneous modalities of delivery (13), although topical viral administration has

been successful in other contexts(14–16). Free flap-mediated immunostimulatory approaches(10) to eradicate microscopic residual disease have previously been described, for example, through the over-expression of interleukin-12 (IL-12)(17–22) and have shown retardation of tumour growth by therapeutic flaps. In this study, we chose to investigate the utility of an alternative gene therapy strategy to treat residual disease: virally delivered enzyme/prodrug therapy (VDEPT). This involves a gain-of-function transfection/infection in target cells with a gene that allows the transfected/infected cell to metabolise a systemically-delivered, non-toxic, pro-drug into a cytotoxic metabolite. In the HSV-TK system that we used, the therapeutic gene encodes thymidine kinase (TK) (derived from the herpes simplex virus (HSV)), an enzyme that converts ganciclovir (pro-drug) into an S-phase specific cytotoxic metabolite (GCV-triphosphate)(23). Previous pre-clinical(24) and Phase I trial(25) data showed early promise with VDEPT systems, however a subsequent Phase III clinical trial in glioblastoma(26) failed to show improvements in survival outcomes following systemic viral administration. The authors concluded that the largest obstacle to therapy was the failure to achieve therapeutically relevant transgene expression at the site of disease. VDEPT may be particularly suited for delivery through a free flap because the production of cytotoxic metabolites will be greatest directly over the tumour bed, atop where the flap sits, and, therefore, be optimally placed to reduce the burden of microscopic disease and the risk of local tumour recurrence.

In this paper, we describe a pre-clinical, small animal model of VDEPT with the HSV-TK/ganciclovir system, delivered *ex vivo*, by an adenoviral vector (Ad.TK), into a free flap. We demonstrate that flaps transfected with Ad.TK mediate a therapeutic effect at the tumour bed despite transgene expression being restricted to flap tissues only. These data provide pre-clinical proof-of-concept for the efficacy of VDEPT delivered through a therapeutic free flap and a strong rationale for further investigation of both VDEPT and other therapeutic strategies by genetically manipulating free flaps.

Materials and Methods

Cell culture

Rat glioma (RG2), human colorectal (HCT116), rat gliosarcoma (9LlacZ) and rat fibroblast (RF) cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with L-arginine (2.5%), foetal bovine serum (FBS) (10%) and penicillin/streptomycin (1%).

Production of replication-defective adenoviral vectors expressing thymidine kinase (Ad.TK) and green fluorescent protein (Ad.GFP)

The thymidine kinase (TK) gene was amplified from pSOM91 (kind gift from Dr Richard Lamb, ICR) by PCR using primers containing restriction sites for BglIII (FW: 5'-AAGATCTATGGCTTCGTACCCCTGCCATC-3') and HindIII (REV: 5'-CTGAAAGCTTTCAGTTAGCCTCCCCATC-3'). The amplified fragment was gel-purified, digested with BglIII and HindIII and subcloned into the adenovirus pShuttle vector (pShuttleCMV-TK). Ad.GFP was kindly donated by Dr Mohan Hingorani (Institute of Cancer Research, London). Recombinant adenoviral constructs were linearised with Pac I and transfected into 293A cells using LipofectamineTM2000 (Life Technologies Ltd, UK).

After infection, cells were incubated at 37 °C for 3–4 days and monitored for cytopathic effect (c.p.e.). Freeze-thaw cycles were repeated until a sufficient quantity of virus had been produced.

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (MTT)

Cell viability was quantified using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. 5×10^3 RG2 cells per well were seeded in 200 μ L 10% DMEM in a 96-well plate. Twenty-four hours later the media was aspirated and cells were inoculated with Ad.TK at multiplicities of infection (MOIs) ranging from 0–100 and 24 hours after this ganciclovir was added to the well to give final concentrations ranging from 0–50 μ M. At the experimental end-point, 20 μ L of MTT (5 mg/mL) was added and the plate incubated for 4-hours at 37 °C after which, crystals were solubilized in 200 μ L DMSO and absorbance measured at 550 nm by a plate reader.

Bystander kill assay

1×10^6 tumour cells (HCT116 and RG2) or non-cancer cells (rat fibroblasts (RF)) were plated in a T25 tissue culture flask on day 0. On day 1, cells were infected with Ad-TK at MOIs of 0, 10 and 50 in a volume of 2 mL DMEM (1% FBS). Forty-eight hours later the inoculum was removed and replaced with fresh DMEM containing ganciclovir (0, 50 or 100 μ M). The following day 5×10^3 RG2 cells were seeded in a 96-well plate in 100 μ L of DMEM and 24 hours later were inoculated with 100 μ L of supernatant from infected cells. MTT assays were performed, as described previously, at 72 and 96 hours after addition of supernatant to RG2 cells.

SIEA flap transfection

Our flap transfection protocol has been described previously(13) but, in brief, superficial inferior epigastric artery (SIEA) flaps (4×2 cm) were marked on the caudal abdomen of Fischer (F344) male rats (250 g) as shown and raised on the SIE vessels under inhalational anaesthesia (Abbott Laboratories, UK). The common femoral (CF) vessels were stripped of adventitia and divided proximal and distal to the origin of the SIE pedicle. The flap was placed in a tissue culture dish for transfection prior to cannulation of the distal common femoral artery (CFA) using a 27 G cannula (John Weiss International, UK). The proximal CF vessels and distal common femoral vein (CFV) were clamped and virus (or PBS for sham infections) injected intra-arterially. The virus was allowed to dwell in the flap for 40 minutes, following which the flap was irrigated with PBS and the proximal CF vessels re-anastomosed. Flaps were inset using 6/0 vicryl rapide (Ethicon, UK). All animals were maintained in accordance with our institution's animal welfare policies and the "humane end-point", as defined by our regulatory framework, was a tumour greater than 2 cm in any dimension.

Microscopic residual disease (MiRD)

SIEA flaps were raised (n=18) and 1×10^7 rat glioma (RG2) cells were injected subcutaneously into the flap immediately prior to transfection as described above. Flaps were treated with PBS (control) or virus (Ad.TK) at an approximate MOI of 10 (1×10^8 PFU)

and $50 (5 \times 10^8 \text{ PFU})$ through the intra-vascular route. Ganciclovir (50 mg/kg i.p.) was administered daily from the first post-operative day until humane end-point, Tumour dimensions were measured using callipers and tumour volume quantified using the formula $V = [\pi \times (\text{length} \times \text{width} \times \text{height})]/6$.

Macroscopic residual disease (MaRD)

RG2 cells (1×10^7) were engrafted below the xiphoid notch ($n=12$) and tumour measurements taken daily until the volume of the tumour reached 1 cm^3 (supplementary figure 2). At this time, the tumour was incompletely excised with a 0.5 cm radial margin to leave a macroscopic residual tumour nodule of 0.1 cm diameter. Following incomplete excision SIEA flaps were raised and treated, *ex vivo*, with PBS (control; $n=6$) or virus (Ad.TK MOI 50; $5 \times 10^8 \text{ PFU}$; $n=6$) and inset over the area of incomplete excision. Flaps were inset and the donor sites closed primarily. Animals were recovered and ganciclovir (50 mg/kg i.p.) was administered daily as described previously, until humane end-point.

Quantitative PCR analysis of thymidine kinase expression within the flap and at the flap-bed interface

SIEA flaps were infected with PBS control ($n = 3$) or Ad.TK (MOI 10; $n=3$; or MOI 50; $n=3$). The animals were culled at 72 hours following infection and the flap, and underlying flap bed, was harvested along with the flap pedicle. RNA was extracted from flap tissue using a commercially available kit (Qiagen, UK) and quantitative reverse transcriptase PCR (RT-PCR) performed. Reaction conditions were 1 cycle of 94°C for 10 minutes, followed by 40 cycles of 30 seconds at 94°C , 1 minute at 60°C and 30 seconds at 72°C . The forward and reverse primers used were: *HSV-1 TK* (Forward ($5'$ - $3'$): GTACCCGAGCCGATGACTTA; Reverse: ($5'$ - $3'$): CCGATATGAGGAGCCAGAAC) and *β -actin* (rat) (Forward ($5'$ - $3'$): AGCCATGTACGTAGCCATCC; Reverse ($5'$ - $3'$): CTCTCAGCTGTGGTGGTGAA).

Immunofluorescent staining for GFP expression in Ad.GFP-infected SIEA flaps

SIEA flaps were infected with Ad.GFP (MOI 10; $n = 9$) or Ad.TK (MOI 10; $n=3$) as a non-GFP encoding vector. Flaps were harvested 72 hours, 1 week or 3 weeks following infection ($n=3$ animals/time point) along with the underlying flap bed and SIEA pedicle. Samples were formalin fixed for 24 hours, embedded in paraffin and sectioned at a thickness of $3 \mu\text{m}$. Sections were de-waxed, permeabilized in Triton 0.2% (Sigma Aldrich, Crawley, UK) and blocked for 1 hour in PBS with bovine serum albumin (1%) and fetal calf serum (2%) at room temperature. Sections were incubated with a polyclonal rabbit anti-GFP primary antibody (1:100 in blocking solution) (Abcam, Cambridge, UK) at 4°C overnight. The following day, sections were washed in PBS and incubated with a goat anti-rabbit 488 FITC-conjugated secondary antibody (1:1000 in blocking buffer) (Life Technologies, Paisley, UK) for 1 hour at room temperature and then washed in PBS with 4',6-diamidino-2-phenylindole (DAPI; 1:50,000). Sections were then washed in PBS and fixed in paraformaldehyde (4%) for 15 minutes. Confocal images were captured on a Leica Microsystems TCS-SP2 confocal. Cell counts were performed at $\times 400$ magnification.

Systemic viral biodistribution

SIEA flaps of F344 rats were infected with adenovirus encoding the *lacZ* gene (Ad-CMV.lacZ) (Clontech, USA) at 1×10^8 PFU. Animals were culled at 5 days post-infection and internal organs (heart, lung, spleen, liver, kidney, testes) harvested and RNA extracted as described earlier. RT-PCR was performed using primers for *lacZ* (Forward (5'-3'): GATCAAATCTGTCGATCCTTCC and reverse (5'-3'): CAAAGACCAGACCGTTCATACA). The PCR product (5 μ L) was used for gel electrophoresis (2% agarose with Tris/Borate/EDTA buffer, 0.8% agarose and 5 μ L ethidium bromide/100 mL).

Statistical Analysis

All statistical analysis was carried out using GraphPad Prism 4 statistical program (San Diego, USA). Univariate analyses were performed using an independent samples t-test or one-way ANOVA (with Bonferroni *post-hoc* analysis) as appropriate. Survival was analysed using the Kaplan-Meier method and survival proportions were analysed using non-parametric log-rank tests (Mantel-Cox and Gehan-Breslow-Wilcoxon). P-values less than 0.05 were regarded as indicating a significant difference.

Results

MTT and Bystander kill assays

We tested the cytotoxic effect of Ad-TK/ganciclovir by MTT assay on a panel of cell lines. We observed minimal cell death attributable to either ganciclovir or Ad-TK infection alone. RG2 cells demonstrated dose-dependent toxicity related to the MOI of Ad-TK with which cells had been infected (figure 1Ai) and all cell lines showed increased cell death with a combination of Ad.TK and ganciclovir (5-50 μ M) (supplementary figure 1A).

Bystander kill assays demonstrated increased cytotoxicity in bystander cells exposed to media taken from cells infected with Ad.TK at MOIs of 10 and 50 and dosed with ganciclovir at concentrations of 50-100 μ M (figure 1B). The observed cytotoxicity was progressive from 72 to 96 h. Bystander kill was observed in RG2 cells treated with supernatant harvested from both cancerous (RG2 and HCT116) and non-cancerous (RF) cell lines (figure 1B). Bystander cytotoxicity was not observed with supernatant taken from cells infected with a control adenoviral vector (Ad-Luc) (figure 1Aii).

Adenoviral gene expression is selective for flap tissues over the flap bed

We performed immunofluorescent staining of sections taken from SIEA flaps infected with Ad.GFP, and the underlying flap beds, at 72 hours post-infection. This demonstrated that GFP expression was more prominent in cephalic flap tissues (mean = 157 GFP positive cells per hpf) (fig 2A) with weaker GFP expression evident in caudal flap tissues (mean = 72 GFP positive cells per hpf) and the SIEA pedicle (mean = 79 GFP positive cells per hpf). Flap bed sections were found to have very little GFP expression (mean = 1.3 GFP positive cells per hpf) and, where present, was observed at the flap-bed interface (fig 2A). Quantitative analysis demonstrated that cephalic flap tissues had significantly greater GFP expression than both caudal flap tissues, SIEA pedicle ($p < 0.0001$) and bed tissues ($p < 0.0001$). There

was no significant difference in GFP expression between cephalic flap tissues and the pedicle and between cephalic and caudal bed tissues (figure 2A and 2C).

Taking the cephalic flap tissues as the area of strongest GFP expression at the 72-hour time point, we performed a time course to investigate how GFP expression changed at 1 and 3 weeks post-infection. A significant reduction in GFP expression was observed by 1 week (mean = 65 GFP positive cells per hpf; $p < 0.0001$) (fig 2B) compared to 72 hours and this had reduced further by 3 weeks (mean = 26 GFP positive cells per hpf; 72 h v. 3 weeks: $p < 0.0001$; 1 week v. 3 weeks: $p < 0.01$) (fig 2B and 2D).

We observed that TK mRNA expression was significantly higher in Ad.TK-infected flaps compared to controls ($F=3.88$; $df=18$; $p=0.025$ (fig 2C). Of note, mRNA expression in cephalic flap tissues ($F=6.41$; $df=8$; $p<0.05$) and the SIEA pedicle ($F=6.41$; $df=7$; $p<0.05$) was greater in flaps infected at an MOI 10 than those infected at MOI 50 (figure 2E). No significant differences in expression were observed between MOI groups in caudal flap tissues.

Biodistribution analysis of *lacZ* RNA expression by PCR showed consistent expression in flap tissues only at 5 days following infection. Inconsistent expression was observed in the spleen in one animal and in the testes of a second animal (figure 2F).

Free flap VDEPT slows the growth of MiRD in flaps engrafted with tumour cells

Following engraftment of RG2 tumour cells in infected flaps, a significant difference was observed in the time taken to develop a palpable tumour between controls (median = 3 days; $n=6$) and Ad.TK-transfected flaps (10 MOI median delay = 14.5 days; 50 MOI median delay = 18.5 days) (Log-rank (Mantel-Cox): $X^2=16.4$; $df=2$; $p=0.0003$). Tumour volume growth was significantly delayed in Ad.TK-infected flaps compared with controls (fig 3A and B; $p<0.05$). Median survival to humane end point was also prolonged in animals with Ad.TK-infected flaps at both 10 MOI (median survival = 28 days) and 50 MOI (median survival = 25 days) compared with controls (figure 3C) (median survival = 21 days; Log-rank (Mantel-Cox): $X^2=15.5$; $df=2$; $p=0.0004$).

Free flap VDEPT prolongs the time to tumour recurrence in a MaRD model

Following incomplete excision of engrafted RG2 tumours, all control animals grew recurrent tumours that propagated from the tumour bed and were incorporated into the flap (figure 1E). In the therapeutic cohort all animals, except one, grew recurrent tumours that initially arose in the areas adjacent to the flap (Figure 1F). Animals with Ad.TK-infected flaps demonstrated longer disease-free survival (median delay to recurrence = 19.5 days; $X^2 = 11.26$; $df = 1$; $p=0.0008$) compared to animals with control flaps (median delay to recurrence = 7 days). Tumour volume growth was also significantly retarded in animals with Ad.TK-infected flaps, with significant prolongations observed in the time taken for tumours to reach 1 cm^3 and the time to humane end point compared with controls (figure 3D and E) ($p<0.0001$). Survival to humane end-point was also significantly prolonged in animals with Ad.TK-infected flaps (figure 3F) (median survival = 30 days; Log-rank (Mantel-Cox): $X^2=12.2$; $df=1$; $p=0.0005$) compared to controls (median survival = 18 days).

Discussion

Free flap gene therapy is attractive as it may circumvent many of the obstacles(27, 28) that limit the efficacy of using systemically-delivered gene therapies, particularly the attainment of therapeutically relevant viral titres at the target site(29). Previous work from our laboratory(13), has shown that the optimal route for introducing a viral vector into a free flap is by intra-arterial injection and that introduction of adenoviral vectors in this manner results in diffuse and durable transgene expression within the flap for up to 28 days. Many therapeutic gene therapy strategies have been proposed for use in conjunction with free flaps(12) and, in this paper, we report the pre-clinical evaluation of a virally directed enzyme prodrug therapy (VDEPT) strategy in the microscopic and macroscopic residual disease settings.

Using an adenoviral backbone, we subcloned the *TK* gene to create the Ad.TK vector and demonstrated both direct cytotoxicity mediated by the Ad.TK/ganciclovir combination on cancer cell lines, and, bystander cytotoxicity that was mediated through both cancerous and non-cancerous cell lines. These data validated the rationale for transfecting the normal tissues of the flap to provide therapy to residual disease on the tumour bed. Of note, bystander toxicity was not observed when the experiments were repeated using a control adenoviral vector (Ad.Luc) confirming that the observed bystander cytotoxicity was specific for the TK/ganciclovir system (figure 1). On the basis of these *in vitro* data we proceeded to *in vivo* studies using MOIs of both 10 and 50 in combination with systemic ganciclovir administration at 50 mg/kg.

Histological analysis of viral GFP expression demonstrated that gene expression was greatest in cephalic flap tissues but was also present in caudal flap tissues and the SIEA pedicle (figures 2A and 2C). Some GFP expression was observed in flap bed tissues and tended to occur at the interface between the flap and the bed; however, the extent of expression was significantly less than flap tissues (mean GFP count flap tissues = 102 cells per hpf v. mean GFP count bed tissues = 1 cell per hpf; $p < 0.0001$). Time course studies demonstrated a significant reduction in adenoviral gene (GFP) expression at 1 and 3 weeks (figures 2B and 2D) ($p < 0.0001$) and, in relative terms, was found to have decreased by 58% at 1 week and 84% by 3 weeks compared to the 72 hour time point. This finding is expected due to the transient nature of adenoviral gene expression and validates our earlier work,(13) which showed that adenoviral gene expression typically ceases by 28 days following flap infection. Using RT-QPCR we quantified viral TK expression within flap tissues (figure 2E) and observed significantly elevated levels of *TK* mRNA tissues of infected SIEA flaps compared to controls ($p < 0.05$). In addition, we observed that the cephalic portion of the flap and pedicle had a significantly greater degree of viral gene expression when infection was performed at an MOI of 10 but not at an MOI of 50. Whilst a comprehensive analysis of this apparent paradox is beyond the scope of this paper, we hypothesise that higher MOIs create a sub-optimal environment for the extravasation of viral particles into the flap tissues. We also postulate that higher degree of viral gene expression seen in cephalic portions of the flap (both histologically and on RT-QPCR) may reflect dominance of flow in the cephalic branch of the SIEA compared to branches that perfuse other portions of the flap. In summary, these data (figure 2) support the selectivity of the VDEPT therapy approach for

targeting flap tissues, rather than the flap bed. However, the transient nature of transgene expression suggests that an adenoviral vector system may not be suitable for translation in the context of a VDEPT approach.

We investigated the therapeutic effect of our VDEPT strategy in the context of both microscopic (MiRD) and macroscopic residual disease (MaRD). Briefly, in MiRD model RG2 tumour cells were inoculated into the flap immediately prior to infection with Ad.TK, whereas in the MaRD model previously engrafted tumours were incompletely excised and flaps infected with Ad.TK were used to reconstruct the resectional defect (supplementary figure 2). In both MiRD and MaRD models we observed retardation in tumour growth and improvement in survival to humane end-point in animals with Ad.TK-infected flaps compared to controls, suggesting a local cytotoxic effect from the infected flap itself (figure 3). In our MaRD studies, all animals, except one, developed tumour recurrences following incomplete tumour excision. Interestingly, we observed that in animals with control flaps recurrences developed immediately under the flap and grew to involve the flap itself (supplementary figure 2) whereas animals with Ad.TK-infected flaps developed recurrences in the areas adjacent to the flap. Furthermore, one animal in the MaRD cohort did not reach the pre-defined humane end point by the end of the experiment (day 50) despite developing a recurrence. Of note, we did not experience any problems with wound healing in animals treated with Ad.TK-infected flaps and ganciclovir, suggesting that this therapeutic approach shows selectivity for tumour growth rather than the normal physiological process of wound healing.

In summary, our data demonstrate pre-clinical proof-of-concept for VDEPT delivered by a free-flap in the management of microscopic and macroscopic residual disease. The period of tumour growth delay that we observed in our model represents an interval where conventional adjuvant therapies, for example radiotherapy, could be added to further improve local control. The ability to use free flaps to deliver targeted therapies is an exciting surgical advance, and there exist many therapeutic strategies that may be employed to deliver tumour bed therapies, or therapy to the flap itself(12). However, successful translation of this concept into the clinical arena will require optimization of vector delivery into flaps, and the choice of vector will be equally paramount to improving therapeutic efficiency and efficacy. For example, recent studies(16) have shown promise in over-coming the problem of short-term transgene expression by using a lentiviral vector that can achieve durable expression within flaps by stably integrating into the host genome. Therapeutic free flaps represent a unique tool in the reconstructive armamentarium of plastic surgeons and, advances in both vector design (30, 31) and delivery systems will yield substantial progress in the future.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

A. i) MTT assay performed at 96 hours following the addition of ganciclovir (0-50 μM) following infection of RG2 cells with Ad.TK at MOIs from 0-100 showing direct cytotoxicity attributable to the Ad.TK/ganciclovir combination. Greater cell death is observed with infection with higher MOIs in combination with ganciclovir. ii) Cytotoxicity assays performed with a control vector (Ad.Luc) did not show any cytotoxicity as a result of viral infection, *per se*, and demonstrated the cytotoxic efficacy of the VDEPT strategy. B) Bystander kill assays performed using supernatant from malignant (RG2 and HCT116) and non-malignant (RF) “producer” cell lines added to target “bystander” cells (RG2). Cell viability was assessed using MTT at 72 hours (i, ii, iii) and 96 hours (iv, v, vi) following the addition of supernatant from producer to bystander cell lines and shows killing in all bystander cell lines with the greatest cytotoxicity observed with Ad.TK at an MOI of 50 and 100 μM ganciclovir.

Figure 2.

A) Immunofluorescent staining for GFP (and DAPI as a nuclear stain) in SIEA flaps (n=3) infected with the Ad.GFP vector at an MOI of 10 72 hours previously. GFP expression was observed to be strongest in the cephalic flap tissues with weaker transgene expression being observed in caudal flap tissues and the SIEA pedicle. B) Time course of GFP expression in SIEA flaps infected with Ad.GFP (MOI 10). Flaps were harvested at 72 hours (n=3), 1 week (n=3) or 3 weeks (n=3). Staining demonstrated a reduction in GFP expression at 1 week post-infection with only very weak GFP expression evident at 3 weeks. Negative staining

controls for this experiment included sections from Ad.GFP-infected flaps that were stained with secondary antibody only and sections taken from flaps infected with a non-GFP encoding adenoviral vector (Ad.TK). C) Histological quantification of GFP expression in flaps infected with Ad.GFP and the corresponding flap bed specimens. Counts were performed at $\times 400$ magnification. Analysis demonstrates significantly greater GFP expression in cephalic flap tissues compared to caudal flap, pedicle, caudal bed and cephalic bed tissues. No significant differences were observed between caudal flap tissues and the SIEA pedicle, and, between caudal and cephalic flap bed tissues. D) Histological quantification of temporal changes in GFP expression in SIEA flaps infected with Ad.GFP. Analysis shows that there is a significant reduction in GFP expression at both 1 and 3 weeks following infection. In relative terms, GFP expression and reduced by 58% at 1 week and 84% at 3 weeks compared to the 72 hour time point. E) Compartmental distribution of Ad.TK viral RNA within infected SIEA flaps (MOI 10 and 50). These data demonstrate that Ad.TK infected flaps exhibit significantly greater viral gene expression ($p < 0.05$) compared to controls and, specifically, that gene expression in the pedicle and cephalic flap tissues are significantly greater when infection is performed at an MOI of 10 compared to an MOI of 50. F) Systemic bio-distribution of Ad.CMV-lacZ adenovirus as shown using RT-PCR at 5 days following SIEA flap infection (1×10^8 PFUs) demonstrates the presence of adenoviral genomic material within infected flaps (arrows) and inconsistent expression within the spleen of one animal and the testis of another animal. [* $p < 0.05$; *** $p < 0.01$; **** $p < 0.0001$].

Figure 3.

Microscopic disease model (MiRD) (A-C). A) Time taken for tumours to reach 1 cm³ in animals with control and Ad.TK-infected flaps (MOI 10 and 50) demonstrating retardation of tumour growth in Ad.TK-infected flaps. B) Tumour volume growth curves of tumours engrafted within control and Ad.TK-infected flaps at MOI 10 and 50 showing retardation of tumour volume growth in the MiRD model. C) Kaplan-Meier survival curve comparing control animals to those with Ad.TK-infected flaps showing an improvement in median survival in the latter group (10 MOI: median survival = 28 days; 50 MOI: median survival = 25 days; controls: median survival = 21 days; p=0.0004). D) Macroscopic residual disease (MaRD) model. Time taken for tumours to reach 1 cm³ and humane endpoint in animals with control and Ad.TK-infected flaps (MOI 50) demonstrating a retardation of tumour growth in Ad.TK-infected flaps (p<0.05). E) Tumour volume growth curves in the MaRD model showing retardation of tumour growth in animals with Ad.TK-infected flaps. F) Improvement in median survival to humane endpoint in MaRD animals treated with Ad.TK-infected flaps (median survival = 30 days) compared to controls (median survival = 18 days) (p=0.0005).