Multi-modal combination gene therapy for malignant glioma using replication-defective HSV vectors

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Herpes simplex virus (HSV) may be modified to produce a non-pathogenic vector that is capable of delivering multiple transgenes simultaneously to cells, both safely and efficiently. We have exploited this property to develop viruses that target glioblastoma, a malignancy that is currently associated with a poor prognosis. Using rationally selected combinations of therapeutic transgenes coupled with gamma-knife radiotherapy, the ablation of experimental tumours in animal models has been demonstrated. Combination gene therapy using replication-defective HSV vectors represents a promising and exciting approach to tackling malignancy in the CNS.

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▼ The annual incidence of malignant glioma, or glioblastoma, is approximately four adults per 100,000 population in the USA[1]. Glioblastoma is associated with a median survival of 4–12 months following diagnosis[2–6]. Treatment is palliative; an invasive tumour margin and a sensitive local environment preclude complete resection, and the cells comprising the tumour seem to be intrinsically resistant to conventional chemotherapy and radiotherapy. Consequently, little impact has been made on the poor prognosis associated with this malignancy, despite recent advances in other fields of therapeutic oncology. The investigation of new antiglioma treatment strategies is therefore important.

Genetic intervention represents a valid and logical approach to developing novel anticancer therapeutics because cancer is a disease caused largely by acquired genetic mutations[7–9]. Gliomas are attractive targets for the delivery of therapeutic transgenes by genetically engineered vectors, because the tumours are highly localized. Although glioma cells are usually seen invading brain tissue at the tumour margin[10], distant metastasis occurs only under unusual circumstances[11,12]. This enables direct inoculation of the tumour or post-operative tumour cavity with recombinant vector, circumventing many challenges currently associated with systemic transgene delivery.

HSV gene therapy vector construction for malignant glioma

Herpes simplex is an enveloped double-stranded DNA virus[13]. It is an attractive gene therapy vector relating to multiple applications, for several reasons. Those relevant to the present discussion are listed in Box 1.

The viral genome is organized into long (U_L) and short (U_S) unique segments flanked by inverted repeats[13] (Fig. 1a). During infection, viral genes are expressed in a tightly regulated, interdependent temporal sequence[13–15] (Fig. 2). Transcription of the five immediate-early (IE) genes, *ICP0*, *ICP4*, *ICP22*, *ICP27* and *ICP47* commences on viral DNA entry to the nucleus. The expression of these genes is regulated by promoters that are responsive to VP16, a viral structural protein that is transported to the host cell nucleus with the viral DNA. VP16 is a potent *trans*-activator that associates with cellular transcription factors and binds to cognate motifs within the IE promoter sequences. Expression of IE genes initiates a cascade of viral gene expression (Fig. 2). Transcription of early (E) genes, which primarily encode enzymes involved in DNA replication, is followed by expression of late (L) genes mainly encoding structural components of the virion[13–15]. Of the IE genes, only *ICP4* and *ICP27* are essential for expression of E and L genes, and hence

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Box 1. Advantages of HSV-1 as a gene therapy vector for malignant glioma

- Broad host cell range; the cellular entry receptors are widely expressed cell surface proteins[a,b].
- Highly infectious – it is possible to transduce 70% glioma cells *in vitro* at a low multiplicity of infection (1.0) with a replication-defective vector[c].
- Non-dividing cells can be efficiently transduced and made to express transgenes.
- Of the 84 known viral genes contained within the 152 kbp genome, approximately half are non-essential for growth in tissue culture. This means that multiple therapeutic transgenes can be accommodated by replacing dispensable viral genes[d]. In the majority of circumstances, this does not adversely affect the ability of the virus to replicate to high titre *in vitro*.
- Recombinant, replication-defective HSV can readily be prepared to high titre and purity without contamination from wild-type recombinants.

Abbreviation: HSV, herpes simplex virus.

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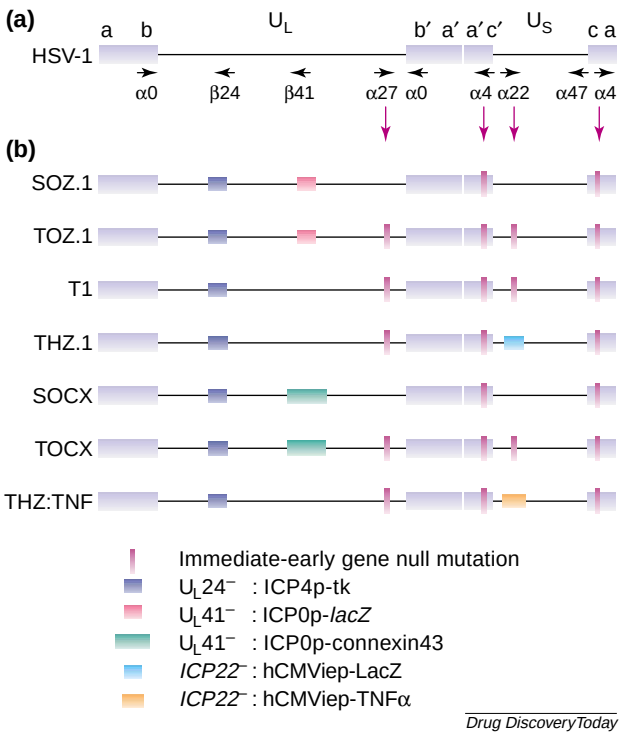

Figure 1. (a) A schematic representation of the HSV-1 genome (not to scale). The inverted repeats flanking the unique long (U_L) and short (U_S) segments of the genome are indicated as ab–b′a′ and a′c′–ca, respectively. The approximate positions and orientations of those HSV-1 genes discussed in the text are shown. **(b)** A series of engineered viruses deleted for immediate-early genes and expressing anti-tumour transgenes were generated. The name of each virus is shown to the left of the schematic; the viruses used in the studies reviewed here are referred to by name throughout the text. The diagrammatic genomic map of each vector is aligned with that of the HSV-1 genome in (a) to facilitate comparison between viruses. Each schematic depicts the positions and types of foreign transgenes inserted into each construct, and which subset of immediate-early genes has been inactivated.

viral replication. The functions of the five IE genes are listed in Table 1.

Toxicity associated with lytic wild-type HSV infection in the brain can be prevented by blocking viral replication. Because E and L gene expression, and therefore replication, is fully dependent on the expression of the essential IE genes, generation of replication-incompetent vectors can be accomplished by disruption of *ICP4* or *ICP27*. For example, a mutant deleted for both copies of the *ICP4* gene is unable to replicate in non-complementing cells[16]. However, with the exception of *ICP47*, the IE gene products are toxic to host cells[17–19]. Infection with an ICP4 null mutant results in extensive cell death in the absence of viral replication. This results from overexpression of other IE gene products, which are negatively regulated by ICP4 (Refs 16,20,21).

To prevent cytotoxicity, a series of vectors has been generated that are multiply deleted for IE genes. Quintuple mutants, null for *ICP0*, *ICP4*, *ICP22*, *ICP27* and *ICP47*, have been produced, are entirely non-toxic and are able to persist in cultured cells for long periods of time[19]. However, these vectors grow poorly *in vitro* and express transgenes at very low levels in the absence of ICP0. Retention of the *trans*-activator ICP0 allows efficient expression of viral genes and transgenes[22], and allows the virus to be prepared to high titre. Recent work has shown that the post-translational processing of ICP0 in neurons is different to that in glia[23]. Although ICP0 mRNA is efficiently expressed in both cell types, it appears that ICP0 selectively undergoes proteolytic degradation in neurons. Because ICP0 does not accumulate in neurons, it might be predicted that a vector carrying an intact *ICP0* gene would not be toxic to these cells. Expression of the ICP0 protein from such a vector in glial cells might confer additional therapeutic benefit in the treatment of glial-derived malignancy by effecting cell

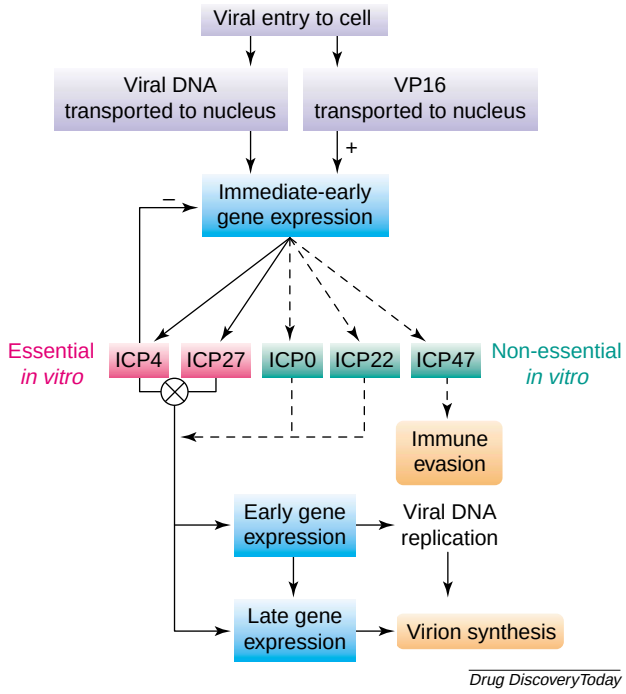

Figure 2. A flow chart depicting the cascade of regulatory events that result in ordered sequential expression of HSV-1 genes during wild-type infection. To proceed to E and L gene expression from IE gene expression, both *ICP4* and *ICP27* must be expressed. Inactivation of either of these genes results in loss of E and L gene products and failure to produce infectious virus. Suitable *ICP4* and *ICP27* expressing cell lines can complement these gene products *in trans* enabling production of high titres of replication-defective virus.

Table 1. Functions of immediate-early gene products

Gene	Essential?	Functions[13,19]
ICP0	No	*Trans* activator of native viral genes and exogenous transgenes[57]
ICP4	Yes	Major transcriptional regulatory protein; necessary for the transition of viral gene expression from the IE to the E phase[16]
ICP22	No	Contributes to efficient L gene expression in a cell-type dependent manner; multiple biochemical functions[58,59]
ICP27	Yes	Regulates processing of viral and host mRNAs; modulates activity of ICP0 and ICP4; contributes to efficient E and L gene expression[60,61]
ICP47	No	Immune evasion – interferes with transporter responsible for loading MHC class I molecules with antigenic peptides[62,63]

Abbreviations: E, early; IE, immediate early; L, late; MHC, major histocompatibility complex.

cycle arrest in tumour cells[24]. Our current view is that ICP0 expression will be advantageous for oncological applications in which transient high-level gene expression is desirable, and limited intratumoural toxicity is not an important issue. Deletion of *ICP47* restores the expression of MHC class I molecules to the surface of the cells, allowing immune surveillance mechanisms to operate. Potentially, this might confer some advantage in gene therapy directed against malignancy, although the utility of this modification is unclear at present. For the majority of work discussed here, triple mutants (*ICP4*$^{-}$: *ICP22*$^{-}$: *ICP27*$^{-}$) were used (Fig. 1b). These vectors show minimal cytotoxicity *in vitro* and *in vivo*, are efficient vehicles for transgene delivery and can be grown efficiently in cells that complement the absence of *ICP4* and *ICP27 in trans*[18,25]. The engineered vectors described in this review are illustrated schematically in Fig. 1, and are referred to by name throughout the text.

The importance of deleting multiple IE genes to reduce viral toxicity and enhance transgene delivery to malignancy was demonstrated in a recent study[20]. A replication-defective virus deleted for *ICP4* alone (SOZ.1) was compared with *ICP4*, *ICP22* and *ICP27* triple null mutants (T1, TOZ.1, THZ.1; Fig. 1b) *in vitro* and *in vivo* (Figs 3,4). The toxicity of the viruses and the ability of the vectors to deliver a suicide gene (see below) to tumour cells were examined *in vitro* and *in vivo*. All viruses were able to infect rat 9L glioma cells efficiently *in vitro*; 100% of the cells were transduced at a multiplicity of infection (MOI) of 10, and 70% of the cells at MOI 1. At MOI 10, cells infected with TOZ.1 showed a reduction in proliferation but normal morphology; 25% of the cells were seen to be undergoing apoptosis. By contrast, 98% of cells infected with SOZ.1 showed apoptosis; extensive cytopathic changes and cell loss were evident (Fig. 3a). In a parallel series of *in vivo* experiments, the ability of the viruses to affect the clinical outcome of an experimental model of glioblastoma was assessed (Figs 3,4). Rats were inoculated intracerebrally with $10^5 \times$ 9L cells and a tumour was established over the subsequent 5 days. The tumours were then stereotactically injected with equivalent doses of the different viruses. Following viral injection, animals were treated with a prodrug (ganciclovir) that is activated by the suicide gene product into a cytolytic anticancer agent. Rats injected with SOZ.1 showed no survival advantage over controls either with or without ganciclovir treatment. Animals treated with T1 showed a clear therapeutic response to ganciclovir, which increased survival time by 50% in some animals (Fig. 3b). Histological examination showed that a localized area of necrosis was evident within tumours infected with the single mutant, but that the area of necrosis

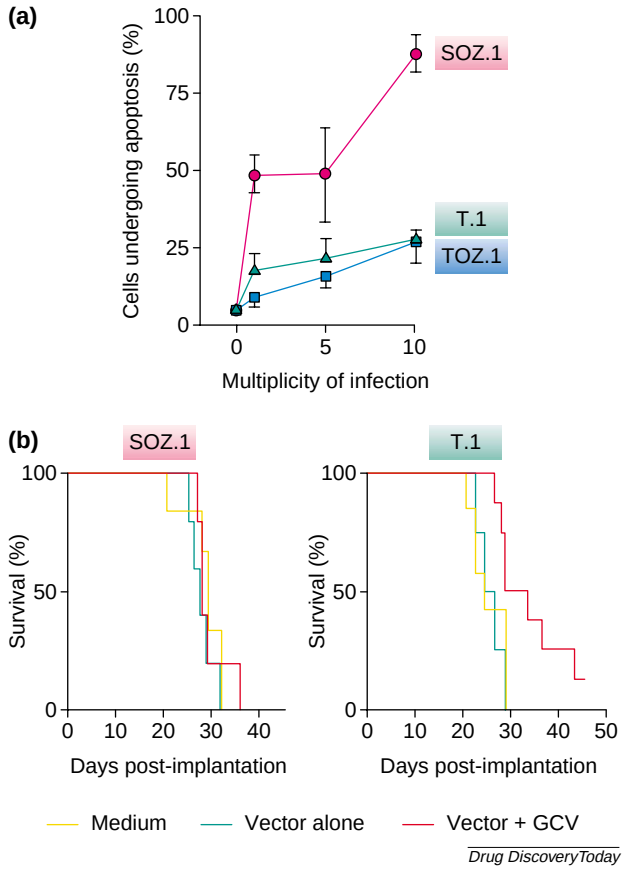

Figure 3. (a) A single IE gene-deleted virus (SOZ.1) was compared with two triple mutants (T.1 and TOZ.1) for cytotoxicity *in vitro*. **(b)** Survival of rats in an orthotopic transplant model of glioma (Fig. 4). Ganciclovir treatment enhanced survival when HSV-TK was expressed from a less toxic virus, implying that the improvement in transgene expression afforded by using a more attenuated vector backbone is an important consideration in designing therapeutic viruses.

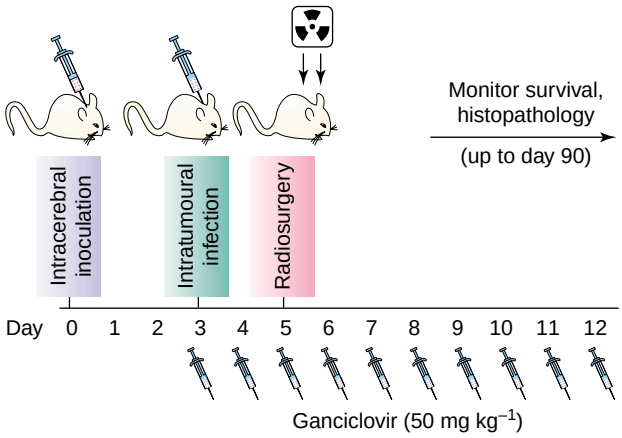

Figure 4. An orthotopic transplant model of glioma. Athymic (nude) mice were inoculated with U87 (human glioma) cells into the striatum on day 1. A tumour was allowed to establish for 3 days, following which the same coordinates were inoculated with the gene therapy vector under study or with a negative control. Ganciclovir was given daily for 10 days following viral delivery via intraperitoneal injection. In protocols involving radiosurgery, this was given 2 days after viral inoculation. The survival of at least eight animals in each treatment group was monitored and is presented as Kaplan-Meier survival curves in Figs 6b, 7b and 8. The data shown in Fig. 3b were generated using a similar protocol, except that rats were inoculated with 9L (rat glioma) cells on day 1, and the tumour was allowed to establish over 5 days before gene therapy and ganciclovir treatment.

was inadequate to affect tumour progression. Thus, tumour lysis resulting from enhanced transgene expression, presumably resulting from diminished toxicity, was superior to direct viral lysis of the glioma cells.

Safety is an important consideration in the development of therapeutic reagents. Several beneficial safety features intrinsic to the vector system described here are listed in Box 2. In addition to deletions of multiple IE genes, which achieve safety and minimize toxicity, it is possible to delete multiple non-essential E and L genes. This enables the insertion of several exogenous sequences. Vectors that express up to five independent expression cassettes have been generated; the expression level of each product seems little affected by the addition of further transgenes[26]. This property might have important implications for the gene therapy of cancer because it seems probable that multiple therapeutic transgenes will be necessary to effectively deal with the heterogeneous and constantly evolving cell populations that comprise most tumours. We have recently reported a series of studies examining *in vitro* and *in vivo* models of glioblastoma, in which we have started to address optimal requirements for combination gene therapy of malignancy[20,26,28,45,46].

Therapeutic transgenes and tumour targeting

A classification of the general types of therapeutic transgenes that can be delivered to malignant gliomas appears in Table 2. Anti-oncogenic interventions seek to correct the molecular defects giving rise to malignant transformation or other tumourigenic processes. Other types of genetic therapy aim to selectively destroy malignant cells. It follows that for this latter group [i.e. suicide gene therapy (SGT), radiosensitization and immunotherapy], some means of targeting is essential to prevent destruction of normal tissue. This can be accomplished in several ways (Fig. 5). The first two targeting mechanisms rely on limiting the expression of a universally toxic gene product to cancer cells, either by targeted viral delivery or by limiting transgene transcription to malignant cells using specific

Box 2. Safety features inherent in the construction of multiply disabled HSV-1 vectors

- The viruses are produced in cell lines that contain minimal gene sequences in common with the defective vector, thus minimizing the likelihood of generating replication-competent revertants during manufacture (thus far, replication-competent virus has not been detected after repeat passaging of vector stock on complementing cells).
- Formation of a transgene-expressing replication-competent strain *in vivo* would require both the presence of replicating wild-type virus and gene therapy vector in the same cell, and multiple recombination events to restore the deleted essential genes. Furthermore, insertion of the transgene at an essential gene locus in the vector prevents its acquisition by wild-type virus. This is because the recombination event necessary to transfer the transgene to the wild-type virus would delete an essential gene and destroy the capacity for replication. These two considerations suggest that the generation of a replicating transgene-expressing virus *in vivo* following gene transfer would be extremely unlikely.
- If a replication-competent mutant were generated, expression of the early gene thymidine kinase would enable appropriate treatment with the antiviral agents acyclovir or ganciclovir.

Abbreviation: HSV, herpes simplex virus.

Table 2. A classification of anti-cancer therapeutic transgenes

Type of transgene	Mechanism of action	Examples
Suicide gene therapy	Toxic to expressing cells	HSV-TK–GCV[27]
Radiosensitization	Enhances toxicity of gamma irradiation in expressing cells	TNF-α[44,45], anti-ATM antisense[48]
Immunotherapy	Stimulates immune response directed against tumour	CD80 (Ref. 25), TNF-α[44,45], GM-CSF[25], IL-2 (Ref. 51), interferon-γ[52], IL-12 (Ref. 53)
Anti-oncogenic	Corrects molecular defects present in tumour	p53 (Ref. 54), anti-VEGF antisense[55,56]

Abbreviations: ATM, ataxia telangiectasia mutated; HSV, herpes simplex virus; GVC, ganciclovir; TK, thymidine kinase; TNF-α, tumour necrosis factor alpha; VEGF, vascular endothelial growth factor.

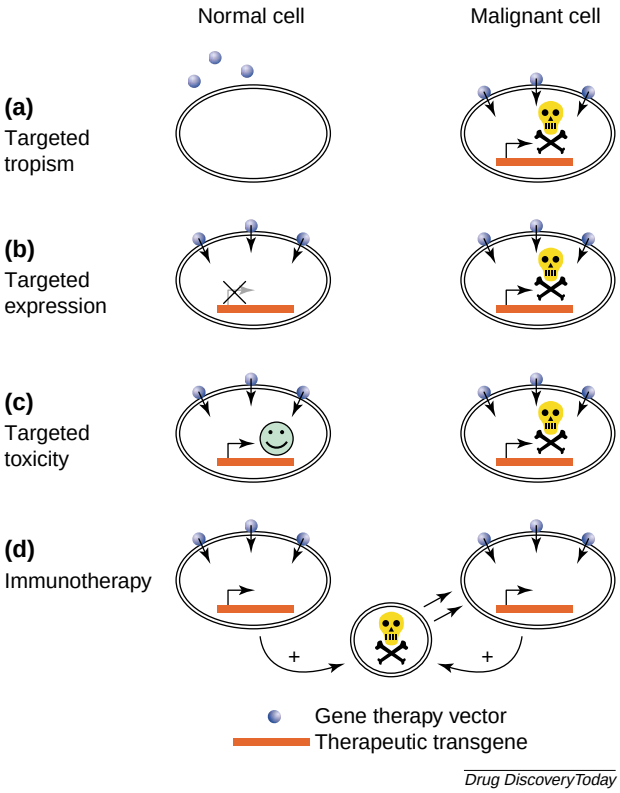

Figure 5. HSV vector-based gene therapy can target tumour in four ways: **(a)** modified vector tropism limits transgene entry into tumour cells; **(b)** transgene enters normal and tumour cells, but tumour-specific *cis*-acting regulatory elements limit transgene expression to tumour cells; **(c)** the gene product is expressed in all cells but is selectively toxic to tumour cells; and **(d)** secretion of soluble mediators or expression of cell surface makers stimulates the immune system to overcome the various mechanisms used by tumour cells to avoid immune recognition.

regulatory elements in the expression cassette. The second two mechanisms depend on differential toxicity of a gene product expressed in both normal and malignant cells. This might be accomplished through direct toxicity to cancer cells or induction of an immune response that is preferentially directed against the tumour.

The first two approaches, modification of the tropism of HSV-1 and identification of glioma-specific regulatory elements, are still in early stages of development. An increasingly detailed understanding of the biochemical mechanisms whereby HSV enters cells has enabled crucial steps to be taken towards targeted tropism[27], although this approach will depend on the identification of tumour-specific cell surface receptors. Promoter-trap experiments are ongoing to identify *cis*-acting regulatory elements that are preferentially active in glioma cells.

Recent work has shown significant tumour targeting by delivery of gene combinations that are preferentially toxic

to tumour cells. The studies discussed below have demonstrated the efficacy of using replication-defective HSV-1 to deliver combinations of therapeutic transgenes to tumours, with clinical benefit in animal models.

Suicide gene therapy and the bystander lysis effect

Herpes simplex thymidine kinase (HSV-TK) is an example of a suicide gene product, in that it is toxic to cells within which it is expressed. The enzyme is encoded by the U_L23 gene of HSV1, and functions to phosphorylate deoxypyrimidines with broad substrate specificity. This latter property allows the conversion of a pro-drug, ganciclovir, into its active form by HSV-TK but not by its cellular counterpart. The phosphorylated form of ganciclovir acts as a defective nucleoside analogue that becomes incorporated into replicating DNA and causes premature strand termination. Activated ganciclovir is toxic only to cells undergoing DNA replication. A degree of cytolytic selectivity is therefore inherent in this approach, with toxicity towards actively dividing tumour cells being much greater than to neurons or quiescent glia.

In the context of replication-deficient HSV vectors, it is important to note that the HSV-TK expression cassette is placed under the transcriptional control of the ICP4 promoter or another IE promoter. This is necessary because IE genes must be expressed to allow transcription of the E gene TK from its native promoter. Essential IE genes are deleted from replication-deficient vectors, which do not express the unmodified forms of any early genes, including HSV-TK.

It is not necessary to transduce all tumour cells with the HSV-TK gene because in many cases cells surrounding transduced cells are killed following ganciclovir administration. This phenomenon is referred to as 'bystander lysis'[28,29]. *In vitro*, bystander lysis is largely attributable to uptake of activated ganciclovir by HSV-TK negative cells[29,30]. The mechanisms responsible for bystander lysis *in vivo* are complex. Passage of activated ganciclovir from HSV-TK positive to HSV-TK negative cells plays a key role, in addition to effects attributable to necrosis-induced inflammation and disruption of vasculature[31–33]. Activated ganciclovir can pass from cell to cell through gap junctions[32,34,35]. These are intercellular channels formed by several proteins, including connexin-43 (Ref. 36). Gliomas are often defective in connexin expression[37] and intercellular gap junctions[38]. It was hypothesized that enhanced gap junction intercellular communication might promote the passage of activated ganciclovir from transduced cells to neighbours within the tumour, thereby augmenting the bystander lysis effect from the HSV-TK–ganciclovir system (Fig. 6a).

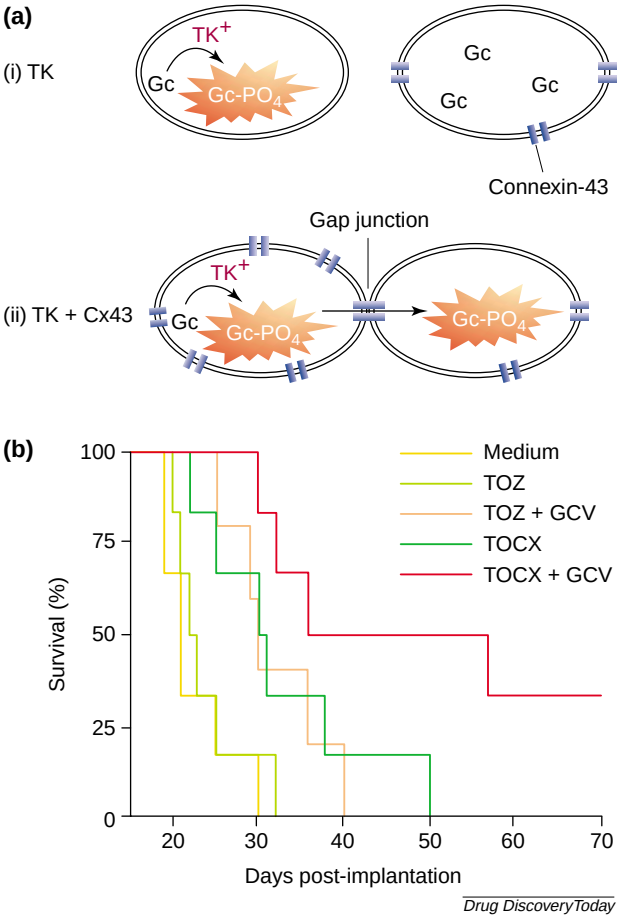

Figure 6. (a) The bystander lysis effect is mediated, in part, by the cell-to-cell spread of activated ganciclovir through gap junctions. (i) Transduction of a small proportion of cells with HSV-TK in the absence of gap junctions leads to the activation of ganciclovir only within the transduced cells. Non-transduced cells are able to escape the toxicity of ganciclovir in the absence of its conversion into the active nucleoside analogue. (ii) *In vitro* studies indicate that, provided a very low basal level of connexin-43 expression is present on HSV-TK$^-$ (non-transduced) recipient cells, enhanced expression of connexin-43 on HSV-TK$^+$ (transduced) donor cells encourages the formation of gap junctions between TK$^-$ and TK$^+$ cells and augments the passage of activated ganciclovir between cells. **(b)** The therapeutic effect of simultaneous TK and Cx43 expression was examined in the mouse orthotopic xenotransplant model (Fig. 4). Cx43 alone has a therapeutic effect similar to HSV-TK–ganciclovir. Combining both types of therapy leads to an augmented therapeutic response.

To test this hypothesis, the anti-tumour effect of TOZ.1 was compared with that of an isogenic vector containing a connexin-43 expression cassette at the U_L41 locus, TOCX[28] (Fig. 1b). Western blot hybridization confirmed that TOCX gave rise to connexin-43 expression in a connexin-43 negative cell line *in vitro*. Significant enhancements in the levels of *in vitro* bystander lysis were observed in cells

transduced with TOCX compared with TOZ. A series of *in vitro* experiments were undertaken in which cells expressing different levels of endogenous connexin-43 were transduced with one of the viruses and then mixed with non-transduced cells. These studies showed that the magnitude of the bystander lysis effect was a function of connexin expression in the TK-positive cell. Thus, provided a basal level of connexin-43 expression was present on HSV-TK$^-$ (non-transduced) recipient cells, enhanced expression of connexin-43 on the surface of HSV-TK$^+$ (transduced) donor cells encouraged the formation of gap junctions between TK$^-$ and TK$^+$ cells. This augmented the passage of activated ganciclovir between cells resulting in an enhanced bystander lysis effect.

These observations constitute a rational basis for attempts to deliver both HSV-TK and connexin-43 simultaneously to a sub-population of tumour cells using single vectors expressing both genes. Nude (athymic, immunodeficient) mice were subject to intracerebral inoculation with 10^5 human glioma cells, and tumours established over 3 days (Fig. 4). The tumours were then stereotactically injected with either TOZ or TOCX, or with medium alone. Half of each study group was treated with ganciclovir to assess the therapeutic effect of SGT with or without connexin-43 expression. Untreated animals died within 30 days from brain tumour. Survival was unaffected by treatment with TK vector alone (TOZ) but was enhanced by treatment with TOZ and ganciclovir. Expression of connexin-43 from TOCX without ganciclovir had an anti-tumour action approximately equivalent to that of TOZ and ganciclovir. A combination of connexin-43 and HSV-TK–ganciclovir therapies (TOCX and ganciclovir) resulted in substantial survival benefit; at the end of the study (70 days), one-third of the TOCX–ganciclovir animals were still alive (Fig. 6b). Thus, a combination of SGT with genes aimed at augmenting SGT bystander lysis results in enhanced *in vivo* efficacy.

Anti-tumour cytokine coexpression

Tumour necrosis factor alpha (TNF-α) is a potent anti-tumour cytokine that demonstrates a range of actions against malignant cells, including the induction of apoptosis via activation of TNF-α receptors, enhancement of HLA antigen expression in tumours and immunomodulatory effects such as induction of NK- and CTL-mediated tumour lysis[39–44]. The molecule is too toxic to deliver systemically[43,44] but the ability of HSV vectors to accommodate multiple transgenes readily enables its incorporation into a locally administered SGT paradigm.

The hypothesis that TNF-α might enhance tumour lysis mediated by TK–ganciclovir was tested by comparison of a replication-deficient HSV-TK expressing virus (THZ.1) with an isogenic vector containing an expression cassette for TNF-α at the *ICP22* locus (TH:TNF)[45] (Fig. 1b). TH:TNF was shown to express biologically active TNF-α by ELISA and viability assays of TNFα-sensitive cell lines using supernatant from infected cultures. Ganciclovir-mediated lysis of TNF-α-sensitive cells *in vitro* following infection was enhanced by the presence of the TNF-α expression cassette when a low proportion of the cells were infected (mimicking the situation *in vivo* after a single dose of vector). When the majority of cells were infected, the cultures were rapidly killed by the expression of TNF-α. Many gliomas, however, are TNF-α resistant.

It was of interest, therefore, to observe that TNF-α-mediated enhancement of HSV-TK–ganciclovir lysis was observed in a TNF-α-insensitive glioma cell line. The mechanism was unclear but presumably arose from sensitization of the cells to one agent as a consequence of exposure to the other (Fig. 7a). *In vivo* studies using the athymic mouse orthotopic xenotransplant tumour model (Fig. 4) confirmed the *in vitro* observations in tumours comprising TNF-α-sensitive cells but showed no additional benefit from TNF-α expression in tumours derived from resistant cells. Although a substantial and significant prolongation of survival was seen with the TNF-α–HSV-TK–ganciclovir regimen compared with negative control, the effect was also observed with the HSV-TK–ganciclovir treatment alone (Fig. 7b). Possible explanations include the observation that the low proportion of tumour cells that were transduced *in vivo* might have favoured detection of the bystander lysis effect from HSV-TK–ganciclovir treatment. Additionally, the direct receptor-mediated lysis effect would have been absent from the TNF-α-resistant cell lines, and the immunomodulatory effects of TNF-α would not be seen in athymic mice. The mechanisms giving rise to TNF-α resistance in glioma cells are of interest; the large cloning capacity of HSV-1 would permit the simultaneous expression of transgenes overcoming the mechanisms whereby glioma cells escape TNF-α-induced cell death. Such vectors are currently under evaluation.

Enhancing tumour sensitivity to γ-irradiation

Fractionated radiotherapy has been shown to confer a small but significant survival benefit to patients with glioblastoma. Unfortunately, the dose of radiotherapy that can be tolerated by the brain (~60 Gray) is inadequate for tumour eradication. To circumvent inherent toxicity problems, techniques have been developed that allow focussing of radiation on the tumour bed, allowing a higher dose to be delivered (radiosurgery). This enables eradication of the central portion of the tumour but does not allow delivery

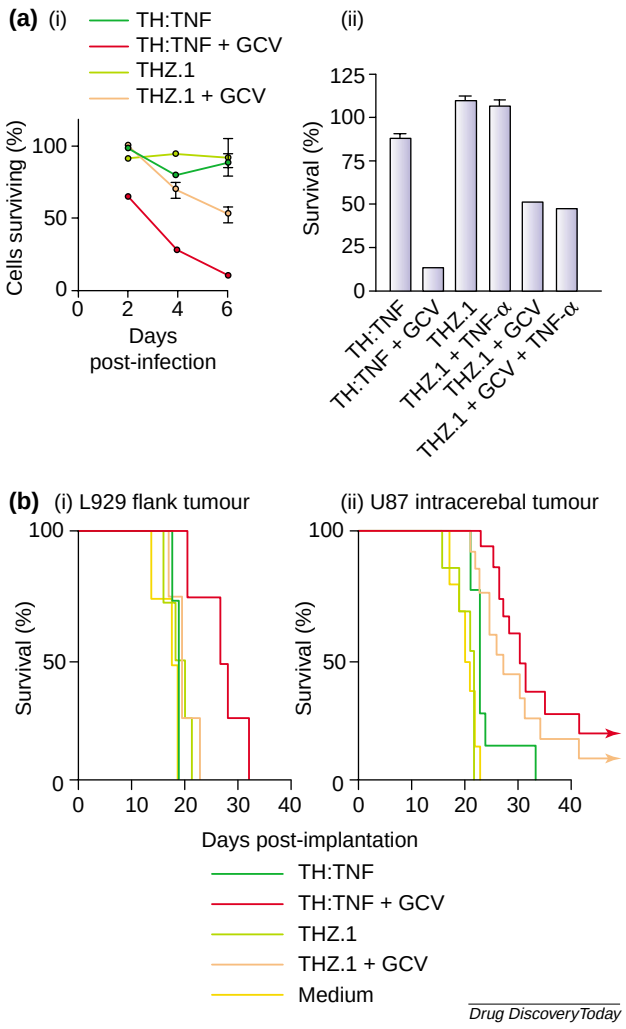

Figure 7. (a) Anti-tumour action of TNF-α. Many glioma cell lines are resistant to the anti-tumour actions of TNF-α. The role of TNF-α in combination therapy was examined *in vitro* using triple IE mutant viruses expressing HSV-TK alone or with TNF-α. (i) Survival of U87MG cells was unaffected by expression of HSV-TK alone or with TNF-α at low MOI (0.1). Ganciclovir treatment, however, caused a substantial degree of cell death in samples transduced with HSV-TK. This effect was greatly augmented by concomitant expression of TNF-α, implying that one treatment modality had sensitized the cells to the effects of the other. (ii) Interestingly, this effect was not recapitulated by administration of exogenous TNF-α. It appears that sensitization to HSV-TK–ganciclovir requires the endogenous expression and intracellular synthesis of TNF. **(b)** *In vivo* therapeutic efficacy of TNF-α expressing viruses. (i) L929 – these cells were chosen because of their intrinsic sensitivity to TNF-α. In this mouse flank tumour model, there is a clear survival advantage in the group treated with the TNF-α coexpressing virus. (ii) By contrast, there was no clear benefit of TNF-α coexpression over an HSV-TK–ganciclovir regime in the mouse orthotopic glioma model.

of an augmented radiation dose to the tumour periphery. Unfortunately, glioma cells are often seen invading the normal tissue surrounding the tumour, often migrating along normal white-matter tracts. This feature of glioma is largely responsible for the inability to effect a surgical cure by resection, and the correspondingly poor prognosis. We have therefore started to examine ways in which the response to radiotherapy can be enhanced by gene delivery in the hope that malignant cells invading the tumour periphery can be eradicated. Two such genes have been evaluated: TNF-α[45,46] and ataxia telangiectasia mutated[47] (*ATM*).

ATM is mutated in the hereditary disease ataxia telangiectasia, which has a pleotrophic phenotype including ataxia, dilated loops of capillaries, lymphoreticular malignancy and susceptibility to radiation-induced cell death[48]. The latter is caused by absence of the ATM protein, which has a pivotal role in the intermediate signalling events linking double-strand DNA breaks to cell cycle arrest and subsequent DNA repair[49]. Abolition of ATM expression in a glioma cell line results in enhanced sensitivity to gamma irradiation[47]. This is an attractive strategy for reducing the radiosensitive threshold of tumour cells because attenuation of ATM function appears to make certain neuronal populations radio-resistant[50]. We are currently evaluating vectors that suppress ATM function in malignant glioma.

It was already known that TNF-α could enhance the therapeutic effect of gamma-knife irradiation in athymic mice. Studies were therefore designed to examine whether or not the individual anti-tumour efficacies of SGT, TNF-α and radiotherapy were additive in combination[46]. The athymic mouse orthotopic xenotransplant tumour model (Fig. 4) and the vectors described in the previous section were used to study this question. A detailed examination of all combinations of the three treatment modalities was undertaken, with or without the administration of ganciclovir. The most important points that emerged were (Fig. 8):

- untreated mice died within 35 days from cerebral tumour;
- both radiotherapy and TK–ganciclovir treatment alone conferred a survival advantage;
- TNF-α enhanced the effect of radiotherapy, whereas SGT did not;
- combining all three treatments led to long-term (>75 days) survival in 89% of the animals; and
- three-quarters of the long-term survivors following combination TNF-α–SGT–γ-irradiation treatment were tumour-free on subsequent histological analysis.

Combination of treatment modalities thus results in improved outcome in this model. Further studies have been undertaken using a combination of radiotherapy with a vector expressing HSV-TK, connexin-43 and TNF-α. Again, the general principle to emerge from these studies is that combination treatment protocols are superior to single interventions (manuscript in preparation).

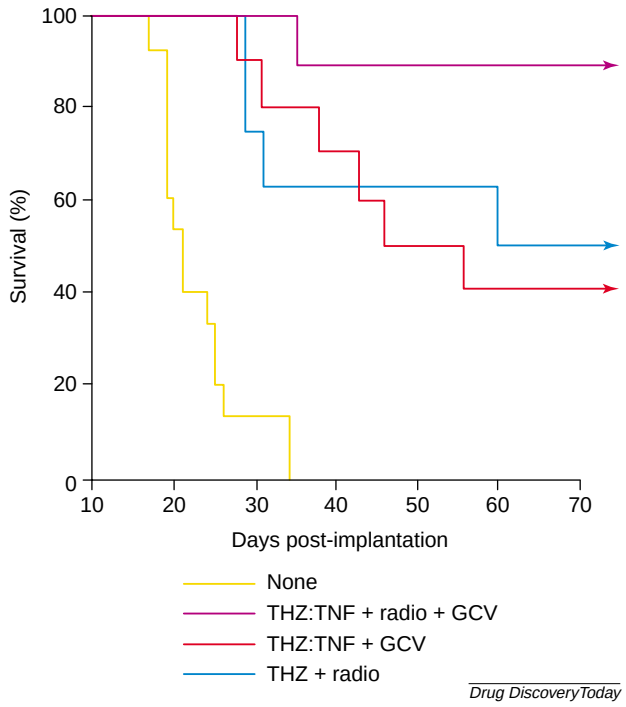

Figure 8. Combination HSV-TK–ganciclovir, TNF-α and radiotherapy treatment is superior to any single intervention or combination of two treatment modalities. The TNF-α coexpressing virus (TH:TNF) was examined in the mouse glioma model shown in Fig. 4 with or without radiotherapy and/or ganciclovir. Both radiotherapy and ganciclovir treatment conferred a survival advantage in the context of tumour infection with TH:TNF. Combining all three treatments led to long-term, tumour-free survival in most of the animals.

Conclusions: future directions

Our experience with experimental models of glioma indicates that combination multi-modality therapies are superior to single interventions, which is not surprising in view of the nature of basic cancer biology. A vector system allowing simultaneous delivery of multiple genes is ideally suited to this application. We have developed replication-defective HSV vectors with many favourable properties for use in tumour gene therapy, including minimal cytotoxicity, effective transgene delivery and expression, and the ability to accommodate multiple therapeutic expression cassettes. Recent studies have established the principle that it is possible to eradicate some experimental tumours in laboratory animals by using combination HSV gene therapy-based approaches. There remains uncertainty regarding the applicability of these models to human tumours, which have evolved over several years in an immunocompetent host and are heterogeneous by the time of diagnosis. These initial studies, however, provide some optimism that tumour cells can be targeted *in vivo*, and form a basis for the continued investigation of this general strategy. Whether or not this approach will have a major impact on the prognosis of malignant glioma is uncertain but it is worth pursuing in the absence of other promising therapeutic strategies.

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