

Herpes Simplex Virus vector-based gene therapy for malignant glioma

Review Article

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Summary

Conventional therapies have made little impact on the poor prognosis associated with malignant glioma. Recent advances in the construction of replication-defective Herpes Simplex Virus (HPV)-based vectors have offered an opportunity to explore the therapeutic affects of simultaneous multiple transgene delivery to these tumours. Identification of co-operative molecular targets has enabled the rational selection of therapeutic transgene combinations. Exploiting the large capacity of HSV for the insertion of multiple transgenes, the high infectivity of HSV for many cell types and the ability to manufacture vectors of high tire and purity, a series of combination gene therapy vectors have been developed and tested in animal models of malignant glioma. Recent work has been established the principle that multi-modal therapies, including both radiosurgery and combination multi-gene therapy, are superior to single molecular interventions. Eradication of some experimental gliomas has been possible using a multi-modal approach, which provides optimism that further developments may yield reagents that prove therapeutically useful in the neuro-oncology clinic.

I. Introduction

Primary CNS neoplasms of adults affect approximately 8.2/100,000 population annually in the USA (Walker et al, 1985). About half of these tumours are highly aggressive malignant gliomas, and are associated with a median survival of four to twelve months following diagnosis (Jubelirer, 1996; Lopez Gonzalez and Sotelo, 2000). Treatment is palliative; despite recent advances in surgery, radiotherapy and chemotherapy, little impact has been made on the poor prognosis associated with this malignancy (West et al, 1983; Kelly et al, 1984; Jubelirer, 1996; Lang et al, 1999; Lopez Gonzalez and Sotelo, 2000). Exploration of novel treatment strategies is therefore of importance.

Cancer is a genetic disease (Collins, 1998; Hill et al, 1999; Hanahan and Weinberg, 2000), oncogenic mutations usually being acquired rather than inherited. Genetic intervention represents a valid and logical approach to developing novel anti-cancer therapeutics. Gliomas are attractive targets for delivery of therapeutic transgenes by genetically engineered vectors; the tumours are highly localised as, although they are usually invasive locally at the tumour margin (McComb and Bigner, 1984), they only metastasise under unusual circumstances (al Rikabi et al, 1997; Hsu et al, 1998). This enables direct inoculation of the tumour or post-operative tumour cavity with recombinant vector, circumventing many challenges currently associated with systemic transgene delivery.

In this review, we consider advances in the construction of herpes simplex-based gene therapy vectors, discuss the types of therapeutic transgenes whose deliver to tumours may be desirable, and review the results from pre-clinical experimental treatment trials using these approaches.

II. HSV as a gene therapy vector

Herpes Simplex is an enveloped double-stranded DNA virus (reviewed in Roizman and Sears, 1996). It is an attractive candidate gene therapy vector relating to multiple applications, for the following reasons:

(i) It has a broad host cell range; the cellular entry receptors HveA (Montgomery et al, 1996) and HveC (Geraghty et al, 1998) are widely expressed cell surface proteins of unknown function.

(ii) It is highly infectious - it is possible to transduce 70% cells *in vitro* at a low multiplicity of infection (1.0), with a replication-defective vector (Moriuchi et al, 2000).

(iii) Non-dividing cells may be efficiently transduced and made to express transgenes

(iv) Of the 84 known viral genes contained within the 152-kilobase pair 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 (Kriskey et al, 1998). In the majority of circumstances, this does not adversely affect the ability of the virus to replicate to high titre *in vitro*.

(v) Recombinant HSV may readily be prepared to high titre and purity without contamination from wild-type recombinants

(vi) The virus can exist in a latent state within nuclei of infected neurons for the lifetime of the host. During latency, the virus adopts a circular or concatemeric configuration, remains episomal, and has minimal effects on host cell metabolism. A limited number of viral genes are chronically expressed during latency; this phenomenon could potentially be exploited for the stable long-term expression of therapeutic transgenes in neurons (Wolfe et al, 1999).

The viral genome is organised into long (U_L) and short (U_S) unique segments flanked by inverted repeats (**Figure 1**; Roizman and Sears, 1996). Genes contained within the unique segments are present at one copy per genome; genes present within the repeats (including ICP0 and ICP4 – see below) are present at two copies per genome. During infection, viral genes are expressed in a tightly regulated, interdependent temporal sequence (Honess and Roizman, 1974; Honess and Roizman, 1975; Roizman and Sears, 1996) (**Figure 2**). Transcription of the five immediate-early (IE) genes, ICP0, ICP4, ICP22, ICP27 and ICP47 commences on viral DNA entry to the nucleus. 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, resulting first in transcription of early (E) genes, which primarily encode enzymes involved in DNA replication, followed by late (L) genes mainly encoding structural components of the virion (Honess and Roizman, 1974; Honess and Roizman, 1975; Roizman and Sears, 1996). Only ICP4 and ICP27 are essential for expression of E and L genes, and hence viral replication. ICP4 is the major regulatory protein of the virus. It functions as a repressor or activator of viral and cellular transcription by contacts with multiple basal factors; it is necessary for the transition of viral transcription from the IE to the E phase (O'Hare et al, 1988; Smith et al, 1993; Gu et al, 1995; Kuddus et al, 1995; Carrozza and DeLuca, 1996). ICP27 regulates the processing of many viral and host mRNAs, and modulates the activity of ICP0 and ICP4. It contributes to efficient E and L gene expression (Hardy and Goldin, 1994; Hardwicke and Goldin, 1994; Brown et al, 1995; Soliman et al, 1997; Mears and Rice, 1998; Sandri Goldin, 1998). The promiscuous *trans*-activator ICP0 contributes to high expression levels of viral genes but is not essential for viral replication *in vitro* (Jordan and Schaffer, 1997; Samaniego et al, 1997). ICP22 contributes to efficient L gene expression in a cell-type dependent manner and has multiple biochemical functions (Poffenberger et al, 1994; Rice et al, 1995; Prod'hon et al, 1996; Leopardi et al, 1997; Bruni and Roizman, 1998). ICP47 does not have a transcriptional regulatory role, but rather has been reported to interfere with the function of a transporter that is responsible for loading MHC class I molecules with antigenic peptides (York et al, 1994; Fruh et al, 1995; Hill et al, 1995; Lacaille and Androlewicz, 1998).

Toxicity associated with lytic wild-type HSV infection in the brain can be prevented by blocking viral replication. As E and L gene expression, and therefore replication, is fully dependent upon the expression of IE genes, generation of replication-incompetent vectors can be accomplished by disruption of one or other essential IE gene, ICP4 or ICP27. For example, an ICP4 null mutant is unable to replicate in non-complementing cells in culture (DeLuca et al, 1985). However, the IE gene products, with the exception of ICP47, are all toxic to host cells (Johnson et al, 1994; Wu et al, 1996; Samaniego et al, 1998). Infection with an ICP4 null mutant results in extensive cell death in the absence of viral replication, which is caused by over-expression of other IE gene products, some of which are negatively regulated by ICP4 (DeLuca et al, 1985; Kriskey et al, 1998; Moriuchi et al, 2000). 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 to cells and are able to persist for long periods of time (Samaniego et al, 1998). However,

these vectors grow poorly in culture 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 (Jordan and Schaffer, 1997), 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 (Chen et al, 2000). It appears that, although ICP0 mRNA is efficiently expressed in both cell types,

ICP0 undergoes proteolytic degradation in neurons. It might be predicted that the use of a vector carrying an intact ICP0 gene would not be toxic to neurons, but may confer additional therapeutic benefit in the treatment of glial-derived malignancy through the differential expression of ICP0 and transgenes in the two cell types. Our current view is that ICP0 expression will be advantageous for oncological applications where

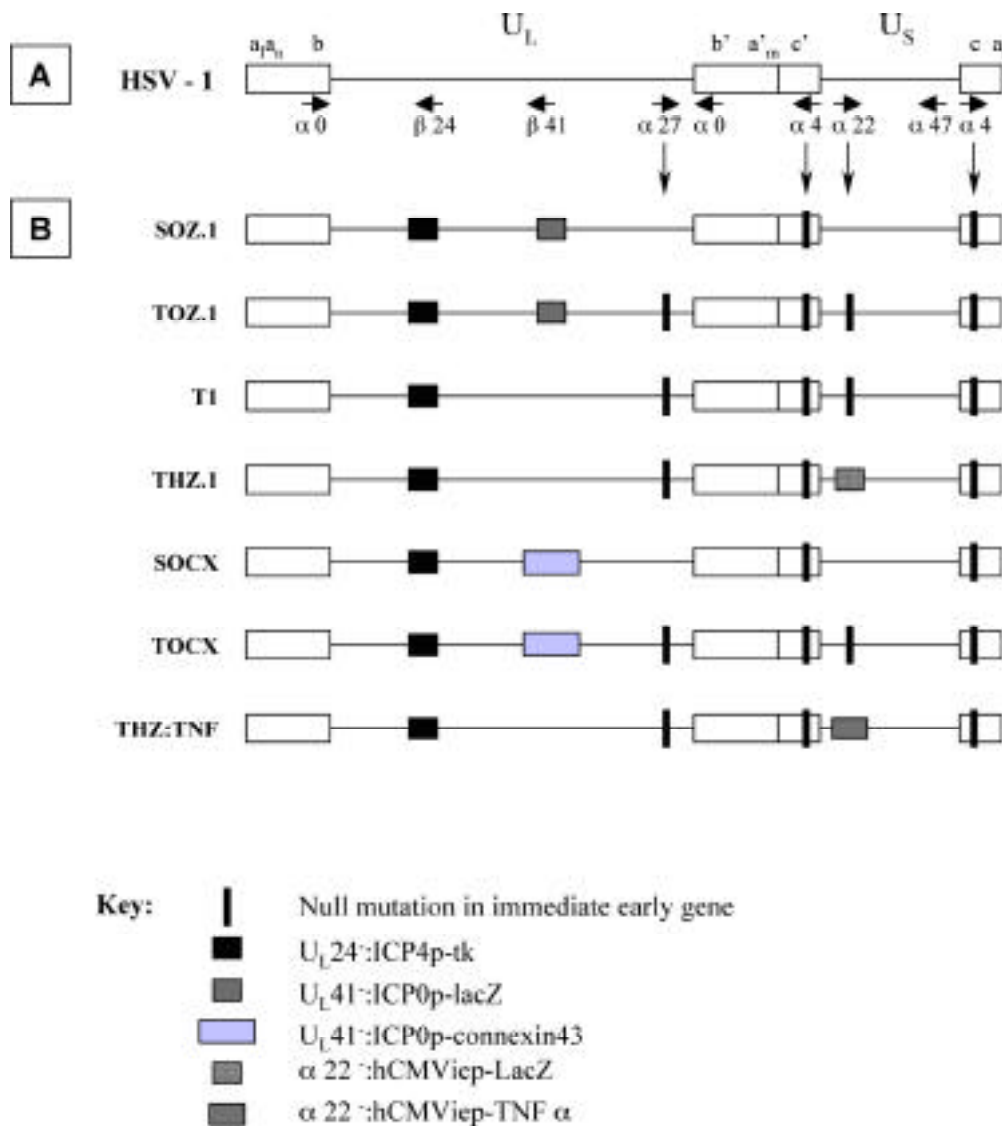


Figure 1 **A.** A schematic representation of the HSV-1 genome (not to scale). The inverted repeats flanking the unique long (U_L) and short (U_S) segments of the genome are indicated as $ab - b'a'$ and $a'c' - ca$, respectively. The approximate positions and orientations of those HSV-1 genes discussed 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 Figure 1A 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.

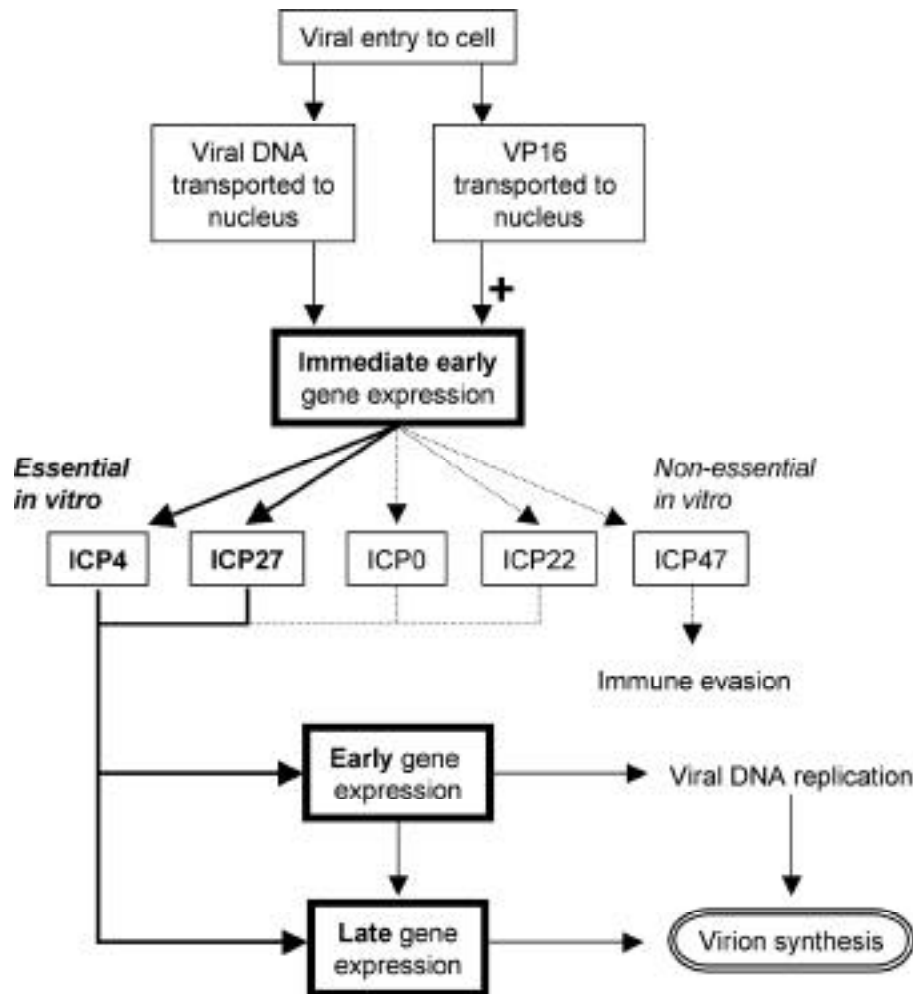


Figure 2. Flow chart depicting the cascade of regulatory events that result in ordered sequential expression of HSV-1 genes during wild-type infection. In order to proceed to E and L gene expression from IE gene expression, both ICP4 and ICP27 must be expressed. Inactivation of either results in loss of E and L gene products and failure to produce infectious virus. Suitable ICP4 and ICP27 expressing cell lines may complement these gene products *in trans*. Full details of the construction of replication-deficient viruses may be found in the text and references.

intratumoural toxicity is not an issue and transient high-level gene expression is desirable. Deletion of ICP47 restores the expression of MHC class I molecules to the surface of the cells. This may potentially confer advantages in the gene therapy of 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 (**Figure 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* (Wu et al, 1996; Wolfe et al, 1999). Safety is an important consideration in the development of therapeutic reagents; in this regard, a number of beneficial features are intrinsic to the vector system described here:

(i) The viruses are produced in cell lines that contain minimal gene sequences in common with the defective vector - it is extremely unlikely that replication-competent revertants will be inadvertently generated during manufacture (thus far, replication-competent virus has not been detected after repeat passaging of vector stock on complementing cells).

(ii) 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 locus in the gene therapy vector prevents its acquisition by wild-type virus; 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 considerations suggest that the generation of a replicating transgene-expressing virus would be extremely unlikely.

(iii) If a replication-competent mutant were generated, expression of the early gene thymidine kinase would enable appropriate treatment with the antiviral agent acyclovir (see below).

In addition to deletions of multiple IE genes, which achieve safety and minimise 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 (Kriskey et al, 1998). This property may have important implications for the gene therapy of cancer, as it seems likely that multiple therapeutic transgenes will be necessary to effectively deal with a disease that is heterogeneous and constantly evolving within an individual patient.

The selection of an appropriate cis-acting regulatory domain to drive expression of each transgenic expression cassette is an important issue for a number of gene therapy applications. For example, in diseases where long-term transgene expression is required, the HSV latency active promoter complex (Goins et al, 1994, 1995; Soares et al, 1996) has been successfully used to effect chronic sustained transcription of the desired therapeutic gene (Lachmann and Efstathiou, 1997). In the context of gene therapy for malignant disease, the goal in many circumstances is transient high level expression of a toxic gene. In this setting, use of viral IE promoters is appropriate, for example the ICP4 promoter or the IE promoter from human cytomegalovirus. The possibility of exploiting glioma-specific promoters to restrict expression of toxic genes to target cells is attractive, but will depend on the identification of appropriate elements.

III. Therapeutic transgenes for cancer

Many different transgenes have been considered for therapeutic application in cancer. This section outlines general principles that are common to the various groups of molecular targets that have been identified, and provides specific examples of each. Types of genes that may be delivered to cancers for therapeutic purposes are conveniently considered in the following categories, although the distinctions are arbitrary and some genes fall into more than one group.

A. Suicide genes

These are genes encoding a product that is toxic to the cells within which it is expressed. An example is herpes simplex thymidine kinase (HSV-TK). 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 active 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, as expression of IE genes is necessary 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, as in many cases cells surrounding transduced cells are killed following ganciclovir administration. This phenomenon is referred to as 'bystander lysis' (Carroll et al, 1997; Marconi et al, 2000). *In vitro*, bystander lysis is largely attributable to uptake of activated ganciclovir by HSV-TK negative cells (Rubsam et al, 1999; Marconi et al, 2000). The mechanisms responsible for bystander lysis *in vivo* are complex, and involve the passage of activated ganciclovir from HSV-TK positive to HSV-TK negative cells in addition to effects attributable to necrosis-induced inflammation and disruption of vasculature (Ram et al, 1994; Hamel et al, 1996; Dilber et al, 1997). Activated ganciclovir may pass from cell to cell through gap junctions (Dilber et al, 1997; Andrade Rozental et al, 2000; Robe et al, 2000). These are intercellular channels formed by a number of proteins including connexin-43 (Nagy and Rash, 2000). As gliomas are often defective in connexin expression (Shinoura et al, 1996) and intercellular gap junctions (Naus et al, 1999), the expression of connexin-43 in these tumours represents one potential strategy whereby the bystander lysis effect may be enhanced (Figure 5 and later section).

B. Genes whose products enhance the susceptibility of the tumour to radiotherapy

Examples of this type of gene include TNF- (Moriuchi et al, 1998; Niranjan et al, 2000), which is discussed below, and ATM, which is mutated in the hereditary disease ataxia telangiectasia. The phenotype of the disease includes ataxia, dilated loops of capillaries, lymphoreticular malignancy and susceptibility to radiation-induced cell death (Smith and Conerly, 1985). 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 (Rotman and Shiloh, 1998). Abolition of ATM expression in a glioma cell line results in enhanced sensitivity to gamma irradiation (Guha et al, 2000). Transient reduction of ATM expression could be achieved during radiotherapy using antisense or ribozyme RNA molecules delivered by a gene therapy vector. This is a potentially useful way of reducing the dose of radiation that is fatal to the tumour cells, enabling reduction of the dose that is received by surrounding tissue.

C. Genes that encode immunomodulatory proteins

Several such genes have been used in anti-cancer vectors, most of which fall into the following groups:

(i) Cell surface receptors or ligands that activate immune surveillance mechanisms to induce lysis of transduced cells – e.g. CD80 (Krisky et al, 1998)

(ii) Soluble mediators that recruit immunocompetent cells to the tumour or activate immunocompetent cells – e.g. TNF (Moriuchi et al, 1998; Niranjana et al, 2000), GM-CSF (Krisky et al, 1998), IL-2 (Colombo et al, 1997), interferon- (Kanno et al, 1999), IL12 (Parker et al, 2000).

D. Genes that correct the molecular defects present within the tumour cell

It has recently become possible to formulate a unifying framework that describes the essential alterations in cell physiology that dictate malignant behaviour (Hanahan and Weinberg, 2000):

- (i) Self-sufficiency in growth signals
- (ii) Insensitivity to anti-growth signals
- (iii) Evasion of apoptosis
- (iv) Limitless replicative potential
- (v) Sustained angiogenesis
- (vi) Tissue invasion and metastasis

The biochemical mechanisms of many of these properties are currently being elucidated, and it may soon be possible to define individual tumours in terms of the four to seven molecular events that govern their development. Indeed, mutations linked to malignant behaviour in glial cells have been identified in several genes, including those for growth factors and their receptors, intracellular messengers, cell cycle proteins, transcription factors, tumour suppresser genes and their regulators. The prospect of identifying the responsible molecular defects in an individual tumour and then stably transducing all of the tumour cells with corrective genes is a daunting one, and not necessarily desirable. Certain types of genetic intervention, however, might be effective

following short-term transgene expression in limited numbers of tumour cells. Examples might include the promotion of physiological death of cells bearing severely mutated genomes by introduction of p53 (Lang et al, 1999), or disruption of neovascularisation (Wesseling et al, 1997) by antisense directed against VEGF (Im et al, 1999; Machein et al, 1999).

IV. Combination treatment of experimental glioma using HSV vectors

A major advantage of HSV as a gene therapy vector is its ability to accommodate multiple transgenes. In a series of studies using *in vitro* and *in vivo* models of glioblastoma, we have demonstrated the feasibility of using HSV as an anti-cancer gene therapy vector, and have started to examine the optimal requirements for combination gene therapy of malignancy. The various vectors used in the studies described here have been listed in **Figure 1B**. By studying the responses of tumour cells to these different vectors, the following principles have been established:

A. Expression of therapeutic transgenes is increased by non-toxic vectors, and results in enhanced anti-tumour properties (Moriuchi et al, 2000)

The *in vitro* and *in vivo* cytolytic properties of HSV-TK expressing replication-defective viruses deleted for ICP4 alone (SOZ.1) were compared with those of ICP4, ICP22 and ICP27 triple null mutants (T1, TOZ.1, THZ.1) (Moriuchi et al, 2000) (**Figure 1B**). All viruses were able to infect rat 9L glial 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. In contrast, 98% of cells infected with SOZ.1 showed apoptosis; extensive cytopathic changes and cell loss were evident (**Figure 4A**). 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 (**Figure 3**). Rats were inoculated intracerebrally with 10^5 x 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. Animals were treated with ganciclovir following viral injection and survival monitored. Rats injected with SOZ.1 showed no survival advantage over controls or following treatment with ganciclovir. Animals treated with T1 showed a clear therapeutic response to ganciclovir, which increased survival time by up to 50% in some animals (**Figure 4B**). Histological examination showed that a localised area of

necrosis was evident within tumours infected with the single mutant, but that the area of necrosis was inadequate to affect tumour progression. Thus, bystander lysis from enhanced transgene expression consequent to reduced toxicity is a superior anti-cancer strategy than direct viral toxicity.

B. Bystander lysis from TK-ganciclovir is enhanced by simultaneous expression of connexin-43 from the same vector (Marconi et al, 2000)

It was hypothesised 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 (**Figure 5A**). 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 (Marconi et al, 2000) (**Figure 1B**). First, it was shown by western blot hybridisation that TOCX gave rise to connexin-43 expression in a connexin-43 experiments using cells with different levels of endogenous connexin-43, that were transduced with either of the viruses and then mixed with non-transduced cells, cell. Thus, above a certain threshold, connexin-43 expression in cells able to activate ganciclovir was sufficient to promote transfer of the nucleoside analogue to cells expressing connexin-43 at very low levels (**Figure 5A**).

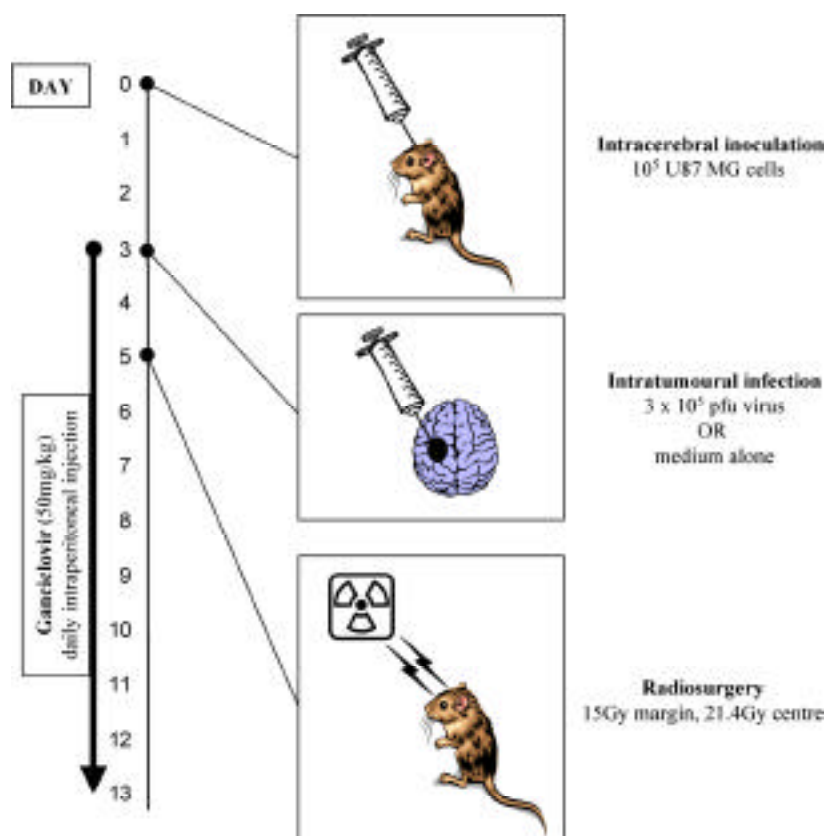


Figure 3. Orthotopic transplant model of glioma. Athymic (nude) mice were inoculated with U87 (human glioma) cells into the striatum on day 1. A tumour was allowed to establish for 3 days, following which the same co-ordinates were inoculated with the gene therapy vector under study, or with a negative 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 8 animals in each treatment group was monitored and is presented as a Kaplan-Meier survival curves in Figures 5B, 6B and 7. The data shown in Figure 4B 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 prior to gene therapy and ganciclovir treatment.

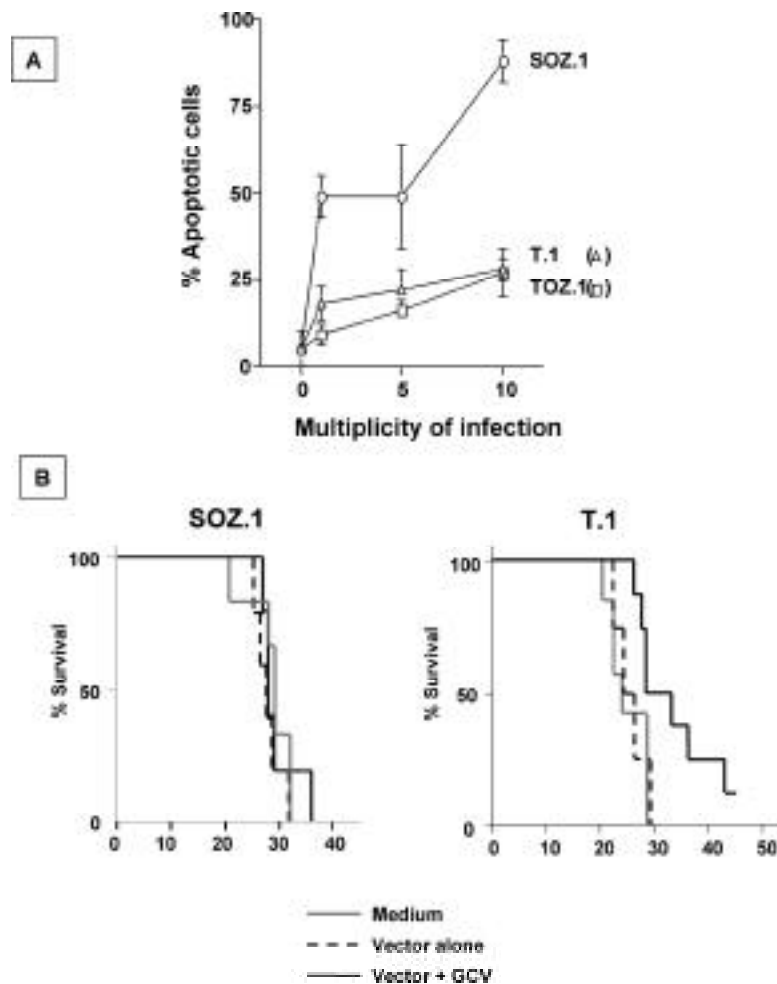


Figure 4. A. A single IE gene deleted virus (SOZ.1) was compared with two triple mutants (T.1 and TOZ.1) for cytotoxicity in vitro. Relative to T.1 and TOZ.1, SOZ.1 causes a substantially higher number of cells to undergo apoptosis at any given multiplicity of infection. This is attributable to the inherent toxicity of ICP22 and ICP27, which are not expressed following infection with the triple mutants. Details of the viruses are shown in Figure 1B. B. Survival of rats in an orthotopic transplant model of glioma (**Figure 3**). Rats were inoculated intracerebrally with 9L glioma cells, then treated with either SOZ.1 or T.1 vector, or negative control medium. Untreated rats died from brain tumour within 30 days. Survival was not affected by administration of vector alone. However, there was significant prolongation of survival in rats treated with ganciclovir, only after T.1 inoculation. As both SOZ.1 and T.1 express the same HSV-TK expression cassette, the superior anti-tumour action of T.1 must have been attributable to differences in the expression of HSV-TK secondary to differences in the toxicity of the two vectors. Thus, the less toxic vector allows better in situ expression of TK and an augmented therapeutic effect following ganciclovir treatment

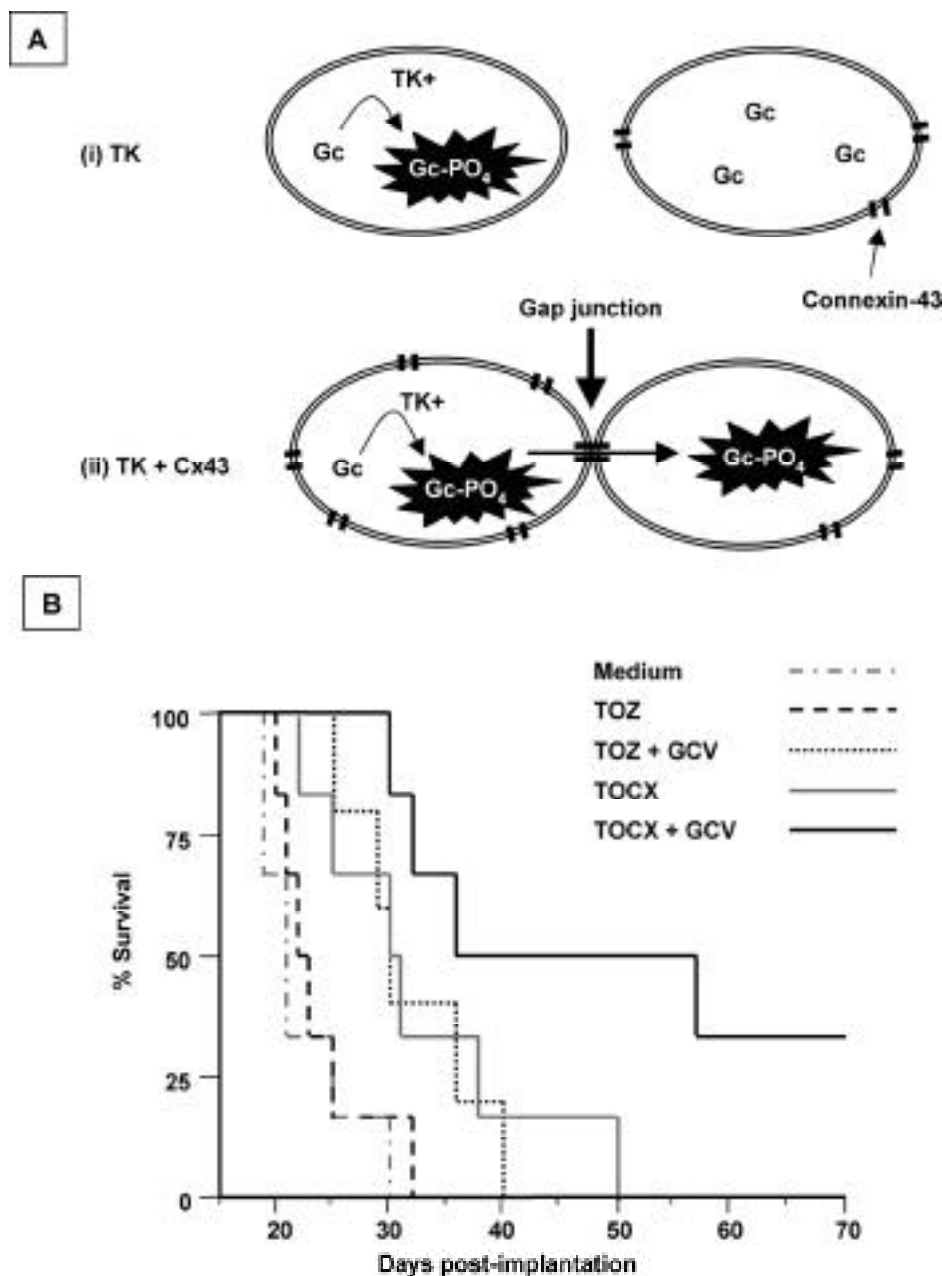


Figure 5 A. The bystander lysis effect is mediated, in part, by cell to cell spread of activated ganciclovir through gap junctions. (i) Transduction of a small proportion of cells with HSV-TK in the absence of gap junctions leads to the activation of ganciclovir only within the transduced cells. Non-transduced 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. This results in an enhanced bystander lysis effect. This observation constitutes 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. **B.** The therapeutic effect of simultaneous TK and Cx43 expression was examined in the mouse orthotopic xenotransplant model described in Figure 3. Negative controls were compared with triple IE mutant vectors expressing either HSV-TK alone or in combination with connexin-43. Details of the viruses are shown in Figure 1B. 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 + ganciclovir. Expression of connexin-43 from TOCX had an anti-tumour action approximately equivalent to that seen with TOZ + ganciclovir. A combination of connexin-43 and HSV-TK/ganciclovir therapies (TOCX + ganciclovir) resulted in substantial survival benefit.

These observations provided a basis for the rational examination of HSV-TK/connexin-43 vectors *in vivo*. Nude (athymic, immunodeficient) mice were subject to intracerebral inoculation with 10^5 human glioma cells, and tumours were allowed to establish for 3 days (Figure 3). The tumours were then stereotactically injected with either TOZ or TOCX, or with medium alone. Half of each treatment group was treated with ganciclovir to assess the therapeutic effect of suicide gene therapy with or without connexin-43 expression. All animals in the control, non-ganciclovir treated or TOZ groups were dead by 50 days into the study, at which point 50% of the TOCX/ganciclovir animals were still alive. At the end of the study (70 days) one-third of the TOCX/ganciclovir animals were alive (Figure 5). Thus, bystander lysis from suicide gene therapy can be augmented substantially and with a clinically therapeutic effect by co-expression of connexin-43 from the same vector.

C. Co-expression of an antitumour cytokine enhances lysis by TK-ganciclovir (Moriuchi et al, 1998).

Tumour necrosis factor alpha (TNF) is a potent antitumour cytokine that demonstrates a range of actions against malignant cells, including the induction of apoptosis via activation of TNF receptors, enhancement of HLA antigen expression in tumours, and immunomodulatory effects such as induction of NK- and CTL-mediated tumour lysis (Scheurich et al, 1986; Pfizenmaier et al, 1987; Ostensen et al, 1989; Rosenblum and Donato, 1989; Cao et al, 1997; Mueller, 1998). The molecule is too toxic to deliver systemically (Rosenblum and Donato, 1989; Mueller, 1998), but the ability of HSV vectors to accommodate multiple transgenes readily enables its incorporation into a locally administered suicide gene therapy paradigm. The hypothesis that TNF 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 at the ICP22 locus (TH:TNF) (Moriuchi et al, 1998) (Figure 1B). TH:TNF was shown to express biologically active TNF, by ELISA and viability assays of TNF-sensitive cells following infection. Ganciclovir-mediated lysis of TNF-sensitive cells *in vitro* following infection was enhanced by the presence of the TNF expression cassette when a low proportion of the cells was 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. Many gliomas, however, are TNF-resistant. It was of interest, therefore, to observe that TNF-mediated enhancement of HSV-TK/ganciclovir lysis was observed in a TNF-insensitive glioma cell line; the mechanism was unclear, but presumably arose from sensitisation of the cells to one

agent as a consequence of exposure to the other (Figure 6A). *In vivo* studies using the athymic mouse orthotopic xenotransplant tumour model (Figure 3) confirmed the *in vitro* observations in tumours composed of TNF-sensitive cells, but showed no additional benefit from TNF expression in tumours derived from resistant cells. Although a substantial and significant prolongation of survival was seen with the TNF/HSV-TK/ganciclovir regimen compared with negative control, the effect was also observed with the HSV-TK/ganciclovir treatment alone (Figure 6B). Possible explanations include the observation that the low proportion of tumour cells that were transduced *in vivo* may 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-resistant cell lines, and the immunomodulatory effects of TNF would not be seen in athymic mice.

D. Co-expression of TNF and TK enhance the therapeutic efficacy of knife radiosurgery, enabling histological and clinical cure in the majority of animals (Niranjan et al, 2000).

Fractionated radiotherapy has been shown to confer a small but significant survival benefit on patients with glioblastoma. Unfortunately, the dose of radiotherapy that may be tolerated by the brain (about 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 augmented radiation doses to be delivered to the tumour periphery where malignant cells are seen invading the surrounding normal tissue, 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. It is therefore of interest to examine ways in which the response to radiotherapy may be enhanced by gene delivery in the hope that malignant cells invading the tumour periphery may be eradicated. It was known that TNF could enhance the therapeutic effect of gamma knife irradiation in athymic mice. Studies were therefore designed to examine whether the individual anti-tumour efficacies of suicide gene therapy, TNF and radiotherapy were additive in combination (Niranjan et al, 2000). The athymic mouse orthotopic xenotransplant tumour model (Figure 3) 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, and the reader is referred to the original reference for the full analysis. The most

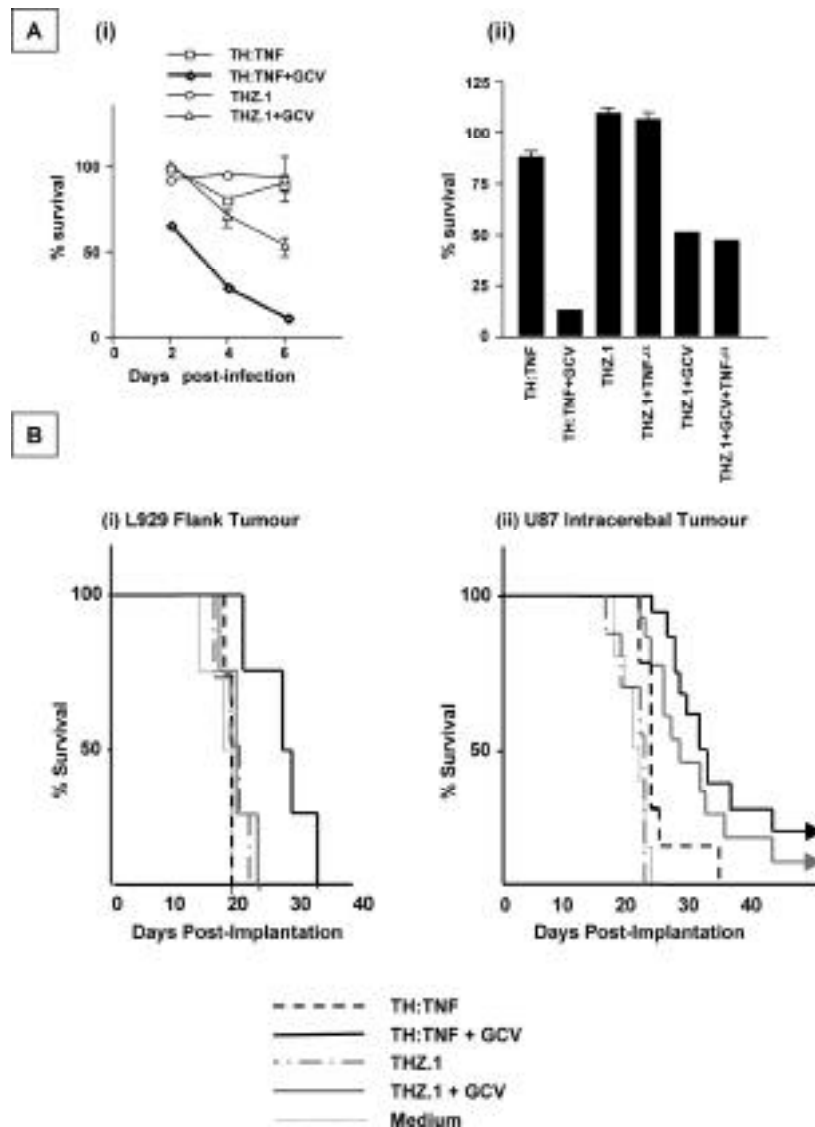


Figure 6 A. Anti-tumour action of TNF- α . A number of gliomas are resistant to the anti-tumour actions of TNF- α . The role of TNF- α in combination therapy with HSV-TK/ganciclovir was examined in vitro using triple IE mutant viruses expressing HSV-TK alone or with TNF- α . Details of the viruses are shown in Figure 1B. (i) As previously reported, 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 sensitised the cells to the effects of the other. (ii) Interestingly, this effect was not recapitulated by administration of exogenous TNF- α . It appears that sensitisation to HSV-TK/ganciclovir requires the endogenous expression and intracellular synthesis of TNF- α . These observations constitute a rational basis for attempts to simultaneously deliver HSV-TK and TNF- α to tumour cells using single vectors that express both genes. **B.** In vivo therapeutic efficacy of TNF- α expressing viruses in (i) mouse flank tumour model and (ii) mouse orthotopic glioma model. (i) L929 cells were chosen because of their intrinsic sensitivity to TNF- α . In this model, there is a clear survival advantage in the group treated with the TNF- α co-expressing virus. (ii) In contrast, there was no clear benefit of TNF- α co-expression over an HSV-TK/ganciclovir regime in the mouse glioma model.

important points that emerged, however, were that (**Figure 7**):

(i) Suicide gene therapy (with or without TNF- α) and radiotherapy alone increased survival relative to no treatment, or administration of either vector alone.

(ii) TNF- α enhanced the effect of radiotherapy, whereas suicide gene therapy did not.

(iii) A combination of the three treatment modalities led to 89% long-term (75 days) survivors, of which 75% were tumour-free. The next best treatment protocol led to

50% survival, and 0% of controls survived beyond 35 days.

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 (Nurel-C). Again, the general principle to emerge from these studies is that combination treatment protocols are superior to single interventions (manuscript in preparation).

V. 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 absence of cytotoxicity, effective transgene delivery and expression, and ability to accommodate multiple therapeutic cassettes. Recent studies have established the principle that it is possible to eradicate 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 a number of years in an immunocompetent host and are heterogeneous by the time of diagnosis. These initial studies, however, provide some optimism that tumour cells may be targeted *in vivo*, and form a basis for the continued investigation of this general strategy. Further studies will be directed at developing more advanced molecular therapeutic agents, and

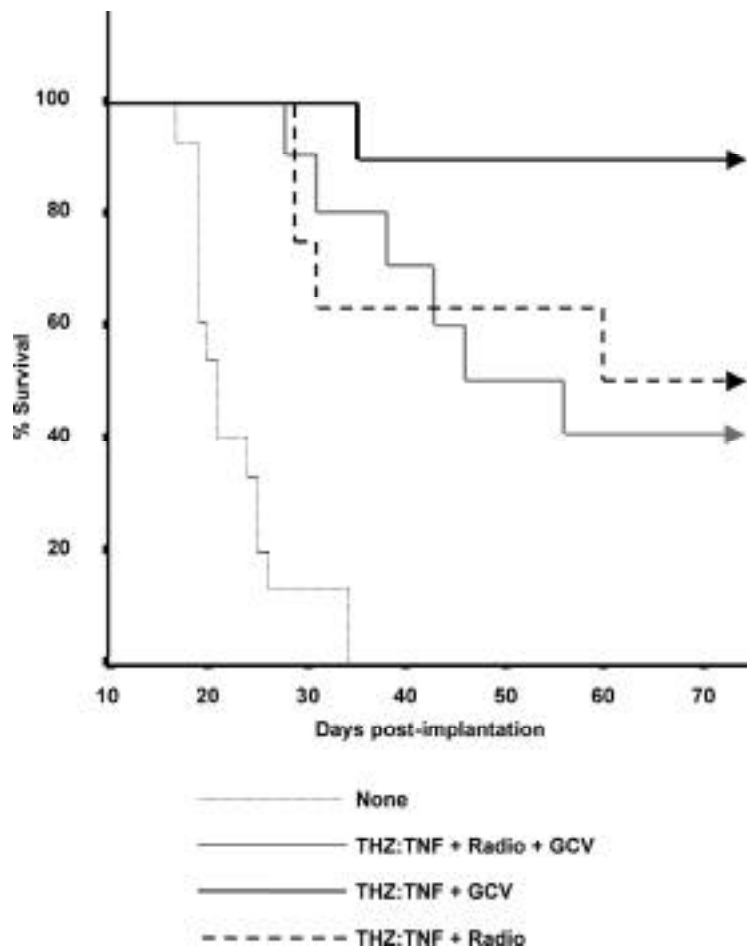


Figure 7. Combination HSV-TK/ganciclovir, TNF- α and radiotherapy treatment is superior to any single intervention or combination of two treatment modalities. The TNF- α co-expressing virus (TH:TNF) was examined in the mouse glioma model shown in Figure 3, with or without radiotherapy and/or ganciclovir. Untreated mice died within 3 days from cerebral tumour. 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.

addressing the efficacy issue in models more closely resembling the human disease. Current work is focussed on improving the vector system further and identifying new molecular targets that might rationally be combined with the strategies described above to disrupt additional aspects of the oncogenic process. The possibility of further enhancing the combinatorial effects of TNF and radiosurgery by downregulation of ATM is being actively pursued, as is the generation of more severely disabled vectors that are likely to be less toxic and better able to deliver their payload. It is envisaged that glioma therapy of the future might involve cytoreductive surgery followed by vector inoculation into the tumour cavity and radiotherapy. The vector might encode genes that allow pharmaceutical lysis of actively dividing cells and their neighbours, enhance the radiosensitivity of tumour cells at the invasive margin, and inhibit neovascularisation of potential areas of recurrence at the tumour margin. Whether this approach will have a major impact on the prognosis of malignant glioma is uncertain, but in the absence of other promising experimental approaches it seems well worth pursuing.

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