Drugs of the Future 1998, 23(4): 401-413

CCC: 0377-8282/98

Review Article

Ganciclovir and the HSV-tk enzyme-prodrug system in cancer therapy

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Introduction

Ganciclovir[1] (GCV) is a comparatively new antiviral drug that has emerged from the structure-activity screening of analogs related to the potent antiherpetic compound aciclovir[2] (ACV) (1, 2). Both molecules are activated by viral thymidine kinases to their corresponding monophosphates and then undergo further phosphorylation by host cell kinases. GCV is activated by thymidine kinases and related enzymes from a range of different viruses and is a superior substrate for both the viral and host cell kinases than ACV (2). The herpes simplex virus-1 (HSV-1) thymidine kinase (HSV-tk) is one of the most efficient viral GCV-phosphorylating enzymes. The triphosphate of GCV is incorporated into DNA and interferes with viral and host cell DNA replication (3, 4). With the emergence of transgenic animal technology and the refinement of gene therapy approaches, the HSV-tk/GCV system has found applications far removed from its early antiviral origins. For example, HSV-tk genes under the control of tissue-specific promoters have been used to selectively ablate whole tissues or organs from transgenic animals (5, 6). Other potential uses include the expression of HSV-tk as a safety feature in cells designed to be introduced into the body (*e.g.*, bone marrow transplantation or cytokine replacement therapy using cytokine-producing cells) (7, 8). The system has even been used to control shoot outgrowth in plants such as *Arabidopsis thaliana* (9). However, most of the scientific effort is currently focusing on application of the HSV-tk/GCV system for the gene therapy of cancer. This review will focus primarily on this latter area, where targeted expression of HSV-tk to tumor cells may allow the selective elimination of neoplastic tissues.

[1]Ganciclovir (GCV) is referred to in the literature by a variety of different names such as 9-(1,3-dihydroxy-2-propoxymethyl)guanine or DHPG; 9-{[2-hydroxy-1-(hydroxymethyl)-ethoxy]-methyl}guanine or HHEMG; 2'-nor-2'-deoxyguanosine or 2'NDG; BWB 759U; BW759; Biolf-62; Cytovene; Cymevene. [2]Aciclovir (ACV) is otherwise known as 9-([2-hydroxyethoxy]-methyl)guanine; acycloguanosine; Zovirax®.

Ganciclovir structure and activity

Nucleoside analogs have long formed one of the mainstays of anticancer, antiviral and antimicrobial chemotherapy. The discovery of the potent antiherpetic drug ACV (1, 2), an acyclic analog of deoxyguanosine/guanosine, marked the beginning of an extensive synthesis effort to optimize the therapeutic value of antiviral guanosine derivatives. ACV now possesses an established position in the treatment of herpes simplex (HSV) and varicella-zoster virus (VZV) infections (10). However, the utility of ACV is limited by its poor aqueous solubility, low oral absorption and limited spectrum of activity. GCV is not subject to many of these drawbacks. The synthesis of GCV was reported independently by a number of laboratories in the early 1980s (11, 12) and its structure is very similar to that of ACV (Fig. 1). Essentially, GCV is guanosine or deoxyguanosine where the pentose ring structure has been broken by deletion of the 2' carbon (Fig. 1). GCV is active against a wide range of viruses, including HSV-1, HSV-2, VZV, cytomegalovirus (CMV), Epstein-Barr virus (EBV) and human herpes virus-6 (HHV-6) (10, 13). This is a great

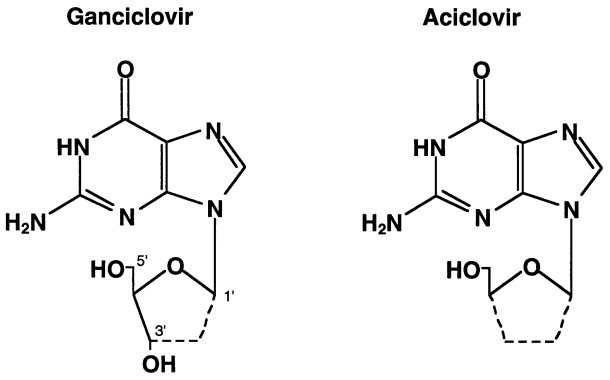

Fig. 1. Structures of ganciclovir and aciclovir have been drawn in a ribose-like conformation to highlight the similarity between these compounds and 2'-deoxyguanosine. The deleted parts of the pentose structure are indicated by stippled bonds and, in accordance with this representation, some of the remaining carbon atoms are labelled as occupying the 1', 3' and 5' positions.

improvement on ACV, the antiviral activity of which essentially is restricted to HSVs and VZV (10).

The HSV-1 tk enzyme

The metabolic and kinetic parameters of the HSV-1 thymidine kinase enzyme have been studied extensively and its crystal structure has recently been determined (4, 14, 15). HSV-tk is part of the phosphorylation cascade which converts deoxythymidine to thymidine triphosphate. HSV-tk is a dimer and, structurally, the core of the viral tk is highly homologous to that of adenylate kinase (14). However, HSV-tk is significantly different from any of the known mammalian thymidine kinases and thus provides an ideal target for chemotherapeutic intervention. The crystal structure has shown that GCV binds to the same site and in a similar configuration as the original substrate, deoxythymidine (14). However, there are some changes in the binding interactions, indicating that the binding pocket is large and flexible enough to allow binding and phosphorylation of a variety of different nucleoside analogs. This promiscuity is not shared by mammalian thymidine kinases and it is thought that this difference provides the spectacular specificity that has been achieved with the HSV-tk/GCV system (10, 14).

Ganciclovir mechanism of action

GCV is a prodrug which requires polyphosphorylation before it can exert its antiproliferative effects. It is an exceedingly poor substrate for animal or plant cell kinases and the presence of viral kinases/phosphotransferases is essential for effective initiation of the activation pathway (16, 17). The catabolic activation of GCV and related acyclic guanosine analogs is shown in Figure 2. GCV is phosphorylated by a number of different viral thymidine kinases of the herpes virus family as well as by an unrelated kinase encoded by the U97 gene product of human cytomegalovirus – a virus which does not encode a dedicated thymidine kinase (18, 19). In all cases, however, GCV is monophosphorylated (GCV-MP) on the 5' carbon of the putative pentose ring (GCV-MP). The thymidine kinase from HSV-1 is one of the most active, and by far the most well-studied, enzymes catalyzing this reaction and is established as the enzyme of choice for geneticallly engineered systems (9, 20, 21).

Only after its initial phosphorylation does GCV become a substrate for cellular kinases. GCV-MP is rapidly converted to GCV di- and triphosphate by the activities of guanosine monophosphate kinase and guanosine diphosphate kinase, respectively (22). The end-product of this polyphosphorylation cascade, GCV-triphosphate (GCV-TP), is responsible for the antiproliferative effects observed. GCV-TP is recognized by both viral and host cell DNA polymerases and incorporated into the extending DNA strand in place of guanine (3, 22). The viral DNA polymerases appear to be somewhat more promiscuous in this respect and it has been shown that viral DNA replication is inhibited more strongly than is the host cell process (3). Unlike ACV-TP, which acts as a chain terminator, GCV-TP is incorporated into elongating DNA strands by virtue of its two hydroxyl groups which can form the required phosphodiester bonds (10, 22, 23).

Whether the delay in DNA polymerase progression alone is responsible for the antiproliferative effects of GCV-TP is unclear. Experiments have shown that in virally infected cells, GCV exposure did not depress the pool size of cellular deoxyribonucleotides, nor did it inhibit the synthesis of viral mRNA species or viral proteins (24). Thus, the antiviral activity of GCV-TP is likely to be mediated by direct inhibition of the viral DNA synthesis apparatus.

In many genetically engineered systems, intracellular production of GCV-TP can trigger apoptotic cell death by interfering with DNA replication (4, 25). The mechanism of action – incorporation of GCV-TP into DNA with a concomitant delay in replication fork progression – is thought to be analogous to that documented for viral polymerases (4, 23, 26). Increased rates of apoptosis have been observed in virally infected or HSV-tk transfected cells after GCV exposure both *in vitro* and *in vivo* (25, 27). *In vivo* this direct cell killing, together with immunological elimination of cells unnaturally arrested by GCV exposure, are the two predominant mechanisms of tissue reduction observed (28, 29).

In vitro efficacy of ganciclovir/tk

One of the first reports demonstrating the efficacy of the tk/GCV system for anticancer activity came from Moolten and coworkers in 1986 (30). They showed that neoplastic BALB/c murine fibroblasts transfected with the HSV-tk gene were 200- to 1000-fold more sensitive to

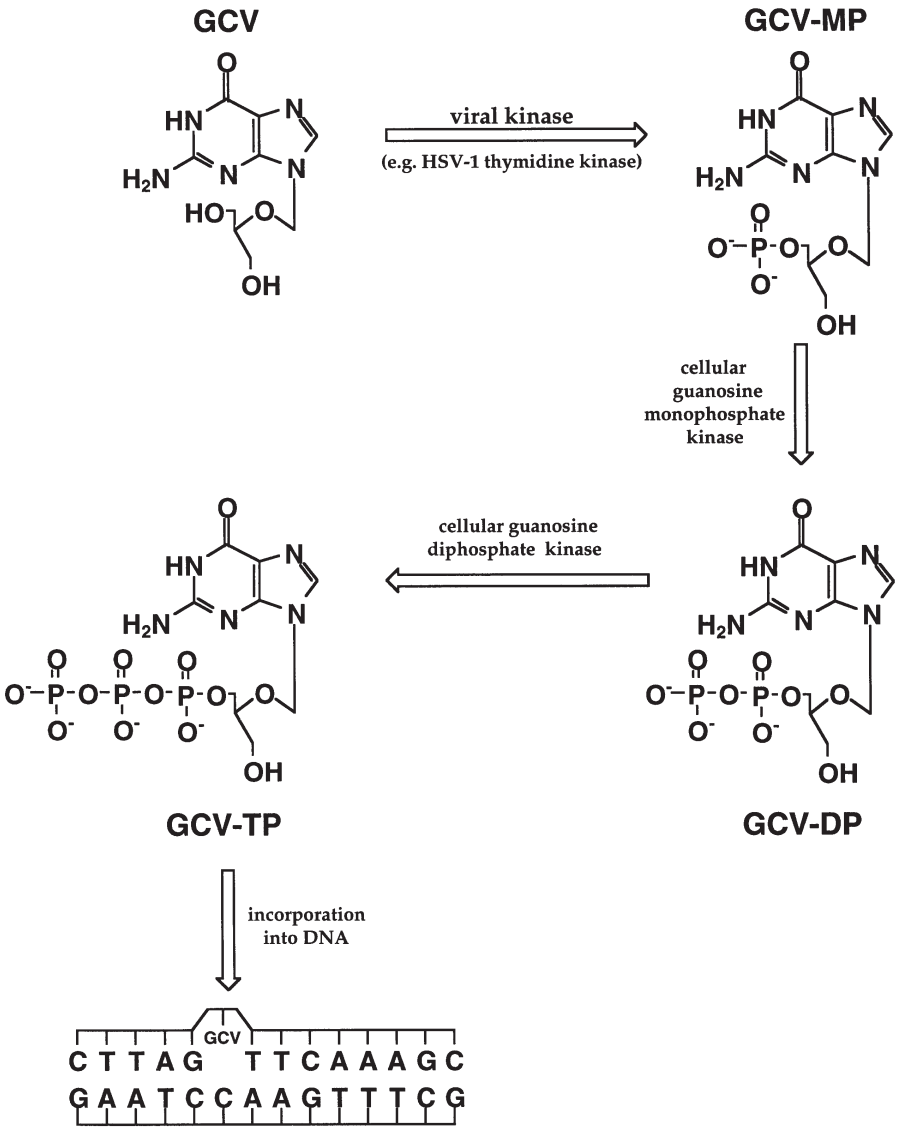

Fig. 2. Activation of ganciclovir by viral thymidine kinase and subsequently by host (cellular) kinases to the cytotoxic form, ganciclovir triphosphate (GCV-TP).

GCV than cells lacking this gene (30). Sensitivity to GCV conferred by tk subsequently has been observed in several other cell lines, for example, murine melanoma (31, 32), rat glioma (33-35), murine and human colon cancer (36), small and non-small lung carcimonas (37, 38) ovarian cancer (39) and human prostate cancer cells (40).

Large differential cytotoxicities between tk transfected and nontransfected cells have been reported in many model systems. Complete cell kill generally is observed at approximately 10 μM GCV concentration or lower with no toxicity being observed in non-HSV-tk expressing cells at this concentration (41-43).

For *in vitro* experiments, a variety of transfection methods have been employed, ranging from simple physical methods, such as calcium phosphate cotransfection, to more complex viral systems. In theory, successful *in vivo* suicide gene therapy would seem to require every malignant cell be transfected with the tk gene. This requirement would place demands on the gene delivery systems which are unachievable at the present time. Fortunately, in light of this inefficiency of current gene delivery techniques, a further important aspect of the direct toxicity of GCV is the well-documented mechanism of "bystander killing".

The bystander effect

The terms "bystander effect" or "bystander killing" describe a phenomenon whereby cells not actually expressing a suicide gene are nevertheless eliminated upon addition of the prodrug. This cell killing is a direct result of the proximity of these untransfected cells to HSV-tk expressing cells. Successful suicide gene therapy

thus appears feasible even in the absence of 100% efficient tumor cell transduction. An extensive bystander effect, both *in vitro* and *in vivo*, has been reported in several model systems with complete tumor regression occurring when as few as 10% of the total cell population expressed the gene (27, 44). In general, particularly *in vitro*, the bystander effect depends on the close proximity of expressing and nonexpressing cells (44, 45), though *in vivo*, immunostimulation may further enhance antitumor activity.

A number of possible mechanisms have been put forward to explain the bystander effect. The release of soluble factors from HSV-tk expressing cells is not believed to be involved (27, 45, 46), but available experimental evidence supports two other possibilities, phagocytosis of apoptotic vesicles from dying cells (46) and the passage of metabolites through gap junctions (44, 45, 47), though the relative contributions of these two processes is likely to vary between cell lines.

A general observation, which would support a role for either gap junctions or intake of apoptotic vesicles, is that direct cell-cell contact is required *in vitro* for an efficient bystander effect to occur (27, 44-46). Early studies using radiolabelled GCV demonstrated that a metabolite of GCV, most probably GCV-TP, is the toxic species transferred between cells (45). Untransformed cells were shown to accumulate radiolabelled GCV from tk-expressing neighbors and to incorporate the nucleotide into their DNA (45). This was suggested to be a form of "metabolic communication" occurring through gap junctions. Gap junctions are intercellular communication channels composed of proteins known as connexins. They permit diffusion of molecules less than 1 KDa in size between cells and therefore should allow passage of phosphorylated GCV, which has a molecular weight of ~ 300 Da. Many transformed cells have lower levels of gap junctions than normal tissue of the same origin and these structures have been reported to be downregulated by tumor promoters and activated oncogenes.

A number of studies have revealed that cells possessing good gap junctional communication showed a better bystander effect than those with less well-developed communication (42, 47). Subsequently, more direct evidence has been provided by experiments in which a human hepatoma cell line with poor gap junctional communication was transfected with cDNAs for connexin genes 32 and 43 (47). Cells stably transfected with the connexin 43 gene showed an improved bystander effect compared with the parental cells. Similarly, when the connexin 32 gene was introduced into such cells under the transcriptional control of the tetracycline transactivator, connexin 32 expressing cells exhibited a greater bystander effect than did those in which it was downregulated (47). An independent set of experiments involving transfection of the connexin 43 or 26 genes into HeLa cells gave similar results and further substantiated these findings (44, 48). Experiments with the gap junction inhibitor, α-glycyrrhetinic acid, indicate that gap junctions are responsible for a large part of the bystander effect (48). In these studies some remaining bystander activity was observed between tk-positive and tk-negative cells when neither expressed the connexin gene. This may reflect some residual gap junctional intercellular communication between the cells or, alternatively, suggests that mechanisms other than gap junction communication may also be involved in the bystander effect. More recently, a series of experiments have shown connexins to be important for the bystander effect not only *in vitro* but also *in vivo* (49, 50).

Freeman *et al.* observed that HSV-tk-transduced cells treated with GCV displayed features of apoptosis (27). Apoptotic vesicles from these dying cells were engulfed by adjacent non-HSV-tk transduced cells, and this was proposed as the mechanism responsible for the bystander effect. In support of this theory, others have observed apoptotic vesicles both *in vitro* and *in vivo* following treatment of HSV-tk-transduced cells with GCV (46). However, even if apoptosis is involved in the mechanism of primary cell death it may not necessarily play a role in the subsequent bystander effect. Thus, some studies report detection of apoptosis and phagocytosis but not within the time frame of the bystander effect (51). In other cases, a bystander effect has been observed in the absence of detectable apoptosis (52), suggesting the importance of other mechanisms.

The precise mechanisms of the bystander effect remain uncertain, and it is likely that a number of different pathways will cooperate *in vivo*. In this regard the involvement of the immune response (29, 31, 53, 54) and tumor ischemia (55) could also be important factors in supplementing the classical bystander phenomenon. Understanding and manipulating these additional factors could, potentially, be very useful in providing means of enhancing the antitumor response to GCV.

An additional point of note is that, while a threshold may be reached in the levels of tk required for optimal kill, increased amounts of tk still may result in an increased bystander effect (35). Thus, if this phenomenon occurs *in vivo*, increasing levels of tk expression as well as the transformation efficiency may increase the antitumor response obtained.

HSV-tk gene delivery and targeting

In order for suicide gene therapy to be efficaceous *in vivo*, the prodrug must be able to reach the tumor in sufficient concentration. Also the activating gene needs to be delivered to the tumor cells where it must be expressed at sufficiently high levels to achieve a therapeutic effect. Clearly, expression of the gene must be selectively targeted to the tumor cells or to the neoplastic cells within the tumor mass. This selectivity can be achieved theoretically in a number of ways, but often relies on targeting to a particular tissue rather than to the tumor *per se*. A detailed description of gene therapy targeting for antitumor therapy is beyond the scope of this review but is covered elsewhere (56, 57). However, the main strategies for achieving selective delivery and expression can be summarized as follows.

Localized administration

The recombinant delivery vector may be injected directly into the tumor itself or alternatively into compartmental areas (*e.g.*, the proximal portal vein during hepatic in-flow occlusion (58)) or into the peritoneal cavity) which facilitates gene delivery to the appropriate target.

Physical targeting

This includes a variety of strategies such as the use of chemical carriers (*e.g.*, liposomes or antibodies), where the carrying agents serve as localizing vectors for the introduced DNA. By such means as modifying liposome coats it is intended to achieve selective and specific delivery to the target cells.

Viral targeting

Viruses are highly evolved for efficient delivery of their genome to host cells and therefore are ideal vectors for transmitting foreign DNA. Various types of virus have been proposed for use in gene therapy, including retroviruses, adenovirus and herpes simplex virus. The characteristics of some viruses confer innate tumor or tissue selectivity. For example, retroviruses infect highly proliferative cells preferentially and this can be exploited where rapidly dividing tumor cells are surrounded by a bed of essentially quiescent normal cells, as is the case for tumors of the CNS (59). Adenoviruses have a wider host range specificity and can transduce both proliferating and nonproliferating cells. Attempts have been made to alter the determinants of tropism in these viruses in order to restrict infectivity to specific subtypes of cells, *e.g.*, by recognition of receptors overexpressed on tumor cells (56).

To date the viruses employed have been, almost exclusively, replication deficient, *i.e.*, able to infect target cells but unable to replicate subsequently. Production of such “disabled” viruses has been made possible by generating “packaging cell lines” which supply the viral proteins necessary for assembly of infective virus. One method of delivering both retroviruses and adenoviruses has been by the injection of virus particles harvested from a packaging cell line directly into the tumor, i.p. or i.v. (31, 60). Other investigators have injected the retroviral packaging line into the tumor in order to increase the efficiency and degree of tissue infection (7, 53, 61, 62). The virus packaging cells should continue to release infectious particles either until they are removed by the host immune system or, as they too are susceptible to elimination by GCV since they express the viral tk, until the administration of the prodrug.

Transcriptional regulation

Selective expression of tk has been achieved by transcriptional regulation (63), whereby the gene is placed under the transcriptional control of tissue- or tumor-specific promoters or locus control regions (LCRs), *e.g.*, the tyrosinase promoter is switched on in melanocytic cells and has been used to target melanoma cells (31, 64, 65). Further examples for a number of currently used promoter targeting strategies are listed in Table I.

Mechanism of ganciclovir toxicity

The mechanism of action of GCV-TP itself imposes an aspect of selectivity as GCV-TP will only be incorporated into DNA during S-phase of the cell cycle (66). This means that GCV-TP will preferentially eliminate highly proliferating tumor cells and not the surrounding quiescent cells. Naturally, therefore, for tumors with a high proportion of more slowly proliferating or quiescent cells, GCV may not be the most effective choice of treatment and underlines the possible need for alternative or combined therapeutic approaches.

In vivo **studies**

Following the demonstration of their efficacy *in vitro*, HSV-tk/GCV studies were extended to a range of *in vivo* systems, including syngeneic and allogeneic tumor models in rodents and nonhuman primates. Initial studies focused on delivering the HSV-tk suicide gene to an established tumor and were aimed at eradicating this by subsequent GCV administration. However, other therapeutic approaches using the HSV-tk/GCV system also are under experimental investigation. These include the prophylactic “mosaic” strategy (30, 67), targeting of tumor vasculature for antiangiogenic treatment and the tumor vaccine approach.

GCV treatment of tumor cells pretransduced *ex vivo* with tk cDNA has induced regression in a variety of malignancies including brain (68), gastric cancer (69) and ovarian carcinoma (70) after implantation into recipient animals and their subsequent treatment with GCV. However, these model systems are very simplistic as it is likely that, with current delivery methods, only a small proportion of the tumor actually will express the therapeutic gene rather than the entire population of cells in these systems. Moreover, the problem of selectivity is not addressed. However, such experiments have provided a means to test the maximal efficacy of the HSV-tk/GCV combination and have allowed the controlled manipulation of conditions to investigate the bystander effect. For example, when 9L glioma cells were implanted *in vivo* at a ratio of 1:1 with 9L HSV-tk-expressing cells, regressions were seen with enhanced survival, confirming a bystander killing mechanism (68).

Studies which more closely mimic the clinical situation have involved *in situ* gene delivery to localized established tumors and metastases. *In situ* gene delivery can be achieved by several methods, the most simple of which is direct injection of plasmid DNA into the tumor either as naked DNA or in combination with nonviral

Table I: Transcriptional targeting of HSV-tk vectors.

Promoter	Tumor/Tissue Target	Vector Type	Specificity	Ref.
α-Fetoprotein (AFP)	Hepatocellular carcinoma (AFP positive)	Retrovirus Adenovirus Adenoassociated virus	*In vivo* *In vitro*	103-106
Carcinoembryonic antigenn (CEA)	CEA positive pancreatic, gastric or lung tumors	Retrovirus Adenovirus	*In vivo* *In vitro*	107-109
DF3/MUC1	Breast carcinoma	Retrovirus	*In vitro*	110
Early growth response gene (EGR-1)	Irradiated cells	Plasmid	*In vitro*	111
Glial fibrillary acidic protein (GFAP) (murine)	Glioma	Retrovirus	*In vitro*	112
Myc-max response element	Myc overexpressing small cell lung cancer cells	Plasmid	*In vitro*	113
Secretory leukoprotease inhibitor (SLP-1)	Lung. breast, oropharyngeal, bladder, endometrial, ovarian and colorectal SPL-1 positive carcinomas	Plasmid-adenovirus-polylysine conjugate	*In vitro*	114
Surfactant protein A (SPA) (human)	SPA positive non-small cell lung cancer	Plasmid-adenovirus-polylysine conjugate	*In vitro*	37
Tyrosinase (murine)	Melanoma	Retrovirus Adenovirus	*In vitro* *In vivo*	31, 64, 115
von Willebrand factor	Endothelial cells	Retrovirus Adenovirus	*In vitro*	116

carriers such as liposomes (71). By this method gene expression in the tumor has been obtained and some antitumor effects have been observed. Most such therapeutic studies, however, have utilized viruses. Retroviral and adenoviral particles injected either directly into the tumor or intravenously have led to antitumour effect in some cases (32, 40, 72-74), but not in others (60, 75). The ultimate goal of any tumor gene therapy is to prevent tumor spread and to eliminate metastases. It is encouraging that a reduction in lung metastases of a B16 murine melanoma model has been obtained following multiple intravenous injections of recombinant retroviruses, where the tk gene was driven by the tyrosinase tissue-specific promoter (31).

Equally, retroviral producer cells have been assessed as a delivery system particularly for the treatment of models of glioma. In preclinical *in vivo* studies, producer cells were injected directly into the tumor and subsequent GCV administration successfully reduced tumor volume and prolonged survival of the injected rats (29, 61, 75-79).

Unfortunately, not all these experimental studies have shown such favorable results. A number have documented no complete responses even when the majority of cells implanted expressed HSV-tk (67, 75, 80). Thus, Vile *et al.* showed no HSV-tk expression or therapeutic effect following liposomal delivery of an HSV-tk vector to B16 melanoma cells (31). Similarly, a lack of response has been noted in some systems based upon the delivery of retroviral particles or producer cells (60, 75). Furthermore, in some cases, even when an apparently complete response was noted, late tumor recurrence was observed (61, 67, 80, 81). It may well be that the potential for recurrence often is missed due to the early termination of animals. Where tumor progression was monitored by magnetic resonance imaging (MRI) over longer time periods, regrowth was seen at a high frequency (81). Recurrence may be due to the survival of cells that have lost tk expression over time *in vivo* or from cells that were quiescent at the time of GCV treatment and thus not responsive to the drug.

An indication of the possible involvement of tumor blood vessels in mediating some of the bystander effect observed with the HSV-tk/GCV system *in vivo* was provided by the experiments of Ram and coworkers (55). They observed that when a tumor was seeded in the presence of retroviral producer cells, infection of the endothelial cells of the tumor blood vessels occurred and hemorrhagic necrosis was noted. In contrast, no transduction of endothelial cells, nor any hemorrhagic necrosis, was seen when tumor cells were pretransduced with the HSV-tk gene. Hemorrhagic necrosis has also been observed by Freeman's group in intraperitoneal tumors following the i.p injection of tk-transduced HCT 116 (human colon carcinoma cells) and the treatment of the animals with GCV. In this case, necrosis occurred centrally within the tumor mass and was hypothesized to be caused by the release of soluble factors (28).

Several experiments have indicated an involvement of the immune system in the antitumor response observed *in vivo* following tk/GCV therapy (29, 31, 53, 54). This immune response is believed to contribute not only to the *in vivo* bystander effect but also to a prolonged

antitumor response. Evidence for the role of the immune system in these effects stems from a number of observations.

(i) Immunodeficient, or chemically immunosuppressed, mice were less responsive to tk/GCV therapy than were their immunocompetent counterparts (31, 82-84). The use of athymic mice in these experiments suggests the importance of T-cells in mediating this response.

(ii) Cytokines and inflammatory cells have been detected in tumors following tk/GCV treatment using rt-PCR, immunohistochemistry and histology. These changes in tumor composition consisted predominantly of expressed interleukins IL-1α and IL-6 (28), and cellular infiltrates of macrophages and $CD8^+$ T-cells (29, 83, 85).

(iii) Upregulation of costimulatory, antigen-presenting and adhesion molecules which could lead to immunostimulation has been observed in some systems. Major histocompatability complex class-I (MHC-I), molecules involved in antigen presentation, were upregulated on tk-transduced, Renca (murine renal carcinoma) cells implanted subcutaneously in BALB/c mice following GCV treatment (85). Furthermore, the costimulatory molecule B7 and adhesion molecule ICAM were upregulated in a tk-expressing i.p. tumor model upon GCV treatment (86).

(iv) The immune response is directed not only against the primary tumor but there is an enhanced systemic antitumor immunity following tk/GCV treatment. Mice treated with tk/GCV for therapy of a primary tumor showed protection against subsequent challenge with either the parental tumor cells or the tk engineered tumor cells (31, 87). This phenomenon has only been observed in immune competent mice. In experiments where colon carcinoma tumor cells bearing the tk gene were seeded in one lobe of the liver, and non-tk expressing colon carcinoma cells in the opposite lobe, regression of both tumors was observed (88), suggesting the importance of the immune system in the rejection process.

It has been suggested that tk/GCV treatment converts the tumor from an immunosuppressive to an immunostimulatory environment (86). Possibly, tumors killed by tk/GCV may be more antigenic because the dying cells release debris which may then be acquired and presented by professional antigen-presenting cells (31, 89). The mechanism of cell death may be important, therefore, in determining the immune response and, as explained above, this could differ according both to tumor type and anatomical location of the cancer. For example, apoptotic cells have been reported to release IL-1 which could stimulate the immune system (28). It seems that expression of the viral tk alone, even in the absence of GCV, may be antigenic and could thus lead to an antitumor immune response (90). These results emphasize that experimental models need to be carefully chosen to allow the generation of relevant data *in vivo*; utilization of animal model systems which at the outset are strongly immunogenic may, for instance, be inappropriate.

Since immune responses are believed to enhance the resultant antitumor effect, various attempts have been made to boost the host immune system by the concomitant transduction of cytokine genes, or genes encoding immune accessory molecules, at the time of attempts to introduce the tk gene. This approach is discussed in more detail below. However, it should be noted that with some delivery systems, it may be advantageous to suppress rather than stimulate the immune response. Where adenoviruses are utilized for gene delivery, for example, the duration of adenoviral expression can be very limited due to immunity against proteins produced by these vectors. Indeed, in apparent contradiction to much of the work cited above, some investigators have shown an improved antitumor effect of tk/GCV in nude rats and ciclosporin-immunosuppressed rats compared with normal animals (47).

Safety and toxicity

Owing to its use as an antiviral agent, GCV has been investigated widely in terms of dose and toxicity both in animal models and in humans, particularly in immunosuppressed patients (91). Early rodent experiments used GCV at 150 mg/kg/injection given i.p. twice daily for 5 consecutive days (30). At this dose tumor regressions were detected with no reported deaths attributable to GCV; others also have used similar concentrations with no significant toxicity (7). However, Caruso *et al.* (53) observed what they believed to be drug-related deaths at this dose and their preliminary toxicity studies determined 75 mg/kg twice daily to be a safe dose in BDIX rats. In nonhuman primates, no drug-related toxic effects were noted following 14 days of GCV treatment at 10 mg/kg/day (7).

In humans, GCV has obtained FDA approval for treatment of cytomegalovirus (CMV) infections of the eye. In general, those HSV-tk gene therapy protocols already in clinical trial employ the known tolerated dose for treatment of CMV retinitis of 5 mg/kg GCV by i.p. infusion twice daily for up to 15 days. This dose should achieve plasma and cerebrospinal fluid (CSF) concentrations of GCV within the range required to kill HSV-tk-expressing cells (7). Although this concentration of drug alone would be expected to give minimal and manageable toxic effects, possible systemic toxicity in combination with HSV-tk is relatively unexplored, although preliminary results from early gene therapy clinical trials indicate no serious drug-related toxicity (62).

Other safety concerns with the HSV-tk system relate to gene delivery and gene expression, which will vary according to the tumor type, location and delivery vehicle used. The major risk is of inappropriate gene expression in nontumor tissues. As discussed previously, this risk can be reduced by employment of appropriate targeting mechanisms. Further protection should be provided by GCV's preferential activity against highly proliferating cells. However, even a low level of transduction of normal tissue is likely to result in GCV-mediated killing of normal cells. As with all suicide gene therapies, cytotoxicity is

conditional on the presence of prodrug, so that stopping prodrug administration and increasing prodrug clearance can be used to control severe toxic effects of drug administration.

Where viruses or viral producer cells are employed as delivery vehicles, additional risks arise including the generation of replication competent virus, insertional mutagenesis (for integrating viruses), survival of producer cells in the host and systemic spread of virus. Discussion of these issues is beyond the scope of this review but they have been described elsewhere (92).

Several preclinical experiments in mice have shown no toxicity following intraperitoneal (7, 93), intravenous (7, 93, 94) or subcutaneous (7, 93, 94) injection of tk-bearing retrovirus producer cells, either before or after GCV treatment. No viral producer cell-specific toxicity was observed when these cells were injected into normal rat brain with and without GCV treatment at 15 mg/kg twice daily for 7 days (59).

In nonhuman primates, no neural toxicity or behavioral changes were observed following intracerebral injection of vector producer cells, although mild reactive gliosis and localized demyelination were observed around the injection site (7). In both rats and primates, injection of retroviral producer cells into brain tissue resulted in edema and reactive gliosis at the injection site within 1 week of injection (75) but this toxic effect could be fully ameliorated by administration of dexamethasone. No significant inflammatory response was observed in either the meninges or brain parenchyma (75).

Taken together, these data suggest that retroviral producer cells injected intratumorally may be safe to use in clinical studies directed against brain tumors.

Clinical trials

A number of clinical protocols for gene therapy of cancer using HSV-tk and GCV have been submitted, approved and initiated, particularly in the United States. Patients have been entered into trials from as early as 1992 (95) but, to date, very few trial results have been published. Most such protocols have focused on the treatment of brain tumors using retroviral producer cells (59, 93, 96) since these cancers often have a poor prognosis. They also provide a good therapeutic target in that brain tumor cells are proliferating whereas normal brain tissue is quiescent allowing retroviruses to target the dividing cells (61). Additionally, the brain is an immunoprivileged site which may allow longer survival of vector producer cells, and thus lead to greater transduction efficiency. Proposals have also been put forward for treatment of melanoma (62), mesothelioma, ovarian cancer (89, 97), multiple myeloma and head and neck cancer using a variety of delivery vehicles.

Suicide gene therapy trials are far more complex in design to those involving standard drug-based chemotherapy. Apart from the implicit aim of generating an antitumor response, the primary aims of most protocols are to assess the efficiency of tumor transduction and to probe for side effects. Tumor evaluations are generally by MRI and CT scan, but additional parameters may need to be incorporated into the assessments. For example, plasma concentrations of GCV were measured and correlated with clinical response in a recent melanoma trial (62).

When retroviral producer cells have been utilized the number injected has varied between protocols, and some trials have escalated the number of cells injected in an attempt to determine whether increased numbers of producer cells correlate with increasing response (62).

The standard GCV schedule is 10 mg/kg/day as either a single i.p. injection or 2 x 5 mg/kg/day for 14-21 days (93), although most trials suggest a treatment period of 14 days.

Among the first protocols to be authorized were those of Oldfield and colleagues for the treatment of malignant brain tumor and Freeman *et al.* for the treatment of ovarian cancer (7, 98). Oldfield *et al.* (7) examined the effect of injecting murine retroviral producer cells bearing the tk gene intratumorally, followed by GCV (5 mg/kg b.i.d. for 14 days) treatment. The specific aims of the study were to assess the toxicity of the approach in human subjects and to determine whether genetic transduction occurred, as well as assessment of the efficacy of the approach. Preliminary reports state that there have now been 2 complete responses and 3 partial responses observed, although with some toxicity (89, 95). A patient in complete remission has also been reported in Freeman's trial for ovarian cancer in which allogenic ovarian tumor cells expressing HSV-tk were injected i.p. before treatment with GCV (89).

The only HSV-tk/GCV clinical trial for which full results have been published to date is for the treatment of glioblastoma multiforme (99). Five patients with recurrent disease, and therefore a short life expectancy, who previously had undergone surgery (5/5; 100%) and radiation therapy (4/5; 80%) were selected for treatment. They were given intratumoural injections of 10^8-10^9 Ψ CRIP amphotropic producer cells, engineered to generate a murine retrovirus carrying the HSV-tk gene driven by the HSV-tk promoter to a titer of 10^5-10^6 colony forming units (cfu)/ml, and 7 days later, GCV was administered at 5 mg/kg intravenously over 1 hour twice daily for 14 days. DNA was extracted from peripheral lymphocytes and examined for the presence of the retroviral *env* gene by PCR. No evidence of retrovirus was detected. Response was assessed by MRI scan before surgery, 2 weeks following treatment and then monthly. No complete remissions were observed as a consequence of this treatment, although limited antitumor effects were noted in 2 of the 5 patients (99). Moreover, there appeared to be some correlation between tumor size and treatment efficacy. Most importantly, there was no evidence of significant toxicity (99).

A recent review, published in the JNCI (95), reports early observations indicating that there has been evidence of gene expression in a number of the clinical trials

and that a few tumor responses have been observed. These tumor responses have not been dramatic (95, 99) but, perhaps more importantly, no life-threatening or highly significant adverse reactions have been noted.

Combined approaches

It seems likely that the tk/GCV system will only be employed along with other forms of therapy including surgery, radiotherapy or chemotherapy. One possibility that has already been explored experimentally *in vivo* is the use of tk/GCV with administration of cytokines or accessory molecules designed to enhance any antitumor immune responses. To date most such studies have utilized IL-2 as the added cytokine. Using syngeneic BALB/c mice, Chen *et al.* (72) obtained an antitumor effect in a hepatic colon carcinoma model following treatment with an adenovirus bearing the tk gene and subsequent administration of GCV. A much greater reduction in tumor size was observed when tumors were treated with both the tk vector and a murine IL-2 vector, although the IL-2 vector alone caused no detectable response (72). However, a prolonged antitumor immune response and protection against further challenge at a distal site was only observed when animals were given both treatment options combined. This protection was found to be tumor cell-specific and involved $CD8^+$ T-cells. Antitumor immunity was not seen in immunodeficient mice. Thus, results suggest that a combined suicide gene and cytokine gene therapy approach may be advantageous for therapy.

Similarly, enhanced tumor regression and a long-lasting antitumor immune response occurred in a subcutaneous Friend erythroleukemia model when the cells were transduced with both tk and interferon-alpha cDNAs, relative to tumors transduced with either one of these genes alone (100). In a glioma model, however, little benefit was observed when IL-2 was combined with tk gene therapy (101). The length and degree of protection conferred against subsequent tumor growth or rechallange does appear to vary depending on the precise model system used.

Future prospects

Preclinical *in vivo* experiments have shown good promise for the use of HSV-tk/GCV in the treatment of cancer. To date, only limited data are available from early clinical studies, and though antitumor responses have been less than dramatic, encouragingly there has been minimal toxicity. Successful clinical application of such gene therapy protocols is likely, therefore, to require a number of improvements. These include the development of improved gene delivery systems based on selective tumor targeting and specific gene expression.

Improvements in the tk enzyme, either by using alternative sources of tk or by mutating HSV-tk for increased substrate affinity and catalytic ability, are being actively pursued. Black and Loeb (102) have modified the HSV-tk gene by site-directed mutagenesis and succeeded in generating HSV-tk mutants with over 40-fold enhanced cellular sensitivity to GCV compared to the wild-type enzyme, probably due to enhanced GCV phosphorylation kinetics (102).

Analog development to identify novel prodrugs with improved characteristics also is a priority. Desired characteristics of such analogs include optimized bystander killing, increased affinity for the viral *versus* cellular tk, improved kinetic parameters, a greater therapeutic ratio and better solubility. With developments in these various facets it is likely that gene therapy approaches based upon tk/GVC treatment will eventually find a place for the treatment of some cancer types.

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