The HSV-TK/GCV gene therapy for brain tumors
Review Article

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Abbreviations: HSV-TK, herpes simplex virus thymidine kinase; GCV, ganciclovir; Tf, transferrin; VPC, vector producer cell; GFAP, glial fibrillary acidic protein; CNS, central nervous system; RCR, replication competent retrovirus; MR, magnetic resonance

Key Words: HSV-TK/GCV, suicide gene therapy, brain tumor, kinase, retroviral vectors, vector producer cells, glioblastoma, bystander effect

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Summary

Herpes simplex virus-thymidine kinase (HSV-TK) gene transduction followed by ganciclovir (GCV) administration is widely used for cancer treatment as a "suicide gene" therapy. The present review describes the HSV-TK/GCV gene therapy for brain tumors (gliomas) through the eyes of a clinician, including a review of preclinical and clinical studies. In particular, the latest data on the first clinical trial are discussed and, moreover, the surgical procedures are depicted. The surgical technique is the most important for neurosurgeons; therefore, its improvement would be beneficial for further clinical developments as well as biological innovation of retroviral vectors or vector producer cells.

I. Introduction

The great advance in molecular biology has been contributing to the development of gene therapy. The gene therapy offers several novel approaches to many diseases, which are difficult to be cured by conventional treatments. In 1990, a 3-year-old girl received retrovirally transduced lymphocytes that produced the enzyme adenosine deaminase, which was absent in her own genome and had rendered her susceptible to life-threatening infections. This was the first case treated with performing the "gene therapy" in a human being. To date there have been more than 200 clinical trials of "gene therapy" in humans (Human Gene Marker/Therapy Clinical Protocols, NIH 1998).

There are many potential advantages of the gene therapy over conventional drug therapies. Gene therapy could eliminate the need for repeated drug administration, risks of immunogenicity, pharmacological tolerance or dose toxicity, and it could be less costly. The great advantage may develop the ability of a target therapy in any disease. On the other hand, an important caution is that gene therapy must be safe and feasible for its potential applications in humans (Crystal, 1995).

In the cancer gene therapy, a number of target genes have been used or are expected to be applied, for example, oncogenes, tumor suppressor genes, immune related genes. Among these, the prodruk genes ("suicide genes") offer a new system for cancer gene therapy.

Here the HSV-TK/GCV (herpes simplex virus thymidine kinase/ganciclovir) gene therapy for brain tumor is reviewed as one of prodrug "suicide gene" therapies. In particular, we emphasize the clinical aspects of this approach including a clinical trial and surgical technique which is expected to become the most important for the clinical application, especially for neurosurgeons.

II. Brain Tumors

A. Glioma and glioblastoma multiforme

Astrocytic tumors are the most common type of intracranial tumor among adults. Astrocytic tumors are classified into four grades according to WHO: (i) pilocytic astrocytoma (grade 1); (ii) astrocytoma (grade 2); (iii)
anaplastic astrocytoma (grade 3); and (iv) glioblastoma multiforme (grade 4). Glioblastoma is the most common of all gliomas and occurs throughout the central nervous system in all decades of life, but most frequently in the fifth decade or above. The incidence rate of glioblastoma is reported to be 2.36 patients per 100,000 persons in USA (Davis et al, 1996). The prognosis of patients with newly diagnosed as well as recurrent glioblastoma is poor. Survival of most patients diagnosed with glioblastoma is less than two years and the tumor recurs most certainly following the initial treatments with the combination of surgery, irradiation and chemotherapy (Ammirati et al, 1987). The median survival time of recurrent glioblastoma is approximately seven months (Ammirati et al, 1987).

B. Therapy of glioblastomas

Up to now the selection of an appropriate therapy for glioblastoma involves surgery, radiation therapy and chemotherapy. Despite a multimodality approach with these therapies, the prognosis remains poor.

The first significant improvement in therapy was achieved thanks to the introduction of radiation (Walker et al, 1980). Radiation improves the median survival time from 14 weeks in patients treated with surgery alone to 40 weeks in those treated with surgery and radiation. Subsequently, chemotherapy has been used during and after radiation therapy. The addition of chemotherapy to surgery and radiation was evaluated in a recent meta-analysis (Fine et al, 1993). Adjuvant chemotherapy did improve survival time compared with surgery and radiation without chemotherapy. However, the survival benefit occurred in patients with earlier-diagnosed and lower grade tumors. In addition, the combinations of chemotherapeutic agents have been studied. Cisplatin showed synergy with nitrosourea in preclinical in vitro models (Durand, 1990). In clinical studies, cisplatin showed activity in high grade gliomas with response rates ranging from 13 to 28% (Spence et al, 1992). Moreover the administration of BCNU and cisplatin with accelerated hyperfractionated radiation therapy is expected to offer higher therapeutic efficacy against high-graded gliomas (Rajkumar et al, 1999).

On the other hand, the regional therapy using the affinity to transferrin (Tf) receptors has been developed (Laske et al, 1997). The targeted protein toxin was transferrin-CRM107 (Tf-CRM107) containing diphtheria toxin (CRM107). Tf-CRM107 potently and specifically kills cells expressing Tf receptors including glioblastoma. At least a 50% reduction in tumor volume occurred in 9 of 15 patients with malignant brain tumors.

III. Gene therapy for brain tumors

A. "Suicide gene" therapy (see chapter IV)

Several systems have been developed for gene therapy of brain tumor (Zlokovic and Apuzzo, 1997). The most feasible and well established approach is the suicide gene therapy, which uses a drug susceptible gene for selective destruction of the tumor cells.

B. Oncogene related therapy (antisense or replacement of genes)

Progression of different brain tumors to more malignant phenotypes involves numerous molecular genetic alterations. The genes affected by these alterations are considered to be those responsible for cell cycling, apoptosis, signal transduction and angiogenesis. Especially used genes could be classified into oncogenes and tumor suppressor genes. Another group contains genes related to DNA replication and repair. Complementary DNA (cDNA) of these genes is being introduced into tumor cells in sense or antisense orientation with the purpose of leading to genetic recovery. Tumor suppressor genes, such as p53, Rb, p21, are expected to have a great effectiveness against gliomas. However, several issues of gene delivery, selectivity, efficacy and even selection of target genes are still unresolved.

C. Other approaches

A number of additional approaches of gene therapy, include anti-angiogenesis, immunotherapy, and toxic gene therapy.

IV. Prodrug "suicide gene" therapy

"Suicide" genes have been introduced into cancer cells to allow their elimination. This approach is also called the enzyme/prodrug system because the suicide gene encodes an enzyme that modifies a nontoxic prodrug into a toxic molecule in the cell. Only the cells bearing the suicide gene will be killed upon the subsequent prodrug treatment.

The strategy of prodrug gene therapy could be summarized as follows: (i) the introduction and selective expression of a gene encoding drug-metabolizing enzyme in target cells; (ii) the prodrug is administered systemically; (iii) the non-toxic prodrug is converted to its active and toxic form in transduced target cells; (iv) the toxic drug damages and finally kills only target cells. The requirements for an ideal prodrug-enzyme system are a combination of a harmless prodrug and a toxic active drug, both of which should be compounds that have been well studied in humans. In addition, the drug-metabolizing enzyme should be of non-mammalian origin, or only expressed in very small quantities in normal human cells. The suicide gene therapy is supposed to have a number of
benefits for cancer therapy including: (i) selective sensitivity to a toxic drug; (ii) short-term gene expression; (iii) induction of a "bystander effect"; and (iv) stimulation of an immune response.

A number of approaches for prodrug-enzyme system have been established to date. The most widely used enzyme/prodrug system is the HSV-TK/GCV, which is currently being tested in 40 clinical trials, mostly for the treatment of cancers (Human Gene Marker/Therapy Clinical Protocols, NIH 1998). There are many other prodrug-enzyme systems with different mechanisms of actions, including, for example, cytosine deaminase/5-fluorocytosine and varicella zoster virus thymidine kinase/6-methoxypurine arabinoside (Rigg and Sikora, 1997).

V. Principle of the HSV-TK/GCV system

A. Vector producer cell (VPC)

The structural genes (gag, pol and env) of retrovirus used to create the vector producer cell (VPC) are derived from Moloney murine leukemia retrovirus (MoMLV). The MoMLV retrovirus is frequently used for gene therapy because its biology is relatively well understood, the vector systems can produce high titers and this virus can infect human cells efficiently. The HSV-TK gene is introduced on the vector plasmid and becomes stably integrated into the packaging cell resulting in a VPC (Figure 1). The VPC has all the components to transduce dividing cells with replication-incompetent virions containing the HSV-TK gene. It should be noted that once the recombinant vector with the HSV-TK gene transduces tumor cells, it cannot replicate because it no longer has access to replication proteins (gag, pol and env) (Ramesh et al, 1998).

![Diagram of HSV-TK/GCV gene therapy](image)

**Figure 1.** The principle of HSV-TK/GCV gene therapy. 1) The VPCs are injected into the tumor-resected cavity and inoculated in the brain parenchyma, where residual tumor cells are expected to be found. 2) The VPCs produce retroviral vectors containing HSV-TK gene. 3) The retroviral vectors infect only tumor cells specifically in the proliferative phase. 4) The HSV-TK gene is integrated into the genome DNA of tumor cells. 5) The anti-virus drug, GCV, is administered intravenously. 6) GCV delivered to HSV-TK positive cells is phosphorylated into GCV triphosphate. 7) The toxic GCV triphosphate kills only HSV-TK positive tumor cells.
B. Inoculation

The VPCs are injected directly into the tumor itself or the brain walls around the excised tumor. The proliferating tumor cells are transduced by vectors containing the HSV-TK gene. Non-proliferating cells, such as neuron, are resistant to transduction. Once the HSV-TK gene is integrated in the genomic DNA of tumor cells, the enzyme thymidine kinase is expressed constitutively. GCV is phosphorylated by the viral thymidine kinase into GCV monophosphate and further phosphorylated to GCV triphosphate by the mammalian thymidine kinase. GCV triphosphate inhibits DNA replication by hindering the activity of DNA polymerase and results in tumor cell death. The viral thymidine kinase is almost 1000-fold more efficient at monophosphorylating GCV compared with the mammalian thymidine kinase (Elion et al, 1977). Bone marrow is the most sensitive to GCV and granulocytopenia and thrombocytopenia are common dose-limiting toxicities of this agent. However, GCV is nontoxic to most non-transduced cells at therapeutic concentrations of the pro-drug (Crumpacker, 1996).

C. "Bystander effect"

In this therapy, indirect antitumor effects occur and non-transduced tumor cells are killed. This phenomenon is termed the "bystander effect" (Freeman et al, 1993). In fact, the rates of tumor cell destruction with HSV-TK/GCV gene therapy exceed those expected with the transduction rates (Samejima et al, 1995). In vitro studies show that HSV-TK-negative tumor cells can be killed when as few as 10% of the population of cultured cells are HSV-TK transduced cells (Freeman et al, 1993). There are several potential mechanisms explaining the bystander effect: (i) The transfer of toxic GCV metabolites from HSV-TK-transduced tumor cells to nearby unmodified cells (Bi et al, 1993). (ii) Release of soluble factors from GCV-exposed HSV-TK-transduced tumor cells and subsequent response by the immune system (Freeman et al, 1993). (iii) Hemorrhagic or ischemic tumor necrosis due to the transfer of the HSV-TK gene to endothelial cells lining the tumor blood vessels (Ram et al, 1993).

VI. Preclinical studies for brain tumors

A. Preclinical trials

Preclinical studies (Table 1) have demonstrated that HSV-TK gene transduction induces sensitivity to GCV and results in killing of tumor cells. In the brain, specific gene transduction into tumor cells is possible because tumor cells usually divide actively. In contrast, brain cells do not usually divide and are resistant to transformation by retroviral vectors (Salmons and Gunzburg, 1993). The other cells in the brain are at minimal risk for transduction; these include glia, endothelial cells, microglia and blood derived cells (leukocytes). Moreover, the lack of a strong immunological response in the brain allows for sufficient survival of xenogeneic VPCs and subsequent transduction of the tumor cells.

Moolten (1986) first reported that HSV-TK gene-induced GCV sensitivity could be demonstrated in vivo using mouse fibroblast 10E2. The TK-positive and negative cells were tested to determine the response exposed to GCV in vivo. The TK-positive tumors treated with GCV demonstrated complete tumor regression. In contrast, the TK-negative tumors after GCV treatment exhibited tumor progression and all mice died.

Table 1 Preclinical trials of HSV-TK/GCV gene therapy in glioma cells; antitumor response (glioma cells containing the TK gene)

<table>
<thead>
<tr>
<th>Author</th>
<th>Model</th>
<th>Glioma Cell</th>
<th>Response</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ezzeddine</td>
<td>in vivo</td>
<td>mouse/C6(TK+)</td>
<td>growth inhibition</td>
</tr>
<tr>
<td>Barba</td>
<td>in vivo</td>
<td>rat/9L(TK+)</td>
<td>100% survival at 90 days</td>
</tr>
<tr>
<td>Kato</td>
<td>in vivo</td>
<td>T98, U251(TK+)</td>
<td>cytotoxicity to GCV (1000-fold)</td>
</tr>
<tr>
<td>Kim</td>
<td>in vivo</td>
<td>U251(TK+)</td>
<td>induction of sensitivity to radiation (1.9-fold)</td>
</tr>
<tr>
<td>LeMay</td>
<td>in vivo</td>
<td>rat/C6(TK+)</td>
<td>induction of GCV permeability by RMP-7 (1.7-2.6-fold)</td>
</tr>
<tr>
<td>Moriuchi</td>
<td>in vivo</td>
<td>U87MG(TK/TNF+)</td>
<td>TNF enhances cytotoxicity</td>
</tr>
<tr>
<td>Vandier</td>
<td>in vivo</td>
<td>U251, C6(TK+)</td>
<td>GFAP promoter enhances sensitivity to GCV (2-fold)</td>
</tr>
</tbody>
</table>
In regard to treatment of glioma, TK-positive glioma cells were sensitive to GCV and tumor growth was reduced in both mice and rats (Ezzeddine et al, 1991; Barba et al, 1993). In vitro studies showed that cytoxicity to GCV was enhanced by 100- to 1000-fold by introducing TK gene (Kato et al, 1994). In addition, TK gene introduction retained sensitivity to radiation (Kim et al, 1994). Recently, induction of sensitivity to GCV has been developed using advanced molecular techniques. Expression of TK gene driven by GFAP (glial fibrillary acidic protein) promoter enhanced sensitivity to GCV by 2-fold (Vandier et al, 1998). The tandem expression of both TK and TNF\(\alpha\) (tumor necrosis factor alpha) genes also induced cytoxicity to GCV by 80% (Moriuchi et al, 1998). On the other hand, a bradykinin analog (RMP-7) induces permeability of GCV into brain parenchyma through the blood-brain barrier (LeMay et al, 1998). Taken together, TK gene transduction into glioma cells induces sensitivity to GCV, which leads a feasibility of the HSV-TK/GCV system for the treatment of glioma.

B. Biological gene transfer (see Table 2)

1. Retrovirus-mediated transfer

Moolten and Wells (1990) demonstrated that retroviral vectors produced by VPCs could successfully transmit the HSV-TK gene into tumor cells and induce tumor regression in response to GCV. Short et al (1990) compared the efficiency of gene transfer using two methods; the procedure of in vivo gene transfer via VPC was much more efficient than that of the direct vector injection. Culver et al (1992) observed complete tumor regression in 11 of 14 rats treated with the same procedure. In addition, the median survival was prolonged 1.6 times for treated rats compared to untreated ones. Ram et al (1993a, 1994) showed the dose dependency of the number of VPCs. Rats transplanted with 9L gliosarcoma cells were inoculated with HSV-TK positive VPCs followed by administration of GCV. Five of 13 (38%), 13 of 18 (72%) and 10 of 12 (83%) rats treated with 1.8x10\(^6\), 3x10\(^6\) and 5x10\(^6\) VPCs experienced complete tumor regression, respectively (Ram et al, 1993a). In addition, the number of tumor vasculature was also reduced by in vivo TK gene transfer and GCV treatment (Ram et al, 1994).

2. Adenovirus-mediated transfer

Some study groups used adenovirus instead of retrovirus. The advantage of using adenovirus is the high efficacy of infection rate into the target cells, approaching nearly 100%. However, the major problem of adenovirus is the induction of a strong immune response against the virus itself as well as the transduced cells expressing viral proteins. In both rats and mice, tumor regression and elongation of survival time were observed (Chen et al, 1994; Perez-Cruet et al, 1994; Maron et al, 1996). Vincent et al (1996) compared the antitumor response between retrovirus and adenovirus. Adenoviral gene transfer showed longer survival rates (39 days vs 26 days). According to Human Gene Marker/Therapy Clinical Protocols of NIH 1998, two protocols of adenovirus-mediated HSV-TK/GCV therapy for brain tumor were approved for clinical trials to date (Human Gene Marker/Therapy Clinical Protocols 98).

<table>
<thead>
<tr>
<th>Author</th>
<th>Model</th>
<th>Adeno-vector</th>
<th>Response</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chen (94)</td>
<td>mouse/C6</td>
<td>ADV/RSV-TK</td>
<td>volume reduction (1/500)</td>
</tr>
<tr>
<td>Perez-Cruet (94)</td>
<td>rat/9L</td>
<td>ADV-</td>
<td>survival prolonged (22-&gt;80~120d)</td>
</tr>
<tr>
<td>Maron (96)</td>
<td>rat/9L</td>
<td>Ad.RSVTK</td>
<td>reduction of tumor volume (97%)</td>
</tr>
<tr>
<td>Vincent (96)</td>
<td>rat/9L</td>
<td>IG.Ad.MLP.TK</td>
<td>survival prolonged (16d-&gt;18d)</td>
</tr>
</tbody>
</table>

Table 2 Preclinical trials of HSV-TK/GCV gene therapy in glioma cells; antitumor response (in vivo TK gene transfer)

<table>
<thead>
<tr>
<th>Author</th>
<th>Model</th>
<th>VPCs</th>
<th>Response</th>
</tr>
</thead>
<tbody>
<tr>
<td>Short (90)</td>
<td>rat/C6</td>
<td>psi2-BAG/-</td>
<td>in vivo VPC transfer&gt;vector injection</td>
</tr>
<tr>
<td>Culver (92)</td>
<td>mouse/9L</td>
<td>PA317/G1NsCTK</td>
<td>reduction of tumor growth (5w; 80%)</td>
</tr>
<tr>
<td>Ram (93)</td>
<td>rat/9L</td>
<td>PA317/G1TkSvNa.90</td>
<td>reduction of tumor growth (28d; 83%)</td>
</tr>
<tr>
<td>Barba (94)</td>
<td>rat/9L</td>
<td>(fibroblast/HSV-TK)</td>
<td>long survival (90d; 22%)</td>
</tr>
<tr>
<td>Ram (94)</td>
<td>rat/9L</td>
<td>PAT24/G1TkSvNa.53</td>
<td>reduction of tumor vasculature (14d; 80%)</td>
</tr>
<tr>
<td>Vincent (96)</td>
<td>rat/9L</td>
<td>PA317/IGRVTK</td>
<td>survival prolonged (15d-&gt;26d)</td>
</tr>
</tbody>
</table>
C. “Bystander effect” in glioma (see Table 3)

Ram et al. (1993a) described that following successful transduction of 10% to 70% of glioma cells, complete tumor ablation occurred in most rats. These data suggested that some indirect effects might induce tumor cell death, termed "bystander effect". The bystander effect was observed in other types of cell lines (colon cancer, fibrosarcoma and so on) treated with HSV-TK/GCV system (Freeman et al., 1993; Bi et al., 1993). Bystander effect also contributes to size reduction of tumor and elongation of survival (Namba et al., 1998). The bystander mechanism is still unknown. The cell-to-cell contact is essential for bystander effect and any soluble factors don’t influence to this mechanism (Samejima and Meruelo, 1995). In addition, bystander effect is mediated with gap junction, especially implied with connexin 43 (Mensil et al., 1996). On the other hand, GCV triphosphate accumulates in neighboring cells, which is supposed to lead bystander killing (Rubsm et al., 1999).

Lyons et al. (1995) demonstrated bystander effect observed in C57BL/6 mice model injected with a mixture of tumor cells and VPCs followed by GCV treatment. In mice received a 10:1 ratio of tumor cells to VPCs, 29% of tumor cells was ablated, even though only 18% of tumor cells were transduced with HSV-TK gene. In addition, retrovirus-mediated gene transfer followed by GCV treatment induced apoptosis in neighboring cells (Colombo et al., 1995; Hamel et al., 1996).

D. Optimal dose of GCV

Ram et al. (1993b) also analyzed the optimal dose of GCV because previous studies used high doses as 300 mg/kg per day intraperitoneally. Such doses would be toxic and not be relevant to human. Three doses of GCV that were employed in this study (10, 20 and 30 mg/kg per day) resulted in comparable tumor regression as assessed by tumor weight. In consequence, the relevant human GCV dose is expected 10 mg/kg per day intravenously.

GCV causes granulocytopenia, thrombocytopenia, azoospermia, and a rise in serum creatinine (Crumpacker, 1996). Among the patients with cytomegalovirus retinitis treated with intravenous GCV (5mg/kg twice a day for three months), 40% developed granulocytopenia (neutrophil count <1000/ml) and 15% developed thrombocytopenia (platelet count<50,000/ml). These symptoms disappeared after GCV administration was discontinued. It was suggested that in case of GCV administration for two weeks in clinical trials, these toxicity symptoms might become less severe.

E. Cytotoxicity of VPC in vivo

Ram et al. (1993b) estimated the safety of the injection of VPCs containing HSV-TK gene or lacZ gene. They concluded that this system is not associated with significant toxicity to the brain and remote organs in mice, rats or monkeys. It should be noted that even in case all of the injected VPCs were able to cross the blood-brain barrier following intracerebral administration, there would be fewer vector particles in relation to the large number of cell receptors in the body and thus the risk to tissues beyond the CNS would be minimal. In regard to the random integration of the provirus into the host genome, it is unlikely that normal cells would undergo insertional mutagenesis. Furthermore, cells containing the HSV-TK gene would be destroyed following GCV administration. In fact, no such mutagenesis was observed.

<table>
<thead>
<tr>
<th>Table 3. Preclinical trials of the HSV-TK gene therapy in glioma cells; &quot;bystander effect&quot;</th>
</tr>
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<tbody>
<tr>
<td>glioma cell containing TK gene</td>
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<table>
<thead>
<tr>
<th>Author</th>
<th>Cell Line</th>
<th>Bystander Mechanism</th>
</tr>
</thead>
<tbody>
<tr>
<td>Samejima (95)</td>
<td>U118MG(TK+) C6(TK+)</td>
<td>• cell-to-cell contact • not mediated with soluble factors</td>
</tr>
<tr>
<td>Namba (98)</td>
<td>rat/9L(TK+)</td>
<td>• (survival elongation) • (size reduction)-</td>
</tr>
<tr>
<td>Rubsm (99)</td>
<td>U251(TK+)</td>
<td>• GCV triphosphate accumulation</td>
</tr>
</tbody>
</table>

| in vivo TK gene transfer |

<table>
<thead>
<tr>
<th>Author</th>
<th>Cell Line</th>
<th>VPCs</th>
<th>Bystander Mechanism</th>
</tr>
</thead>
<tbody>
<tr>
<td>Colombo (95)</td>
<td>U87</td>
<td>PA317/G1NsCVTK</td>
<td>• apoptosis (nuclear segmentation)</td>
</tr>
<tr>
<td>Hamel (96)</td>
<td>9L</td>
<td>PA317/HSVTK</td>
<td>• apoptosis (inhibited by BCL2)</td>
</tr>
</tbody>
</table>
1. Studies in mice using intravenous administration of VPCs

Intravenous administration of VPCs in mice was used to determine whether VPCs could be trapped in lungs and cause transduction of the respiratory epithelium and diffuse destruction of lung parenchyma after GCV treatment (Ram et al, 1993b). Histological examination of lung, spleen, thymus, liver, intestine and bone marrow did not reveal toxicity nor necrosis and inflammation. Some of the treated mice were followed for more than seven months and no toxicity was observed.

2. Studies in rats using direct brain injection of VPCs

The HSV-TK VPCs were injected into the deep white matter of rat cerebrum, treated with GCV and sacrificed (Ram et al, 1993b). A mild and transient response including weakness, weight loss, somnolence and dehydration was evident during the initial 24 to 48 hours after VPC injection; however, this effect was reversible. A similar response was seen at the first day of GCV therapy. This response is expected to arise from an immune reaction to the xenogeneic cells. Histological examination of brain tissue showed moderate edema around the injection site. Moreover, administration of dexamethasone completely abolished this response in all treated rats.

3. Studies in monkeys using direct brain injection of VPCs

Five monkeys were used to investigate the impact of intracerebral injection of VPCs followed by GCV therapy (Ram et al, 1993b). The monkeys received a stereotactic intracerebral injection of 1x10^7 HSV-TK VPCs in the deep white matter of the right frontal lobe. GCV was administered to monkeys, and physical and neurological examinations were performed daily. In addition, magnetic resonance imaging, blood analysis and cerebrospinal fluid analyses were conducted. There was no evidence of CNS toxicity following neurological examinations, nor changes in motor or behavioral activity. A few endothelial cells at the injection site were transduced. At the injection site, mild reactive gliosis without edema or pathological changes in surrounding brain tissue was observed. Localized demyelination was limited to the injection site and did not increase in size in response to GCV administration. There was no evidence of VPC proliferation in the brain. The VPCs were observed in the brain at two weeks but not at three weeks, similar to the rat model. Analysis of CSF revealed normal levels of protein and glucose, and negative bacteriological cultures. At 270 days post-injection of VPCs, no detrimental effects or alterations of baseline neurological function were noted.

VII. Clinical trial for malignant brain tumor

Up to date 226 protocols of clinical trials using gene therapy have been approved by NIH. Eight out of 226 are protocols using the HSV-TK/GCV gene therapy for brain tumors (Table 4). The data of the first study (phase I; GLI-0100) were published in 1997 being the only one for malignant brain tumors (Ram et al, 1997).

A. Surgical procedure for stereotactic administration of VPC

Stereotactic administration permits direct treatment of the initial tumor after biopsy. However, this procedure is limited to small-size tumors (Ram et al, 1997). Ram described that the antitumor response was observed in small tumors (1.4±0.5 ml) but not in larger-size tumors (7.4±5.8 ml). To determine an efficacy for large-size tumors, the second clinical trial of injecting VPCs into the resected tumor (newly diagnosed) cavity is conducted (phase III).

B. Preparation of VPC suspension

The VPCs, suspended in Plasma Lyte-A or Ringer's Lactate Solution at a concentration of 1x10^8 cells/ml, are inoculated slowly at sites distributed as evenly as possible around the tumor or to the wall of the resection cavity. To avoid settling of cells, the cell suspension should be gently and frequently massaged prior to drawing up into the syringe for injection.

C. Study design of the first clinical trial (GBI-0100) (Ram et al, 1995, 1997)

The first clinical trial using HSV-TK/GCV system was designed to determine: (i) the safety of intratumoral delivery of VPCs into human brain tumors; and (ii) the short and long term efficacy. This study was a small study of 15 patients with malignant brain tumors; 12 had malignant glioma (9 with glioblastoma) and 3 had secondary metastatic brain tumors (2 with melanoma and 1 with breast cancer). Patients received intratumoral stereotactic injection of TK-positive VPCs (murine PA317/G1TkSvNa.53). Seven days after the inoculation, GCV was administered at 5 mg/kg twice a day for 14 days.

D. Antitumor response on clinical trial

Thirteen of 15 patients (87%) were included in this trial with 16 evaluable tumors (Ram et al, 1995, 1997). Five lesions (31%) in four patients (29%) had either a partial response (three lesions) or complete response (two lesions). The enhancing volume of these five small tumors
decreased from 1.4±0.5 ml before therapy to 0.4±0.5 ml after therapy. The five tumors were smaller at treatment than the nonresponsive tumors (1.4 ml vs 7.4 ml).

E. Clinical safety of the trial
Two patients had intratumoral hemorrhage and neurological deficits from the procedures used to take biopsies and implant VPCs (Ram et al, 1995, 1997). One of two had completely recovery of deficits. Another patient required removal of hemorrhage. Two more patients had an increase in the frequency of preexisting seizures immediately after VPC injection. One patient was suffering from seizures in the immediate period after VPC implantation. Anticonvulsant medications were able to control seizures in three patients.

F. Biosafety of clinical trials
Replication competent retrovirus (RCR) was not detected in any of the 79 blood samples from the 13 patients in this trial (Ram et al, 1995; 1997). In addition, the vector DNA was not detected in any of blood samples using PCR assay. Antibodies reacting with retrovirus core protein p30 were not detected in any of the same samples. Multiple serum samples from each of 15 patients were analyzed for the presence of antibodies to the injected murine VPC using flow cytometric analysis. Ten of 15 patients produced a readily detectable increase in VPC-binding antibody, which peaked at 66 days after VPC inoculation.

VIII. Conclusion
The HSV-TK/GCV gene therapy promises one of the advanced therapies for brain tumors as well as for a variety of other cancers. As with all types of gene therapy, this approach might develop as a safe and effective clinical modality.

One of the important points of a gene therapy approach is to select a target gene, which could become the most effective molecular tool against a tumor. Another point is to choose a gene delivery protocol, which could achieve specific and selective integration of a target gene into tumor cells. In our opinion, the best advantage would be to use a single target gene for gene therapy because of convenience of manipulation. In this point of view, the suicide gene therapy using HSV-TK gene is expected to be almost ideal. However, this approach has several points for improvement before it becomes a practical treatment modality for brain tumors. In the near future, many problems might be resolved establishing gene therapy as a strong treatment not only for brain tumors but for other malignancies alike.

XI. Surgical technique of injection
In contrast to the stereotactic injection, a multi-injection into the wide surface of tumor is needed for larger tumors. In general, the size of glioblastoma at diagnosis is usually about 30 ml. This multi-injection will be more convenient for the practical technique of VPC inoculation. Here we demonstrate a) surgical procedure for multi-injectional administration of VPC, b) characteristic MRI features of VPC implantation and c) injection technique and pitfalls, from our own experience of a phase II clinical trial (GLI-B201).

A. Surgical procedure for multi-injectional administration of VPC (Adachi, 1998) (Figure 2)

1. Preparation: This procedure is the same as that of the stereotactic injection (see chapter VII-B).

2. Marking: It is required to create a grid with clips to mark 1-cm apart line on the tumor cavity. This grid would be helpful for surgeons to inject at 1-cm intervals. In our experiences, it should be better to use Dubtamp® sheet, which is easy to see 1-cm² square at a glance, to place on the cavity surface and to save the operation time.

3. Sites: In general, the number of injection is needed 50 to 100-site in 30 cm³-volume tumor cavity. It is recommended 100-sites to increase an efficacy of infection to tumor cells. However, the number of sites depends on the tumor size, tumor location, operation time even more technique of an operator.

4. Volume: The volume per injection site is determined by dividing the total volume of cells by the number of injection sites. Actually, 0.1 ml of VPC suspension is usually injected into a site, which means 1x107 cells per one site. The depth of the injection should be approximately 1 to 2 cm to the resection margin without injury of eloquent region or entering the ventricle. It is recommended that the elastic needle should be marked every 1 cm.

5. Reflux: At the surgeon's discretion, a hemostatic sponge may be placed over the opening of the cavity to impede extravasation of VPCs into the subarachnoid space.

6. Infiltration: The histological examination showed the infiltration of VPCs along the needle tract. At four days after injection, fibroblast cells were seen 3 to 4 mm from the injection tract (Ram 97). Injection of VPC suspension at 1-cm intervals allows proximity to tumor cells for transduction.
Figure 2. Injection technique. The VPCs suspension is directly injected into the tumor-resected cavity. The tip of the needle is usually placed at the depth 1.5~2.0 cm from the cavity edge.

Figure 3. Typical MRI finding after VPC inoculation. Gd-enhanced MR image shows needle tracts, which radiate from the tumor-resected cavity. This finding is termed "Uni" sign ("Uni" means sea urchin in Japanese).
Figure 4. Pitfalls of VPC injection. **Left**: The unwilling injection into ventricle or basal ganglia. The great care should be taken for the depth of injection near to ventricle or basal ganglia. If the ventricle is opened, the VPCs suspension flows away in CSF space which results in low inoculation. **Middle**: The blind corner. The cortex side is difficult to be injected because of the blind space for surgeons. This can be resolved by bending the needle. **Right**: "Reflux". During the injection procedure, a significant quantity of the VPC suspension is lost through "reflux". The reflux phenomenon could be prevented by slowing and pumping injection while pulling out the syringe.

**B. Characteristic MRI features of VPC implantation**

A transient increase in the volume of the enhanced tumor is observed on MRI in most patients immediately after intratumoral VPC placement. Contrast enhancement of the needle tracts in the brain which the needles passed to inject the VPCs occurred and subsided 4-8 weeks after the injection. This finding is termed "tract sign" or "Uni" sigh (Uni=sea urchin in Japanese), which shows the radiation shape of several tracts from the resected cavity (Figure 3).

**C. Pitfalls of injection (Figure 4)**

In case of the multi-injection, one must pay an attention to following procedures; 1) injection into ventricle or basal ganglia, 2) injection into the blind corner and 3) reflux of the VPC suspension.

1. **Ventricle and basal ganglia**

Glioblastoma invades diffusely into the white matter, especially near the ventricle or basal ganglia. If retroviral particles could infiltrate into these regions, invaded tumor cells could be killed. The deeper injection is better for transfection, however, one must not penetrate ventricles to avoid diffusion of VPCs or not inject suspension into basal ganglia not to influence neurological deficits.

2. **Blind corner of cortex**

Glioma grows in the white matter and occupies subcortex regions. Neurosurgeons remove tumor in the white matter, which lead to make the sharp angle corner under the cortex. It is difficult that VPCs suspension is injected into this region using conventional method with the straight needle. We always use the angled needle for these blind corners.

3. "**Reflux"**

During the injection procedure, a significant quantity of the VPC suspension is lost through "reflux". In general, as much as 50% of the injected volume is refluxed out into the tumor cavity, however, it depends on technique of operators. The reflux phenomenon could be prevented by slowing and pumping injection while pulling out the syringe. This method actually takes at least 30 sec per one injection. In the near future, an injection technique should be improved and developed to less labor, less time and more homogeneous inoculation.

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