Intrathecal Gene Therapy for Malignant Leptomeningeal Neoplasia

Zvi Ram, Stuart Walbridge, Eric M. Oshiro, John J. Viola, Yawen Chiang, Stephan N. Mueller, R. Michael Blaese, and Edward H. Oldfield

ABSTRACT

In meningeal carcinomatosis, retroviral-vector-producer cells can be introduced into the thecal sac and circulate in the cerebrospinal fluid to reach malignant tumor cells in the leptomeninges, release vector particles, and selectively infect and transfer a gene of interest to these cells. Gene transfer experiments with the lacZ gene and in vitro retroviral titers showed that retroviral vectors can survive in the cerebrospinal fluid, retain their infectivity, and successfully transduce tumor cells. To examine the potential of intrathecal gene therapy, we evaluated the antitumor efficacy of in situ transduction with the herpes simplex-thymidine kinase gene followed by ganciclovir therapy in a murine model of leptomeningeal neoplasia. Fischer rats were inoculated via a subarachnoid catheter implanted at the upper thoracic level, and thymidine kinase vector-producer cells were injected into the subarachnoid space the day of tumor inoculation. Seven days later, rats received ganciclovir for 14 days by daily i.p. injections (30 mg/kg/ml) or intrathecal injections (25 mg/kg or 600 μg/kg) for 14 days. To evaluate possible enhancement of tumor eradication by the ability of helper virus to package the vector in the cells and further extend gene transfer, additional rats received thymidine kinase vector-producer cells that had been previously coinfected with a replication-competent retrovirus (4070A). In all groups, control rats received i.p. or intrathecal saline injections.

Ganciclovir administration i.p. resulted in significant prolongation of survival in rats given injections of thymidine kinase vector-producer cells. Injection of producer cells coinfected with the 4070A retrovirus did not improve antitumor efficacy. Intrathecal administration of ganciclovir (low and high doses) did not extend survival; histological examination of the spinal cords showed elimination of the infiltrative tumor in the leptomeninges, but residual tumor mass was present at the inoculation site, consistent with limited penetration of topical ganciclovir into the tumor.

These results support the potential application of gene therapy using the thymidine kinase/ganciclovir approach for treatment of meningeal carcinomatosis.

INTRODUCTION

Meningeal carcinomatosis, which occurs in 5–20% of all cancer patients, results most commonly from metastatic spread of solid cancers, such as lung, breast, and melanoma, to the leptomeningeal coverings of the brain and spinal cord (1–5). Standard therapy for meningeal carcinomatosis is comprised of radiation therapy and intrathecal administration of chemotherapy. The need to irradiate the entire neuroaxis may cause profound bone marrow suppression and limit the tolerable dose of systemic or intrathecal chemotherapy (1).

We, and others, have recently described a novel approach to treat experimental solid brain tumors by in vivo retroviral-mediated transfer of HStk into tumor cells, which confers sensitivity to the antiviral drug ganciclovir (6–10). Ganciclovir is preferentially phosphorylated by transduced tumor cells and interferes with DNA synthesis. Gene transfer is achieved by infection of tumor cells with murine retroviral vectors carrying the HStk and integration of this gene into the genome of the host cell. These vectors are continuously produced by murine vector-producer cells that are injected into the tumor mass. Since retroviruses can only infect cells that are actively synthesizing DNA (replicating cells), a preferential transduction of tumor cells is achieved. This approach is now being evaluated in a clinical trial (11). However, whether distribution of the vector in solid tissue will be sufficient to affect tumor in infiltrated brains remains to be established.

Meningeal carcinomatosis may provide an ideal setting for the application of this approach. If vector-producer cells, after injection into the subarachnoid space, circulate in the CSF, continuously releasing retroviral vector particles, these should contact tumor-infiltrated leptomeninges, transduce the replicating tumor cells, and enable tumor eradication with systemic administration of ganciclovir.

We evaluated the feasibility of applying this approach for the treatment of experimental malignant leptomeningeal neoplasia in rats.

MATERIALS AND METHODS

Vectors and Cell Cultures. The HStk (G1TkSvNa.53 and G1TkSvNa.7) vectors (Genetic Therapy, Inc., Gaithersburg, MD) have a G418 backbone derived from the Moloney murine leukemia virus. Each vector contains HStk just downstream of the 5' long terminal repeat and uses this long terminal repeat as its promoter. The Simian virus-40 early promoter serves as an internal promoter for the neomycin phosphotransferase gene, NeoR, which confers resistance to the neomycin analogue G418. The vector is packaged by the amphotropic retroviral-vector-producer cell line PA317, which is derived from NIH3T3 cells and has a titer of 1.0 x 10⁶ to 1.0 x 10⁷ colony-forming units/ml on NIH3T3 cells. The β-galactosidase vector (G1BgsNa.29), the lacZ gene replaces HStk. Its producer cell line has a titer of 0.5 x 10⁶ colony-forming units/ml on NIH3T3 cells. The cell lines were negative for replication-competent virus by 5'-assay.

The cloned vector-producer cell lines were maintained in culture in Dulbecco modified Eagle's medium with 10% fetal bovine serum (HyClone Laboratories, Inc., Logan, UT), 2 mM L-glutamine (GIBCO BRL, Gaithersburg, MD), 50 units/ml penicillin (GIBCO BRL, Gaithersburg, MD), 50 μg/ml streptomycin (GIBCO), and 2.5 μg/ml Fungizone (ICN Biomedicals, Inc., Costa Mesa, CA). The vector-producer cells were grown in T-175 flasks. Cells were harvested prior to intrathecal injection by incubation in 0.05% trypsin-EDTA (GIBCO) for 5–10 min at 37°C. The cells were collected in Hanks' balanced salt solution (Biofluids, Inc., Rockville, MD), washed twice, and resuspended at 8 x 10⁶ cells/ml for injection.

For infection of the HStk replication-incompetent vector-producer cells with the replication-competent retrovirus, 4070A, virus-containing supernatant was filtered through a 0.22-μm filter onto a monolayer of HStk producer cells. Two passages of the culture were allowed to achieve uniform infection of the producer cells with the 4070A retrovirus. The titer of the G1TkSvNa.53 HStk vector-producer cells was unaffected by coinfection with the 4070A retrovirus. The titer of the replication-competent virus was not determined.
5% CO₂, for 24 h or until nearly confluent). Duplicate wells were exposed to growth medium containing 8 µg/ml polybrene and then incubated for 60 mm vector, supernatant samples of known titer from producer cells for the supernatants used as positive control (undiluted with CSF) had a neomycin-resistant gene titer between 1 X 10⁸ and 4 X 10¹⁰ particles/ml (PA317/G1TkSvNa.53). The dishes were incubated at 32°C and 5% CO₂. The supernatants were transduced with the neomycin-resistant gene and thus protected from the toxic effect of G418) were visible microscopically. The cells were then stained with methylene blue and the colonies were counted. The titer per ml at each dilution was determined by calculating the average number of colonies in the wells, multiplying by the dilution factor, and dividing by 3 ml. The average dilution was determined by calculating the average number of colonies in the wells, multiplying by the dilution factor, and dividing by 3 ml. The average dilution was determined by multiplying the average of the titers for all dilutions. A sterile PE-10 tube was inserted into the upper thoracic subarachnoid space via the cisterna magna, secured in the s.c. soft tissue, and pierced through the skin with a steel rod. The rats were then observed for 5—7 days, during which any rat that developed neurological deficits was excluded from the study. Rats were reanesthetized, using inhalation anesthesia (N₂O:O₂:halothane mixture), and 8 X 10⁶ HStk (G1TkSvNa.53) vector-producer cells (n = 100), 8 X 10⁶ HStk vector-producer cells (n = 100), 8 X 10⁶ HStk vector-producer cells coinfected with the 4070A replication-competent retrovirus (n = 28), 8 X 10⁶ HStk (G1TkSvNa.7 clone) vector-producer cells (n = 25), or 8 X 10⁶ β-galactosidase producer cells (n = 6) were then injected in a similar way, and the catheter was flushed with an additional 10 µl of PBS and sealed with a steel rod. Rats given injections of the β-galactosidase vector were sacrificed on days 3, 6, and 10 after cell injection. The brain and spinal cord were removed, and histological sections were stained with the X-Gal histochemical technique to identify cells expressing β-galactosidase (7). Rats that had received intrathecal injection of HStk vector-producer cells (both clones), or HStk vector-producer cells coinfected with the 4070A virus, began treatment with GCV 7 days later. Ganciclovir (active drug, Syntex; Syntex, Palo Alto, CA) was administered daily by i.p. injections (30 mg/kg; 1 ml PBS) or intrathecal injections (25 µg/kg or 600 µg/kg; 10 µl PBS) for 14 days. The low intrathecal dose was chosen to achieve a CSF concentration of 5-10 µg/ml, which had been shown to be the effective antitumor concentration in in vitro studies (7, 8). The higher concentration was chosen to deliver a great excess of the drug into the subarachnoid space. The details of the different study groups are summarized in Table 1. An additional 4 rats in each treatment group (2 treated with GCV, 2 treated with saline; total, 16 rats) were sacrificed after 7 days of treatment and the spinal cords were examined histologically. These rats were not included in the survival statistics.

Rats were observed daily for development of neurological deficits, which were almost invariably manifest as rapidly progressing paraparesis and paraplegia leading to death within 12 to 24 h. Animals that became moribund and were unable to access food and water were sacrificed in accordance with the NIH guidelines for euthanasia of experimental animals.

**Statistical Analysis.** The Mantel-Haenszel test (13) was used to compare survival between GCV-treated and saline-treated rats in the survival experiments. Since independent experiments were performed, statistical analyses were performed only within the same experimental group (Table 1). The study was approved by the NIH Animal Care and Use Committee and was performed according to the NIH guidelines for the care of laboratory animals.

**RESULTS**

**In Vitro Assay of Retroviral Titer after Exposure to Human CSF**

No CPE were seen in the murine vector-producer cells at 10 and or 1, 4, 24, and 48 h after exposure to human CSF. HStk vector production by the producer cells (both clones) was not affected by exposure to human CSF (Table 2).

Similarly, free HStk vector titer (from supernatants of known titer) was not affected by CSF during exposure for 60 min prior to overnight incubation over NIH3T3 cells. The results are listed in Table 3.

**Transduction Studies with the β-Galactosidase Gene**

Qualitative assessment of spinal cord sections from rats that had been inoculated with intrathecal 9L tumor and injected with β-galactosidase vector-producer cells revealed gene expression in the majority of tumor cells at the 3 time points at which animals were sacrificed (3, 6, and 10 days after producer cell injection). The highest expression (or accumulation) of the enzyme was detected in the tumor mass

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**Table 1 Experimental groups, treatment parameters, and statistical significance of Mantel-Haenszel statistics in evaluation of antitumor efficacy of intrathecal injection of HStk producer cells (G1TkSvNa.53 and G1TkSvNa.7) and ganciclovir therapy**

<table>
<thead>
<tr>
<th>Experimental group</th>
<th>Vector-producer cells used</th>
<th>No. of saline-treated rats</th>
<th>No. of GCV-treated rats</th>
<th>GCV dose/day; mode of administration</th>
<th>p value (M-H)</th>
<th>Mean (median) days of survival, saline</th>
<th>Mean (median) days of survival, GCV</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>G1TkSvNa.53</td>
<td>10</td>
<td>13</td>
<td>30 µg/kg; i.p.</td>
<td>0.000</td>
<td>14.3 (14)</td>
<td>21.8 (21)</td>
</tr>
<tr>
<td>2</td>
<td>G1TkSvNa.53</td>
<td>9</td>
<td>11</td>
<td>25 µg/kg; intrathecal</td>
<td>0.434</td>
<td>19.1 (21)</td>
<td>20.2 (20)</td>
</tr>
<tr>
<td>3</td>
<td>G1TkSvNa.53</td>
<td>10</td>
<td>13</td>
<td>600 µg/kg; intrathecal</td>
<td>0.608</td>
<td>18.7 (19)</td>
<td>19.6 (19)</td>
</tr>
<tr>
<td>4</td>
<td>G1TkSvNa.53</td>
<td>12</td>
<td>12</td>
<td>30 µg/kg; i.p.</td>
<td>0.153</td>
<td>15 (14)</td>
<td>19 (19)</td>
</tr>
<tr>
<td>5</td>
<td>G1TkSvNa.7</td>
<td>9</td>
<td>13</td>
<td>30 µg/kg; i.p.</td>
<td>0.017</td>
<td>15.8 (16)</td>
<td>17.9 (18)</td>
</tr>
<tr>
<td>6</td>
<td>G1TkSvNa.7</td>
<td>10</td>
<td>15</td>
<td>30 µg/kg; i.p.</td>
<td>0.003</td>
<td>15.6 (16)</td>
<td>19.5 (20)</td>
</tr>
</tbody>
</table>


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**Table 2 Retroviral vector formation by 2 clones of HStk vector producer cells (PA317/G1TkSvNa.53 and PA317/G1TkSvNa.7) after exposure to increasing concentrations of human CSF**

<table>
<thead>
<tr>
<th>Cell line</th>
<th>% CSF</th>
<th>G418-resistant titer (x 10⁶)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PA317/G1TkSvNa.53</td>
<td>0</td>
<td>1.1</td>
</tr>
<tr>
<td>5</td>
<td>1.0</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>0.9</td>
<td></td>
</tr>
<tr>
<td>25</td>
<td>1.9</td>
<td></td>
</tr>
<tr>
<td>50</td>
<td>1.9</td>
<td></td>
</tr>
<tr>
<td>75</td>
<td>1.8</td>
<td></td>
</tr>
<tr>
<td>PA317/G1TkSvNa.7</td>
<td>0</td>
<td>46</td>
</tr>
<tr>
<td>5</td>
<td>22</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>28</td>
<td></td>
</tr>
<tr>
<td>25</td>
<td>20</td>
<td></td>
</tr>
<tr>
<td>50</td>
<td>31</td>
<td></td>
</tr>
<tr>
<td>75</td>
<td>34</td>
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</tbody>
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near the tip of the implanted catheter, where most of the histologically distinguished producer cells could also be seen. Maximal staining of transduced tumor cells occurred on day 6 after cell injection (Fig. 1a) and was unchanged in specimens obtained 10 days after tumor and producer cell injection. No transduction of nontumoral cells in the covering or substance of the spinal cord or brain occurred. In contrast to tumors injected with β-galactosidase vector-producer cells, tumors that had been injected with HStk vector-producer cells were completely negative for β-galactosidase expression (Fig. 1b).

Survival Studies

Intrathecal HStk Vector-Producer Cells (Clone G1TkSvNa.53) and i.p. Ganciclovir. Survival was significantly extended when i.p.

Table 3 Titers of free retroviral vectors (PA317/G1TkSvNa.53 and PA317/G1TkSvNa.7) after coincubation with human CSF

<table>
<thead>
<tr>
<th>Supernatant</th>
<th>Dilution</th>
<th>Final (calculated) titer (× 10^5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>G1TkSvNa.53</td>
<td>Undiluted</td>
<td>4.0</td>
</tr>
<tr>
<td></td>
<td>X2 with CSF</td>
<td>6.0</td>
</tr>
<tr>
<td></td>
<td>X2 with medium</td>
<td>3.6</td>
</tr>
<tr>
<td>T1Tk1SvNa.7</td>
<td>Undiluted</td>
<td>90</td>
</tr>
<tr>
<td></td>
<td>X2 with CSF</td>
<td>140</td>
</tr>
<tr>
<td></td>
<td>X2 with medium</td>
<td>140</td>
</tr>
<tr>
<td></td>
<td>X10 with CSF</td>
<td>90</td>
</tr>
<tr>
<td></td>
<td>X10 with medium</td>
<td>120</td>
</tr>
</tbody>
</table>

Fig. 1. a, leptomeningeal tumor mass surrounding the cavity of the implanted catheter 6 days after injection of β-galactosidase vector-producer cells showing the producer cells (arrow) and diffuse cytoplasmic β-galactosidase expression in the majority of the 9L tumor cells; b, leptomeningeal tumor cells after injection of HStk vector-producer cells show no cytoplasmic β-galactosidase activity (X-Gal stain, hematoxyline counterstain; × 400).

GCV was administered compared to the saline-treated rats (22 ± 3 days versus 14 ± 2 days; mean ± SD, P < 0.0001) (see Fig. 3a). Spinal cord specimens taken from GCV-treated rats after 7 days of therapy showed no macroscopic tumor in the subarachnoid space, while saline-treated rats had large tumor deposits (Fig. 2a and b).

Microscopic tumor remnants or incomplete tumor eradication was
probably responsible for the subsequent relapse and death of the GCV-treated rats. In some rats, hemorrhagic necrosis of the tumor was also observed.

Intrathecal HS\textit{k} Vector-Producer Cells Coinfected with 4070A Retrovirus. In this group, we evaluated whether coinfection of HS\textit{k} vector-producer cells with a replication-competent retrovirus would enhance antitumor effect. Survival was extended in the GCV-treated rats compared to saline-treated rats, but did not reach a statistically significant level (19 ± 4 versus 15 ± 1; mean ± SD, \( P = 0.153 \)) (Fig. 3b). The histological appearance of the spinal cord was similar in both groups.

Intrathecal HS\textit{k} Vector-Producer Cells and Intrathecal Ganciclovir. Intrathecal administration of GCV did not extend survival compared to intrathecal saline administration at 25 \( \mu \)g/kg (\( P = 0.4 \)) or 600 \( \mu \)g/kg (\( P = 0.6 \)) (Fig. 3c and d). Histological examination of the spinal cords demonstrated that the thin infiltrating layers of tumor, which invaded the leptomeninges in the saline-treated rats, were completely eradicated in the GCV-treated animals. However, the larger tumor mass around the tip of the implanted catheter was not affected by intrathecal GCV (Fig. 2c) and subsequently produced paraplegia and death. These observations indicate that intrathecal GCV can penetrate a solid tumor mass only to a limited extent. This limited penetration was not enhanced by administering high-dose GCV.

Comparison between Survival of Rats Treated with Intrathecal Injection of G1TkSvNa.53 or G1Tk1SvN.7 HS\textit{k} Vector-Producer Cells and i.p. Ganciclovir. To evaluate the antitumor efficacy of G1TkSvNa.53 clone of HS\textit{k} vector-producer cells, which have a higher titer of retroviral particles than the G1TkSvNa.53 clone, rats were treated in a separate experiment with intrathecal injection of each HS\textit{k} producer cell clone and subsequent i.p. ganciclovir therapy. Survival was extended in both groups compared to saline-treated rats (18 ± 3 versus 16 ± 1, \( P < 0.05 \) for G1TkSvNa.53; 20 ± 4 versus 16 ± 3, \( P < 0.005 \) for G1Tk1SvN.7) (Fig. 4). Although treatment with the clone 7 cells appeared superior to treatment with clone 53 cells, it was not statistically significant (\( P < 0.09 \)).

DISCUSSION

Tumor infiltration of the leptomeningal coverings of the brain and spinal cord presents a unique therapeutic challenge. Irradiation and chemotherapy result in only a limited tumor response, marginal extension of
survival, and significant morbidity. Retroviral-mediated gene therapy provides an attractive treatment option for investigation. Since the retroviral vectors described here are replication-incompetent, and thus unable to propagate infection and gene transfer from one tumor cell to another, efficient distribution of the vector is crucial to maximize gene transfer into as many tumor cells as possible. Efficient delivery of the vector is achieved by injection of the vector-producer cells into the CSF. The retroviral HStk vector particles are continuously released from the vector-producer cells and reach the surface of the tumor-infiltrated meninges. Selective transfer of the suicide gene into the proliferating tumor then sensitizes it to the effects of ganciclovir.

In vitro measurements of retroviral titers in supernatants from HStk vector-producer cells revealed no decrease in vector production after 48 h of exposure of the producer cells to various concentrations of ganciclovir, but not when the drug is administered intrathecally, even at a very high concentration. Histological examination of spinal cords from rats with leptomeningeal neoplasia that had received intrathecal GCV demonstrated almost complete eradication of the thin layers of tumor cells, which characteristically infiltrate the circumference of the spinal cord in our model of leptomeningeal neoplasia. However, the larger tumor mass surrounding the implanted catheter was almost unaffected when GCV was administered intrathecally. This suggests that the penetration of topical GCV is limited to a few cell layers.

Bystander effect, a process through which nontransduced tumor cells in the vicinity of HStk-transduced cells can be killed by GCV, has been described previously (7–9). One of the major components of the bystander effect has been linked to transfer of phosphorylated GCV from transduced to contiguous nontransduced cells via gap junctions.4 When topical GCV, after intrathecal delivery, interacts with the superficial layers of tumor cells in the leptomeninges, this bystander effect may account for the eradication of this layer of infiltrating tumor at the surface of the spinal cord. That the main tumor mass could not be eradicated by topical GCV suggests that the gap junction-mediated bystander effect is limited to a few cell layers from its point of origin.

Infection of HStk-transduced C6 tumor cells with a wild-type Molony murine leukemia virus enhances the antitumor efficacy of Molony-GCV therapy in vitro and in animal models of brain tumors (14, 15). In culture, infected cells had a 300-fold increase in sensitivity to GCV. The authors speculated, but did not demonstrate, that infection of HStk-transduced tumor cells with a replication-competent retrovirus enhances the effect of GCV and extends gene transfer by the ability of the helper virus to package the therapeutic vector in tumor cells (pseudotyping) and further enhances gene transfer to contiguous tumor cells. We evaluated the use of HStk vector-producer cells coinfected with a replication-competent Molony murine leukemia retrovirus in a model of leptomeningeal neoplasia, but found no enhancement of survival compared to treatment with HStk vector-producer cells alone. Differences in the tumor models (leptomeningeal tumor versus solid tumor) or the tumor cells used (9L versus C6) may have accounted for the difference in the results of the studies. An additional difference is that we did not use tumor cells that had been pretreated with the HStk gene and only then exposed to the wild-type virus prior to tumor inoculation.

Because of the rapid doubling time of the 9L tumor cells and the limited survival of animals in our model of leptomeningeal neoplasia, the extension of survival observed in rats treated with i.p. GCV represents a significant kill of tumor cells. However, apart from an occasional rat with long-term survival, all rats eventually succumbed to the meningeal tumor. This may be explained by residual small tumor deposits that were not identified in the specimens obtained 7 days after GCV therapy. In addition, in the setting of leptomeningeal neoplasia, where multiple, noncontiguous deposits of tumor cells occur, the bystander effect, which depends on contact between cells, may not be as effective as in a setting of solid tumors treated with the HStk/GCV approach. Thus, the clinical application of this approach may require repeated intrathecal administrations of HStk vector-producer cells and systemic GCV. The titer of the vector that is released from the HStk vector-producer cells, i.e., the number of viral particles available to transduce tumor cells, may be an important factor in achieving effective antitumor response. Although survival was extended somewhat with intrathecal injection of G1TkSvNa.53 clone of HStk vector-producer cells compared to the lower-titer G1TkSvNa.53 clone, the difference in survival was not statistically significant. Furthermore, various treatment parameters, such as the optimal number of HStk vector-producer cells that need to be injected, the period of time necessary for maximal gene transfer before GCV, and the duration of GCV therapy, are still unknown and need to be established in further studies.

Intrathecal delivery of vector-producer cells and transfer of a suicide gene to diffusely infiltrating leptomeningeal cancer cells resulted in significant prolongation of survival after systemic therapy with GCV. The results indicate that a similar approach merits further investigation for the treatment of this complication in cancer patients.

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