Tumor cells expressing the herpes simplex virus–thymidine kinase gene in the treatment of Walker 256 meningeal neoplasia in rats

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A promising strategy in the treatment of neoplastic meningitis involves the use of herpes simplex virus–thymidine kinase (HSV-tk)–modified cells. In these experiments the authors used cells expressing HSV-tk to treat meningeal carcinomatosis in the rat Walker 256 model. Intrathecal injection of $2 \times 10^7$ Walker cells resulted in a median survival time of 15 days. Up to 80% of animals implanted with HSV-tk–modified Walker cells (Walker-tk+) and treated with ganciclovir showed long-term survival (120 days or more), whereas the remaining animals died from tumor growth between 37 and 44 days after implantation. Tumor cells from an animal in which the treatment failed were cultured in vitro and were shown to be still sensitive to ganciclovir. However, continuous ganciclovir administration for 6 weeks rather than 2 weeks did not improve survival. Histopathological studies confirmed leptomeningeal infiltration in the untreated Walker or Walker-tk+ animals. Walker-tk+ cells were mixed with Walker cells in 1:1, 10:1, or 50:1 ratios, respectively, and implanted intrathecally; the animals were treated with ganciclovir. All groups of treated animals had long-term survivors, with 40% of the rats in the 10:1 and 50:1 groups demonstrating long-term survival and absence of microscopic tumors in the brain or spinal cord. Similarly, murine fibroblast HSV-tk virus–producer cells improved survival. Walker-tk+ cells were better than fibroblast-producer cells in improving the survival of animals with Walker tumors at low (1:1) but not at high (10:1) effector-to-target cell ratios. Repeated intrathecal administration of Walker-tk+ cells resulted in inhibition of established Walker tumors. The authors conclude that Walker-tk+ cells are at least as effective as murine virus–producer cells and could be used in the treatment of meningeal neoplasia.

KEY WORDS • brain tumor • carcinomatous meningitis • ganciclovir • gene therapy • herpes simplex virus–thymidine kinase gene

Neoplastic meningitis occurs in 8% of patients with cancer of the central nervous system (CNS) and is associated with a dismal prognosis, with a mean survival of 3 to 6 months. A promising approach in the treatment of CNS neoplasia involves tumor cell sensitization to nucleoside analogs such as ganciclovir by the herpes simplex virus–thymidine kinase (HSV-tk) gene. The HSV-tk gene can be transduced into tumor cells by retroviral vectors resulting in phosphorylation of ganciclovir, which leads to inhibition of DNA synthesis and to tumor cell death. Although retroviral HSV-tk transduction of every tumor cell is currently impossible, complete tumor eradication has been observed in several animal models. To explain the death of nontransduced tumor cells, a bystander effect was hypothesized and has been shown to be mediated by HSV-tk–modified tumor cells in the presence of ganciclovir.

Previous work has shown that HSV-tk virus–producer cells can be successfully used to prolong survival of ganciclovir-treated rats with 9L leptomeningeal neoplasia. However, 9L gliosarcoma forms a focal mass that results in spinal cord compression and to a lesser degree infiltrates the leptomeninges. In fact, in the study by Ram, et al., HSV-tk retroviral virus–producer cells were able to eliminate the leptomeningeal disease but not the focal tumors that subsequently caused animal death.

In our study we used the Walker 256 carcinosarcoma model of meningeal neoplasia, which results in diffuse leptomeningeal tumor infiltration without focal tumors. Our in vitro results indicate that Walker-tk+ cells exert a greater cytotoxic effect on nonmodified Walker cells than do murine fibroblast virus–producer (3T3 VP-tk+) cells. To extend these in vitro observations we used Walker-tk+ cells and compared them to 3T3 VP-tk+ cells in the treatment of Walker 256 carcinomatous meningitis in rats. We observed tumor inhibition when HSV-tk–modified tumor cells or 3T3 VP-tk+ cells were premixed with target Walker cells followed by ganciclovir administration. As in our in vitro study, Walker-tk+ cells exerted a greater
tumorcidal effect at low but not at high effector-to-target cell ratios, as compared to fibroblast virus–producer cells. We then showed that in situ treatment of established Walker leptomeningeal tumors with Walker-tk+ cells significantly improved survival after multiple injections of effector cells.

Materials and Methods

Cell Lines

The rat Walker 256 carcinosarcoma cell line and its derivatives (Walker-tk+ and Walker VP-tk+) were maintained in a RPMI-based culture medium (RPMI-1640, with 0.1 mM minimum essential medium (MEM) nonessential amino acids solution, 1 mM MEM sodium pyruvate solution, 10% fetal calf serum, 2 mM MEM-glutamine, 25 U/ml penicillin, 25 μg/ml streptomycin, 25 μg/ml fungizone, and 50 μg/ml gentamicin). The 3T3 VP-tk+ cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) with 10% bovine serum, 2 mM MEM-glutamine, and 100 U/ml penicillin.

Vector Plasmids and HSV-tk–Modified Cell Lines

The STK plasmid vector consists of the HSV-tk gene under the control of the simian virus 40 early region promoter and enhancer inserted into the LNL6 plasmid vector.14 The STK vector also contains the neomycin phosphotransferase gene (neoR), which confers resistance to the neomycin analog G418. The pPAM3 plasmid contains a packaging-defective Moloney leukemia virus genome in which packaging and other sequences have been deleted to prevent production of replication-competent virus.11,12 This plasmid expresses viral genome products that function in trans to produce virus containing vector genome.

The 3T3 VP-tk+ cell line was generated by calcium phosphate transfection with the STK vector of the amphotropic murine fibroblast packaging line GP+envAm12, and selected by addition of 0.4 mg/ml of G418 to the media.12,13 The Walker virus–producer line (Walker VP-tk+) was generated by cotransfection of the Walker cells with the STK and pPAM3 plasmid vectors (1:10 molar ratio, respectively), followed by G418 selection (0.4 mg/ml). Both producer cell lines were negative for replication-competent (helper) virus, as determined by the absence of infectious neoR retrovirus in the medium of infected murine 3T3 fibroblasts.4 Viral titers ranged from 103 to 105 cfu/ml for the 3T3 VP-tk+ cells and 106 to 107 cfu/ml for the Walker VP-tk+ cells. Colonies resistant to G418 were derived from the Walker-tk+ and (parent) Walker-tk+ cells were plated in duplicate (2 x 105 cells/ml) and selected by addition of 0.4 mg/ml G418. Cells were then suspended with 1 ml of 0.05% trypsin–ethylene diamine tetracetic acid and counted 8 days after plating.

In Vivo Experiments

In vivo experiments were approved by the Animal Research Committee (protocol No. 137-92) and were performed according to the National Institutes of Health guidelines for care of laboratory animals. Sprague-Dawley rats weighing approximately 200 g were anesthetized with a subcutaneous injection of a mixture containing ketamine HCl 100 mg/ml, xylazine HCl 20 mg/ml, and acepromazine 10 mg/ml, mixed in a 30:6:1 ratio, respectively. Each animal received 0.5 to 0.7 ml/kg of the anesthetic mixture; then they were placed in a stereotactic frame and immobilized via ear bars. Sterile technique was used throughout the procedure. After preparing the rats with betadine solution, a 1-cm midline incision was made in the suboccipital region. Muscles were retracted from the occipital bone, and the atlantooccipital membrane was exposed between the foramen magnum and the posterior arch of C-1. The atlantooccipital membrane and dura were sharply incised in the midline, revealing the obex. Under microscopic magnification, a 7-cm PE-10 polyethylene catheter was inserted dorsal to the spinal cord to the level of the lumbar cistern. Approximately 30 μl of cerebrospinal fluid (CSF) was withdrawn and 2 x 107 Walker 256 tumor cells suspended in a volume of 10 μl of Hank’s buffered saline solution (HBSS) were slowly injected followed by flushing of the catheter with 10 μl of CSF. The volume of the PE-10 catheter used for the intrathecal injection is approximately 5 μl.

In experiments in which the Walker cells were premixed with effector cells (Walker-tk+, Walker VP-tk+, and 3T3 VP-tk+ cells) prior to the intrathecal injection, the number of Walker cells was kept constant (2 x 107 cells/animal). The effector cells (2 x 105, 2 x 106, and 107 cells/animal) were premixed with Walker cells at 1:1, 10:1, and 50:1 ratios, respectively. The volume of infusion was kept constant at 10 μl except for the 50:1 group, which received 20 μl. Each experimental group usually consisted of six animals. The muscles and skin were reaproximated with interrupted 4-0 silk sutures after the removal of the PE-10 catheter. Animals were watched until fully recovered. Only animals that appeared neurologically intact (approximately 95% of the animals that underwent operation) were used for the study. A course of ganciclovir was started 3 days after tumor cell implantation and was continued for 2 weeks. Ganciclovir was administered by intraperitoneal injection twice daily at a dose of 30 mg/kg per day. In one experiment, in which the effect of continuous administration was assessed, ganciclovir was administered for a total of 6 weeks.

In the in situ treatment of established Walker leptomeningeal tumors, the surgical technique was modified so that a permanent 7-cm PE-10 catheter was inserted in the lumbar cistern. Walker cells (5 x 105/animal) in 10 μl of HBSS were injected through the catheter and the catheter was flushed with 10 μl of heparin (100 U/ml) to prevent clotting. A small loose knot in the tubing coated with cranoplasty cement was used to anchor the catheter in the suboccipital region. The muscles were closed with interrupted 5-0 silk sutures over the cranioplasty. The catheter was subcutaneously tunneled by passing it through a 16-gauge needle and exited through the skin on the back of the neck. The tube, which protruded approximately 4 to 5 cm from the skin, was occluded with a short length of sterilized stainless steel wire. Animals were housed one per cage for the duration of the injections (10 days) to protect the catheter. They received dexamethasone (0.5 mg/kg/day) and chloramphenicol (50 mg/kg/day) in their drinking water for 10 days, assuming that each animal consumes 20 ml of water per day. Three days after intrathecal implantation of the Walker cells, animals were randomized and received intrathecal injections of 5 x 107 Walker-tk+ cells in 10 μl of HBSS, then flushed with 10 μl of heparin 100 U/ml, once (single injection group) or every 2 days for a total of three injections (multiple injection groups). The rats were observed daily and moribund paraplegics were sacrificed.

Histological Studies

A total of 41 animals were sacrificed for histological examination. Animals were sacrificed when they were paraplegic and unable to reach food and water or when they were long-term survivors after the completion of the experiment. One or two animals from each experimental group were examined histologically to confirm the
The HSV-tk Gene and Ganciclovir Sensitivity to Walker Cells in Vivo

Walker-tk+ cells were prepared from Walker cells using the STK retroviral vector and were shown to be sensitive to ganciclovir in vitro. \(^{25}\) We found that Walker-tk+ cells were less tumorigenic than their parent Walker cells (median survival 20 vs. 14.5 days, \(p < 0.05\); Fig. 1). Administration of ganciclovir did not significantly affect the survival of animals with intracranially implanted wild-type Walker tumors (median survival 15.5 vs. 14.5 days, \(p = 0.18\)). However, administration of ganciclovir resulted in long-term survival in 50% of the animals implanted with \(2 \times 10^5\) Walker-tk+ cells. The fact that some ganciclovir-treated animals succumbed to tumors after inoculation with Walker-tk+ cells was not surprising given that resistance of HSV-tk+ cells to ganciclovir has been observed in vivo in other tumor models. \(^{13,14}\) Unexpectedly, a larger tumor burden following intracranial injection of \(10^5\) Walker-tk+ cells (50 times the number of Walker-tk+ cells used before), further improved long-term survival of the ganciclovir-treated animals (80% vs. 50%, \(p < 0.05\); Fig. 1). Walker HSV-tk virus-producer cells (2 \times 10^5 Walker VP-tk+) alone were also sensitive to ganciclovir in vivo, with 50% of the animals showing long-term survival.

Results

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Analysis of a Recurrent Walker-tk+ Tumor

To better understand the mechanism of ganciclovir resistance, we reestablished in culture the Walker-tk+ cells from a rat in which ganciclovir treatment failed. After several passages in vitro, a pure tumor cell population was obtained (rWalker-tk+) with the same morphological appearance as the Walker-tk+ cells. A portion of these cells demonstrated sensitivity to G418 or ganciclovir, in contrast to the parent Walker-tk+ cells, which were only sensitive to ganciclovir (Fig. 3). When the rWalker-tk+ cells were premixed with wild-type Walker cells in vitro, they were able to exert a bystander cytotoxic effect in the presence of ganciclovir (our unpublished data). These results suggest that the rWalker-tk+ cell population may be a mixture of cells that have lost or mutationally inactivated the STK vector (G418-sensitive/ganciclovir-resistant cells) and cells that have maintained expression of neoR and HSV-tk genes (G418-resistant/ganciclovir-sensitive cells).

Tumoricidal Effect of Walker-tk+ Cells on Wild-Type Walker Cells in Vivo

To evaluate the antitumor effect of Walker-tk+ cells in vivo we injected animals with a mixture of 1:1, 10:1, and 50:1 Walker-tk+/Walker cells, respectively. The number of wild-type Walker cells was kept constant (2 \times 10^5/animal) for every mixture group. All animals were treated with ganciclovir for 14 days, except for the control group, which received an equal number of Walker and Walker-tk+ cells (1:1) and was not treated with ganciclovir. All control animals died within 18 days of tumor cell inoculation. In contrast, rats that received 1, 10, or 50 times more Walker-tk+ cells than Walker cells had prolonged survival when treated with ganciclovir as compared to the control.

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There were one of five, two of five, and two of five long-term survivors in the 1:1, 10:1, and 50:1 groups, respectively (Fig. 4). Histological examination of animals that received a mixture of Walker-tk+ and Walker cells and that were sacrificed 3 or 7 days after ganciclovir treatment showed a diminution in the tumor cell population with time (Fig. 2C–F). However, even at 7 days after ganciclovir administration there were tumor cells present in the leptomeninges. No gross or microscopic tumors were seen in the long-term survivors (Fig. 2G, H).

**Tumoricidal Effect of Walker-tk+, Walker VP-tk+, and 3T3 VP-tk+ Cells in Vivo**

Our previous *in vitro* studies showed that Walker-tk+ cells had greater bystander cytocidal effect on wild-type Walker tumor cells than did 3T3 VP-tk+ cells. To extend these *in vitro* observations, we compared the tumoricidal effect of Walker-tk+, Walker VP-tk+, and 3T3 VP-tk+ cells on Walker 256–induced carcinomatous meningitis in rats. Walker VP-tk+ cells produce HSV-tk+ retroviral vectors with titers ranging from $10^3$ to $10^5$ cfu/ml, which are approximately 100 times less than the corresponding 3T3 VP-tk+ titers. An excess of 10 times more effector than target Walker cells were used in all groups to achieve maximum cytocidal effect. Implantation of Walker cells with Walker-tk+, Walker VP-tk+, or 3T3 VP-tk+ cells resulted in no statistically significant difference in survival among the three effector groups (median survival was 24.5, 27, and 24 days, respectively; Fig. 5 left). However, there were three of seven long-term survivors in...
the Walker-tk+ group, one of six in the Walker VP-tk+ group, and one of nine in the 3T3 VP-tk+ group. These results indicate that at a high effector-to-target cell ratio (10:1), Walker-tk+, Walker VP-tk+, and 3T3 VP-tk+ cells had similar tumor-inhibiting effect on Walker-induced carcinomatous meningitis. At lower effector-to-target cell ratios (1:1), a statistically significant difference in the efficacy of the three different HSV-tk effector cell lines was observed. All animal groups that received effector HSV-tk+ cells showed statistically significant prolongation of survival when compared to the control Walker group (Fig. 5 right, p < 0.05). We also found a statistically significant prolongation of survival in animals treated with Walker-tk+ or Walker VP-tk+ cells as compared to animals treated with 3T3 VP-tk+ cells (Walker-tk+ vs. 3T3 VP-tk+, p < 0.05; Walker VP-tk+ vs. 3T3 VP-tk+, p < 0.05). There was no significant difference in survival between the Walker-tk+ and the Walker VP-tk+ groups (p = 0.49). When the corresponding treatment groups from the 1:1 experiment were compared to the 10:1 experiment, no difference in survival was observed.

One Walker group was treated with Walker-tk+ cells and ganciclovir for 6 weeks instead of the usual 2 weeks in an attempt to eliminate the survival of small numbers of Walker-tk+ cells observed occasionally after a 2-week ganciclovir treatment. There was no statistically significant difference in survival when ganciclovir was administered continuously for 6 weeks instead of 2 weeks in animals treated with Walker-tk+ cells (data not shown).

In Situ Treatment of Established Walker Leptomeningeal Carcinomatosis

In the previous experiments, Walker cells were pre-mixed in different ratios with HSV-tk+ effector cells to determine the maximum therapeutic effect of those cells under ideal conditions. To assess the efficacy of Walker-tk+ cells in in situ treatment of Walker leptomeningeal neoplasia, we treated 3-day-old Walker tumors with either a single or multiple (that is, × 3) injections of Walker-tk+ cells. Ganciclovir administration was started 24 hours after the first Walker-tk+ injection and was continued for a period of 14 days. A single Walker-tk+ cell injection resulted in a median survival of 17 days, which was not statistically different from the 12 days observed in the control Walker group (Fig. 6). However, multiple injections of Walker-tk+ cells resulted in statistically significant prolongation of survival as compared to the Walker control or the single injection groups (p < 0.05). No long-term survivors were seen in any of these groups.

Discussion

Neoplastic meningitis can result from leptomeningeal dissemination of a variety of cancers. The majority of patients with leptomeningeal disease have a dismal prognosis despite chemotherapy and radiation therapy; mean survival times range from 3 months for patients with metastatic carcinoma to 12 months for those with primary CNS tumors such as medulloblastoma.10,15,17,21 Gene therapy of carcinomatous meningitis is a promising new therapeutic approach. Previous studies showed that retroviral virus-producer 3T3 VP-tk+ cells can survive in the CSF, release virus particles, and infect 9L tumor cells, with maximum transduction occurring 6 days after producer-cell injection.20 These 3T3 VP-tk+ cells were also used to treat an equal number of tumor cells in the 9L gliosarcoma model of leptomeningeal neoplasia in rats. A statistically significant prolongation of survival was seen in ganciclovir-treated animals receiving HSV-tk virus–producer cells.20 Although the results of that study are encouraging, it is important to consider the following points: 1) intrathecal injection of 9L gliosarcoma cells resulted in a focal tumor mass surrounding the intrathecal catheter at
the site of injection, resulting in spinal cord compression and death even when the leptomeningeal tumor was histo-
logically eliminated with treatment; 2) no long-term sur-
vivors were seen; and 3) virus-producer cells were inject-
ed right after tumor cell inoculation, a situation clearly
different from \textit{in situ} treatment of established tumors.\textsuperscript{20} In
this study we used the Walker 256 carcinosarcoma model
of meningeal neoplasia, which, in contrast to 9L glioma
cells, resulted in disseminated meningeal spread without
the formation of focal tumors.\textsuperscript{9}

Intrathecally implanted wild-type Walker cells were
100\% tumorigenic and their growth was not affected by
intraperitoneal administration of ganciclovir. Walker cells
engineered to express the HSV-tk gene were also 100\% tumorigenic but grew significantly more slowly than the
parent Walker cells (Fig. 1). This could be due to in-
creased antigenicity of Walker-tk+ cells secondary to the
expression of the HSV-tk gene by the tumor cells.\textsuperscript{2} The
reduced tumorigenicity of HSV-tk expressing tumor cells
was also observed in the C19 mouse melanoma model.\textsuperscript{25}
Walker-tk+ cells were partially sensitive to ganciclovir \textit{in vivo}, despite the fact that they were 100\% sensitive to ganci-
clovir \textit{in vitro}. One obvious explanation is that Walker-
tk+ cells were kept under G418 selection \textit{in vitro} but not \textit{in vivo}. Resistance to ganciclovir has been observed in
other HSV-tk+ tumor cell lines and has been attributed to
HSV-tk gene loss or methylation, inadequate ganciclovir
concentrations, or presence of noncycling HSV-tk+ cells
during the period of ganciclovir administration.\textsuperscript{13,14} Sensi-
tivity to ganciclovir, as determined by long-term sur-
vival, increased from 50\% to 80\% when the number of
intrathecally implanted Walker-tk+ cells was increased by
50-fold (Fig. 1). This increase in ganciclovir sensitivity
could be due to closer contact of Walker-tk+ with rever-
tant or noncycling Walker-tk+ cells and greater potential
for elimination due to an enhanced bystander effect. The
bystander effect is augmented by cell proximity.\textsuperscript{7,25} This
was shown by a stronger cytocidal effect in dense cell cul-
tures than in sparse ones.

Walker-tk+ cells from a rat in which ganciclovir treat-
ment failed were established \textit{in vitro} and were shown to
maintain some degree of ganciclovir sensitivity, although
significantly less than the parent Walker-tk+ cells (Fig. 3).
We theorized that a more prolonged ganciclovir adminis-
tration might be beneficial. However, in application, we
observed no difference in the bystander tumoricidal effect
of Walker-tk+ cells \textit{in vivo} when ganciclovir was given con-
tinuously for 6 instead of 2 weeks (data not shown).
Walker-tk+ cells exerted tumor inhibition in vivo in ganciclovir-treated rats when they were premixed with wild-type Walker cells. Although increased efficacy was observed with increasing numbers of Walker-tk+ cells, this trend was not statistically significant (Fig. 4). It is possible that the bystander tumoricidal effect reaches a certain potency or “saturation” at high effector-to-target cell ratios and that there is little to gain by further increasing the ratio of the effector cells. Our results are in agreement with other investigators, who reported no difference in survival when animals received 1 or 10 times more 3T3 VP-tk+ cells.2

We found that 3T3 VP-tk+ cells also exerted a tumor-inhibiting effect in vivo after ganciclovir administration. When 3T3 VP-tk+, Walker-tk+, and Walker VP-tk+ cells were compared with respect to their tumoricidal effect on Walker cells in vivo, no statistically significant difference was found between the three effector cell groups at 1:1 effector-to-target cell ratios (Fig. 5 left). This is probably due to the excess of effector over target cells resulting in saturation of the tumoricidal effect. However, when a lower number of effector cells was mixed with Walker cells (1:1 effector-to-target cell ratio), the Walker-tk+ and Walker VP-tk+ groups were superior to the 3T3 VP-tk+ group with regard to animal survival (Fig. 5 right). This is consistent with our in vitro results, in which Walker-tk+ cells were superior to the 3T3 VP-tk+ cells at low effector-to-target cell ratios and as effective as the 3T3 VP-tk+ cells at high effector-to-target cell ratios.24 This difference in the bystander effect between HSV-tk–modified tumor cells and the 3T3 VP-tk+ cells can be exploited in the treatment of human CNS malignancies by allowing a lower number or fewer injections of effector HSV-tk–modified tumor cells to be used. However, significant variation in the sensitivity to the bystander effect is known to exist among different tumor cell lines; thus, it is uncertain whether the observed superiority of HSV-tk–modified tumor cells over 3T3 VP-tk+ cells at low effector-to-target cell ratios can be generalized to other tumor models.

In situ treatment of established Walker tumors showed an improvement in survival and an advantage in using repeated intrathecal administration of Walker-tk+ cells similar to current use of multicyle chemotherapy regimens. Multiple injections of Walker-tk+ cells were given in the presence of circulating ganciclovir because of the rapid growth of the Walker tumor cells and the narrow therapeutic window. Repeated injections of HSV-tk+ cells improved survival more than a single injection despite the presence of ganciclovir. This underscores the need for persistent presence of the effector cells to ensure long “contact” time with the target tumor cells. Although long-term survivors were seen when Walker cells were premixed with Walker-tk+ cells, no such cases were seen in the in situ treatment of established Walker tumor with Walker-tk+ cells. Similar results were observed by Ram, et al.,20 in the 9L model of neoplastic meningitis, results that were less successful than those observed by the same investigators in the 9L intracerebral glioma model.4 This indicates a limitation of the current gene therapy approaches in the treatment of widespread disseminated tumors such as leptomeningeal tumors in contrast to more contained, local intracerebral lesions.

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References


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