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In Vivo Gene Transfer with Retroviral Vector–Producer Cells for Treatment of Experimental Brain Tumors

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Direct in situ introduction of exogenous genes into proliferating tumors could provide an effective therapeutic approach for treatment of localized tumors. Rats with a cerebral glioma were given an intratumoral stereotaxic injection of murine fibroblasts that were producing a retroviral vector in which the herpes simplex thymidine kinase (HS-tk) gene had been inserted. After 5 days during which the HS-tk retroviral vectors that were produced in situ transduced the neighboring proliferating glioma cells, the rats were treated with the anti-herpes drug ganciclovir. Gliomas in the ganciclovir- and vector-treated rats regressed completely both macroscopically and microscopically. This technique exploits what was previously considered to be a disadvantage of retroviral vectors—that is, their inability to transfer genes into nondividing cells. Instead, this feature of retroviruses is used to target gene delivery to dividing tumor cells and to spare nondividing neural tissue.

Direct transfer of specific tumor suppressor genes, of genes that encode a particular toxic product, or of genes whose products specifically induce apoptosis in the tumor cells could be important approaches for treating malignancy. Retroviral-mediated gene transfer, the current method of choice for clinical gene transfer, has been limited in its usefulness because murine retroviruses stably integrate their genes only in target cells that are actively synthesizing DNA (*1*). Thus, attempts at retrovirus-mediated gene transfer into cell types that are usually in G_0, such as the totipotent bone marrow stem cell, have had only limited success. We considered the possibility that the requirement for target cell DNA synthesis could be exploited to limit gene delivery to proliferating tumor cells present in an organ in which the resident normal cells are not proliferating. The optimal method of gene transfer to a growing tumor would provide for the continuous delivery of retroviral particles into the immediate local environment of the tumor so that when an individual tumor cell enters DNA synthesis, a vector particle is available to transduce it. Continuous local infusion of retroviral vector–containing supernatants might be possible, although technically difficult. Our approach was to infiltrate the tumor mass with cells engineered to actively produce retroviral vector particles so that continuous production of the vector occurred within the tumor mass in situ.

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We designed our initial experiments to determine if retroviral-mediated gene transfer after reimplantation of mixtures of tumor cells with vector-producing fibroblasts could be successfully accomplished in vivo. Mice were inoculated subcutaneously with fibrosarcoma cells mixed with either control fibroblasts that expressed the neomycin resistance gene (NeoR) (3T3 cells not producing retroviral vectors) or 3T3 cells that produce the NeoR retroviral vector (PA317) (*2*). To measure the efficiency of gene transfer in vivo, we resected the tumors after 4 weeks, reestablished them in culture, and then tested them in a clonogenic assay for expression of the NeoR gene by culture with the neomycin analog G418 (Table 1). No G418-resistant tumor colonies were recovered from any animal that received control fibroblasts. By contrast, 63 ± 9% (mean ± SEM) of the tumor cells recovered from animals injected with the tumor mixed with retroviral vector–producing fibroblasts grew as G418-resistant colonies in a clonogenic assay, which indicated that in vivo gene transfer into the proliferating tumor cells had been accomplished. Southern (DNA) blot analysis for the NeoR vector and a direct enzyme assay for neomycin phosphotransferase were positive in all these G418-resistant tumor cell populations (*3*). These experiments clearly indicate that proliferating tumor cells can be successfully transduced in vivo if mixed with retroviral vector–producing cells.

Several gene transfer systems might be useful for the local treatment of cancer. Increasing the immunogenicity of tumors by causing local cytokine production or by enhancement of major histocompatibility complex antigen expression can lead to local antitumor effect (*4*). Another approach is the introduction of a gene that

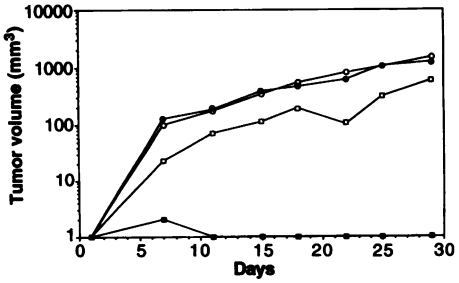

Fig. 1. The elimination of tumors in GCV-treated mice with fibrosarcomas derived from mixtures of 1×10^6 tumor cells and 2×10^6 HS-tk retroviral vector–producing fibroblasts. Control groups consisted of mice injected with 1×10^6 tumor cells mixed with 2×10^6 NeoR-transduced 3T3 cells (LNL6) (○), NeoR retroviral vector–producing fibroblasts (PA317/LNL6) (●), or 3T3 cells transduced with the HS-tk retroviral vector PA317-G1NsCTK (□) (a generous gift of Genetic Therapy, Gaithersburg, Maryland). G1NsCTK is a modified LNL6 vector that contains an HS-tk gene under the control of a cytomegalovirus promoter inserted 3′ to the NeoR gene (■). This vector-producer line produces ~1×10^5 vector colony-forming units per milliliter in culture and is free of replication-competent virus. The sensitivity of the nonproducer HS-tk–transduced 3T3 cells and HS-tk–producer cells to GCV in vitro was similar. Three days after cell injection, the mice were treated for 6 days with GCV twice daily at 150 mg per kilogram of body weight (Syntex, Palo Alto, California). The tumors were measured twice weekly with calipers in three dimensions.

encodes a susceptibility factor that would make the tumor sensitive to a chemotherapeutic agent. Preferably, the tumor would be rendered sensitive to a drug that is ordinarily not toxic to the target organ or to other tissues. At least two such systems have been described. Cells modified to contain the herpes simplex thymidine kinase (HS-tk) gene become sensitive to treatment with the antiviral agent ganciclovir (GCV), whereas normal cells are unaffected by this agent (5). Similarly, cells modified to express the bacterial enzyme cytosine deaminase convert the ordinarily nontoxic drug 5′-fluorocytosine to the cytotoxic compound 5-fluorouracil, which kills the gene-modified cells (6).

To determine if the HS-tk gene–transfer system would be capable of eliminating tumors in our model, we injected mice subcutaneously with mixtures of tumor cells and control fibroblasts or with fibroblasts engineered to produce HS-tk retroviral vectors. Three days after cell implantation, small growing tumors became visible, and we began to treat the mice twice daily with GCV. In control mice bearing tumors mixed with control fibroblasts or fibroblasts that produced only a NeoR vector (Fig. 1), GCV treatment had no effect on tumor growth. Tumors in mice injected with mixtures of tumor and 3T3–HS-tk cells and subsequently treated with GCV showed a modest slowing of growth, which suggested a mild antitumor effect. In contrast, tumors derived from a mixture of tumor cells and HS-tk retroviral vector–producing fibroblasts regressed rapidly and completely with GCV treatment and the animals remained tumor-free until the experiment was terminated on day 29. This result was somewhat unexpected because the efficiency of in vivo gene transfer to tumor in this type of experiment had been less than 100% (Table 1).

To further explore this observation, we injected normal mice subcutaneously with wild-type tumor cells mixed at various ratios with tumor cells that had been transduced with, and that were expressing, the HS-tk gene. Treatment with GCV produced complete tumor regression in nearly all animals containing 50:50 tumor mixtures and in some animals bearing tumor mixtures consisting of as few as 10% HS-tk–expressing cells (Table 2). The mechanism mediating this "bystander" tumor killing is not fully understood. Nearby normal tissues were apparently only minimally affected by the "bystander" effect because the normal skin overlying the tumors and the muscles underlying them were not damaged when the tumor was completely destroyed during GCV treatment (7).

Because of this profound "bystander" antitumor effect, we tested whether direct in situ injection of a growing tumor with fibroblasts that produce the HS-tk retrovirus vector might be able to transfer the HS-tk gene to a sufficient fraction of tumor cells to cause regression of an already established cancer. Fisher 344 rats carrying a 9L glioma (8) previously implanted in the right cerebral hemisphere were injected intratumorally with HS-tk retroviral vector–producing murine fibroblasts by stereotaxic guidance. After 5 days to allow time for the murine fibroblasts to continuously produce retroviral vectors capable of transducing the neighboring proliferating glioma cells, GCV was administered intraperitoneally to the tumor-bearing rats twice daily for 5 days. In recipients in which the gliomas were injected with saline or control vector–producing fibroblasts, GCV treatment had no effect on tumor growth. The tumors rapidly progressed in size and grew out of the brain along the needle tract. In contrast, complete macroscopic and microscopic tumor regression was observed in 11 of 14 of the GCV-treated rats (Fig. 2). In three rats, microscopic examination showed residual tumors composed of viable and necrotic malignant cells without evidence of residual vector-producer cells. Adjacent brain tissue, which should not integrate the

Table 1. Clonogenic assay of tumors regrown from mice 4 weeks after inoculation. We used a clonogenic assay to test for the presence of NeoR-expressing tumor cells in mice previously injected with mixtures of untransduced tumor and fibroblasts that produced NeoR-containing retroviral vectors. Murine fibrosarcoma (MCA 205) cells (10^6) (*12*) mixed with an equal number of murine fibroblasts (3T3) were injected subcutaneously into syngeneic C57BL/6 6- to 8-week-old female mice (*12*). One group received tumor cells and 3T3 cells transduced with the retroviral vector LNL6 (NeoR nonproducer). A second group was given tumor cells mixed with LNL6 retroviral vector–producing PA317 fibroblasts. LNL6 produces a NeoR-containing retroviral vector with a titer of ~10^6 cells per milliliter that is free of replication-competent virus (*2*). Tumors grew in all animals. After 4 weeks, the tumors were harvested from three mice in each group, recultured, and tested for transfer of neomycin resistance in a clonogenic assay at 500 cells per well seeded in a six-well Costar microculture plate ± G418 (1.0 mg/ml active concentration; Geneticin, Gibco). The number of growing colonies was counted after 7 days. The cloning efficiency of tumor cells from each group cultured in the absence of G418 was 8 to 20%.

Inoculation	Mouse	Colonies (*n*)	
		No G418	G418 (1.0 mg/ml)
Tumor + control fibroblasts	907	82	0
	908	78	0
	909	84	0
Tumor + vector-producer cells	438	100	79
	441	42	25
	442	84	41

Table 2. The effect of GCV on the incidence of tumor growth in mice injected with mixtures of tumor cells transduced with the HS-tk gene and wild-type tumor cells. Adult C57BL/6 mice were injected subcutaneously with a total of 1×10^5 tumor cells. This representative experiment includes data from three groups of five mice, each with either an adenocarcinoma (MC 38) or one of two distinct fibrosarcomas (MCA 102 or 205) (*12*). There was no difference in response between the different tumor types. Beginning on day 4 after tumor injection, each animal was treated intraperitoneally twice daily with GCV (150 mg per kilogram of body weight) for 7 days. The incidence of tumors in groups of control mice that were not treated with GCV was similar whether or not the tumor had been transduced with the HS-tk gene. Tumors were palpable by 4 weeks in all control animals injected with 5×10^4 wild-type tumor cells (*13*).

HS-tk tumor cells (%)	Palpable tumors	
	2 weeks	5 weeks
100	0/15	2/15
50	0/15	1/15
10	3/15	6/15
0	12/15	15/15

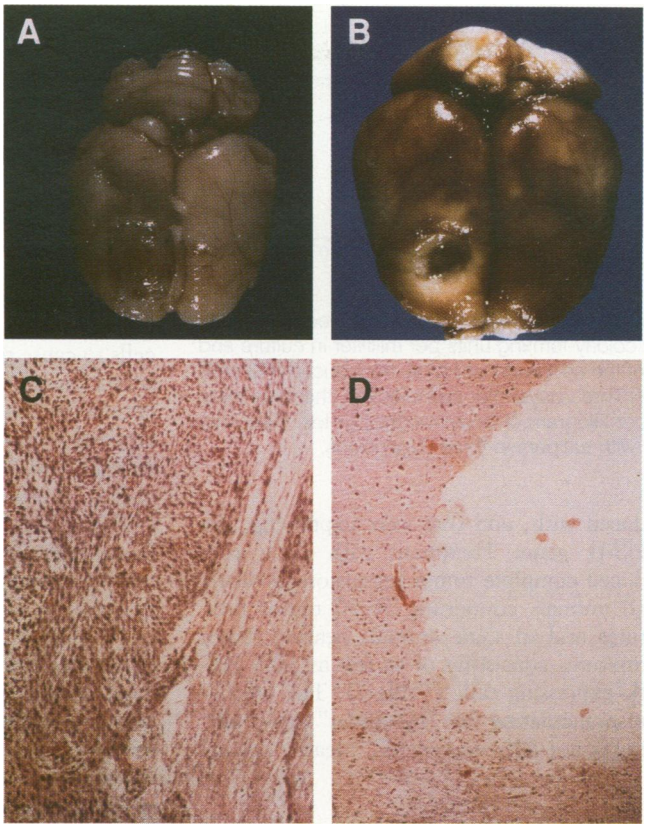

Fig. 2. Gross and microscopic morphology of rat brains with the 9L glioma after stereotaxic injection with control or HS-tk retroviral vector-producer cells and treatment with GCV. Fisher 344 rats weighing 230 to 350 g were anesthetized and placed in a stereotaxic apparatus (*11*). Syngeneic 9L gliosarcoma cells (4×10^4) were inoculated into the right cerebral hemisphere, a dose of tumor which results in 100% lethality by 4 weeks. Five days later, the same stereotaxic coordinates were used to directly inject saline ($n = 4$) or 3×10^6 PA317/HS-tk producer line cells ($n = 14$) into the growing 9L tumor. Other controls included 9L tumors injected with fibroblasts that produced retroviral vectors that did not contain the HS-tk gene. Five days later, treatment was started with intraperitoneal injections of GCV at 150 mg per kilogram of body weight twice daily for 5 days. The rats were then killed to determine antitumor effect. (**A**) The brain from a control rat that was treated with an injection of saline into the tumor. (**B**) The brain from a rat that was treated with an injection of PA317/HS-tk producer cells into the tumor. (**C**) A microscopic section of the brain from a control rat that was treated with an injection of saline into the tumor. (**D**) A microscopic section of the brain from a rat that was treated with an injection of PA317/HS-tk producer cells into the tumor.

retroviral vector or express the HS-tk enzyme, was not detectably harmed.

These studies were designed primarily to evaluate the potential efficacy of in situ gene transfer in the treatment of localized malignancy. Because of perioperative mortality in the rat brain tumor model, we have not generated long-term survival statistics that compare the various treatment groups. However, several rats treated with HS-tk producer cells and GCV survived tumor-free for over 100 days, whereas all control animals in these experiments had died by 35 days, which suggests that this procedure has the potential to be curative (*7*).

Brain tumors have several features that make them particularly attractive for this approach to treatment (*9*). Neurons and most other cellular elements in the brain are stable and do not regularly synthesize DNA. Vascular endothelial cells in the brain may be cycling at a low rate, but among those most likely to be cycling are cells that respond to signals promoting angiogenesis in the vicinity of the tumor. Such vessels would most likely be part of the blood supply of the tumor and, therefore, their destruction would be desirable. Thus, within the brain, the principal mitotically active cells will be those within the tumor or cells necessary for its support. Brain tumors are often localized and yet are inoperable because of their location and their relation to adjacent critical structures. Another advantage of the brain for this technique is that it is a relatively immunologically privileged site and could permit the histoincompatible retroviral vector-producer cells to persist and expose the tumor cells to potential transduction for a long time without immunologic rejection (*10*).

A possible undesirable consequence of this treatment could be systemic toxic effects because other proliferating tissues might also take up the HS-tk vector and then be destroyed during GCV treatment. However, we observed no systemic toxicity in the animals that received this treatment protocol during the 30-day period. In control experiments, target tissues that contained rapidly proliferating cells, such as the intestinal epithelium, the thymus, and bone marrow, were unaffected in GCV-treated animals that had been previously injected intravenously or intraperitoneally with the HS-tk retroviral vector–producer cells (*7*). Because most cells of the body express receptors for amphotropic retroviruses, any vector particle that escapes the local environment of the tumor should be quickly bound to another cell, even though most cells would not be in cycle and would, therefore, be unable to integrate the genes carried by the vector. These data support the potential clinical application of this technique for the treatment of brain tumors in humans. The procedure should be applicable to other gene systems and tumors at different anatomic sites.

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