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Adenoviral Thymidine Kinase Prodrug Gene Therapy Inhibits Sarcoma Growth *in Vivo*

Howard M. Ross, M.D.,* Edward A. Hirschowitz, M.D.,† Thomas J. Russi, M.D.,† Ronald G. Crystal, M.D.,† Sumihiko Nawata, M.D.,* Michael E. Burt, M.D., Ph.D.,* Murray F. Brennan, M.D.,* and Jonathan J. Lewis, M.D., Ph.D.*,[1]

*Department of Surgery, Memorial Sloan–Kettering Cancer Center, and †Department of Pulmonary Critical Care Medicine, Cornell University Medical Center, New York, New York 10021

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Local recurrence of sarcoma is due to residual tumor cells remaining after surgical resection and is associated with decreased survival. We implemented adenoviral-mediated transfer of the herpes simplex thymidine kinase (HSTK) gene with subsequent ganciclovir (GCV) administration to treat a model of residual sarcoma. [^{3}H]Thymidine uptake in MCA sarcoma cells was determined after infection with replication incompetent adenovirus of the AdMLP.HSTK construct in the presence of GCV. *In vivo* efficacy was evaluated in a model of residual sarcoma when 9 mg of MCA tumor was implanted into the latissimus muscle of Fischer 344 rats. Three days after implantation, animals were randomized to receive AdMLP.HSTK, AdCMV.Null, or viral suspension buffer intratumorally. From Day 4, animals were administered b.i.d. GCV (50 mg/kg) or saline ip. Tumors were excised on Day 14 and weighed. Statistical analysis was by Mann–Whitney *U* test. *In vitro:* [^{3}H]-thymidine incorporation was significantly decreased in MCA sarcoma cells infected with AdMLP.HSTK in the presence of GCV ($P < 0.05$). *In vivo:* Growth of MCA sarcoma treated with AdMLP.HSTK and GCV was significantly inhibited. Final tumor weights in the AdMLP.HSTK/GCV group were lower than all control groups ($P < 0.05$). A significant antitumor growth effect on MCA sarcoma was seen with adenoviral-mediated transfer of the HSTK gene and GCV administration, both *in vitro* and in an *in vivo* model of residual disease. This prodrug gene therapy strategy warrants investigation as an adjuvant modality in the management of sarcoma.

INTRODUCTION

Local recurrence of soft tissue sarcoma poses a significant clinical problem and is associated with decreased survival [1, 2]. Multimodality therapy has reduced the incidence of local recurrence but rates remain high [3]. Patients with retroperitoneal sarcoma treated with resectional surgery can have a 40% rate of local recurrence [4]. Local recurrence in patients with extremity sarcoma treated with limb sparing surgery and adjuvant brachytherapy ranges from 10 to 35% [3]. Local recurrence in both the abdomen and extremity is due to residual tumor cells remaining after surgical resection and adjuvant treatment. This study explored the local application of prodrug gene therapy to the growth of MCA sarcoma in a model of residual disease.

Prodrug gene therapy for cancer involves the activation of an anticancer agent via the expression of a foreign transferred gene. We utilized an adenovirus vector to transfer the herpes simplex viral thymidine kinase (HSTK) gene. Adenovirus has been used to transfer foreign genes into mammalian cells to affect tumor growth in multiple animal models [5–7]. Adenovirus can be genetically engineered to be replication incompetent and to contain up to a 7.5-kb exogenous gene. Adenoviral infection generates a strong inflammatory response and this may be helpful in eliciting an immune response toward tumor cells [8].

Transfer of the HSTK gene with subsequent ganciclovir (GCV) administration yields cell death via a mechanism described in 1986 [9]. Expression of the HSTK gene produces the viral enzyme (HSTK), which monophosphorylates the nucleoside analog prodrug GCV. Mammalian cellular enzymes then triphosphorylate the monophosphorylated GCV. The incorporation of triphosphorylated GCV into DNA results in chain termination and ultimately causes cell death [10]. An observed phenomenon associated with the HSTK/ GCV paradigm is the "bystander effect" [11]. This phenomenon involves the death of non-HSTK-transfected cells adjacent to HSTK-transfected cells, and allows regression of an entire tumor when only a fraction of the cells comprising the tumor are genetically altered.

In this study we examined the effect of adenoviral-mediated HSTK gene transfer with subsequent GCV administration on MCA sarcoma tumor growth *in vitro* and in an *in vivo* model of residual disease.

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[1] To whom correspondence should be addressed at Department of Surgery, Box 126, 1275 York Avenue New York, NY 10021.

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MATERIALS AND METHODS

Adenoviral Vectors

Construction of AdMLP.HSTK. A recombinant replication-deficient adenovirus (AdMLP.HSTK) was constructed with the HSV tk gene downstream of the adenovirus major late promoter. The 1.8-kb cDNA encoding HSV tk was first ligated into an adenovirus shuttle plasmid. The shuttle plasmid based on pBluescript II SK contained a multiple cloning site flanked on the 5′ end by the left inverted terminal repeat of adenovirus, the E1A enhancer, along with the adenovirus major late promoter and tripartite leader of adenovirus type 2. On the 3′ end of the multiple cloning site there was a 3-kb region homologous to adenovirus type 5. A linearized fragment of this recombinant of this recombinant plasmid was cotransfected into 293 cells (American Type Culture Collection) along with *Cla*I cut adenovirus DNA from the E3 deletion mutant Ad-dl327. Single plaques were amplified in 293 cells. Recombinant virus was screened by PCR amplification for the presence of HSV tk and the absence of the E1 region to demonstrate successful homologous recombination. A single PCR-positive plaque, AdMLP.HSTK, was amplified in 293 cells and subsequently purified on cesium gradients. Viral stocks in excess of 10^{10} plaque forming units (pfu) per milliliter of Ad.MLP.HSTK were prepared and titered in 293 cells [12].

Construction of AdCMV.Null. A control vector of replication-incompetent adenovirus containing an expression cassette with a CMV promoter without the cDNA insert for HSTK was constructed using the same methods.

Construction of AdCMV.LacZ. A replication incompetent adenoviral vector containing the marker gene, β-galactosidase, under control of the CMV promoter was constructed as previously described [13].

Tritiated Thymidine Uptake Studies

Tritiated thymidine uptake was utilized as a measure of cellular proliferation. The ability of recombinant adenovirus containing the HSTK gene to affect the viability of methylcholanthrene-induced rat rhabdomyosarcoma cells in the presence of GCV (Cytovene, Ganciclovir sodium, Syntex Laboratories, Inc., Palo Alto, CA) was assessed by [^{3}H]thymidine uptake studies. The first *in vitro* experiment evaluated the impact of increasing multiplicity of viral infection (m.o.i.) with subsequent GCV administration on MCA sarcoma cell DNA synthesis. In triplicate plates, 10^5 MCA sarcoma cells were incubated in RPMI, 2% fetal calf serum in the presence of AdMLP.HSTK or AdCMV.Null at 0, 10, 50, and 100 m.o.i. for 24 hr. GCV was then added to the infected cells to a concentration of 10 μg/ml for 48 hr. Cells were incubated for 6 hr with [^{3}H]thymidine (0.5 μCi/ well; DuPont New England Nuclear, Boston, MA). The medium was aspirated, cells were washed three times with phosphate-buffered saline followed three times with ice-cold 5% trichloroacetic acid. Two hundred proof ethyl alcohol was added to the cells and they were then aspirated. The cells were allowed to air-dry for 15 min and then lysed with 500 μl 0.1 *N* NaOH. pH was neutralized with 0.1 *N* HCl and the amount of [^{3}H]-thymidine was quantified in a scintillation counter.

The second *in vitro* experiment determined the effect on MCA sarcoma cells of increasing doses of GCV at an m.o.i. of 100 and on MCA cells without prior viral exposure. This experiment was conducted to evaluate GCV toxicity *in vitro.* In triplicate wells, 10^5 MCA sarcoma cells were infected with either AdMLP.HSTK or AdCMV.Null at an m.o.i. of 100 for 24 hr. One group of wells was not exposed to virus. GCV was then added to the wells at concentrations of 0, 5, and 10 μg/ml for 48 hr. [^{3}H]Thymidine uptake was evaluated as above.

In Vivo Foreign Gene Expression

To evaluate the ability to transfer a foreign gene via recombinant adenovirus infection into MCA tumor growing in the flank of the Fischer rat, β-galactosidase expression was investigated. Two Fischer 344 rats were anesthetized with pentobarbital sodium (Wyeth Laboratories Inc., Philadelphia, PA) 50 mg/kg intraperitoneally. After betadine skin preparation, a 1-cm skin incision was made over the left latissimus muscle. Forceps and scissors were used to create a small cavity in the muscle. A 9-mg MCA sarcoma tumor specimen, excised from a passage animal, was placed into the cavity. A 5-O silk suture was placed around the tumor to facilitate later identification. The skin was then stapled closed and animals were allowed to recover from anesthesia. Three days were allowed to pass to enable the tumor to implant.

On Day 3, animals were anesthetized with pentobarbital sodium, their prior incision was opened with gentle traction, and the incorporated tumor was identified. Under direct vision intratumoral injection of 10^9 pfu of AdCMV.LacZ in 50 μl of viral suspension buffer was performed. The skin was closed and animals were allowed to recover from anesthesia. On Day 5, 48 hr post-AdCMV.LacZ infection, animals were sacrificed via CO_2 inhalation and their tumors were excised with a 1-cm margin of surrounding muscle. The excised flank was fixed in 10% Formalin on ice for 1 hr. The tissue was then placed in X Gal reagent for 4 hr at 37°C. The presence of expressed β-galactosidase protein was revealed in the development of a blue chromophore. The presence of tumor expressing the β-galactosidase protein was confirmed visually.

In Vivo Residual Tumor Model

In vivo experiments assessed MCA sarcoma tumor growth in a model of residual disease. Consistent tumor establishment was seen in 170/171 rats implanted with tumor in experiments conducted in our laboratory. This model attempted to approximate clinical postresection sarcoma. On Day 0, 41 Fischer rats underwent tumor implantation on Day 0 as described in the β-galactosidase expression section. On Day 3, animals were anesthetized with pentobarbital sodium, their prior incision was opened with gentle traction, and the incorporated tumor was identified. Animals were randomized to receive intratumoral injection of AdMLP.HSTK 10^9 pfu, AdCMV.Null 10^9 pfu, or viral suspension buffer in 50 μl total volume through a 28-gauge needle. Starting on Day 4 and continuing through Day 14, the animals were administered GCV (50 mg/kg) b.i.d. or saline ip. Tumors were excised and weighed on Day 14. The randomization of animals to both intratumoral treatment and subsequent ip administration of GCV or saline resulted in the creation of five experimental groups. The treatment group consisted of 12 animals that received intratumoral AdMLP.HSTK and then GCV ip. Control groups were designed to evaluate the individual effects of AdMLP.HSTK intratumoral injection, intratumoral viral suspension buffer injection, and GCV ip administration. The effect of combined AdCMV.Null intratumoral injection with subsequent GCV ip administration and the impact of intratumoral viral suspension buffer injection followed by ip saline were also evaluated.

Animal Care

Animals (male Fischer 344 rats (F344), 200–250 g, Charles River, Kingston, NY) were treated in accordance with the "Principles of Laboratory Animal Care" formulated by the National Society of Medical Research and the NIH "Guide for the Care and Use of Laboratory Animals" NIH Publication 86-23, revised 1985). Experiments were approved by the Institutional Animal Care and use Committee (IACUC), Memorial Sloan–Kettering Cancer Center. All rats were allowed access to standard laboratory rat chow (Purina Rat Chow, St. Louis, MO).

Statistical Analysis

Comparison of counts per minute of incorporated tritiated thymidine into MCA sarcoma cells under various conditions and Day 14 tumor weights of animals in different groups was by the nonparametric test to evaluate means, Mann–Whitney *U.* Significance was defined as $P < 0.05$.

RESULTS

In Vitro

In vitro assessment of an antiproliferative effect of AdMLP.HSTK and GCV revealed a significant de-

crease in [^{3}H]thymidine incorporation as the multiplicity of AdMLP.HSTK infection increased from 0 to 100 in the presence of GCV ($P < 0.05$). There was not a significant decrease in [^{3}H]thymidine incorporation as the m.o.i. of AdCMV.Null increased from 0 to 100 in the presence of GCV (Fig. 1).

Increasing GCV concentrations had a significant antiproliferative effect on MCA sarcoma cells infected with AdMLP.HSTK. Ganciclovir administration to MCA sarcoma cells without prior viral infection did not result in a significant change in [^{3}H]thymidine incorporation. There was no decrease in [^{3}H]thymidine incorporation of MCA sarcoma cells as GCV was increased from 0 to 5 μg/ml. A small but significant decrease in [^{3}H]thymidine incorporation was evident in cells infected with AdCMV.Null without GCV and with those treated with 5 and 10 μg/ml GCV (Fig. 2).

In Vivo

MCA sarcoma tumor growing in the flanks of the Fisher rat expressed the β-galactosidase protein after infection with AdCMV.LacZ. The blue chromophore, resultant from expressed β-galactosidase protein and exposure to X-gal reagent, was evident in the tumor nodule. The percentage transfection based on visual estimation from cross sections of tumor after B-Gal staining was between 30 and 50%. This quantification is only an approximation. (Fig. 3).

The mean tumor weight after excision on Day 14 is illustrated in Table 1. Tumors in the group of animals that received intratumoral AdMLP.HSTK with subsequent b.i.d. ip GCV (AdMLP.HSTK/GCV) weighed 3.8 g ($\pm$ 0.6 g). The mean tumor weight of animals in this group (AdMLP.HSTK/GCV) was significantly smaller than the mean tumor weights of animals in each of the other control groups ($P < 0.05$). Control groups consisted of animals who received intratumoral AdMLP.HSTK and then ip saline (AdMLP.HSTK/saline), AdCMV.Null intratumorally with subsequent GCV (AdCMV.Null/GCV), intratumoral viral suspension buffer, and then subsequent GCV (buffer/GCV), and animals who received intratumoral viral suspension buffer and then ip saline (buffer/saline).

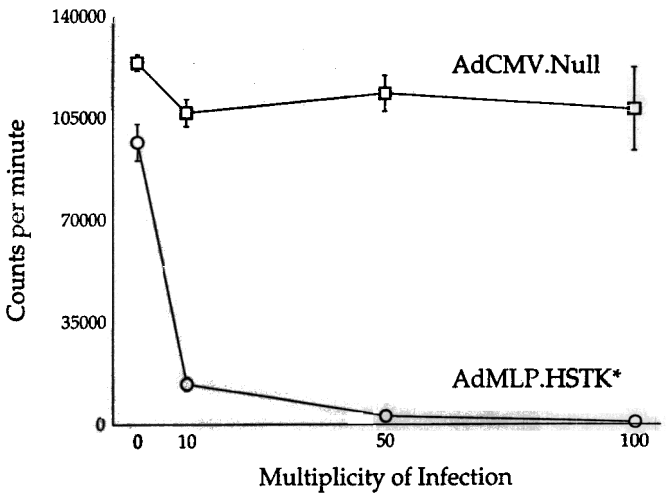

FIG. 1. The effect of increasing multiplicity of viral infection (m.o.i.) and constant GCV dose on MCA sarcoma cell [^{3}H]thymidine incorporation. Error bars reflect standard deviation. A significant antiproliferative effect was revealed as m.o.i. increased from 0 to 100 ($P < 0.05$ for each increase in m.o.i.) of cells infected with AdMLP.HSTK prior to GCV.

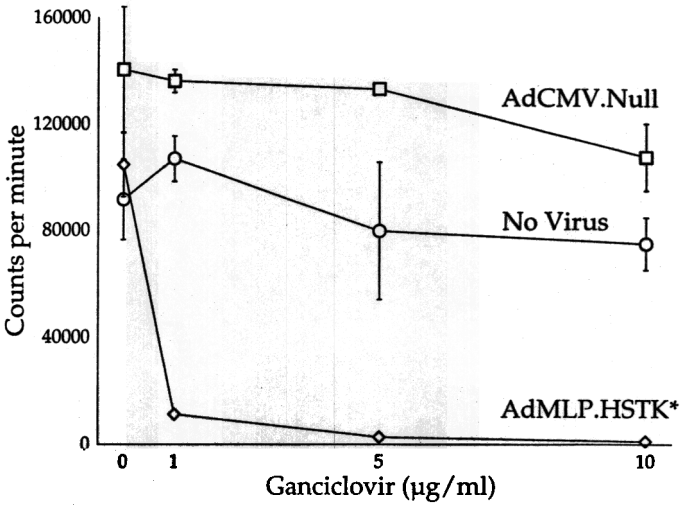

FIG. 2. The effect of increasing GCV dose on MCA sarcoma cells infected with AdMLP.HSTK, AdCMV.Null, or without prior adenoviral infection. [^{3}H]Thymidine incorporation decreased as GCV dose was increased in cells infected with AdMLP.HSTK ($P < 0.05$ for each increase in GCV). Error bars reflect standard deviation.

DISCUSSION

Despite optimal multimodality therapy, the problem of soft tissue sarcoma recurrence persists. This study demonstrated the ability of adenoviral-mediated prodrug gene therapy to diminish the growth of sarcoma *in vitro* and in an *in vivo* model of residual disease. The ability to treat *in vivo* sarcoma with a herpes simplex thymidine kinase/ganciclovir prodrug gene therapy strategy is novel.

A scenario where the HSTK/GCV paradigm could be employed clinically is to treat the tumor bed remaining after excision of a soft tissue sarcoma. Local recurrence of sarcoma must occur because of residual tumor cells remaining after operation. The tumor bed of a resected sarcoma has features that may enhance the success of prodrug gene therapy. These characteristics are the minimal amount of residual disease that can exist and the superficial nature of these residual cells. A small amount of residual disease augments the likelihood of successful therapy. Superficial, nonbulky disease facilitates adenovirus access to tumor cells. The "bystander effect" may prevent the need for every tumor cell to be infected with virus. The induced death of cells adjacent to adenovirus-infected cells maximizes potential antitumor efficacy.

Evaluating tumor treatment in a model which approximated clinical postresection sarcoma was important. Our model of residual sarcoma was created on the principle that a tumor must have an established

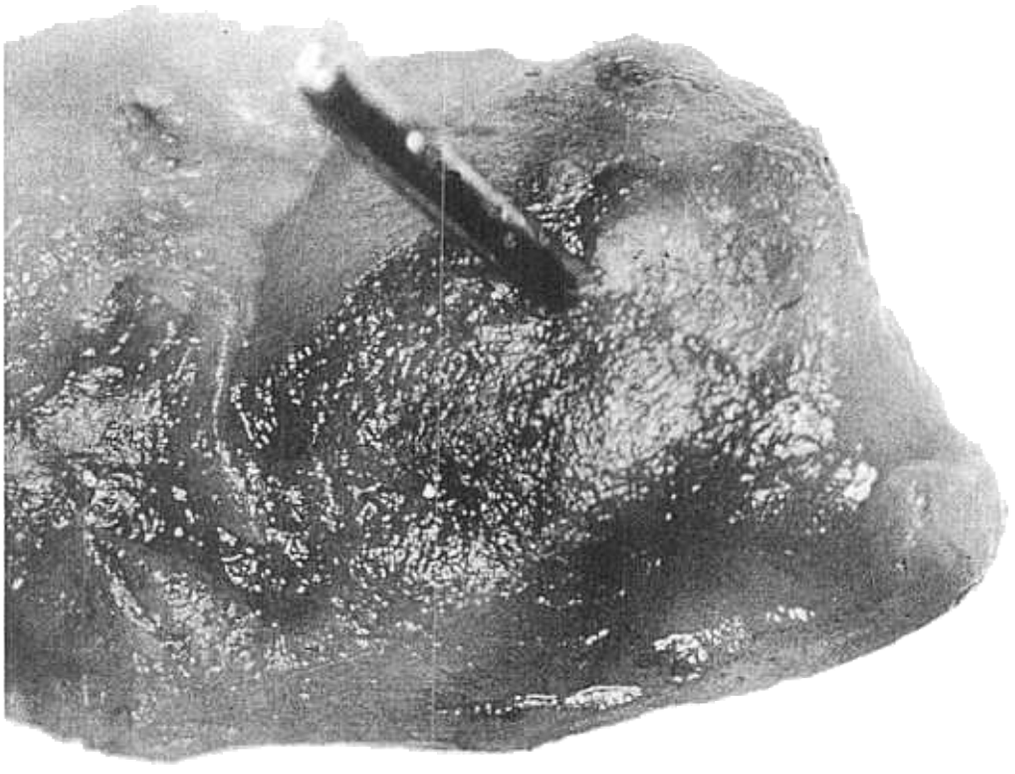

FIG. 3. Photograph of MCA sarcoma tumor expressing the blue chromophore, indicating β-galactosidase production. The metal pin entering the tumor indicates the path of the initial intratumoral injection.

blood supply to achieve rapid growth [14]. In this model of residual disease, MCA sarcoma tumor implanted in the latissimus muscle exhibited rapid consistent growth from Day 3 after implantation. In order to affect tumor growth and not tumor implantation, treatment with intratumoral adenovirus injection was started on Day 3.

Adenoviral infection and GCV administration exhibited independent antitumor growth effects in our study. Adenovirus infection is known to be cytopathic to cells *in vivo*. Adenoviral infection elicits a strong inflammatory response of predominantly cytotoxic T ($CD8^+$) lymphocytes [8]. Tumor growth inhibition by GCV has been seen in other studies [6]. MCA sarcoma cells were sensitive to elevated doses of GCV *in vitro* as revealed by decreased [^{3}H]thymidine incorporation. The weight gain of animals in the GCV alone group was less over the study length than animals not receiving GCV but not significantly. We chose to treat animals with GCV for 10 days to maximize antitumor effects of the combination of GCV and TK, understanding that transgene expression was probably less than 10 days. GCV had an antianabolic effect on the rats that could be related to both daily and total doses received. We do not have an explanation for the antitumor growth effect.

TABLE 1

Day 14 Tumor Weights after Excision

Group	Tumor weight (mean ± SEM in g)
AdMLP.HSTK/GCV (n = 12)	3.8 ± 0.6*
AdMLP.HSTK/saline (n = 9)	6.9 ± 1.2
AdCMV.Null/GCV (n = 9)	8.3 ± 1.8
Buffer/GCV (n = 7)	7.2 ± 1.2
Buffer/saline (n = 4)	19.7 ± 2.2

Note. SEM, standard error of the mean.
* $P < 0.05$ vs all control groups.

Clinically, the dimensions of a postresection field after sarcoma excision can measure 20 × 20 cm. The ability to treat residual tumor cells over a large field with adenoviral mediated prodrug gene therapy presents a challenge. Further, although treated tumors were smaller at the end of the treatment period, they were not eradicated. We believe this indicates that the treatment was able to kill a proportion of the initial tumor cells but cells remaining after transgene expression stopped and cells not infected with the TK gene grow normally. Optimization of both vector and prodrug are necessary and are currently under study in our laboratory. Strategies include more specifically targeting tumor cells through promoter manipulation and discovering means to enhance immune recognition of tumor. The demonstration of adenovirus-mediated herpes simplex thymidine kinase prodrug gene therapy to inhibit *in vivo* tumor growth has encouraged us to pursue further study of this modality as an adjuvant treatment of human soft tissue sarcoma.

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