Evaluation of HSV-tk Gene Therapy in a Rat Model of Chemically Induced Hepatocellular Carcinoma by Intratumoral and Intrahepatic Artery Routes

René Gerolami, Jorge Cardoso, Maïté Lewin, Marie-Pierre Bralet, Antonio Sa Cunha, Olivier Clément, Christian Bréchot, and Phuong-Lan Tran

INSMER U370, Necker Faculty of Medicine, 75730 Paris [R. G., A. S. C., B. P.-L. T.]; Laboratoire de Recherche en Chimurgie, INSERM 94–05, Hopital Cochin, 75014 Paris [J. C.]; Laboratoire de Recherche en Imagerie, INSERM U494, Necker Faculty of Medicine, 75730 Paris [M. L., O. C.]; and Service d’Anatomie Pathologique, Hopital Beaujon, 92110 Clichy [M.-P. B.], France

ABSTRACT

Transfer of the herpes simplex virus-thymidine kinase (HSV-tk) gene followed by the administration of ganciclovir (GCV) into hepatocellular carcinoma (HCC)-derived cell lines either in vitro or transplanted into nude mice has been shown to provide a potential strategy for HSV-tk-based gene therapy of HCC. We report herein an analysis of the antitumor efficacy of two recombinant adenoviruses (Ads), Ad.CMVtk and Ad.AFPtk, in a relevant model of multifocal hepatic lesions induced in rats by a potent alkylating chemical carcinogen, diethylnitrosamine. Two routes of administration of the Ad were studied: intratumoral and intrahepatic artery injections. Both recombinant Ads, Ad.CMVtk and Ad.AFPtk, express the HSV-tk gene under the control of the early enhancer/promoter cytomegalovirus and α-fetoprotein regulatory gene sequences, respectively. The antitumor response was assessed by magnetic resonance imaging and by autopsy and histological analysis following postmortem. Tumor growth cessation was demonstrated by magnetic resonance imaging in large tumor nodules of size 5–8 mm treated by intratumoral administration of 2 × 10⁹ pfu Ad.CMVtk plus i.p. treatment with GCV. We also show an antitumor effect in small tumor nodules of size <3 mm treated with 2 × 10⁹ pfu Ad.CMVtk plus GCV by the intrahepatic artery route, albeit associated with an adverse toxicity. In vivo targeting of the HSV-tk gene to diethylnitrosamine-induced HCC cells with the recombinant Ad.AFPtk suppresses the hepatic toxicity in the nontumoral liver. The lower antitumor response would argue for the use of multiple injections of such adenoviral constructs. These observations may lead to potential approaches for designing gene therapy destined for early treatment of dysplastic nodules or advanced HCC in cirrhosis.

INTRODUCTION

Several approaches aimed at in vitro and in vivo inhibition of HCC tumor growth have shown promising results in terms of prospects for gene therapy in primary liver cancer. Adenoviral- or retroviral-mediated transfer of sequences encoding for the HSV-tk gene expression, but they clearly do not enable us to address the specific problems encountered in human HCC due to the pattern of tumor vascularization.

To address this issue, we and others have been investigating rat HCC induced by DEN injection. In this classical model of liver carcinogenesis, HCC develops against a background of increased liver cell proliferation in immunocompetent animals (16, 17). We have investigated in detail this model and have shown that, as with human tumors, HCC displays predominant arterial vascularization, thus pointing to the need for selective IHA or IT injections (18). We have also shown the overall low transduction rate of tumor cells, although IHA injection of small nodules and dysplastic nontumor areas yielded higher efficacy (18).

Although encouraging, such observations do not address a major issue in gene therapy designed for primary liver cancer, namely that it develops in most cases against a background of CAH and cirrhosis (10, 11). HCC, the most prevalent histological form of primary liver cancer, indeed occurs with a yearly rate of 3–5% in patients with cirrhosis (10). Although present therapies of human HCC tumors, including liver transplantation, have been improved, the cure rate for this disease still remains low. In contrast with normal liver, the vascularization of cirrhotic livers is predominantly arterial, and this feature is reinforced in HCC-bearing patients, in whom up to 70% of the blood supply is dependent on the hepatic artery (12, 13). Cirrhosis and HCC tumors also show the so-called “sinusoid capillarization” whereby the normal fenestration of liver sinusoidal endothelial cells is abrogated (14, 15). The necrotic and inflammatory lesions that drive CAH and cirrhosis stimulate liver cell DNA replication. Hence, this latter process may favor targeting of toxic compounds to nontumor cells, such as those generated upon HSV-tk transduction and related toxicity mediated by phosphorylated GCV. Finally, given the generally poor liver function observed in patients with advanced liver cirrhosis, the potentially toxic effects of gene transfer may have marked deleterious consequences. These observations stress the need, when designing vectors and routes of administration, for the use of relevant animal models. Tumors that develop in immunodeficient mice are extremely useful for investigating the parameters of the therapeutic effect of HSV-tk gene expression, but they clearly do not achieve the specific problems encountered in human HCC due to the pattern of tumor vascularization.
demonstrated that this hepatoma-specific expression of murine and human AFP regulatory sequences is retained in adeno- and retroviral vectors (19–21). However, the efficacy and toxicity of this approach in an in vivo model such as DEN-induced rat HCC have not yet been evaluated. The present study includes an analysis of antitumoral efficacy and eventual related toxicity in HSV-tk/GCV gene therapy. Our results lead us to propose different modalities in gene therapy for the early treatment of dysplastic nodules or advanced HCC in cirrhotic patients.

MATERIALS AND METHODS

Construction of Recombinant Ads

The construction of pAd.AFPtk and of the recombinant Ad.AFPtk,2 are performed as described by Tran et al. (22). Infectious viruses were purified by plaques, and recombinant Ad was tested for HSV-tk gene expression using a titer cell proliferation assay in the presence of absence of GCV.

The E1-deleted recombinant Ad.CMVlacZ and Ad.CMVtk containing, respectively, Escherichia coli lacZ and HSV-tk genes, were kindly provided by Drs C. Qian and J. Prieto (5).

All recombinant Ads were amplified on 293 cells and purified by double cesium chloride density gradient ultracentrifugation. Titers of the adenoviral stocks were determined by plaque assay on 293 cells.

Cell Culture

Two AFP-producing human hepatoma cell lines ( HepG2 and HuH7) and a human cervical cancer cell line (HeLa) were grown in DMEM (Life Technologies, Inc., Gaithersburg, MD) containing 8% FCS, 2 mM glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin.

To test the sensitivity to GCV of infected cells, HepG2, HeLa cells were plated in triplicate wells into a 96-well plate, and various MOIs of Ad.AFPtk were infected. Twenty h after infection, increasing concentrations (0, 1, 5, 10, and 20 μg/ml) of GCV (Synthex Laboratories, Inc., Palo Alto, CA) were added. Cell proliferation was then measured with a nonradioactive cell proliferation assay according to the manufacturer’s protocol (CellTiter 96 AQueous Non-Radioactive Cell Proliferation Assay; Promega, Madison, WI).

The percentage of surviving cells is presented as a percentage of the absorbance observed in GCV-treated cells divided by that of cells without GCV treatment (mean ± SD).

Tumor Induction and Tumor Size Assessment

Male Wistar rats (6 weeks old, 150–180 g) were purchased from Iffa Credo (l’Arbresle, France). They were acclimatized for 15 days before DEN treatment. Rats were treated at a dose of 10 mg/kg/day of DEN (Sigma) for 60 days (Figs 1 and 2). They received their dose of DEN in drinking water at 100 mg/liter from a fresh DEN solution prepared weekly. Multifocal hepatic lesions, including dysplastic nodules and tumors, were apparent in 60% of the animals 10 weeks after beginning exposure to the carcinogen.

MRI was performed on rats deeply anesthetized with i.p. ketamine (0.5 ml/kg) and xylazine (0.5 ml/kg; Rhoˆne Poulenc, France) using a 1.5 Tesla (T) magnet (Signa, General Electric, Milwaukee, WI) with a radiofrequency coil adapted for rat experiments. A T2-weighted spin-echo sequence was used with a resolution of 0.92 × 0.92 mm2; acquisition time = 4 sec; excitation time = 1000 ms; field of view = 40–60 mm; matrix size = 256 × 256; and slice thickness = 2 mm. T2-weighted images were compared to the arterial blood flow interruption varied between 5 and 15 min. On day 77, all rats received a dose of 50 mg/kg/day GCV diluted in saline buffer by i.p. administration started on the following day and lasted from days 82 to 91. Evaluation of tumor growth by MRI was assessed on day 94, 3 days after the last GCV dose, and all animals were sacrificed on day 95. During GCV treatment, the death of one rat in Ad.CMVtk + GCV-treated rats was reported in each experiment. Autopsy revealed i.p. hemorrhage, and histological examination showed evidence of acute hepatitis. In control groups, one case of death was observed in saline-injected rats related to a hemorrhage of the injected tumor. No deaths were reported in Ad.CMVlacZ-treated rats.

**Ad.AFPtk.** Two independent experiments were performed in the groups of tumor-bearing rats treated with Ad.AFPtk. As a typical example, 12 tumor-bearing rats were randomly distributed into two groups and treated with either 5 × 109 pfu Ad.AFPtk (n = 8) or saline buffer (n = 4). The treatment schedule was the same as previously described in Fig. 1A. No clinical signs or morbidity were observed during GCV treatment. Deaths of Ad.AFPtk-treated (four of eight) and saline-injected (one of four) rats were essentially due to their advanced pathology, with a bleeding tumor provoking i.p. hemorrhage.

**Ad.CMVtk.** Two independent experiments were performed with recombinant Ad.CMVtk as scheduled in Fig. 1B. Tumor-bearing rats with multiple tumors of size < 3 mm were selected by MRI on day 75 and randomly assigned to various groups. They were laparatomized under ether anesthesia on day 76. After resection of the hepatic pedicles, the celiac artery was clamped. A 30 1/2-gauge needle was introduced into the hepatic artery via the gastroduodenal artery. Recombinant Ad.CMVtk or Ad.CMVlacZ or saline buffer was then injected in a volume of 400 μl, followed by an equivalent volume of saline buffer. After injections of both solutions, the gastroduodenal artery was ligated, and blood flow into the hepatic artery was re-established. The duration of the arterial blood flow interruption varied between 5 and 15 min. On day 77, all rats received a dose of 50 mg/kg/day GCV diluted in saline buffer by i.p. administration on day 10. The first experiment included 21 rats injected with either a dose of 2 × 106 pfu Ad.CMVtk (n = 8), 106 pfu Ad.CMVtk (n = 7), 2 × 105 pfu Ad.CMVlacZ (n = 3), or saline buffer (n = 3); the second experiment was repeated with 14 rats injected with either a dose of 2 × 105 pfu Ad.CMVtk (n = 7), 2 × 105 pfu Ad.CMVlacZ (n = 4), or saline buffer (n = 3). Follow-up of the experiments included controls of the morbidity, mortality, and a second MRI on day 105. In both experiments, treatment with HSV-tk/GCV induces early toxicity manifested by mild to severe hepatitis. Mortality recorded was due to HSV-tk-related toxicity and/or to the advanced pathology. All rats were kept for survival recording up to day 125. The surviving rats were then killed. The evaluation of the antitumor response was

In Vivo Gene Therapy

All animal experiments were reviewed and approved by institutional committees for animal care and use and for recombinant DNA research (Minist`ere de l’Agriculture, Paris, France).

Evaluation of the efficacy of HSV-tk gene therapy was performed on DEN-treated rats with tumors of size 5–8 mm or < 3 mm. Two recombinant Ads, Ad.CMVtk and Ad.AFPtk, were assessed. Control animals received either Ad.CMVlacZ, Ad.AFPlacZ, or saline buffer. Two experimental schedules were designed for IT and IHA administrations as shown in Fig. 1, A and B, respectively.

Administration of Recombinant Ads Via the IT Route

**Ad.CMVtk.** Two independent experiments on HSV-tk/GCV efficacy upon IT administration of recombinant Ad were performed in tumor-bearing rats using Ad.CMVtk and Ad.CMVlacZ, or saline buffer. The experimental schedule is shown in Fig. 1A. The rats were monitored by MRI on day 80 to select homogeneous groups of animals with advanced tumors of 5–8 mm in size. On the following day (day 81), they were randomized and laparatomized under ether anesthesia. Then the selected tumor nodule received a volume of 100 μl containing either recombinant Ad or saline buffer as follows: in the first experiment, seven rats received injections of 2 × 106 pfu Ad.CMVtk, and five rats received injections of 2 × 107 pfu Ad.CMVlacZ; the second experiment included eight rats receiving injections of 2 × 107 pfu Ad.CMVtk and seven rats receiving injections of saline buffer. In both experiments, GCV treatment by the i.p. route started on the following day and lasted from days 82 to 91. Evaluation of tumor growth by MRI was assessed on day 94, 3 days after the last GCV dose, and all animals were sacrificed on day 95. During GCV treatment, the death of one rat in Ad.CMVtk + GCV-treated rats was reported in each experiment. Autopsy revealed i.p. hemorrhage, and histological examination showed evidence of acute hepatitis. In control groups, one case of death was observed in saline-injected rats related to a hemorrhage of the injected tumor. No deaths were reported in Ad.CMVlacZ-treated rats.

**Ad.AFPtk.** Two independent experiments were performed in the groups of tumor-bearing rats treated with Ad.AFPtk. As a typical example, 12 tumor-bearing rats were randomly distributed into two groups and treated with either 5 × 109 pfu Ad.AFPtk (n = 8) or saline buffer (n = 4). The treatment schedule was the same as previously described in Fig. 1A. No clinical signs or morbidity were observed during GCV treatment. Deaths of Ad.AFPtk-treated (four of eight) and saline-injected (one of four) rats were essentially due to their advanced pathology, with a bleeding tumor provoking i.p. hemorrhage.

Selective Administration of Recombinant Ads Via the IHA Route

**Ad.CMVtk.** Two independent experiments were performed with recombinant Ad.CMVtk as scheduled in Fig. 1B. Tumor-bearing rats with multiple tumors of size <3 mm were selected by MRI on day 75 and randomly assigned to various groups. They were laparatomized under ether anesthesia on day 76. After resection of the hepatic pedicles, the celiac artery was clamped. A 30 1/2-gauge needle was introduced into the hepatic artery via the gastroduodenal artery. Recombinant Ad.CMVtk or Ad.CMVlacZ or saline buffer was then injected in a volume of 400 μl, followed by an equivalent volume of saline buffer. After injections of both solutions, the gastroduodenal artery was ligated, and blood flow into the hepatic artery was re-established. The duration of the arterial blood flow interruption varied between 5 and 15 min. On day 77, all rats received a dose of 50 mg/kg/day GCV diluted in saline buffer by i.p. administration on day 10. The first experiment included 21 rats injected with either a dose of 2 × 106 pfu Ad.CMVtk (n = 8), 106 pfu Ad.CMVtk (n = 7), 2 × 105 pfu Ad.CMVlacZ (n = 3), or saline buffer (n = 3); the second experiment was repeated with 14 rats injected with either a dose of 2 × 105 pfu Ad.CMVtk (n = 7), 2 × 105 pfu Ad.CMVlacZ (n = 4), or saline buffer (n = 3). Follow-up of the experiments included controls of the morbidity, mortality, and a second MRI on day 105. In both experiments, treatment with HSV-tk/GCV induces early toxicity manifested by mild to severe hepatitis. Mortality recorded was due to HSV-tk-related toxicity and/or to the advanced pathology. All rats were kept for survival recording up to day 125. The surviving rats were then killed. The evaluation of the antitumor response was

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assessed by recording their body and liver weights and the number of tumor nodules. Because tumor growth was variable, the number of tumor nodules included tumor nodule sizes from 1–10 mm.

Ad.AFP<sub>tk</sub>. The evaluation of the efficiency of Ad.AFP<sub>tk</sub> + GCV on tumor growth and comparison with that of Ad.CMV<sub>tk</sub> + GCV was assessed by three experiments. A typical experiment is reported herein: tumor-bearing rats with homogeneous tumor sizes <3 mm, as monitored by MRI, were randomly assigned to various groups on day 75. After being ether-anesthetized and laparotomized, they were injected by the IHA route with either a dose of $5 \times 10^9$ pfu Ad.AFP<sub>tk</sub> ($n = 6$), $10^9$ pfu Ad.CMV<sub>tk</sub> ($n = 4$), or $5 \times 10^9$ pfu Ad.AFP<sub>lacZ</sub> ($n = 6$), or saline buffer ($n = 6$). No morbidity or mortality was observed in Ad.AFP<sub>tk</sub>-injected rats. No sign of toxicity was observed in Ad.AFP<sub>tk</sub>-, Ad.AFP<sub>lacZ</sub>- or saline-treated rats. Instead, high toxicity was observed in Ad.CMV<sub>tk</sub>/GCV-treated rats during GCV treatment, leading to death of all them. Autopsy of deceased animals in the control groups showed that it was essentially due to their advanced pathology because histological examination did not reveal evidence of hepatitis. The others were kept for survival recording. They were then sacrificed. Their liver weights were noted, and the number of tumor nodules was recorded.

**Toxicity Study in Tumor-free Rats**

Twenty-five male Wistar rats (10–12 weeks old; 300 g) were ether-anesthetized and laparotomized. They were injected through the hepatic artery with a dose of either recombinant Ad $2 \times 10^9$ pfu Ad.CMV<sub>tk</sub> ($n = 15$) or $2 \times 10^9$ pfu Ad.CMV<sub>lacZ</sub> ($n = 10$), as described above. The following day, 10 of 15 Ad.CMV<sub>tk</sub>- and 5 of 10 Ad.CMV<sub>lacZ</sub>-treated rats were treated with GCV at a dose of 50 mg/kg/day for 10 days. The remaining rats were left without GCV administration. Three days after the end of GCV treatment, blood samples were collected for assay of liver serum enzymes (AST and ALT), and all animals were sacrificed for macroscopic examination. The liver and various organs were removed and processed for histology.

![Fig. 1. A, experimental schedule of administration of recombinant Ad by the IT route and GCV treatment. Treatment with DEN and MRI schedules are also depicted. B, experimental schedule of administration of recombinant Ad by the IHA route and GCV treatment. Treatment with DEN and MRI schedules are also depicted.](image1.png)

![Fig. 2. T2-weighed magnetic resonance images of two representative rat livers showing tumor nodules before and after treatment with $2 \times 10^9$ pfu Ad.CMV<sub>tk</sub> + GCV. A and C, day 80 before treatment; B and D, day 94 after IT injection of $2 \times 10^9$ pfu Ad.CMV<sub>tk</sub> and i.p. GCV treatment. For each rat, the treated tumor is indicated (arrowhead). In both cases, a reduction in tumor size is depicted on images B and D: image B, $-73\%$ of tumor growth, and image D, $-53\%$ of tumor growth, respectively (see Table 1). Growth of the surrounding untreated tumors was increased: image B, $+92\%$ of tumor growth, and image D, $+67$ and $+71\%$ of tumor growth, respectively (see Table 1).](image2.png)
Histology

Liver samples from tumoral and nontumoral areas of each DEN-treated rat, and liver samples as well as various organs (spleen, intestine, pancreas, kidney, and lung) from tumor-free rats were collected on the day of sacrifice or after death of the animal. They were fixed in 4% formaldehyde and then embedded in paraffin. Staining of 3-μm-thick sections was performed with H&E.

Statistics

Comparisons of the survival rate, number of tumors, liver weight, and tumor growth between rats from different groups were performed using the nonparametric distribution Mann and Whitney test.

RESULTS

Tumor Development

Male Wistar rats were treated with a daily dose of 10 mg of DEN/kg of body weight for 2 months. Multiple microscopic hepatic lesions arose in 60% of rats 8 weeks after the first DEN dose, in agreement with a previous report (23). The development of neoplastic lesions arose in 60% of rats 8 weeks after the first DEN dose, in experimental conditions of dose and duration of treatment, 40% of tumor-bearing rats had tumor sizes of 5–8 mm and <3 mm. Histological examination of these neoplastic nodules (dysplastic and HCC nodules) showed that they presented all features of cellular alterations characteristic of a process of hepatocarcinogenesis (not shown), as previously described (24).

Table 1  IT administration of recombinant Ad

<table>
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<td>Untreated</td>
<td>Treated</td>
</tr>
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<td>+66</td>
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*NM, nonmeasurable tumor because its size is below the detection threshold.

*P < 0.001.

*P < 0.05.

Tumor Growth Delay following IT Administration of Ad.CMVtk + GCV

The efficacy of HSV-tk/GCV treatments on DEN-induced rat HCC was first evaluated by IT injection of large hepatic tumor nodules of size 5–8 mm in diameter with either 2 × 10^5 pfu Ad.CMVtk, 2 × 10^6 pfu Ad.CMVLacZ, or saline buffer into each selected tumor of each rat. The tumor sizes of injected and noninjected tumors were evaluated by MRI 14 days after initiation of the treatment. Fig. 2 depicts representative magnetic resonance images of two individual rats showing, for each animal, an antitumor response after treatment with 2 × 10^5 pfu Ad.CMVtk + 50 mg/kg/day GCV, whereas no reduction in tumor size was noted in surrounding untreated tumors. Table 1 pools together the results of two experiments (experiments I and II). Each value represents separately the percentage of tumor growth calculated for each treated and surrounding untreated tumor in each animal from each group of rats. In some rats, one or two noninjected tumors were measured, whereas in others, the noninjected tumors were not measurable due to their size below the detection threshold.

In the two experiments, the results showed that Ad.CMVtk-treated tumors responded to HSV-tk + GCV treatments as follows: 5 of 13 tumors had significant regression (−22 to −73% of tumor growth), 4 tumors had pronounced growth cessation (+10–29% of tumor growth), and growth of 3 tumors was delayed (+56–90% of tumor growth). In control groups, growth of Ad.CMVLacZ-treated tumors fluctuated from +53 to +196% of tumor growth, and that of saline-injected tumors fluctuated from +20 to +880%. Likewise, growth of the surrounding untreated tumors from all rats unevenly fluctuated. The mean values of tumor growth determined for all treated and untreated tumors in each group of rats were, respectively, +5% of tumor growth in Ad.CMVtk-treated tumors, +125% in Ad.CMVLacZ-treated tu
mors, and +221% in saline-injected tumors ($P < 0.001$; Table 1). Table 1 also showed no difference in the mean values of tumor growth between all untreated tumors and saline-injected tumors.

Histological sections from Ad.CMVtk + GCV-treated tumors showed a combination of apoptosis, necrosis, and inflammatory infiltration (Fig. 3, A and B). The inflammatory response was moderate in Ad.CMVtk + GCV-treated tumors and was composed predominantly of mononuclear cells (Fig. 3A). No evidence of hepatic lesion was observed in the nontumoral liver parenchyma (not shown). No inflammatory infiltrate was observed in any control groups. In conclusion, treatment of DEN-induced HCC tumors of size 5–8 mm by IT administration of Ad.CMVtk + GCV elicited tumor growth cessation with low toxicity.

**Efficacy of Ad.CMVtk and GCV Treatment on Tumor Growth following IHA Administration of the Recombinant Virus and Survival**

All rats had multiple macroscopic tumor nodules with varying sizes from 1 to 2 mm diameter. By contrast with treatment of advanced tumors by IT administration of the viral vector, we evaluated the effect of HSV-tk + 50 mg/kg/day GCV treatment on small hepatic tumor nodules by IHA administration of Ad.CMVtk. The results obtained in the two experiments, A and B, were pooled together for statistical analyses. Table 2 reports the effect of Ad.CMVtk/GCV on tumor burden as measured by liver weight 50 days after initiation of the treatment. Both groups of animals that were treated with either $2 \times 10^9$ or $10^9$ pfu Ad.CMVtk + GCV showed a decrease in tumor burden, as measured by weight. In contrast, control rats injected with Ad.CMVlacZ + GCV had an increase in liver weight similar to that of saline-injected rats. The latter observation can be correlated with the number of tumors listed for each liver (Table 2). The mean number of tumors in rats treated with $2 \times 10^9$ pfu Ad.CMVtk was 8.8, and that of rats treated with $10^9$ pfu was 16.4, as compared to a mean value of 29 in Ad.CMVlacZ-treated rats and 27 for the saline-injected rats ($P < 0.001$). This demonstrates that the high Ad.CMVtk dose had a more efficient therapeutic effect.

However, early HSV-tk/GCV-related toxicity in Ad.CMVtk + GCV-treated rats was reported 5 days after the beginning of GCV treatment. The deaths of 10 of 15 rats treated with $2 \times 10^9$ pfu Ad.CMVtk + GCV were recorded 20 days after initiation of GCV treatment, compared with the death of two of seven rats treated with $10^9$ pfu Ad.CMVtk + GCV (Table 2). Deaths in the control groups of rats injected with either $2 \times 10^9$ pfu Ad.CMVlacZ + GCV or saline + GCV were essentially due to their advanced pathology. The survival rate between the two groups of Ad.CMVtk + GCV-treated rats was not significantly different ($P > 0.09$), whereas the survival rate between rats treated with $2 \times 10^9$ pfu Ad.CMVtk + GCV and that of $2 \times 10^9$ pfu Ad.CMVlacZ + GCV or saline-injected rats was significantly different ($P < 0.05$). Thus, high doses of Ad.CMVtk + GCV were found to be toxic for the animals.

Histological examination of liver sections from the surviving Ad.CMVtk-treated rats showed remnants of hepatic tumors associated with a few dysplastic areas, and the structure of large areas of liver...
AFP-producing cells.

Growth and Survival.

A scheduled in Fig. 1. IT injection of Ad.AFP was performed in vivo gene in DEN-induced HCC in rats, expression of the targeted HSV-tk gene resulted in GCV killing of only Ad.AFP-treated rats. No mortality was reported in Ad.AFP-treated rats, whereas all four Ad.CMV-tk + GCV-treated rats died during GCV treatment. Data show a weak antitumoral effect in Ad.AFP-treated rats, as compared to that observed in Ad.CMVtk/GCV-treated rats.

In conclusion, targeting HSV-tk to HCC under the control of AFP regulatory sequences abolished the hepatic toxicity induced in the nontumoral liver parenchyma by HSV-tk/GCV. The limited antitumor effect observed in rats, which were injected with a single treatment dose of recombinant Ad by IT or IHA routes, should advocate the use of multiple injections of recombinant Ad.

Targeted Expression of the HSV-tk Gene to HCC Cells

In Vitro Biological Activity of Recombinant Ad.AFPtk Containing the HSV-tk Gene Under the Control of the Rat AFP Gene Promoter. Previous results from our laboratory showed the need for specific targeting of the recombinant Ad.AFPlacZ to HCC cells (20). We therefore constructed a recombinant Ad, Ad.AFPtk, containing the HSV-tk gene controlled by the rat AFP gene regulatory sequences. Two human HCC cell lines, HuH7 and HepG2, and a non-HCC cell line, HeLa, were used as target cells for the recombinant Ad infection. At a MOI of 500, all HCC and HeLa cells transduced with Ad.AFPtk showed cytopathic effects. After infection with adenoviral vector Ad.AFPtk, cells were treated with varying doses of GCV for 5 days, and the number of viable cells was determined by a cell proliferation assay. The AFP-producing HuH7 and HepG2 cells were more sensitive to this effect than HeLa cells. HuH7 cells infected with Ad.AFPtk displayed GCV sensitivity at a concentration as low as 5 μg/ml, at a MOI of 30 (Fig. 4). HeLa cells infected with Ad.AFP.tk did not show any GCV sensitivity (Fig. 4). These results indicate that Ad.AFPtk-mediated transfer of the HSV-tk gene resulted in GCV killing of only AFP-producing cells.

In Vivo Efficacy of Ad.AFPtk and GCV Treatments on Tumor Growth and Survival. To ascertain the transduction efficiency and expression of the targeted HSV-tk gene in DEN-induced HCC in rats, we carried out in vivo treatment of HCC tumors using IT or IHA injection of the recombinant Ad.AFPtk.

Antitumor therapy of DEN-induced HCC tumors was designed as scheduled in Fig. 1A. IT injection of Ad.AFPtk was performed in 5-mm HCC tumor nodules. Each designated tumor received either a dose of 10^10 pfu Ad.AFPtk or saline buffer, followed by i.p.-treatment with 50 mg/kg/day of GCV. The results displayed as experiment III in Table 1 show a pronounced growth cessation in two tumors (+2 and +23% of tumor growth), whereas growth of the other two tumors was simply delayed. A mean value of +66% of tumor growth in Ad.AFPtk-treated tumors was compared with the mean values of +521% of tumor growth in saline-injected tumors and +296% in untreated tumors (P < 0.05). Histological examination of Ad.AFPtk + GCV-treated tumors showed moderate cell apoptosis and necrosis, with a limited inflammatory response composed predominantly of mononuclear cells (Fig. 3C).

Rats bearing tumors with size <3 mm were also treated by the IHA route with either a single dose of 5 × 10^9 pfu Ad.AFPtk or a single dose of 10^10 pfu Ad.CMVtk. Control rats received, by the IHA route, either a dose of 5 × 10^9 pfu Ad.AFPlacZ or saline buffer. A 5-fold dose of the recombinant Ad.AFPtk was used, as compared to that of Ad.CMVtk. All rats were then treated by i.p. injection of GCV for 10 days. The results are summarized in Table 2 (experiment C). No toxic effect was observed in Ad.AFPtk- and Ad.AFPlacZ-treated rats. No mortality was reported in Ad.AFPtk-treated rats, whereas all four Ad.CMVtk + GCV-treated rats died during GCV treatment. Data show a weak antitumoral effect in Ad.AFPtk-treated rats, as compared to that observed in Ad.CMVtk/GCV-treated rats.

In conclusion, targeting HSV-tk to HCC under the control of AFP regulatory sequences abolished the hepatic toxicity induced in the nontumoral liver parenchyma by HSV-tk/GCV. The limited antitumor effect observed in rats, which were injected with a single treatment dose of recombinant Ad by IT or IHA routes, should advocate the use of multiple injections of recombinant Ad.

Toxicity of Ad.CMVtk and GCV in Tumor-free Rats

Histological examination of liver sections from DEN-treated rats and injected with Ad.CMVtk/GCV by the IHA route showed evidence of severe hepatitis, including apoptotic and necrotic hepatocytes with acidophilic bodies in the nontumoral liver, as well as ballooning hepatocytes (Fig. 5A). This toxic effect was much less observed in animals treated by direct IT injection of Ad.CMVtk. The enhanced and dose-dependent toxicity of the higher dose was likely due to transduction of normal hepatic cells by Ad.CMVtk and resultant toxicity mediated by phosphorylated GCV. Our present data show that absence of toxicity in the nontumoral liver parenchyma was obtained when HSV-tk was placed under the control of AFP regulatory sequences (not shown).

To further analyze HSV-tk/GCV-induced toxicity, we examined Ad.CMVtk and Ad.CMVlacZ effects in tumor-free rats in the presence or absence of GCV. Normal rats were treated by IHA injection with either 2 × 10^7 pfu Ad.CMVtk or 2 × 10^8 pfu Ad.CMVlacZ and i.p. injection of GCV for 10 days, or treated with the recombinant Ad alone. Adverse toxic effects due to HSV-tk + GCV were manifested 5 days after the beginning of GCV treatment. In addition to polyuria, they showed signs of discomfort, unhealthy coat, and loss of body weight (30% of body weight loss in Ad.CMVtk-treated rats; not shown). Four of 10 rats treated with Ad.CMVtk + GCV died during GCV treatment, i.e., between days 5 and 10. Conversely, no mortality was reported in Ad.CMVlacZ ± GCV- and Ad.CMVtk alone-treated rats. On the killing day, macroscopic aspects of the livers of Ad.CMVtk-treated rats appeared atrophic and yellowish. The evaluation of HSV-tk/GCV toxicity was studied by assaying liver serum enzymes and histological examination. Fig. 6, A-C displays the mean values of serum levels of AST and ALT, and the liver weight of the various groups of Ad-treated ± GCV rats. It showed, 14 days after
initiation of the treatment, an increase in serum liver enzymes in Ad.CMVtk + GCV-treated rats accompanied by a decrease in liver weight, indicating severe liver damage.

Histological examination of liver sections showed evidence of acute severe hepatitis in the four deceased rats. Histology showed necrotic hepatocytes with acidophilic bodies. There was few macro- or microvesicular steatosis. These findings were associated with a moderate inflammatory infiltrate of mononuclear cells (Fig. 5B). This severe hepatitis was diffused in all of the liver lobes. In the remaining Ad.CMVtk + GCV-treated rats, severe (n = 4) and moderate hepatitis (n = 2) were recorded. The spleen of Ad.CMVtk-treated rats were atrophic, whereas in the other organs (intestine, pancreas, kidney, or lung), no abnormality was reported. Finally, Table 3 summarizes the scoring of HSV-tk/GCV-related toxicity, as observed in all experiments performed with either tumor-free or DEN-induced HCC rats, following treatment with either Ad.CMVtk + GCV or Ad.AFPtk + GCV by the IHA and IT routes. We conclude that low toxicity of HSV-tk + GCV could be obtained by using IT administration of recombinant Ad and by targeting HSV-tk gene expression to HCC.

**DISCUSSION**

HSV-tk/GCV suicide gene therapy for HCC is presently being experimented in tumors transplanted into nude mice or syngeneic rats (2–4, 25, 26). It is, however, important to emphasize that such an approach far from reflects the in vivo situation. DEN-induced liver carcinogenesis provides a well controlled experimental model of cancer whereby the multifocal hepatic tumors depend on dose and duration of carcinogen exposure. Thus, the development of HCC in such a model should resemble that developed in humans. In vivo treatment of the multifocal DEN-induced HCC tumors was investigated by intraportal injection of the recombinant Ad containing HSV-tk (5). But the antitumoral efficacy of the procedure was accompanied by an important hepatic toxicity and a high mortality rate.

The results presented here may lead to an additional approach on the in vivo feasibility and impact of the HSV-tk/GCV suicide gene therapy for primary liver cancer. Our experiments provide strong support that the transfer of HSV-tk/GCV into DEN-induced rat HCC
by IT and IHA injections of Ad.CMV\textsubscript{tk} elicited an antitumor response in tumor nodules, including small and large tumors, as assessed by MRI and histological analyses. We showed that antitumor efficacy associated with low toxicity was obtained by IT administration of Ad.CMV\textsubscript{tk} into large tumor nodules and GCV treatment. Our results also stressed the major problem of adverse toxicity of HSV-\textit{tk} + GCV treatment despite the use of the selective IHA instead of the intraportal route of administration (5). Finally, we showed that targeting the HSV-\textit{tk} gene to HCC with Ad.AFP\textsubscript{tk} under the control of the AFP gene promoter suppressed adverse toxicity in the nontumoral liver parenchyma. Nonetheless, cell targeting with a tissue-specific promoter also induced a decrease in the antitumoral efficacy.

Because of the heterogeneity of tumor nodules in HCC induced by DEN treatment in rats, monitoring their sizes by MRI is an important step in the evaluation of the tumor growth. Thus, the combination of MRI with the liver weight, and histological examination at necropsy provide potential tools for its assessment. Moreover, the MRI approach allowed us to locate and select large tumor nodules for a direct IT injection into the tumor. Thus far, the growth of the selected tumor node can be analyzed and compared to that of untreated nodules, acting as internal controls. In addition, we were able to establish a reproducible selective IHA injection procedure that parallels therapeutic strategies presently used in humans for chemotherapy and chemoembolization.

Direct IT injection of large tumor nodules (5–8 mm) with $2 \times 10^9$ pfu Ad.CMV\textsubscript{tk} and GCV treatment revealed a significant bystander effect within the tumoral mass, leading to a tumor growth arrest, as compared to that of untreated nodules. It clearly elicited less toxicity in the nontumoral liver parenchyma than that observed by IHA administration of the recombinant Ad, although signs of mild hepatitis were present. The absence of antitumor effects on untreated tumor nodules suggests that, in this model of HCC, there are only a few IT arterial anastomoses to allow leakage of the vector and/or toxic metabolites generated into other tumor areas. This observation varies from those made in rats injected with the HCC-derived cell line (25). In this model, \textit{tk} gene expression was retrovirally transduced \textit{ex vivo} in rat McA-RH8994 tumor cells before transplantation into the right hepatic lobe of syngeneic rats, whereas nonexpressing \textit{tk} cells were transplanted into the left hepatic lobe of the same animals. These authors showed that following treatment with 150 mg/kg/day GCV, not only \textit{Tk}\textsuperscript{+} tumors but also \textit{Tk}\textsuperscript{-} tumors regressed. They reported that the distant bystander effect observed was likely immunemediated. Such observations were also reported in another model of rat using \textit{ex vivo}-transduced HCC cells before transplantation into syngeneic rats. In both models, the introduction of transduced tumor cells \textit{in vivo} could induce efficiently a tumor-specific immunity that was associated to CD8 T cells (27). Instead, in the model of HCC tumors induced \textit{in situ} in the rat liver, the activation of the immune response against transduced and nontransduced tumor cells would require more complex immune mechanisms.

Intrahepatic arterial injection of Ad.CMV\textsubscript{tk} plus i.p. injection of GCV led to a significant antitumoral effect in small tumor nodules $<3$ mm. The antitumor response observed in small nodules was clearly due to \textit{tk} gene expression and was not observed in Ad.CMVlacZ\textsubscript{2} and saline-treated animals. It was also dose-dependent because the dose of $2 \times 10^9$ pfu Ad.CMV\textsubscript{tk} showed significantly higher efficacy, as compared to the dose of $10^9$ pfu. We also attempted to treat large tumor nodules $>5$ mm by IHA injection of $2 \times 10^9$ pfu Ad.CMV\textsubscript{tk} plus i.p. injection of GCV. The low antitumor response of large tumor nodules is in accordance with our previous report that, despite the use of selective IHA injection, the transduction efficiency of large HCC nodules remains extremely low (18). However, doses of $2 \times 10^9$ and $10^9$ pfu Ad.CMV\textsubscript{tk} both induced hepatitis, although the toxicity of the former dose was high because 10 of 15 of the Ad.CMV\textsubscript{tk}-treated animals died during GCV treatment. This toxic effect was likely due to the use of a strong and ubiquitous CMV enhancer/promoter to drive expression of the \textit{tk} gene in both tumoral and nontumoral cells. GCV metabolites generated from \textit{tk} expression should theoretically show toxic effects only in cells that replicate their DNA (28). The high replication rate of nonmalignant hepatocytes induced by DEN treatment could, in part, account for the observed toxicity in our experiments and others (5). However, in agreement with a previous report, we demonstrate that Ad.CMV\textsubscript{tk} + GCV also induces significant alterations in liver functions of tumor-free normal rats leading to severe hepatitis and high mortality rate (Fig. 6; Ref. 29). Similar results of HSV-\textit{tk}/GCV-related toxicity for resting cells have also been reported by several laboratories (30–32). An histological score of hepatotoxicity in both tumor-free and tumor-bearing rats showed that it ranged from moderate to severe hepatitis and included the usual pattern of HSV-\textit{tk}/GCV-induced toxicity with apoptotic and necrotic liver cells (Table 3 and Fig. 5, A and B). Given the extremely low proportion of replicating normal liver cells, the mechanisms implicated in this toxicity still remain unclear, but this observation has important implications for the design of clinical gene therapy.

Finally, the present study shows that the hepatic toxicity elicited in the nontumoral liver parenchyma could be practically eliminated by the use of the AFP regulatory sequences targeting \textit{tk} gene expression to hepatoma cells. We did observe an absence of toxicity upon IHA injection of the Ad.AFP\textsubscript{tk} vector and GCV administration, although up to $5 \times 10^9$ pfu was used. On the other hand, the tissue-specific promoter produced a lower antitumoral response in both IT and IHA administrations of the recombinant virus (Tables 1 and 2). Two

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\(^a\) H+: mild hepatitis; H++: moderate hepatitis; H+++: acute severe hepatitis.

\(^6\) R. Gerolami and P. L. Tran, unpublished results.

\(^7\) Unpublished results.
explanations could account for such results. It is obvious that the AFP promoter sequence is markedly less efficient, both in vitro and in vivo, as compared with the CMV promoter; thus, the intracellular amount of thyminde kinase might not be sufficient to generate complete toxic and bystander effects. Furthermore, Ohguchi et al. (33) have reported the heterogeneity of the AFP expression as revealed by immunohistochemistry and in situ hybridization within human HCC. It is not excluded that the same process would also occur in the DEN-induced rat HCC and contribute to the low antitumor response. Therefore, the absence of the hepatic toxicity in the nonmalignant liver parenchyma will allow multiple injections of Ad.AFPΔtk for in vivo treatment of HCC. Bramson et al. (34) have reported that preexisting immunity to Ad in the Ad-immune mice did not prevent tumor regression following IT administration of a recombinant Ad expressing the cytokine IL-12, but inhibits virus dissemination. This should be examined in the DEN-induced rat model under HSV-1k gene therapy. Our histological results showed that within a short term following Ad.CMVΔtk or Ad.AFPΔtk injection and GCV treatment, the transduced tumors underwent rapidly an apoptotic and necrotic process. In addition, the inflammatory response composed of mononuclear cells may result in antitumor effects independent of the transgene.

In conclusion, both IT and IHA injections of the recombinant Ad for in vivo treatment of HCC appear to be two potential and feasible routes of administration for human clinical trials. The combination of direct IT injections of large nodules with IHA injections allowing “sterilization” of small foci of dysplastic, “preneoplastic” liver cells would provide an attractive strategy for treatment of the multifocal tumor lesions in most primary liver cancer. Our data emphasizes the need, in the future, to further delineate hepatoma-specific regulatory sequences to improve targeting efficacy.

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Evaluation of HSV-\textit{tk} Gene Therapy in a Rat Model of Chemically Induced Hepatocellular Carcinoma by Intratumoral and Intrahepatic Artery Routes

René Gerolami, Jorge Cardoso, Maïté Lewin, et al.


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