Virus-free Transfer of the Herpes Simplex Virus Thymidine Kinase Gene Followed by Ganciclovir Treatment Induces Tumor Cell Death

V. Calvez, O. Rixe, P. Wang, R. Mouawad, C. Soubrane, A. Ghoumari, O. Verola, D. Khayat, and F. Colbère-Garapin


ABSTRACT

We report virus-free transfer of a “suicide” gene into tumoral cells. The system can be used in vitro or in vivo to induce tumor cell death. A plasmid carrying the herpes simplex virus thymidine kinase (HSV-TK) gene with its 5′- and 3′-flanking regions was used both alone and in liposomes to transduce B16 cells. In vitro, a 5-day treatment with ganciclovir after transfection with the HSV-TK gene in liposomes induced a significant lysis of B16 melanoma cells as assessed by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide test. The efficacy of transfection was determined using liposomes harboring the β-galactosidase reporter gene and was around 10%. Thus, the cytotoxicity observed resulted presumably from a large bystander effect. In vivo, direct transfer of the TK DNA into established B16 melanoma tumors in C57B6 mice followed by i.p. ganciclovir treatment induced a 50% reduction of tumor weight after 8 days and an increased necrosis. Despite the use of the nonspecific strong TK promoter, no necrosis was detected in normal tissues surrounding the tumor or elsewhere. Thus, this system of tumor transfection, which does not involve any viral vector, is safe and straightforward and seems to be suitable for testing in clinical trials.

INTRODUCTION

Gene transfer technology is an exciting new approach to the treatment and cure of cancers. This methodology is developing in several directions in which different types of genes are transferred: (a) suicide genes such as HSV-TK and cytosine deaminase using GCV and 5-fluorocytosine, respectively as substrates (1, 2); their metabolites are toxic and thus induce death of transfected cells (3); (b) genes coding for cytokines such as tumor necrosis factor or interleukin 2, which enhance the antitumoral activity of cellular immune effectors (4, 5); (c) heterologous human leukocyte antigen-encoding genes, which increase tumor immunogenicity (6); (d) the multidrug resistance gene transferred into myeloid cells as a way to circumvent toxicity for normal tissues (7); and (e) growth factor genes (i.e., interleukin 4, granulocyte colony-stimulating factor, or granulocyte-macrophage colony-stimulating factor), which stimulate WBC and platelets (8, 9, 10). Several other approaches and concepts are being explored currently.

There also has been much work on vectors. Retroviral vectors show high efficacy of integration specific to dividing cells but have various limitations. The maximum size of the gene transferred is 8 kb; titers in cell culture are low, limiting large-scale production; and they could potentially cis-activate proto-oncogenes (11). Adenoviral vectors are also useful for gene transfer (12, 13). Virus-free gene transfer systems using asialo glycoproteins or liposomes are being developed currently (14, 15).

Since the HSV-TK gene was cloned (16), its applications have been developed extensively. It has been used routinely as a marker for cell selection following transfection. Recently, it has become one of the main tools for gene therapy of cancer as a suicide gene (17, 18) and for other diseases as stenosis after arterial injury (19, 20).

Transfer of the TK gene into a cell followed by GCV treatment induces cell death. One of the features that gives this system high efficiency is the bystander effect (21). A low level of gene transfer is compensated by the diffusion through gap junctions of toxic metabolites to the neighboring tumor cells. Recombinant retrovirus are usually used to transduce the HSV-TK gene (22). This approach is efficient, and clinical trials have begun. We investigated whether direct virus-free transfer of the HSV-TK gene could confer GCV sensitivity to tumor cells. We report the efficiency of direct injection of a plasmid containing the HSV-TK gene, with its 5′ and 3′ flanking regions, into melanoma tumors of mice.

MATERIALS AND METHODS

Plasmids. In the 6.4-kb plasmid pAG0 (16), a 2-kb HSV DNA fragment carrying the HSV-TK gene with its 5′ and 3′ transcriptional signals was inserted in the clockwise orientation...
into the PvuII site of pBR 322. The plasmid pTKβ contains the β-galactosidase gene under the control of TK gene transcriptional signals (23). Plasmids were purified using a commercial column chromatography method (Qiagen kit) according to the manufacturer's recommendations and then extracted by phenol and chloroform. Endotoxin contamination was quantified by the BioWhittaker LAL kinetic test. As a negative control in our experiments, the HSV-TK gene was disabled by overnight digestions at 37°C with restriction enzymes EcoRI and BglII (Biolabs) at a concentration of 1 unit/µg pAG0. The absence of undigested or partially digested plasmid was then verified by agarose gel electrophoresis.

Cell Lines. B16 murine melanoma cells were grown in Eagle's MEM (GIBCO) supplemented with 10% heat-inactivated FCS and 2 mM L-glutamine. They were maintained at 37°C in a humidified atmosphere of 95% air and 5% CO2. All cultures were mycoplasma free.

PCR. One µg DNA extracted from injected tumors, lung, liver, spleen, brain, and serum was tested by PCR for HSV1-TK gene sequences. Plasmid pAG0 (1 ng) was used as a positive control. Oligonucleotides 5'-CAGCGTCTGCGTTCGACC-3' and 5'-CCATGCGGCTGAATGAG-3' were used as primers (synthesized at the Pasteur Institute and purified by PAGE) in PCR reactions using an Appligene DNA thermal cycler.

Liposomes. Lipofectamine (GIBCO-BRL) is a 3:1 (w/w) liposome formulation of the polycationic lipid 2,3-dioleyloxy-N-[2(2-sperminecarboxamido)ethyl]-N,N-dimethyl-1-propanaminium trifluoroacetate and the neutral lipid dioleoyl phosphatidylethanolamine in membrane-filtered water.

In Vitro DNA Transfection and Cytotoxicity Assay. For detection of β-galactosidase activity in infected cells, cultures were fixed with 3% paraformaldehyde in PBS for 10 min at room temperature. After washing with PBS, cells were stained with 1 mg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactoside (BrdU) in PBS for 60 min. They were then washed with PBS, stained with 200 µl MEM were mixed immediately before injection. For pure DNA transfer, 10 µg pAG0 was resuspended in 200 µl 0.15 m NaCl. In two different experiments, pAG0 in liposomes or pure pAG0 was injected by needle directly into the melanoma nodule at day 11. A second identical injection was performed at day 14. From day 14, mice were given daily i.p. GCV (Syntex) at a dose of 120 mg/kg body weight/day. All mice were sacrificed at day 22, and tumors were removed. Tumors were weighed and were fixed in Bouin's solution. A blinded, semi-quantitative pathological analysis was performed to quantify the percentage of viability and necrosis in the tumors.

Statistical Analysis. The statistical significance of the differences between treated groups and controls was determined by the nonparametric Mann-Whitney test. Differences were considered significant at P ≤ 0.05 (25).

RESULTS

In Vitro Assays. In contrast to what may happen in vivo in the muscle (26), simple addition of DNA to cells cultured in vitro does not result in DNA transfection. Therefore, we used a liposome transfection technique to compare the results of in vitro and in vivo assays. Optimal conditions for transfection of B16 melanoma cells by liposomes were determined using the β-galactosidase reporter gene by varying the ratio of liposome:DNA. Optimal conditions were 100 µg DNA in 1 µl H2O mixed with 7 µl lipofectamine reagent and 200 µl cell culture medium; 10% of the cells were transfected (Fig. 1). B16 cells were transfected with the TK gene on pAG0 (16) in liposomes. The toxicity of GCV for the transfected tumor cells was determined. GCV had a dramatic cytotoxic effect. The concentration inducing a 50% decrease in cell growth was 15 µg/ml (Fig. 2). At a GCV concentration of 200 µg/ml, 65% of B16 cells were killed, whereas more than 85% of mock-transfected cells survived under the same conditions. The fact that 65% of pAG0-transfected tumor cells could be killed, whereas only 10% of cells were expressing a transfected gene (under the control of the same HSV-TK transcription signals), is indicative of a bystander effect, as defined previously (17). It was demonstrated that metabolic cooperation can account for this effect (18). Exposure of B16 melanoma cells to various concentrations of lipofectamine (from 1 to 200 µg/ml) did not reveal any significant cytotoxic effect. Furthermore, B16 cell viability was not affected by exposure to the DNA-liposome complex alone. Thus, toxicity was due to transfection with pAG0 followed by GCV treatment. The absolute number of cells decreased only after treatment with pAG0 in liposomes plus GCV. Thus, this effect was not only the effect of a decrease of cell proliferation but also the result of direct cytotoxicity.

In Vivo Assays. In vivo assays were performed either with a mixture of plasmid DNA in liposomes or with plasmid DNA alone. Due to the highly polymorphous nature of tumors, tumor weight was chosen as a measure of tumor growth 22 days after injection of B16 cells. Tumor growth was lower in the group of mice treated with pAG0 in liposomes and GCV (group 1) than in all other groups (B16 cells without any treatment, pAG0 in liposomes without GCV treatment, GCV treatment alone, and liposomes without DNA). GCV alone had a slight effect on tumor growth (Tables 1 and 2), but it was nonsignif-
Fig. 1 B16 melanoma cells transfected in vitro by the β-galactosidase reporter gene in liposomes. Positive cells are stained black.

![Graph showing cell survival vs GCV concentration]

Fig. 2 In vitro cytotoxicity of GCV on pAG0-transfected B16 cells. The cells were either transfected with pAG0 in liposomes (○) or mock-transfected with liposomes alone (□) before being treated with GCV. Cell viability was determined by a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide test after a 5-day treatment (see "Materials and Methods").

Table 1 In vivo effect of transfection with pAG0 and liposomes followed by GCV treatment

<table>
<thead>
<tr>
<th>No. of animals at day 0/22</th>
<th>Injected material</th>
<th>GCV treatment (g)</th>
<th>Tumor weight (g)</th>
<th>P*</th>
</tr>
</thead>
<tbody>
<tr>
<td>22/14</td>
<td>pAG0/lip</td>
<td>+</td>
<td>5.12 ± 3.42</td>
<td></td>
</tr>
<tr>
<td>20/9</td>
<td>pAG0/lip</td>
<td>-</td>
<td>11.9 ± 2.16</td>
<td>0.0001</td>
</tr>
<tr>
<td>20/9</td>
<td>Lip alone</td>
<td>-</td>
<td>10.03 ± 3.92</td>
<td>0.0046</td>
</tr>
<tr>
<td>20/8</td>
<td>pAG0/lip</td>
<td>+</td>
<td>9.97 ± 2.94</td>
<td>0.0031</td>
</tr>
<tr>
<td>20/12</td>
<td>Lip alone</td>
<td>+</td>
<td>7.76 ± 1.81</td>
<td>0.035</td>
</tr>
</tbody>
</table>

* Mann-Whitney statistical analysis was performed as described (25).

Table 1 In vivo effect of transfection with pAG0 and liposomes followed by GCV treatment.

The reduction of tumor weight following injection with pAG0 in liposomes and GCV treatment is statistically different from that obtained with GCV alone (P < 0.005), according to a Mann-Whitney analysis (25). In mice treated with pAG0 in liposomes, median tumor weight after GCV treatment was 5.12 g. Median tumor weight in the control group without any treatment was 9.97 g (Table 1). Necroses were more important in the first group than in the controls.

pAG0 injected without liposomes had a similar effect. The median tumor weight in the pAG0- and GCV-treated group was about one-half that of controls (Table 2). Neither the injection of a control plasmid (pBR 322; Table 2 nor the injection of a disabled HSV-TK gene resulting from a complete digestion of the pAG0 plasmid with EcoRI and BglII (not shown) had a detectable effect on tumor growth.

The animals were examined after sacrifice. No macroscopic necrosis was observed in any normal tissue (lung, skin, liver, spleen, or brain in mice treated with pAG0 and GCV). Furthermore, PCR failed to detect the presence of the transfected HSV-TK gene in lung, liver, spleen, brain, and serum; in contrast, the HSV-TK gene was detected by PCR in transfected tumor cells (data not shown). A higher percentage (80–90%) of
Treatment of Melanoma by HSV-TK DNA and GCV

**Table 2 In vivo effect of naked DNA injection followed by GCV treatment**

<table>
<thead>
<tr>
<th>No. of animals at day 0/22</th>
<th>Injected plasmid</th>
<th>GCV treatment</th>
<th>Tumor weight (g)</th>
<th>P*</th>
</tr>
</thead>
<tbody>
<tr>
<td>26/18 pAG0 +</td>
<td>3.86 ± 2.2</td>
<td>0.0009</td>
<td></td>
<td></td>
</tr>
<tr>
<td>24/18 pAG0 –</td>
<td>7.22 ± 2.4</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>18/12 pBR322b +</td>
<td>7.91 ± 3.7</td>
<td>0.0038</td>
<td></td>
<td></td>
</tr>
<tr>
<td>18/12 pBR322 –</td>
<td>8.54 ± 2.7</td>
<td>0.0007</td>
<td></td>
<td></td>
</tr>
<tr>
<td>23/18 +</td>
<td>8.06 ± 2.4</td>
<td>0.0001</td>
<td></td>
<td></td>
</tr>
<tr>
<td>24/17 +</td>
<td>6.50 ± 2.3</td>
<td>0.0053</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*a* Mann-Whitney statistical analysis was performed as described (25).

**DISCUSSION**

The prognosis for metastatic malignant melanoma is poor, partly because conventional therapies (chemotherapy and immunotherapy) are largely ineffective. The demonstration that the HSV-TK gene could be used as a suicide gene to make tumor cells susceptible to antiviral drugs such as GCV brought new hope for the treatment of human tumors (22). This gene has been used to transduce cells in vivo using viral vectors, especially retroviral vectors. Despite the advantages of this system, potential risks associated with viruses, even if defective, cannot be eliminated completely. To develop another type of vector to transfer the suicide TK gene into tumors, we tested a system that does not use any viral vector: injection of purified plasmid DNA or DNA in liposomes.

*In vitro* GCV inhibited the growth of melanoma cells transfected by pAG0 (carrying the TK gene) in liposomes strongly, even under conditions in which expression of the foreign DNA was detected in only 10% of cells. The toxicity toward untransfected tumor cells in the vicinity of transfected cells has been called the bystander effect; this effect was described first in cells transformed by retroviruses harboring the TK gene (17). It was attributed to the transfer of phosphorylated GCV from cell to cell through gap junctions (21, 27). The inhibition of tumor cell growth in *vitro* was dependent both on transfection with a functional TK gene and on treatment of cells with therapeutic doses of GCV.

Recently, Nabel et al. (6) used liposomes to transfer a HLA-B7 gene into human melanoma tumors, showing the efficacy and safety of such a transfection in *vivo*. In our animal model, a mean reduction of almost 50% in the rate of tumoral growth was obtained when TK transfection with liposomes was followed by GCV treatment for 8 days. We used very unfavorable conditions deliberately. In some previous reports, the tumor cells were transfected in *vitro*, allowing the selection of 100% of HSV-TK-positive cells before injection into animals (28). In our *in vivo* experiments, the B16 tumor cells in mice were transfected in *situ*, and thus the conditions used were closer to those of human gene therapy. The treatment was performed when the tumors were already large to see whether it would still be efficient. Indeed, in the group that received no treatment, 60% of the animals died before the end of the experiment, compared with 36.4% in the group that received the TK gene in liposomes followed by GCV treatment (Table 1). The enhancement of the life span of animals treated with pAG0 and GCV was confirmed further in another experiment in which mice received pAG0 or, as a negative control, the TK gene disabled by restriction enzyme digestion. There was a slight reduction in tumor growth in mice given GCV without DNA injection, but it was not significant according to the P value of our statistical test (25). Transfection with the TK gene without GCV treatment or injection of liposomes without DNA had no effect on tumor growth, demonstrating the requirement of both the TK gene and the antiviral drug. Two injections of liposomes harboring pAG0 were sufficient to deliver the TK gene into enough cells for GCV treatment to reduce tumor growth by one-half due to the bystander effect.

The results obtained in *vivo* with simple injection of pure DNA were quite surprising. The effects were similar to those of liposome transfection. The direct injection of pure DNA has been developed for the expression of foreign proteins (24, 26, 29, 31). It is, however, remarkable that naked DNA, without any protection against nucleases, was in our study able to penetrate into many cells of a large and dense tumor. The use of liposomes is thus not necessary to transfer the TK gene into tumoral cells in *vivo*.

Our results indicate that viral vectors are not necessarily required for efficient gene transfer in *vivo*, and that a circular plasmid, naked or associated with liposomes, can induce the biochemical transformation of tumor cells successfully. The advantages of using a plasmid for transfection are numerous. It is very safe and very easy to use. It eliminates the problems of packaging cell lines, possible leakiness, and low titers of retroviral vectors. Because the plasmid is purified from *Escherichia coli*, this system eliminates the problems inherent to, and the cost of, using animal cell cultures and FCS. Although the reactions of the organism against the retroviral vectors and the packaging lines seemed to be very limited (30), it is highly probable that the injection of pure DNA should be well tolerated. However, one would not expect tumor elimination. We used short-term experiments, 22 days long, because mice were injected with high doses of tumor cells. Using rats instead of mice, and lower amounts of tumor cells, a significant antitumor immunity following HSV-TK and GCV treatments was demonstrated previously (29). It is probable that our conditions could be improved further. The next developments of this model will focus on specific expression of the TK gene in tumoral cells. The specificity could be conferred by a tissue-dependent, trans-activated promoter.

This study is the rationale for a future clinical trial. Using naked pAG0 injected into melanoma s.c. metastases followed by i.v. GCV administration, the purpose of this trial will be to evaluate the safety and efficacy of this vector (naked DNA) after injection into a tumor. Finally, naked DNA could be an easy way to administrate a gene systematically, requiring specific targeting for the treatment of a metastatic disease.

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*References and footnotes are not included in the natural text.*
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