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Original Paper

Direct Gene Transfer of a Plasmid Carrying the Herpes Simplex Virus-Thymidine Kinase Gene (HSV-TK) in Transplanted Murine Melanoma: *In Vivo* Study

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The aim of the study was to use a virus-free system to transfer the Herpes Simplex Virus-thymidine kinase (HSV-TK) gene in mice bearing melanoma tumours. B16 F1 murine melanoma cells were injected subcutaneously. On days 11 and 14, an intratumoral injection of either naked plasmid containing the HSV-TK gene (pAG0) or pAG0–lipofectamine complexes was given. Ganciclovir (120 mg/kg/day) was given for 5 days starting on day 14. Tumour weight reduction (40–50%) was observed in treated animals versus different control groups. Moreover, histopathological analysis on tumours showed large areas of cavitary necrosis (85%) in treated groups compared to controls (10%). Using a simple and safe method, the results presented here demonstrated that virus-free mediated delivery of the HSV-TK gene is efficient *in vivo* in murine malignant melanoma.

Key words: malignant melanoma, gene therapy, *in vivo*, vectors, HSV-TK gene
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INTRODUCTION

MANY MALIGNANCIES remain resistant to established conventional treatments among which malignant metastatic melanoma, with its ominous prognosis, has led to a large number of clinical treatment trials including chemotherapy [1, 2], immunotherapy [3, 4] and more recently chemoimmunotherapy [5, 6].

Molecular therapies for cancer are currently being developed in which tumour cells are transduced with a variety of genes introduced by several delivery systems including, in most cases, viruses such as retrovirus or adenovirus. Although modified viruses have served as vectors for gene transfer, their ability to recombine or interact with endogenous viruses (adenovirus) and to activate human oncogenes (retrovirus) has raised great concerns regarding their safety for *in vivo* gene transfers. Thus, great attention is currently being given to the development of virus-free systems. Non-viral vectors would provide an important and potentially safer alternative, but their efficiency in delivery genes to human cells has yet to be demonstrated.

Since Colbère-Garapin and associates [7] cloned, in a plasmid (pAG0), the Herpes Simplex Virus-thymidine kinase (HSV-TK) gene, the use of this "suicide gene" has been routinely used as a marker for cell selection following transfection [8]. Suicide genes produce enzymes that convert non-toxic drugs into cytotoxic compounds and the production of such enzymes within the genetically-modified cells results in site-specific cytotoxicity. Thus, HSV-TK converts ganciclovir (GCV), a guanosine analogue, into toxic triphosphates disrupting DNA synthesis and leading to the transduced cell death. The TK gene transfer has been achieved in several malignant cell types [9–11].

We report here the efficiency of the direct intratumoral injection of the plasmid pAG0 in mice bearing melanoma tumours. Two different methods of gene delivery were investigated, either naked DNA or DNA–lipofectamine complexes.

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MATERIALS AND METHODS

Animals

Male C57B1/6 mice were purchased from Charles River© (Orléans, France) and were fed *ad libitum* a standard diet (Extra-Labo©, Provins, France). The average body weight was 25 g.

Cell lines

B16 F1 murine melanoma cells were purchased from American Type Culture Collection, ATCC (Maryland, U.S.A.) and maintened in Dulbecco's Modified Eagle's medium (DMEM) (Gibco BRL©, Cergy, France) supplemented with 10% heat-inactivated fetal calf serum and 2mM L-glutamine. They were maintained at 37°C in a humidified atmosphere of 95% air, 5% CO_2. All cultures were found to be mycoplasma-free.

Plasmids

The Herpes Simplex Virus type 1-thymidine kinae (HSV1-TK) gene carried by a 2 kilobase (kb) HSV DNA fragment was inserted in a clockwise orientation into the Pvu II site of pBR 322. The recombinant plasmid pAG0 has 6.4 Kb and harbours the entire HSV-TK gene with its 5′ and 3′ transcriptional signals. The pTKβ contains the β galactosidase gene driven by the HSV-TK 5′ flanking region and served as control of TK gene transcriptional signals. All the plasmids were grown in YT medium containing ampicillin (100 μg/ml) and purified using a commercially available column chromatography method (Qiagen Kit, Coger, France) according to the manufacturer's recommendations.

Liposome–DNA complex preparation

Lipofectamine (2 mg/ml) was purchased from Gibco BRL©, Cergy, France.

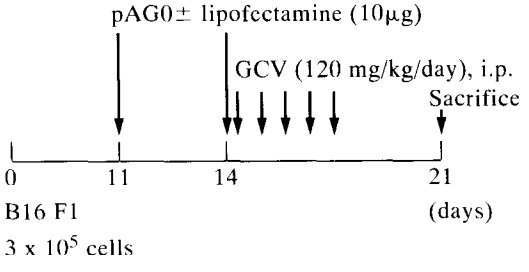
DNA–lipofectamine complexes were prepared by adding a solution of 10 μg plasmid DNA in 50 μl saline solution to 10 μg of lipofectamine in 50 μl saline solution. This DNA–liposome mixture was then incubated for 45 min at room temperature before intratumoral injection.

In vivo *experiments*

Three experiments were performed (initial experiment + two repeats).

Treated groups

3×10^5 B16 F1 cells (100 μl) were injected s.c. into the flank of mice at day 0. DNA treatment was given according to the following schedule:



At day 11, all the mice harboured tumours, with a median size of 1 × 1 ± 0.3 cm.

For naked DNA transfer, 10 μg of pAG0 were resuspended in 100 μl of NaCl 0.15 M. 61 mice received an intratumoral injection on days 11 and 14.

For liposome mediated transfection, DNA–lipofactamine complexes were prepared immediately before injection as above described. Thirty-six mice received an intratumoral injection of DNA–lipofectamine complexes (10 μg) on days 11 and 14.

From day 14, all the mice were given daily i.p. injections (200 μl) of ganciclovir (Syntex©, France) at the dose of 120 mg/kg of body weight for 5 days. All the mice were sacrificed at day 21 and tumours were dissected out and weighed. Tumours were fixed in Formol solution (10%). Histopathological analysis was performed to quantify percentage of necrosis.

In three further experiments, using naked DNA, we studied the same protocol using 100 μg DNA plasmid instead of 20 μg. In the second one, ganciclovir was given at the dose of 60 mg/kg/day instead of 120. The third one where animals were not sacrificed, was used for the survival study.

Control groups

Seventy-four mice received B16 F1 cells alone without GCV treatment; 59 mice received B16 F1 cells + GCV; 33 mice received B16 F1 cells + naked DNA without GCV; 32 mice received B16 F1 cells + DNA–lipofectamine complexes without GCV; 20 mice received B16 F1 cells + lipofectamine alone without GCV.

Histopathological study

Tumours were serially cut every 2 mm and paraffin embedded. Sections were stained with haematoxylin and eosin. Slides were examined with a micrometric ocular and the percentage of necrotic areas was determined.

Statistical analysis

Statistical significance of the differences between treated groups and controls was determined by the non-parametric Mann–Whitney test. For survival study, the Chi-square test with Yates' correction was performed. Differences were considered as significant at $P \leq 0.05$.

RESULTS

They were expressed as mean ± SEM of tumour weight (g).

Naked DNA

In the experiment using pAG0 alone (Figure 1) a significant decrease in tumour weight was observed for the group receiving DNA-TK and GCV (mean tumour weight, 5.48 ± 0.5 g) compared to those that received only DNA-TK (mean tumour weight, 9.38 ± 0.7, $P < 0.0001$). Similarly, a difference was seen between the DNA-TK–GCV treated group and other control groups (B16 F1 cells alone, 8.95 ± 0.5 g, $P < 0.0001$; or B16 F1 cells + GCV, 7.16 ± 0.4 g, $P = 0.0097$).

DNA–lipofectamine complexes

In the experiment using DNA–lipofectamine complexes (Figure 2), we also observed a significant difference in tumour weight between the group receiving LIPO-TK and GCV (4.63 ± 0.5 g) and those receiving only LIPO-TK (9.34 ± 0.8 g; $P < 0.0001$). Moreover, an additional group receiving lipofectamine alone showed a mean tumour weight of 10.03 ± 1.30 g ($P = 0.0018$), while the transfer of a control plasmid pBR 322 without any insert had no detectable effect on tumour growth (data not shown).

Using an intratumoral injection of 100 μg of naked DNA

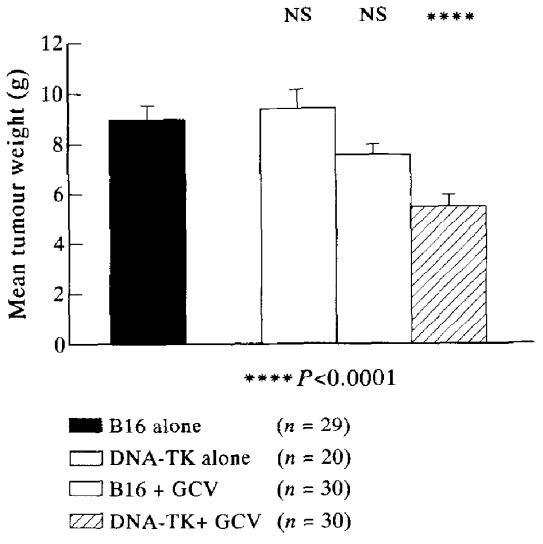


Figure 1. The effect of naked DNA-TK and GCV on mean tumour weight.

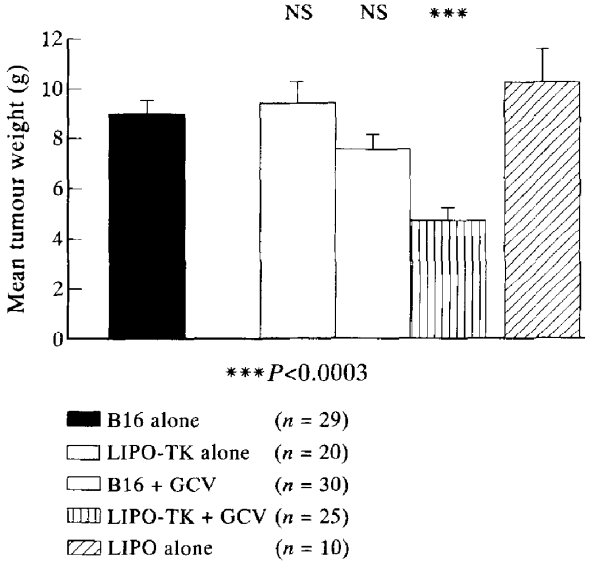


Figure 2. The effect of DNA–liposome complex and GCV on mean tumour weight.

plasmid, we found a significant difference ($P = 0.001$) in tumour weight between the group receiving DNA-TK and GCV (6.93 ± 0.6 g) and those who received only DNA-TK (9.85 ± 1.3 g) (Figure 3). With 20 μg of naked DNA and 60 mg/kg/day of ganciclovir, we again observed a significant decrease in tumour weight between the treated group (7.17 ± 0.6 g) and controls (11.33 ± 0.95 g, $P = 0.0012$; or 10.91 ± 0.53 g, $P = 0.0003$) (Figure 4).

Histopathological analysis (Figure 5) showed large areas of cavitary necrosis (85%) in treated groups compared to the non-treated ones (10%).

The percentage of survival was significantly ($P = 0.01$) higher in those treated with naked DNA and GCV from day 21 compared to controls (Table 1).

DISCUSSION

The results presented here show that it is possible to insert new genetic material into tumour cells using a virus-free

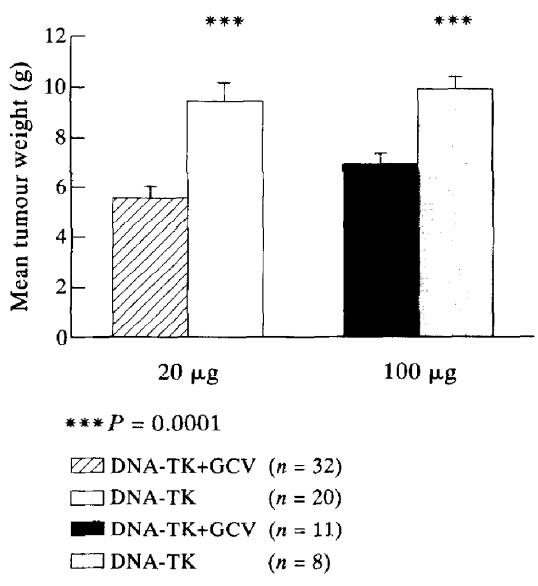


Figure 3. The effect of different levels of naked DNA on mean tumour weight.

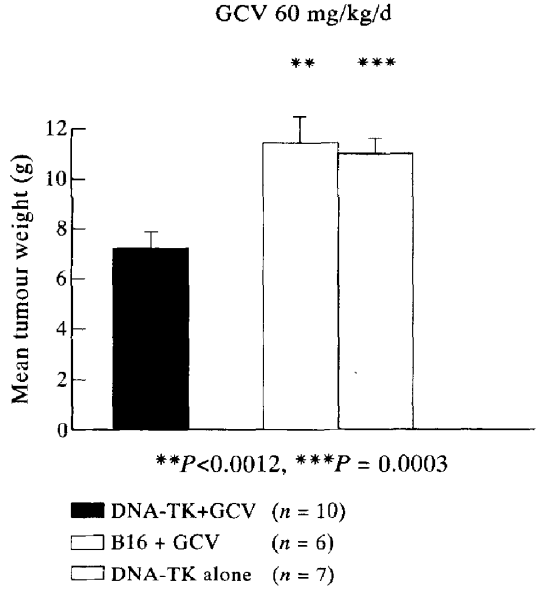


Figure 4. The effect of a lower concentration of GCV in mice treated with naked DNA on mean tumour weight.

transfer system. Until now, most studies [12 14] reported *in vitro* and/or *in vivo* retroviral or adenoviral mediated gene transfer. However, virus-free systems are now under investigation [15–17] since non-viral vectors offer some advantages, including a quick and easy method of gene preparation, simplicity, economy and safety.

We report here the *in vivo* virus-free transfer of a plasmid (pAG0) carrying the Herpes Simplex Virus-thymidine kinase (HSV-TK) gene to transplanted murine B16 F1 melanoma. This transfection followed by ganciclovir treatment leads to the transduced cell death. We investigated either a direct intratumoral injection of naked DNA–plasmid or DNA–lipofectamine complexes in order to compare the transfection percentage and the efficiency of tumour cell killing. Interest-

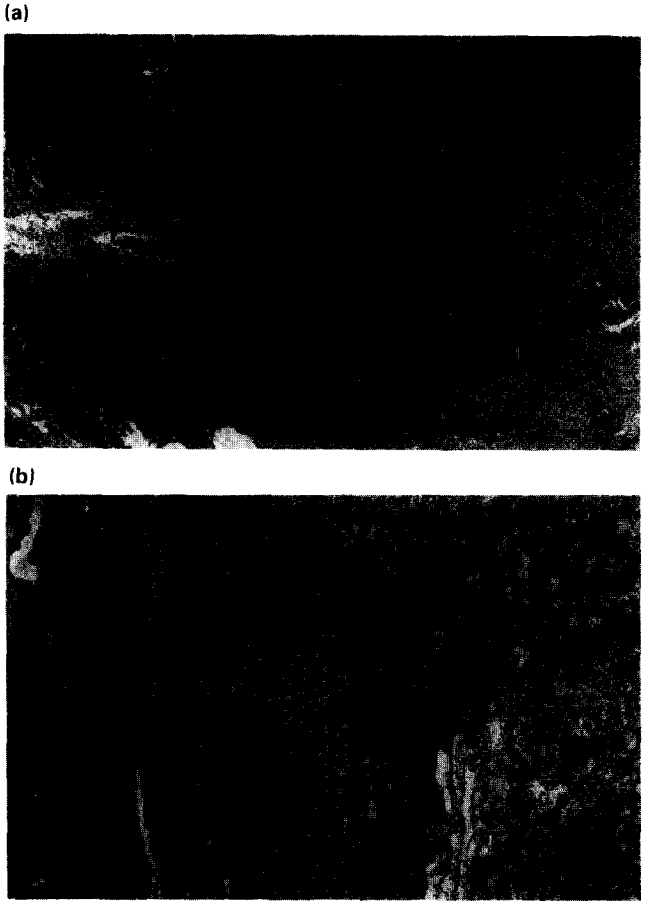


Figure 5. H & E (haematoxylin and eosin) staining of paraffin-embedded section from B16 transplanted tumour. (a) Non-treated; (b) treated by naked DNA-TK + GCV. Magnification × 40.

Table 1. *Percentage of survival in treated group by naked DNA + GCV compared to controls (combination of DNA alone, GCV alone and B16 F1 alone)*

	Treated		Controls	
	n	%	*n*	%
Day 0	42	100	76	100
Day 14	42	100	71	92
Day 21	23	55*	22	28
Day 22	9	22**	3	4
Days 23–28	5	11*	0	0

n = number of surviving animals.
*$P < 0.05$. **$P = 0.014$.

ingly, we found approximately the same tumour weight reduction with both systems 42 and 50%, respectively.

Vile and Hart [18] reported a direct intratumoral injection of naked Tyrosinase-TK plasmid DNA in established B16 F1 tumour mice followed by ganciclovir treatment. They also obtained a tumour weight reduction in the treated group compared to the different controls. Similarly, Plautz and associates [19] studied the immune response following intratumoral injections of a foreign naked MHC gene into malignant murine tumours *in vivo*. They showed that the expression of the murine class I H-2K^S gene within the CT26 mouse colon adenocarcinoma or the MCA 106 fibrosarcoma induced a cytotoxic T-cell response attenuating tumour growth. Nabel and associates [15] demonstrated the feasibility, safety and therapeutic potential of direct gene transfer with DNA–liposome complexes in metastatic melanoma patients. They treated subcutaneous metastatic tumour nodules in 5 patients using intratumoral injection of HLA-B7 DNA–liposome complexes. Plasmid DNA was detected within biopsies of treated tumours nodules 3–7 days after injection, but was not found in the serum at any time in all 5 patients. One patient demonstrated regression of injected nodules which was accompanied by a regression of distant metastasis (lung).

To investigate how critical the amount of the DNA injected in order to produce a detectable therapeutic response is, we used 100 μg of DNA–plasmid instead of 20 μg. Surprisingly, we did not observe any increase of antitumoral activity compared to the lower concentration.

Whatever the gene transfer system used, we and others [8, 20, 21] have noted tumour cell killing *in vitro* as well as *in vivo* despite the very small percentage of transduced cells. Kolberg [22] described this phenomenon and called it "the bystander effect". Freeman and associates [23] reported that *in vitro* HSV-TK positive cells were toxic to unmodified tumour cells in the presence of ganciclovir inducing their own process of apoptotic cell death. The toxic effect of HSV-TK positive cells on HSV-TK negative cells was reproduced in an *in vivo* murine model. This phenomenon might be explained by an intimate cell–cell contact necessary for the effect to occur involving cell–cell transfer of particulate substances or toxic molecules (triphosphorylated ganciclovir) through nearby gap junctions.

In our study, the presence of necrosis in treated tumours suggests specific tumour cell lysis induced by the injection of the plasmid followed by GCV treatment and the bystander effect.

Concerning the ganciclovir treatment, most studies [18, 24] have reported the use of 100–150 mg/kg/day for 5–7 days. In our study, we used 120 mg/kg/day for 5 days and, surprisingly, we observed a slight effect of GCV in the control group receiving B16 F1 cells alone, resulting in a lower mean tumour weight than other control groups without GCV. This fact might be explained by a toxic effect of GCV on tumoral cells. This toxic effect resulting from high concentrations has been previously described *in vitro* [25] as well as *in vivo*. Thus, we used a lower concentration of GCV (60 mg/kg/day) and eliminated this non-specific activity, without having a significant decrease in the transduced tumoral response (34%) (Figure 4).

Very recently, Hickman and colleagues [17] reported gene expression following direct injection of naked DNA into liver of both rats and cats: they demonstrated the efficiency of this method for hepatic gene delivery and suggested its future potential for development of gene therapy.

Therefore, it seems that many diseases, including malignancies resistant to conventional treatments, will benefit from gene therapy. Several clinical trials have started for brain tumours, ovarian cancer, malignant melanoma or non small cell lung cancer using, in most cases, retroviral or adenoviral mediated gene transfer. Our results demonstrate that virus-free mediated delivery of the TK gene is efficient in malignant melanoma using a simple and safe method. This is the rationale for a clinical trial to be started in the near future.

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