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Repeated cycles of retrovirus-mediated HSVtk gene transfer plus ganciclovir increase survival of rats with peritoneal carcinomatosis

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Peritoneal carcinomatosis is a common clinical situation that requires novel therapeutic approaches. We investigated the efficiency of an HSVtk gene therapy for the treatment of peritoneal carcinomatosis induced in syngeneic rats by DHD/K12 colon carcinoma cells. In this setting, the efficiency of two different retrovirus producing cell lines (GP+AmEnv12 and FLYA13) was compared. Rats treated with a single injection of retrovirus producing cells followed by a 5-day course of ganciclovir treatment showed an increased survival as compared with control animals. Animals treated with three injections of producing cells, each followed by a 4–5-day course of ganciclovir treatment, showed an increased survival as compared with control rats and with those treated with a single cycle of retrovirus producing cells plus ganciclovir. However, only a few animals remained tumor-free after day 180. There was no difference between the two producing cell lines in any of the experiments. RT-PCR demonstrated a faint expression of the tk transgene in the liver, spleen, epiploon, bowels and the lung of the animals injected with the HSVtk producing cells, reflecting most likely the transduction of only a limited number of cells.

Keywords: *gene therapy; thymidine kinase; peritoneal carcinomatosis; colorectal cancer; retrovirus; ganciclovir*

Introduction

Peritoneal dissemination is a common clinical situation in patients suffering from ovarian or digestive primary neoplasms.[1] Surgical resection of extensive peritoneal carcinomatosis is impossible. Moreover, most of these cancers, including colorectal carcinoma, are poorly reactive to systemic chemotherapy.[2]

Suicide gene therapy consists in the intracellular delivery of a gene coding for an enzyme which transforms a prodrug into a cytotoxic product.[3,4] Thymidine kinase from the herpes simplex virus type 1 (HSV*tk*) is the best studied and most used suicide gene. It can efficiently phosphorylate nucleoside analogs such as ganciclovir (GCV) into monophosphorylated molecules. Phosphorylation by cellular kinases leads then to the formation of the cytotoxic ganciclovir-triphosphate which is incorporated into DNA and inhibits DNA polymerase.[5–7] Of great therapeutic interest, untransduced adjacent cells are also sensitive to the GCV-triphosphate cytotoxic effect ('bystander effect').[8–14]

For *in vivo* transduction of HSV*tk* gene, retroviral vectors have been successfully used in numerous tumor models.[15–17] In this strategy, the requirement of active DNA synthesis for retrovirus replication limits the HSV*tk* gene delivery to proliferating tumor cells. Such an approach is currently used in several clinical trials, mostly for the treatment of brain tumors[18,19] and more recently for the treatment of cancers disseminated in anatomical cavities.[20,21]

We and others have recently demonstrated the efficiency of an HSV*tk*-mediated gene therapy for peritoneal carcinomatosis induced by colorectal adenocarcinoma cells in a syngeneic immunocompetent animal model.[22,23]

The aim of the present work was to study the therapeutic effect of retrovirus-mediated transfer of the HSV*tk* gene in the same model of peritoneal carcinomatosis induced by colorectal adenocarcinoma cells in syngeneic rats. In these experiments, we demonstrated an increase survival of the animals treated with multiple intraperitoneal injections of HSV*tk* retrovirus producing cells followed each time by a GCV treatment.

Results

Retrovirus producing cell lines

In this report, two different packaging cell lines were used, GP+AmEnv12 and FLYA13. The FLYA13 cell line has been described as a highly efficient retrovirus producing line, generating high titers of retroviruses resistant to inactivation by human serum.[24] We developed a HSV*tk* retrovirus producing cell line FLYA13-TK by transfection of a retroviral vector, derived from the Moloney murine leukemia retrovirus (Mo-MuLV). The second HSV*tk* retroviral producer cell line GP+AmEnv12-TK was a generous gift from Dr C-M Calberg (Laboratory of Virology, University of Liège, Belgium). Using G418

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resistance as a marker, we measured the HSV*tk* retroviral particles produced by both cell lines and reproducibly found a titer of 1×10^6 p.f.u./ml for GP+AmEnv12-TK cells and 2×10^5 p.f.u./ml for FLYA13-TK cells.

Survival of rats after a single i.p. injection of retrovirus producing cell lines

We investigated the efficiency of HSV*tk*-based gene therapy in a model of peritoneal carcinomatosis induced by DHD/K12 colorectal adenocarcinoma cells in syngeneic BD-IX rats. As previously reported, ganciclovir treatment of animals injected i.p. with stably transfected DHD/K12-TK cells allowed a significant reduction of tumor volume, as well as prolonged survivals.[22]

BD-IX rats i.p. injected at day 0 with 10^6 DHD/K12 cells developed a peritoneal carcinomatosis before day 14 and died of peritoneal dissemination within 60 days.[22] HSV*tk* retrovirus producing cells (GP+AmEnv12-TK or FLYA13-TK) were injected at day 9 and a GCV (150 mg/kg) treatment was administered once daily from day 14 for 5 days (Figure 1a, 10 animals for each group). Simultaneously, a control goup (five animals) which had also been injected with HSV*tk* retrovirus producing cells, was treated according to the same protocol with 10 ml of HBSS (Hank's balanced salt solution). The animals injected with the HSV*tk* retrovirus producer FLYA13-TK cells and treated with HBSS died between days 42 and 51 (median survival: 48 days) (Figure 1b). Animals injected with HSV*tk* retrovirus producer FLYA13-TK cells and treated with GCV showed a significantly prolonged survival as compared with the HBSS-treated animals (log-rank test, $P < 0.01$; median of survival, 52 days). The five animals injected with HSV*tk* retrovirus producer GP-AmEnv12-TK cells and treated with HBSS died between days 42 and 51 (median survival, 45 days) (Figure 1b). Animals injected with HSV*tk* retrovirus producer GP+AmEnv12-TK cells and treated with GCV showed a significantly prolonged survival as compared with the HBSS-treated animals (log-rank test, $P < 0.05$; median of survival, 55 days). However, in each of the GCV-treated group, only one animal out of 10 showed a long survival and was free of tumors at day 240 (Figure 1b). We did not observe any significant difference between the results obtained after treatment with the different retrovirus producing cell lines.

Survival of rats after three i.p. injections of retrovirus producing cell lines

To improve the efficiency of the suicide gene treatment, BD-IX rats were i.p. injected at day 0 with 10^6 DHD/K12 cells and the HSV*tk* retrovirus producing cells (GP+AmEnv12-TK or FLYA13-TK) were injected at days 9, 18 and 25. For the treated groups (10 animals for each group), daily 150 mg/kg GCV i.p. injections were performed from day 14 to 17, from day 21 to 24 and from day 28 to 32 (Figure 2a). At the same time, control groups

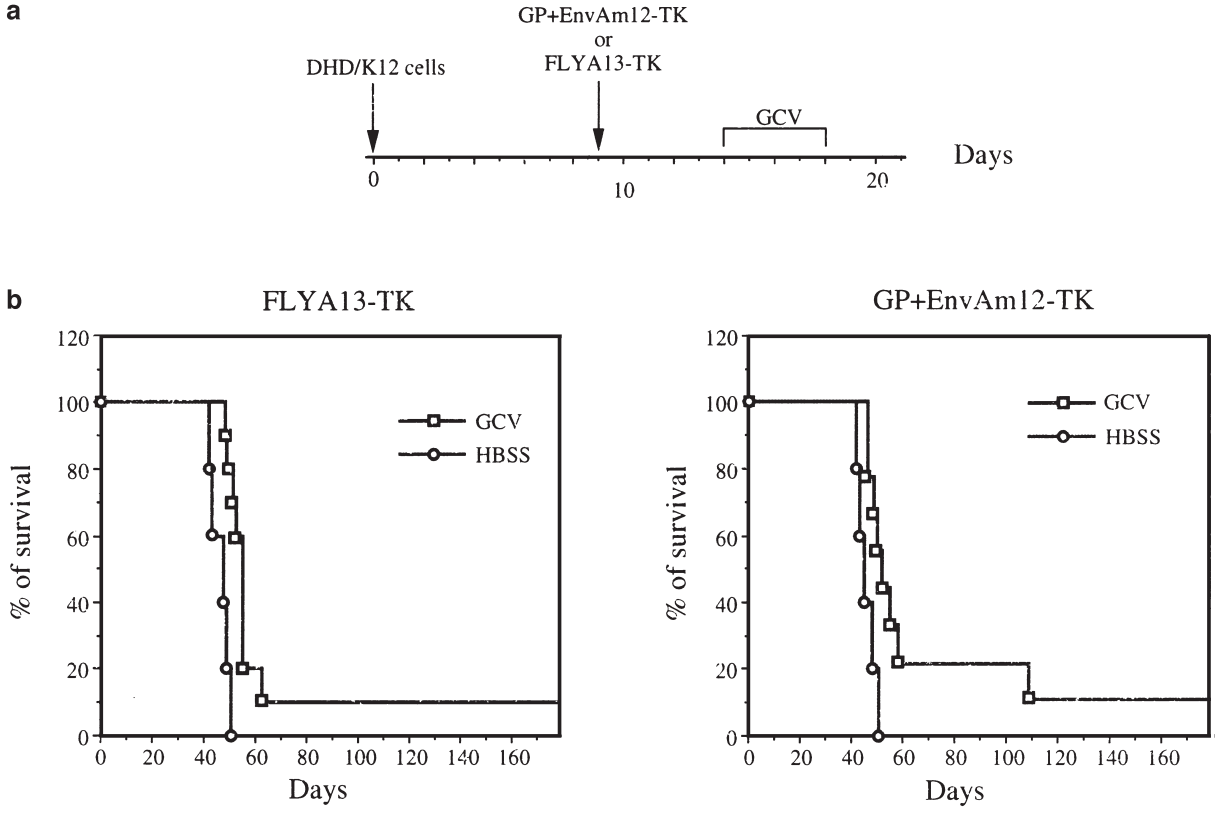

Figure 1 *Survival of rats after a single i.p. injection of retrovirus producing FLYA13-TK or GP+EnvAm12-TK cells and treatment with GCV. (a) Protocol for i.p. single delivery of HSVtk retrovirus producing cells and GCV treatment. The rats were i.p. injected with 10^6 DHD/K12 cells at day 0. The HSVtk retrovirus producing cells FLYA13-TK or GP+EnvAm12-TK were i.p. injected at day 9. For the GCV-treated group, the treatment started at day 14 for 5 days with 150 mg/kg of GCV once daily, while at the same time, a control group was injected with 10 ml of HBSS once daily. (b) Survival curves: 10 or five animals were respectively monitored in the GCV and in the control groups. In the FLYA13-TK and GP+EnvAm12-TK GCV-treated groups, one rat was still alive and tumor-free at day 240.*

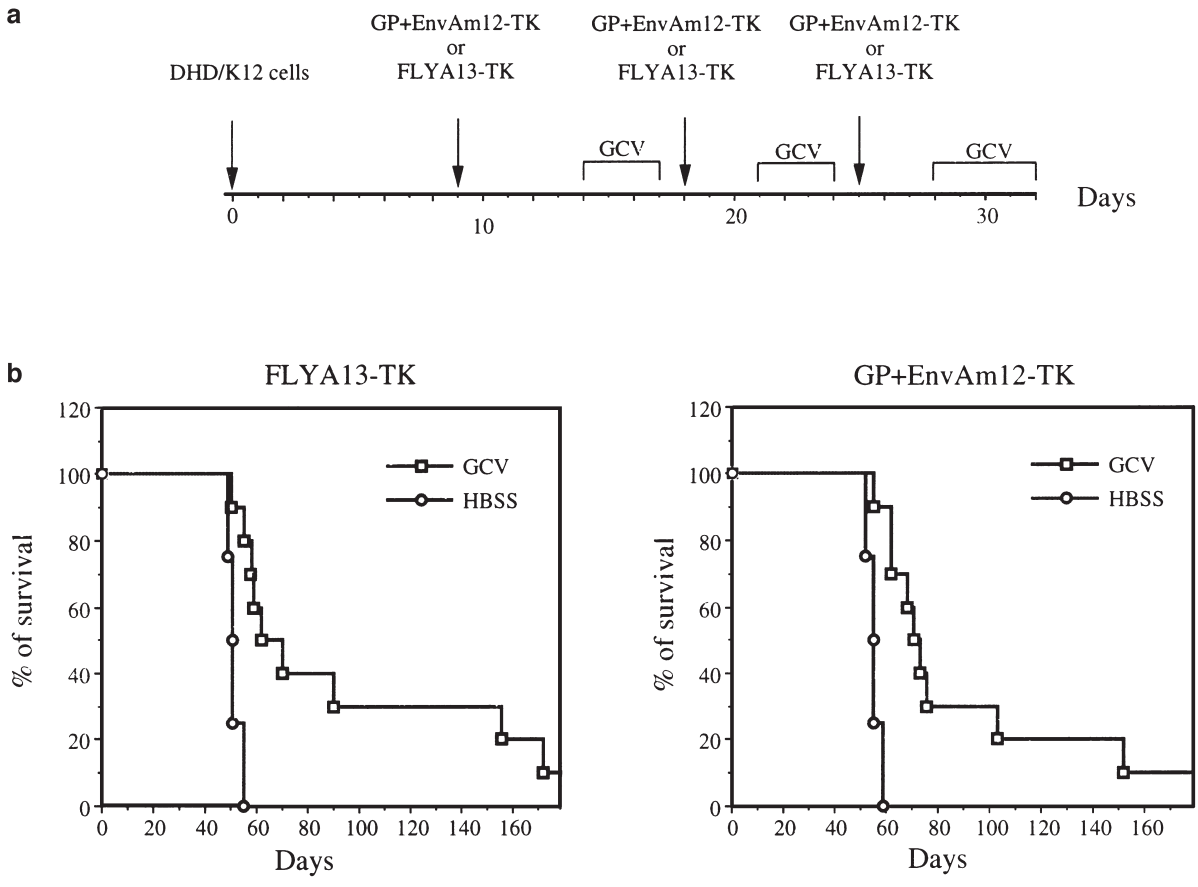

Figure 2 *Survival of rats after three i.p. injections of HSVtk retrovirus producing FLYA13-TK or GP+EnvAm12-TK cells. Each injection was followed by a GCV treatment. (a) Protocol for i.p. multiple delivery of HSVtk retrovirus producing cells lines and GCV treatment. Each group was i.p. injected with 10^6 DHD/K12 cells at day 0. The HSVtk retrovirus producing FLYA13-TK or GP+EnvAm12-TK cells were i.p. injected at day 9, 18 and 25. For the GCV-treated group, the first treatment was administered from day 14 to 17, the second from day 21 to 24 and the third from day 28 to 32, with 150 mg/kg of GCV once daily. At the same time, the control group received 10 ml of HBSS once daily. (b) Survival curves: 10 or five animals were respectively monitored in the GCV and in the control groups. In both GCV-treated groups FLYA13-TK and GP+EnvAm12-TK, one rat was still alive and tumor-free at day 240.*

(five animals in each group) were injected with the *tk* producing cells and treated according to the same protocol with 10 ml of HBSS (Hank's balanced salt solution). The five animals injected with HSV*tk* retrovirus producing FLYA13-TK cells and treated with HBSS died between day 49 and 55 (median survival, 51 days) (Figure 2b). Animals repeatedly injected with HSV*tk* retrovirus producing FLYA13-TK cells and treated with GCV showed a significantly prolonged survival as compared with the HBSS-treated animals (log-rank test, $P < 0.01$; median survival, 70 days). The five animals injected with HSV*tk* retrovirus producer GP-AmEnv12-TK cells and treated with HBSS died between day 52 and 59 (median survival, 55 days) (Figure 2b) while animals repeatedly injected with the same producing cells and treated with GCV showed a significantly prolonged survival (log-rank test, $P < 0.001$; median survival, 73 days). However, in each of the GCV-treated group, only one animal out of 10 showed a long survival and was free of tumors at day 240 (Figure 2b). In these conditions, we did not observe any difference between the groups of animals treated by the two retrovirus producing cell lines.

We compared the outcome of animals treated with a single or with three cycles of retrovirus-mediated *tk* gene transfer plus GCV. As shown in Figure 3, the animals which received three injections of FLYA13-TK or GP+AmEnv12-TK producing cells followed by GCV had a significantly better survival than the animals treated with a single course of producing cells plus GCV ($P < 0.05$).

In vivo distribution of HSVtk retroviruses after intraperitoneal injection of HSVtk retrovirus producing cells

The biodistribution of HSV*tk* retroviruses following single or multiple intraperitoneal injections of HSV*tk* retrovirus producing cells (GP+AmEnv12-TK or FLYA13-TK) is an important consideration for the safety of the treatment.

The expression of HSV*tk* mRNA in various samples of organs from differently treated rats was investigated by RT-PCR followed by Southern blotting and *tk*-DNA probe detection. The levels of *tk* mRNA were evaluated in various tissues of peritoneal carcinomatosis bearing rats which received either a single or a triple i.p. administration of HSV*tk* producing cells. In these experiments, two animals were analyzed for each condition.

After a single i.p. administration of HSV*tk* producing

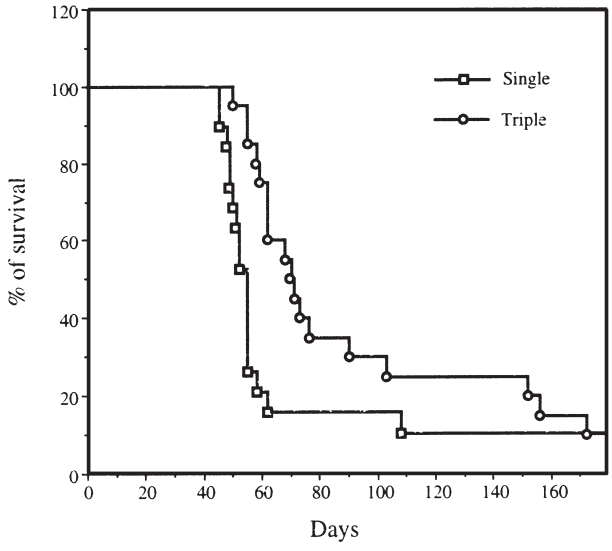

Figure 3 *Comparison of single versus multiple cycles of tk-retrovirus infections plus GCV treatment. Survival curves were established for the 20 animals treated with a single injection of retrovirus producing cells (FLYA13-TK or GP+EnvAm12-TK) and for the 20 animals treated with three cycles of TK-retrovirus plus GCV.*

cells (GP+AmEnv12-TK or FLYA13-TK), a weak but detectable expression of the transgene was detected in liver, spleen, epiploon, bowels and lungs (Figure 4a). When compared with the signal (Figure 4a, lane +) obtained with DHD/K12 cells stably transfected with the *tk* gene, those observed in the different tissues indicated that only a small proportion of cells were transduced by the HSV*tk* retrovirus. However, a strong expression of the *tk* transgene was detected in the epiploon of one of the animals injected with Gp+AmEnv12-TK cells (Figure 4a, lower panel).

Similarly, after three i.p. administrations of HSV*tk* producing cells (GP+AmEnv12-TK or FLYA13-TK), the expression of the transgene was also detected by RT-PCR in the same organs (Figure 4b). The HSV*tk* expression was clearly higher after multiple injections of the producing cells than after a single injection (compare Figure 4a and b) but remained much lower than that observed in control *tk* expressing DHD/K12 cells (Figure 4b, lane +) indicating that only a small proportion of the cells from these tissues had captured the transgene.

Discussion

Peritoneal carcinomatosis remains a major clinical problem as neither surgery nor chemotherapy can eradicate tumor cells disseminated in the peritoneal cavity. Novel therapeutic approaches are thus required to treat these tumors which constitute a suitable model for gene therapy. Indeed, intraperitoneal administration of viruses or virus producing cells should confine gene transduction to the peritoneal cavity. We therefore investigated whether a *tk*-based suicide gene therapy could treat peritoneal carcinomatosis induced by DHD/K12 colorectal adenocarcinoma cells in syngeneic BDIX rats.

In this model, rats i.p. injected with DHD/K12 cells developed a macroscopic peritoneal carcinomatosis within 12 days. The animals were then injected once or three-fold with retrovirus producing cells, each administration being followed, 3 to 5 days later to allow *tk* gene expression, by a GCV treatment.

The efficiency of *in vivo* retrovirus-mediated gene delivery depends on virus titer and on virus stability. Therefore, we chose to compare the efficiency of two retrovirus producing cell lines. In our hands, the retrovirus titer obtained with the FLYA13-TK cells was lower than with the GP+AmEnv12-TK cells, although the FLYA13 has been reported to generate high titers of retroviruses.[24] *In vivo*, we did not observe any difference in the outcome of animals treated with one cell line or the other. This could mean that the virus titer does not influence the therapeutic benefit in our model or that the lower titer observed with the FLYA13-TK cells is compensated by the production of more stable viruses. Alternatively, the killing of allogeneic tumor cells might induce a local inflammatory reaction and could participate in the bystander effect. In this hypothesis, one could imagine that such a nonspecific stimulation of the immune system might be more important with human FLYA13 cells than with murine GP+AmEnv12 cells and that such a difference could partially explain the observed results. Ongoing experiments comparing the efficiency of liposomes and retrovirus producer cells will help to define the putative role of an immune bystander effect generated by the killing of allogeneic producer cells.

Rats treated with a single injection of producing cells followed by a 5-day course of GCV showed an increased survival when compared with control animals. However, it is clear from a number of experiments that a single injection of producing cells would lead to the transduction of only a small proportion of tumor cells. Therefore, we repeated three times the injection of producing cells followed each time by a GCV treatment, hoping to improve the efficacy of the treatment. In these conditions, animals again showed an increased survival as compared with control animals. Moreover, their survival curves were better than those observed after a single cycle. These data thus demonstrated that repeated cycles of producing cell injections followed by GCV treatments were more efficient than a single cycle and that such an approach will have to be considered for the design of future clinical trials.

Our results compare favorably with other data obtained after *in vivo* delivery of the HSV*tk* gene. Indeed, several authors reported a poor efficiency of such an approach and questioned whether the suicide gene alone would be able to improve the outcome of animals or patients suffering from cancers.[25–27] Several reasons could account for our favorable data. First, the peritoneal cavity should allow a large distribution of the retrovirus producing cells and an efficient infection of a number of small size peritoneal tumor nodes while producing cells injected in a solid tumor would only be able to deliver the gene to a low number of neighboring tumor cells. Secondly, the DHD/K12 cells could demonstrate a very efficient bystander effect, allowing the diffusion of the phosphorylated GCV from cell to cell through the gap junctions. Our previous *in vivo* data showed that i.p. injections of 20% of *tk* expressing DHD/K12 cells combined with untransfected DHD/K12 cells allowed an increased survival after GCV treatment.[22] Also, as our model is based on immunocompetent animals, an

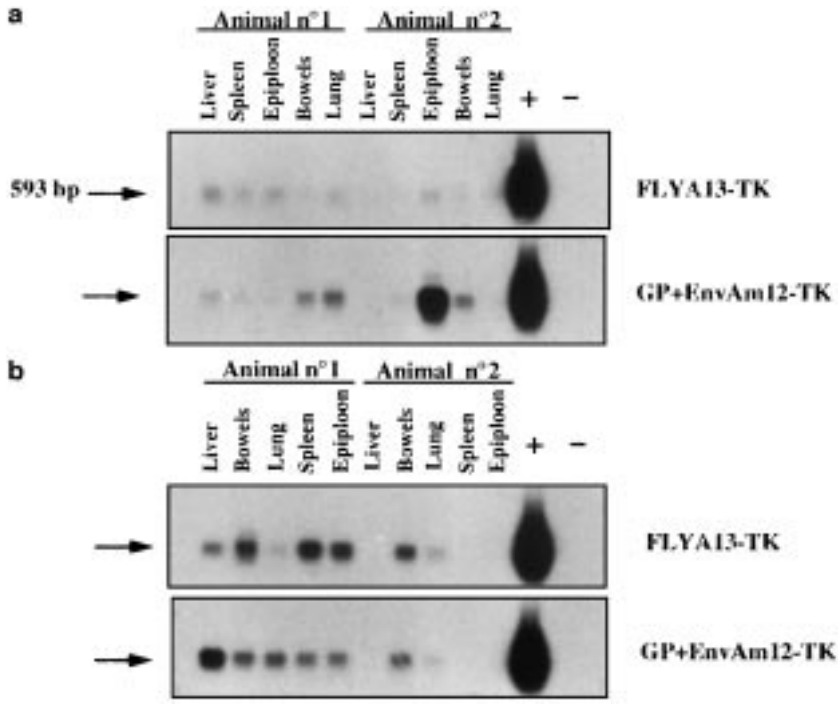

Figure 4 *tk Gene expression in various organs following single or multiple i.p. injections of HSVtk retrovirus producing cells. The expression of HSVtk mRNA in various organs was investigated by RT-PCR followed by Southern blotting revealed by a tk probe after one or three administrations of HSVtk producing cells into the peritoneal cavity of rats with established peritoneal carcinomatosis. Two animals were studied for each condition. (a) Animals received a single i.p. injection of HSVtk retrovirus producing FLYA13-TK or GP+EnvAm12-TK cells. Samples of tissue from different organs were collected 5 days after injection of producing cells. Total RNA was isolated and RT-PCR were performed with primers designed to amplify the HSVtk message (593 bp). The PCR products were analyzed by Southern blotting probed with a specific ^{32}P labeled tk DNA probe. The organs analyzed and the cell lines injected are indicated in the Figure. (b) Animals received three injections of HSVtk retrovirus producing FLYA13-TK or GP+EnvAm12-TK cells. At day 28, 3 days after the last injection of producing cells, the organs were harvested and total RNA was isolated and analyzed as in (a) for tk gene expression. In a and b, lane (–) shows the negative control (PCR performed on water) and lane (+) shows the RT-PCR performed on the DHD/K12-TK stably transfected cells. The presence of HSVtk mRNA was detected in all the organs analyzed.*

immunological antitumor reaction could have played a role in delaying tumor progression or even in eradicating the tumor in a few animals after the HSV*tk*/GCV treatment. Other authors had previously demonstrated an immune response following HSV*tk* gene therapy.[13,28–32]

However, despite these favorable results, only 10% of the treated animals were still alive and tumor-free at day 240, confirming that the *tk* gene alone will only rarely eradicate established solid tumors. In the future, it will thus be necessary to combine the *tk* gene or other suicide genes therapy system with additional therapeutic approaches acting by other mechanisms on the cancer cells.[23,26]

The distribution of the *tk* mRNA in the organism was studied by RT-PCR. In the peritoneal cavity itself the distribution appeared to be quite heterogenous as one sample from the epiploon showed a high expression of the transgene while other samples demonstrated only a faint expression (Figure 3). This is quite consistent with the data obtained in our laboratory with a *LacZ* retrovirus. After i.p. injection of *LacZ* retrovirus producing cells, a few peritoneal tumor nodes showed a high expression of the transgene while others had apparently remained untransduced or poorly transduced (data not shown). The strong signal observed on Figure 3a is probably related to one of these highly transduced tumor nodes. This heterogenous distribution of the transgene in tumor nodes is another argument in favor of repeated injections of retrovirus producing cells.

RT-PCR analysis demonstrated a low expression of the transgene in several intra- and extra-abdominal organs. This observation was expected given the high number of injected producing cells and the communications existing between the peritoneal cavity and the rest of the organism. Similar observations had previously been reported with adenoviruses.[33] However, a comparison of the *tk* mRNA expression observed in these organs with the level of expression detected in stably transfected DHD/K12 cells indicates that only a very small number of cells have probably been transduced in these organs. Therefore, any significant toxicity should not be expected after such a treatment and was not observed in our experiments. However, the levels of the expression in these organs is higher after three injections of retrovirus producing cells than after a single one, indicating that, if repeated producing cells injections increase the efficacy of *tk* gene therapy, they also might increase the risk of toxicity.

Materials and methods

Cell lines and retroviral vector

DHD/K12/Prob cells were cultured in DMEM-modified Dulbecco's medium (Life Technologies, Gaithersburg, MD, USA) supplemented with 5% fetal bovine serum (FBS), 1% glutamine 200 mm, 1% Hepes 1 m, 1% arginine 0.55 mm and antibiotics.

The packaging cell lines were the GP+EnvAm12 line derived from murine NIH3T3 fibroblasts[34] and the FLYA13 line derived from human HT1080 fibrosarcoma cells[24] (FLYA13 was obtained from European Collection of Cell Cultures, Salisbury, UK). GP+EnvAm12 and FLYA13 cells were cultured in DMEM-modified Dulbecco's medium (Life Technologies) supplemented with 10% fetal bovine serum (FBS) (Life Technologies), antibiotics and 1% glutamine 200 mm (Life Technologies).

The retroviral vector, derived from a murine retrovirus (Mo-MuLV), was a generous gift from Dr C-M Calberg (Laboratory of Virology, University of Liège, Belgium). This vector contains the neomycin resistance gene driven by the first LTR and the HSV1-*tk* gene driven by a SV40 promoter.

Animal treatment and in vivo transduction

For single *in vivo* transduction, BD-IX rats were injected i.p. at day 0 with 10^6 DHD/K12 tumoral cells. On day 9, the animals were i.p. injected with 30×10^6 retrovirus producing cells per animal (FLYA13-TK cells or GP+EnvAm-TK cells) (Figure 1a). They were then separated in two groups and treated for 5 days, starting at day 14, either by i.p. injections of GCV (150 mg/kg) diluted in Hank's balanced salt solution (HBSS; Gibco BRL) or of the HBSS buffer alone.

For triple *in vivo* transductions, BD-IX rats were injected i.p. at day 0 with 10^6 DHD/K12 tumoral cells. The animals received three intraperitoneal injections of 30×10^6 retrovirus producing cells (FLYA13-TK cells or GP+EnvAm-TK cells) at days 9, 18 and 25 (Figure 2a). They were separated in two groups and treated either with GCV (150 mg/kg from day 14 to 17, from day 21 to 24 and from day 28 to 32) or with HBSS alone according to the same schedule (Figure 2a).

Statistics

Kaplan–Meier curves were established for each group and survivals were compared with the log-rank test.

RT-PCR for HSVtk expression

tk Gene expression in various organs after a single or three-fold *in vivo* transduction was investigated by RT-PCR followed by Southern blotting. Tissue samples from various organs (liver, bowels, lung, spleen and epiploon) were collected 5 days after the injection of producing cells (day 14) for the single transduction group (two animals) and 3 days after the last injection (day 28) for the triple transduction group (two animals).

Total cellular RNA was extracted by Tripure reagent (Boehringer Mannheim, Mannheim, Germany). One microgram of RNA was reverse transcribed (Superscript II; Life Technologies, Gaithersburg, MD, USA) with a specific *tk*-3′ primer (5′-ACAGGGTAAATAACGTGTC-3′). After purification by filtration on a spin column (GlassMax DNA Isolation Spin Cartridge System; Life Technologies), the cDNA product was amplified with the *tk* primers 5′-ATGGCTTCGTACCCCTG-3′ and 5′-AAGGTCGGCGGGATGAG-3′. A 35 cycle amplification was performed (denaturation at 95°C for 1 min, annealing at 50°C for 1 min, and elongation at 72°C for 2 min) followed by a 4 min final elongation at 72°C. The RT-PCR products (593 bp) were transferred on a nylon membrane (Quiabrane; Qiagen, Hilden, Germany) and analyzed by Southern blotting. The *tk*-DNA probe was a ^{32}P randomly labeled RT-PCR product (593 bp) amplified from stably transfected DHD/K12-*tk* cells.

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