

Combined suicide gene therapy for pancreatic peritoneal carcinomatosis using BGTC liposomes

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Peritoneal dissemination is a common end-stage complication of pancreatic cancer for which novel therapeutic modalities are actively investigated, as there is no current effective therapy. Thus, we evaluated, in a mouse model of pancreatic peritoneal carcinomatosis, the therapeutic potential of a novel nonviral gene therapy approach consisting of bis-guanidinium-tren-cholesterol (BGTC)-mediated lipofection of a combined suicide gene system. Human BxPC-3 pancreatic cells secreting the carcinoembryonic antigen (CEA) tumor marker were injected into the peritoneal cavity of nude mice. After 8 days, intraperitoneal (i.p.) lipofection was performed using BGTC/DOPE cationic liposomes complexed with plasmids encoding the two prodrug-activating enzymes Herpes Simplex Virus thymidine kinase and *Escherichia coli* cytosine deaminase, the latter being expressed from a bicistronic cassette also encoding *E. coli* uracil phosphoribosyltransferase. Administration of the lipoplexes was followed by treatment with the corresponding prodrugs ganciclovir and 5-fluorocytosine. The results presented herein demonstrate that BGTC/DOPE liposomes can efficiently mediate gene transfection into peritoneal tumor nodules. Indeed, HSV-TK mRNA was detected in tumor nodule tissues by semiquantitative reverse transcription-polymerase chain reaction analysis. In addition, green fluorescent protein (GFP) fluorescence and X-gal staining were observed in the peritoneal tumor foci following lipofection of the corresponding EGFP and LacZ reporter genes. These expression analyses also showed that transgene expression lasted for about 2 weeks and was preferential for the tumor nodules, this tumor preference being in good agreement with the absence of obvious treatment-related toxicity. Most importantly, mice receiving the full treatment scheme (BGTC liposomes, suicide genes and prodrugs) had significantly lower serum CEA levels than those of the various control groups, a finding indicating that peritoneal carcinomatosis progression was strongly reduced in these mice. In conclusion, our results demonstrate the therapeutic efficiency of BGTC-mediated i.p. lipofection of a combined suicide gene system in a mouse peritoneal carcinomatosis model and suggest that BGTC-based prodrug-activating gene therapy approaches may constitute a potential treatment modality for patients with peritoneal carcinomatosis and minimal residual disease.

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Pancreatic adenocarcinoma is a major cause of cancer deaths. Indeed, it is a highly aggressive disease characterized by early local invasion and distant spread that progresses in a relatively symptom-free manner. Thus, the vast majority of patients has already advanced disease at the time of diagnosis and are therefore not amenable to surgical resection, which offers at present the best chance of cure.^{1–4} Moreover, even after presumed curative surgery, local recurrence, lymph node involvement and spreading to the liver and/or the peritoneal cavity occur frequently. Thus, as adjuvant chemotherapy and radiotherapy approaches are ineffective,^{5–7} patients

with this devastating disease have a very poor prognosis, and pancreatic cancer remains one of the few cancers where death rate nearly equals incidence. Median survival after diagnosis is only around 4–6 months and the reported overall 5-year survival rates average 1–2%.^{1,6,7} Thus, as the current conventional treatment is ineffective, there is an urgent need for new adjuvant therapeutic modalities, including gene therapy approaches, in particular for the treatment of peritoneal carcinomatosis, which is a frequent fatal end-stage complication.

Several cancer gene therapy approaches are currently under development, including strategies to correct the primary genetic defects, suppress angiogenesis, enhance the immune response and confer increased chemosensitivity to tumor cells.^{8–12} The latter approach, suicide gene therapy — also termed gene-directed enzyme prodrug therapy (GDPET) — involves transfection into the tumor cells of nonmammalian genes encoding enzymes

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that convert nontoxic prodrugs into toxic active metabolites.^{13–18} Such prodrug-activating strategies are especially appealing for killing tumor cells because of the large therapeutic index that can be achieved, a high concentration of toxic drug being produced only at the tumor site. Another attractive feature of suicide gene therapy is that there is a local bystander effect, the activated drug being able to act on neighboring nontransfected tumor cells. Over the last 15 years, a dozen different prodrug activation schemes have been developed. Among them, activation of ganciclovir (GCV) by herpes simplex virus thymidine kinase (HSV-TK) and activation of 5-fluorocytosine (5-FC) by *Escherichia coli* cytosine deaminase (eCD) have been extensively studied. Promising results of several experimental cancer gene therapy studies with a single suicide gene/prodrug system (either HSV-TK/GCV or eCD/5-FC) have suggested that single suicide approaches may constitute efficient therapeutic modalities for the treatment of cancer in man, and consequently such strategies are at present tested in clinical trials. However, single prodrug-activation systems do often not induce complete tumor regression or avoid tumor recurrence subsequently to the termination of prodrug administration. Thus, these limitations have more recently led to the use of combined approaches in which prodrug activation schemes are combined with each other in a synergistic manner.^{16,19–21}

Nonviral vectors have emerged as attractive alternatives to recombinant viruses for gene therapy.^{22–27} Indeed, synthetic gene delivery systems are free of the problems associated with the use of recombinant viruses, including safety concerns and practical issues relating to bulk production and quality control. Thus, the search for efficient and easy-to-use nonviral vectors has resulted in the development of various methods for nonviral gene delivery. Among the synthetic vectors, cationic lipids are particularly attractive as it is possible to synthesize a wide variety of well-characterized reagents with favorable features such as biodegradability and low toxicity.²² Accordingly, various cationic lipid-based gene delivery systems have been developed over the last decade and used for cancer gene therapy, including in the clinical setting.²⁸ In particular, intraperitoneal (i.p.) *in vivo* lipofection has been evaluated for the treatment of pancreatic peritoneal carcinomatosis in the mouse.²⁹ However, it is generally agreed that the efficiency of cationic lipid-mediated *in vivo* gene transfer is rather low. Thus, others and we have directed our efforts towards the development of novel cationic lipid systems. Over the last several years, we have developed cholesterol derivatives characterized by polar head groups with guanidinium functions.^{30–32} Bis-guanidinium-tren-cholesterol (BGTC), the prototype of this novel class of cationic lipids, is a multivalent T-shaped lipid with two guanidinium groups (providing DNA binding strength) and a carbamoyl linker. BGTC/DOPE liposomes were highly efficient for gene transfection *in vitro* into a variety of a mammalian cell lines.³⁰ We also found that BGTC/DOPE liposomes mediate efficient gene transfection into the mouse respiratory epithelium *in vivo*, the lipoplexes being

instilled either directly into the trachea or into the nasal cavity.^{33,34}

The aim of the present work was thus to assess the therapeutic potential of a novel nonviral gene therapy approach consisting of BGTC-mediated *in vivo* lipofection of a combined suicide gene system for the treatment of pancreatic peritoneal carcinomatosis. I.p. administration of BGTC/DOPE liposomes complexed with plasmids expressing the two suicide genes HSV-TK and eCD followed by injection of the prodrugs GCV, and 5-FC was used for treating an experimental pancreatic peritoneal carcinomatosis induced in nude mice by i.p. injection of human pancreatic BxPC-3 cells that secrete the carcinoembryonic antigen (CEA) tumor marker. We herein report that BGTC/DOPE liposomes mediate efficient gene transfection into the pancreatic tumor nodules disseminated in the peritoneal cavity. Most importantly, we also show that BGTC-mediated double suicide gene therapy can reduce significantly the progression of peritoneal carcinomatosis as indicated by the low levels of the CEA tumor marker in the sera of mice receiving the full treatment scheme (liposomes, suicide genes and prodrugs). To the best of our knowledge, this work is the first experimental study to use the evolution of a systemic tumor marker to evaluate the therapeutic potential of *in vivo* lipofection for peritoneal carcinomatosis.

Materials and methods

Plasmids and transfection reagents

Plasmid pEGFP-N1, where enhanced green fluorescent protein is under the control of the CMV immediate/early (CMV-IE) promoter, was purchased from Clontech (Ozyme, Saint Quentin, France). pCMV-Luc, where the firefly *Photinus pyralis* luciferase reporter gene is driven by the CMV-IE promoter, was obtained from Promega (Charbonnières, France). Plasmid pCAG-LacZ, where the bacterial LacZ gene is under the control of the CAG promoter (consisting of the CMV immediate early enhancer and the chicken β -actin/rabbit β -globin hybrid promoter) was kindly provided by M Terada (Tokyo, Japan).²⁹

Plasmid pCUT contains the eCD and uracil phosphoribosyltransferase (UPRT) genes in a bicistronic cassette driven by the CMV-IE promoter, whereas the HSV-TK gene is under the control of a CAG promoter. The plasmid pCUT was constructed in three steps in pIRES plasmid (Clontech). First, eCD was PCR amplified using *Pfu* turbo DNA polymerase (Stratagene, Amsterdam, The Netherlands) and specific oligonucleotides (forward: 5'-GCCTCGAGTGACGCATCTGGAGGCTAACAAATGTCG-3'; reverse: 5'-GCGAATTC GGCATAACTAAGCTCGCTGTAAC-3') with flanking *XhoI* and *EcoRI* restriction sites (underlined) from genomic DNA library of *E. coli* (strain B; ATCC11303). The resulting PCR product was digested with *XhoI/EcoRI* and directionally cloned into the *XhoI/EcoRI* sites of the mcsA of pIRES, leading to pCD-IRES plasmid. Next, the blunt-ended UPRT cDNA was generated by PCR as reported by

Kawamura et al,³⁵ and inserted into the blunt-ended *Xba*I restriction site of the *mcsB* of *peCD-IRES* plasmid downstream of the encephalomyocarditis virus internal ribosome entry site (IRES), leading to *peCD-IRES-UPRT* plasmid. Finally, the entire HSV-TK eukaryotic expression cassette, containing the CAG promoter, the HSV-TK gene and the rabbit β -globin polyadenylation signal, was removed from the plasmid *pCtkN2* (kindly provided by M Terada, Tokyo, Japan) and subcloned into the unique *Bgl*III site of *peCD-IRES-UPRT* plasmid, generating *peCD-IRES-UPRT-HSV/TK* which is herein called *pCUT*.

All plasmids were amplified in TG2 *E. coli* bacteria and purified using the endotoxin-free mega-preparation kit (Qiagen, Courtaboeuf, France). DNA concentration was measured by UV absorption at 260 nm. $OD_{260/280}$ ratios were 1.8–1.9. Plasmid integrity and purity was confirmed by 1% agarose gel electrophoresis. The final concentration of the different plasmids was adjusted to 2–3 $\mu\text{g}/\mu\text{l}$ in endotoxin-free water and the plasmids were stored at -20°C .

Cationic BGTC/DOPE (molar ratio 3/2) liposomes were prepared as previously described at a total lipid concentration of 3.5 mg/ml.³⁰ Branched polyethylenimine (PEI25 from Sigma-Aldrich, Saint Quentin-Fallavier, France) with an average molecular weight of 25 kDa was used as 100 mM (4.5 mg/ml) aqueous stock solution. Linear 22-kDa PEI (Exgen500 from Euromedex, Strasbourg, France) (5.47 mM, 0.4 mg/ml) was used as recommended by the manufacturer.

In vitro experiments

Cell culture and in vitro transfection. The human pancreatic carcinoma cell line BxPC-3 was cultured in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum and penicillin (100 U/ml) plus streptomycin (100 $\mu\text{g}/\text{ml}$) at 37°C in 5% $\text{CO}_2/95\%$ air. Cell culture products were purchased from Invitrogen-Life Technologies (Cergy Pontoise, France).

To assess their *in vitro* transfectability, BxPC-3 cells were transiently transfected with *pCMV-Luc* plasmid using different reagents. BGTC-DOPE/DNA lipoplexes characterized by a range of positive charge ratios (+/− = 1 to 8) were prepared by mixing the plasmid DNA with required amounts of liposomes as previously described.^{30,36} For transfection, BxPc-3 cells were seeded in six-well dishes for 24 hours before transfection and used at 60–80% confluence. Cells were transfected for 4 hours in serum-free medium with 2 μg of *pCMV-Luc* complexed with BGTC/DOPE liposomes, branched PEI25 (N/P = 10) or Exgen500 (N/P = 6). The cells were then incubated in standard culture conditions for 48 hours prior to monitoring of luciferase activity.

Luciferase assay. Luciferase activity was measured in cell lysates using the Luciferase Assay System (Promega, Charbonnières, France). Cells were washed twice with PBS to remove serum and 100 μl of reporter lysis buffer was added to each well prior maintenance at 4°C for

15–30 minutes. Lysed cells were scraped into 1.5 ml Eppendorf tubes, vortexed briefly, and centrifuged at high speed for 5 minutes at 4°C to remove cellular debris. Supernatants were stored at -80°C . Cell lysates (20 μl) were dispensed into ELISA plates, mixed with 100 μl of Luciferase Assay Reagent and luciferase activity was measured using a luminometer (BMG Labotechnologies, Champigny-sur-Marne, France).

Sensitivity to GCV/5-FC of cultured BxPC-3 cells transfected with pCUT/BGTC-DOPE. Cells were seeded at 3×10^5 cells/well in triplicate into 24-well plates and incubated overnight to allow cell adherence and then transfected with *pCUT/BGTC-DOPE*. After 24 hours, the cells were left untreated or were treated with 50 $\mu\text{g}/\text{ml}$ 5-FC (Sigma) and 25 $\mu\text{g}/\text{ml}$ GCV (Roche, Neuilly-Sur-Seine, France), and further incubated at 37°C in standard cell culture conditions. Culture medium was then removed at different time points and aliquots were used for monitoring the CEA concentration by an enzyme-linked immunosorbent assay (ELISA) (Institut de Chimie Biologique, Hôpital Civil, Strasbourg, France). In parallel, cell survival was determined using a colorimetric assay which measures the ability of viable cells to reduce a soluble yellow 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium (MTT) to an insoluble formazan precipitate. Here, the culture medium was discarded and replaced with 100 μl of MTT (1 mg/ml). The dishes were then incubated for another 4 hours at 37°C and centrifuged. Next, the medium was replaced with 100 μl of dimethyl sulfoxide, the dishes were shaken for 20 minutes and absorbance at 570 nm was determined for each well using a microplate reader (Bio-Rad laboratories model 550). Two independent experiments were performed in triplicate. Cell survival was calculated as the percentage of viable cells in comparison to nontransfected cells treated with the prodrugs.

In vivo experiments

Animals. Pathogen-free, 7–8-week-old female athymic NMRI-nu (nu/nu) nude mice (Elevage Janvier, Le Genest, France) were allowed to acclimate for at least 2 weeks before starting the experiments. Animals were maintained under specific pathogen-free conditions and procedures were performed in accordance with recommendations for the proper care and use of laboratory animals.

Induction of pancreatic peritoneal carcinomatosis. Growing pancreatic BxPC-3 cells were trypsinized, washed and resuspended in RPMI-1640 medium supplemented with antibiotics (100 U/ml penicillin, 100 $\mu\text{g}/\text{ml}$ streptomycin). To induce peritoneal carcinomatosis, nude mice were inoculated i.p. with 10^7 viable BxPC-3 cells in 1 ml of RPMI-1640. In pilot experiments, the animals developed within 8–12 days a peritoneal carcinomatosis easily detectable with a binocular stereomicroscope.

Preparation of DNA complexes for i.p. lipofection. DNA complexes characterized by a mean positive charge ratio of 4 were prepared by mixing equal volumes (200 μ l) of plasmid DNA solution and BGTC/DOPE liposomes (molar ratio 3:2, total lipid concentration of 3.5 mg/ml). Thus, plasmid DNA was diluted in 200 μ l PBS containing 5% (w/v) glucose and mixed with the required amount of BGTC/DOPE liposomes. Practically, when preparing complexes with 100 μ g of DNA, 200 μ l of BGTC/DOPE liposomes were mixed with the plasmid solution (as 1 μ l of BGTC/DOPE liposomes is approximately 6 nmol of positive charges, whereas 1 μ g of DNA is 3 nmol of negatively charged phosphate). When preparing complexes from 50 μ g DNA, 100 μ l of glucose-containing PBS was added to the required 100 μ l of liposomes before mixing with the plasmid. The resulting solution was intensively vortexed and incubated for 20 minutes at room temperature. Because of the colloidal instability of highly concentrated solutions of lipoplexes, the BGTC/DOPE/DNA complexes were further diluted to a final volume of 800 μ l with PBS containing 5% (w/v) glucose, vortexed and immediately administered i.p.

In vivo experiments with suicide genes. At 8 days after i.p. inoculation of tumor cells, the mice were randomly allocated to four experimental treatment groups (groups G1–G4) as indicated in Table 1 and all mice (except G4 mice) were injected i.p. with either BGTC/DOPE/pCUT lipoplexes (G1 and G2 mice) or naked pCUT DNA (G3 mice). The lipoplexes prepared as indicated above (or naked DNA in 5% (w/v) glucose-containing PBS) were administered i.p. for three successive days at a dosage of 100 μ g DNA per mouse the first day and 50 μ g DNA the second and third days. At 1 day after the first DNA administration, all mice (except G2 mice) were i.p. injected for 21 successive days with GCV (250 mg/kg/day) plus 5-FC (500 mg/kg/day), the G2 mice being injected with NaCl 0.9%. The characteristics of the four treatment groups of mice are indicated in Table 1, and Figure 1 shows the treatment schedule. Finally, pCUT/BGTC–DOPE lipoplexes were also administered to tumor-free (not inoculated) mice ($n = 7$) in order to help to assess the preferential transfection of tumor cells.

In vivo experiments with reporter genes. In a second set of *in vivo* experiments, plasmids expressing reporter genes

Table 1 Characteristics of the different treatment groups

	G1	G2	G3	G4
DNA	+	+	+	–
BGTC/DOPE	+	+	–	+
GCV/5-FC	+	–	+	+
NaCl 0.9%	–	+	–	–

At 8 days after i.p. inoculation of BxPC-3 pancreatic tumor cells, the mice were divided into four groups (G1–G4, $n \geq 6$) and subjected to the various treatments indicated above according to the treatment schedule shown in Figure 1. G1: full treatment group, G2: suicide system control group, G3: naked DNA control group, G4: liposome and prodrug toxicity control group.

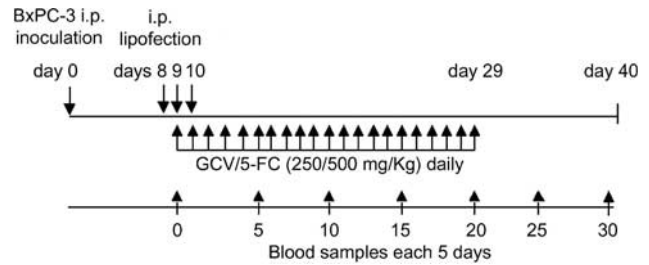


Figure 1 Schematic schedule of the experimental protocol involving induction of pancreatic peritoneal carcinomatosis in athymic mice, followed by BGTC-mediated i.p. suicide gene lipofection and prodrug treatment. At 8 days after i.p. injection of BxPC-3 pancreatic tumor cells, the mice were randomly affected to four treatment groups (G1–G4, $n \geq 6$). The treatment schedule for G1 mice receiving the full treatment scheme is shown here. BGTC/DOPE liposomes complexed with pCUT plasmid were injected i.p. for three successive days. Administration (i.p.) of the prodrugs GCV (250 mg/kg) and 5-FC (500 mg/kg) was started 1 day after the first lipofection and was for 21 successive days. Blood samples were collected at 5-day intervals for 1 month. Groups G2–G4 mice received the treatments indicated in Table 1 following a similar schedule.

were i.p. administered (either complexed with BGTC–DOPE liposomes or naked) to mice with established pancreatic peritoneal carcinomatosis. Preparation of the lipoplexes and i.p. administrations were as indicated above for suicide genes. In experiments with the EGFP reporter gene, the mice received either BGTC–DOPE/pEGFP-N1 lipoplexes ($n = 15$) or naked pEGFP-N1 plasmid ($n = 10$). When using the LacZ reporter gene, the mice were i.p. injected with either BGTC–DOPE/pCAG-LacZ lipoplexes ($n = 2$) or naked pCAG-LacZ ($n = 2$). Control tumor-free mice were also injected with either BGTC–DOPE/pEGFP-N1 ($n = 2$) or BGTC–DOPE/LacZ ($n = 2$) lipoplexes.

RT-PCR analysis of HSV-TK expression in vivo. In order to assess suicide gene expression *in vivo*, reverse transcriptase-polymerase chain reaction (RT-PCR) was used to study HSV-TK expression in G1–G3 mice. Two mice per group were killed at days 2, 5, 15 and 30 after the first DNA administration. Tumor nodules were sampled by careful dissection from various abdominal organs bearing tumor nodules (pancreas, small gut, abdominal wall, genital tract, mesentery, lymph nodes), snap-frozen in liquid nitrogen and stored at -80°C until assayed for gene expression. A sample of liver tissue macroscopically free of tumor nodules was also obtained. In healthy (not inoculated) mice injected with BGTC–DOPE/pCUT lipoplexes ($n = 3$), biopsies originating from the same organs were excised after 5 days and used as a control of gene transfection into normal tissue.

Total RNA was isolated from the different tissue samples using TRIzol reagent (Life Technologies), pooled and subsequently treated with RNase-free DNaseI to avoid contamination with plasmid or genomic DNA. For cDNA synthesis, 5 μ g of purified total RNA was reverse transcribed for 1 hour at 42°C using 200 U of SuperScript reverse transcriptase II and random hexamer primers

(Invitrogen-Life Technologies). A measure of 1–5 μ l of RT product was subjected to PCR amplification. Each amplification was performed twice using independent RT reactions to confirm the results and the reproducibility.

HSV-TK-specific primers (sense: 5'-GGCGTGAAA-CTCCCGCACCT-3'; antisense: 5'-CCGCGTTTATGA-ACAAACGA-3') were used to amplify a 1280-bp fragment. RT-PCR analysis of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA levels was performed as a control. GAPDH-specific primers (sense: 5'-ACCA-CAGTCCATGCCATCAC-3'; antisense: 5'-TCCACCA-CCCTGTTGCTGTA-3') were used to PCR amplify a 450-bp fragment. All the primers were purchased from Invitrogen-Life Technologies. PCR was performed using 5 μ l of RT reaction, 0.8 mM of each sense and antisense primer, 0.8 mM dNTP, 2 mM MgCl₂ and 2 U of *Taq* DNA polymerase (final volume 50 μ l). For amplification, each PCR mixture was subjected to 35 cycles of denaturing 45 seconds at 94°C, annealing 45 seconds at 60°C and extension 2 minutes at 72°C using a PT100 apparatus (MJ Research, Inc.). Equal volumes of PCR products were analyzed on a 1.5–2% agarose gel containing ethidium bromide.

Assay for GFP expression in vivo. In mice with established peritoneal carcinomatosis and i.p. injected with pEGFP-N1 DNA (either complexed with BGTC/DOPE liposomes or naked), tumor tissue samples were also obtained from various abdominal organs scattered with tumor nodules at days 2, 5, 10, 15 and 20 after DNA injection (two mice per day group), snap-frozen and stored at –80°C until assayed. Samples were also obtained from the same organs of tumor-free animals injected with EGFP-N1 complexes. The samples were then thawed in presence of luciferase reporter lysis buffer (Promega, Charbonnières, France) supplemented with protease inhibitor cocktail (Roche Diagnostics, Meyland, France). Tissues were homogenized on ice using a Polytron homogenizer. The samples were transferred to Eppendorf tubes, subjected to three freeze–thaw cycles and centrifuged at 12,000 rpm at 4°C for 10 minutes. The supernatants were recovered and directly assayed for GFP fluorescence by spectrofluorometry (excitation wavelength: 479 nm; emission wavelength: 507 nm) using a Shimadzu RF-5000 apparatus (Roucaire, Les Ulis, France). Data were expressed as relative fluorescence units (RFU) per milligram protein, the protein concentration being determined by the bicinchoninic acid assay (Tebu, Le Perray-en-Yvelines, France).

In vivo expression pattern of β -galactosidase. At 5 days after the first i.p. injection of Lac-Z DNA (complexed with BGTC/DOPE liposomes or naked as indicated above), all mice were killed under anesthesia by exsanguination. A small incision was made in the abdominal wall and the digestive cavity was washed twice with ice-cold PBS. After laparotomy, the entire digestive tract, the peritoneal lining of the abdominal wall and the genital tract were excised, placed in Petri dishes, washed in PBS,

incubated in a DNase solution in PBS for 15 minutes and washed again with PBS. The abdominal organs were then fixed in 2% formaldehyde for 2 hours, washed with PBS, and finally stained *in toto* with a X-gal solution for 24 hours at 37°C. The X-gal solution was freshly prepared by mixing a solution containing 3.5 mM potassium ferricyanide, 3.5 mM potassium ferrocyanide, 2 mM MgCl₂, 0.01% sodium deoxycholate, and 2% of a solution containing 40 mg of 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-gal) in 1 ml of dimethylformamide at pH 7.3.

Serum CEA levels. Blood samples were collected from the tail vein of mice each 5 days for 30 days as indicated in Figure 1. The serum CEA levels were determined by ELISA.

Statistical analysis

All data are presented as mean values \pm SEM. Differences were analyzed with the Student's *t*-test or analysis of variance (ANOVA). A *P*-value of <.05 was considered to indicate a statistically significant difference between groups.

Results

In vitro transfection of human pancreatic BxPC-3 cells using BGTC/DOPE liposomes

First, we undertook to verify that BGTC/DOPE liposomes could efficiently transfect human pancreatic BxPC-3 cells *in vitro*, as these cells will constitute the target cells in the subsequent *in vivo* studies. Various amounts of BGTC/DOPE liposomes were complexed with the luciferase-expressing plasmid pCMV-Luc and the resulting lipoplexes were used to transfect the BxPC-3 cells as indicated in Materials and methods. The transfection results are indicated in Figure 2a where luciferase reporter gene expression at 48 hours post-transfection is shown as a function of the charge ratio of the lipoplexes. To determine the theoretical mean charge ratio of the lipoplexes, we took into account that 1 μ g of DNA is 3 nmol of negative charges and assumed that only the two guanidinium groups (and not the tertiary amine) of BGTC are positively charged at neutral pH. Clearly, high levels of luciferase expression were observed, a finding demonstrating that BGTC/DOPE liposomes mediate efficient transfection of the human BxPC-3 pancreatic cancer cells. These levels were actually similar to those previously observed when transfecting a variety of mammalian cell lines with BGTC/DOPE liposomes.³⁰ The dose–response curve was roughly bell-shaped, luciferase expression being highest with lipoplexes characterized by a charge ratio of approximately 4. We and others have already reported that the decline in transfection efficiency at high charge ratio may reflect some toxic effects of the cationic lipid.^{30,36–39} As shown in Figure 2b, the transfection efficacy of BGTC/DOPE liposomes was, in fact, also similar to that of Exgen500, a linear form of

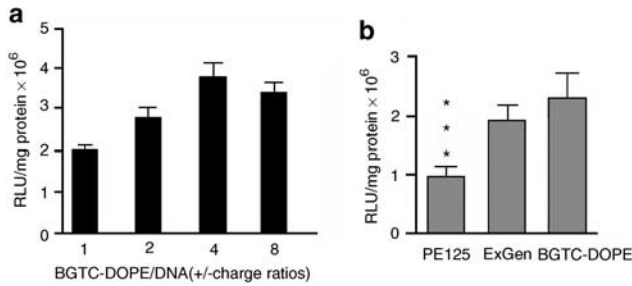


Figure 2 Luciferase activity in transfected human pancreatic BxPC-3 cells. Transient transfections of BxPC-3 cells were performed as indicated in Materials and methods using 2 μ g of pCMV-Luc plasmid DNA. (a) Transfection activity of BGTC/DOPE/DNA lipoplexes characterized by various mean charge ratios. (b) Transfection activity of branched PEI-25 (N/P=10) and Exgen500 (N/P=6) polyplexes compared with BGTC/DOPE lipoplexes (+/- 4) as described in Materials and methods. Luciferase activity was measured 48 hours after transfection and the results are shown as means \pm SEM of five independent experiments (RLU, relative light units) *** P < .001.

polyethylenimine (PEI) with an average molecular mass of 22 kDa, which has been shown to be a highly efficient reagent for *in vitro* and *in vivo* gene transfection.^{40,41} Of note, branched PEI25 exhibited a significantly lower transfection activity (P < .001). This is in agreement with previous studies indicating that linear PEI22 had greater transfection efficiency than branched PEI25.⁴² Taken together, these results basically showed that BGTC/DOPE liposomes could satisfactorily transfect BxPC-3 cells. In addition, as it is generally agreed that such an *in vitro* screening can be used for establishing starting values for *in vivo* studies, we chose to use (*in vitro* optimal) BGTC/DOPE lipoplexes with a charge ratio of 4 for the subsequent *in vivo* experiments with the murine pancreatic peritoneal carcinomatosis model.

Next, we used BGTC/DOPE liposomes for transferring the suicide genes-expressing plasmid pCUT into cultured BxPC-3 cells (and also COS-7 cells) to verify its correct functioning. As expected, in these transient transfection experiments, expression of the HSV-TK and eCD suicide genes as well as of the UPRT gene was easily detected at the RNA level by RT-PCR analysis, the eCD protein being in addition also detected by Western blotting using a monoclonal anti-eCD antibody⁴³ (data not shown). Finally, in these preliminary *in vitro* experiments, we also assessed secretion of the CEA tumor marker by BxPC-3 cells and other pancreatic cancer cell lines (SOJ6, HA-HPC2, Panc1). These pancreatic cell lines were cultured for 48 hours and the CEA levels in the culture medium was then evaluated with an ELISA assay (see Materials and methods). The highest CEA levels were observed with BxPC-3 cells (50 \pm 12 ng/ml) and SOJ6 cells (37 \pm 9 ng/ml), the other cell lines secreting much lower levels of CEA. Thus, these data showed that BxPC-3 cells secreted significant levels of the CEA tumor marker and suggested that extension of BxPC-3 peritoneal carcinomatosis *in vivo* may be monitored by measuring the CEA levels in the serum of the animals.

In vitro sensitivity of BxPC-3 cells to GCV/5-FC

To confirm the gene transfer efficiency of BGTC-DOPE liposomes and test our suicide system *in vitro*, we compared the sensitivity of parental and pCUT-transfected BxPC-3 cells to the prodrugs 5-FC and GCV. The pancreatic tumor cells were transfected with pCUT/BGTC-DOPE and subsequently exposed to GCV/5-FC as indicated in Material and methods. The parental cells were resistant to the prodrugs, whereas transfected cells were markedly sensitive to GCV/5-FC treatment in a time-dependent manner as measured by the MTT assay (Fig 3a). In the same experiments, we also evaluated the CEA level in the cell culture medium. In the treated parental as well as in untreated transfected BxPC-3 cells, the CEA levels increased, being maximum after 72 hours.

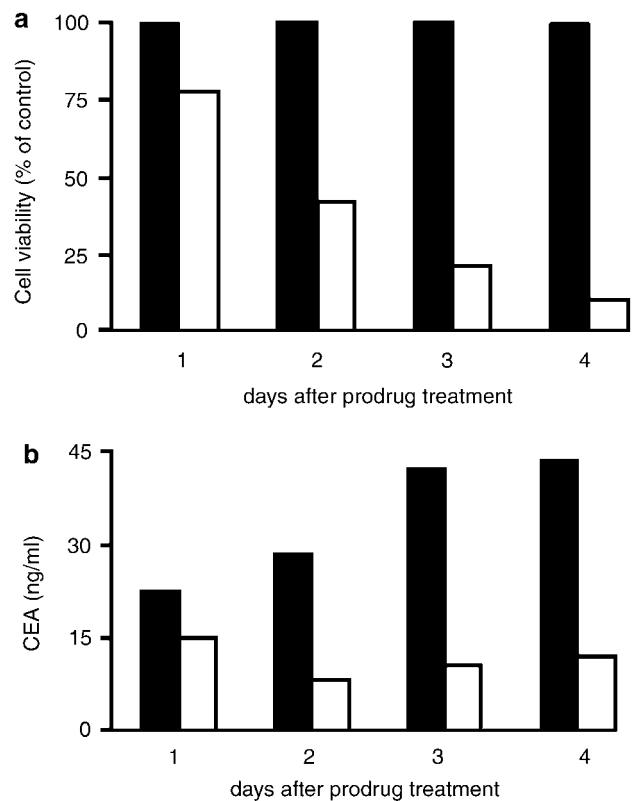


Figure 3 (a) *In vitro* sensitivity of pancreatic tumor cells to GCV and 5-FC treatment. BxPC-3 cells were transfected with pCUT/BGTC-DOPE lipoplexes, and after 24 hours the cell cultures were either left untreated (black bars) or were treated (white bars) with GCV (25 μ g/ml) and 5-FC (50 μ g/ml). Cells were harvested at the indicated time points and cell survival was measured by MTT assay. Treated nontransfected cells were used as controls. Data represent the mean of two independent experiments carried out with triplicate samples. (b) CEA concentration in the cell culture medium after GCV and 5-FC treatment. In the same *in vitro* experiments with BxPC-3 cells, aliquots of culture medium were assayed for CEA using an ELISA assay. An elevated secretion of CEA was detected in untreated transfected BxPC-3 cells (black bars). The CEA accumulation was increased in a time-dependent manner. On the contrary, CEA concentration decreased in the culture medium of transfected and GCV/5-FC treated cells (white bars). Data are representative of two separate experiments carried out with triplicate samples.

On the contrary, GCV/5-FC treatment of transfected cells caused a marked decrease in the CEA level in the cell culture medium (Fig 3b). As the CEA level is directly correlated with the number of living cells, such a decrease reflects the antitumor cytotoxic effect of our combined suicide gene/prodrug system.

Inhibition of pancreatic peritoneal carcinomatosis progression by BGTC-mediated lipofection of suicide genes and prodrug treatment

In order to assess the usefulness of a BGTC-based suicide gene therapy approach for the *in vivo* treatment of pancreatic cancer, we next examined whether direct BGTC-mediated i.p. lipofection of pCUT plasmids followed by treatment with the prodrugs GCV and 5-FC could inhibit tumor progression in a mouse model of pancreatic peritoneal carcinomatosis. As indicated in Materials and methods, the mouse pancreatic peritoneal carcinomatosis model was established via i.p. inoculation of CEA-secreting human pancreatic BxPC-3 cells. Preliminary experiments indicated that a peritoneal carcinomatosis developed in the animals within 8–12 days (data not shown). Thus, in our *in vivo* suicide gene therapy experiments, the mice were divided into four groups at day 8 following i.p. inoculation of the BxPC-3 cells and subjected to various treatment schemes (Table 1).

As BxPC-3 cells secrete the CEA tumor marker, the CEA level in the serum of the animals was used as an indicator of the extension of peritoneal invasion by the pancreatic tumor cells. As shown in Figure 4, the serum CEA levels in mice receiving the full treatment (G1 mice) remained very low (almost unchanged) for the first 2 weeks after initiation of the treatment but did however increase afterwards, reaching moderate levels at 1 month.

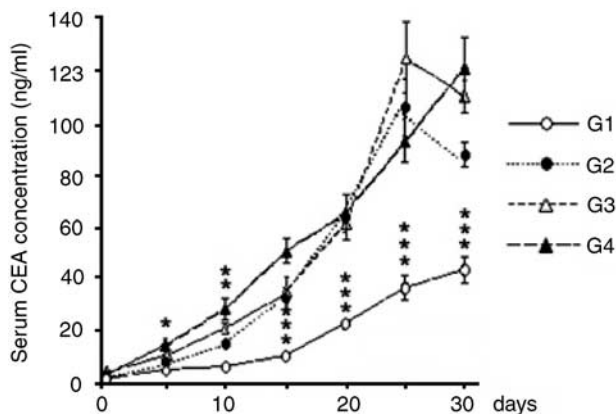


Figure 4 Evolution of serum CEA levels in mice with pancreatic peritoneal carcinomatosis subjected to various treatment regimens. G1 mice received the full treatment scheme, whereas G2–G4 mice formed different control groups (see text and Table 1). Blood samples were obtained at various time points as indicated in Figure 1 (day 0 being here the first day of blood sampling) and serum CEA levels (ng/ml) were measured by an ELISA assay. Data are shown as mean values \pm SEM ($n \geq 6$). Statistically significant differences for G1 mice versus G2–G4 mice are indicated at P -values: * $P < .05$, ** $P < .01$, *** $P < .001$.

On the contrary, the serum CEA levels in the mice of the different control groups increased steadily, very high levels being reached at 1 month. Accordingly, the serum CEA levels in G1 mice were significantly lower than those observed in the various control groups throughout the experiment ($P < .01$ at days 5–15 and $P < .001$ at days 20–30) and there was no statistically significant difference between the diverse control groups. These data also indicate that both suicide gene expression (comparison of G1 mice with G2 mice) and BGTC/DOPE liposomes (G1 mice versus G3 mice) played critical roles here and that the reduced serum CEA levels in G1 mice were not related to toxic effects of BGTC/DOPE liposomes and/or prodrugs (comparison of G1 mice with G4 mice). In addition, ascites formation seemed to be reduced in G1 mice when compared with control mice. Finally, the full treatment may have had an impact on the survival of the animals, as two G1-mice were still alive at day 70 whereas all the control mice were dead within 50 days. Taken together, these results demonstrate that BGTC-mediated i.p. lipofection of suicide genes can transiently reduce progression of pancreatic peritoneal carcinomatosis in the mouse.

Efficacy of BGTC-mediated i.p. lipofection

In order to substantiate at the molecular level the observed therapeutic efficiency of BGTC-mediated i.p. lipofection of suicide genes, we first used RT-PCR analysis to demonstrate *in vivo* suicide gene transfection into the tumor nodules disseminated in the peritoneal cavity. Indeed, such an RNA-based assessment has the advantage over direct PCR analysis to avoid overestimation of the gene transfer efficiency due to DNA complexes sticking to the surface of the tumor cells. In addition, RT-PCR analysis also allows to evaluate the duration of transgene expression.

As shown in Figures 5a and b, RT-PCR analysis detected, at days 2 and 5 after lipofection, high levels of HSV-TK mRNA in various i.p. tumor nodule tissues in G1 mice (Fig 5a) as well as in G2 mice (Fig 5b). Figure 5b also shows that HSV-TK expression was not detected in tumor-free liver tissue. In addition, HSV-TK expression was not or hardly detected in normal tissues obtained from tumor-free mice at day 5 after lipofection (Fig 5d). These data demonstrate gene transfection into the tumor nodules and also suggest that there was a tumor cell preference of BGTC-mediated lipofection. Interestingly, as regards duration of suicide gene expression, it should be noted here that RT-PCR analysis no longer detected HSV-TK mRNA at day 15 in the tumor tissues of G1 mice receiving the full treatment scheme (Fig 5a). On the contrary, in G2 mice not treated with prodrugs, a low HSV-TK signal was, however, still detected by RT-PCR at day 15 after lipofection (Fig 5b); such an increased duration of suicide gene expression may be due to the fact that the transfected tumor cells were not killed here by activated prodrugs. Finally, after i.p. administration of naked DNA, low levels of HSV-TK mRNA were detected only at day 2, such a low and highly transient HSV-TK

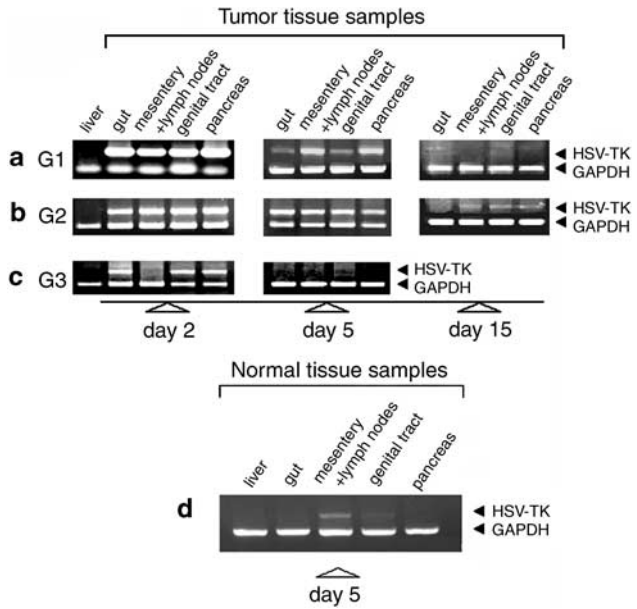


Figure 5 RT-PCR analysis of HSV-TK mRNA in tumor and normal tissue. The upper part (a–c) of the figure shows the results obtained from samples of tumor-bearing animals from the different groups described in Table 1. The lower part (d) shows the results obtained from samples of tumor-free animals. The GAPDH expression was used as an internal control. (a) G1: the mice received pCUT/BGTC–DOPE lipoplexes followed by prodrug treatment. (b) G2: the mice received pCUT/BGTC–DOPE lipoplexes without prodrug injection. (c) G3: the mice received naked pCUT DNA. (d) Control tumor-free mice which received pCUT/BGTC–DOPE lipoplexes.

expression probably explaining the aforementioned absence of an obvious therapeutic effect in G3 (naked DNA control) mice (Fig 5c).

In order to further assess the *in vivo* gene transfection efficiency, we also performed i.p. lipofection experiments with plasmids expressing reporter genes. First, pEGFP-N1 DNA plasmid expressing the green fluorescent protein (GFP) was lipofected under similar conditions and GFP fluorescence levels in tumor nodule tissues were measured to evaluate the efficiency of gene transfection quantitatively. As shown in Figure 6, significant levels of GFP fluorescence were observed in various tumor tissues at day 2 postlipofection, the highest levels being detected in tissue homogenates from mesenteric nodules and lymph nodes. In contrast, only very low levels of GFP fluorescence were observed in the tumor nodules after i.p. administration of naked (uncomplexed) pEGFP-N1 plasmid. Similarly, in control experiments where pEGFP-N1 plasmid was lipofected into tumor-free mice, no GFP fluorescence was detected in several normal tissues (data not shown). These results again demonstrate that BGTC-mediated i.p. lipofection is efficient for gene transfection into disseminated i.p. tumor nodules, and that BGTC/DOPE liposomes unequivocally play a crucial role. In order to attempt to evaluate the percentage of tumor cells lipofected *in vivo*, we compared the GFP fluorescence levels in the tumor nodules to those observed with BxPC-3 populations containing known proportions

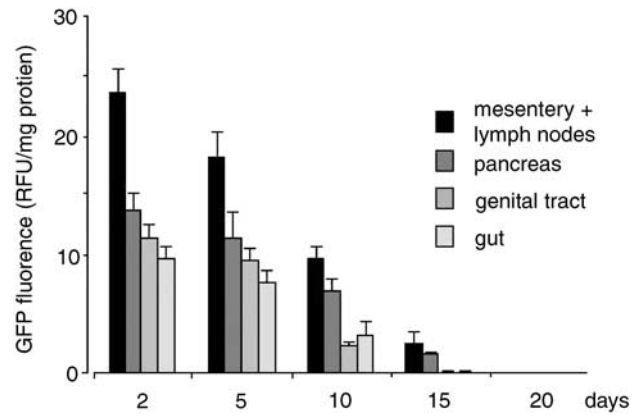


Figure 6 Time course of GFP expression after i.p. injections of BGTC/DOPE/pEGFP-N1 lipoplexes. The relative fluorescence levels were determined 2, 5 and 10 days after lipofection in tumor samples from different abdominal organs with scattered tumor nodules. Results are expressed as mean (\pm SEM) RFU/mg protein.

of GFP-expressing cells obtained by mixing parental untransfected cells with pEGFP-N1 transfected cells, as previously described.⁴⁴ Such a comparison allowed us to estimate that at least 5–10% of the tumor cells were transfected under our experimental conditions as tumor nodules harvested at an early stage of peritoneal carcinomatosis might have been contaminated with normal tissue. Finally, Figure 6 also indicates that GFP fluorescence of the different tumor nodule tissues decreased during the weeks following lipofection, reaching low levels (or becoming even undetectable in some tumor tissues) at day 15. Such a decrease in GFP expression did, in fact, parallel that in HSV-TK expression (see above). Thus, in order to get some insights into the temporal relationships between duration of transgene expression and the aforementioned transient therapeutic efficiency, we compared the evolution of GFP fluorescence in tumor tissue *in vivo* (which reflects gene transfection) with the evolution of the serum CEA level (which is indicative of the extension of peritoneal carcinomatosis in G1 mice receiving full treatment). Figure 7 shows that the serum CEA levels began to increase strongly (at day 15) when the GFP fluorescence became very low, the reciprocal evolution of the two quantitative parameters being demonstrated by the existence of an inverse correlation ($r = 1$, $P < .001$) between them. This finding basically suggests that tumor escape from the treatment occurred when the gene transfer efficiency became too low. It should be noted here that the number of transfected cells in mice lipofected with the suicide genes and treated with the prodrugs may decrease even faster than that in mice transfected with the GFP reporter gene, because prodrug treatment kills the tumor cells expressing the suicide genes, whereas loss of GFP expression may simply reflect tumor progression (as it is probably due to dilution of the plasmid DNA in the regrowing tumors). Accordingly, as already indicated above, duration of HSV-TK expression was also shorter in the G1 mice receiving the full treatment than in G2

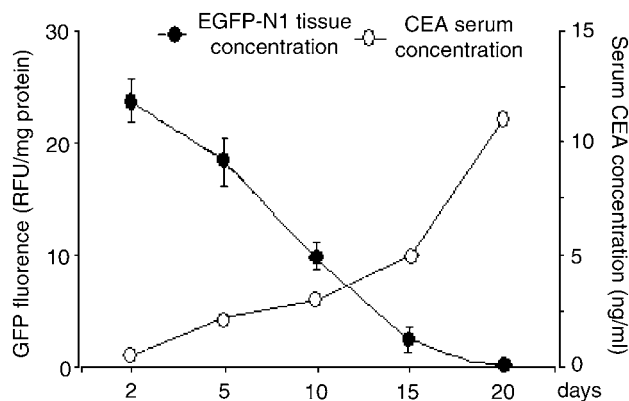


Figure 7 Comparison of the evolution of GFP fluorescence levels in tumor nodule biopsies with that of serum CEA levels in G1 mice receiving full treatment. GFP fluorescence levels were assessed by spectrofluorometric measurements and serum CEA levels were determined by an ELISA assay as indicated in Materials and methods. An inverse correlation ($r=1$, $P<.001$) was observed between the two parameters.

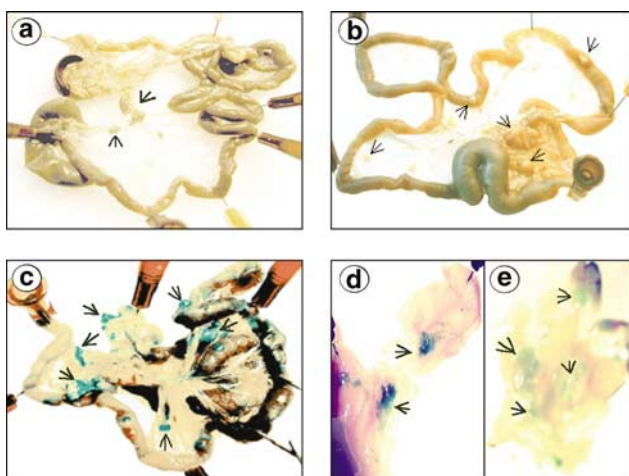


Figure 8 Representative β -galactosidase expression pattern (detected by *in toto* X-gal staining) in the peritoneal cavity of normal mice (a) and mice with established peritoneal carcinomatosis (b–e). The X-gal staining reaction was performed at day 5 post-DNA injection. Arrows in (a)–(e) indicate either tumor nodules, or the presence of β -gal positive staining. (a) No X-gal staining in the whole peritoneal cavity after administration of pCAG-LacZ/BGTC–DOPE lipoplexes in tumor-free mice. (b) No X-gal staining in the whole peritoneal cavity after administration of naked pCAG-LacZ. (c) Clusters of dark blue, β -gal positive cells observed at multiple sites throughout the peritoneum, in particular in the pancreas and in the mesentery. (d) X-gal staining of tumor nodules on the peritoneal lining of the anterior abdominal wall. (e) X-gal staining of tumor nodules on the genital tract.

mice lipofected with the suicide genes but not treated with prodrugs (in which the suicide gene can in fact be considered as a reporter gene).

Finally, we also performed i.p. lipofection experiments with BGTC/DOPE liposomes complexed with β -galactosidase-expressing pCAG-LacZ plasmid in order to further

monitor *in vivo* gene transfection. Indeed, X-gal staining is a widely used method for evaluating qualitatively the pattern of transgene expression *in vivo*. When the abdominal organs were harvested at day 5 postlipofection and stained *in toto* with X-gal, macroscopic examination showed no staining in tumor-free mice (Fig 8a), whereas we observed an intense blue staining of the tumor nodules on various organs in the peritoneal cavity, including the pancreas, mesentery, lymph nodes, intestine, genital tract and peritoneal lining of the anterior abdominal wall (Fig 8c–e). In contrast, macroscopic examination of the peritoneal cavity in control mice injected with naked DNA did not show any blue staining of the tumor nodules (Fig 8b). These data, which are in agreement with the HSV-TK RT-PCR analysis results and GFP fluorescence measurements, confirmed that BGTC-mediated i.p. lipofection allows transfection of tumor nodules at various sites in the peritoneal cavity and also suggested that there was a tumor cell preference of BGTC-mediated i.p. lipofection. Because of the low sensitivity of X-gal staining, one cannot exclude the possibility that normal organs may also be transfected, albeit at a lower level than the tumor cells. However, the negative RT-PCR of HSV-TK expression in normal tissue from tumor-free mice (see above) provides additional evidence for a preferential transfection of the tumor cells via i.p. lipofection with BGTC/DOPE liposomes.

Discussion

The major goal of our work was to study whether the high efficiency of *in vitro* gene transfection by BGTC/DOPE liposomes and the high antitumor activity of our combined suicide gene/prodrug system with pancreatic tumor cells *in vitro* can be extended to an *in vivo* application. Taken together, our results show in a nude mouse model of pancreatic peritoneal carcinomatosis that BGTC/DOPE liposomes can mediate efficient gene transfection into the disseminated peritoneal tumor nodules and that BGTC-mediated i.p. lipofection of suicide genes can transiently reduce disease progression.

As a result of the unsatisfactory results of current therapy, pancreatic peritoneal carcinomatosis represents an ideal target for new therapeutic approaches such as gene therapy. Although it is generally agreed that nonviral vectors are less efficient than recombinant viruses, several nonviral gene delivery systems have already been evaluated for gene transfection into tumors, especially as they raise none of the safety concerns of viral vectors for human gene therapy.^{23–25} Although nonviral vectors have been used for *in situ* administration into macroscopic tumors or even via systemic injection^{45–48} the peritoneal cavity represents a particularly attractive target for nonviral vectors as it allows a more efficient diffusion of the DNA complexes when compared to the micro-environment of solid tumors. Thus, i.p. injection of DNA complexes might enable efficient *in vivo* gene transfection into small tumors disseminated in the peritoneal cavity.

Cationic polymers as well as cationic lipids have already been used for gene transfer into peritoneal tumor nodules.^{29,49,50} For example, it has recently been reported that direct i.p. administration of PEI polyplexes was an efficient and safe method for *in vivo* transfection of reporter genes into i.p. tumors.⁵⁰ As concerns cationic lipids, the cationic lipopolyamine dioctadecylamidoglycylspermine (DOGS) has been found to permit transgene expression in about 10% of the tumor cells in a mouse model of pancreatic peritoneal carcinomatosis.²⁹ In order to develop more efficient cationic lipid systems, we have directed our efforts over the last several years toward the development of cationic lipids with novel cationic moieties. Indeed, although the structure/activity relationships of cationic lipids are not well understood, it is generally agreed that the nature of the positive head group impacts on their transfection activity. We have previously already reported the transfection properties of cationic lipid formulations containing BGTC, a cationic cholesterol derivative characterized by a polar head group with two guanidinium functions.^{30,51} We have, in particular, found that BGTC/DOPE cationic liposomes permit efficient gene transfection into the normal mouse airway epithelium *in vivo*.³³ The results presented herein indicate that BGTC/DOPE liposomes can also mediate transfection of pancreatic cancer cells *in vitro* and *in vivo* in peritoneal carcinomatosis. Of note, loco-regional gene transfer to the respiratory epithelium (via instillation into the airways) is to some extent similar to gene transfection into small tumor nodules disseminated in the peritoneal cavity, as both can be viewed as a two-dimensional transfection with minimal extracellular barriers. Our data also invite to work out improved BGTC-based formulations for i.p. lipofection (characterized by optimal charge and lipid/colipid ratios, the presence of other additives than DOPE, etc.) in order to increase the efficiency of gene transfection into the tumor nodules. Enhanced i.p. lipofection may also be obtained by use of higher doses of lipoplexes, whose administration may require colloidal stabilization via adequate polyethylene glycol (PEG) derivatives, which should ideally be equipped with ligands (e.g. folate, transferrin) for tumor cell targeting. It should be stressed here that we have already reported that incorporation of appropriate lipophilic PEG derivatives allowed to prepare highly concentrated solutions of colloidally stable BGTC/DOPE lipoplexes that permitted enhanced transfection of the mouse airways *in vivo* via intranasal instillation.³⁴ In addition, we have recently observed that incorporation of such lipophilic PEG derivatives allows efficient gene transfection into the mouse respiratory epithelium when using aminoglycoside-derived cationic lipids.³⁹

Our results also indicate that the various transgenes (HSV-TK, EGFP, LacZ) were preferentially expressed in the peritoneal tumor tissues. Accordingly, there was no obvious toxicity or organ damage. Various mechanisms may underlie this apparent tumor preference of BGTC-mediated i.p. lipofection. Such tumor preference has actually already been observed following i.p. transfection with other nonviral vector systems.^{29,49,50} First, the tumor

nodules stick on the peritoneal outside layer and are therefore directly accessible to the lipoplexes injected into the peritoneal space (via infiltration through their surface). In contrast, intra-abdominal organs are embedded in a continuous peritoneal epithelial lining, which may constitute an effective barrier against the entry of the DNA complexes through their surface. Second, the highly proliferating cancer cells in the tumor tissues (especially at an early stage of tumor growth) are probably more readily transfected with nonviral vectors than normal cells with low mitogenic activity.^{33,52,53} Indeed, it is generally agreed that the breakdown of the nuclear membrane during mitosis plays a crucial role in nonviral gene transfection as the transferred plasmid DNA does not easily enter the nuclear compartment during interphase.^{22,32,37} In addition, the transgene may be more promptly expressed in highly cycling cancer cells than in normal cells.⁵⁴ Third, with regard to suicide gene expression, another element possibly accounting for the apparent tumor preference is that the HSV-TK/GCV system kills only dividing cells as GCV triphosphates interfere with DNA replication. Finally, the high transgene expression observed in the hypertrophic mesenteric lymph nodes suggests that the BGTC/DOPE/DNA lipoplexes may also enter the lymphatic vessels. This lymph node hypertrophy may result not only from metastatic invasion but may also reflect an immune response that it might be highly interesting to investigate. It was indeed reported that suicide gene expression was able to induce an immune response.^{43,55}

Most importantly, our work also shows that BGTC-mediated i.p. lipofection of suicide genes and treatment with the corresponding prodrugs can transiently reduce the progression of peritoneal carcinomatosis. In other words, the efficacy (5–10%) of gene transfection into the tumor nodules was sufficient to lead to a significant therapeutic efficiency of the particular suicide gene therapy strategy used herein consisting of an UPRT-enhanced double suicide approach combining HSV-TK/GCV with eCD/5-FC. The HSV-TK/GCV system induces cell death only in dividing cells as HSV-TK forms nucleotide-like precursors (GCV triphosphates) that block DNA replication. In contrast, the eCD enzyme converts the prodrug 5-FC into 5-fluorouracil (5-FU) whose intracellular metabolites kill the cells, including arrested cells.¹⁶ Pharmacological synergism between the two activation modalities has been demonstrated,^{19–21} its mechanism being believed to involve reduction of cellular thymidine levels (via 5-FU-mediated depletion of cellular thymidylate) and subsequent freeing of HSV-TK active sites for GCV binding.²⁰ In addition, the double suicide gene plasmid pCUT also expresses *E. coli* UPRT, an enzyme enhancing the activity of the eCD/5-FC system as it converts very efficiently 5-FU into its toxic metabolites. UPRT transfection constitutes, in fact, a chemosensitization (drug enhancement) strategy whose use with the eCD/5-FC prodrug-activation system or with direct 5-FU administration has been shown to increase the antitumor effects.^{35,56} Most importantly, our UPRT-enhanced dual suicide gene therapy approach probably also had a particularly strong *in vivo* bystander effect capable of

counterbalancing the relatively limited efficiency of i.p. lipofection. Indeed, whereas the HSV-TK bystander effect has been shown to require cell-to-cell contact, the eCD/5-FC system has a strong bystander effect that does not require cell-to-cell contact, its mechanism involving facilitated diffusion of 5-FU.^{16,57} Finally, it should be stressed that the therapeutic effect observed here was transient and that there was no cure. As the disappearance of transgene expression (around days 15–20) was correlated with an increase in the serum CEA levels in mice receiving the full treatment, it may be hypothesized that the delayed intraperitoneal tumor progression was caused by the growth, when all the transfected tumor cells had been killed, of the remaining untransfected cancer cells that had escaped the bystander effect. Thus, a more durable therapeutic effect may be obtained by repeated i.p. lipofections at a 15–20-day interval. The apparent low toxicity of BGTC formulations should allow to perform such repetitive i.p. lipofections, which may result in complete tumor eradication via increased duration of the activity of the suicide gene system.

In conclusion, the present study shows, in a mouse model of pancreatic peritoneal carcinomatosis, that BGTC/DOPE cationic liposomes offer a convenient and effective means for gene transfection into small intraperitoneally disseminated tumor nodules and that BGTC-mediated i.p. lipofection of an UPRT-enhanced combined HSV-TK/eCD suicide gene system (followed by administration of the corresponding prodrugs GCV and 5-FC) can reduce and delay progression of the disease. Our work also suggests that BGTC-based prodrug activating gene therapy strategies may constitute a future treatment modality for patients with intraperitoneally disseminated cancer and minimal residual disease.

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