Efficient electrogene therapy for pancreatic adenocarcinoma treatment using the bacterial purine nucleoside phosphorylase suicide gene with fludarabine

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Abstract. The aim of this study was to demonstrate the potential of electrogene therapy with the bacterial purine nucleoside phosphorylase gene (ePNP), on pancreatic carcinoma (PC) large tumors. The *in vivo* electroporation (EP) conditions and efficacy were investigated on both subcutaneous xenografts of human PC cells in immunocompromised mice and orthotopic intrapancreatic grafts of rat PC cells in syngenic rats. After intratumoral injection of naked plasmid DNA, EP was performed using a two-needle array with 25-msec pulses and either a 300 V/cm field strength for subcutaneous or a 500 V/cm field strength for orthotopic PC, parameters providing the best electrotransfer as reflected by the measurements of both luciferase activity and ePNP mRNA. As expected, tumors developed sensitivity to prodrug treatment (6-methylpurine deoxyribose or fludarabine phosphate). We observed both significant inhibition of tumor growth and extended survival of treated mice. In fact, after prodrug treatment, PC growth in the subcutaneous model was delayed by 50-70% for ePNP-expressing tumors. In an orthotopic pancreatic tumor model, the animal survival was significantly prolonged after ePNP electrogene transfer followed by fludarabine treatment, with one animal out of 10 being tumor-free 6 months thereafter. The current study demonstrates for the first time on PC the *in vivo* feasibility of electrogene transfer and its therapeutic efficiency using the suicide gene/prodrug system, ePNP/fludarabine. These findings suggest that electrogene therapy strategy must be considered for pancreatic cancer treatment, particularly at advanced stages of the disease.

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Abbreviations: PC, pancreatic carcinoma; ePNP, purine nucleoside phosphorylase gene of *Escherichia coli*; HSV-tk, herpes simplex virus thymidine kinase; GCV, ganciclovir; MePdR, 6-methylpurine deoxyribose; MeP, 6-methylpurine; CD/5-FC, cytosine deaminase/5-fluorocytosine

Key words: electroporation, electrogene therapy, *E. coli* purine nucleoside phosphorylase, pancreatic adenocarcinoma, suicide gene therapy

Introduction

As a matter of evidence pancreatic carcinoma is still a frightening disease with an appalling dismal prognosis. Apart from the few patients who can be treated by pancreatic resection, all others fail to be cured by conventional current therapies (i.e. chemotherapy and radiotherapy). In such cases, gene therapy approaches appear to be the most promising. Among them is the genetically directed enzyme/prodrug approach, also called suicide gene therapy.

A chemical compound (the prodrug) is administered in the general circulation (such as intraperitoneally), gene transfer is then used to target the expression of a foreign enzyme that can convert it into a toxic metabolite. Gene transfer is used to express foreign enzymes that convert into a toxic metabolite a chemical compound (the prodrug) that is administered separately (1). If the bacterial enzyme is expressed only in malignant cells, this clever delivery system will allow a targeted *in situ* chemotherapy.

Several genetically directed enzyme/prodrug systems are now under investigation as tumor killing procedures. Among them are the well-investigated herpes simplex virus thymidine kinase (HSV-tk)/ganciclovir (GCV) and the cytosine deaminase/5-fluorocytosine (CD/5-FC) systems (2). These suicide genes, which act merely on DNA replication, are poorly efficient on slowly growing tumors. Moreover, HSV-tk induces a poor bystander effect as GCV nucleotides diffuse weakly across cell membranes and fail to kill non-proliferating tumor cells (3). A new suicide gene system, the *E. coli* purine nucleoside phosphorylase (ePNP)/6-methylpurine deoxyribose (MePdR), described originally by Sorscher *et al* (4) and applied on a pancreatic tumor model by our team (5), exhibits on the contrary a very strong bystander effect (6). Differing from human or mammalian PNP, this bacterial enzyme converts the non-toxic prodrug MePdR into a very toxic metabolite. Indeed the 6-methylpurine (MeP) impairs DNA, RNA and protein synthesis (7,8) and can kill both dividing and non-dividing cells, in contrast to most

conventional anti-tumor agents (9). Therefore, MeP could be used against solid tumors with a reduced proliferation rate. As this toxic purine derivative diffuses freely across cell membranes, it mediates an efficient bystander effect resulting in an effective killing of neighboring cells that do not express ePNP. This ability might allow tumor eradication and constitutes a major advantage, as currently available vectors for *in vivo* gene delivery have very low efficiency, transducing <10% of the target cells. As such, the genetically directed enzyme/prodrug ePNP/MePdR approach constitutes a very attractive candidate as a weapon against pancreatic cancer. However, one of the most challenging concerns for successful 'suicide gene therapy' remains DNA delivery to the tumors.

The current methods for *in vivo* gene transfer include viral and non-viral vectors (10,11). The viral vectors, which provide generally an efficient gene transfer, are the most widely used tools. However, side-effects and complications such as non-specific inflammation or stimulation of cell-mediated immunity are possible (12,13). These immune reactions could impair more or less recurrent administration. More appalling is the risk of toxicity linked to chronic over expression or insertional mutagenesis when using recombinant retroviruses.

Another problem is that the maximal DNA size that can be delivered with regular vectors is limited to 5 Kb, a DNA size too small for many genes of interest. Finally, good manufacturing practice requirements are at least so demanding that they increase both the cost and time required for development of viral delivery systems. Alternative approaches have consequently received increasing attention.

Despite the lower efficiency in gene delivery than viral vectors, the non-viral approaches have many advantages. First, they allow transferring large DNA inserts. Second, compliance with good manufacturing practice is easier and less expensive than for viral vectors. Third, they can be considered as safe systems for clinical applications as they avoid the risk of unintentional replication or infectious spread of the DNA construct. Moreover, owing to the absence of foreign structural proteins, they could be less immunogenic than viral vectors. Consequently, these non-viral vectors would allow recurrent gene deliveries. Their development remains, however, limited by a relatively low rate of gene transfer *in vivo*. Current *in vivo* non-viral gene transfer methods include injection of naked plasmid DNA encoding for the gene of interest directly into the selected tissue (14). To overcome the low gene transfer efficiency of this direct local application, several studies have used *in vivo* electroporation (EP) technology.

EP is a well-established laboratory technique for *in vitro* gene delivery into cultured cells (15). It was shown that *in vivo* EP is a safe, non-toxic delivery system and can be used successfully for efficient delivery of naked plasmid DNA to many different tissues. This non-viral method introduces exogenous molecules into cells by electric pulses and may be considered as clinically relevant for DNA transfer (16). EP is assumed to induce transient cell membrane permeation through the creation of small and reversible pores allowing DNA electrophoresis into the cells (17).

Electrogene therapy of cancer has been evaluated using expression plasmids for several genes of the immune system such as interleukin-12 alone (18,19) or in combination with interleukin-18 (20), GM-CSF (21) or IFNα (22), for tumor suppressor genes such as p53 (23), angiogenesis inhibitors such as endostatin (24) or with HSV-tk suicide gene alone (25) or combined with interleukin-12 (26).

Surgical removal of pancreatic tumors offers the best chance for a cure. Unfortunately, pancreatic tumors are often inoperable because they may be too large, or have grown into major blood vessels or other vital structures. Therefore, we hypothesized that chemo and gene electroporation may offer patients a non-surgical option that preserves healthy tissue and can be repeated. We recently demonstrated that suicide gene prodrug/system (ePNP/MePdR) has a powerful anti-tumor activity with an extremely potent bystander effect (4,5).

Thus, in the current study, we investigated whether the administration of an electroporation protocol for ePNP suicide gene followed by fludarabine prodrug treatment will result in regression of pancreatic tumors and long-term animal survival.

We first determined the optimal conditions for electrogene transfer in pancreatic tumor xenografts. The efficiency of electrogene therapy with the ePNP/MePdR system was then investigated on human pancreatic tumors grafted subcutaneously into immunocompromised mouse flanks to allow assessing tumor growth evolution. Finally, to be close to clinical conditions, this electrogene therapy strategy was analyzed using an orthotopic pancreatic cancer model in immunocompetent Lewis rats. Electrogene transfer efficiency was determined in both cases through reporter and therapeutic gene expression as well as by tumor volume and animal survival evolution.

Materials and methods

Recombinant plasmids. The recombinant pCAG-Luc was constructed in our laboratory using the pGL3-enhancer (Promega, Charbonnières, France). The CAG promoter (the cytomegalovirus immediate-early enhancer and the modified chicken ß-actin promoters) was recovered from pCAGGS expression plasmid (obtained from Miyazaki Jun-ichi, Osaka, Japan) and inserted unidirectionally downstream of the luciferase coding sequence. The recombinant pCAG-ePNP/Neo and pCAG-LacZ/Neo plasmids were established as previously described (5). All plasmid DNAs were amplified using Qiagen Plasmid Endo-free Mega-Prep Kit (Qiagen, Courtaboeuf, France).

Cell lines and cell culture conditions. The human pancreatic cancer cell line BxPC-3 (ECCAC-Sigma, Saint Quentin Fallavier, France) and the stably ePNP-transfected BxPC-3 (5) were cultured as monolayers in RPMI-1640 Glutamax supplemented with 10% heat-inactivated fetal calf serum, 2 mM L-glutamine and antibiotics (100 U/ml penicillin, 100 μg/ml streptomycin and 10 mg/ml gentamicin). The ductular rat pancreatic cancer cell line HA-RPC, developed in our laboratory (27) from a chemically induced pancreatic adenocarcinoma as described by Pettengill *et al* (28), grew in Dulbecco's modified Eagle's medium supplemented in RPMI medium as indicated above. The cell cultures were maintained at 37˚C in a 5% CO_2 humidified incubator. Stock cultures were

passed weekly and supplied with fresh medium every 3 days. All cell culture reagents were purchased from Invitrogen-Life Technologies (Cergy Pontoise, France). The cell lines were routinely examined to be free of mycoplasma infection.

Pancreatic adenocarcinoma models

Animals. Eight-week-old female athymic NMRI (nu/nu) nude mice (Elevage Janvier, Le Genest, France) were kept under specific pathogen-free conditions, in alternating 12-h periods of light and dark, and given sterile commercial food and water *ad libitum*. Male Lewis rats (Elevage Janvier), weighing 200-220 g and maintained under standard laboratory conditions (22±2˚C and alternating 12-h periods of light and dark) were used for orthotopic grafting of syngenic HA-RPC tumor cells. Experiments were carried out in compliance with the 'Guidelines for Animal Experiments of Service Véterinaires du Ministère de l'Agriculture-France'.

Subcutaneous xenografts of BxPC-3 human pancreatic cancer cells. Tumor xenografts were established using the parental and the ePNP-transduced BxPC-3 cells. An inoculum of 0.2 ml containing $1x10^7$ tumor cells was subcutaneously injected in the dorsal flank using a 23-gauge needle under isoflurane anesthesia (Forene, Abbott, Rungis, France). Subcutaneous (s.c.) tumor growth was assessed by calipers. The tumor volume was calculated according to the following formula: $V = (\pi \alpha b^2/6$ where V is the volume, α the largest diameter and b the smallest diameter. Experiments were carried out on tumors of an average volume of 100-110 mm^3.

Orthotopic grafts of rat HA-RPC pancreatic tumor cells. For an orthotopic pancreatic tumor model, Lewis rats were anesthetized with isoflurane gas and a suspension of syngenic HA-RPC tumor cells ($1x10^7$) was injected under a small laparotomy in the pancreatic tail (also called left pancreatic lobe) using a 23-gauge needle, as previously described (27,29). A double layer suture closed the abdominal incision at length. Experiments were carried out 3 weeks later, a time interval at which tumors are becoming palpable, under anesthesia that is consistent with a volume of 0.5-1.0 cm^3 as previously ascertained (29).

Determination of the optimal parameters for pancreatic tumor electroporation. In a first attempt we determined the optimal parameters for an efficient electrogene transfer in pancreatic tumors. For human BxPC-3 subcutaneous tumors, a percutaneous intratumoral injection of 50 μg of pCAG-Luc (vector expressing luciferase reporter gene) in 50 μl of PBS was performed under anesthesia using a 25-gauge needle. Five min after plasmid injection and mechanical massage, tumors were pulsed from a T820 square-wave electroporator (BTX, San Diego, CA) fitted with a 0.5-cm diameter array of two needle electrodes. Ten square-wave pulses at 950-msec intervals were delivered at a frequency of 1 Hz with either 25- or 50-msec pulse length and 150 or 250 V. Accordingly, 20 animals were electroporated in total in batches of 5, treated respectively with 150 V/25 msec, 150 V/50 msec, 250 V/25 msec and 250 V/50 msec. Regarding HA-RPC orthotopic tumors, pCAG-Luc plasmid (50 μg) was injected directly into the tumor, through a small midline laparotomy and the same parameters were also evaluated on four groups of 5 animals each.

All mice and rats were sacrificed under anaesthesia for tumor removal 3 days after the EP session. Tumors were grinded in a cell lysis buffer (Promega) for luciferase assays and in TRIzol reagent (Invitrogen-Life Technologies) for RNA extraction. Cell debris was removed by centrifugation after three freeze/thaw cycles. Luciferase assays were performed using the luciferase assay system (Promega) and a Lumistar luminometer (BMG Labtechnologies, Champigny sur Marne, France). The amount of proteins was measured by BCA protein assay kit (Pierce, Interchim, Montluçon, France) and the results were expressed in relative light units (RLU) per milligram of protein.

Comparison of three non-viral methods for gene transfer. The efficiency of intratumoral electrogene transfer of 50 μg of pCAG-Luc plasmid was compared to those achieved using either naked DNA (50 μg of in 50 μl of PBS) or DNA/PEI complexes (100 μl with a ratio of 0.5 μg DNA per μl of jetPEI™ , according to the manufacturer's recommendation). PEI was a gift of Qbiogene (Illkirch-Graffenstaden, France). Gene transfers were carried out always under anesthesia on both subcutaneous and orthotopic tumor models. Twenty animals were used for each tumor localization. They were randomly assigned to one of the following treatments: naked DNA injection, DNA/PEI complex injection and EP with either 300 V/cm or 500 V/cm electric field with a 25-msec pulse. Tumors were removed 3 days after intratumoral injection of the pCAG-Luc plasmid and luciferase activities were assayed as described above.

Assessment of electrotransfer efficiency in tumors using an expression plasmid for ePNP. The parameters giving the best efficiency of transduction determined in the previous experiment were used for EP (i.e. 300 V/cm for subcutaneous BxPC-3 tumors and 500 V/cm for orthotopic HA-RPC tumors). Mice bearing an s.c. tumor (n=10) and rats bearing an orthotopic tumor (n=10) were sacrificed under anaesthesia for tumor removal 3 days after electrotransfer of the expression plasmid (either pCAG-ePNP/Neo in 5 animals or pCAG-LacZ/Neo as a control in the 5 others).

The transcriptional activity of the ePNP gene was evaluated by RT-PCR of ePNP mRNA and compared to constitutive G3PDH (glyceraldehyde-3-phosphate dehydrogenase) mRNA as an internal control. Briefly, after total RNA extraction by TRIzol reagent (Invitrogen) and reverse transcription, cDNA was amplified by PCR using Taq DNA polymerase (Invitrogen). Specific ePNP and G3PDH primers were used to amplify respectively a 700-bp and 450-bp fragment. PCR products were resolved in 1.5% ethidium bromide-stained agarose gels and visualized by UV light. Images of agarose gels were stored as electronic TIF files and analyzed with the image analysis software ImageQuantTL, version 5.1 (Molecular Dynamics, Sunnyvale, CA). Signal intensity was measured for each band using the ImageQuant volume integration tool. The relative values of the corresponding G3PDH bands were used to normalize the ePNP mRNA levels. The ratio ePNP/G3PDG was calculated for ePNP expression level determination.

In vivo evaluation of electrogene therapy efficiency through tumor sensitivity to prodrug treatment. The ability of EP to achieve gene transfer and the efficacy of ePNP electrogene therapy were investigated through tumor responsiveness to prodrug supply. Investigations were carried out on both s.c. BxPC-3 tumors xenografted in immunocompromised mice and on orthotopic HA-RPC tumors grafted in Lewis rat pancreas.

Subcutaneously xenografted BxPC-3 tumors in immunocompromised mice. Electrogene transfer efficiency was evaluated in 18 mice bearing a BxPC-3 s.c tumor, using the recombinant pCAG-LacZ/Neo plasmid (reporter gene) in 6 animals and the pCAG-ePNP/Neo plasmid (suicide gene) in 6 others. The 6 remaining mice (without EP or DNA injection) served as a control group. Five minutes after intratumoral injection of DNA (50 μg), EP was performed under anesthesia using the optimal EP parameters determined in preliminary experiments, i.e. pulse of 25-msec length and nominal field strength of 300 V/cm. After a first sequence of 10 pulses, a 45˚ clockwise rotation of the electrodes was performed before the next ten-pulse sequence. Twenty-four hours thereafter, the prodrug treatment was initiated in all animals including the controls. An intraperitoneal injection of 15 mg/kg of MePdR (Invitrogen), corresponding to 3.5 mg/m^2, was carried out once a day for 5 successive days. One week after the first EP session, a second electrogene transfer was performed with the same amount of plasmid DNA, followed by a 5-day prodrug supply.

In order to evaluate the gene delivery efficiency of this EP protocol, three other experimental groups of 6 mice were added. In these experimental groups, immunocompromised mice were injected with mixtures of BxPC-3 cells (10^7 cells in total) including increasing amounts of tumor cells expressing constitutively ePNP (ePNP$^+$) (i.e. 10^5, 10^6, and 10^7 cells stably transfected with the ePNP gene) in order to mimic a 1, 10 and 100% transduction rate. As soon as tumors reached an average volume of 110 mm^3, mice were also daily injected with MePdR for two successive sessions of 5 days.

Accordingly, six groups of 6 animals each were used in this study of subcutaneous tumors. Tumor sizes were determined twice a week for 5 weeks.

Orthotopic HA-RPC tumors grafted in Lewis rat pancreas. Electrogene transfer efficiency was evaluated in 50 Lewis rats bearing an orthotopic HA-RPC tumor. When these intrapancreatic tumors became detectable through abdominal palpation (i.e. 3 weeks after orthotopic injection), 20 rats were randomly assigned to two control groups of 10 rats each, one without any management and the other undergoing solely a midline laparotomy under anesthesia. A single EP session was carried out under anesthesia on the 30 remaining rats. The tail of the pancreas was exteriorized through a small midline laparotomy. The measured tumor sizes were between 0.4 and 1.1 cm^3 with an average volume of 745±84 mm^3. Either 50 μl of phosphate buffer (for 10 animals) or 100 μg of DNA (pCAG-LacZ/Neo plasmid for 10 animals and pCAG-ePNP/Neo plasmid for 10 others) was injected into the tumors 5 min prior to EP. Electrodes (0.5 cm large) were inserted directly into the tumors, all of which had a diameter >0.5 cm. EP consisted of 10 pulses of 25-msec length with a nominal field strength of 500 V/cm. After a 45˚-clockwise rotation of the electrodes, a second 10-pulse sequence was performed. A double-layer suture closed the laparotomy at length. The prodrug treatment was initiated 2 days thereafter in all animals that underwent EP. An intraperitoneal injection of 2.5 mg/kg of fludarabine phosphate (Fludara®, Schering SA, Lys-Lez-Lannoy, France), corresponding to a dosage of 5 mg/m^2, was carried out twice a day for 5 successive days. Survival of the animals was followed over a 6-month period.

Statistical analysis. Mean and standard error of the mean were calculated. Data of the *in vivo* investigations were analyzed using the ANOVA test for mean comparison and the Student-Neumann-Keuls test for multiple comparisons. A difference between the values was considered significant when $P<0.05$. Instat 2.00® Macintosh software (GraphPad Software, San Diego, CA) was used. The survival curves were generated using the Kaplan-Meier method, and the differences between the curves were compared by the log rank test. P-value <0.05 was considered to be statistically significant.

Results

Determination of the optimal parameters for electroporation. These experiments were performed using a 50-μg DNA injection with the pCAG-Luc plasmid. The tumors were harvested 3 days after EP and luciferase assays were carried out. The efficiency of EP depends mainly on both electrical field strength (V/cm) and duration (pulse length). The pulse lengths of 25 and 50 msec were tested. The best luciferase activity was obtained with a 25-msec pulse (30% more than with 50 msec, $P<0.05$). Electric field strengths were then investigated with this pulse length.

In the case of subcutaneous BxPC-3 tumors xenografted in mice, luciferase activities obtained with 300- and 500-V/cm electric field strengths were not significantly different (2100±177 vs. 2143±180 RLU/mg of protein). To avoid a possible occurrence of tumor burns with the highest voltage, we applied a 300-V/cm electric field with a 25-msec pulse length for *in vivo* experiments on subcutaneous pancreatic tumors. As for orthotopic HA-RPC tumors grafted in the pancreas of syngenic rats the 500-V/cm electric field strength induced a 2-fold higher luciferase activity than 300 V/cm (1097±103 vs. 492±70 RLU/mg of protein, $P<0.01$). Thus, the highest voltage was applied for further *in vivo* experiments on orthotopic pancreatic tumors. The electrogene transfer was almost 2-fold less efficient in orthotopic than in subcutaneous tumors.

Enhancement of transgene expression in pancreatic tumors by electroporation. To determine the best non-viral method for gene transduction, we compared luciferase activities in both subcutaneous and orthotopic tumors after intratumoral delivery of 50 μg of pCAG-Luc plasmid performed as a 'naked' DNA injection, a DNA/PEI complex injection and a naked DNA injection followed by an EP session, using either a 300- or a 500-V/cm electric field. Subcutaneous BxPC-3 tumors and orthotopic HA-RPC tumors were harvested 3 days later and assayed for luciferase activity.

Table I. *In vivo* transduction efficiency of non-viral methods.[a]

Tumor cell line	Tumor graft localization	Naked DNA	DNA/PEI complexes	Electroporation 300 V/cm	Electroporation 500 V/cm
BxPC-3	Dorsal flank in mouse	81±3	315±27	2091±145	2136±185
HA-RPC	Pancreas in rat	33±1	132±7	484±67	1114±101

[a]Data, expressed in relative light units ($x10^3$) per mg of protein, are means (± SEM) of 5 animals for each experiment. Tumor samples were harvested 72 h after transduction with pCAG-Luc plasmid *in vivo*.

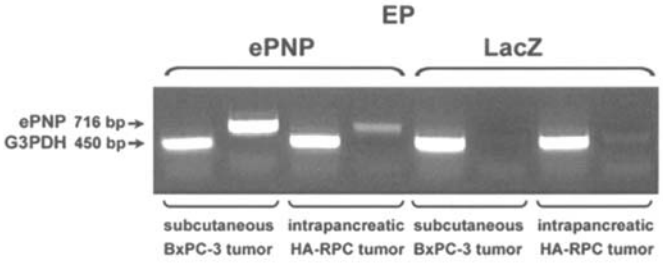

Figure 1. Digital photograph of an agarose gel showing the result of RT-PCR for ePNP and G3PDH mRNA from subcutaneous BxPC-3 and intrapancreatic HA-RPC tumors, removed 3 days after electroporation with either pCAG-LacZ (LacZ) or pCAG-ePNP (ePNP) plasmid. ePNP mRNA was missing after LacZ electrogene transfer in both kinds of tumors and seemed more abundant in BxPC-3 than in HA-RPC tumors after ePNP electrogene transfer.

Naked DNA injection in subcutaneous BxPC-3 tumors xenografted in mice led to a weak luciferase activity (Table I). The use of PEI induced a 4-fold increase in luciferase activity (P<0.001), but the best result was obtained after EP [a 7-fold increase in comparison with the DNA/PEI group (P<0.001)]. Obviously, EP appeared as the most potent non-viral method in this situation.

In the case of orthotopic HA-RPC tumors in syngenic rats, luciferase activity was very weak after the naked DNA injection (Table I). The addition of PEI also improved gene transfer [by 4-fold (P<0.001)]. However as observed in the subcutaneous BxPC-3 tumors experiments, EP appeared as the most potent non-viral technique, with an 8-fold increase in luciferase activity in comparison with the DNA/PEI group (P<0.001) when using a 500-V/cm electric field.

Assessment of in vivo ePNP electrogene transfer efficiency in tumors. Electrogene transfer of ePNP or LacZ plasmids in subcutaneous and orthotopic pancreatic tumors was carried out according to the previously determined parameters. The tumors were removed three days after the EP session. The expression of the genes was investigated through RT-PCR. ePNP mRNA was only detected after ePNP transfer (Fig. 1). The results of the semi-quantitative analysis of PCR products revealed a 2-fold higher content in subcutaneous BxPC-3 than in orthotopic HA-RPC tumors (Fig. 2).

Efficiency of ePNP electrogene therapy on subcutaneous tumor growth. The efficiency of electrogene therapy was evaluated by a survey of tumor size evolution after ePNP gene transfer. Control animals received only injections of

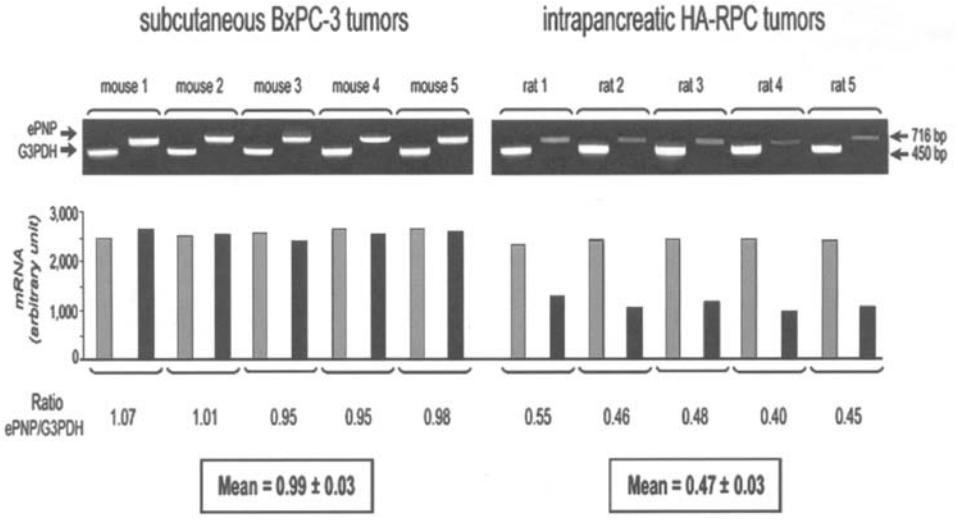

Figure 2. Results of the semi-quantitative analysis of ePNP mRNA in 5 human BxPC-3 tumors and in 5 rat HA-RPC tumors, sampled 3 days after ePNP electrogene transfer. Signal intensities, expressed in arbitrary units, were measured on each band of the agarose gel images using the volume integration tool of the ImageQuantTL software. The corresponding G3PDH mRNA values were used to normalize the results and ePNP mRNA amounts were expressed as a ratio of ePNP to G3PDH RT-PCR products.

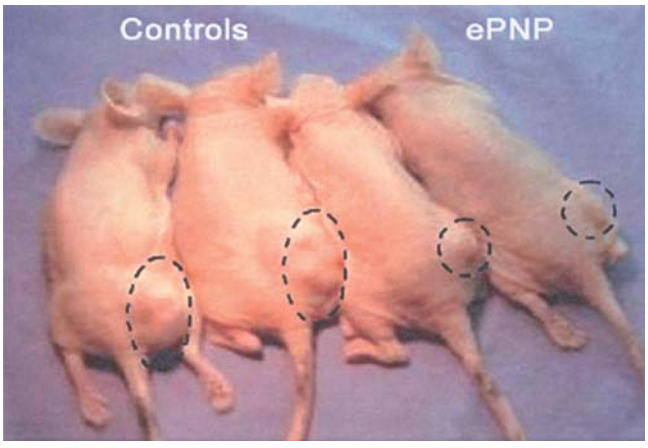

Figure 3. Representative photograph of the effect of ePNP electrogene therapy on subcutaneous BxPC-3 pancreatic tumors 4 weeks after MePdR treatment. The 4 tumor-bearing mice were under isoflurane anesthesia. The left side of the photograph displays 2 control mice without any electrogene transfer (controls) and the right side two mice after electrogene transfer with ePNP plasmid (ePNP). Tumors are surrounded by a dotted circle and look obviously reduced in the ePNP electrogene therapy animals with less than half of the volume of the control tumors.

the MePdR prodrug. Animals treated by LacZ electrogene transfer served as controls of the potential anti-tumor effect of EP per se. A first EP session was performed on tumors with an average volume of 110-mm^3. MePdR was supplied for 5 days and followed by a second electrogene transfer plus MePdR treatment. This therapeutic schedule induced a dramatic decrease of tumor growth after ePNP electrogene transfer in comparison with controls, as illustrated on a representative photograph of mice, 4 weeks after the first EP (Fig. 3).

Precise tumor size measurements confirmed this therapeutic effect. The electrogene therapy by using an expression plasmid for ePNP retarded by 50-70% ($p<0.001$ vs. control) the pancreatic tumor growth throughout the experiment (Fig. 4A). Surprisingly, the electrogene transfer of LacZ also reduced tumor growth by almost 50% ($P<0.001$ vs. control) 4 weeks after the first EP and by 25% ($P<0.01$) at the end of the experiment (Fig. 4A). Thus, electrogene transfer of a bacterial DNA by itself had a non-negligible anti-tumor effect. Indeed, the same reduction was observed after electrogene transfer of ePNP without any prodrug supply (data not shown). Nevertheless, only ePNP electrogene therapy combined with prodrug treatment gave the most important tumor reduction, as it was significantly higher than the retardation linked to the electrogene transfer procedure ($p<0.01$ vs. LacZ electrogene transfer at day 37). This improvement was clearly linked to MePdR supply, as tumors started to grow again only after the last injection of the prodrug (Fig. 4A).

The efficiency of ePNP electrogene therapy was evaluated with another experiment in which the effects of the same amount of prodrug on tumors were studied with pre-determined transduction rates of ePNP. The tumors were generated by s.c. injections of parental BxPC-3-cells mixed with increasing amounts of BxPC-3 stably expressing the ePNP gene (1, 10 and 100% ePNP$^+$ cells/inocula). The tumor growth retardation achieved on the 1% ePNP$^+$ tumor inocula by prodrug injections (Fig. 4B) was dramatically lower than that obtained with ePNP-EGT (9% vs. 50% of the control, $P<0.001$). With 10% ePNP$^+$ cells in the tumor inocula, the tumor growth retardation was higher (62% vs. 50%, $P<0.05$) at the end of the experiment. Altogether, these results indicate that pancreatic tumor cells are sensitive to ePNP-EGT. However, they also suggest that the transduction achieved by EP would affect <10% of the cells in the tumor.

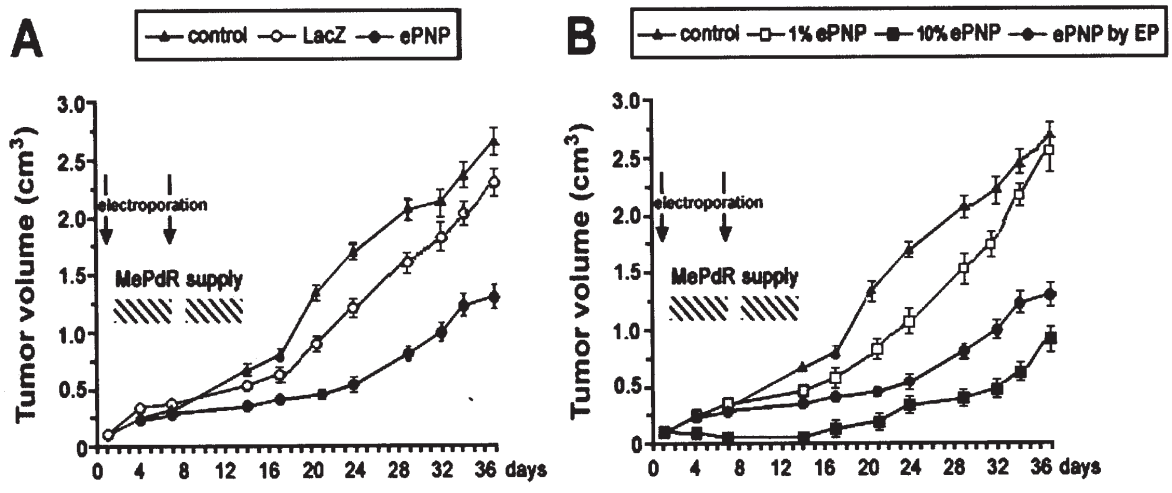

Figure 4. Effect of ePNP electrogene therapy on tumor growth. Antitumor activity was evaluated on subcutaneous BxPC-3 tumors in immunocompromised mice after 2 recurrent EP sessions at days 1 and 7 (shown by arrows), each of them followed by a 5-day treatment with the prodrug (specified by hatched rectangles). All the groups received 3.5 mg/m^2/day of MePdR. Tumor volumes, determined by caliper measurements, were plotted as mean ± SEM of 6 animals. (A) Comparison of tumor reductions induced by ePNP and LacZ electrogene transfer to animals receiving only the prodrug injection (control). Tumor suppressions were statistically significant for both ePNP ($P<0.001$) and LacZ ($P<0.01$) electrogene transfers when compared with the control group. (B) Comparison of the efficiency of ePNP electrogene therapy (ePNP by EP) with the tumor suppressions achieved by the MePdR prodrug on xenografts of tumor cell inocula containing 0 (control), 1% (1% ePNP) and 10% (10% ePNP) of BxPC-3 cells transduced *ex vivo* with the ePNP plasmid. The tumor suppression observed at the end of the experiment with ePNP electrogene therapy was significantly different from those observed in both 10% ($P<0.05$) and 1% ($P<0.01$) ePNP-transduced tumors.

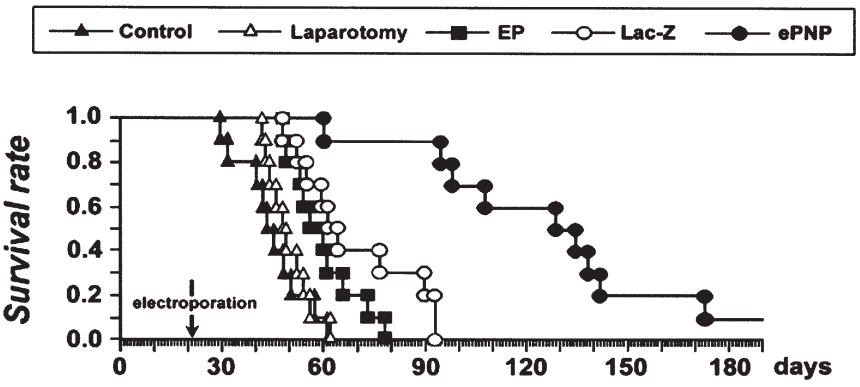

Figure 5. Survival curves of rats with intrapancreatic tumors. Variations in survival induced by ePNP and LacZ electrogene transfer were evaluated on orthotopic HA-RPC tumors in syngenic Lewis rats and compared with control animals kept without any treatment (control), submitted to laparotomy alone (laparotomy) or associated to EP of phosphate buffer (EP). A single EP session was performed at day 21 (arrow), followed by a 5-day treatment with fludarabine phosphate (10 mg/m^2/day). Survival was significantly improved for ePNP and LacZ electrogene transfers ($P<0.001$, log-rank test) as after EP alone ($P<0.01$, log-rank test) when compared with the control groups. Moreover, the comparison of survivals induced respectively by ePNP electrogene therapy and LacZ electrogene transfer revealed an obvious advantage for ePNP ($P<0.001$, log rank test), with one animal still alive 6 months after tumor induction.

Assessment of ePNP electrogene therapy efficiency with orthotopically grafted HA-RPC tumors. The feasibility of ePNP electrogene therapy was also evaluated on an intrapancreatic model, physiologically more relevant. The animal survival as an end-point provides more conclusive data in terms of therapeutic benefits for humans. In this study, we used fludarabine as a prodrug for ePNP despite its lower antitumoral activity compared to MePdR (6,30). This choice was related to the difficulty to obtain the MePdR compound and because fludarabine is already used in clinical trials.

In addition to a control group undergoing no treatment at all, we used another control submitted to only laparotomy plus tumor exteriorization without EP. To determine the effect of EP alone on survival, we included a control group in which tumors were injected with phosphate buffer prior to EP. The animals of the 2 other electroporated groups differed only by the type of DNA injected within the tumors (ePNP vs. LacZ). The three groups submitted to EP received injection of prodrug for 5 days.

Our results indicate that all the animals of the control groups with or without laparotomy died within 61 days (Fig. 5). The survival rate of animals receiving LacZ electrogene transfer was slightly enhanced. This effect could be explained by the bacterial protein origin, LacZ, which can induce a slight immunogenic effect. For the group of rats bearing tumors and receiving ePNP electrogene transfer and prodrug treatment, the survival rate was drastically improved. In this group, one rat was still alive 190 days after tumor induction. The median survival of this group was 126 days ($P<0.001$ vs. LacZ) and survival was significantly greater than for the group submitted to LacZ electrogene transfer ($P<0.001$, log-rank test). These results indicate that, despite a slight tumor effect linked to the technique, the electrogene therapy of pancreatic carcinoma with the ePNP/fludarabine combination improved animal survival considerably.

Discussion

The objective of this study was to assess if fludarabine/ePNP electrogene therapy may be relevant for treatment of pancreatic adenocarcinoma. Indeed, among the gene-directed prodrug activation therapies giving attractive results in cancer treatment (31), the ePNP/fludarabine (or MePdR) can be considered as one of the most potent suicide gene/prodrug systems (4,5). However, concerning other gene therapeutic approaches, the drawback of this suicide gene therapy remains the low rate of gene transfer already achieved by available methods. Preclinical and clinical studies using the widely applied adenoviral vector are disappointing in terms of cancer gene therapy, with low numbers of tumor cells targeted due to a defect in the primary and/or secondary receptors required for adenoviral infection (32). As a consequence, the amount of adenoviral particles needed *in vivo* to reach an adequate level of gene transfer in poorly permissive tumor cells would be flawed by a non-negligible vector-related toxicity, such as a severe liver damage. Over the last 5 years, non-viral methods of gene transfer have been increasingly investigated (33). Among the physical methods of plasmid delivery, EP achieves an anatomic targeting of the gene of interest circumventing the risk of systemic toxicity encountered with adenoviral transfection (34). Indeed, this non-viral method of gene transfer is only responsible per se for some local tissue damage, for instance cell necrosis through tumor heating by too high a voltage (34,35). Electrogene transfer constitutes an alternative for transduction of adenoviral-resistant tumors (34).

According to the gene transfer potentiality reported in the literature (33,36), we decided to combine for the first time electrogene transfer with ePNP suicide gene as a potential pancreatic cancer therapy. Indeed, the potential and the advantages of electrogene therapy have never been tested on pancreatic adenocarcinoma with any of the suicide genes or other anti-cancer agents. Our experiments were carried out on two different pancreatic tumor systems, one consisting of human ductal cancer cells implanted subcutaneously in immunocompromised mice and the other of rat ductal carcinoma cells implanted directly in the pancreas of syngenic immunocompetent rats (orthotopic model).

As a prerequisite prior to any experimental therapeutic studies, we had to determine the parameters for an optimal electrogene transfer within pancreatic tumors. If all kinds of

tissues or tumors can be transduced with foreign DNA using EP (33,37), each of them has its own optimal parameters (35,37). Electrogene transfer depends on both electric field strength and pulse duration (35,37). Conversely to electrochemotherapy that needs high voltages (1,100-1,300 V/cm) and short pulses (100 msec), a low electric field strength (50-400 V/cm) and a long pulse (20-50 msec) are more suitable for gene transfer (25,35,38). In the current study, the best plasmid-based gene transfer was achieved with a relatively short pulse length (25 msec), corroborating the previous findings of Molnar *et al* (35). However the strength of the electric field required for an optimal reporter gene transfer in pancreatic tumors was different in the two models (300 vs. 500 V/cm). This is probably linked to the changes in both pancreatic tumor cell type and localization (35).

As EP is assumed to be one of the most potent tools for gene transfer, we first compared it with other physical methods of plasmid delivery using a luciferase reporter gene system in pancreatic tumors. As expected, electrogene transfer of luciferase expression plasmid was clearly more efficient than injections of the sole naked DNA (30-fold less) or of PEI/DNA complexes (8-fold less). It should be noted, that gene transfer efficiency in tumors was lower in orthotopic than in subcutaneous tumor localization, in the present study. Indeed, luciferase activity was reduced by almost two-thirds after injections of naked DNA and PEI/DNA complexes. This disparity could result from a difference in tumor cell types and/or perhaps from the release of some deleterious nucleases under pancreatic gland injury. Electrogene transfer appeared also 2-fold less efficient in an orthotopic location. As previously demonstrated (35,39), this disparity is as likely the result of a change in pancreatic tumor type as of changes in species, age and weight of the tumor recipients.

Electrogene therapy using the expression plasmid for the ePNP gene followed by fludarabine treatment inhibited drastically the growth of human pancreatic tumor xenografts in immunocompromised mice with a two-third reduction of the average volume compared with controls. This tumor reduction is more important than the one achieved by Goto *et al* (25) on a murine colic tumor model after electrogene transfer of the HSV-tk suicide gene. Indeed, with the same initial tumor size, the same amount of injected DNA and also two recurrent EP sessions, these authors reported an almost half reduction of the average tumor volume (25). Despite better experimental conditions, namely a more convenient EP device fitted with a 6-electrode array (vs. only 2 in the present study), a 1-month supply of the prodrug (vs. 2 recurrent 5-day injections in our experiment) and the use of an immunocompetent tumor model (25), their electrogene therapy was less effective than ours. This improvement is likely the result of the higher bystander effect of the ePNP/MePdR system (4,5,40) outlining the potential of such a suicide gene.

Noteworthy is that electrogene transfer of the expression plasmid for LacZ induced a 25% suppression in the tumor growth. This result could be considered as a proper effect of the EP and attributed to the bacterial protein origin (LacZ). However, it was reported in a recent paper by Durieux *et al* (41) that tissue damage after EP is closely related to the presence of plasmid DNA and also that it is made worst when the LacZ reporter gene is expressed. This enhancement of tissue injury was observed in immunocompetent animals (41) and should result, as suggested by Heller and Coppola (42), from the expression of bacterial-derived DNA with several CpG motifs.

Tumor growth was not completely suppressed by ePNP electrogene therapy on this human tumor model. This drawback could be explained by a too low efficiency in tumor cell transduction. To evaluate this efficiency, we compared the reduction of tumor growth induced by prodrug treatment after EP with ePNP plasmid with those of xenografts comprising an increasing percentage of stably expressing ePNP cells. The EP-mediated transduction rate of BxPC-3 human tumors was estimated to be approximately 10%. Lohr *et al* (43) obtained 3 to 8% of cell transduction after electrotransfer of green fluorescent protein in murine melanoma cells.

The *in vivo* potential of fludarabine/ePNP electrogene therapy was also evaluated on a syngenic orthotopic model of ductal pancreatic cancer (27) with a natural history involving tumor spread, lymph node involvement and liver plus lung metastasis (29). Such an immunocompetent model with an initial intrapancreatic tumor is more representative of the clinical situation. Moreover, EP was performed only when the tumors became detectable through abdominal palpation, reproducing in part the clinical reality in which pancreatic cancer is not detected at an early stage. In this way, EP was performed on large tumors, mostly >1 cm in diameter, rendering impossible a full EP of the whole tumor mass. With always the concern of clinical adequacy we performed a single EP session, using as the prodrug a clinically approved compound widely applied in the treatment of hematologic malignancies, fludarabine phosphate, and assessed the effect on survival. In our experiment, EP that requires surgery in this particular location of the tumor, did not cause by itself any post-operative morbidity. In these *in vivo* experimental conditions, ePNP electrogene therapy with fludarabine treatment appears again to be highly efficient on tumor evolution, extending significantly the median survival, with 1 animal out of 10 being considered disease-free 6 months thereafter. Noteworthy is that this successful experiment was carried out with a low prodrug dosage (i.e. 10 mg/m^2/day) for 5 days, whilst others provided 75 mg (44), 135 mg (45,46) and even 600 mg/m^2/day of fludarabine (47) for 5 days.

As observed for subcutaneous xenografts of human pancreatic tumors, the electrogene transfer of the LacZ reporter gene also had an effect on tumor evolution, confirming a deleterious effect of foreign DNA transfer via EP (41). Survival was significantly increased in comparison with all control groups, even vs. the group that underwent EP with phosphate buffer. One animal survived up to 6 months after tumor induction versus only 2 months for the controls. This effect was more obvious than in immunocompromised mice. It seems likely to be the consequence of bacterial-derived DNA expression (41) involving cell-mediated immunity as recently described after EP (48). Noteworthy is the fact that in our experiment EP was able to induce alone a significant improvement in survival, likely as a result of tumor injury by relatively high electric field strength. As a matter of evidence, at least part of the anti-tumor effect observed in the Lac-Z group was the result of direct cell injury.

EP appears as a safe gene transfer tool, without any morbidity such as fever, weight loss or digestive disorders, that could by applied for electrogene therapy of pancreatic cancer using the ePNP/fludarabine suicide gene system. It should be possible to improve the electrogene transfer efficiency in pancreatic tumors, by using a 6-needle array as employed in some experimental studies (25,26,42,43,49) applying the electric field strength on the whole volume and not only on a single plane between two electrodes, as in the current experiment. Six-needle array should increase the global electric conductivity of a basically heterogeneous tumor, mixing fibrotic, necrotic and proliferative areas of different electric diffusions (50). The electric conductivity itself can also be improved by intratumoral injection of hyaluronidase, 2 h prior to electrogene transfer, as demonstrated by Molnar *et al* (35). These authors reported a 150-370% increase in the transduction efficiency of a reporter gene (35). Similarly, as the reduced electrotransfer of ePNP within the orthotopically-implanted tumor could be due in part to some constitutive pancreatic DNAse activities, we suggest adding a repressor of DNAse activity such as EDTA or G-actin to the hyaluronidase injection. The therapeutic results of electrogene therapy using the ePNP/fludarabine system can also be improved through an expended period of drug supply, up to one month as shown by Goto *et al* (25) with the HSV-tk/GCV suicide gene system, allowing a long-term production of toxic metabolites and a better tumor eradication. In the same way, higher prodrug availability, up to 75-150 mg/m^2/day of fludarabine in some studies (9,44), may also enhance ePNP efficiency. However, as the dosage of fludarabine for each month of treatment is only 25 mg/m^2/day for 5 successive days in human beings, we suggest the same dosage for animal experiments, as used by Mohr *et al* on mouse hepatocarcinoma (45). Electrogene therapy may also be improved using a continuous prodrug infusion with intraperitoneal osmotic Alzet® pump reproducing the continuous chronic prodrug delivery performed in humans through daily recurrent perfusions. Finally, it may be useful to practice recurrent 5-day sessions of prodrug administration, since it was recently demonstrated that part of the foreign DNA transferred through EP was integrated *in fine* into host genomic DNA (51,52) achieving a stable transduction and a long-term expression of the therapeutic gene (51).

As electrogene transfer in a pancreatic tumor needs a surgical approach, it cannot be proposed in a first intent to manage presumably resectable tumors of grade 1 or 2, which are suitable for curative debunking surgery. However, ePNP electrogene therapy can be proposed as an adjuvant treatment for non-resectable pancreatic cancer, besides conventional chemotherapy. Performed with the help of the nowadays widely applied minimal invasive surgery, it should considerably improve survival, as observed in our *in vivo* study on orthotopical pancreatic tumors in rats. It must be emphasized that our results were obtained on tumors of mostly >1 cm in diameter, which were too large to be fully transduced through EP. Thus, the efficiency of ePNP electrogene therapy in such a situation is clearly linked to the high bystander effect of the ePNP/fludarabine system, already described in several *in vivo* studies (5,40,44-47). Suicide gene systems are also able to induce a systemic anti-tumor response, called 'vaccination based on cellular suicide' (53). So, electrogene therapy with ePNP can be efficient against tumor spreading and metastases development by the addition of this immune stimulation with the concomitant so-called bystander effect. It would be, moreover, feasible to boost the immune response by adding genes encoding for cytokines, as already achieved by O'Malley *et al* with the HSV-tk/GCV system (54). This ability to combine different cDNA encoding different factors with suicide genes (26,55) emphasizes the potential of the EP approach in cancer treatment.

In conclusion, the current study shows that ePNP electrogene therapy is highly efficient on both human and rat pancreatic tumors. Fludarabine phosphate, already applied in clinical practice, can be efficiently used as the prodrug. It remains, however, indubitable that the performances of electrogene transfer must be improved as a prerequisite before human application, for instance by more convenient devices and hyaluronidase addition. Regarding the current disappointing evolution of human pancreatic cancer, we believe that the feasibility and usefulness of EP already demonstrated by clinical trials of electro-chemotherapy for antimitotic local delivery (56), the impressive survival increase reported in this experimental study and the current availability to perform DNA injection and EP through the endoscopes used for abdominal minimal invasive surgery make a quick development of ePNP electrogene therapy for non-resectable pancreatic cancer in willing patients possible.

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