Antitumor Effect of in Vivo Somatostatin Receptor Subtype 2 Gene Transfer in Primary and Metastatic Pancreatic Cancer Models

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ABSTRACT

Our previous studies conducted in pancreatic cancer models established in nude mice and hamsters revealed that cloned somatostatin receptor subtype 2 (sst2) gene expression induced both antioncogenic and local antitumor bystander effects in vivo. In the present study, in vivo gene transfer of sst2 was investigated in two transplantable models of primary and metastatic pancreatic carcinoma developed in hamsters. LacZ reporter or mouse sst2 genes were expressed by means of two different delivery agents: an adenoaviral vector and a synthetic polycationic carrier [linear polyethyleneimine (PEI)]. sst2 was injected into either exponentially growing pancreatic primary tumors or hepatic metastases, and then transgene expression and tumor progression were investigated 5–6 days after gene transfer. Molecular mechanisms involved in the inhibition of tumor growth were also analyzed. Both adenovirus- and PEI-mediated in vivo gene transfer in primary pancreatic tumors induced an increase of β-galactosidase activity and expression of sst2 transgene mRNA (100% and 86% of tumors for adenovirus and PEI vector, respectively). Adenoviral vector-based sst2 gene transfer resulted in significant reduction of pancreatic cancer cell growth (P < 0.05). Using PEI vector, both pancreatic primary tumor growth and metastatic tumor growth were also significantly slackened as compared with both LacZ-treated and untreated control groups (P < 0.02). Moreover, the proliferative index decreased significantly (P < 0.005), whereas apoptosis increased (P < 0.005) in tumors transferred with sst2 gene. The increase of apoptosis correlated with an activation of the caspase-3 and poly(ADP-ribose) polymerase pathways. We concluded that in both primary and metastatic pancreatic cancer models, the synthetic gene delivery system can achieve in vivo sst2 gene transfer and results in a significant antitumor effect characterized by an increase of apoptosis and an inhibition of cell proliferation. This new strategy of gene therapy allows the restoration of expression of an antioncogenic molecule and could be promising for the treatment of advanced pancreatic cancer.

INTRODUCTION

Pancreatic ductal carcinoma is, at present, the fifth leading cause of cancer-related deaths in Western countries. Because of late diagnosis due to a long silent clinical phase during tumor development and the absence of early markers of the disease, the prognosis of this cancer is very poor (1). Up to now, the only curative treatment for pancreatic cancer is surgical resection. Unfortunately, surgery for curative purposes is possible in only 10–15% of cases, and among patients undergoing surgery, only 5% have a 5-year survival. The overall 5-year survival rate for pancreatic carcinoma is no more than 3%. At the time of diagnosis, 15–30% of patients with pancreatic adenocarcinoma have extensive metastasis, primarily to the liver, peritoneum, and lymph system, and are therefore not amenable to resection. Conventional chemotherapy (the mainstay 5-fluorouracil and, more recently, gemcitabine) and radiotherapy approaches have been used to improve patient survival but are relatively ineffective. Thus, to improve the prognosis, there is a need to develop a new modality of treatment for pancreatic cancer. Gene therapy strategies may provide therapeutic benefits with a more favorable risk:benefit ratio than the current conventional treatments.

Many groups are investigating gene therapy for pancreatic cancer. Several experimental reports demonstrated the efficacy of in vivo gene-directed enzyme prodrug therapy such as the herpes simplex virus thymidine kinase/ganciclovir (2, 3), Escherichia coli cytosine deaminase/5-fluorocytosin (4), or uracil phosphoribosyltransferase/5-fluorouracil systems (5). Moreover, a gene therapy strategy for pancreatic carcinoma using reintroduction of antioncogenic molecules such as p16Ink4a or p53 tumor suppressor gene is more likely to be successful (6). With regard to immunotherapy (immunomodulatory genes), experimental studies have involved gene transfer of cytokines such as human interleukin 2 or interleukin 1B (7).

We recently proposed a new gene therapy approach for pancreatic cancer based on the antioncogenic properties of the sst2 receptor gene. Somatostatin is a cyclic neuropeptide that negatively regulates a number of processes such as epithelial cell proliferation and exocrine and endocrine secretions. Studies have shown that somatostatin and its stable analogues suppress the growth of various normal and cancer cells (8). This peptide exerts a direct antiproliferative effect mediated by specific cell surface receptors. Five subtypes of somatostatin receptors have been cloned from human, mouse, and rat (9). Among them, the subtypes sst1, sst2, and sst5 are responsible for the antiproliferative effect of somatostatin and its analogues in vitro (10, 11). In parallel, we observed that a loss of sst2 gene expression occurs in human pancreatic adenocarcinomas and in most of pancreatic cancer derived cell lines (12). These observations correlate well with the results of immunohistochemistry and in vivo scintigraphy, which show an absence of somatostatin-binding sites in human pancreatic adenocarcinomas (13, 14). We thus hypothesized that sst2 receptor loss in pancreatic cancer could confer growth advantage in these tumors, which is in agreement with the fact that in patients with exocrine pancreatic cancer, somatostatin analogue treatment is inefficient. Correction of the sst2 gene deficit in human pancreatic cancer cells (15) confirms this hypothesis. In vitro cell growth and in vitro tumorigenicity were significantly reduced in sst2-expressing cells. In experiments conducted in athymic mice, we demonstrated that sst2 reexpression was responsible for a dramatic decrease in tumor growth. Local and distant antitumoral bystander effects were also observed (16). Furthermore, we recently conducted studies in a...
transplantable model of pancreatic carcinoma in hamsters demonstrating that reexpression of sst2 gene caused inhibition of primary tumor growth and metastatic progression (17). All these results strongly suggest that the sst2 gene acts as a tumor suppressor in pancreatic cancer cells. Its transfer may represent a novel therapeutic approach to this cancer. Our previous studies were based on ex vivo approaches using cellular clones transfected with sst2 or control genes (16, 17). A rational preclinical approach to gene therapy for pancreatic cancer requires in vivo gene transfer experiments, and we tested in this way both viral and synthetic vectors to transduce sst2 gene in either primary or metastatic tumors.

The transplantable model of pancreatic carcinoma established in hamsters (17) and a new transplantable model of hepatic metastasis developed by us were used in the present study. We observed that in vivo sst2 gene transfer resulted in significant inhibition of growth progression for both pancreatic primary tumor and hepatic metastases. This therapeutic effect required an antitumor bystander effect, whose molecular mechanisms were investigated here.

**MATERIALS AND METHODS**

Reagents

ExGen 500/PEI in vivo was from Euromedex (Souffelweyersheim, France). Oligonucleotides, RNAble, and SuperScript II reverse transcriptase were from Eurobio (Les Ulis, France) and Invitrogen, respectively. RNasin was from Promega (Charbonnières, France). Taq polymerase and DNase I were from Boehringer Mannheim France (Meylan, France).

Plasmids

The murine sst2 cDNA, subcloned into pCMV6c vector, was kindly provided by G. L. Bell (Howard Hughes Medical Institute, The University of Chicago, Chicago, IL). Control plasmid pGLCMV-β-gal was a gift from H. Paris (INSERM U388, Toulouse, France). All plasmids were purified using the Endofree system (Qiagen, Courtaboeuf, France).

**Construction and Preparation of Recombinant Adenoviruses**

The Ad.RSV.nlsLacZ adenoviral vector used was a recombinant human serotype 5 replication-deficient adenovirus containing nuclear-targeted *E. coli* β-galactosidase (LacZ) reporter gene driven by the Rous sarcoma virus long terminal repeat promoter.

Replication-deficient (ΔE1,E3) adenoviral vector expressing murine sst2 cDNA under the transcriptional control of the human CMV promotor was constructed with the AdEasy System (Quantum Biotechnologies; Ref. 18). Briefly, the 1.2-kb Xhol-XhoI fragment of msst2 cDNA was subcloned into XhoI-digested shuttle vector pAdTrack-CMV. This vector (Ad.CMV.sst2.GFP) also independently expressed enhanced GFP (Clontech). The adenoviral vector Ad.CMV.sst2.GFP expressing GFP alone was a gift from B. Coudere (INSERM U563, Institut Claudius Regaud, Toulouse, France). Ad.CMV.sst2.GFP vector was produced by the Vector Core of the University Hospital of Nantes, France (Ph. Moullier and N. Ferry).

**Tumor Cell Lines**

The hamster pancreatic carcinoma PC1-0 cells [derived from pancreatic ductal carcinoma induced by N-nitrosobis(2-oxopropanol)lamine in hamsters] were cultured in RPMI 1640 containing 5% FCS, fungizone, penicillin, streptomycin, and L-glutamine (Invitrogen, Cergy Pontoise, France) as described previously (17).

**Animal Models and Experimental Protocol**

Five-week-old male Syrian golden hamsters (weighing 70–80 g) were obtained from Harlan France (Gannat, France). The animals were acclimatized in a temperature-controlled room under a 12-h light/12-h dark schedule and received pellet diet and water. Hamsters were anesthetized for all surgical procedures by an i.p. injection of pentobarbital (60 mg/kg, i.p.).

**Pancreatic Ductal Carcinoma Allograft.** PC1-0 cells were orthotopically implanted into hamsters as described previously (17). Briefly, after minilaparotomically 5 × 10⁶ PC1-0 cells resuspended in 100 μl of FCS-free RPMI 1640 were injected into the tail of the pancreas by means of sterile 29-gauge lymphography catheter set (Vygon, Ecouen, France). Intrapancreatic allografts grew rapidly and were palpable 9–13 days postimplantation. On day 9 after allograft, tumor volume was determined after median laparotomy, and intratumoral gene transfer was performed with either a synthetic carrier or recombinant adenoviruses. At 1–6 days after transfer, animals were euthanized, tumor volume was measured, and primary tumors were sampled.

**Hepatic Metastasis Model of Pancreatic Carcinoma.** After a median minilaparotomy, 5 × 10⁶ PC1-0 cells resuspended in 100 μl of FCS-free RPMI 1640 were injected into the portal vein using a sterile 29-gauge lymphography catheter set (Vygon), under an optical microscope (KAPS-SoM82, Bordeaux, France). At day 15 post-cell inoculation, after laparotomy, an hepatic metastasis was selected as a target for in vivo gene transfer and located using liver cartography. Tumor volume was determined, and intratumoral gene transfer was performed using the synthetic carrier, PEI. At 5 days after transfer (day 20), animals were euthanized, and tumor volume of the target metastasis was measured before it was sampled.

Tumor volumes were determined by the formula $V = \frac{W^2 \times L}{2}$, where $L$ = length (mm) and $W$ = width (mm), using width as the smaller dimension. TGP was calculated according to the following formula: $TGP(\%) = \left(\frac{T \times 10^3}{t}\right) - 100$, where $T =$ the volume of the tumor at the beginning of the gene transfer, and $t =$ volume of the same tumor at the end of the therapy.

For each experiment, primary tumors or metastases were dissected and fixed in formalin for immunohistochemical studies. Half of tumors were also immediately harvested in cold isopentane, frozen in liquid nitrogen, and kept at −80°C for subsequent immunoblotting, β-galactosidase activity, or RT-PCR assay.

Each treated or control group comprised four animals, and experiments were subsequently repeated on a different batch of animals. All experiments have been performed according to the rules of the animal ethical committee. Two sets of gene transfer experiments were conducted as described below.

**In Vivo Gene Transfer**

**In Vivo Gene Transfer Using Recombinant Adenovirus.** During the exponential growth phase of primary pancreatic tumors, 10⁹ pfu of sst2 adenoviruses were given to the animal intratumorally in a total volume of 100 μl by means of a sterile 29-gauge needle. Control animals received adenovirus Ad.RSV.nlsLacZ or Ad.CMV.GFP using the same concentrations and injection protocols.

**In Vivo Gene Transfer Using the Synthetic Carrier PEI.** Linear polymers of ethyleneimine (PEI or L-PEI) with a mean molecular mass of 22 kDa were used as in vivo transfection reagent. PEI-DNA complexes nitrogen:DNA phosphate of 10 equivalents were prepared in 5% (w/v) glucose (19, 20). PEI-DNA complexes so obtained were injected into tumor using a sterile 29-gauge lymphography catheter set with a flow rate of 25 μl/min. A total of 10 μg of pCMV6c-mss2 or pGLCMV-LacZ cDNA were injected to each animal.

**Determination of Transgene Expression**

**Total β-Galactosidase Activity in Tumor Tissues and X-Gal Staining in Tumor Sections.** β-Galactosidase activity was quantified in tumor tissue using a chemiluminescence assay. Frozen tumor sample was homogenized in a cell lysis buffer (Promega). Homogenates were centrifuged at 10,000 rpm for 10 min at 4°C and then incubated for 1 h at 37°C with o-nitrophenyl β-D-galactopyranoside (Sigma, St. Louis, MO) in a 96-well microtiter plate, and chemiluminescence was measured using a luminometer. β-Galactosidase activity was standardized on the protein content of the lysates (protein assay kit; Bio-Rad, Hercules, CA). The other half of each frozen tumor was cryosectioned into 14-μm-thick sections. The tumor sections were fixed for 10 min in a 2% formaldehyde solution. After rinsing in PBS, cryosections were incubated overnight at 37°C in a PBS solution containing 5 mM potassium ferriyanide, 5 mM potassium ferrocyanide, 2 mM magnesium chloride, and 1 mg/ml X-Gal (Sigma). The slides were washed three times in PBS and counterstained with Mayer’s hematoxylin.

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Imaging Transgene Expression in Allograft Tumors after Adenovirus-mediated Gene Transfer. The adenoviral recombinant vector independently encodes both mouse sst2 and enhanced GFP genes. Frozen Ad.CMV.sst2.GFP-transduced tumor tissues were cryosectioned and fixed in freshly prepared paraformaldehyde solution [2% in PBS (pH 7.4)] for 10 min at room temperature. The slides were rinsed three times with PBS and mounted under a fluorescent mounting medium (Fluoprep; Dako Corp., Carpinteria, CA). GFP reporter gene expression was observed using a fluorescence microscope coupled to an image analyzer (Biocom, France).

RNA Isolation and RT-PCR Assays. Tumor tissue was homogenized mechanically in RNAble, and total RNA was extracted by the modified procedure of Chomczynski and Sacchi as described previously (12). RNase-free Dnase I treatment and PCR were performed using specific sense and antisense primers for mouse sst2 receptor as described previously (12, 15).

Immunohistochemical Staining of Proliferative Cells

After tumor growth, the half of each tumors was harvested and fixed in formalin. Four-μm-thick sections were prepared from paraffin-embedded specimens. Immunohistochemical reactions were performed as described previously (16) using the hors eradish peroxidase-conjugated system and anti-PCNA (mouse anti-PCNA, clone PC10; purchased from Dako Corp.). Immunoperoxidase immunostaining using the DAKO EnVision+ system (Dako Corp.) was performed to augment the PCNA immunostaining intensity. The immunostaining was developed with the use of 3,3'-diaminobenzidine solution (Sigma) as chromogen. Slides were counterstained with Mayer’s hematoxylin. Negative control slides were prepared by replacing the primary antibody with PBS.

In Situ TUNEL Staining for the Detection of Apoptotic Cells

TUNEL staining was performed using in situ cell death detection kit apopDETEK (Enzo Diagnostics, Inc., Farmingdale, NY), according to the manufacturer’s instructions with minor modifications. Slides were counterstained with Mayer’s hematoxylin. Some sections stained with no TUNEL mixture were used as negative control. The resulting cell morphology was observed with an optical microscope.

Assessment of DNA Fragmentation

DNA fragmentation was detected by gel electrophoresis using the Apoptotic DNA Ladder Kit (Roche) according to the manufacturer’s instructions. Briefly, 6 days after gene transfer, primary tumors were removed and mechanically homogenized in lysis buffer, DNA was then isolated, and the resulting DNA ladder was visualized on a 1.5% agarose/ethidium bromide gel.

Image Processing

The histological cuts after immunohistochemistry labeling (PCNA) or TUNEL reaction are visualized with an optical microscope coupled to a VisioLab2000 image analyzer (Biocom Paris, France). This system allows the quantification of proliferating cells or cells undergoing apoptosis. Measurements are made at high magnification (×40) on 15–20 fields representing at least 5000 cells/tumor for apoptosis and on 8–10 fields representing at least 1000 cells for proliferation index (16).

Immunoblotting Analysis of Caspase-3 Activation and Cleavage of PARP

Primary tumors were dissected mechanically in liquid nitrogen, and aggregates were solubilized in lysis buffer (50 mM HEPES, 150 mM NaCl, 10 mM EDTA, 10 mM Na3PO4, 100 mM NaF, and 2 mM sodium orthovanadate (pH 7.4)) containing 0.01% soybean trypsin inhibitor and 1 mM phenylmethylsulfonyl fluoride in the presence of 20 μg/ml aprotinin and 20 μg/ml leupeptin (Sigma, Saint-Quentin Fallavier, France). The mixture was gently agitated for 20 min at 4°C and centrifuged at 12,000 rpm for 10 min. For immunoblotting, 100 μg of soluble proteins were resolved through 7.5% or 12% SDS-polyacrylamide gels, transferred to a nitrocellulose membrane, and immunoblotted with goat polyclonal antihuman caspase-3 (sc-1224; Santa Cruz Biotechnology) or rabbit polyclonal anti-PARP (Roche) antibodies. Immunoreactive proteins were visualized by the enhanced chemiluminescence immunodetection system (Super Signal kit; Pierce) as described in Refs. 17 and 21.

Statistical Analysis

The results are expressed as mean ± SE. Paired or unpaired Student’s t test was used to compare data. *P < 0.05 was considered significant.

RESULTS

In Vivo Gene Transfer Efficiency Using Adenoviral- or PEI-mediated Transfection in Pancreatic Primary Tumors

To examine the transduction efficiency of PEI- or adenovirus-mediated in vivo gene transfer into tumors, 107 pfu of Ad.RSV.LacZ adenosiviruses or PEI:DNA pCMVLacZ complexes were injected directly in primary pancreatic tumor mass. Tumors were removed from animals at days 1, 2, and 6 after gene transfer, and then we quantitatively evaluated LacZ expression in primary tumors by measuring the total β-galactosidase activity using a chemiluminescence assay. As shown in Fig. 1, using PEI at the optimal PEI:DNA ratio (20), β-galactosidase activity was maximal at day 1, and it was detectable up to day 6 in tumor extracts. Levels of β-galactosidase activity were higher in tumors of animals injected with adenoviruses as compared with tumors in animals injected with PEI (maximal at day 2); β-galactosidase levels were 5-fold higher and detected up to day 6 (P < 0.01). These quantitative results were confirmed by X-Gal staining: in animals that received an intratumoral injection of PEI-DNA pCMVLacZ complexes both in primary and metastasic pancreatic cancer models, quantification revealed that about 2% of tumors cells were stained. In contrast, marked X-Gal staining-positive areas were observed in tumors that received an intratumoral injection of adenovirus Ad.RSVLacZ, with 10–15% positive transduced tumor cells (data not shown).

Detection of Transgene Expression after in Vivo Adenovirus- and PEI-mediated Gene Transfer

We were able to easily identify tumor cells successfully infected by adenovector by detecting expression of enhanced GFP on primary
Fig. 2. Detection of mouse sst2 transgene expression in primary and metastatic pancreatic adenocarcinoma models. A, light-based detection of reporter GFP after gene transfer with adenovector Ad.CMV.sst2.GFP. Primary tumors were removed from animals on day 6 and frozen in nitrogen. Light emission was achieved after excitation of the GFP protein with blue light on frozen tissue sections. The micrograph is representative of at least five different Ad.RSV.LacZ or Ad.CMV.sst2.GFP-transduced tumors (magnification, $\times 200$). Left panel corresponds to Ad.RSV.LacZ-transduced tumor section, right panel corresponds to Ad.CMV.sst2.GFP-transduced tumor section, and GFP expression is reflected by green-colored cytoplasm. B, tumors were removed from animals on days 2, 6, and 5 in primary carcinoma and hepatic metastatic models, respectively. Total RNA was extracted, and RT-PCR analysis was performed from LacZ and sst2 tumors. The PCR products resulting from specific primers for mouse sst2 (expected length, 661 bp) were analyzed on polyacrylamide gel. Lane M, DNA size markers (pGEM markers; Promega). RT-PCR carried out in the absence of reverse transcriptase during reverse transcription procedure were negative. Results are representative of at least six different PEI-transduced tumors.

Antitumoral Effect of Adenovirus-mediated sst2 Gene Transfer in the Orthotopic Pancreatic Carcinoma Model

On day 9 after intrapancreatic inoculation of PC1-0 cells, adenoviruses carrying sst2 and/or GFP genes were administered intratumorally, and tumor volume was measured 6 days later (day 15 after tumor implantation). As shown in Fig. 3, TGP after Ad.CMV.sst2.GFP injection was slowed down as compared with those of control tumors injected with Ad.GFP or with vehicle ($P < 0.005$). Primary pancreatic tumor volume in hamsters treated with Ad.CMV.sst2.GFP (529 ± 98 mm$^3$) was significantly reduced as compared with those in hamsters treated with Ad.CMV.LacZ.GFP (972 ± 123 mm$^3$) or in untreated animals (1385 ± 282 mm$^3$; data not shown; $P < 0.01$). In addition, we observed that 40% of tumors transferred with sst2 were stabilized without any tumor volume increase.

Antitumoral Effect of PEI-mediated sst2 Gene Transfer in Both Primary and Metastatic Pancreatic Carcinoma Models

To examine the usefulness of synthetic vector administration, the PEI-DNA complexes were also administered intratumorally to the hamsters at day 9 after intrapancreatic tumor cell inoculation, and animals were sacrificed 6 days later. For our newly created metastatic model, PEI-DNA complexes were administered intratumorally at 15 days after intraportal tumor cell inoculation, and animals were sacrificed 5 days later. The volume of transplanted pancreatic tumors or hepatic metastasis was measured before and after in vivo sst2 gene transfer. No significant difference was
Mechanisms Involved in the Antitumoral Effect of PEI-mediated sst2 Gene Transfer in Both Primary and Metastatic Pancreatic Carcinoma Models

Antiproliferative Effect of in Vivo sst2 Gene Transfer. PCNA has been recognized as an auxiliary protein for DNA polymerase δ and as an endogenous histological marker for the late G1-S phase of the cell cycle. We then analyzed the proliferative index on histological sections from pancreatic cancer allograft and tumor metastases using nuclear immunodetection of the PCNA antibody. As shown in Fig. 5, a significant decrease of immunodetected PCNA in cell cycling tumor cells (PCNA labeling index) was observed in tumors (primary or metastatic) injected with the sst2 gene compared with control tumors injected with the LacZ gene. Representative micrographs of PCNA labeling in pancreatic hepatic metastases are illustrated in Fig. 6, A and B.

Proapoptotic Effect of in Vivo sst2 Gene Transfer. To investigate whether the sst2 gene therapy-induced antiproliferative effect results from an induction of apoptosis, we attempted to detect typical DNA ladders, a hallmark of apoptotic cells, on purified nucleic acids from primary tumor samples. DNA fragmentation was observed in both sst2- and control LacZ-expressing tumors (Fig. 7A). A quantification of apoptosis was then performed using TUNEL in situ labeling on histological sections from pancreatic cancer allograft and metastases. As shown in Fig. 7B, the percentage of apoptotic cells was significantly increased in both primary and metastatic tumors injected with the sst2 gene as compared with control tumors injected with the LacZ gene. Representative micrographs of TUNEL analysis in pancreatic primary tumor are shown in Fig. 6, C and D.

To characterize whether caspases, which are the major protease mediators of the classical apoptosis pathways, are involved in the sst2 gene transfer-induced proapoptotic effect, we measured the modulation of caspase-3 activity and the expression of PARP caspase target proteins of the DNA repair system by Western blot. Western blot analysis showed an activation of caspase-3 in 67% of sst2-expressing tumors (six of nine tumors were positive). The M, 32,000 precursor form of caspase-3 was more efficiently cleaved into the active M, 11,000 shorter fragment in sst2- versus control LacZ-expressing tumors (22%, two of nine tumors were positive), indicating that the effector procaspase-3 is specifically activated in tumors that received the PEI-DNA sst2 complexes. Representative results are shown in Fig. 7C (top panel). Consistently, in the same tumor samples, the caspase-3 substrate PARP is also cleaved into shorter fragments in 75% of sst2 tumors versus 25% of LacZ tumors (Fig. 7C, bottom panel).
clear staining is represented by after intraportal PC1-0 cell inoculation. PCNA nu-
and sst2 (PCNA labeling index). of PCNA using monoclonal PC10 PCNA antibody
(black arrows). Right panels, TUNEL reaction (ap-
opoptosis). C and D, LacZ control (C) and sst2 pri-
mary tumors (D) generated 15 days after intrapan-
creatic PC1.0 cells inoculation. Positive nuclear
reactions appear as dark brown nuclei (small black arrows). Tissues are counterstained with Mayer’s
hematoxylin (magnification, ×200).

DISCUSSION

Transfer of molecules carrying antioncogenic properties could be a promising therapeutic strategy for exocrine pancreatic cancer. Gene therapy approaches for pancreatic cancer should be aimed at the treatment of not only primary tumors but also the proximal and distant metastases that often occur in this type of carcinoma. sst2, the expression of which is frequently lost in human pancreatic carcinomas, evoked an antioncogenic effect when reexpressed in vitro or in vivo in pancreatic exocrine cancer cells (15, 17). In the present study, we used transplantable models of primary and metastatic pancreatic carcinoma established in Syrian Golden hamsters to evaluate the therapeutic efficacy of in vivo sst2 gene transfer on pancreatic carcinoma. We demonstrated that the direct intratumoral sst2 gene transfer is responsible for a significant antitumor effect in both models of pancreatic carcinoma.

Despite a low transgene expression rate, we observed a significant inhibition of tumor growth in sst2-transduced tumors. A sst2-induced bystander effect might therefore be responsible for this therapeutic effect. A bystander effect has been described in gene-directed enzyme prodrug therapy. The mechanism is not fully characterized, but we can put forward some hypothesis for our gene therapy approach with the sst2 gene. The first explanation is that apoptosis can contribute to local bystander killing effect. Indeed, in ex vivo experiments conducted in nude mice xenografted with human pancreatic cancer cells, we observed that mixed tumors (containing only 25% sst2-expressing cells) displayed a significant inhibition of tumor growth as compared with control tumors (16). Interestingly, we detected a similar increase of apoptosis in both mixed tumors and tumors containing 100% of sst2-expressing cells (16). We therefore concluded that apoptosis can diffuse from the transduced cells to the untransduced neighboring cells. In the present work conducted on orthotopic primary pancreatic cancers, we also detected apoptosis in tumor injected with sst2 gene. A significant increase of the apoptotic index was measured by the TUNEL reaction, demonstrating an activation of caspase-3 and PARP pathways that are major protease mediators of the late phase of apoptosis. These pathways could be specifically activated in sst2-
expressing cells as a result of recombinant sst2 receptor occupation by mature somatostatin. Indeed, sst2 gene transfection in the pancreatic cancer cell lines BxPC-3, Capan-1, and PC1-0 (which lack the sst2 receptor) has been shown to induce a sst2-activated negative autocrine loop, through the activation of endogenous somatostatin, which consequently activates sst2 (15, 17). Finally, bystander effect might result from an uptake by the neighboring untransduced tumor cells of apoptotic vesicles that contain hydrolases or enzymes activated by programmed cell death, including caspase-3 and PARP (22).

However, mechanisms other than apoptosis might be involved in sst2-mediated bystander killing. We observed that in vivo, sst2 gene transfer was accompanied by a significant decrease in the PCNA proliferative index. This is in agreement with our previous ex vivo experiments conducted in nude mice xenografted with mixed tumors. This inhibition could be explained in two ways: (a) the receptor sst2 is known to mediate G_1-G_s cell cycle arrest in transduced cells and inhibits proliferation (21); and (b) the secreted somatostatin by sst2-expressing cells could also inhibit, via a paracrine or endocrine pathway, local angiogenesis and/or secretion of growth factors that positively control the untransduced cell proliferation. Moreover, as we described previously, locally produced somatostatin may also up-regulate the somatostatin receptor subtypes sst1 or sst5 in untrans-
duced cells; these subtypes are also known to mediate the antiprolif-
erative effect of the native peptides somatostatin 14 and 28 (11, 16).
gene delivery was realized by means of a micropump, allowing low DNA-PEI complexes adapted to intratumoral injection. Gene intratumorally, we performed gene transfer using a mode of delivery of gene (20). Complexes produced a high and long-lasting expression of the transgene (5-fold) and a more significant reduction of sst2 expression in most of sst2 tumor samples (Blot: anti-PARP). This might be another argument to support that in vivo sst2 gene transfer specifically generated an antitumor effect.

PEI has been chosen because of its high efficiency for gene delivery in pancreatic cancer cells both in vitro (17) and in vivo (23). In our study, we performed gene transfer using a mode of delivery of DNA-PEI complexes adapted to intratumoral injection. Gene intratumoral delivery was realized by means of a micropump, allowing low delivery of complexes into tumor mass. Indeed, Coll et al. (20) analyzed the influence of the mode of intratumoral delivery using a micropump versus a normal, rapid injection using a syringe. In contrast to syringe-injected PEI complexes, micropump-injected PEI complexes produced a high and long-lasting expression of the transgene (20).

Using the LacZ reporter gene, we estimated that about 2% of tumor cells were transfected and expressed transgene for at least 6 days. Such experimental in vivo data were also observed in s.c. tumors of lung carcinoma, and the level of reporter gene was constant for 15 days (20). Furthermore, we were able to detect transgene expression by RT-PCR in both primary and metastatic tumors 5–6 days after injection. In a similar manner, Aoki et al. (23) detected the presence of injected DNA in various organs 7 days after the i.p. injection, and transgene expression decreased in all organs except for the brain 3 months later. In the present model, due to the fast growth of primary tumors and their metastases (17), we could not perform long-term experiments because control animals had to be sacrificed before day 20 after cell inoculation. However, in the nude xenograft pancreatic cancer model, we recently observed that in vivo PEI-sst2 gene transfer generated a significant and sustained antitumor effect over 12 days after intratumoral gene delivery.

Despite their low efficiency in comparison with viral vectors, cationic vectors retain high attractiveness in gene therapy because of their theoretical safety profile. PEI should fulfill both requirements for gene therapy in humans. (a) It is easy to prepare and handle, and it is an inexpensive in vivo transfection reagent. (b) It is an inert and innocuous compound with no or low toxicity; no mortality was observed in injected animals, and no necrosis was observed at the site of injection (24, 25). (c) It raises none of the concerns of viral vectors for human gene therapy and is not associated with the possibility of generating a replication-competent virus, and there is no immune reaction such as those experienced for adenovirus vector. (d) Multiple injections will be less problematic than virus-based gene transfer because repeated intra-pancreatic adenovirus injection increases cytotoxic, lymphoproliferative, and cytokine release activity (26). (e) Finally, contrary to cationic lipids, PEI may better protect DNA

Fig. 7. Proapoptotic effect of in vivo sst2 gene transfer. A, DNA fragmentation visualized by DNA ladder formation. Six days after gene transfer, DNA from primary pancreatic tumor was extracted following the protocol provided by the manufacturer. Results are representative of five different tumor samples. B, histological sections of pancreatic primary carcinoma and hepatic metastasis were analyzed for apoptosis by TUNEL reaction staining. The percentage of labeled nuclei was measured at high magnification (×40) using the VisioLab 2000 analysis system on 12–15 successive high-power fields representing at least 5000 cells/tumor. Hatched bars, primary pancreatic tumors; dotted bars, hepatic metastasis. Values are means ± SE (5 tumors/group were analyzed). Difference between LacZ- and sst2 gene-injected tumors: *, P < 0.002; #, P < 0.005. C, effect of sst2 gene transfer on apoptosis effector protease caspase-3 activation. Tissue homogenates from tumors were subjected to Western blot analysis using anti-caspase-3 and anti-PARP antibodies after injection of intratumoral PEI-DNA complexes. The M, 32,000 precursor form of the effector caspase-3 is cleaved to the M, 11,000 active fragment in most of sst2 tumor samples (Blot: anti-caspase-3). The cleavage of the full-length PARP (M, 116,000) is demonstrated by a decrease of expression of the full-length PARP and is also detected in most sst2 versus LacZ tumors (Blot: anti-PARP). sst2 expression is responsible for the activation by cleavage of apoptosis effector protease caspase-3 in correlation with apoptosis increasing after sst2 gene transfer (9 tumor samples/group were analyzed).
because of its best stability in pancreatic environment, known to be rich in enzymes such as cholesterol esterase that could digest other nonviral lipidic vectors.

In conclusion, PEI-formulated DNA can efficiently and stably transfect tumor pancreatic cells \textit{in vivo}. Because of the efficient expression and absence of toxicity, the intratumoral injection of \textit{sst2}-PEI complexes is expected to play an important role in future treatment of both primary and metastatic pancreatic tumors.

In summary, we have demonstrated that transfer of \textit{sst2} into both primary and metastatic pancreatic cancer models resulted in a significant inhibition of tumor growth \textit{in vivo}. The antioncogenic effect that results from \textit{sst2} reexpression is accompanied by a strong bystander effect on untransduced cells, which undergo apoptotic cell death and inhibition of cell proliferation. \textit{sst2} gene therapy as a new strategy for pancreatic cancer requires long-term and stable expression of the \textit{sst2} receptor to obtain a durable antitumor effect. Repeated \textit{in vivo} intra-tumor transfer of \textit{sst2} gene using cationic vector should be performed because of the absence of toxicity.

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Fabienne Vernejoul, Patrick Faure, Naoual Benali, et al.


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