RESEARCH ARTICLE

Polyethylenimine-mediated gene transfer into pancreatic tumor dissemination in the murine peritoneal cavity

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Although peritoneal dissemination of cancer cells often occurs at the advanced stages of pancreatic, gastric or ovarian cancers, no effective therapy has been established. Cationic lipid-mediated gene transfer into peritoneal dissemination may offer a prospect of safe therapies, but vector improvements are needed with regard to the efficiency and specificity of the gene transfer. In this study, the intraperitoneal injection of plasmid DNA:polyethylenimine (PEI) complexes into mice was evaluated as a gene delivery system for the peritoneal disseminations. The luciferase and β-galactosidase genes were used as marker genes. PEI was more efficient than the cationic lipids examined in this study in vivo, and the transgene was preferentially expressed in the tumors. Although PCR analysis showed that the injected DNA was delivered to various organs, the distributed DNA became undetectable by 6 months after the gene transfer. Blood chemistry and histological analysis showed no significant toxicity in the injected mice. This study demonstrated that the intraperitoneal injection of DNA:PEI is a promising delivery method to transduce a gene into disseminated cancer nodules in the peritoneal cavity. Gene Therapy (2001) **8**, 508–514.

Introduction

Adenocarcinoma of the pancreas is one of the most difficult cancers to treat at present.[1] By the time of diagnosis, most patients have extensive metastasis, primarily to the liver, peritoneum and lymph system. Peritoneal dissemination is also one of the most frequent modes of recurrence after surgery.[1,2] Moreover, it is often associated with intractable ascites leading to substantial impairment of the patient's quality of life. Yet, no effective therapy has so far been established to alleviate this devastating and often fatal end-stage condition.[3,4] Thus, there is an urgent need to develop a new modality of treatment for peritoneal dissemination of 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 the gene therapy for peritoneal metastasis; several reports demonstrated the efficacy of suicide gene therapy with the gene transfer of herpes simplex virus thymidine kinase (HSVtk) gene and cytosine deaminase gene using various vectors.[5–8] As regards an apoptosis-inducing gene therapy, the treatment of adenovirus encoding p53 gene effectively suppressed the growth of peritoneal tumors in ovarian can-

cer model,[9] and the administration of BAX-expressing adenovirus was capable of eradicating >99% of ovarian tumor implants.[10] A phase I trial suggested that adenovirus-mediated gene therapy using anti-erbB2-directed intrabody is feasible in the human ovarian cancer.[11] As an anti-angiogenesis strategy, intraperitoneal transduction of a soluble Flt-1 gene using HVJ-liposome suppressed peritoneal metastasis in mice.[12] Tanaka et al[13] reported the immune gene therapy by in vivo transfer of IL-6 gene using adenovirus vector in SCID-PBL/hu mice. We have been developing lipofection as a method for in vivo delivery of therapeutic genes such as antisense K-ras and HSVtk gene into peritoneal dissemination of pancreatic cancer.[14,15] DNA-mediated transfection has major advantages in that it raises none of the concerns of viral vectors for human gene therapy; it is not associated with the possibility of generating a replication competent virus, there is no acute toxicity or immune reaction such as those experienced for adenovirus vectors, it is more stable in vivo than retroviral vectors, and multiple injection will be less problematic than a virus-based gene transfer.[16,17] However, nonviral gene delivery techniques do not compare favorably with viral vectors on the basis of efficiency of gene transfer to a cell. More efficient vectors are needed to improve the low gene transfer efficiency.

Among nonviral vectors, two main classes of molecules have been used: (1) cationic lipids such as N[1-(2,3-dioleyloxy)propyl]-n,n,n-trimethylammonium chloride (DOTMA), dioctadecylamidoglycylspermine (DOGS) or

1,2-dimyristyloxypropyl-3-dimethyl-hydroxyethyl ammonium bromide (DMRIE);[18–20] and (2) polymeric DNA-binding cations such as poly-L-lysine, protamine, cationized albumin, and more recently, polyethylenimine (PEI).[21,22] PEI is the organic macromolecule with the highest cationic-charge-density potential; every third atom is an amino nitrogen that can be protonated, which makes the polymeric network an effective 'proton sponge' at virtually any pH.[21] In this study, we first compared the gene transfection efficiency between a linear form of PEI and several widely used cationic liposomes *in vitro* and found that PEI shows the best overall transfection efficiency in pancreatic cancer cells. The data prompted us to investigate whether an *in vivo* gene transfer into intraperitoneally disseminated tumors could be effectively achieved by PEI. This study demonstrated that the direct intraperitoneal injection of DNA:PEI complexes is an efficient and safe method for *in vivo* gene transfer into intraperitoneal tumors.

Results

Comparison of gene transfer efficiency between PEI and cationic liposomes

To compare the relative efficiencies of PEI and various cationic liposomes in transfecting pancreatic cancer cell lines, we evaluated the expression of the reporter gene, luciferase. The PEI exhibited higher luciferase activity than other cationic liposomes in three of four cell lines (Figure 1a). In Panc-1 cells, DMRIE/cholesterol showed
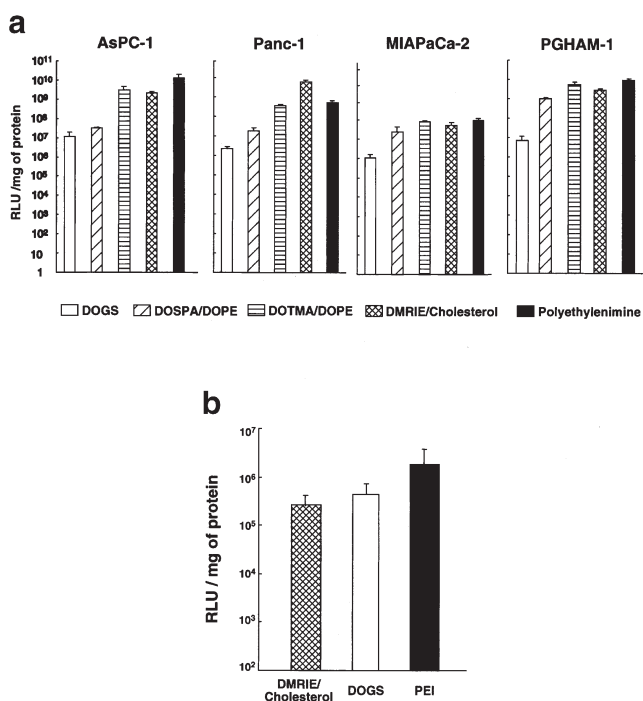
Figure 1 Comparison of gene transfer efficiency between PEI and cationic liposomes. (a) The luciferase activity following transfection of pancreatic cancer cell lines in vitro. Two micrograms of pCAG-luci was transfected using PEI or several cationic liposomes. The assays (performed in triplicate) were repeated a minimum of two times and average numbers plotted with standard deviation. Results were expressed as light unit per mg of cell protein. (b) Expression of luciferase in tumors on the mesentery after the intraperitoneal injection of DNA:cationic vector complexes. DNA:cationic vector complexes were injected three times into the peritoneal cavity of three mice with peritoneal dissemination.

the highest luciferase expression and approximately the same activity was observed with PEI and DOTMA/DOPE. The *in vivo* gene transfer into intraperitoneally disseminated tumors was then examined after intraperitoneal injection of plasmid DNA:PEI complexes. We compared PEI with DMRIE/cholesterol or DOGS, because DMRIE/cholesterol exhibited a higher transfection efficiency among the cationic liposomes *in vitro* (Figure 1a) and gene therapy experiments using DOGS were successful in mouse models of pancreatic peritoneal tumors, as previously reported.[14,15] AsPC-1 cells were transplanted to the peritoneal cavity of nude mice, and pCAG-luci was injected as a complex, either with PEI, DMRIE/cholesterol or DOGS. As shown in Figure 1b, PEI was more efficient than cationic lipids in transducing the luciferase gene into the peritoneal dissemination of mice.

Gene transfer into intraperitoneally disseminated tumors using DNA:PEI complexes

As shown in Figure 2a, repeated injections of pCAG-luci DNA:PEI complexes increased the magnitude of transgene expression in tumors on the mesentery. Three injections increased the luciferase expression more than 10-fold compared with that obtained using a single injection. Comparison of luciferase expression between pCAG-luci DNA:PEI complex and the naked plasmid pCAG-luci DNA demonstrated that PEI enhanced the gene transfer efficiency approximately 100–1000-fold compared with the naked plasmid, confirming that PEI is practically essential to mediate gene transfer (Figure 2b). To define the tissue distribution of the gene expression, luciferase activity was analyzed in many organs after the intraperitoneal injection of pCAG-luci DNA:PEI complexes for a total of three times. High luciferase activities ($1.2–1.4 \times 10^6$ RLU/mg protein) were observed only in tumors on the mesentery and pancreas, and low activities were detected in some organs such as spleen, stomach, and skeletal muscle. Other organs such as the brain, lung, heart, liver, kidney, testis, and small intestine did not show any luciferase activity (Figure 2b, left). The luciferase activity was maximal at day 1, and it was detectable up to day 7 in tumors on the mesentery. In the spleen, the luciferase activity was detected up to day 4 (Figure 2c).

To analyze the distribution of gene delivery in the tissue further, pCAG-lacZ DNA:PEI complexes were injected into murine peritoneal cavity, and β-galactosidase expression was examined in tumors on the mesentery and other organs. Blue-stained cells were observed only in tumors on the mesentery (Figure 2d), but not in other normal organs including the peritoneum and renal capsule. We think the discrepancy between luciferase assay and X-gal staining is explained by the low sensitivity of X-gal.[15]

Peritoneal barrier against PEI-mediated gene transfer

We tried to understand a part of the mechanisms for tumor-preferential expression of a transgene following intraperitoneal injection of DNA:PEI complexes. Unlike the tumor nodules disseminated on the peritoneum, normal organs in the peritoneal cavity are covered by the peritoneum and underlying connective tissue (Figure 3a, arrow), which may constitute a barrier against the entry of DNA:PEI complexes through the surface of organs. To test this premise, the peritoneum and an underlying fibrous layer (renal capsule) covering the right kidney
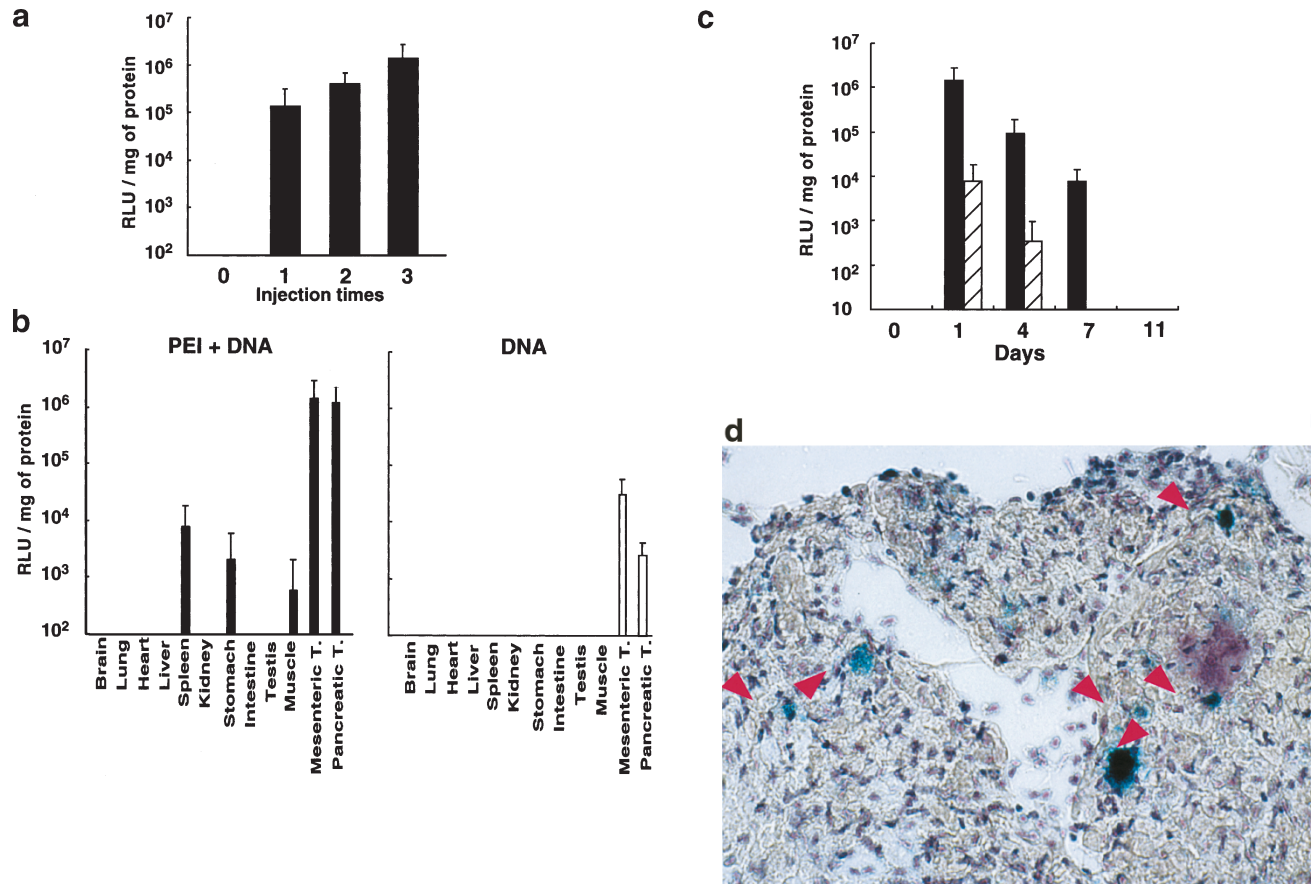
Figure 2 *Luciferase expression in the organs after the intraperitoneal injection of DNA:PEI complexes. After intraperitoneal transplantation of AsPC-1 cells, Balb/c nude mice were intraperitoneally injected with pCAG-luci complexed with PEI. Results were expressed as light unit per mg of tissue protein. (a) Dose dependency of transgene expression in tumors on the mesentery. The pCAG-luci:PEI complexes were injected into the peritoneal cavity either once, or two or three times (n = 3) at 12 h intervals. For each group, the luciferase activity of tumors on the mesentery was assayed 24 h after the last injection. (b) Tissue distribution of a transgene expression. (Left) pCAG-luci:PEI complexes were injected three times into the peritoneal cavity of five mice. (Right) pCAG-luci plasmids were injected three times into the peritoneal cavity of five mice. Mesenteric T., tumors on the mesentery; pancreatic T., tumors on the pancreas. (c) Time course of luciferase expression. After three injections of pCAG-luci:PEI complexes, the expression levels of the luciferase gene were assayed 1, 4, 7 and 11 days later in the spleen and in tumors on the mesentery. Black box, tumors on the mesentery (n = 3); hatched box, the spleen (n = 3). (d) Histochemical demonstration of the β-galactosidase activity in intraperitoneal tumors. After intraperitoneal transplantation of AsPC-1 cells, a Balb/c nude mouse was intraperitoneally injected with pCAG-lacZ complexed with PEI 3 times. Arrows: X-gal-positive, blue-stained cell. Counter staining by hematoxylin and eosin (×100).*

were surgically peeled off (Figure 3b), and then pCAG-luci:PEI complexes were instilled directly on to the surface of the renal parenchyma. As a control, the same *in vivo* gene transfer was attempted on the left kidney (Figure 3c, 1st column) with an intact surface. No luciferase expression was detected in the left kidney, whereas the right kidney showed a significant luciferase activity (2.4×10^3 RLU/mg protein) (Figure 3c, 2nd column). Next, the transgene expression within the cells of the peritoneal lining was assayed. The intraperitoneal injection of pCAG-luci:PEI complexes produced a relatively high luciferase activity (1.0×10^5 RLU/mg protein) in the peritoneum and an underlying fibrous layer (renal capsule) covering the kidney (Figure 3c, 3rd column). Although the gene transfer into the kidney with or without peritoneal barrier was examined by the instillation of pCAG-lacZ DNA:PEI complexes, blue-stained cells were not detected in the kidney parenchyma and capsule.

Persistence of distributed DNA and its toxicity

To determine organ distribution of injected DNA and time course of its clearance, the presence of DNA was examined in various organs by PCR analysis after the intraperitoneal injection of DNA:PEI complexes. The sensitivity of the PCR analysis was estimated to be about one copy per 10^2–10^3 genomes. The PCR analysis of DNA from various organs showed that the injected DNA was present in all organs 7 days after the injection. The band intensities of the luciferase gene decreased in all organs except for the brain 3 months later, and the transgene was detectable in none of the organs 6 months later (Figure 4). Furthermore, to characterize the potential toxicity of intraperitoneally injected DNA:PEI, 100 μg of pCAG-luci complexed with PEI (N/P = 7) was intraperitoneally injected into Balb/c mice (n = 5) three times at 12-h intervals. Animals were killed 1, 3 and 10 days after the injection to evaluate blood chemistry and histology of major
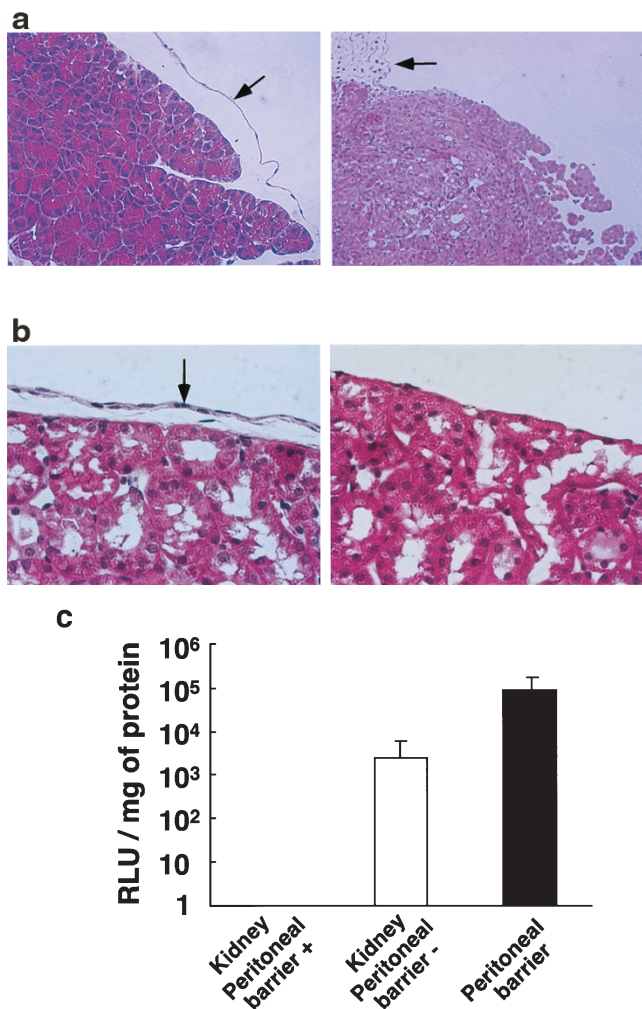
Figure 3 Gene transfer into the kidney by instillation of DNA:PEI complexes. (a) Representative sections of the pancreas and tumors on the pancreas. The pancreas (left) and tumor nodules on the pancreas (right) were stained with hematoxylin and eosin (×100). Arrow: peritoneum. (b, c) Gene transfer into the renal parenchyma by the instillation of DNA:PEI complexes. The kidneys were stained with hematoxylin and eosin (arrow) (b) (×200). The peritoneum and underlying fibrous component (capsule) on the right kidney was peeled off (b, right), then 50 μg of pCAG-luci complexed with PEI (N/P = 7) were instilled on to the kidney (c, 2nd column). As a control, the same dose of pCAG-luci:PEI complexes was also instilled on to the left kidney with those tissues (b, left; c, 1st column) at the same time. After incubation of 30 min, the abdomen was closed, and 24 h later the luciferase activity was assayed (c; n = 5). On the other hand, 100 μg of pCAG-luci complexed with PEI was intraperitoneally injected, and 24 h later the peritoneum and an underlying fibrous layer (renal capsule) covering the kidney were assayed for luciferase activity (c, 3rd column; n = 5).

organs. No significant toxicity was observed with blood chemistry (Table 1), hematoxylin- and eosin-stained sections and monitoring animal body weights (data not shown).

Discussion

PEI is one of the polycations that have been used successfully to transduce genes into living cells. Many groups have been investigating the *in vivo* gene transfer of DNA: PEI complexes by various routes. It was reported that widespread expression was obtained in neurons and glia

after the direct injection into the brain or intraventricular injection of DNA:PEI.[22,23] DNA:PEI complexes effectively transduced pseudocystic tumors when they are injected in the cyst.[24] Intravenous injection led to efficient gene transfer into the normal lung epithelial cells and hepatocytes,[24–28] but subcutaneous tumors as well as lung metastases were not transfected by systemic injection.[23] To the best of our knowledge, this paper is a first report evaluating PEI as a gene delivery system into peritoneal dissemination by intraperitoneal injection. This study showed that the intraperitoneal injection of DNA:PEI complexes achieves a tumor-preferential transgene expression (Figure 2b and d).

Cationic liposomes have also been reported to transduce a gene into intraperitoneal tumors in a tumor-preferential manner, but the mechanism for this tumor targetability is not elucidated.[15,29,30] The DNA:polycation complexes might enter the lymphatics, then the blood circulation, to be conveyed to various organs.[31] Another possible route of gene transfer is the direct entry of the DNA:PEI complexes to the organ parenchyma through its surface. The organs in the peritoneal cavity are normally covered with the peritoneum and subperitoneal connective tissue, whereas the tumor nodules disseminated over the peritoneal surfaces are devoid of such coverings, since the interaction of cancer cells with the peritoneum induces disruption and exfoliation of the mesothelial cells in the early process of peritonal metastasis.[32,33] The peritoneum and underlying fibrous component may act as a barrier against the entry of DNA:PEI complexes through the surface of organs. Interestingly, relatively high luciferase activity was detected within the cells of the peritoneum and underlying fibrous layer (renal capsule), and a significant luciferase activity was observed in the kidney parenchyma without the peritoneal barrier. It appears that the peritoneal lining captures the plasmid DNA:PEI complexes and prevents the spread of the gene transfer into the underlying organ parenchyma. The kidney with peritoneal barrier showed no luciferase activity, regardless of the effective gene transfer into renal coverings. This may be explained by the large volume ratio of kidney parenchyma to peritoneal barrier, which means many more untransduced cells in the organ core than transduced cells in the peritoneal lining. An additional element which may account for the apparent tumor-preference is that the transduced gene is more readily expressed in rapidly proliferating cells such as cancer cells than in normal cells with low mitogenic activity.[30,34,35] Since transfected DNA is probably transported inefficiently through the nuclear membrane during interphase, it is likely that the breakdown of the nuclear membrane during mitosis is crucial to efficient entrance of the transfected DNA into the nucleus.[36,37]

Issues pertaining to the toxicity, kinetics and biodistribution of DNA:PEI complexes need to be addressed. Concern has been raised regarding the inadvertent introduction of DNA into normal cells. Consistent with our previous study with a liposome DOGS,[14] PCR analysis showed that the intraperitoneally injected DNA was delivered to various organs. However, the luciferase assay showed faint or no expression of a gene in normal organs irrespective of the presence of plasmid DNA. Our previous RT-PCR analysis to detect a transgene expression revealed similar results.[15] Thus, it is plausible that DNA detected by PCR analysis might be the par-
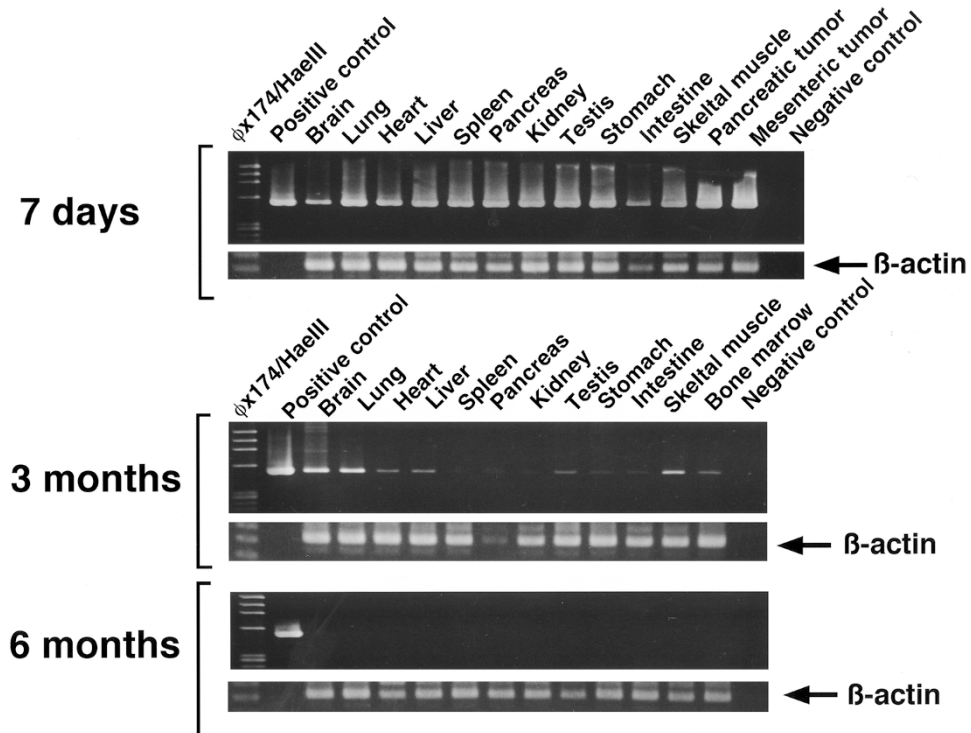
Figure 4 Organ distribution of injected DNA and time course of its clearance after intraperitoneal injection of DNA:PEI. The plasmid DNA complexed with PEI was intraperitonelly injected into Balb/c mice. The genomic DNA was analyzed by PCR for luciferase transgene or β-actin (marked) from various organs (the brain, lung, heart, liver, pancreas, spleen, kidney, testis, stomach, small intestine, skeletal muscle, bone marrow, tumors on the pancreas and the mesentery) 7 days (upper), 3 months (middle) and 6 months (lower) later. Positive control, pGL3 control plasmid; negative control, no DNA.

Table 1 Evaluation of selected blood chemistries after intraperitoneal injection

	PBS	DNA:PEI		
		1 day	3 day	10 day
T. protein (mg/ml)	4.95 ± 0.42	4.68 ± 0.22	4.77 ± 0.36	4.92 ± 0.19
Albumin (mg/ml)	2.95 ± 0.13	2.93 ± 0.10	2.88 ± 0.12	3.20 ± 0.06
T. bilirubin (mg/ml)	0.60 ± 0.56	0.28 ± 0.26	0.40 ± 0.32	0.43 ± 0.42
BUN (mg/dl)	15.3 ± 0.45	15.7 ± 1.88	17.9 ± 1.35	21.8 ± 1.98
Creatinine (mg/ml)	0.1 ± 0	0.1 ± 0	0.1 ± 0	0.1 ± 0
GOT (IU/I)	101.8 ± 74.1	114.2 ± 79.77	108.3 ± 56.08	55.5 ± 9.09
GPT (IU/I)	40.0 ± 4.00	35.8 ± 7.27	31.3 ± 8.21	34.0 ± 9.72
LDH (IU/I)	989.0 ± 742.5	522.8 ± 222.2	758.2 ± 315.4	648.3 ± 402.0
ALP (IU/I)	263.0 ± 57.2	227.8 ± 31.9	263.3 ± 21.0	336.5 ± 31.7

Blood chemistry values were analyzed (SRL, Tokyo, Japan), and mean values and standard deviations are shown for five mice in each group 1, 3 and 10 days after the injection of plasmid DNA:PEI complexes. Control is PBS-injected mice ($n = 8$).

tially degraded fragments of plasmid or DNA attached to the surface of organs. Moreover, the injected DNA was no longer evident up to 6 months after injection, and no significant abnormality was observed in blood chemistry and histological sections of major organs. Because of the efficient tumor-preferential expression and the lack of toxicity, the intraperitoneal injection of DNA:PEI complexes is expected to play an important role in future treatment of peritoneal dissemination.

Materials and methods

Plasmid
The firefly luciferase gene and β-galactosidase gene were used as reporter genes to monitor the result of gene transfer. The luciferase expression plasmid (pCAG-luci) and β-galactosidase expression plasmid (pCAG-lacZ) were constructed by subcloning *Photinus pyralis* luciferase or *E. coli* β-galactosidase cDNA to pCAGGS, respectively. The

pCAGGS vector expresses an inserted DNA by its CAG promoter, which consists of the cytomegalovirus immediate–early enhancer sequence and the chicken β-actin/rabbit β-globin hybrid promoter.[38,39]

Cells

AsPC-1, MIAPaCa-2 and Panc-1, human pancreatic cancer cell lines, were obtained from the American Tissue Culture Collection (Bethesday, MD, USA). PGHAM-1, a hamster pancreatic cancer cell line, was a gift from Dr E Uchida (Nippon Medical School, Tokyo, Japan).[40] All pancreatic cancer cell lines were grown in RPMI-1640 medium (GIBCO BRL, Gaithersburg, MD, USA) supplemented with 10% fetal bovine serum and antibiotics.

PEI

A linear form of polyethylenimine (PEI) of molecular weight 22 kDa was synthesized by acid hydrolysis of 50 kDa poly (2-ethyl-2-oxazoline)s (Sigma-Aldrich, St Louis, MO, USA). The ultimate elementary analysis showed that the composition of synthesized PEI was C (%): 55.36, H (%): 11.63, N (%): 31.37, which was almost the same for the calculated composition (C: 55.81, H: 11.63, N: 32.56).

Assay for luciferase activity in vitro

Twenty-four hours before transfection, cells were seeded at 2×10^5 cells per well in a six-well plate, and each well received 2 μg of pCAG-luci. Transfection with N[1-(2, 3-dioleyloxy)propyl]-n,n,n-trimethylammonium chloride (DOTMA)/dioleoyl phoaphatidylethenolamine (DOPE) (Lipofectin; GIBCO BRL), 2, 3-dioleyloxy-N-[2(sperminecarboxamido)ethyl]-N,N-dimethyl-1-propanaminium trifluoroacetate (DOSPA)/DOPE (LiopfectAMINE; GIBCO BRL), or 1, 2-dimyristyloxypropyl-3-dimethyl-hydroxyethyl ammonium bromide (DMRIE)/cholesterol (DMRIE-C; GIBCO BRL) and dioctadecylamidoglycylspermine (DOGS) (Transfectam; BioSepra, Marlborough, MA, USA) were set up following optimal conditions suggested by the manufacturers' protocols. Since the transfection efficiency of DNA:PEI complexes was maximal at 6.75 to 9 equivalents of PEI nitrogen per DNA phosphate (N/P) (data not shown), we used 9 N/P in the *in vitro* gene transfer. Twenty-four hours after transfection, cells were lysed with 100 μl of cell lysis buffer (25 mM Tris-phosphate, pH 7.8, 2 mM DTT, 2 mM EDTA, 10% glycerol, 1% Triton-X) and incubated at room temperature for 20 min. Three microliters of cell lysate was mixed with 100 μl of Luciferase Assay Reagent (PicaGene; Toyo Ink, Tokyo, Japan), and the light unit of the luciferase activity was measured using a luminometer (MiniLumat LB 9506; EG&G Berthold, Vilvoorde, Belgium).

In vivo gene transfer into peritoneal dissemination

A linear form of polyethylenimine, and cationic liposomes such as DMRIE-C or DOGS, were used as *in vivo* transfection agents. Twenty-one days after the transplantation of 1×10^6 AsPC-1 cells into the peritoneal cavity of male Balb/c nude mice (Charles River Japan, Kanagawa, Japan), the multiple peritoneal nodules about 2–3 mm in diameter were formed. The mice were then intraperitoneally injected with the pCAG-luci or pCAG-lacZ complexed with PEI, DOGS or DMRIE/cholesterol. For complex formation, the following amounts of each vector were used: for PEI transfection solution, 100 μg of plasmid was diluted in 300 μl of 5% glucose solution, and

the 2.1 mmol of a linear form of PEI (7 N/P) was diluted in 300 μl of 5% glucose solution, and then the PEI solution was added to the plasmid solution; for DOGS transfection solution, 100 μg of plasmid was diluted in 300 μl of a 0.15 M NaCl solution, and the 400 nmol of DOGS ethanolic solution was diluted in 350 μl of 0.15 M NaCl solution, and then the DOGS solution was added to the plasmid solution; for DMRIE/cholesterol transfection solution, 100 μg of plasmid was diluted in 300 μl of 10 mM phosphate buffered saline (PBS), and 490 nmol of DMRIE/cholesterol was added to the plasmid solution. After 10 min, the resulting mixture was injected into the intraperitoneal cavity. The mice were killed 24 h after the last injection, and tissue samples were obtained from tumor nodules on the pancreas and mesentery, and from various normal organs (brain, heart, lung, liver, spleen, pancreas, kidney, stomach, intestine, testis, muscle and bone marrow).

Assay for luciferase activity in vivo

The frozen tissues were pulverized using liquid nitrogen, an equal volume of tissue lysis buffer (125 mM Tris-phosphate, pH 7.8, 10 mM DTT, 10 mM EDTA, 50% glycerol, 5% Triton-X, 0.2 mM phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, 1 μg/ml leupeptin) was added, and incubated on ice for 20 min. Twenty microliters of supernatants was mixed with 100 μl of Luciferase Assay Reagent (PicaGene), and the light unit of the luciferase activity was assayed using a luminometer (MiniLumat LB 9506). We calculated the mean value plus two standard deviations of each organ and tumor in untreated mice as background values ($n = 5$), and then subtracted background values from each luciferase value of samples in DNA:PEI-injected mice. It was confirmed that the tissue extract of various organs does not inhibit the activity of luciferase enzyme (data not shown).

Assay for β-galactosidase in vivo

After intraperitoneal transplantation of AsPC-1 cells, a Balb/c nude mouse was intraperitoneally injected with 100 μg of pCAG-lacZ complexed with PEI three times. Forty-eight hours later, tumors in the mesentery were fixed with 0.25% glutaraldehyde, and developed in a substrate solution [5 mM $K_3Fe(CN)_6$, 5 mM $K_4(CN)_6$, 2 mM $MgCl_2$, and 1 mg/ml X-gal] at 37°C for 16 h.

PCR analysis of the injected DNA

PCR analysis was performed to evaluate the organ distribution of intraperitoneally injected DNA and the time course of its clearance. One hundred micrograms of luciferase expression plasmid (pBL3 control vector; Promega, Madison, WI, USA) complexed with 2.1 mmol of PEI was injected into Balb/c mice (Charles River Japan) for a total of three times at 12-h intervals. The genomic DNA was extracted from tumors and various normal organs 7 days, 3 months and 6 months after the injection. The 0.1 μg of DNA was subjected to PCR analysis using two primers specific to the luciferase specific primers (Luci-L, 5'-GCGCCATTCTATCCGCTGGA-3'; Luci-R, 5'-CTATCGAAGGACTCTGGCAC-3'), which should yield a 530-bp fragment. We performed PCR for 30 cycles at 94°C for 1 min, 60°C for 30 s, and 72°C for 2 min, and the PCR products were electrophoresed on a 2.0% agarose gel.

Acknowledgements

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