Cancer-Specific Transgene Expression Mediated by Systemic Injection of Nanoparticles

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Abstract

The lack of safe and efficient systemic gene delivery vectors has largely reduced the potential of gene therapy in the clinic. Previously, we have reported that polypropyleneimine dendrimer PPGI3/DNA nanoparticles are capable of tumor transfection upon systemic administration in tumor-bearing mice. To be safely applicable in the clinic, it is crucial to investigate the colloidal stability of nanoparticles and to monitor the exact biodistribution of gene transfer in the whole body of the live subject. Our biophysical characterization shows that dendrimers, when complexed with DNA, are capable of forming spontaneously in solution a supramolecular assembly that possesses all the features required to diffuse in experimental tumors through the enhanced permeability and retention effect. We show that these nanoparticles are of sizes ranging from 33 to 286 nm depending on the DNA concentration, with a colloidal stable and well-organized fingerprint-like structure in which DNA molecules are condensed with an even periodicity of 2.8 nm. Whole-body nuclear imaging using small-animal nano-single-photon emission computed tomography/computer tomography scanner and the human Na+/I- symporter (NIS) as reporter gene shows unique and highly specific tumor targeting with no detection of gene transfer in any of the other tissues of tumor-bearing mice. Tumor-selective transgene expression was confirmed by quantitative reverse transcription-PCR at autopsy of scanned animals, whereas genomic PCR showed that the tumor sites are the predominant sites of nanoparticle accumulation. Considering that NIS imaging of transgene expression has been recently validated in humans, our data highlight the potential of these nanoparticles as a new formulation for cancer gene therapy. [Cancer Res 2009;69(6):2655–62]

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Introduction

To be efficient against cancer, the ideal antitumoral agent would have to target malignant cells throughout the body while sparing normal tissues. Cancer gene therapy has thus far been limited by the lack of systemically active, cancer-specific delivery vectors (1), especially in the field of nonviral, synthetic gene delivery vectors (2–4). In this context, we have recently described that nanoparticles composed of the polypropyleneimine dendrimers of third generation (PPGI3), when complexed with DNA, are capable of efficient gene transfer to tumor deposits, upon systemic injection. Importantly, when a therapeutic transgene was used, marked antitumor activity was observed, leading, in some experiments, to the “cure” of all animals treated (5, 6).

Key to the furthering clinical development of this technology and its safe application in patients is the ability to monitor gene transfer in the live subject, using a minimally invasive method. This is of particular importance considering the toxic nature of the therapeutic transgenes required for cancer gene therapy. Whole-body nuclear imaging of gene transfer in the live subject has been described (7) and requires a reporter gene and a radiolabeled tracer. One possible strategy exploits the Na/I symporter (NIS) as a reporter gene and radio-halogen as tracer. NIS is endogenously expressed in the thyroid and stomach and, to a lower extent, in the salivary glands, breast, and thymus (8). In the thyroid, NIS promotes iodide concentration, and radio-iodide uptake is used in the detection and, in some specific cases, in the treatment of disseminated thyroid carcinomas (9). In gene therapy experiments, ectopic expression of NIS has been shown to allow accumulation of radioactive iodide in tissues for noninvasive imaging (10–12). Moreover, ectopic NIS expression is unlikely to interfere with the cell biochemistry. Different modalities have been reported to image NIS-expressing tissues, including positron emission tomography (PET), scintigraphic imaging, and single photon emission computed tomography (SPECT; refs. 13–16). The imaging of NIS-expressing tissues is particularly versatile because NIS can promote cellular uptake of different radioisotopes: 125I− (SPECT), 124I− (PET), 99m-TcO4− (SPECT), and 131I− (scintigraphic imaging; refs. 8, 11). This imaging method has very recently been validated in humans (17).

Another consideration that is key to the translation of PPGI3/DNA complexes to the clinic is the biophysical characterization of the nanoparticles. Delivery of polymer-drug conjugates containing doxorubicin (18), paclitaxel (19), or camptothecin (20) to tumors has been described and some of these compounds...
are in phase III clinical trials (21). These complexes have been shown to remain for long periods in the blood stream and accumulate passively in tumor tissues through a phenomenon referred to as the enhanced permeability and retention effect (EPR). The EPR effect first described by Matsumura and Maeda (22, 23) constitutes an important mechanism by which "objects" with adapted size and surface characteristics can extravasate through the endothelial fenestration of tumor blood tissues, usually larger and more disorganized than normal blood tissues. As a result, these "objects" are preferentially retained in the tumors and, thus, the pharmacokinetic and pharmacodynamic properties of the associated-drugs are enhanced compared with administration of the drug alone (24, 25). Nanoparticles exploiting the EPR effect for cancer-selective delivery of nucleic acids would be highly desirable.

In this context, the aim of the present study is (a) to determine whether the biophysical characteristics of PPIG3/DNA complexes are compatible with the EPR effect, (b) to assess whether NIS-imaging can be used to visualize nanoparticle-mediated gene transfer, and (c) to determine the pattern of gene expression allowed by these nanoparticles in tumor-bearing mice.

Materials and Methods

Nanoparticles and plasmid DNA. The nanoparticles used in this study are constituted of PPIG3 and prepared in 5% dextrose solution as previously described (5). The NIS cDNA was amplified by PCR from pTGW10 plasmid (13) using the primers 5'-CTCTAGAGTGGAGCCTGGAGACC GG-GGAACGG-3' (forward) and 5'-CTCTAGAGCTGGCTT GTGGCTATATC-3' (reverse) and subcloned into the pcDNA3.1/V5- His-TOPO expression plasmid DNA (Invitrogen).

Dynamic light scattering, fluorescence, and cryo-transmission electron microscopy studies. Dynamic light scattering, fluorescence, and cryo-electron transmission microscopy (cryo-TEM) studies were done as previously described (26, 27). PPIG3/DNA nanoparticles were prepared by combining increased concentration of PPIG3 dendrimer (0-5 µg/mL) with a fixed-standardized concentration of DNA plasmid (10 µg/mL) in 1 mL of 5% dextrose. After 10-min incubation at room temperature, the hydrodynamic mean diameter of nanoparticles formed was determined by dynamic light scattering using the intensity mean size (Z average) with a sample refractive index of 1.39 and a viscosity of 0.89 cP. The system was routinely calibrated using a 220-nm standard (Malvern Instrument). Data presented are the mean diameters ± SE of nanoparticles measured from three independent experiments. To evaluate the level of DNA condensation, 5 nmol/L final concentration of ethidium bromide was added to the tubes, and fluorescence measurement assessed 5 min later at 600 nm on a Kontron SFM25 spectrophotometer (Kontron). Structural information and size of PPIG3/DNA nanoparticles were determined by cryo-TEM assay. PPIG3 dendrimer (1 µg/mL) was combined with DNA plasmid at the indicated concentration and let to assemble for 10 min. A 5-µL aliquot was deposited onto a holey carbon-coated copper grid and plunged into a liquid ethane bath cooled with liquid nitrogen. The grids containing the frozen samples were thereafter mounted onto a Gatan 626 cryoholder, and samples observed with a Tecnai F20 FEI transmission electron microscope, operating at 200 kV. Low-dose images were recorded at a nominal magnification of ×50,000 with a 2k × 2k USCI1000 slow-scan charge-coupled device camera (Gatan). To determine formation of highly ordered phases between the PPIG3 dendrimer and DNA molecules, periodic information within the PPIG3/DNA nanoparticle structures was investigated using the Image J analysis program applied to the pictures from the cryo-TEM analysis. The procedure consists of computing the Fourier transform and in displaying the power spectrum image. DNA spacing is calculated from measurements of distance of the first-order peaks based on the fact that in Fourier space this distance is proportional to the inverse length of DNA spacing.

Cell lines. All cell lines used were obtained from Cancer Research UK Cell Services. The cell lines A431, HeLa, and HeP-2 were grown in DMEM, supplemented with 10% of fetal bovine serum and antibiotics. The SCC25 cell line was cultured in a 1:1 mixture of DMEM and Ham’s F-12 medium, supplemented with 10% fetal bovine serum and 400 ng/mL hydrocortisone.

Animals. All experiments were conducted with appropriate ethical approval and in accordance with the Guidance on the Operation of the Animals (Scientific Procedures) Act 1986 (House of Commons 1990) of the UK Home Office regulations. Six- to eight-week-old female BALB/c nu/nu or C3H mice (Harlan) were allowed to acclimatize for 1 wk and kept in individual ventilated caged with free access to food and water. Tumor cells (10⁶) were implanted subcutaneously in the right flanks of mice and kept until tumors reached > 200 mm³. PPIG3 dendrimer and plasmid DNA encoding NIS were then complexed in 250 µL of a 5% dextrose solution at a ratio of 5:1 (w/w) with 50 µg of plasmid DNA and 250 µg of dendrimer (5). The final concentration of nanoparticle components was therefore 200 µg/mL for the plasmid DNA and 1 µg/mL for the PPIG3 dendrimers. After incubation for 10 min at room temperature, the resulting PPIG3/DNA nanoparticles were injected intratumorally or i.v. into the tail vein of mice bearing tumors (n = 3). Control mice were treated with PBS (n = 3), PPIG3 nanoparticles (n = 3), or plasmid DNA alone (n = 3). Animals were weighed every 5 d; aspartate aminotransferase (AST) and alanine aminotransferase (ALT) serum transaminase activity levels were determined from blood samples collected at the indicated times in the text. Histologic examinations of liver, lungs, spleen, and kidney were done 5 d after the systemic administration of the PPIG3/DNA nanoparticles.

Nano-SPECT/computer tomography imaging. Small-animal SPECT/ computer tomography (CT) scanning was done as previously described (28). Twenty-four hours after administration of the PPIG3/DNA nanoparticles, animals were anesthetized and then given an i.v. injection of 18.5Mbq of ⁹⁰Tc. Alternatively, different scans were done on the same cohort of animals 10, 24, 48, and 72 h after administration of the PPIG3/DNA nanoparticles. Mice were then positioned inside the SPECT/CT scanner (Bioscan, Inc.). The SPECT image was obtained, and acquisition time depended on specific radioactivity levels in each mouse over the limits of the scan to obtain 100,000 cps. A CT scan was taken at the same levels as the SPECT scan and all images were reconstructed with MEDIOSOFT software (Medical Imaging Systems). Fusion of SPECT and CT images was carried out using PMOD software (Medical Imaging Systems).

Quantitative reverse transcriptase-PCR and genomic PCR. To detect NIS mRNA expression in tissues, scanned animals were sacrificed and the tumor, liver, spleen, lung, kidney, and bone marrow tissues were harvested. Tissues were homogenized and total RNA was prepared using the RNeasy Mini kit according to the manufacturer’s instructions (Qiagen). Reverse transcription followed by quantitative PCR was done from 2 µg of total RNA extracted as previously described (28). To detect plasmid DNA in tissues, quantitative genomic PCR was done. Tissues were harvested and genomic DNA was extracted using DNeasy kit according to the manufacturer’s protocol (Qiagen). DNA concentration and purity were determined using NanoDrop spectrophotometer (NanoDrop Technologies). Five nanograms of DNA sample were used in each quantitative PCR reaction in triplicate. The PCR conditions and primers were the same as those used for the reverse transcription-PCR (RT-PCR) described above. Negative controls included PCR reaction without the cDNA or the genomic DNA.

Statistical analysis. The experiments were done at least thrice in triplicate. Errors bars represent SE. One-way ANOVA followed by the Bonferroni posttest (GraphPad Prism software) was used to determine statistical significance for the following experiments: quantitative RT-PCR; genomic PCR; body weight and serum transaminase activity measurements; and nanoparticle uptake, luciferase activity, and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assays.

Results

Colloidal stability of the PPIG3/DNA nanoparticles by the light dynamic scattering method. We investigated the physicochemical properties of the supramolecular assemblies of the
nanoparticles obtained by mixing the PPIG3 dendrimer with a plasmid DNA encoding NIS. Dynamic light scattering was first used to assess the colloidal stability of the formed nanoparticles as a function of the charge ratio. To calculate the mean theoretical charge ratio, we assumed that 1 μg of plasmid DNA is 3 nmol of negatively charged phosphate and that 16 positive charges are displayed by the PPIG3 dendrimer according to its chemical formulation and molecular weight (PPIG3, 1.67 kDa). Increasing concentrations of PPIG3 dendrimer (0–3 μg/μL) were left to assemble to a constant concentration of DNA plasmid (10 μg/mL) for 10 minutes before determining the size of the formed nanoparticles using a Zetasizer instrument. Results in Fig. 1 show that the PPIG3/DNA nanoparticles exhibit a three-zone model of colloidal stability depending on the cationic vector/DNA charge ratio used. In zone A (i.e., for a charge ratio <1), we found colloidal stable, negatively charged complexes with a mean diameter of 107 ± 17 nm (n = 3). In zone B, characterized by dendrimer/DNA charge ratios ranging from 1 to 1.5, the complexes were neutral, large, and colloidal unstable as evidenced by the observation that their diameter increased up to 402 ± 56 nm (n = 3). Finally, in zone C (i.e., for a charge ratio above 1.5), we observed colloidal stable, positively charged complexes with a mean diameter of 86 ± 9 nm (n = 3). These results show that PPIG3/DNA nanoparticles followed the well-known three-stage model of colloidal stability described elsewhere (3, 26, 27, 29).

Next, we investigated the level of DNA condensation using fluorescence measurement after addition of ethidium bromide in all samples. The percentage of free DNA or DNA bound loosely to PPIG3 dendrimer was then estimated at 600 nm. As expected (Fig. 1), the fluorescence intensity decreased as the dendrimer/DNA charge ratio increased from 100% in zone A to a low minimal value in zones B and C. The very low level of fluorescence intensity in zones B and C indicates that DNA molecules in these zones are firmly packed within the nanoparticle structure, preventing their interaction with ethidium bromide molecules and, consequently, with any other components of the extracellular media.

Structural features of the PPIG3/DNA nanoparticles by cryo-TEM imaging and the Fourier transform method. The structure of the PPIG3/DNA nanoparticles was finally examined using cryo-electron microscopy (Fig. 2). Cryo-TEM allows visualizing nanoparticles with their native contrast without the need of dehydration or staining procedures. Therefore, when combined to Image J software and the Fourier method, cryo-TEM images provide accurate information about the structure and the size of nanoparticles. Plasmid DNAs were mixed with the PPIG3 dendrimers (1 μg/μL), and the size of nanoparticles was determined

Figure 1. Colloidal stability of the PPIG3/DNA nanoparticles as a function of their charge ratio. Complexes were obtained by mixing the PPIG3 dendrimer at the required concentrations with plasmid DNA at the fixed standardized concentration of 10 μg/mL. Dynamic light scattering analysis (solid lines) was done to assess colloidal stability of the complexes. Ethidium bromide fluorescence measurements (dashed lines) were also done for each tube to evaluate the level of nucleic acid entrapment within the PPIG3/DNA nanoparticle structure. Size determination and fluorescence measurements were done after 10 min of complexation. An arbitrary value of 400 nm was attributed to complexes that were colloidal unstable. Inset, a schematic representation of zones A, B, and C. Complexes from zones A and C were stable, whereas those from zone B were colloidal unstable, with their diameter increasing to up to 400 nm. Representative of three independent experiments.

Figure 2. Cryo-TEM visualization of dendrimer/DNA complexes. A, field of view of PPIG3/DNA nanoparticles at 100 μg/mL showing round-shaped structures (black arrows). Gallery of cryo-TEM images of PPIG3/DNA nanoparticles at 100 μg/mL (B–D) and 50 μg/mL (E–H) revealing a fingerprint pattern of DNA organization within dendrimer/DNA complexes whose sizes vary from 38 nm (E, arrow) up to 255 nm (D). Bar, 1 μm (A); 50 nm (B–H).
individually. At a DNA concentration of 100 µg/mL, corresponding to a PPIG3 dendrimer/DNA charge ratio of 16, globular structures ranging in size from 103 to 255 nm were observed (Fig. 2A–D). When the DNA concentration was halved (50 µg/mL) with the same dendrimer/DNA charge ratio, the same globular structure was observed, but the size of particles was smaller, ranging from 38 to 152 nm (Fig. 2E–H). The average size of the PPIG3/DNA nanoparticles was 78 ± 14 nm (7 particles analyzed) for a DNA concentration of 50 µg/mL, 150 ± 26 nm (7 particles analyzed) for a DNA concentration of 100 µg/mL, and 286 ± 41 nm (7 particles analyzed) for a DNA concentration of 200 µg/mL. At higher DNA concentrations, the complex precipitated. These results show that the size of PPIG3/DNA nanoparticles was directly dependent on the DNA loaded and led, as shown in the gallery of Fig. 2, to the formation of compact, concentric, and homogeneous structures with a fingerprint-like aspect. Image analysis based on the use of Fourier transform showed that despite the lack of a highly ordered organization, a repeat distance of ~2.8 nm corresponding to DNA strand packing was detected. Taken together, these data show that the PPIG3 dendrimer when complexed with plasmid DNA displays a set of biophysical characteristics that are suitable for a tumor-specific gene transfer mediated by the EPR effect (22, 23).

Visualizing of nanoparticle-mediated gene transfer upon intratumoral injection. To monitor the exact localization and amplitude of gene transfer in the whole body of tumor-bearing mice, we first examined gene transfer on intratumoral injection of the nanoparticles. Mice with subcutaneous A431 tumors were injected with PPIG3 dendrimers (250 µg) complexed with a plasmid DNA encoding NIS (50 µg), forming, as shown above, nanoparticles averaging 286 nm (± 41) in size. Twenty-four hours later, mice were scanned. Fusion of the SPECT and CT images revealed that the 99mTcO4 tracer accumulated in the thyroid gland and the stomach (Fig. 3A) as a result of endogenous expression of the NIS gene (10–13) and in the tumor (Fig. 3A) as a result of gene transfer. Control, intratumoral injections of PBS, DNA, or PPIG3 alone failed to result in radiotracer accumulation in the tumor whereas signals not related to gene transfer (thyroid and stomach) were detected (data not shown). Quantitative RT-PCR done on tumor biopsies harvested at autopsy of the scanned animals revealed the presence of NIS-specific mRNA (48.2 ± 8.2 pg/µg tRNA), confirming in vivo gene transfer upon intratumoral injection of the PPIG3/DNA nanoparticles. By contrast, a very low presence of NIS-specific mRNA was found in control mice treated with PBS (0.28 ± 0.06 pg/µg tRNA), DNA (1.25 ± 0.32 pg/µg tRNA), or PPIG3 alone (0.62 ± 0.16 pg/µg tRNA).

Visualizing of nanoparticle-mediated gene transfer upon systemic injection in immunodeficient tumor-bearing mice. To examine the pattern of gene transfer obtained upon systemic injection, mice with subcutaneous Hep-2, A431, and HeLa xenograft tumors (Fig. 4A) were injected in the tail vein with nanoparticles (250 µg) complexed with the plasmid encoding NIS (50 µg). Twenty-four hours later, mice were scanned. Fusion of the SPECT and CT images revealed that the 99mTcO4 tracer accumulated in the thyroid and stomach as a result of endogenous NIS expression (data not shown) and in the tumors (Fig. 4A). Careful examination of all projections of the tomographic sections failed to detect any other gene-transfer–related signal (data not shown), suggesting that systemic nanoparticle delivery resulted in tumor-specific gene transfer. To confirm this hypothesis, quantitative RT-PCR was done on tumor biopsies taken from the scanned animals. Results showed that NIS-specific mRNA was below the threshold of detection in the liver, spleen, kidney, heart, and lung in all tumor-bearing mice scanned (data not shown). By contrast, NIS-specific mRNA could be quantified in tumor samples from Hep-2 (Fig. 4B; 31.66 ± 5.8 ng/µg tRNA), A431 (27.2 ± 4.2 ng/µg tRNA), and HeLa cells (29.6 ± 6.4 ng/µg tRNA). Taken together, these experiments show that systemic injection of the nanoparticles results in tumor-selective transgene expression.

We then assessed whether systemic gene delivery mediated by the nanoparticles could result in gene transfer in more than one tumor deposit. Mice with subcutaneous Hep-2, A431, and HeLa xenograft tumors implanted in both flanks were injected systemically with the PPIG3 dendrimers (250 µg) complexed with the DNA plasmid encoding NIS (50 µg). Twenty-four hours later, mice were scanned and fusion of the SPECT and CT images was done. Result in Fig. 4A shows a coronal image in which the two Hep-2 tumors...
are in the same tomographic section. The signals observed in the two tumors show that both have been transfected by the PPIG3/DNA nanoparticles. Similar scans were obtained with subcutaneous A431 and HeLa tumors (data not shown). The only additional signals detected were in the thyroid and the stomach as a result of endogenous NIS expression (data not shown). Quantitative RT-PCR (Fig. 4C) analysis on tumor biopsies taken from the scanned animals showed that gene transfer in bilateral tumors was in the same order of magnitude: Hep-2 (right: 26.5 ± 4.7 ng/µg tRNA; left: 31.5 ± 6.1 ng/µg tRNA), HeLa (right: 25.5 ± 5.2 ng/µg tRNA; left: 32.5 ± 8.1 ng/µg tRNA), and A431 (right: 41 ± 8.1 ng/µg tRNA; left: 31.0 ± 8.0 ng/µg tRNA). In control mice injected with PBS, DNA, or PPIG3 alone, NIS-specific signal was below the threshold of detection of the PCR assay (data not shown). Altogether, these data show that gene transfer mediated by the nanoparticles is tumor specific and can reach at least two independent tumor deposits.

**Visualization of nanoparticle-mediated gene transfer upon systemic injection in immunocompetent tumor-bearing mice.**

To assess whether the nanoparticle-mediated gene transfer remains cancer selective in an immunocompetent mouse model, C3H mice bearing subcutaneous SCC25 tumors were i.v. injected with the PPIG3 nanoparticles (250 µg) complexed with the plasmid encoding NIS (50 µg). Mice were scanned 24 hours later. Fusion of the SPECT and CT images revealed that, as in the case of an immunodeficient mouse model, injection of the PPIG3/DNA nanoparticles results in radiotracer uptake in the tumor (Fig. 5A). In addition, different scans were done on the same cohort 10, 24, 48, and 72 hours after the PPIG3/DNA nanoparticle injection. In these experiments and at each time point, the animals were anesthetized and scanned to verify that no residual 99mTcO4− from the previous scan remained in the tumor. The animals were then injected with the radiotracer, and the data acquired. Tumors were then outlined and the radioactivity was calculated in the volume of interest. Result in Fig. 5B shows that the mean peak of radiotracer accumulation (0.24 ± 0.021 MBq) is obtained 24 hours after PPIG3/DNA nanoparticle injection, showing the transient nature of this gene transfer technology. In control mice injected with PBS, DNA, or PPIG3 alone, the radioactivity value was <0.001 (data not shown).

To assess the homing of nanoparticles in mice, quantitative PCR specific for the plasmid DNA was done on total DNA extracted from various tissues of C3H mice bearing SCC25 tumors injected systemically with the PPIG3/DNA nanoparticles (250 µg/50 µg, w/w). Result in Fig. 5C shows a low but detectable PCR signal in the stomach (0.0012 ± 0.0002 pg equivalent plasmid), thyroid (0.002 ± 0.0001 pg equivalent plasmid), kidneys (0.0002 ± 0.0001 pg equivalent plasmid), and bone marrow (0.0009 ± 0.0002 pg equivalent plasmid). A higher signal was detected in the liver (0.0091 ± 0.0006 pg equivalent plasmid), spleen (0.0033 ± 0.0004 pg equivalent plasmid), and lungs (0.0042 ± 0.0018 pg equivalent plasmid). In tumors, this signal was increased more than 20-fold (0.192 ± 0.0017 pg equivalent plasmid) compared with the liver. These data show that the plasmid predominantly deposits in the tumors upon injection of the nanoparticles.
Evaluation of acute toxicity in mice and macrophage uptake in vitro. To evaluate acute toxicity, body weight measurement, serum AST and ALT transaminase activity levels, and tissues histology were determined in immunocompetent mice upon systemic injection of the PPIG3/DNA nanoparticles (250 μg/50 μg, w/w). Results in Supplementary Fig. S1B show a mild but significant ($P < 0.01$) increase in serum AST and ALT transaminase activity levels 24 hours after the administration. These values went back to normal level 2 days later. This does not seem to affect the overall health of the animals because no variation in body weight (Supplementary Fig. S1A) and no loss of tissue integrity were detected in animals (Supplementary Fig. S1C).

Finally, to assess a possible capture of the PPIG3/DNA nanoparticles by macrophages of the mononuclear phagocyte system of mice, murine J774 A1 macrophage cell line was cultured for 24 hours in the presence of the PPIG3/DNA nanoparticles prepared by mixing the PPIG3 nanoparticles (250 μg) with an expression plasmid coding for the F-Luciferase gene (50 μg), which was previously labeled with the fluorescent DNA intercalating agent YOYO-1. Data from Supplementary Fig. S2A show that 29.4 ± 9.37% of J774 A1 cells were capable of taking up nanoparticles as shown by the presence of the fluorescent dye within the cytoplasm of these cells (Supplementary Fig. S2A). However, no transgene expression (Supplementary Fig. S2B) or cellular toxicity (Supplementary Fig. S2C) was observed following the uptake of the PPIG3/DNA nanoparticles by the macrophages. These in vitro results tend to suggest that the PPIG3/DNA nanoparticles might be internalized by macrophages in vivo. However, this uptake is low and does not result in transgene expression.

Discussion

In this report, we show for the first time tumor-specific gene transfer on systemic injection of nanoparticles of dendrimers in tumor-bearing animal. Using nuclear whole-body imaging and NIS as reporter gene, we have been able to detect a specific and unique radiotracer uptake in tumors of both immunodeficient and immunocompetent mice, whereas no signal was detected in normal tissues of the animals. Importantly, quantitative RT-PCR on different organs harvested at autopsy of the scanned animals provided the same conclusions. A NIS-specific RT-PCR signal was detected in tumor sites, whereas no signal above the detection threshold was detected in kidney, liver, spleen, heart, and lungs, known to retain nonspecifically synthetic or viral particles (30–32).

Nanoparticle biodistribution was indirectly measured by quantitative PCR on DNA extracted from various tissues 24 hours after the systemic injection. No trace of NIS plasmid DNA was detected in the stomach, thyroid, kidney, and bone marrow, whereas a weak but detectable signal was measured in the liver, spleen, and lungs. In the tumor, this signal reached 20-fold that of the liver, suggesting an accumulation of the nanoparticle in the malignant lesion. This property seems to be unique to the PPIG3/DNA nanoparticles because synthetic vectors are usually accumulated in abundance in the lungs, kidney, and liver 1 hour following administration and can be detectable for up to 3 days (30–32). This extratumoral accumulation can in turn induce severe toxicity, which limits its...
applications in humans. By contrast, no loss of animal body weight or signs of long-lasting or serious toxicity were observed upon systemic injection of the PPIG3/DNA nanoparticles.

A possible explanation for this remarkable selectivity would be the EPR effect. Conceptually, EPR exploits the aberrant biology of solid tumors and, in particular, the architecture of their vasculature, which is irregular in shape, dilated, and contains endothelial cells with unusually large fenestrations (22, 23). These characteristics, combined with a poor lymphatic drainage, result in the fact that macromolecules with high blood circulation time and specific features such as charge and size will be preferentially accumulated in tumors. This accumulation has been shown to be restricted to high molecular weight macromolecules with a slow clearance, and this concept has given rise to at least 11 clinical trails testing the efficacy of polymer-drug conjugates (4, 18–20).

Results from biophysical characterization show that the PPIG3/DNA nanoparticles exhibit a set of physicochemical features that are suitable with a passive diffusion of particles in tumors through the EPR effect. First, the colloidal stability of supramolecular assemblies formed between the dendrimers and plasmid DNA follows the well-known three-stage model of colloidal stability described with other synthetic vectors (3, 4, 26, 27). This colloidal stability and the high positive charge of the particle are classic characteristics, combined with a poorly lymphatic drainage, result in the formation of monodispersed, nonaggregated particles, which could sequentially promote internalization via endocytosis or pynocytosis formation of nanoparticles. Consequently, particles of the same size of 286 ± 41 nm in size with a cutoff value of 50 kDa, to diffuse through the tumor, e.g., tumor necrosis factor (TNF) receptors testing the efficacy of polymer-drug conjugates (4, 18–20).

In conclusion, we provide evidence that polypropylenimine dendrimer PPIG3, when complexed with plasmid DNA, formed colloidal stable self-assembly nanoparticles that possess the biophysical properties compatible with features required for passive accumulation of particles in tumor through the EPR effect. In the context of systemic delivery, molecular imaging provides a whole-body, minimally invasive method of transgene expression. This unique information obtained by in vivo molecular imaging revealed that PPIG3/DNA nanoparticles-mediated gene transfer in tumors is transient and reaches its peak 24 hours after administration. Considering that NIS imaging of transgene expression has been recently validated in humans (17), our data highlight the potential of these nanoparticles as a new formulation for cancer gene therapy. In this context, the utilization of plasmids with a therapeutic expression cassette (a cancer-cytotoxic cytokine that would diffuse through the tumor, e.g., tumor necrosis factor α) combined with an imaging expression cassette (in which NIS expression is driven by a strong, ubiquitous promoter) may provide therapeutic benefits for patients with metastatic tumors.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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References

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