Image-Guided Tumor-Selective Radioiodine Therapy of Liver Cancer After Systemic Nonviral Delivery of the Sodium Iodide Symporter Gene

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Abstract

We reported the induction of tumor-selective iodide uptake and therapeutic efficacy of ^{131}I in a hepatocellular carcinoma (HCC) xenograft mouse model, using novel polyplexes based on linear polyethylenimine (LPEI), shielded by polyethylene glycol (PEG), and coupled with the epidermal growth factor receptor-specific peptide GE11 (LPEI-PEG-GE11). The aim of the current study in the same HCC model was to evaluate the potential of biodegradable nanoparticle vectors based on pseudodendritic oligoamines (G2-HD-OEI) for systemic sodium iodide symporter (NIS) gene delivery and to compare efficiency and tumor specificity with LPEI-PEG-GE11. Transfection of HCC cells with NIS cDNA, using G2-HD-OEI, resulted in a 44-fold increase in iodide uptake *in vitro* as compared with a 22-fold increase using LPEI-PEG-GE11. After intravenous application of G2-HD-OEI/NIS HCC tumors accumulated 6–11% ID/g ^{123}I (percentage of the injected dose per gram tumor tissue) with an effective half-life of 10 hr (tumor-absorbed dose, 281 mGy/MBq) as measured by ^{123}I scintigraphic gamma camera or single-photon emission computed tomography (SPECT CT) imaging, as compared with 6.5–9% ID/g with an effective half-life of only 6 hr (tumor-absorbed dose, 47 mGy/MBq) for LPEI-PEG-GE11. After only two cycles of G2-HD-OEI/NIS/ ^{131}I application, a significant delay in tumor growth was observed with markedly improved survival. A similar degree of therapeutic efficacy had been observed after four cycles of LPEI-PEG-GE11/ ^{131}I . These results clearly demonstrate that biodegradable nanoparticles based on OEI-grafted oligoamines show increased efficiency for systemic NIS gene transfer in an HCC model with similar tumor selectivity as compared with LPEI-PEG-GE11, and therefore represent a promising strategy for NIS-mediated radioiodine therapy of HCC.

Introduction

HEPATOCELLULAR CARCINOMA (HCC) is the fifth most common cancer worldwide and third most common cause of cancer mortality (Shariff *et al.*, 2009). Because of limited response to conventional chemo- or radiotherapy, surgery is currently the only potentially curative therapy available for patients with resectable disease. Despite a variety of alternative therapeutic options, including locally ablative therapies, such as radiofrequency thermal ablation and chemoembolization, the prognosis for advanced HCC has

remained poor. Thus, in addition to novel strategies for early diagnosis, new therapeutic strategies must be explored, such as multikinase inhibitors, immunotherapy, as well as gene therapy (Gerolami *et al.*, 2003).

To investigate an innovative, alternative therapeutic approach, in an earlier study we examined the feasibility of ^{131}I therapy of HCC after stable transfection with the sodium iodide symporter (NIS), using a mouse α -fetoprotein (AFP) promoter construct to target NIS expression to HCC cells (Willhauck *et al.*, 2008b). NIS is a transmembrane glycoprotein that mediates the uptake of iodide into thyroid follicular

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cells (Dai *et al.*, 1996; Smanik *et al.*, 1996). The presence of NIS at the basolateral membrane of thyroid follicular cells has been exploited for many years for diagnostic imaging purposes as well as for ablative therapy of differentiated thyroid cancer using radioactive iodide (^{131}I). This noninvasive therapy has proven to be a safe and effective treatment for thyroid cancer, even in advanced metastatic disease (Van Nostrand and Wartofsky, 2007). To extend the use of NIS-mediated radioiodine therapy to other types of cancer, we have proven the feasibility of extrathyroidal radioiodine therapy after induction of iodide uptake by *ex vivo* NIS transfection or local adenoviral *in vivo* NIS gene transfer using tissue-specific promoters, such as the prostate-specific antigen (PSA) promoter, the carcinoembryonic antigen (CEA) promoter, and the calcitonin promoter, to specifically target NIS expression to prostate, colon, and medullary thyroid cancer, respectively (Spitzweg *et al.*, 1999, 2000, 2001, 2007; Cengic *et al.*, 2005; Scholz *et al.*, 2005; Willhauck *et al.*, 2007, 2008a). In the liver cancer model we applied the AFP promoter for HCC-specific delivery of the NIS gene and demonstrated tumor-specific iodide uptake activity that allowed a therapeutic effect of ^{131}I in stable NIS-expressing HCC xenografts and after local adenoviral NIS gene transfer (Willhauck *et al.*, 2008b; Klutz *et al.*, 2011b).

After proof of principle of the NIS gene therapy concept in liver cancer in our studies outlined above and by other investigators after retroviral or local/regional adenoviral NIS gene delivery (Faivre *et al.*, 2004; Chen *et al.*, 2006; Herve *et al.*, 2008), the next crucial step toward clinical application must be the evaluation of gene delivery vehicles that allow tumor-selective transgene expression in the presence of a sufficiently high transduction efficiency after systemic application to be able to reach disseminated tumor manifestations. In the evaluation of systemic application of gene delivery vectors, the dual function of NIS as therapy and reporter gene provides the advantage of detailed characterization and direct monitoring of *in vivo* vector biodistribution as well as localization, level, and duration of transgene expression, which have been recognized as critical elements in the design of clinical gene therapy trials (Spitzweg and Morris, 2002; Dingli *et al.*, 2003; Baril *et al.*, 2010). Several research groups, including our own, have demonstrated the potential of NIS as reporter gene in various applications, showing that *in vivo* imaging of radioiodine accumulation by ^{123}I or technetium-99m ($^{99\text{m}}\text{Tc}$) scintigraphy as well as ^{123}I single-photon emission computed tomography computed tomography (SPECT CT) fusion or ^{124}I positron emission tomography (PET) imaging correlates well with the results of *ex vivo* gamma counter measurements as well as NIS mRNA and protein analysis (Spitzweg *et al.*, 1999, 2000, 2001, 2007; Dingli *et al.*, 2003; Groot-Wassink *et al.*, 2004; Dwyer *et al.*, 2005; Blechacz *et al.*, 2006; Carlson *et al.*, 2006, 2009; Goel *et al.*, 2007; Merron *et al.*, 2007; Willhauck *et al.*, 2007, 2008a-c; Klutz *et al.*, 2009; Baril *et al.*, 2010; Li *et al.*, 2010; Penheiter *et al.*, 2010; Trujillo *et al.*, 2010; Watanabe *et al.*, 2010).

With the aim of systemic delivery of the NIS gene we have reported a nonviral NIS gene delivery approach in the HCC xenograft model, in which linear polyethylenimine (LPEI) was shielded by attachment of polyethylene glycol (PEG) and coupled with the synthetic epidermal growth factor receptor (EGFR)-specific peptide GE11 for actively targeting the NIS gene to EGFR-overexpressing HCC cells (Klutz *et al.*,

2011a). We have demonstrated high transduction efficiency of LPEI-PEG-GE11/NIS resulting in significant EGFR-specific iodide accumulation *in vitro* and *in vivo*, which was high enough for a significant delay of tumor growth associated with prolonged survival after three or four cycles of polyplex application followed by ^{131}I therapy (Klutz *et al.*, 2011a).

Specifically for systemic delivery of therapeutic genes we have further developed biodegradable synthetic vectors based on low molecular weight polycations cross-linked either via ester or disulfide bonds (Kloeckner *et al.*, 2006; Russ *et al.*, 2008a,b). In a syngeneic neuroblastoma mouse model we have demonstrated the high potential of synthetic biodegradable polymeric vectors based on pseudodendritic oligoethylenimine (OEI)-grafted polypropylenimine dendrimers (G2-HD-OEI) for tumor-specific delivery of the NIS gene. After intravenous application of NIS-conjugated polyplexes NIS-mediated radioiodine accumulation was restricted mainly to the tumor and sufficiently high for a significant delay of tumor growth associated with improved survival (Klutz *et al.*, 2009). The syngeneic Neuro2A mouse model develops exceptionally well-vascularized tumors and is an ideal animal model to demonstrate the effect of passive tumor targeting using macromolecular drug carriers. Because of the imperfect and leaky tumor vasculature combined with inadequate lymphatic drainage, gene carriers can efficiently accumulate in the tumor tissue (Zintchenko *et al.*, 2009). Before pursuing application of G2-HD-OEI in a clinical setting it is therefore essential to confirm their potential for systemic gene delivery in clinically more relevant human xenograft models. Hence, in the current study we have applied the concept of systemic NIS gene delivery with biodegradable OEI-grafted polypropylenimine dendrimers in a human HCC xenograft mouse model and compared transduction efficiency, tumor specificity, and therapeutic efficacy with the results obtained with LPEI-PEG-GE11 (Klutz *et al.*, 2011a). On the basis of its dual function as reporter and therapy gene, NIS was used for noninvasive imaging of vector biodistribution by ^{123}I scintigraphy and ^{123}I SPECT-CT imaging followed by assessment of the therapy response after application of ^{131}I .

Materials and Methods

Cell culture

The human hepatoma cell line (HuH7) (0403; Japanese Collection of Research Bioresources [JCRB] Cell Bank, Tokyo, Japan) was cultured in Dulbecco's modified Eagle's medium (DMEM)-F12 medium (Invitrogen, Karlsruhe, Germany) supplemented with 10% fetal bovine serum (v/v) (PAA, Cölbe, Germany), 5% L-glutamine (Invitrogen), and 1% penicillin-streptomycin. Cells were maintained at 37°C and 5% CO₂ in an incubator with 95% humidity. The cell culture medium was replaced every other day and cells were passaged at 85% confluency.

Plasmids and polymers

The expression vector CMV-NIS-pcDNA3 (pCMV-NIS) carrying the full-length NIS cDNA coupled to the cytomegalovirus (CMV) promoter was kindly provided by S.M. Jhiang (Ohio State University, Columbus, OH). As a control, NIS cDNA was removed with *EcoRI* and religated into the

same expression vector in the antisense direction (pCMV-antisense-NIS).

G2-HD-OEI was synthesized as described previously and used as a 5-mg/ml stock solution (Russ *et al.*, 2008b). LPEI-PEG-GE11 was synthesized as described previously (Klutzn *et al.*, 2011a).

Polyplex formation

Plasmid DNA was condensed with polymers at the indicated conjugate-to-plasmid (c/p) ratios (w/w) in HEPES-buffered glucose (HBG: 20 mM HEPES, 5% glucose [w/v]; pH 7.4) and incubated at room temperature for 20 min prior to use described previously. Final DNA concentrations of polyplexes for *in vitro* studies were 4 or 2 μ g/ml; for *in vivo* studies it was 200 μ g/ml (Russ *et al.*, 2008b; Klutz *et al.*, 2011a).

Transient transfection

For *in vitro* transfection experiments, HuH7 cells were grown to 60–80% confluency. Cells were incubated for 4 hr with polyplexes in the absence of serum and antibiotics followed by incubation with growth medium for 24 hr. Transfection efficiency was evaluated by measurement of iodide uptake activity as described below.

^{125}I uptake assay

After transfections, iodide uptake of HuH7 cells was determined under steady state conditions as described previously (Spitzweg *et al.*, 1999). Results were normalized to cell survival measured by cell viability assay (see below) and expressed as counts per minute (cpm) divided by the absorbance at 490 nm ($A_{490\text{nm}}$).

Cell viability assay

Cell viability was measured with a commercially available MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt] assay (Promega, Mannheim, Germany) according to the manufacturer's recommendations as described previously (Willhauck *et al.*, 2007).

Establishment of HuH7 xenografts

HuH7 xenografts were established in female CD-1 *nu/nu* mice (Charles River, Sulzfeld, Germany) by subcutaneous injection of 5×10^6 HuH7 cells suspended in 100 μ l of phosphate-buffered saline (PBS) into the flank region. Animals were maintained under specific pathogen-free conditions with access to mouse chow and water *ad libitum*. The experimental protocol was approved by the regional governmental commission for animals (Regierung von Oberbayern).

NIS gene transfer and radioiodine biodistribution studies *in vivo*

Experiments started when tumors had reached a tumor size of 8–10 mm and after a 10-day pretreatment with L-T₄ (L-thyroxin Henning; Sanofi-Aventis, Frankfurt, Germany) (5 mg/liter) in their drinking water to maximize radioiodine uptake in the tumor and reduce iodide uptake by the thyroid gland. For systemic *in vivo* gene transfer polyplexes (c/p ratio, 2) were applied via the tail vein at a DNA dose of

2.5 mg/kg (i.e., for a 20-g mouse, 250 μ l of polyplex in HBG buffer at 200 μ g of DNA per milliliter), either NIS-containing polyplexes (G2-HD-OEI/NIS) or polyplexes with the control vector (G2-HD-OEI/antisense-NIS). Two groups of mice were established and treated as follows: (1) intravenous injection of G2-HD-OEI/NIS ($n=24$); and (2) intravenous injection of G2-HD-OEI/antisense-NIS (control vector) ($n=9$). As an additional control, in mice treated with G2-HD-OEI/NIS ($n=9$) the specific NIS-inhibitor sodium perchlorate (NaClO_4 , 2 mg per mouse) was injected intraperitoneally 30 min before ^{123}I administration. Twenty-four hours after polyplex application, mice were injected intraperitoneally with 18.5 MBq (0.5 mCi) of ^{123}I , radioiodine biodistribution was monitored by serial imaging on a gamma camera (e.cam; Siemens, Munich, Germany) equipped with an ultra-extra high-resolution (UXHR) collimator (^{123}I) as described previously (Willhauck *et al.*, 2007). Because intraperitoneal injections are performed quickly without requiring anesthesia and are well tolerated, radioiodine, which is absorbed from the peritoneal cavity within a few minutes, is routinely applied via the peritoneal route by many research groups including our own (Boland *et al.*, 2000; Spitzweg *et al.*, 2000, 2001; Willhauck *et al.*, 2007, 2008b,c; Li *et al.*, 2010; Penheiter *et al.*, 2010). Regions of interest were quantified and expressed as a fraction of the total amount of applied radioiodine per gram of tumor tissue. The retention time within the tumor was determined by serial scanning after radioiodine injection, and dosimetric calculations were performed according to the concept of medical internal radiation dose (MIRD), with the dose factor of the Radiation Dose Assessment Resource (RADAR) group (www.doseinfo-radar.com).

SPECT CT imaging

For SPECT CT imaging the same groups of mice were prepared as outlined above, and 24 hr after polyplex application mice were injected intraperitoneally with 50 MBq (1.35 mCi) of ^{123}I , followed by monitoring of radioiodine biodistribution by serial imaging (1, 3, and 5 hr after ^{123}I application) on a NanoSPECT/CT (Mediso, Budapest, Hungary). CT scans were taken covering the same field of view (FOV) as the SPECT scans. Total scan time was between 30 and 40 min, with 48 projections and a scan time of 20 sec per projection in the case of the SPECT scans.

The SPECT component of the NanoSPECT/CT uses four detector heads, each comprising a thallium-activated sodium iodide [NaI(Tl)] crystal with a size of 262 mm $\times$ 255 mm $\times$ 6.35 mm. It gives an axial field of view of 20 mm and, with the medium-resolution aperture/collimator (nine pinholes per head) that was used in this study, has a spatial resolution of about 1.2 mm. The CT component employs a continuously operating miniature microfocus X-ray tube with a maximal anode current of less than 0.2 mA. The detector has an active area of 98.6 $\times$ 49.2 mm and consists of 1024 $\times$ 2048 pixels. It provides an axial field of view of 45 mm and a maximal spatial resolution of 48 μ m. The color scale was adjusted so that the reference tissue had the same appearance in both images and the uptake in the tumors could be easily compared visually.

Analysis of radioiodine biodistribution *ex vivo*

For *ex vivo* biodistribution studies, mice were injected with G2-HD-OEI/NIS ($n=24$) or G2-HD-OEI/antisense-NIS

($n=9$) as described above followed by intraperitoneal injection of 18.5 MBq of ^{123}I 24 hr later. A subgroup of NIS-transduced mice ($n=9$) was treated with sodium perchlorate before ^{123}I administration as an additional control. Two, 6, and 12 hr after ^{123}I injection, mice were killed and tumors as well as organs of interest were dissected and weighed, and radioiodine uptake was measured with a gamma counter (five NIS-transduced animals per time point [G2-HD-OEI/NIS] and three mice of each control). Results are reported as the percentage of injected dose per organ (% ID/organ).

Analysis of NIS mRNA expression using quantitative real-time PCR

Total RNA was isolated from HuH7 tumors or other tissues, using an RNeasy mini kit (Qiagen, Hilden, Germany) according to the manufacturer's recommendations, and quantitative real-time PCR (qPCR) was performed as described previously (Klutze *et al.*, 2009).

Immunohistochemical analysis of NIS protein expression

Immunohistochemical staining of frozen tissue sections derived from HuH7 tumors after systemic NIS gene delivery was performed with a mouse monoclonal antibody directed against amino acid residues 468–643 of human NIS (kindly provided by J.C. Morris, Mayo Clinic, Rochester, MN) as described previously (Spitzweg *et al.*, 2007).

Radioiodine therapy study in vivo

After a 10-day L-T₄ pretreatment as described above, two groups of mice were established, receiving 55.5 MBq of ^{131}I as a single intraperitoneal injection 24 hr after systemic application of G2-HD-OEI/NIS ($n=6$) or G2-HD-OEI/antisense-NIS ($n=6$), respectively. As control, two further groups of mice were treated with saline instead of ^{131}I after injection of either G2-HD-OEI/NIS ($n=6$) or G2-HD-OEI/antisense-NIS ($n=6$). An additional control group was treated with saline only ($n=6$). The treatment consisting of systemic polyplex application followed by ^{131}I or saline application after 24 hr was repeated once on days 7 and 8. Tumor sizes were measured before treatment and daily thereafter for up to 30 days. Tumor volume was estimated according to the following equation: tumor volume = length $\times$ width $\times$ height $\times$ 0.52.

Indirect immunofluorescence assay

Indirect immunofluorescence staining was performed on frozen tissues with an antibody against human Ki67 (Abcam, Cambridge, UK) and an antibody against mouse CD31 (BD Biosciences, Heidelberg, Germany) as described previously (Willhauck *et al.*, 2007).

Statistical methods

All *in vitro* experiments were carried out in triplicate. Results are represented as mean $\pm$ SD of triplicates. Statistical significance was tested by Student *t* test.

Results

Iodide uptake studies in vitro

Transfection conditions using G2-HD-OEI/NIS were optimized in HuH7 cells by measurement of perchlorate-

sensitive iodide uptake activity 24 hr after polyplex application. We found that the optimal conjugate-to-plasmid (c/p) ratio was 2, which resulted in the highest transfection efficiency at low cytotoxicity (Fig. 1A). This ratio was used in all subsequent experiments. Twenty-four hours after transfection with G2-HD-OEI/NIS, HuH7 cells showed a 44-fold increase in ^{125}I accumulation as compared with cells preincubated with the competitive NIS inhibitor perchlorate (Fig. 1B). Furthermore, no perchlorate-sensitive iodide uptake above background levels was observed in cells transfected with the control vector G2-HD-OEI/antisense-NIS (Fig. 1B). In comparison, the optimal c/p ratio for LPEI-PEG-GE11/NIS was 0.8, which resulted in a 22-fold increase in radioiodide accumulation 24 hr after transfection with LPEI-PEG-GE11/NIS (Fig. 1A) as compared with cells treated with perchlorate. Results of iodide uptake were normalized to cell

FIG. 1. (A) Iodide uptake activity of HuH7 cells after transfection with various conjugate/plasmid (c/p) ratios of G2-HD-OEI/NIS and LPEI-PEG-GE11/NIS. G2-HD-OEI/NIS showed the highest iodide uptake activity at a c/p ratio of 2 and LPEI-PEG-GE11/NIS at a c/p ratio of 0.8, respectively. (B) HuH7 cells transfected with G2-HD-OEI/NIS (c/p ratio, 2) showed a 44-fold increase in perchlorate-sensitive ^{125}I accumulation. In contrast, no perchlorate-sensitive iodide uptake above background level was observed in cells transfected with control vector or without DNA (** $p < 0.001$).

survival measured by MTS assay, which was not altered after polyplex-mediated NIS gene transfer (Fig. 1A and B).

In vivo radioiodine biodistribution studies

To investigate the iodide uptake activity in HuH7 xenografts after systemic *in vivo* NIS gene transfer, ^{123}I distribution was monitored by gamma camera imaging in tumor-bearing mice 24 hr after G2-HD-OEI/DNA administration. Whereas no radioiodine accumulation was detected in tumors after application of G2-HD-OEI/antisense-NIS (Fig. 2C), significant radioiodine uptake was observed in 80% (12 of 15) of HuH7 tumors after systemic injection of G2-HD-OEI/NIS (Fig. 2A), in addition to physiological radioiodine accumulation in thyroid, stomach, and bladder (Fig. 2A and C). As determined by serial scanning, 6–11% ID/g ^{123}I (percentage of the injected dose per gram of tumor tissue) was accumulated in NIS-transduced xenograft tumors with a biological half-life of 11 hr. Considering a tumor mass of 1 g and an effective half-life of 10 hr for ^{131}I , a tumor-absorbed dose of 281 mGy/MBq of ^{131}I was calculated (Fig. 2D). To confirm that tumoral radioiodine uptake was indeed NIS mediated, a subset of G2-HD-OEI/NIS-injected mice ($n=9$) received sodium perchlorate. In all experiments a single injection of sodium perchlorate completely blocked tumoral radioiodine accumulation in addition to abolishing physiological iodide uptake in stomach and thyroid gland (Fig. 2B). Moreover, no significant radioiodine uptake was

observed in nontarget organs, including lungs, liver, kidney, or spleen, which confirms the tumor specificity of nanoparticle-mediated NIS gene delivery. In comparison, after LPEI-PEG-GE11-mediated NIS gene transfer HuH7 xenografts accumulated slightly less radioiodine (6.5–9% ID/g ^{123}I) with a shorter effective half-life of 6 hr, which resulted in a significantly lower tumor-absorbed dose of 47 mGy/MBq of ^{131}I (Klutetz *et al.*, 2011a).

In a subset of mice, radioiodine biodistribution was also monitored by ^{123}I SPECT CT imaging after intraperitoneal injection of 50 MBq of ^{123}I (Fig. 3). Tumor-selective iodide accumulation was confirmed after systemic G2-HD-OEI/NIS application (Fig. 3A) whereas no significant radioiodine accumulation was detected in tumors after application of G2-HD-OEI/antisense-NIS (Fig. 3B). Moreover, SPECT CT imaging allowed a more detailed three-dimensional analysis of tumoral iodide accumulation, revealing inhomogeneous iodide accumulation appearing as clusters of iodide uptake throughout the tumor. A maximal tumoral iodide uptake of approximately 7% ID/g was measured in mice after systemic application of G2-HD-OEI/NIS, as compared with the control groups injected with the control polyplexes G2-HD-OEI/antisense-NIS (2.5% ID/g) or after application of sodium perchlorate (3.2% ID/g) (Fig. 3C).

In addition to tumoral iodide uptake, significant radioiodine accumulation was observed in tissues physiologically expressing NIS, including stomach, bladder, and thyroid.

FIG. 2. ^{123}I scans of nude mice harboring HuH7 tumors 4 hr after intraperitoneal injection of 18.5 MBq of ^{123}I after G2-HD-OEI-mediated NIS gene delivery (A). Whereas mice treated with control vectors (G2-HD-OEI/antisense-NIS) showed no tumoral radioiodine uptake (C), treatment with G2-HD-OEI/NIS induced significant tumor-specific iodide accumulation in HuH7 tumors (A), which was completely abolished on pretreatment with NaClO_4 (B). Radioiodine was also accumulated physiologically in thyroid, stomach, and bladder (A and C). Time course of ^{123}I accumulation in HuH7 tumors after systemic polyplex-mediated NIS gene delivery, using ^{123}I scintigraphy (D). Maximal tumoral radioiodine uptake was 6–11% ID/g for ^{123}I with an average effective $t_{1/2}$ of 10 hr for ^{131}I .

FIG. 3. ^{123}I SPECT CT scan of tumor-bearing nude mice 5 hr after intraperitoneal injection of 50 MBq of ^{123}I after G2-HD-OEI-mediated NIS gene delivery (A) showed a tumor-specific iodide accumulation. In contrast, mice treated with control vectors (G2-HD-OEI/antisense-NIS) showed no significant tumoral radioiodine uptake (B). The color scale was adjusted so that the reference tissue had the same appearance in both images and the uptake in the tumors could be easily compared visually. Time course of tumoral iodide accumulation as determined by SPECT/CT scans (C). A maximal radioiodine uptake of 7% ID/g tumor was measured.

Ex vivo radioiodine biodistribution studies

Ex vivo biodistribution analysis confirmed significant iodide uptake in tumors after systemic NIS gene transfer using G2-HD-OEI/NIS (Fig. 4A–C). While NIS-transduced HuH7 tumors accumulated approximately 3.5% ID/organ ^{123}I even 12 hr after radioiodine injection, mock-transduced tumors showed no significant radioiodine uptake. In comparison, after LPEI-PEG-GE11-mediated NIS gene transfer, 12 hr after radioiodine application only 1.5% ID/organ ^{123}I remained in the tumor (Klutz *et al.*, 2011a). It is noteworthy that the average tumor weight in these experiments was approximately 0.9 g. In both groups, thyroid gland and stomach accumulated approximately 41 and 38% ^{123}I ID/organ, resulting from endogenous expression of NIS in thyroid and stomach. It is important to point out that, because of the exquisite regulation of thyroidal NIS expression by thyroid-stimulating hormone (TSH), ^{123}I accumulation in the thyroid gland can effectively be downregulated by thyroid hormone treatment as shown in humans (Wapnir *et al.*, 2004). In addition, iodide accumulation in the stomach is mostly a result of pooling of gastric juices, which is more prominent in mouse experiments than is usually seen in humans because of anesthesia for a prolonged period during imaging proce-

dures (Spitzweg and Morris, 2002; Klutz *et al.*, 2009). Administration of perchlorate in mice injected with G2-HD-OEI-NIS significantly blocked iodide uptake in tumors and in physiologically NIS-expressing tissues including thyroid gland and stomach (Figs. 2B and 4). In addition, no significant radioiodine uptake above the background level was observed in nontarget organs, including lung, liver, kidney, and spleen, confirming the tumor specificity of G2-HD-OEI (Fig. 4; see also Figs. 2A and 3A).

Analysis of NIS mRNA expression by quantitative real-time PCR analysis

NIS mRNA expression levels were analyzed in various tissues by quantitative real-time PCR (qPCR) 24 hr after NIS gene transfer (Fig. 5A). Only a low background level of NIS mRNA expression was detected in untreated tumors or tumors after application of G2-HD-OEI/antisense-NIS. In contrast, a significant level of NIS gene expression was induced in HuH7 tumors after systemic injection of G2-HD-OEI/NIS (Fig. 5A). As expected, administration of the competitive NIS inhibitor sodium perchlorate had no influence on NIS mRNA expression in NIS-transduced tumors. Furthermore, no significant NIS mRNA expression above background levels was detected in nontarget organs, such as liver and lung, after systemic application of G2-HD-OEI/NIS or G2-HD-OEI/antisense-NIS.

Analysis of NIS protein expression in HuH7 xenografts

Immunohistochemical analysis of HuH7 tumors revealed a heterogeneous staining pattern with clusters of primarily membrane-associated NIS-specific immunoreactivity in tumors after systemic application of G2-HD-OEI/NIS (Fig. 5B, arrows). In contrast, tumors treated with G2-HD-OEI/antisense-NIS (Fig. 5C) or untreated tumors (data not shown) showed no NIS-specific immunoreactivity. Parallel control slides with the primary and secondary antibodies replaced in turn by PBS and isotype-matched nonimmune immunoglobulin were negative (data not shown).

Radioiodine therapy studies after in vivo NIS gene transfer

Twenty-four hours after systemic administration of G2-HD-OEI/NIS or G2-HD-OEI/antisense-NIS polyplexes, a therapeutic dose of 55.5 MBq (1.5 mCi) of ^{131}I was injected intraperitoneally. As control, saline was injected instead of radioiodine. This cycle consisting of systemic NIS gene transfer followed by radioiodine or saline administration was repeated once on days 7 and 8 (Fig. 6A). As an additional control, tumor growth of mice injected only with saline was assessed.

Mice treated with G2-HD-OEI/NIS or G2-HD-OEI/antisense-NIS followed by application of saline and mice treated with G2-HD-OEI/antisense-NIS followed by application of ^{131}I , as well as saline-treated mice, showed exponential tumor growth. In contrast, NIS-transduced (G2-HD-OEI/NIS) and ^{131}I -treated tumors showed a significant delay in tumor growth (Fig. 6A). Whereas all mice in the control groups had to be killed within 2 weeks of the onset of experiments, because of excessive tumor growth, 100% of the mice treated with ^{131}I after injection of G2-HD-OEI/NIS survived

FIG. 4. Evaluation of iodide biodistribution *ex vivo* after injection of 18.5 MBq of ^{123}I . Whereas tumors in NIS-transduced mice showed high perchlorate-sensitive iodide uptake activity (up to $6.6 \pm 1.95\%$ ID/organ), nontarget organs revealed no significant radioiodine accumulation. No radioiodine accumulation was measured after injection of control vectors. Results are reported as the percentage of injected dose per organ $\pm$ SD.

approximately 3 weeks and 50% survived up to 4 weeks (Fig. 6B). Importantly, none of these mice showed major adverse effects of radionuclide or polyplex treatment in terms of lethargy or respiratory failure. However, a minor body weight loss of 3–5% was observed in mice after systemic administration of polyplexes.

In comparison, after LPEI-PEG-GE11-mediated NIS gene delivery, four cycles of LPEI-PEG-GE11/NIS followed by ^{131}I application were needed to achieve a similar degree of tumor growth inhibition as after two cycles of G2-HD-OEI/NIS combined with ^{131}I (Klut *et al.*, 2011a).

Immunofluorescence analysis

Three to 4 weeks after treatment immunofluorescence analysis using a Ki67-specific antibody (green) and an antibody against CD31 (red, labeling blood vessels) showed

significantly lower intratumoral blood vessel density and proliferation index in NIS-transduced tumors (G2-HD-OEI/NIS) (Fig. 7A and C) as compared with mock-transduced (G2-HD-OEI/antisense-NIS) tumors (Fig. 7B and D) after ^{131}I therapy.

Discussion

In the present study we investigated the efficacy of synthetic nanoparticle vectors (G2-HD-OEI) to achieve tumor-selective NIS-mediated radioiodine accumulation in an HCC mouse model. After confirmation of high transduction efficiency *in vitro*, intravenous application of NIS-conjugated G2-HD-OEI in nude mice carrying HCC xenografts was demonstrated to result in tumor-selective radioiodine accumulation, which was high enough for a significant therapeutic effect after application of ^{131}I .

FIG. 5. (A) Analysis of human NIS mRNA expression in HuH7 tumors and nontarget organs by qPCR. A significant level of NIS mRNA expression was induced in HuH7 tumors after systemic NIS gene transfer with or without sodium perchlorate pretreatment. Only a low background level of NIS mRNA expression was detected in untreated tumors, which was set as 1 arbitrary unit. Moreover, no significant NIS expression above the background level was found in tumors after application of G2-HD-OEI/antisense-NIS, or in nontarget organs, such as liver and lung. Results are reported as NIS-to-GAPDH ratios. **(B and C)** Immunohistochemical staining of HuH7 tumors 24 hr after G2-HD-OEI/NIS application showed clusters of primarily membrane-associated NIS-specific immunoreactivity (**B**, arrows). In contrast, HuH7 tumors treated with the control plasmid (G2-HD-OEI/antisense-NIS) did not reveal NIS-specific immunoreactivity (**C**). Original magnification, ×200.

NIS represents one of the oldest and most successful targets for molecular imaging and targeted radionuclide therapy. Cloning and characterization of the NIS gene have therefore allowed the development of the NIS gene therapy concept based on NIS gene transfer into nonthyroidal tumor

cells, followed by diagnostic and therapeutic application of radioiodine (Dai *et al.*, 1996; Smanik *et al.*, 1996; Hingorani *et al.*, 2010).

One of the major challenges on the way to efficient application of the NIS gene therapy concept in the clinical

FIG. 6. Radioiodine treatment of HuH7 tumors after systemic polyplex-mediated NIS gene transfer *in vivo*. Twenty-four hours after intravenous polyplex injection (long arrows), 55.5 MBq of ¹³¹I or saline was injected (short arrows). This treatment cycle was repeated once on days 7 and 8. ¹³¹I therapy after systemic G2-HD-OEI/NIS application resulted in a significant delay in tumor growth (**A**, *p < 0.05), which was associated with markedly improved survival (**B**, Kaplan-Meier plot) as compared with the control groups that were injected with saline only, with G2-HD-OEI/NIS followed by saline application, or with G2-HD-OEI/antisense-NIS followed by saline or ¹³¹I.

FIG. 7. Immunofluorescence analysis using a Ki67-specific antibody (green) and an antibody against CD31 (red, labeling blood vessels) showed significantly decreased proliferation and blood vessel density in NIS-transduced tumors (A and C) after ^{131}I treatment as compared with mock-transduced tumors (B and D). Slides were counterstained with DAPI nuclear stain. Original magnification: (A and B) $\times 100$; (C and D) $\times 200$.

setting of metastatic cancer concerns optimal tumor targeting in the presence of low toxicity and sufficiently high transduction efficiency after systemic administration of gene delivery vectors. Only a limited number of studies have investigated systemic NIS gene delivery approaches with the aim of NIS-targeted radionuclide therapy of metastatic disease (Dingli *et al.*, 2004; Goel *et al.*, 2007; Klutz *et al.*, 2009; Liu *et al.*, 2010).

In the present study we used a promising nonviral gene delivery system for tumor-targeted NIS gene transfer in a human HCC xenograft model. Branched polyocations based on OEI-grafted polypropylenimine dendrimers (G2-HD-OEI) have been characterized as biodegradable synthetic gene delivery vectors with high *in vivo* transduction efficiency and remarkable intrinsic tumor affinity in the presence of low toxicity (Russ *et al.*, 2008b; Klutz *et al.*, 2009).

G2-HD-OEI complexed with the human NIS cDNA under the control of the unspecific CMV promoter revealed high transfection efficiency *in vitro*, resulting in a 44-fold increase in iodide uptake activity in HuH7 cells. After systemic application of NIS-conjugated G2-HD-OEI via the tail vein *in vivo*, 80% of HuH7 tumors showed tumor-specific iodide accumulation as determined by ^{123}I scintigraphic gamma camera imaging, with accumulation of approximately 6–11% ID/g and an effective half-life of 10 hr for ^{131}I . In contrast, mice pretreated with the competitive NIS inhibitor sodium perchlorate or mice injected with control vectors showed no tumoral iodide uptake, confirming that the observed radioiodine accumulation in the tumors was mediated by functional NIS expression. One explanation for the remarkable tumor selectivity of G2-HD-OEI used in this study is the so-called “enhanced permeability and retention” (EPR) effect. Because of large endothelial fenestrations in tumor vasculature combined with poor lymphatic drainage, circulating macromolecules can preferentially accumulate in solid tu-

mors (Matsumura and Maeda, 1986; Iyer *et al.*, 2006). We and others also observed an intrinsic affinity of well-vascularized tumors for polyocations, as their removal from tumor tissue is prevented because of their affinity for tumor cells and tumor matrix (Smrekar *et al.*, 2003; Dufes *et al.*, 2005; Schwerdt *et al.*, 2008). We demonstrated that polyplex organ distribution and transgene expression do not necessarily correlate (Navarro *et al.*, 2010). Although considerable amounts of polyplexes can be entrapped in nontarget organs such as lung and liver, expression is limited to tumor tissue. This can be explained by additional selectivity being achieved by the mitotic activity of tumor cells, which is advantageous for polyplex-mediated transgene expression (Brunner *et al.*, 2000). In a recent study, in the same HCC mouse model we used GE11, an EGFR-specific ligand, for additional active tumor targeting by coupling GE11 to PEG-shielded LPEI (LPEI-PEG-GE11), as the “gold standard” of PEI-based gene carriers (Klutz *et al.*, 2011a). After systemic application of LPEI-PEG-GE11/NIS polyplexes, HuH7 xenografts accumulated slightly lower amounts of iodide (6.5–9% ID/g ^{123}I), but with a lower effective half-life of only 6 hr, which resulted in a smaller tumor-absorbed dose of 47 mGy/MBq of ^{131}I (Klutz *et al.*, 2011a). This difference in tumor-absorbed dose is a result of the lower effective half-life for ^{131}I , and might also be caused by decreased transduction efficiency of LPEI-PEG-GE11. LPEI *per se* shows excellent transduction efficiency due to an intrinsic endosomolytic mechanism, the so-called “proton sponge effect” (Akinc *et al.*, 2005). The polymer protonation results in chloride influx and osmotic swelling of the endosome, followed by membrane destabilization, which in concert is responsible for the high transfection efficiency (Sonawane *et al.*, 2003). Not only LPEI but also oligoethylenimine (OEI)-based polymers are characterized by this intrinsic endosomolytic mechanism (Sonawane *et al.*, 2003). The major drawback of LPEI, however, is its significant toxicity after systemic application,

a result of acute and long-term toxic effects (Chollet *et al.*, 2002) caused at least in part by its high transgene expression in the lungs, due to pronounced aggregation with erythrocytes (Russ *et al.*, 2008a; Klutz *et al.*, 2011a). A technique to reduce the unspecific toxicities and prolonged blood circulation time is shielding of polyplexes by PEGylation. Unfortunately, at the same time as shielding improves the properties of polyplexes for systemic application it appears to reduce its cell transfection activity (Oupicky *et al.*, 2002; Ogris *et al.*, 2003). The reduced cell surface interaction by PEGylation has been overcome by the attachment of targeting ligands. However, the transfection efficiency of the targeted PEG vectors often still does not reach the level achieved with untargeted gene vectors, most probably due to an attenuated endosomolytic effect (Guo *et al.*, 1999; Rudolph *et al.*, 2002), which at least in part explains the lower transduction efficiency of LPEI-PEG-GE11 as compared with G2-HD-OEI, resulting in decreased tumoral iodide accumulation with significantly reduced iodide retention time. On the other hand, LPEI-PEG-GE11 offers the advantage of additional active tumor targeting by coupling the tumor-specific ligand GE11, which could theoretically also be attached to G2-HD-OEI. Taken together, both systems are in principle suitable for targeting the NIS gene to experimental tumors implanted in mice. For the G2-HD-OEI polymer a hypervascularized tumor is a precondition for successful tumor accumulation, whereas the LPEI-PEG-GE11 conjugate is in principle able to promote efficient receptor-mediated uptake into tumor cells by active ligand-mediated tumor targeting, which could be of even greater importance in tumors with a lower level of vascularization.

In addition to ^{123}I gamma camera imaging we have used small-animal whole body SPECT CT imaging in a subset of animals, using ^{123}I as a radiotracer. The cross-sectional fusion imaging techniques such as SPECT CT provide a useful means to improve three-dimensional spatial resolution and separate the overlapping regions of radioiodine uptake *in vivo*, thereby allowing a more robust biodistribution analysis. In our study ^{123}I -SPECT CT imaging allowed a more detailed three-dimensional analysis of NIS-mediated radioiodine accumulation, which appeared inhomogeneous in clusters of iodide uptake throughout the tumor. The examination of all projections of the SPECT CT images failed to detect any other NIS gene transfer-related signals, suggesting that systemic NIS gene transfer using G2-HD-OEI is highly tumor specific. In addition, our data are consistent with several studies demonstrating the sensitivity of micro-SPECT CT for imaging and quantitation of NIS-mediated radionuclide uptake (Marsee *et al.*, 2004; Carlson *et al.*, 2006, 2009; Merron *et al.*, 2007; Chisholm *et al.*, 2009; Peerlinck *et al.*, 2009; Penheiter *et al.*, 2010).

Moreover, ^{123}I scintigraphic and SPECT CT imaging studies were confirmed by *ex vivo* biodistribution experiments revealing significant tumoral radioiodine accumulation, whereas no significant iodide uptake was measured in non-target organs. Tumor-specific NIS expression was further confirmed by real-time qPCR as well as NIS-specific immunoreactivity, which was primarily membrane associated with an inhomogeneous, patchy staining pattern, and therefore nicely correlates with the clusters of iodide accumulation detected by ^{123}I -SPECT CT imaging. Because of the limited polyplex spread in the tumor resulting in the inhomogeneous transgene expression, nonviral gene delivery is ideally com-

bined with therapy genes that are able to provide a bystander effect. The path length of up to 2.4 mm of the β particles emitted by ^{131}I causes a significant crossfire effect after NIS gene transfer, resulting in a bystander effect, which makes NIS an ideal candidate gene for synthetic vector-based systemic cancer gene therapy (Dingli *et al.*, 2003).

Most importantly, systemic polyplex-mediated NIS gene transfer resulted in tumor-specific radioiodine uptake activity in HuH7 tumor-bearing mice which was sufficiently high for a significant therapeutic effect of ^{131}I . After two cycles of systemic polyplex application followed by ^{131}I injection, tumor-bearing mice showed a significant delay of tumor growth associated with markedly prolonged survival. Using LPEI-PEG-GE11 in our former study, four cycles of polyplex/ ^{131}I application were needed to achieve a similar degree of tumor growth inhibition, which is a logical consequence of the significantly lower tumor-absorbed dose that was obtained after systemic application of LPEI-PEG-GE11/NIS as compared with G2-HD-OEI/NIS (Klutz *et al.*, 2011a).

In addition, immunofluorescence analysis showed a significantly reduced proliferation and blood vessel density after systemic polyplex-mediated NIS gene transfer followed by ^{131}I application, suggesting radiation-induced tumor stroma cell damage in addition to tumor cell death.

In conclusion, the data in the current study correlate well with the data acquired in the syngeneic neuroblastoma mouse model (Klutz *et al.*, 2009), demonstrating that the application of these synthetic nanoparticles based on OEI-grafted pseudodendritic oligoamines for systemic NIS gene delivery is not restricted to a specific tumor model, but is suitable for all cancers with hypervascularized tumors. Using NIS as reporter gene, this study allowed detailed characterization of the *in vivo* biodistribution of polyplex-mediated functional NIS expression by ^{123}I scintigraphic gamma camera and ^{123}I -SPECT CT imaging, which is an essential prerequisite for exact and safe planning and monitoring of clinical gene therapy trials with the aim of individualization of the NIS gene therapy concept in the clinical setting. This study therefore opens the exciting prospect of NIS-targeted radioiodine therapy of disseminated HCC, using polyplexes based on biodegradable polymers for systemic NIS gene delivery.

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Author Disclosure Statement

No competing financial interest exists.

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