0021-972X/05/$15.00/0
Printed in U.S.A.

The Journal of Clinical Endocrinology & Metabolism 90(8):4457–4464

doi: 10.1210/jc.2004-2140

A Novel Therapeutic Strategy for Medullary Thyroid Cancer Based on Radioiodine Therapy following Tissue-Specific Sodium Iodide Symporter Gene Expression

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Context: In contrast to papillary and follicular thyroid cancer, medullary thyroid cancer (MTC) remains difficult to treat due to its unresponsiveness to radioiodine therapy and its limited responsiveness to chemo- and radiotherapy.

Objective: To investigate an alternative therapeutic approach, we examined the feasibility of radioiodine therapy of MTC after human sodium iodide symporter (hNIS) gene transfer using the calcitonin promoter to target hNIS gene expression to MTC cells (TT).

Design: TT cells were stably transfected with an expression vector, in which hNIS cDNA was coupled to the calcitonin promoter. Functional hNIS expression was confirmed by iodide accumulation assays, Northern and Western blot analysis, immunostaining, and *in vitro* clonogenic assay.

Results: hNIS-transfected TT cells showed perchlorate-sensitive iodide uptake, accumulating 125-I about 12-fold *in vitro* with organification of 4% of accumulated iodide resulting in a significant decrease in iodide efflux. NIS protein expression was confirmed by Western blot analysis using a monoclonal hNIS-specific antibody, which revealed a major band of a molecular mass of 80–90 kDa. In addition, immunostaining of hNIS-transfected TT cells revealed hNIS-specific immunoreactivity, which was primarily membrane associated. In an *in vitro* clonogenic assay, 84% of NIS-transfected TT cells were killed by exposure to 131-I, whereas only about 0.6% of control cells were killed.

Conclusions: A therapeutic effect of 131-I has been demonstrated in MTC cells after induction of tissue-specific iodide uptake activity by calcitonin promoter-directed hNIS expression. This study demonstrates the potential of NIS as a therapeutic gene, allowing radioiodine therapy of MTC after tissue-specific NIS gene transfer. **(*J Clin Endocrinol Metab* 90: 4457–4464, 2005)**

MEDULLARY THYROID CANCER (MTC) originates from the neuroendocrine-derived calcitonin-secreting parafollicular thyroidal C cells and accounts for approximately 5–10% of all thyroid cancers (1). MTC cells synthesize and secrete high levels of calcitonin and other substances, including calcitonin gene-related peptide, carcinoembryonic antigen, neuron-specific enolase, and chromogranin A, with calcitonin representing the most specific and therefore widely employed diagnostic tumor marker for MTC (2). MTC occurs in sporadic (approximately 75%) and hereditary/familial forms with autosomal dominant trait (approximately 25%). In childhood MTC represents 10% of thyroid malignancy, in which it is almost always hereditary. Prognosis and effectiveness of currently available treatment options mainly depend on tumor stage at the time of diagnosis resulting in a 10-yr overall survival rate of about 75% (2). Primary treatment of MTC is surgical removal of all neoplastic tissue with very limited therapeutic options for residual or recurrent disease due to only minor effectiveness of radio- and chemotherapy (3). Fascinating new perspectives for treatment of MTC have been opened by innovative gene therapeutic approaches, including corrective, immunomodulatory, and cytoreductive suicide gene/prodrug strategies (4).

Cloning and characterization of the sodium iodide symporter (NIS) gene has provided us with a promising novel diagnostic and therapeutic gene (5–7). Successful radioiodine therapy of follicular cell-derived thyroid cancer is based on expression of NIS, a key plasma membrane glycoprotein with 13 putative transmembrane domains mediating active iodide transport in the thyroid gland (8). Delivery of the NIS gene to extrathyroidal or dedifferentiated thyroid tumor cells resulting in functional membrane-associated NIS protein expression therefore offers the possibility of therapeutic application of radioiodine, which has been successfully used for treatment of follicular cell-derived thyroid cancer for more than 60 yr (9).

Using various gene delivery techniques based on viral and nonviral vectors, several investigators induced radioiodine accumulation *in vitro* and *in vivo* in dedifferentiated thyroid carcinoma and other cancer cells after NIS gene expression (10–23).

To enhance tumor selectivity, tissue- or tumor-specific promoters can be used to transcriptionally target therapeutic gene expression to tumor cells, thereby maximizing therapeutic efficacy and minimizing toxic side effects (24). In an

First Published Online June 7, 2005

Abbreviations: Cal-1, TT cells stably transfected with Cal-pEGFP-1; CalN-1, TT cells stably transfected with Cal/NIS-pEGFP-1; HBSS, Hank's balanced salt solution; h, human; MTC, medullary thyroid cancer; NIS, sodium iodide symporter; pEGFP, enhanced green fluorescent protein plasmid; PSA, prostate-specific antigen; SDS, sodium dodecyl sulfate; TT, human MTC cell line.

JCEM is published monthly by The Endocrine Society (http://www.endo-society.org), the foremost professional society serving the endocrine community.

earlier study, we used the prostate-specific antigen (PSA) promoter to target NIS expression to prostate cancer cells. PSA promoter-driven NIS expression resulted in high levels of prostate-specific radioiodine accumulation that allowed a significant therapeutic effect of 131-I *in vitro* and *in vivo* (25–27).

In the current study, we chose MTC as an attractive thyroidal tumor model to investigate the potential of the NIS gene as a novel therapeutic gene using the calcitonin promoter to drive NIS expression, thereby achieving tumor specificity.

Materials and Methods

Cell culture

The human MTC cell line TT (kindly provided by Dr. C. Hoang-Vu, Martin-Luther-University, Halle, Germany) was grown in F-12K medium, the human breast cancer cell line MCF-7 was grown in MEM medium, the human adrenocortical cancer cell line NCI-H295R was grown in RPMI 1640 medium, and the human hepatocellular carcinoma cell line HepG2 was grown in DMEM medium; each medium was supplemented with 10% fetal bovine serum and penicillin G (2 U/ml)/streptomycin sulfate (100 mg/ml) (Life Technologies, Inc., Karlsruhe, Germany). Cells were maintained in a 5% CO_2-95% air atmosphere at 37 C with a change of medium every third day and were passaged every 7 d using 0.5 g trypsin/0.2 g EDTA (Life Technologies).

Plasmid construct

The full-length NIS cDNA was removed from the pcDNA3 expression vector (kindly provided by Dr. S. M. Jhiang, Ohio State University, Columbus, OH) by restriction digestion using *Hin*dIII and *Not*I, agarose gel purified, and ligated into vector phosphorylated enhanced green fluorescent protein plasmid (pEGFP)-1 (Clontech Laboratories, Inc., Heidelberg, Germany). pEGFP-1 was precut with *Hin*dIII and *Not*I restriction enzymes, thereby removing the 800-bp EGFP fragment. After restriction digestion of NIS-pEGFP-1 with *Hin*dIII and dephosphorylation, a 1425-bp calcitonin promoter fragment (kindly provided by Dr. R. F. Gagel, University of Texas, M. D. Anderson Cancer Center, Houston, TX) was ligated into NIS-pEGFP-1. The resulting plasmid construct containing full-length NIS cDNA coupled to the calcitonin promoter (Cal/NIS-pEGFP-1) was agarose gel purified and confirmed by DNA sequencing. A control vector was designed by ligation of the calcitonin promoter fragment into the *Xho*I and *Hin*dIII site of pEGFP-1 (Cal-pEGFP-1).

Transient transfections

The human MTC cell line TT and control cancer cell lines MCF-7, NCI-H295R, and HepG2 were used in transfection experiments. Before transfections, cells were grown to a 50–70% confluency and transfected with Cal/NIS-pEGFP-1 or the control vector Cal-pEGFP-1, using LipofectAMINE Plus reagent (Life Technologies) under serum-free conditions according to the manufacturer's recommendations. After transfections, cells were incubated for 48 h in growth medium. All groups of cells were prepared in triplicate for transfections, which were performed at least three times.

Stable transfection

TT cells were stably transfected with Cal/NIS-pEGFP-1 or the control vector Cal-pEGFP-1 using LipofectAMINE Plus reagent under serum-free conditions as described above. Selection was performed with 0.8 mg/ml geneticin (Life Technologies) in F-12K medium containing 10% fetal bovine serum. Surviving clones were isolated and subjected to screening for iodide uptake activity. Three stably transfected cell lines termed CalN-1, -2, and -3 (Cal/NIS-pEGFP-1) that showed highest levels of iodide accumulation among approximately 20 colonies screened were obtained. In addition, three stably transfected TT cell lines for the control vector Cal-pEGFP-1 were obtained (Cal-1 to -3).

Iodide uptake studies

Uptake of 125-I by transfected TT cells and control cancer cell lines was determined at steady-state conditions as described by Weiss *et al.* (28). In brief, cells were plated on six-well plates (6×10^5cells/well) and after transfections, iodide uptake studies were performed in Hank's balanced salt solution (HBSS) supplemented with 10 μM NaI, 0.1μCi Na 125-I/ml, and 10 mM HEPES (pH 7.3). Then 100 μM $KClO_4$ was added to control wells. After incubation for 45 min, trapped iodide was removed from cells by a 20-min incubation in 1 N NaOH and measured by gamma counting.

Measurement of iodide organification

Cells, grown in 12-well plates, were incubated for 2 h at 37 C with HBSS supplemented with 10 μM NaI, 0.1 μCi Na 125-I/ml, and 10 mM HEPES (pH 7.3). Contents of organified iodide in the cells were determined by trichloroacetic acid precipitation as described by Urabe *et al.* (29).

Iodide efflux studies

Efflux of 125-I was determined as described by Weiss *et al.* (28). In brief, cells were plated on 12-well plates (3×10^5 cells/well) and incubated with HBSS supplemented with 10 μM NaI, 0.1 μCi Na 125-I/ml, and 10 mM HEPES (pH 7.3) at 37 C for 45 min. Medium was then replaced every 4 min with fresh HBSS. The content of 125-I in the collected supernatant was measured by a γ-counter. After the last time point, trapped 125-I was removed from cells by a 20-min incubation in 1 N NaOH and measured by a γ-counter.

Membrane preparation

After transfection with Cal/NIS-pEGFP-1 or the control vector Cal-pEGFP-1, cell membranes were prepared from TT cells by a modification of a previously described procedure (30). In brief, cells plated on 100-mm dishes were washed with PBS, harvested, and resuspended in buffer A [250 mM sucrose; 10 mM HEPES (pH 7.5); 1 mM EDTA; 10 μg/ml leupeptin; 10 μg/ml aprotinin; and 1 mM phenylmethanesulfonyl fluoride]. The homogenate was centrifuged twice at $500 \times g$ for 15 min at 4 C. After centrifugations, 100 μl 1 M Na_2CO_3/ml buffer A was added to the supernatant and incubated at 4 C for 45 min with continuous shaking. Then a further centrifugation at $100{,}000 \times g$ was performed for 15 min, and the pellet was resuspended in an appropriate volume of buffer B [250 mM sucrose; 10 mM HEPES (pH 7.5); and 1 mM $MgCl_2$]. Protein concentrations were determined by a protein assay (Bio-Rad DC protein assay, Bio-Rad Laboratories, Munich, Germany).

Western blot analysis

Aliquots of membranes (20 μg) prepared from transfected TT cells were reduced by incubation with 0.5 M dithiothreitol for 10 min at 70 C and loaded on 4–12% bis-Tris-HCl-buffered polyacrylamide gels. After gel electrophoresis for 1 h, proteins were transferred to nitrocellulose membranes using electroblotting. After blotting, membranes were preincubated for 1 h in 5% low-fat dried milk in 20 mM Tris, 137 mM NaCl, and 0.1% Tween 20 to block nonspecific binding sites. Membranes were then incubated with a mouse monoclonal antibody directed against amino acid residues 468–643 of human (h)NIS (dilution, 1:3000) (31) for 2 h at room temperature. After washing with 20 mM Tris, 137 mM NaCl, and 0.1% Tween 20, horseradish peroxidase-labeled goat-antimouse antibody was applied (dilution, 1:5000) for 1 h at room temperature before incubation with enhanced chemiluminescence Western blotting detection reagents (Amersham, Braunschweig, Germany) for 1 min. Exposures were made at room temperature for approximately 1 min using Kodak BIOMAX MR film (Sigma, Munich, Germany). Prestained protein molecular weight standards (Life Technologies) were run in the same gels for comparison of molecular weight and estimation of transfer efficiency.

Northern blot analysis

Total RNA was isolated from TT cells stably transfected with Cal/NIS-pEGFP-1 (CalN-1) and the control vector Cal-pEGFP-1 (Cal-1) using

the RNeasy mini kit (QIAGEN, Hildesheim, Germany). Ten micrograms of total RNA were electrophoresed on a 1% agarose gel containing 2 M formaldehyde and transferred to a positively charged nylon membrane (PALL, Gelman Laboratory, Dreieich, Germany). The human NIS gene-specific cDNA fragment (nucleotides 1184–1667) was radiolabeled with [α-^{32}P] deoxyadenosine-5′-triphosphate by random priming (Amersham) and used as hybridization probe. Blots were prehybridized for 30 min at 68 C in hybridization solution (Express Hyb solution, Clontech Laboratories), followed by hybridization at 68 C for 1 h. Blots were then rinsed four times in 2× saline sodium citrate/0.05% sodium dodecyl sulfate (SDS) at room temperature for 10 min and twice in 0.1× saline sodium citrate/0.1% SDS at 50 C for 20 min, respectively. Exposures were made at −80 C for 48 h using Kodak X-OMAT AR films (Sigma, Germany). To strip off the NIS cDNA probe, blots were treated in 0.5% SDS at 95 C for 10 min and were reprobed with a human β-actin cDNA probe to monitor RNA integrity and quantity. Computer-assisted densitometric analysis of band intensities was performed, and NIS measurements were normalized for β-actin signal intensity (data not shown).

Immunocytochemical staining

Immunocytochemical staining was performed using the Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA). Stably transfected TT cells (CalN-1 and Cal-1) were plated directly onto one-chamber slides and grown to 60% confluence. Monolayers were washed with PBS and air dried at room temperature overnight followed by incubation at 40 C for 45 min. Air-dried slides were rehydrated in PBS and preincubated for 45 min with blocking serum to inhibit nonspecific binding. Cell monolayers were then incubated with the mouse monoclonal antibody mentioned above at a dilution of 1:3000 for 90 min at room temperature. Cell monolayers were washed and incubated with biotin-conjugated antimouse Ig for 60 min at room temperature, followed by incubation with preformed avidin and biotinylated horseradish peroxidase macromolecular complex. Diaminobenzidine was used as the chromogene and yielded a bluish-black precipitate indicative of hNIS-specific immunoreactivity. Parallel monolayers with the primary and secondary antibodies replaced in turn by PBS, and isotype-matched nonimmune IgGs were examined to assure specificity and exclude cross-reactivities between the antibodies and conjugates used.

In vitro clonogenic assay

TT cells stably transfected with the expression vector (CalN-1) or the control vector (Cal-1) were incubated for 7 h with 0.8 mCi Na131-I in HBSS supplemented with 10 μM NaI and 10 mM HEPES (pH 7.3). After incubation with radioiodine, cells were trypsinized and plated in quadruplicates at cell densities of 3,000, 7,000, 10,000, and 30,000 cells/well in 12-well plates. Four weeks later, after colony development, cells were fixed with methanol and stained with crystal violet, and colonies containing more than 50 cells were counted. Parallel experiments were performed for each cell line using HBSS without 131-I, and all values were adjusted for plating efficiency. The percentage of survival represents the percentage of cell colonies after 131-I treatment, compared with mock treatment with HBSS. Results are expressed as mean ± SEM of quadruplicates. Statistical significance was tested using Student's *t* test.

Statistical methods

All experiments were carried out in triplicates or quadruplicates. Results are presented as means ± SEM. Statistical significance was tested using Student's *t* test. Results shown are representative of three experiments performed under the same conditions.

Results

Iodide accumulation studies after transient and stable transfection with hNIS cDNA

Iodide uptake was measured in stably transfected TT cells (Fig. 1A) as well as transiently transfected TT cells and control cancer cell lines MCF-7, NCI-H295R, and HepG2 (Fig. 1B) after liposome-mediated transfection with Cal/NIS-pEGFP-1 or the control vector Cal-pEGFP-1. The stably transfected TT cell line CalN-1 (Cal/NIS-pEGFP-1) concentrated 125-I about 12-fold (Fig. 1A), whereas no perchlorate-sensitive iodide uptake above background level was observed in cells transfected with the control vector Cal-pEGFP-1 (Cal-1) (Fig. 1A). In CalN-1 cells, iodide accumulation reached half-maximal levels within 5–10 min and became saturated at 30–40 min (Fig. 2). In addition, transiently transfected TT cells concentrated 125-I about 1.7-fold (Fig. 1B), whereas transient transfection of control cancer cells without calcitonin expression (MCF-7, NCI-H295R, HepG2) with Cal/NIS-pEGFP-1 did not result in NIS-specific iodide accumulation (Fig. 1B).

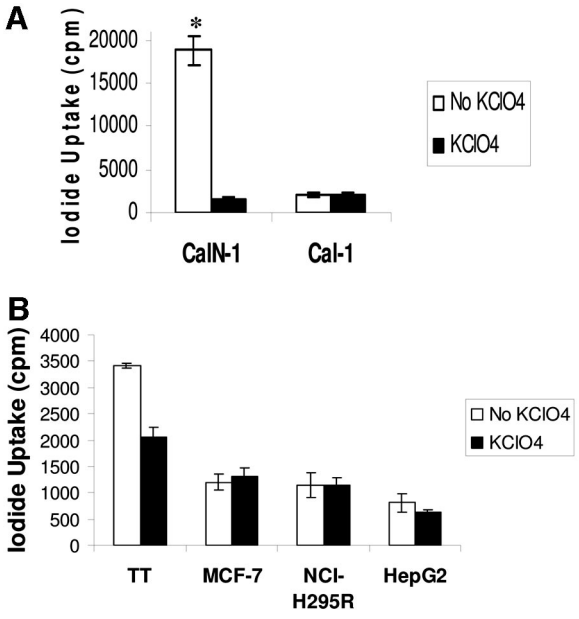

FIG. 1. Iodide accumulation studies. Iodide uptake was measured in TT cells (A and B) after transfection with Cal/NIS-pEGFP-1 (CalN-1) or the control vector Cal-pEGFP-1 (Cal-1) and control cancer cell lines MCF-7, NCI-H295R and HepG2 after transfection with Cal/NIS-pEGFP-1 (B). TT cells stably transfected with Cal/NIS-pEGFP-1 (CalN-1) revealed perchlorate-sensitive iodide accumulation and concentrated 125-I about 12-fold (*, $P < 0.0001$). No perchlorate-sensitive iodide uptake above background level was observed in cells transfected with the control vector Cal-pEGFP-1 (Cal-1) (A). TT cells transiently transfected with Cal/NIS-pEGFP-1 accumulated 125-I about 1.7-fold, whereas transient transfection of control cancer cells with Cal/NIS-pEGFP-1 did not result in NIS-specific iodide accumulation (B). Results represent means ± SEM of triplicate experiments, and are expressed as amount of iodide accumulation in counts per minute. A, lane 1, TT, Cal/NIS-pEGFP-1; lane 2, TT, Cal/NIS-pEGFP-1, with perchlorate; lane 3, TT, Cal-pEGFP-1; lane 4, TT, Cal-pEGFP-1, with perchlorate. B, lane 1, TT, Cal/NIS-pEGFP-1; lane 2, TT, Cal/NIS-pEGFP-1, with perchlorate; lane 3, MCF-7, Cal/NIS-pEGFP-1; lane 4, MCF-7, Cal/NIS-pEGFP-1, with perchlorate; lane 5, NCI-H295R, Cal/NIS-pEGFP-1; lane 6, NCI-H295R, Cal/NIS-pEGFP-1, with perchlorate; lane 7, HepG2, Cal/NIS-pEGFP-1; lane 8, HepG2, Cal/NIS-pEGFP-1, with perchlorate.

Iodide efflux studies and measurement of iodide organification after stable transfection with hNIS cDNA

Iodide efflux was studied *in vitro* in TT cells after stable transfection with hNIS cDNA under the control of the calcitonin promoter (CalN-1 cell line) in the presence or absence of perchlorate, in comparison with LNCaP cells stably ex-

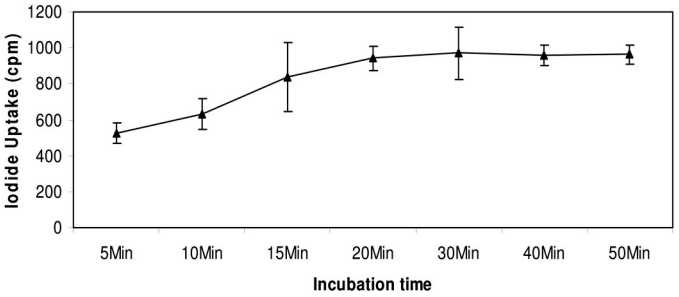

FIG. 2. Time course. Time course of iodide uptake in TT cells stably transfected with Cal/NIS-pEGFP-1. Iodide accumulation reached half-maximal levels within 5–10 min and became saturated at 30–40 min. Results represent means ± SEM of triplicate experiments and are expressed as amount of iodide accumulation in counts per minute.

pressing hNIS under the control of the PSA promoter (NP-1 cell line) (25, 26). In contrast to NP-1 cells, in which approximately 80% of the accumulated 125-I was released into the medium during the initial 10 min, in CalN-1 cells only approximately 40% of the accumulated 125-I was released into the medium during the initial 10 min; 20% of the accumulated radioiodine remained in the cells after 60 min (Fig. 3A).

To investigate whether trapped iodide is organified in CalN-1, iodide organification was measured by trichloroacetic acid precipitation of cell lysate after the incubation of the cells with 0.1 μCi Na 125-I/ml for 2 h. As shown in Fig. 3B, approximately 4% of accumulated 125-I became organified in CalN-1 cells.

Western blot analysis

After stable transfection with Cal/NIS-pEGFP-1 and Cal-pEGFP-1, respectively, NIS protein expression was determined in TT cells by Western blot analysis (Fig. 4). Western blotting of membrane proteins derived from CalN-1 cells using a mouse monoclonal antibody that recognizes the carboxy terminus of hNIS revealed a major band of a molecular mass of approximately 80–90 kDa (lane 2), and two minor

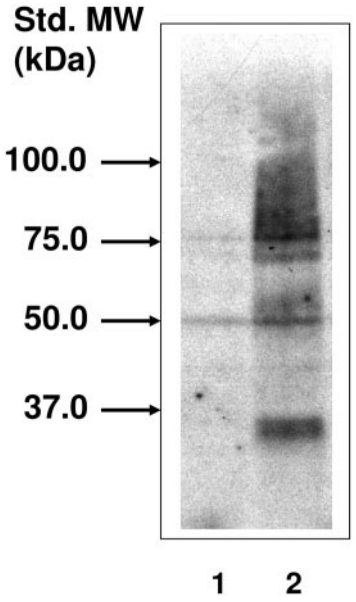

FIG. 4. Western blot analysis. Western blot analysis of membrane proteins derived from TT cells after stable transfection with Cal-pEGFP-1 (Cal-1; lane 1) and Cal/NIS-pEGFP-1 (CalN-1; lane 2), respectively, was performed using a mouse monoclonal NIS-specific antibody. NIS protein was detected as a major band of a molecular mass of approximately 80–90 kDa (lane 2), and two minor bands at approximately 55 kDa [deglycosylated NIS protein (25)] and at approximately 30 kDa [degradation fragments of NIS protein (31)], which were not detected in Cal-1 cells (lane 1). MW, Molecular weight.

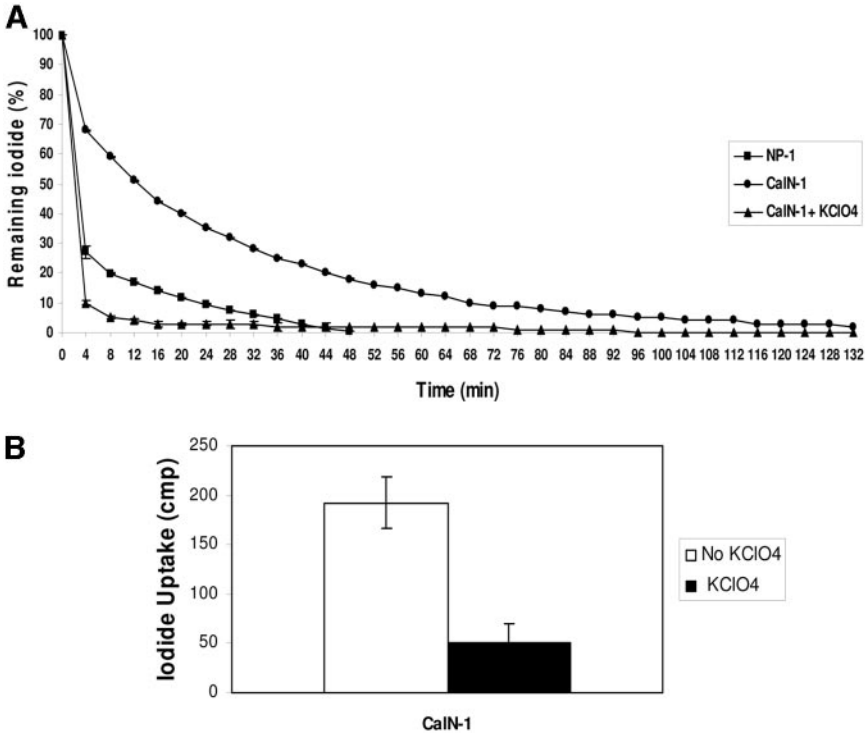

FIG. 3. Iodide efflux and organification. A, Iodide efflux was studied *in vitro* in TT cells after stable transfection with hNIS cDNA under the control of the calcitonin promoter (CalN-1 cell line) in comparison with LNCaP cells stably expressing hNIS under the control of the PSA promoter (NP-1 cell line). In contrast to NP-1 cells, in which approximately 80% of the accumulated 125-I was released into the medium during the initial 10 min, in CalN-1 cells only approximately 40% of the accumulated 125-I was released into the medium during the initial 10 min; 20% of the accumulated radioiodine remained in the cells after 60 min. Results represent means ± SEM of triplicate experiments. B, Iodide organification in TT cells after calcitonin promoter-directed NIS gene transfer (CalN-1) was measured by trichloroacetic acid precipitation. Approximately 4% of accumulated 125-I became organified in CalN-1 cells. Nonspecific binding of 125-I was determined in the presence of $KClO_4$. Results are expressed as means ± SEM of triplicate experiments.

bands at approximately 55 kDa [deglycosylated NIS protein (25)] and approximately 30 kDa [degradation fragments of NIS protein (31)], which were not detected in Cal-1 cells (lane 1).

Northern blot analysis

Human NIS mRNA expression was examined by high-stringency Northern blot analysis using a 32-P-labeled hNIS-specific cDNA probe (Fig. 5). NIS mRNA was detected as a single species of approximately 4 kb in total RNA derived from CalN-1 cells (lane 1), which was not detected in Cal-1 cells (lane 2).

Immunocytochemical staining

Using a highly sensitive immunostaining technique and a mouse monoclonal hNIS-specific antibody, distinct, primarily membrane-associated (*arrows*) NIS-specific immunoreactivity was detected in CalN-1 cells (Fig. 6, A and B). In contrast, Cal-1 cells (Fig. 6C) did not show NIS-specific immunoreactivity. In addition, parallel control slides with the primary and secondary antibodies replaced in turn by PBS and isotype-matched nonimmune Ig were negative (Fig. 6D).

In vitro clonogenic assay

A clonogenic assay was performed to determine whether NIS-transfected TT cells could be selectively killed by 131-I treatment. CalN-1 and Cal-1 cells were incubated in HBSS containing 0.8 mCi (29.6 MBq) 131-I for 7 h. Control cells were treated in parallel with HBSS without 131-I. Whereas only about 0.6% of NIS-negative Cal-1 cells were killed by exposure to 131-I, approximately 84% of CalN-1 cells were killed (Fig. 7). These data indicate that a sufficiently high dose of radiation was achieved in NIS-transfected TT cells to result in cell killing at a dose that spared control TT cells that are not able to trap iodine.

Discussion

Iodide-trapping activity in the thyroid gland due to expression of the NIS plays a key role in diagnosis and therapy

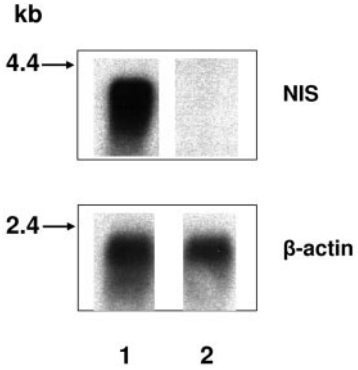

FIG. 5. Northern blot analysis. Northern blot analysis of mRNA derived from TT cells after stable transfection with Cal/NIS-pEGFP-1 (CalN-1; lane 1) and Cal-pEGFP-1 (Cal-1; lane 2), respectively, using a 32-P-labeled NIS-specific cDNA probe. NIS mRNA was detected as a single species of approximately 4 kb in total RNA derived from CalN-1 cells (*upper panel*, lane 1), which was not detected in Cal-1 cells (*upper panel*, lane 2). Hybridization with β-actin probe served as a control (*lower panel*).

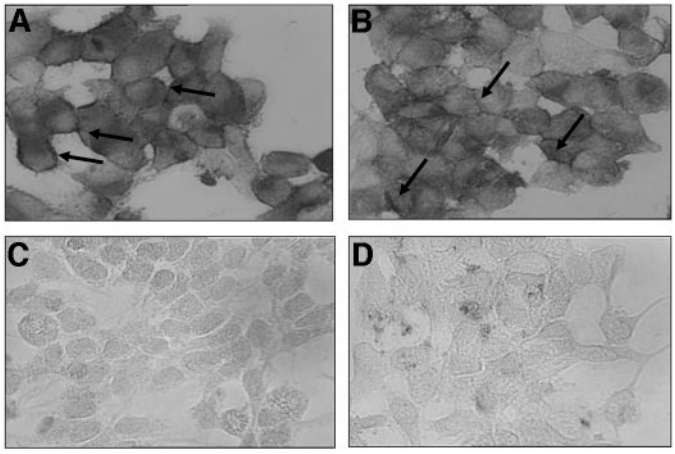

FIG. 6. Immunocytochemistry. Immunocytochemical staining of stably transfected TT cells using a mouse monoclonal NIS-specific antibody. Distinct, primarily membrane-associated (*arrows*) NIS-specific immunoreactivity was detected in TT cells stably transfected with Cal/NIS-pEGFP-1 (A and B). In contrast, TT cells stably transfected with the control vector Cal-pEGFP-1 (C) did not show NIS-specific immunoreactivity. In addition, parallel control slides with the primary and secondary antibodies replaced in turn by PBS and isotype-matched nonimmune Ig were negative (D).

of follicular cell-derived (papillary and follicular) thyroid carcinomas and their metastases (32). Functional expression of NIS in papillary and follicular thyroid carcinomas offers the possibility of effective imaging as well as therapeutic destruction of tumors by 131-I application resulting in an excellent prognosis in patients with thyroid cancer, with 10-yr survival rates of approximately 90–95% (9). Follicular-cell derived thyroid carcinomas are usually treated by total or near-total thyroidectomy, followed by 131-I ablation of the thyroid remnant and occult microscopic carcinomas. Subsequent postablative 131-I total body scanning can diagnose local and metastatic residual and recurrent disease, which can then be treated by further 131-I ablation. Recurrence rates are significantly higher in patients treated with surgery and TSH suppression by thyroxine alone, compared with those who also receive radioiodine treatment (9). In contrast, MTC remains difficult to treat due to lacking iodide accumulation

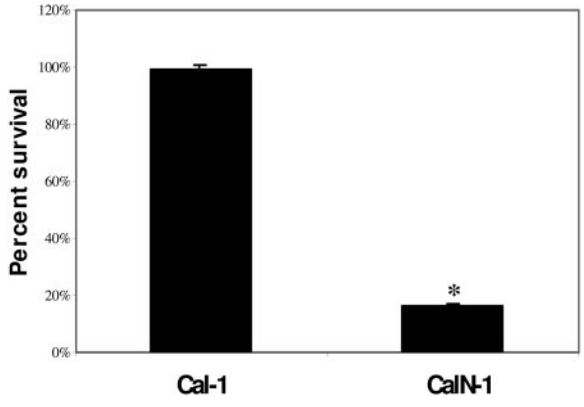

FIG. 7. Clonogenic assay. In an *in vitro* clonogenic assay, CalN-1 and Cal-1 cells were exposed to 0.8 mCi 131-I for 7 h. Although only about 0.6% of Cal-1 cells were killed by exposure to 131-I, approximately 84% of CalN-1 cells were killed. *, $P < 0.0001$.

and therefore unresponsiveness to radioiodine therapy, and, similar to other forms of follicular cell-derived thyroid cancer, limited efficacy of chemotherapy and external beam radiation. The mainstay of therapy is surgical resection, including total thyroidectomy associated with lymph node dissection, which is often unsatisfactory due to advanced, recurrent, or metastatic disease. Therefore, additional therapeutic options, in particular systemic approaches, are urgently needed for improved management of metastatic MTC, including gene therapeutic strategies.

Gene therapy is particularly attractive for the treatment of MTC because of the potential to restrict expression of therapeutic genes to the target cell population by application of tissue-specific promoters, such as the calcitonin promoter, thereby maximizing tissue-specific cytotoxicity and reducing extratumoral side effects. In addition, with the possibility of complete thyroid hormone replacement therapy, the thyroid gland represents a dispensable organ, which allows the clinician to pursue therapeutic strategies, including gene therapy, that might ablate normal as well as malignant thyroid cells.

A variety of gene therapy approaches has been examined in MTC, such as corrective gene therapy aiming at inhibition of oncogenic RET signaling by expression of a dominant-negative RET mutant (33, 34), cytoreductive gene therapy using the Herpes simplex virus thymidine kinase/ganciclovir system (35–37) or the purine nucleoside phosphorylase/fludarabine system (38), and immunomodulatory gene therapy including IL-2 and IL-12 gene transfer alone or in combination with suicide gene therapy (39–43).

Based on the highly effective application of radioiodine that has been used for more than 60 yr in the management of follicular cell-derived thyroid cancer due to endogenous NIS expression, cloning, and characterization of the NIS gene has led to the development of a novel radioisotope concentrator gene therapy strategy aiming at targeted NIS expression in cancer cells, making them susceptible to therapeutic destruction by the β-energy emitted by 131-I (7). In contrast to other gene therapeutic approaches, the NIS gene as novel therapeutic gene offers the possibility to extend an already established and highly effective anticancer strategy, 131-I therapy of follicular cell-derived thyroid cancer, to the treatment of other cancer types, including MTC. A further characteristic of NIS gene therapy, enhancing therapeutic efficacy, is its association with a significant bystander effect based on the cross-fire effect of the β-emitter 131-I with a path length of 0.2–2.4 mm (mean 0.4 mm), resulting in a cytotoxic effect in not only NIS-transduced but also surrounding nontransduced cancer cells, as shown by Dingli *et al.* in multiple myeloma cells after lentivirus-mediated NIS expression (17, 44). To achieve maximal tumor-specific cytotoxicity with minimal toxicity in healthy organs, in addition to a systemic therapeutic effect with the ultimate goal of treatment of metastatic disease, tumor-specific targeting of therapeutic genes is desirable. Besides the possibility of tumor-selective targeting of the delivery vector to surface receptors on tumor cells by vector targeting technology, transcriptional targeting by application of tumor-specific promoters represents an important and well-established component of systemic gene therapeutic approaches (24).

In an earlier study, we explored the efficacy of NIS gene transfer to induce accumulation as well as a therapeutic effect of 131-I in prostate cancer (LNCaP) cells and used the PSA promoter to target NIS gene expression to prostate cells (24, 45–47). Prostate cell-specific iodide uptake activity was induced resulting in a dramatic therapeutic effect of 131-I in NIS-transfected LNCaP cells *in vitro* as well as *in vivo* in LNCaP cell xenografts (25–27). These studies clearly showed for the first time that tissue-specific NIS gene expression into nonthyroidal tumors is capable of inducing the accumulation of therapeutically effective radioiodine doses *in vitro* and *in vivo* and might therefore represent an effective and potentially curative therapy for tumors without endogenous iodide accumulation.

In the current study, we chose MTC as a thyroidal tumor model unresponsive to 131-I therapy due to lacking iodide accumulation to investigate the potential of the NIS gene as a therapeutic gene. To target NIS gene expression to MTC cells, we applied a tumor-specific promoter, the calcitonin promoter. Expression of calcitonin is thought to be largely restricted by promoter and splicing specificity to C cells and MTC, which secrete high levels of the polypeptide hormone calcitonin, a highly sensitive and specific serum marker of persistent or recurrent MTC (36). Because the calcitonin gene is regulated in a tissue-specific manner, the calcitonin promoter represents an ideal means for C cell-specific gene expression and has therefore been used in a variety of studies to target therapeutic genes to MTC (36–38, 43, 48).

To drive hNIS expression, we used a 1425-bp calcitonin promoter fragment, including a distal *cis*-acting tissue-specific enhancer element (nucleotides -1060 to -905 upstream from the transcription start site), which has been demonstrated to be responsible for cell-specific expression of the calcitonin gene. We thereby induced tumor-specific radioiodine accumulation in human MTC cells that concentrated 125-I approximately 12-fold, which is about half of the iodide uptake activity induced in LNCaP cells stably transfected with NIS under the control of the PSA promoter in our recent study (25). MTC cell specificity of NIS expression was evidenced by lack of iodide uptake activity in control cancer cells (breast, adrenal, and liver cancer cells) after calcitonin promoter-mediated NIS gene expression. Interestingly, approximately 4% of accumulated radioiodine was organified in TT cells, resulting in a significant decrease in iodide efflux, compared with LNCaP cells stably expressing NIS under the control of the PSA promoter, which did not organify iodide and showed rapid iodide efflux *in vitro* (25, 26). Finally, the amount of trapped and retained 131-I was shown to be sufficiently high to elicit a significant therapeutic effect in an *in vitro* clonogenic assay with selective killing of approximately 84% of NIS-expressing TT cells, compared with only 0.6% of control cells. Despite a lower level of radioiodine concentrating activity in TT cells, compared with LNCaP cells after tissue-specific NIS gene expression, the therapeutic effect of 131-I in TT cells was similar to the therapeutic effect in LNCaP cells, in which approximately 75% of cells were killed by 131-I in an *in vitro* clonogenic assay (26). This might be due to decreased iodide efflux of TT cells resulting, at least in part, from organification of a part of the accumulated iodide.

In the thyroid gland, NIS-mediated iodide uptake at the

basolateral membrane is followed by apical iodide efflux into the colloid mediated by pendrin, a chloride/iodide transporter, thyroid peroxidase-catalyzed oxidation and incorporation into tyrosyl residues along the thyroglobulin backbone, a complex reaction at the cell/colloid interface that is termed iodide organification. The term organification refers to the incorporation of iodide into organic molecules, which results in an increased retention time of trapped iodide in the thyroid gland. Interestingly, several studies suggest that organification of iodide does not occur exclusively in thyroid tissue (49, 50). In addition to thyroid peroxidase in the thyroid gland, peroxidases in extrathyroidal tissues such as lacto-, myelo-, and eosinophil peroxidase have been demonstrated to organify iodide (51). In the mammary gland, iodide is bound to tyrosyl residues of caseins and other milk proteins, and this organification process has been shown to correlate with peroxidase activity (52–54). These data support the idea that iodide organification is not restricted to thyroid tissue. The exact role of iodide trapping and organification in extrathyroidal tissues is not known but may include antioxidative and antiproliferative effects, as demonstrated for certain iodolipids in the thyroid gland (55). Our study is the first report of organification of iodide in MTC cells after NIS-mediated iodide trapping. The functional role and underlying mechanisms are currently unknown and have to be addressed in future studies. However, after NIS gene expression, iodide organification in MTC cells represents a significant advantage for therapeutic application of 131-I because it is capable of increasing the achieved radiation dose due to enhanced retention time and biological half-life of 131-I in the target tissue.

In conclusion, a therapeutic effect of 131-I has been demonstrated in MTC cells after induction of tumor-specific iodide uptake and organification by calcitonin promoter-directed NIS expression. Provided that further studies will confirm radioiodine accumulation and therapeutic efficacy of 131-I in the *in vivo* situation, these data demonstrate the potential of NIS as a therapeutic gene, allowing radioiodine therapy of MTC after tumor-specific NIS gene expression. Because the ultimate goal of this project is to establish a novel therapeutic approach for metastatic MTC, we have begun to develop adenoviral vectors carrying the NIS gene linked to the calcitonin promoter allowing tumor-specific *in vivo* NIS gene expression as a next crucial step toward therapeutic application of NIS gene transfer followed by radioiodine therapy of MTC in a clinical setting.

Acknowledgments

The authors are grateful to Dr. S. M. Jhiang (Ohio State University, Columbus, OH) for supplying the full-length hNIS cDNA and Dr. R. F. Gagel (University of Texas, M. D. Anderson Cancer Center, Houston, TX) for supplying the calcitonin promoter.

Received October 29, 2004. Accepted May 26, 2005.

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This work was supported by Grants Sp 581/3-1, Sp 581/3-2, Sp581/4-1, and Sp 581/4-2 (to C.S.) from the German Research Council (Deutsche Forschungsgemeinschaft, Bonn, Germany) and Mayo Foundation Prostate Cancer SPORE Grant CA91956 (to J.C.M.).

This work represents a portion of M. Schütz's medical thesis at Munich Medical School, Ludwig-Maximilians-University, Munich, Germany.

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