Radioisotope Concentrator Gene Therapy Using the Sodium/Iodide Symporter Gene

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ABSTRACT

We demonstrate a novel method of concentrating radiation for tumor imaging or killing. The rat sodium/iodide symporter gene (rNIS) was cloned into a retroviral vector for transfer into cancer cells to mimic the iodide uptake of thyroid follicular cells. In vitro iodide transport shows that the symporter functions similarly in rNIS-transduced tumor cells and rat thyroid follicular cells. rNIS-transduced and control nontransduced (NV) human A375 melanoma xenografts established in vivo in athymic nude mice were imaged using a gamma camera after i.p. injections of 123I. The rNIS-transduced human A375 melanoma tumors are visually distinguishable from and accumulate significantly more radioiodines than NV tumors. In vitro clonogenic assays confirm efficacy and clearly show that rNIS-transduced A375 human melanoma, BNL.1 ME murine transformed liver, CT26 murine colon carcinoma, and IGROV human ovarian carcinoma can be selectively killed by the induced accumulation of 131I. Thus, NIS-based gene therapy may have both diagnostic and therapeutic applications for cancer.

INTRODUCTION

New anticancer approaches with less systemic toxicity need to be developed. Cancer that recurs after radiation therapy or chemotherapy represents a difficult clinical challenge. Patients often cannot tolerate further external beam radiotherapy without being subjected to prohibitive toxicity. In this setting, an ideal therapy would allow more local and effective treatment to better palliate these significant lesions without life-threatening side effects. Toward this end, investigators are exploring molecular approaches that are based on selective transfer of therapeutic genes to tumor cells (1, 2) or promoter specificity to localize transgene expression to tumor cells (3, 4). Such experiments are in the early stages of development but show some promise. We took a unique approach to tumor control by retroviral transfer of the rNIS gene, expressed in the thyroid gland and other tissues including the salivary glands, mammary glands, and gastric mucosa (5). This symporter facilitates intracellular iodide accumulation. Expression of the gene in tumor cells, followed by administration of 123I or technetium pertechnetate (99Tc), might provide a novel means of diagnostic imaging of tumors in vivo. Additionally, if NIS expression in cancer cells can approach or exceed that of typical thyroid tumors, then accumulation of 131I can result in up to 50,000 cGy dose of ionizing radiation, which far exceeds that achievable by external beam radiotherapy (6). Thus, radiotherapy with 131I in a manner analogous to thyroid treatment protocols presently used in humans should kill NIS-expressing cells.

The NIS facilitates the accumulation of iodide by thyroid follicular cells to concentrations 20–40-fold over the plasma levels. This is essential for the production of the thyroid hormones thyroxine (T4) and tri-iodothyronine (T3), which are important for growth, development, and metabolism of most tissues (7). Iodide uptake is competitively inhibited in vitro and in vivo by the anions thiocyanate and perchlorate (7, 8). Both the rat and human NIS genes were cloned recently and have been shown to be functional in vitro when expressed in cells from different species (9–11).

The cDNA nucleotide sequence of the rat gene (rNIS) contains an open reading frame of 1854 nucleotides that codes for a protein of 618 amino acids with a predicted molecular weight of Mr 65,000 (9). Secondary structure and hydrophobic profile predict an intrinsic membrane protein with 12 membrane-spanning domains (9). Sequence comparisons to other sodium-dependent cotransporters indicate that the rNIS is most homologous (25% amino acid identity) to the human Na+/glucose cotransporter (9). Indirect immuno-fluorescence using a polyclonal anti-COOH terminus antibody in permeabilized FRTL-5 cells confirmed the cytosolic location of the COOH terminus of the rNIS (12). The cDNA nucleotide sequence of the human gene (hNIS) contains an open reading frame of 1928 nucleotides that codes for a protein of 643 amino acids with an expected molecular weight of Mr 69,000 (11). The predicted hNIS amino acid sequence has 84% identity and 92% similarity with the rNIS (11). The hNIS is also predicted to have 12 transmembrane-spanning domains, where the sequences are most highly conserved (11). Additionally, three charged residues thought to be important for iodide uptake in the rNIS (Asp16, Glu79, and Arg208) are also conserved (9, 11).

The iodide-concentrating ability of thyroid cells allows for diagnostic radionuclide thyroid imaging with 123I and 99Tc. Intense radiotherapy of the thyroid using 131I can provide local radiation with minimal (if any) systemic side effects, except the need for thyroid supplements (13). We demonstrate that retroviral transfer of the rNIS gene into human and murine tumor cells results in highly significant iodide uptake. rNIS-mediated accumulation of 123I or 99Tc by tumors provides a means for diagnostic imaging of solid tumors. Additionally, rNIS-mediated accumulation of 131I is an innovative approach for concentrating a lethal dose of radiation in tumor cells and a new category of gene therapy for cancer.

MATERIALS AND METHODS

Cloning the rNIS Gene into a Retroviral Vector. RNA was extracted (RNasey kit; Qiagen) from the rat FRTL-5 (American Type Culture Collection CRL-8305) thyroid cell line (14). cDNA was prepared from mRNA by the reverse transcription-PCR method and used as a template to PCR amplify the rNIS gene using the manufacturer’s method (Clontech, Palo Alto, CA) with two oligonucleotide primers: (a) a 5' 29-mer ggc ggg tac cga gtc acc tgt ctc cat g that anneals at position 90 of the reported sequence (9) and encodes both the ATG initiation codon and a 5' KpnI restriction endonuclease site, and (b) a 3' 31-mer tgg aga cca acc tac ctg ggc ggg gtc caa g that anneals at position 1950 and encodes the stop codon and a 3' XhoI restriction endonuclease site. A 1.8-kb DNA fragment was obtained and cloned into the KpnI/XhoI sites of the episomal expression vector pREP7 (Invitrogen). The rNIS gene was then cloned into the LXS/N retroviral backbone that contains a multicloning site and the neo* gene under SV40 promoter control (15). This vector is referred to as LNISN. Supernatants from the previously described LTKOSN vector producer cell line carrying the herpes thymidine kinase gene were used to transduce tumor cells as negative controls (16).

Cell Culture and Cell Lines. Cells were maintained in RPMI 1640 (Life Technologies, Inc.) with 10% fetal bovine serum and 1% penicillin/strepto-
mycinc-glutamine (Life Technologies, Inc.) at 37°C, 5% CO2. The PA317 packaging cell line was transfected with LNISN and selected in G418 (1 mg/ml) for 14 days (15). Supernatants from these cells containing LNISN retroviral particles were used to transduce four cell lines: A375 human melanoma (17), BNL.1 ME mouse transformed liver (18), CT26 mouse colon carcinoma (19), and IGROV human ovarian adenocarcinoma, kindly provided by J. Bernard (20). Transduced cells were selected for G418 resistance as described previously (16).

Iodide Uptake Experiments. Both time course and kinetic experiments were performed using the methods of Weiss et al. (21). Cells (5 x 10^6) were seeded into 24-well dishes in 0.5 ml of RPMI 1640. After an 18–24-h incubation period at 37°C with 5.0% CO2, the medium was aspirated, and cells were washed with B-HBSS (Life Technologies, Inc.; Ref. 21). Iodide uptake was initiated by adding 0.5 ml of B-HBSS containing 0.057–0.345 mM NaI (146–860 nCi/ml). Incubations (37°C, 5% CO2) were performed using the methods of Weiss et al. (21) (21). Cells (5 x 10^6)

Iodide Efflux Determinations in FRTL-5 and Tumor Cells. Cells were plated as described for uptake experiments. Cells were incubated as described above in B-HBSS with 30–100 μM NaI and 0.0575–0.345 mM Na125I (146–860 nCi/ml) for 60 min. The radioactive medium was removed, cells were washed twice with B-HBSS, and 0.5 ml fresh (iodide-free) B-HBSS was applied and incubated as described above. At varying time points, the B-HBSS was removed, and the cells were solubilized in Triton X-100 and analyzed by a gamma counter.

In Vivo Imaging and Quantitation of Accumulated Radionuclides in Explanted rNIS-transduced A375 Human Melanoma Tumors. Tumors were established in athymic nude mice (Harlan Sprague Dawley, Indianapolis, IN) by intradermal injection of 5 x 10^6 rNIS-transduced A375 cells on the left ventral hind limb and a similar number of NV tumor cells on the right ventral hind limb. By 30 days after injection, tumors had reached ~10 mm in diameter. Mice were injected i.p. with 0.2 ml of a saline solution containing 8 μCi of 123I (Synco, Des Moines, IA), which releases 159 KeV gamma energy. Sixty-two min after injection, a 5-min planar image was obtained using a Park Isocam II camera (Park Medical Systems, Montreal, Canada) with a low energy, high resolution collimator. Immediately afterward, the mice were euthanized, and the tumors were carefully dissected and weighed. The amount of 123I accumulated by the tumors was quantitated using a Ludlam model 2200 Scaler Ratemeter and single channel well analyzer (Ludlam Measurements, Inc., Sweetwater, TX).

In Vitro Clonogenic Assays to Demonstrate 131I-mediated Killing of rNIS-transduced Murine and Human Tumors. Cells were grown to ~50% confluence in T80 flasks (Nunc) with RPMI 1640 medium supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin/c-glutamine. The cells were then washed with HBSS and allowed to incubate...
7 h (5% CO₂ at 37°C) after the addition of 10 ml of 100 μCi/ml Na¹³¹I (DuPont NEN, Boston, MA) in 30 μM NaI in B-HBSS (21). Then each cell line was washed twice with B-HBSS, trypsinized, counted, and plated at densities of 125, 250, and 1000 cells/well in six-well plates in quadruplicate. The cells were placed in 5% CO₂ at 37°C for 5–8 days. Then each plate was fixed with 3:1 methanol:acetic acid and stained with crystal violet, the colonies were counted, and the mean and SD were calculated (22). Each cell line, NIS-transduced or NV, had cells exposed to ¹³¹I (experimental) and cells exposed to only B-HBSS (control). All values were adjusted for plating efficiency before comparisons were made. The percentage of survival represents the percentage of cell colonies after ¹³¹I treatment compared with mock treatment with B-HBSS.

RESULTS

Transduction with the rNIS Gene Is Sufficient to Confer Perchlorate-sensitive Iodide Uptake by Tumor Cells. We cloned the rNIS gene from the FRTL-5 cell line into pREP7 and confirmed a gene sequence identical to that reported (9). The rNIS gene was cloned into the LXSN retroviral backbone (15). This vector is referred to as LNISN. Four cell lines (A375 human melanoma, IGROV human ovarian adenocarcinoma, BNL.1 ME mouse transformed liver, and CT26 mouse colon carcinoma) were transduced with LNISN retroviral vector and selected for G418 resistance. To mimic conditions that might result from in vivo gene transfer into tumors, we used mixed populations of transduced cells and did not study individual tumor cell clones as reported previously (10). Therefore, iodide uptake measurements represent the cell population average. Uptake was measured by incubating the cells with 30 μM NaI and 0.057 nM Na¹²⁵I (146 nCi/ml) in B-HBSS for 1 h (Fig. 1). Uptake in rNIS-transduced cells is between 8- and 21-fold that observed in nontransduced lines (NV) and 9–35-fold that of lines transduced with the LTKOSN retroviral vector containing the herpes simplex virus thymidine kinase gene (TKO). Additionally, uptake is inhibited 8–11-fold by 30 μM perchlorate, a competitive inhibitor of iodide uptake (7, 10, 11, 21, 23). These results clearly demonstrate rNIS-dependent iodide uptake by the tumor cells. They are consistent with our results obtained with FRTL-5 cells (Fig. 2A) and data reported previously (9–11, 14, 21).

Iodide Uptake Into rNIS-expressing Cells Is Rapid. The time course of iodide uptake was measured by incubating the tumor cell lines for 5–120 min with 30 μM NaI and 0.115 nM Na¹²⁵I (294 nCi/ml) in B-HBSS (Fig. 3). Consistent with our results with FRTL-5 cells (Fig. 2B), those reported previously (21), and results with other NIS-transfected lines (9, 10), the iodide accumulation reaches a steady state within 20–30 min after iodide is added. Furthermore, iodide is rapidly lost from FRTL-5 and rNIS-transduced tumor cells. Iodide efflux was examined by replacing the radioiodide-containing B-HBSS with iodide-free B-HBSS after a 1-h incubation. Accumulated iodide is rapidly lost from the tumor cells (Fig. 4) and FRTL-5 cells (Fig. 2C; Refs. 10 and 21). Efflux still occurred when cells were placed on ice, suggesting a passive process. The saturation kinetics of iodide uptake were determined for FRTL-5 cells and tumor lines (Figs. 1D and 5, 7–10, 11, 21).
respectively). The initial velocity of iodide uptake was determined at 5 min in B-HBSS with iodide concentrations of 5–300 μM. Lineweaver-Burk double-reciprocal plots (24) yield the $K_m$ and $V_{max}$ values. The calculated $K_m$ values (Fig. 2D) are similar to values reported previously for FRTL-5 cells (9, 10) and are also consistent with data from other rNIS-expressing mammalian cell lines (9, 10). Our $V_{max}$ values are between 23 and 75% of that seen for FRTL-5 cells, which likely indicates that fewer symporter molecules are functional per cell. These results clearly demonstrate that the NIS functions similarly in both tumor cell lines and FRTL-5 cells.

rNIS-transduced Tumors Can Be Visualized in Vivo Using a Gamma Camera. Athymic nude mice were injected intradermally with $5 \times 10^6$ A375 human melanoma cells. When the tumors reached ~10 mm in diameter, as seen in Fig. 6A (30 days after injection), the mice were injected i.p. with 8 μCi of $^{123}$I, which approximates the human equivalent dose (for imaging the thyroid) per kg for a 25-g mouse (25). Sixty-two min after the isotope was injected, a 5-min planar image of the mouse was obtained (Fig. 6B). The image shows in vivo uptake in expected sites including the salivary glands, thyroid, stomach, and bladder. Importantly, the NIS-transduced tumor is readily visible with an intensity similar to the thyroid gland, whereas the nontransduced tumor is not seen. Such results strikingly demonstrate the clear utility of the rNIS gene to image nonthyroid tumors.

A375 Tumor Cells Transduced with LNISN Vector Accumulate Significantly More Radionuclides in Vivo Than Nontransduced Tumor Cells. After imaging was completed, the athymic nude mice described above were euthanized, and the A375 human melanoma xenografts were carefully dissected and analyzed directly for accumulated $^{123}$I. The tumors were weighed and assayed three times for radioiodide. The rNIS-transduced tumor weighed 1.26 g and yielded $1.77 \pm 0.001 \times 10^6$ cpm (1403 cpm/mg). The NV tumor weighed 0.38 g and yielded $0.0771 \pm 0.0004 \times 10^6$ cpm (203 cpm/mg). Therefore, the relative uptake of $^{123}$I into the rNIS-transduced tumor mass was 6.9-fold greater than for the NV tumor. These results demonstrate that A375 NIS-transduced tumors accumulate significantly more radionuclides than NV tumors in vivo.

rNIS-transduced Tumor Cells Can Be Selectively Killed with $^{131}$I. A clonogenic assay was used to determine whether rNIS-transduced cells could be selectively killed by treatment with $^{131}$I. rNIS-transduced and NV tumor cell lines were exposed to B-HBSS containing 100 μCi/ml $^{131}$I (133 nM Na$^{131}$I) with 30 μM NaI. Control cells were treated in parallel with HBSS without $^{131}$I. After 7 h, cells were washed, harvested, and plated at low densities. Colony formation was assessed between 5 and 8 days after plating. Although only 10–17% of the NV cells were killed by exposure to $^{131}$I, between 56 and 69% of the rNIS-transduced cells were killed (Fig. 7). The results are highly significant for tumor cells growing in a monolayer because a great proportion of the $\beta$ energy emitted from accumulated $^{131}$I is deposited outside of the monolayer cells. The results demonstrate that rNIS-transduced cell lines are selectively killed by treatment with $^{131}$I.
DISCUSSION

We studied the characteristics of the rNIS gene expressed in human and murine tumor cell lines. After a 60-min incubation in B-HBSS containing 0.115 nM $^{125}$I, 30 μM NaI (294 nCi/ml), the external iodide concentration was reduced to 0 μM by replacing the radiolabeled medium with iodide-free B-HBSS. A375 human melanoma, BNL.1 ME transformed mouse liver, CT26 mouse colon carcinoma, and IGROV human ovarian adenocarcinoma cells are shown in A–D, respectively. Plotted values are the means of four determinations; bars, SD.

V_{max}s varied between cell lines, and this might reflect the number of symporters functionally expressed. With retroviral transfer into tumor cells, the integration occurs at a variety of sites, usually associated with open chromatin (26). The integration sites may affect the level of transgene expression, depending upon the proximity of enhancer or suppressor elements that exert cis-control at the integration site. Therefore, some cells may have significantly greater rNIS expression. Our results represent the average uptake for a mixed population of transduced cells. This model more closely mimics the potential result of an in vivo transduced population than a selected, individual (optimally rNIS-expressing) cell clone. One group has reported the imaging of a differentiated thyroid cancer after transfection of the rNIS gene into FRTL rat thyroid cells that did not concentrate iodine. (27). In this report, an attempt was made to kill the transfected thyroid cells with $^{131}$I, but no effect on tumor growth was observed, and they suggested that the short effective half-life of $^{131}$I in the tumor was responsible for their observed lack of efficacy. In contrast, our experiments used human and murine tumor cells and demonstrated clear antitumor efficacy in vitro.

In vivo imaging and ex vivo quantitation experiments indicate that $^{125}$I accumulation occurs in rNIS-transduced A375 tumors; these are easily distinguished from nontransduced tumors in vivo (Fig. 6). Our results suggest that NIS gene therapy may have applications for in vivo diagnostic imaging. A developing effective and specific means of imaging tumors involves the use of tumor-specific monoclonal antibodies (28). However, these are only specific to a relatively limited subset of malignancies including lymphoma, ovarian, prostate, colorectal, and small-cell lung cancer (29). Additionally, multiple administrations of mouse antibodies can lead to a human anti-mouse antibody response, which can interfere with localization (30–32) and possibly induce allergic responses (33). Imaging with the NIS gene using tumor-specific promoters (3, 4) will compete with these established antibody approaches when promoter elements can be engineered to achieve a difficult task, high level expression specificity in target tumors.

Efficacy experiments show selective killing of up to 64% of rNIS-transduced tumor cells plated in monolayer after $^{131}$I accumulation (Fig. 7). The in vitro cell culture methods provide suboptimal conditions to kill cells by rNIS-mediated $^{131}$I uptake because most of the β energy from accumulated iodide is deposited outside of the cell (34).
The dose from accumulated $^{131}$I is affected by cell density, radius, and the stopping power of the $\beta$ particle energy emitted by $^{131}$I; the absorbed dose can be simplified to the following equation: 
\[
\text{Percent absorbed} = 100(1 - e^{-m t}),
\]
where $m$ refers to the stopping power for $\beta$ energy (3.009 m$^2$/kg) $\times$ cell density (approximately $1.3 \times 10^3$ kg/m$^3$) and $t$ is the cell radius in meters (34, 35). The absorbed dose from accumulated $^{131}$I increases exponentially as the radius of a tumor mass increases. Thus, a 0.5-mm tumor mass in vivo could receive a significantly higher proportion of the possible $\beta$-energy dose (>90%) from accumulated $^{131}$I than our monolayer cells (<4%). Nonetheless, even under these suboptimal conditions, selective rNIS-dependent killing by treatment with $^{131}$I is observed (Fig. 7). NIS-mediated radioiodide accumulation should be more effective in vivo, because many tumors will have diameters significantly greater than that of monolayer cells.

To further develop the hypothesis that this method will be more effective for cell masses with radii larger than a single cell, we are presently determining $^{131}$I efficacy using NIS-transduced tumor spheroids grown in soft agar (36) and in mice (both of which have substantially larger radii than monolayer cells). Additionally, bystander radiation killing will be an attractive component of this NIS-mediated system because the $\beta$ energy from $^{131}$I can travel ~1.2 mm from the point of radionuclide decay in tissues (35). The increased radii of the tumor masses in the tumor spheroid and in vivo systems will allow a greater deposition of energy into neighboring cells. Furthermore, because energy can be effectively deposited in neighboring cells that may not have the rNIS, gene delivery to cells in a tumor mass may not require 100% gene transfer efficiency. Even a conservative interpretation of our results suggests that the NIS gene therapy approach represents a promising method for antitumor radiotherapy. Once the complex issues involving the regulation of NIS expression and/or targeting are addressed, the method could potentially transform any type of malignancy into one as treatable as thyroid cancer. We are examining the use of different promoters and delivery vehicle systems in an effort to increase expression levels of the NIS gene in tumor cells.

Nonthyroid, rNIS-expressing cells do not organify iodide like many thyroid cancers, which means that the concentration of internalized iodide will drop proportionally to the external iodide concentration (Fig. 4). Therefore, an important question becomes whether thyroid carcinomas that are effectively treated by $^{131}$I therapy can concentrate and/or organify radioiodine. Previous investigators have demonstrated that thyroid carcinoma often has a defect in the ability to accumulate iodide compared with the normal thyroid gland (37, 38). Furthermore,

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**Fig. 5. Saturation kinetics: the effect of external iodide concentration on iodide uptake in human and murine tumor cells.** The external iodide concentration ranged from 5 to 300 $\mu$M, and initial velocity was determined at 5 min. The data are graphed as Lineweaver-Burk plots (24). A375 human melanoma, BNL.1 ME transformed mouse liver, CT26 mouse colon carcinoma, and IGROV human ovarian adenocarcinoma cells are shown in A–D, respectively.
only variable amounts of organification occur within thyroid carcinomas (37, 39). Consistent with these observations, it has been shown recently that whereas NIS mRNA and protein levels are actually increased in some papillary thyroid carcinomas, they concentrate much less iodide than normal thyroid tissue (40). Rare cases have been reported where metastatic lesions from thyroid carcinoma lead to a state of thyrotoxicosis from overproduction of tri-iodothyronine or thyroxine (41). Such cases where tumors can synthesize thyroid hormones are very unusual (42). Therefore, the majority of thyroid carcinomas do not have significant thyroid hormone synthetic ability and hence do not organify normal amounts of iodide. Although the relative amount of iodide organification is much less than normal thyroid tissue, it may still be substantially greater than for other solid neoplasms, such as melanoma and ovarian cancer (as demonstrated in this report). Another key issue is the relative radiosensitivity of the histological type of tumor, which clearly varies among malignant cell types (43). Further study on solid tumors in animals will be required to determine the relationship between iodide uptake and efficacy for nonthyroid tumors.

The reduction or elimination of systemic toxicities would represent a substantial gain for patients with recurrent cancer. A variety of efficient gene delivery methods are in development in human clinical trials. (44) Our approach should be adaptable to other gene delivery methods and hopefully will allow the development of human clinical trials using the NIS gene as an antitumor agent. This new tumor-killing strategy should have several significant advantages over present treatment approaches for recurrent, localized cancer lesions. The transition to the clinic should be facilitated by the fact that $^{131}$I uptake protocols are already routinely used in humans.

**ACKNOWLEDGMENTS**

We thank Dr. M. D. Enger of Iowa State University for laboratory space and helpful discussions; Dr. E. Grollman of the NIH for helpful discussions and the FRTL-5 cell line; K. Kerns, R.S.O., W. Landowski, S. Simpson, and T. Zimmerman from Iowa State University for technical help and invaluable discussions; and Dr. J. Berger at Iowa Methodist Medical Center for help with imaging. Drs. A. Russo, T. Seregina, and R. Panchal helped with manuscript review.

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*Cancer Res* 1999;59:661-668.

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