

Treatment of Prostate Cancer by Radioiodine Therapy after Tissue-specific Expression of the Sodium Iodide Symporter¹

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ABSTRACT

Causing prostate cancer cells to express functionally active sodium iodide symporter (NIS) by targeted *NIS* gene transfer might offer the possibility of radioiodine therapy of prostate cancer. Therefore, we investigated radioiodine accumulation and therapeutic effectiveness of ¹³¹I in *NIS*-transfected prostate cancer cells *in vitro* and *in vivo*. The human prostatic adenocarcinoma cell line LNCaP was stably transfected with *NIS* cDNA under the control of the prostate-specific antigen promoter. The stably transfected LNCaP cell line NP-1 showed perchlorate-sensitive, androgen-dependent iodide uptake *in vitro* that resulted in selective killing of these cells by ¹³¹I in an *in vitro* clonogenic assay. Xenografts were established in athymic nude mice and imaged using a gamma camera after i.p. injection of 500 μ Ci of ¹²³I. In contrast to the *NIS*-negative control tumors (P-1) which showed no *in vivo* uptake of ¹²³I, NP-1 tumors accumulated 25–30% of the total ¹²³I administered with a biological half-life of 45 h. In addition, *NIS* protein expression in LNCaP cell xenografts was confirmed by Western blot analysis and immunohistochemistry. After a single i.p. application of a therapeutic ¹³¹I dose (3 mCi), significant tumor reduction was achieved in NP-1 tumors in the therapy group compared with P-1 tumors and tumors in the control group. In conclusion, a therapeutic effect of ¹³¹I has been demonstrated in prostate cancer cells after induction of tissue-specific iodide uptake activity by prostate-specific antigen promoter-directed *NIS* expression *in vitro* and *in vivo*. This study demonstrates the potential of *NIS* as a novel therapeutic gene for nonthyroidal cancers, in particular prostate cancer.

INTRODUCTION

Prostate cancer is an important health issue in American men, representing the second leading cause of cancer death (1). Because no curative therapy for metastatic prostate cancer exists, novel therapeutic strategies are urgently needed. In marked contrast, metastatic thyroid cancer can be effectively managed, even in advanced cases, because of the ability of thyroidal cells to trap and concentrate iodine, making therapy with radioactive iodine possible and highly effective (2). Recently, the mechanism mediating iodide uptake across the basolateral membrane of thyroid follicular cells has been clarified by the cloning and characterization of the sodium iodide symporter, *NIS* (3–5). *NIS* is an intrinsic membrane glycoprotein with 13 putative transmembrane domains and is responsible for the ability of the thyroid gland to transport and concentrate iodide approximately 20–40-fold above plasma concentration (6).

A novel form of gene therapy, using *NIS* gene transfer to induce iodide accumulation activity in prostate cancer cells by expression of functionally active *NIS*, would therefore extend the utility of radioiodine therapy to the treatment of prostate cancer. To minimize

extratumoral toxicity, a tissue-specific promoter, such as the PSA³ promoter, may be used to provide selective, prostate-specific *NIS* gene expression (7–9). The PSA promoter has been extensively characterized in recent years and has been shown to be responsible for prostate-specific and androgen-regulated expression of PSA, a serine protease of 237 amino acids, that is mainly expressed within the epithelial lining and acini of the prostate gland (10–13).

We reported recently the induction of tissue-specific, androgen-dependent iodide uptake activity in prostate cancer cells *in vitro* by PSA promoter-directed *NIS* gene delivery (14). The androgen-sensitive human prostatic adenocarcinoma cell line LNCaP was stably transfected with an expression vector in which full-length *NIS* cDNA had been coupled to a 6-kb PSA promoter fragment (14, 15). The stably transfected LNCaP cell line NP-1 showed perchlorate-sensitive, androgen-dependent iodide uptake activity, whereas no iodide uptake activity was detected in LNCaP cells transfected with the control vectors. The magnitude of iodide uptake in NP-1 cells concentrating ¹²⁵I ~50-fold was highly encouraging and suggested that a therapeutic effect of accumulated radioiodine (14) could be achieved. Although these *in vitro* data suggested the feasibility of the concept of *NIS* gene transfer as a first step toward radioiodine therapy of prostate cancer, its utility required direct demonstration. Therefore, the aim of our current study was to investigate radioiodine accumulation in *NIS*-transfected LNCaP cell xenografts *in vivo* and to examine the therapeutic effectiveness of ¹³¹I *in vitro* and *in vivo* for prostate cancer cells.

MATERIALS AND METHODS

Plasmid Constructs. The expression and control vectors have been generated as described previously (14). The resulting expression plasmid construct contained full-length *NIS* cDNA coupled to the 6-kb PSA promoter fragment (Ref. 15; *NIS*/PSA-pEGFP-1). Two control vectors were designed containing *NIS* cDNA without the PSA promoter (*NIS*-pEGFP-1) and the PSA promoter without *NIS* cDNA (PSA-pEGFP-1).

Establishment of Stable Transfected LNCaP Cell Lines. Stable transfection of LNCaP cells was performed as described previously (14). In brief, the androgen-sensitive human prostatic adenocarcinoma cell line LNCaP was transfected with *NIS*/PSA-pEGFP-1 and the control vectors *NIS*-pEGFP-1 and PSA-pEGFP-1, respectively, using LipofectAMINE Plus Reagent (Life Technologies, Inc., Gaithersburg, MD). Selection was performed with geneticin, and surviving clones were isolated and subjected to screening for androgen-dependent iodide uptake activity. NP-1, the clone with the highest androgen-dependent iodide uptake activity, was chosen for the following studies, as well as the stably transfected (PSA-pEGFP-1) control cell line P-1 (14).

In Vitro Clonogenic Assay. LNCaP cells stably transfected with the expression vector (NP-1) or the control vector (P-1) were incubated for 7 h with 0.8 mCi Na¹³¹I in HBSS supplemented with 10 μ M NaI and 10 mM HEPES at pH 7.3. After incubation with radioiodine, cells were trypsinized and plated in quadruplicates at cell densities of 1000, 2000, 3000, 5000, and 7000 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 >50 cells were counted. Parallel experiments were performed for each cell line

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³ The abbreviations used are: PSA, prostate-specific antigen; hNIS, human sodium iodide symporter.

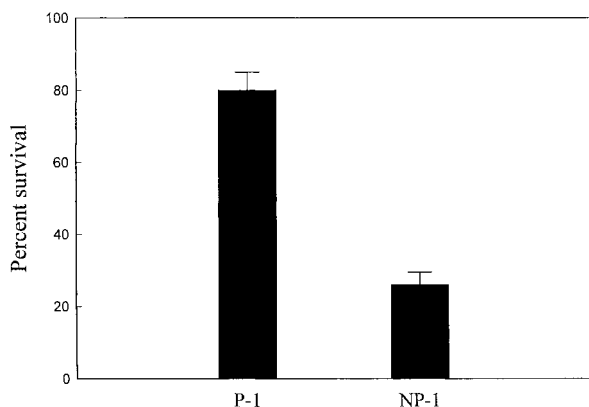


Fig. 1. Although only ~20% of LNCaP cells stably transfected with the control vector PSA/pEGFP-1 (P-1) were killed by exposure to ^{131}I , ~75% of *NIS*-transfected LNCaP cells (NP-1) cells were killed in an *in vitro* clonogenic assay, thereby demonstrating that NP-1 cells are selectively killed by ^{131}I . Results are expressed as means of quadruplicate experiments; bars, SE.

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 \pm SE of quadruplicates. Statistical significance was tested using Student's *t* test.

Establishment of LNCaP Cell Xenotransplants. Xenotransplants derived from NP-1 (right flank) and P-1 (left flank) were established in male BALB/c *nu/nu* mice (Harlan Sprague Dawley, Indianapolis, IN) by s.c. injection of 1×10^6 cells suspended in 0.25 ml of RPMI 1640 and 0.25 ml of Matrigel Basement Membrane Matrix (Becton Dickinson, Bedford, MA). Nude mice were maintained in our facility under specific pathogen-free conditions with access to mouse chow and water *ad libitum*. All experiments were performed in accordance with the Institutional Animal Care and Use Committee guidelines of the Mayo Clinic (Rochester, MN).

Western Blot Analysis. Membrane proteins were prepared from LNCaP cell xenografts as described previously (14) and subjected to electrophoresis on a 4–12% Bis-Tris-HCl buffered polyacrylamide gel. After transfer of proteins to nitrocellulose membranes by electroblotting, membranes were preincubated in 5% low fat dried milk in TBS-T (20 mM Tris, 137 mM NaCl, and 0.1% Tween 20). Western blot analysis was performed using a mouse monoclonal antibody directed against amino acid residues 468–643 of human *NIS* (dilution 1:3000; Ref. 16) as described previously (14).

Immunohistochemistry. Immunohistochemical staining of frozen tissue sections derived from LNCaP cell xenografts was performed using the Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA). After fixation of tissue sections in cold acetone, inhibition of endogenous peroxidase activity, and blocking of nonspecific binding with blocking serum for 30 min, slides were incubated with the mouse monoclonal hNIS antibody at a dilution of 1:1600 (16) for 90 min. Tissue sections were incubated with biotin-conjugated antimouse-immunoglobulin for 30 min at room temperature, followed by incubation with preformed avidin and biotinylated horseradish peroxidase macromolecular complex. Diaminobenzidine was used as the chromogen (bluish-black precipitate). Parallel control slides were examined with the primary and secondary antibodies replaced in turn by PBS and isotype matched nonimmune IgGs.

Iodide Uptake Studies *in Vivo*. Eight to 12 weeks after s.c. injection of transfected LNCaP cells, when tumors had reached ~10 mm in diameter, mice were switched to a low-iodine diet and received T4 supplementation (5 mg/l) in their drinking water for 2 weeks to maximize radioiodine uptake in the tumor and reduce uptake by the thyroid gland. After i.p. injection of 500 μCi ^{123}I , radioiodine imaging was performed using a gamma camera (Helix system; Elscint, Inc., Haifa, Israel). Regions of uptake have been quantified and expressed as a fraction of the total amount of the applied radioiodine. Iodide retention time within the tumor was determined by serial scanning after radioiodine injection, and dosimetric calculations were performed.

Radioiodine Therapy Study *in Vivo*. Xenografts of NP-1 and P-1 cells were established in four groups of mice (five mice in each group) as described above.

One group of mice (group 1) was administered 3 mCi ^{131}I by a single i.p. injection after 8–10 weeks of tumor growth (late tumors), and another group of mice (group 2) was administered 3 mCi of ^{131}I after 4–6 weeks of tumor growth (early tumors). The other two groups of five mice were administered saline by i.p. injections and were used as controls. Tumors were measured before administration of radioiodine and weekly thereafter. Tumor volume was estimated using the formula: tumor volume = length \times width \times height \times 0.52 (17). All mice were followed for a total of 6 weeks. Tumor volumes of NP-1 tumors in the therapy groups were compared with tumor volumes of P-1 tumors in the therapy groups and tumor volumes of NP-1 and P-1 tumors in the control groups. Statistical significance was tested using Student's *t* test (for unpaired samples: when examining between treated and untreated mice; and for paired samples: when examining tumors within the same mouse).

RESULTS

***In Vitro* Clonogenic Assay.** A clonogenic assay was performed to determine whether *NIS*-transfected LNCaP cells can be selectively killed by ^{131}I treatment. NP-1 and P-1 cells were incubated in HBSS containing 0.8 mCi ^{131}I for 7 h. Control cells were treated in parallel with HBSS without ^{131}I . Although only ~20% of the P-1 cells were killed by exposure to ^{131}I , ~75% of NP-1 cells were killed (Fig. 1). These data indicate that a sufficiently high dose of radiation was achieved in *NIS*-transfected LNCaP cells to result in cell killing at a dose that spared control LNCaP cells that are not able to trap iodine.

Establishment of LNCaP Cell Xenotransplants and Iodide Uptake Studies *in Vivo*. Eight to 12 weeks after s.c. injection of LNCaP cells, when tumors had reached a tumor size of ~10 mm in diameter, 500 μCi ^{123}I were injected i.p. ($n = 6$), and radioiodine uptake by the tumors was monitored and quantified by imaging with a gamma camera. In contrast to the control P-1 tumors, which showed no *in vivo* uptake of radioiodine, NP-1 tumors accumulated 25–30% of the total radioiodine dose administered (Fig. 2). The maximum radioiodine uptake with an average of 27.5% occurred around 15–20 h after ^{123}I injection. The average biological half-life of accumulated radioiodine in LNCaP cell xenotransplants was 45 h with a minimum of 30 h and a maximum of 61.5 h (Fig. 3).

Considering a tumor mass of 1 g, a biological half-life of 45 h for accumulated radioiodine and an effective half-life of 37 h for ^{131}I , the absorbed dose to the tumor was calculated to be ~350 rads after an i.p. application of a 1 mCi dose of ^{131}I (37 MBq). In comparison, the average absorbed dose to stomach was ~138 rads/mCi ^{131}I and approximately 450 rads/mCi ^{131}I to the thyroid gland.

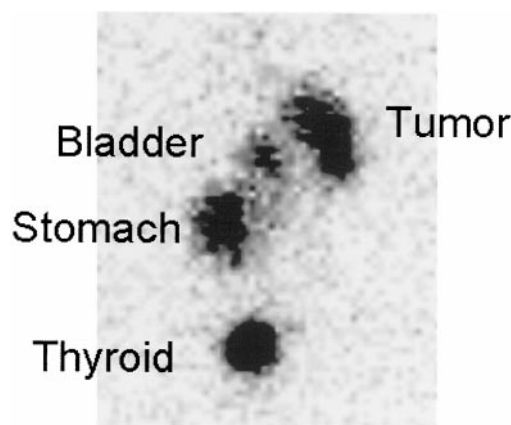


Fig. 2. ^{123}I scan of a mouse harboring NP-1 and P-1 xenografts 24 h after i.p. injection of 500 μCi of ^{123}I . Although the *NIS*-expressing NP-1 tumors trapped about 25–30% of the total radioiodine administered (right), *NIS* negative control P-1 tumors did not show radioiodine uptake (left). ^{123}I was also accumulated physiologically in the bladder, stomach, and the thyroid gland.

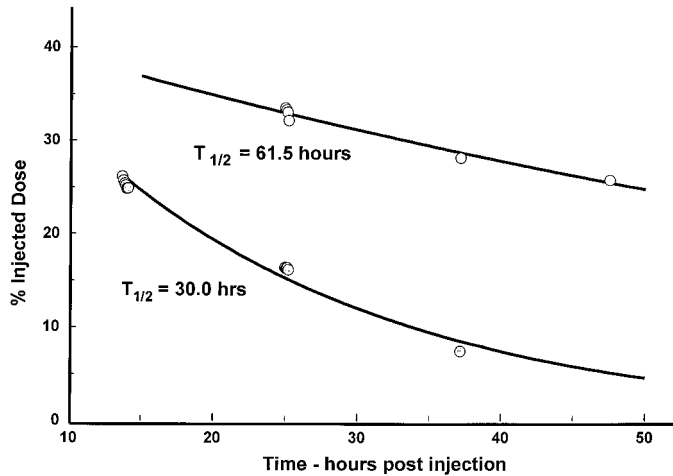


Fig. 3. Radioiodine washout in NP-1 xenografts after i.p. injection of 500 μCi of ^{123}I . The curves shown represent the two extremes observed in a total of six examined mice. An average biological half-life of 45 h has been calculated.

Western Blot Analysis. Using a mouse monoclonal hNIS-specific antibody, Western blot analysis of membrane proteins derived from NP-1 cell xenografts revealed a band of a molecular weight of $M_r \sim 90,000$ [consistent with previously reported NIS protein (16)], which was not detected in P-1 cell xenografts (data not shown).

Immunohistochemical Staining. Immunostaining of frozen tissue sections derived from LNCaP cell xenografts using a mouse monoclonal hNIS antibody revealed marked cell membrane-associated hNIS-specific immunoreactivity in LNCaP cells in NP-1 cell xenografts (Fig. 4A). In contrast, LNCaP cells in P-1 cell xenografts did not show hNIS-specific immunoreactivity (Fig. 4B). Control slides stained with primary and secondary antibodies replaced in turn by

PBS and isotype-matched nonimmune mouse immunoglobulin were consistently negative (Fig. 4C).

Radioiodine Therapy Study *in Vivo*. As demonstrated in Fig. 5, the NP-1 and P-1 tumors in the control groups (saline injection) as well as P-1 tumors in the therapy groups continued their growth unabated throughout the observation period. In contrast, NP-1 tumors in therapy group 1 (late tumors; mean tumor volume, $177 \pm 72 \text{ mm}^3$) demonstrated dramatic reduction in tumor volumes. All five tumors demonstrated $>50\%$ reduction in volume, and two of five tumors became undetectable, indicating complete response (mean tumor volume reduction, $95 \pm 3\%$; $P < 0.01$; Fig. 5A). Therapy group 2 (early tumors; mean tumor volume, $43 \pm 25 \text{ mm}^3$) showed an even more prominent ($P < 0.05$) therapeutic response in that 60% of the NP-1 tumors demonstrated complete response (mean tumor volume reduction, $99 \pm 0.6\%$; $P < 0.01$; Fig. 5B). None of the mice demonstrated adverse effects of the radioiodine therapy.

DISCUSSION

Thyroid cancer, even in advanced metastatic disease, can be very effectively treated by radioiodine therapy. This is possible because of thyroidal expression of the NIS (2, 6). Functioning thyroid cancer metastases can be detected and treated by administering radioiodine, while avoiding adverse effects of ionizing radiation to other organs, which do not express NIS and thus do not concentrate radioiodine. The high success rate of radioiodine therapy is reflected in the low mortality of patients suffering from metastatic thyroid cancer who are treated with ^{131}I (3%) as compared with those who are not (12%). Even young patients with diffuse pulmonary metastases at initial presentation can be successfully treated by ^{131}I , achieving a 10-year survival of $>80\%$ (2).

Cloning and characterization of the *hNIS* gene offers the possibility

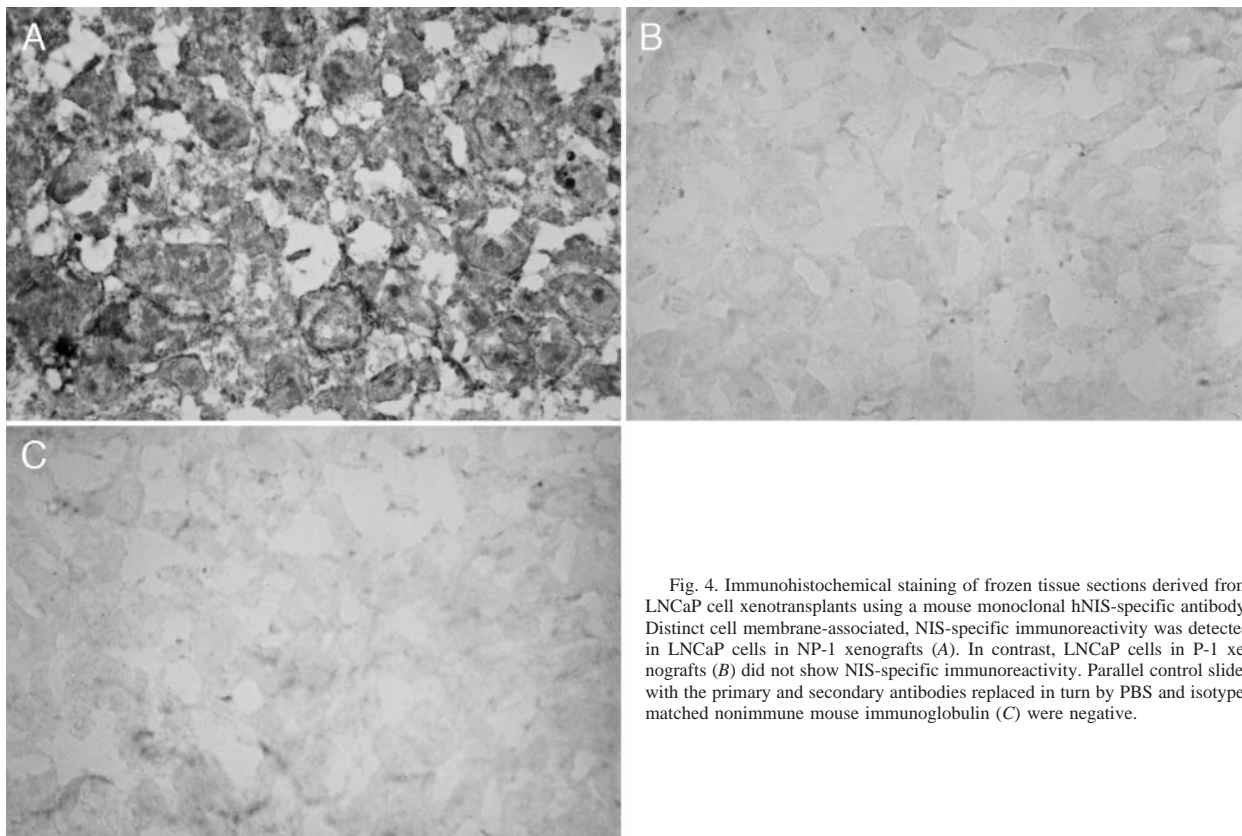


Fig. 4. Immunohistochemical staining of frozen tissue sections derived from LNCaP cell xenografts using a mouse monoclonal hNIS-specific antibody. Distinct cell membrane-associated, NIS-specific immunoreactivity was detected in LNCaP cells in NP-1 xenografts (A). In contrast, LNCaP cells in P-1 xenografts (B) did not show NIS-specific immunoreactivity. Parallel control slides with the primary and secondary antibodies replaced in turn by PBS and isotype-matched nonimmune mouse immunoglobulin (C) were negative.

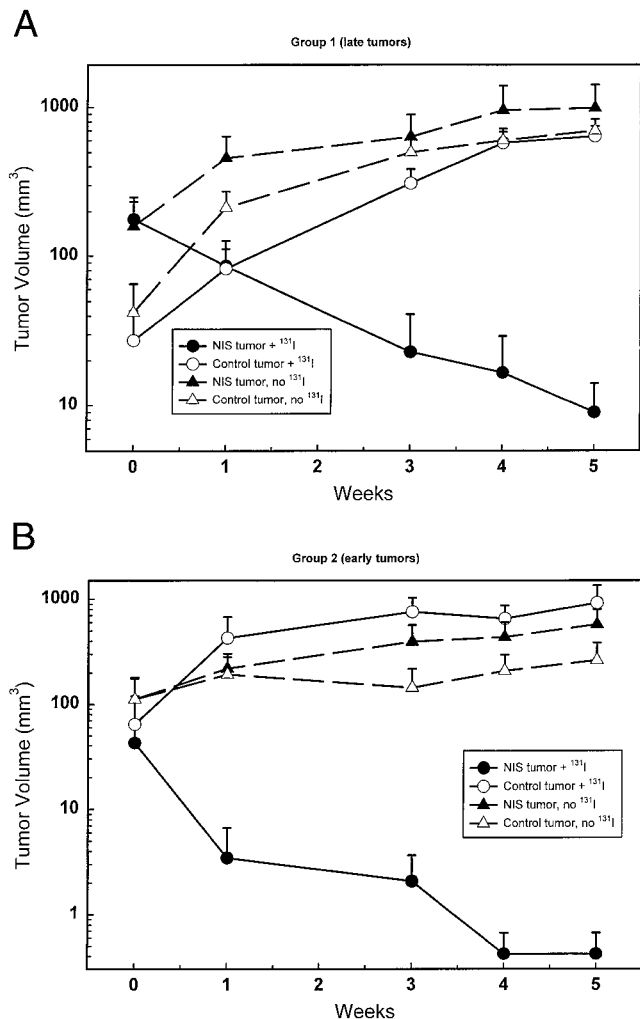


Fig. 5. Growth of NP-1 (●, ▲) and P-1 (○, △) xenografts in nude mice after injection of 3 mCi of ¹³¹I (—) or saline (---). Group 1 (A) was administered ¹³¹I after 8–10 weeks of tumor growth (late tumors), whereas group 2 (B) was administered ¹³¹I after 4–6 weeks of tumor growth (early tumors). Bars, SE.

of *NIS* gene transfer into nonthyroidal tumor cells, thereby inducing radioiodine accumulation and making imaging and treatment of nonthyroidal tumors with radioiodine possible (3, 4, 6). This could offer a highly effective therapy that is remarkably free of adverse affects, except for transient and usually mild sialadenitis and depression of bone marrow activity (2, 18). Because thyroidal *NIS* expression is exquisitely thyroid-stimulating hormone sensitive (19, 20), pretreating the patients with thyroid hormone would suppress thyroid-stimulating hormone levels and thyroidal ¹³¹I uptake and thereby prevent hypothyroidism. Even if thyroidal radioiodine uptake would cause hypothyroidism, patients could be easily and inexpensively managed by thyroid hormone replacement therapy.

Recently, expression of functionally active *NIS* was reported in human glioma cells using adenovirus-mediated gene delivery (21). Shimura *et al.* (22) reported transfection of malignantly transformed rat thyroid cells (FRTL-5 cells), which normally do not concentrate iodide, with a rat *NIS* cDNA expression vector. The resulting rat *NIS*-expressing FRTL-5 cell line accumulated ¹²⁵I *in vitro* and *in vivo* (22). Furthermore, Mandell *et al.* (23) demonstrated *in vitro* and *in vivo* iodide accumulation in several cancer cell lines, including melanoma, liver, colon carcinoma, and ovarian carcinoma cell lines, after transfection with the rat *NIS* gene. These data demonstrate the potential of *NIS* gene transfer to induce iodide accumulation activity in

tumor cells, although the therapeutic efficacy of accumulated ¹³¹I remains to be investigated.

The purpose of the present study was to evaluate the efficacy of prostate-specific *NIS* gene transfer as a novel form of gene therapy in which tissue-specific radioiodine accumulation is induced in prostate cancer cells. To maximize intratumoral cytotoxicity and minimize extratumoral side effects, we used a prostate tissue-specific promoter to target the *NIS* gene selectively to prostate cancer cells. With the *PSA* gene being strictly regulated in a tissue-specific and androgen-dependent manner, the *PSA* promoter, which has been extensively characterized in recent years (10–13), provides an efficient means for prostate cell-specific, androgen-regulated gene delivery (7–9). Recently, using the *PSA* promoter, antisense gene delivery targeting DNA polymerase- α and topoisomerase II α was shown to inhibit cell growth specifically in human prostate cancer cells. In contrast, no cytotoxicity was observed in five control nonprostatic cell lines (24).

In our experiments, we stably transfected the human prostatic adenocarcinoma cell line LNCaP with an expression vector containing full-length *NIS* cDNA coupled to a 6-kb promoter fragment that has been shown recently to mimic, in transgenic mice, the prostate-specific and androgen-regulated expression of the endogenous *PSA* gene in humans (15). Prostate cell-specific, androgen-regulated iodide uptake activity was demonstrated in LNCaP cell lines stably expressing *NIS* under the control of the *PSA* promoter *in vitro* (14). In the current study, the amount of accumulated ¹³¹I has been shown to be sufficiently high to selectively kill *NIS*-transfected LNCaP cells in an *in vitro* clonogenic assay. These results are highly significant for transfected prostate cancer cells growing in a monolayer, considering that a great proportion of the β -energy emitted from accumulated ¹³¹I is deposited outside from the monolayer. Radioiodine accumulation has further been confirmed *in vivo* in *NIS*-transfected LNCaP cell xenografts that accumulated 25–30% of the total administered radioiodine with a biological half-life of 45 h. *NIS* protein expression was confirmed by Western blot analysis and immunohistochemistry. Considering a tumor mass of 1 g, a tumor dose of 350 rad/mCi ¹³¹I has been calculated, which is comparable with therapeutic doses achieved in thyroid metastases during radioiodine therapy with a 100–150 mCi ¹³¹I therapy dose (25). According to the dosimetry studies, a single therapeutic ¹³¹I dose of 3 mCi was administered and shown to elicit a dramatic therapeutic response in *NIS*-transfected LNCaP cell xenografts with an average volume reduction of >90% and complete tumor regression in up to 60% of the tumors.

In conclusion, a therapeutic effect of ¹³¹I has been demonstrated in prostate cancer cells after induction of tissue-specific iodide uptake activity by *PSA* promoter-directed *NIS* expression *in vitro* and *in vivo*. This study clearly demonstrates the potential of *NIS* to serve as a novel therapeutic gene in the therapy of nonthyroidal cancers, in particular metastatic prostate cancer.

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