Prostate-specific Antigen (PSA) Promoter-driven Androgen-inducible Expression of Sodium Iodide Symporter in Prostate Cancer Cell Lines

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ABSTRACT

Currently, no curative therapy for metastatic prostate cancer exists. Causing prostate cancer cells to express functionally active sodium iodide symporter (NIS) would enable those cells to concentrate iodide from plasma and might offer the ability to treat prostate cancer with radioiodine. Therefore, the aim of our study was to achieve tissue-specific expression of full-length human NIS (hNIS) cDNA in the androgen-sensitive human prostatic adenocarcinoma cell line LNCaP and in subcell lines C4, C4-2, and C4-2b in vitro. For this purpose, an expression vector was generated in which full-length hNIS cDNA coupled to the prostate-specific antigen (PSA) promoter has been ligated into the pEGFP-1 vector (NIS/PSA-pEGFP-1). The PSA promoter is responsible for androgen-dependent expression of PSA in benign and malignant prostate cells and was therefore used to mediate androgen-dependent prostate-specific expression of NIS. In addition, two control vectors were designed, which consist of the pEGFP-1 vector containing the PSA promoter without NIS cDNA (PSA-pEGFP-1) and NIS cDNA without the PSA promoter (NIS-pEGFP-1). Prostate cancer cells were transiently transfected with each of the above-described expression vectors, incubated with or without androgen (mibolerone) for 48 h, and monitored for iodide uptake activity. In addition, stably transfected LNCaP cell lines were established for each vector. Prostate cells transfected with NIS/PSA-pEGFP-1 showed perchlorate-sensitive, androgen-dependent iodide uptake in a range comparable to that observed in control cell lines transfected with hNIS cDNA. Perchlorate-sensitive iodide uptake was not observed in cells transfected with NIS/PSA-pEGFP-1 and treated without androgen or in cells transfected with the control vectors. In addition, prostate cancer cell lines without PSA expression (PC-3 and DU-145) did not show iodide uptake activity when transfected with NIS/PSA-pEGFP-1. Western blotting of LNCaP and C4-2b cell membranes transfected with NIS/PSA-pEGFP-1 using a monoclonal antibody that recognizes the COOH-terminus of hNIS revealed a band with a molecular weight of 90,000 that was not detected in androgen-deprived cells or in cells transfected with the control vectors, as well as a minor band at M, 150,000, in transiently transfected LNCaP cell membranes. In conclusion, tissue-specific androgen-dependent iodide uptake activity has been induced in prostate cancer cells by PSA promoter-directed NIS expression. This study represents an initial step toward therapy of prostate cancer with radioiodine.

INTRODUCTION

Currently, there is no curative therapy for metastatic prostate cancer, which represents the second leading cause of cancer death in men in the United States (1). In contrast, thyroid cancer can be effectively treated, even in advanced cases, by radioactive iodine administration because of the unique ability of thyroidal cells to concentrate iodine from plasma. This iodine-trapping activity is due to thyroidal expression of the NIS. NIS is responsible for the ability of the thyroid gland to transport and concentrate iodide about 20- to 40-fold, which represents the first step in the production of thyroid hormones (2). NIS expression in thyroid cancer cells allows imaging and therapy of metastatic thyroid disease by administration of 131I while avoiding adverse effects of ionizing radiation on other organs that do not express NIS and thus do not concentrate radioiodine.

Recently, cloning of rNIS from a Fisher rat thyroid cell line (FRTL-5)-derived cDNA and of its human homologue (hNIS) from a human thyroid cDNA library have been reported (3, 4). Following cloning and characterization (3–10), the NIS gene, which encodes a protein of 643 amino acids, has been successfully expressed in several nontyroidal cell lines, such as COS-7 cells and Chinese hamster ovary cells (11–13).

Gene delivery causing prostate cancer cells to express functionally active NIS would enable those cells to concentrate iodine from plasma and would offer the possibility of treating prostate cancer with radioiodine. To minimize toxicity in this setting, the induction of NIS gene expression should be limited to prostate cancer cells by use of a tissue-specific promoter. Therefore, we chose the PSA promoter to provide selective, prostate-specific NIS gene expression. PSA, a serum protease of 237 amino acids that is mainly expressed within the epithelial lining and acini of the prostate gland and is up-regulated by androgens, has emerged as a very useful marker for monitoring and detecting prostate cancer because of its tissue specificity (14). The aim of our study was to achieve tissue-specific androgen-inducible expression of full-length hNIS cDNA in human prostate cancer cell lines by generating an expression vector in which full-length hNIS cDNA has been coupled to the PSA promoter. Functional NIS protein expression has been tested by Western blot analysis and immunocytochemistry, accompanied by measurement of iodide uptake.

MATERIALS AND METHODS

Plasmid Constructs. 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 HindIII and NotI, agarose gel-purified, and ligated into vector pEGFP-1 (Clontech, San Diego, CA). pEGFP-1 was precut with HindIII and NotI restriction enzymes, thereby removing the 800-bp EGFP fragment. The resulting NIS cDNA-containing pEGFP-1 vector (NIS-pEGFP-1) was used as a control vector for transfection studies. After restriction digestion of NIS-pEGFP-1 with HindIII and dephosphorylation, the 6-kb PSA promoter fragment (15) was ligated into NIS-pEGFP-1. The resulting plasmid construct containing full-length NIS cDNA coupled to the PSA promoter (NIS/PSA-pEGFP-1) was agarose gel-purified and confirmed by DNA sequencing. An additional control vector was designed by ligation of the PSA promoter fragment into the HindIII site of pEGFP-1 (PSA-pEGFP-1).

Transient Transfections. The androgen-sensitive human prostatic adenocarcinoma cell line LNCaP (16) and the subcell lines C4, C4-2, and C4-2b (Refs. 17 and 18), which produce osseous prostate cancer metastases in nude...
mice with increasing tumorigenicity and metastasizing activity, were used in transfection experiments. In control transfections, two androgen-independent prostate cancer cell lines without PSA expression (PC-3 and DU-145) were studied. LNCaP, PC-3, and DU-145 cells were grown in 5% FBS-RPMI 1640, whereas subcell lines C4, C4-2, and C4-2b were grown in DMEM/Ham’s F-12 medium supplemented with 5% FBS and a five-hormone mixture (5 μg/ml insulin, 13.65 pg/ml T3, 5 μg/ml apo-transferrin, 0.224 μg/ml biotin, and 25 μg/ml adrenalin). Cells were maintained in a 5% CO₂/95% air atmosphere at 37°C with a change of medium every third day and passed every 7 days. Before transfections, cells were grown to 50–70% confluency. Cells were transfected with NIS/PSA-pEGFP-1 or the control vectors NIS-pEGFP-1 and PSA-pEGFP-1, respectively, using LipofectAMINE Plus Reagent (Life Technologies, Inc., Gaithersburg, MD) under serum-free conditions, according to the manufacturer’s recommendations. After transfections, cells were incubated for 48 h with 10% charcoal-stripped FBS-containing growth medium with or without 3.2 nm mibolerone, a synthetic androgen. All groups of cells were prepared in triplicate for transfections, which were performed at least three times.

Establishment of Stable Transfected LNCaP Cell Lines. LNCaP cells were transfected with NIS/PSA-pEGFP-1 and the control vectors NIS-pEGFP-1 and PSA-pEGFP-1, respectively, using LipofectAMINE Plus Reagent under serum-free conditions as described above. Selection was performed with 400 μg/ml Geneticin (true concentration, Life Technologies, Inc.) in RPMI 1640 containing 10% FBS for approximately 3 weeks from the day after transfection. Surviving clones were isolated and subjected to screening for androgen-dependent iodide uptake activity. Five stably transfected cell lines termed NP-1, -2, -3, -4, and -5 (NIS/PSA-pEGFP-1) that showed the highest levels of androgen-dependent iodide uptake among the 30 colonies screened were obtained. In addition, five stably transfected LNCaP cell lines for each control vector were obtained [N-1, -2, -3, -4, and -5 (NIS-pEGFP-1); P-1, -2, -3, -4, and -5 (PSA-pEGFP-1)].

Membrane Preparation. After transfection with NIS/PSA-pEGFP-1 or the control vectors NIS-pEGFP-1 and PSA-pEGFP-1, respectively, cell membranes were prepared from LNCaP cells and the subcell line C4-2b by a modification of a previously described procedure (19). 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 phenylmethylsulfonyl fluoride]. The homogenate was centrifuged twice at 500 × g for 15 min at 4°C. After centrifugations, 100 μl of 1 M Na₂CO₃/ml buffer A was added to the supernatant and incubated at 4°C for 45 min with continuous shaking. An additional centrifugation at 100,000 × 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 MgCl₂]. Protein concentrations were determined by a protein assay (Bio-Rad DC protein assay).

Iodide Uptake Studies. Uptake of 125I by transfected LNCaP cells; subcell lines C4, C4-2, and C4-2b cells; and control prostate cancer cell lines PC-3 and DU-145 was evaluated at steady-state conditions as described by Weiss et al. (20). In brief, cells were plated on 6-well plates (2 × 10⁵ cells/well), and after transfections, iodide uptake studies were performed in HBSS supplemented with 10 μM NaI, 0.1 μCi of Na¹²⁵I/ml and 10 mM HEPES at pH 7.3. KCIO₄ (100 μM) was added to control wells. Trapped iodide was removed from cells by a 20-min incubation in 1 N NaOH and measured by γ-counting.

Western Blot Analysis. For Western blot analysis, the NuPAGE electrophoresis system (NOVEX, San Diego, CA) was used. Aliquots of membranes (20 μg) prepared from transfected LNCaP and C4-2b cells were reduced by incubation with 0.5 M DTT 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 TBS-Τ (20 mM Tris, 137 mM NaCl, and 0.1% Tween-20) to block nonspec-

Fig. 1. Iodide uptake was measured in transiently transfected LNCaP cells (A), subcell line C4-2b (B), and stably transfected LNCaP cells (C) after transfection with NIS/PSA-pEGFP-1 or the control vectors NIS-pEGFP-1 and PSA-pEGFP-1. Lane 1, NIS/PSA-pEGFP-1, incubation without androgen; Lane 2, NIS/PSA-pEGFP-1, incubation with androgen; Lane 3, NIS-pEGFP-1; Lane 4, PSA-pEGFP-1; Lane 5, NIS/PSA-pEGFP-1, treatment with perchlorate.
RESULTS

Iodide Uptake Studies. Iodide uptake was measured in transiently (Fig. 1A) and stably transfected LNCaP cells (Fig. 1C); transiently transfected subcell lines C4, C4-2, and C4-2b (Fig. 1B); and prostate cancer control cell lines PC-3 and DU-145 after liposome-mediated transfection with NIS/PSA-pEGFP-1 or the control vectors NIS-pEGFP-1 and PSA-pEGFP-1, respectively. LNCaP cells and subcell lines transfected with NIS/PSA-pEGFP-1 revealed perchlorate-sensitive, androgen-induced iodide uptake. Considering cell number and cell volume, the stably transfected LNCaP cell line NP-1 concentrates 125I about 60-fold. On average, the five stably transfected LNCaP cell lines (NP-1, -2, -3, -4, and -5) concentrate 125I about 50-fold. No perchlorate-sensitive iodide uptake above the background level was observed in cells transfected with NIS/PSA-pEGFP-1 when incubated without androgen or in cells transfected with the control vectors (Fig. 1, A–C). In addition, neither PC-3 nor DU-145 cells transfected with either NIS/PSA-pEGFP-1 or any of the control vectors showed perchlorate-sensitive iodide uptake (data not shown).

Western Blot Analysis. NIS protein expression in transiently (Fig. 2A) and stably transfected LNCaP cells (Fig. 2C) and the subcell line C4-2b (Fig. 2B) transfected with NIS/PSA-pEGFP-1 or the control vectors NIS-pEGFP-1 and PSA-pEGFP-1, respectively, was determined by Western blot analysis. Western blotting of LNCaP and monolayers were then incubated with the mouse monoclonal antibody mentioned above at a dilution of 1:2400 for 90 min at room temperature. Cell monolayers were washed and incubated with biotin-conjugated antinmouse 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, and it yielded a bluish-black precipitate indicative of hNIS-specific immunoreactivity. Slides were counterstained with malachite green for 5 min before mounting. Parallel monolayers with the primary and secondary antibodies replaced in turn by PBS and isotype-matched nonimmune IgGs were examined to assure specificity and to exclude cross-reactivities between the antibodies and conjugates used.

Immunocytochemical Staining. Immunocytochemical staining was performed using the Vectastain Elite ABC Kit (Vector Laboratories, Burlingame, CA). Transient transfected LNCaP cells were plated directly onto 1-chamber slides and grown to 60% confluence. Monolayers were washed with PBS and fixed in 100% methanol for 10 min at 4°C. Air-dried slides were rehydrated in PBS and preincubated for 20 min with blocking serum to inhibit nonspecific binding. Cells specific binding sites. Membranes were then incubated with a mouse monoclonal antibody directed against amino acid residues 468–643 of hNIS (dilution, 1:5000) for 1 h at room temperature before incubation with enhanced chemiluminescence Western blotting detection reagents (Amersham, Arlington Heights, IL) for 1 min. Exposures were made at room temperature for approximately 1 min using Kodak BIOMAX MR films. Prestained protein molecular weight standards (Life Technologies, Inc.) were run in the same gels for comparison of molecular weight and estimation of transfer efficiency.

For deglycosylation of the membrane proteins, 20 μg of membrane fractions were denatured in the denaturing buffer (0.5% SDS and 1% β-mercaptoethanol) for 30 min at 37°C. Denatured proteins were treated with 1 μl (500 units) of N-glycosidase-F (New England Biolabs, Beverly, MA) in 50 mM sodium phosphate buffer (pH 7.5) containing 1% NP40 at 37°C overnight. The deglycosylation reaction was quenched by adding the same volume of reducing sample buffer [0.125 M Tris-HCl (pH 6.8), 4% SDS, 10% β-mercaptoethanol, and 20% glycerol].

Immunocytochemical Staining. Immunocytochemical staining was performed using the Vectastain Elite ABC Kit (Vector Laboratories, Burlingame, CA). Transient transfected LNCaP cells were plated directly onto 1-chamber slides and grown to 60% confluence. Monolayers were washed with PBS and fixed in 100% methanol for 10 min at 4°C. Air-dried slides were rehydrated in PBS and preincubated for 20 min with blocking serum to inhibit nonspecific binding. Cell
C4-2b cell membranes transfected with NIS/PSA-pEGFP-1 using a mouse monoclonal antibody that recognizes the COOH terminus of hNIS revealed a band with a molecular weight of approximately 90,000, which was not detected in androgen-deprived cells transfected with NIS/PSA-pEGFP-1, and a minor band at approximately \( M_r \) 150,000 in transiently transfected LNCaP cell membranes. In addition, using Western blot analysis, no NIS protein expression was demonstrated in LNCaP and C4-2b cells transfected with the control vectors NIS-pEGFP-1 or PSA-pEGFP-1. The molecular mass of deglycosylated hNIS proteins in transiently and stably transfected LNCaP cells (Fig. 3) and in C4-2b cells was approximately 55 kDa, which represents the molecular mass of hNIS in human thyroid tissue (21).

**Immunocytochemical Staining.** Using a highly sensitive immunostaining technique and a mouse monoclonal hNIS-specific antibody, distinct hNIS-specific immunoreactivity was detected in approximately 40% of methanol-fixed LNCaP cells 48 h after transient transfection with NIS/PSA-pEGFP-1 (Fig. 4A). In contrast, LNCaP cells transfected with NIS/PSA-pEGFP-1 and incubated without androgen did not show hNIS-specific immunoreactivity (Fig. 4B). In addition, no hNIS-specific immunoreactivity was detected in LNCaP cells transfected with the control vectors NIS-pEGFP-1 and PSA-pEGFP-1, respectively (Fig. 4, C and D). Control monolayers stained with primary and secondary antibodies replaced in turn by PBS and isotype-matched nonimmune mouse immunoglobulin were consistently negative (data not shown).

### DISCUSSION

To date, curative therapy for prostate cancer exists only for early stages and nonmetastatic disease, whereas patients with metastatic prostate cancer have a poor survival rate. Although androgen ablation at least leads to a partial remission in about 70–80% of prostate cancer patients, tumor recurrence is very likely after several months or years (1). Because prostate cancer is the second leading cause of cancer death in American men, novel therapeutic strategies for treatment of prostate cancer are urgently needed.

The ultimate goal of cancer therapy is a maximum of tissue-specific cytotoxicity with a minimum of toxic side effects in nonmalignant cells. An additional requirement for a successful cancer therapy is the elimination of metastatic cancer cells in addition to treatment of the local tumor. Gene therapy using tissue-specific promoters provides a way of selectively targeting therapeutic genes to malignant cells (22). For example, directing gene expression specifically to melanoma cells has been reported both *in vitro* and *in vivo* using two melanocytic cell-specific promoters, the 5' flanking regions of the tyrosinase and tyrosinase-related protein genes. Melanocyte-specific transcription of those genes is responsible for tissue-specific synthesis of melanin (22, 23). This serves as a method of maximizing cytotoxicity to the target tissue where the gene of interest is expressed and minimizing exposure to cells that do not express it.

In the case of prostate tissue, PSA represents a tissue-specific...
glycoprotein that is synthetized and secreted mainly by epithelial cells lining the acini and ducts of the prostate gland (14). Because the PSA gene is strictly regulated in a tissue-specific manner, the PSA promoter, which has been extensively characterized in the recent years (14, 24–26), might be an ideal means for prostate cell-specific gene delivery (27–30). Recently, using the PSA promoter, antisense gene delivery targeting DNA polymerase -α and topoisomerase IIα was shown to inhibit cell growth specifically in human prostate cancer cells. In contrast, no cytotoxicity was observed in five control nonprostatic cell lines (31). These data support the use of the PSA promoter for targeting cytotoxic therapy to prostate cancer cells.

Thyroidal expression of the NIS is responsible for highly effective treatment of thyroid cancer, even in advanced metastatic disease, by radioactive iodine administration. Recent cloning and characterization of the hNIS gene (3–10) offers the possibility of NIS gene delivery into nontumoral thyroid cells, thereby allowing those cells to trap radioiodine. In addition to successful transfection of COS-7 and Chinese hamster ovary cells with the hNIS gene (11–13), expression of functionally active NIS has been reported very recently in human glioma cells using adenovirus-mediated gene delivery (32). Shimura et al. (33) reported transfection of malignantly transformed rat thyroid cells (FRTL-5 cells), which normally do not concentrate iodide, with a rNIS -cDNA expression vector. The resulting rNIS-expressing FRTL-5 cell line accumulated 125I in vitro and in vivo. Those data suggest that NIS gene transfer might restore the efficacy of radioiodine therapy in undifferentiated thyroid cancers as well as in malignant tumors derived from nontumoral tissues.

Causing prostate cancer cells to express functionally active NIS in a prostate cell-specific manner might offer the ability to treat prostate cancer with radioidine, a specific and potentially curative therapy. Therefore, in our experiments, we transfected human prostate cancer cell lines (LNCaP, C-4, C-4-2, and C-4-2b) with an expression vector containing full-length NIS cDNA coupled to a 6-kb promoter fragment that has recently been shown to mimic, in transgenic mice, the prostate-specific and androgen-regulated expression of the endogenous PSA gene in humans (15). Transfection was followed by incubation in androgen-supplemented growth medium. Prostate cell-specific iodide uptake activity was demonstrated in each of the above-mentioned prostate cancer cell lines. In addition, we established LNCaP cell lines stably expressing NIS under the control of the PSA promoter. 125I is concentrated by these stably transfected LNCaP cell lines about 50-fold, which exceeds the iodide concentrating activity in thyroid cells (2). NIS protein expression was confirmed by Western blot analysis and immunocytochemistry. This PSA promoter-driven NIS gene expression was specific for PSA-expressing prostate cells, as evidenced by the lack of iodide uptake activity in transfected PC-3 and DU-145 cells, which represent androgen-independent prostate cancer cell lines that do not express PSA. Thus, even prostate cells that do not express PSA will not express NIS using our construct.

In addition to its specific expression in prostate tissue, PSA is further characterized by androgen regulation (14, 34, 35). Induction by androgen of PSA mRNA expression has been shown to be primarily due to transcriptional activation (36). In addition to a functional ARE located in the proximal region of the PSA promoter (24), another ARE in the 5′ upstream region of the PSA gene has recently been identified (26). Both AREs have been shown to cooperate, thereby maximizing the androgen induction of PSA gene expression. In our experiments, which included both AREs, PSA promoter-driven NIS gene expression was absolutely androgen dependent. Human prostate cancer cells transfected with NIS/PSA-pEGFP-1 and incubated with growth medium that was not supplemented with the synthetic androgen mibolerone did not reveal iodide uptake activity. In addition, NIS protein expression was not detected by Western blot analysis or immunocytochemistry. This androgen dependency of the PSA promoter might offer an important tool to control NIS gene delivery into prostate cancer cells by the addition or withdrawal of androgen in future experiments including in vivo expression.

In conclusion, prostate tissue-specific androgen-dependent iodide uptake activity has been induced in prostate cancer cell lines using PSA promoter-driven NIS gene delivery. Additional investigations are needed to explore the utility and limitations of prostate cell-specific NIS gene transfer as a first step toward therapy of prostate cancer with radioiodine, a concept that has been shown to be feasible by our data. Currently, we are examining whether transfected prostate cancer cells are able to organify trapped radioiodine, which would increase the therapeutic response from the radionuclide. However, the magnitude of iodide uptake activity obtained in prostate cancer cells, which concentrate radioiodine about 50-fold after PSA promoter-directed NIS gene delivery, is encouraging and suggests that the achieved radioiodine concentration may be sufficiently high to allow a therapeutically effective therapy.

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