Probasin Promoter (ARR2PB)-Driven, Prostate-Specific Expression of the Human Sodium Iodide Symporter (h-NIS) for Targeted Radioiodine Therapy of Prostate Cancer

Hideaki Kakinuma, Elizabeth R. Bergert, Christine Spitzweg, John C. Cheville, Michael M. Lieber, and John C. Morris

1Departments of Endocrinology, 2Urology, and 3Laboratory Medicine and Pathology, Mayo Clinic, Rochester, Minnesota, and 4Department of Internal Medicine II, Klinikum Grosshadern, Ludwig-Maximilians-University, Munich, Germany

ABSTRACT

Prostate cancer is one of the most promising candidates for sodium iodide symporter (NIS)-mediated gene therapy. Adenovirus-mediated expression of NIS that is driven by prostate-specific promoters induces generous radioiodine accumulation in prostate cancer cells that may be used for therapy with $^{131}$I. We have recently developed a replication-deficient adenovirus carrying the human NIS cDNA linked to a composite probasin promoter, ARR2PB, aiming toward specific expression of the human NIS gene (h-NIS) in prostate tissue for targeted radioactive iodide therapy of prostate cancer (Ad-ARR2PB/hNIS). The ability of Ad-ARR2PB/hNIS to cause NIS expression in tumor cells was characterized by iodide uptake assay and compared with Ad-CMV/hNIS in which the h-NIS expression is driven by the cytomegalovirus (CMV) promoter. Androgen-dependent prostate cancer cell lines (LNCaP) and non-prostate origin tumor cell lines (SNU449, MCF-7, HCT116, OVCAR-3, and Panc-1) were infected with the viral constructs, and perchlorate-sensitive 125I uptake and NIS protein expression were measured. Ad-ARR2PB/hNIS-infected LNCaP cells showed androgen-dependent and perchlorate-sensitive iodide uptake. Iodide accumulation in LNCaP cells infected with Ad-ARR2PB/hNIS, followed by incubation with synthetic androgen, was 5.3-fold increased compared with those coincubated with perchlorate (15,184 ± 1,173 cpm versus 2,837 ± 187 cpm). Ad-ARR2PB/hNIS-infected LNCaP cells revealed a 3.2-fold increase of iodide accumulation compared with those infected with Ad-CMV/hNIS (multiplicity of infection = 30). Iodide uptake in a panel of non-prostate tumor cell lines infected with Ad-ARR2PB/hNIS was no more than 2,500 cpm, demonstrating the tissue specificity of this construct. These results indicate that Ad-ARR2PB/hNIS can be used to achieve high-magnitude and tissue-specific expression of h-NIS in prostate tissue and is a promising candidate for cancer gene therapy of prostate cancer.

INTRODUCTION

Although it is detected earlier than in the pre-PSA era, prostate cancer remains the second leading cause of cancer mortality in the American male (1). The prognosis of patients with locally relapsed and metastatic prostate cancer is discouraging despite initial response to androgen deprivation (2). Therefore, new therapeutics are needed urgently for prostate cancer. Cytoreductive gene therapy represents a promising approach for treatment of prostate cancer (3, 4). Therefore, new therapeutics are needed urgently for prostate cancer. Cytoreductive gene therapy represents a promising approach for treatment of prostate cancer. In previous work, we have demonstrated the potential of h-NIS as a novel therapeutic gene for prostate cancer (5–15). An 11.5-kb fragment of the PB promoter achieved high levels of transgene expression in prostate cancer cells (15). The ARR2PB composite promoter, which was modified to contain two androgen response elements (ARR) of the PB promoter, was developed by Zhang et al. (17). This small 0.5-kb PB promoter fragment not only maintains reliable prostate specificity but also provides very high transgene expression in transgenic mice (17). However, the utility of this new promoter construct for targeting gene therapy of prostate cancer was unclear. Here we have developed and characterized an adenovirus carrying h-NIS linked to ARR2PB for targeted radiiodine therapy of prostate cancer.

MATERIALS AND METHODS

cDNA Cloning of h-NIS. h-NIS cDNA was cloned from human thyroid tissue by the reverse transcription-PCR method. Total RNA was isolated from human thyroid tissue (surgical waste). Single-stranded oligo(dT)-primed cDNA was generated using Superscript II Reverse Transcriptase (Life Technologies, Inc., Gaithersburg, MD). A PCR primer set was designed to amplify the entire coding region of h-NIS cDNA (Forward, 5’-TATGGATCCGCCTC-CAGCACCTCAT; Reverse, 5’-ATAGAATTTCTACATCCAGGTTGT-CAGTC; Refs. 5, 6). PCR was carried out in a 50-μl aliquot containing 4 μl of each cDNA template, 50 pmol of each primer, 125 μM deoxynucleotide triphosphates, 1 unit of Expand High Fidelity PCR polymerase (Roche, Mannheim, Germany), and 5 μl of 10× reaction buffer supplied by the manufacturer. PCR amplification conditions were 10 min of initial denaturation at 95°C, followed by 40 cycles of 30 s at 95°C, 60 s at 60°C, and 3 min at 72°C and a 7-min of final extension at 72°C. PCR product was ligated into the pcDNA3 plasmid (Invitrogen, Carlsbad, CA) using the Rapid DNA Ligation kit (Roche), and the sequence of the insert was determined by DANN sequencing.

Recombinant Adenovirus Production. VQ/Sk and VQ/Sk-CMV were used as shuttle vector plasmids that contained the adenovirus backbone (obtained from Vira Quest Inc., North Liberty, IA). The former includes no
promoter sequence, whereas the latter contains the CMV promoter sequence. The h-NIS cDNA fragment was subcloned into VQ5k and VQ5k-CMV to give VQ5k-hNIS and VQ5k-CMV/hNIS, respectively. For prostate-specific expression of h-NIS, we used ARR2PB (17). ARR2PB was subcloned into the VQ5k-hNIS immediately upstream of h-NIS to give VQ5k-ARR2PB/hNIS. Replication-deficient human recombinant type 5 adenovirus carrying h-NIS linked to ARR2PB (Ad-ARR2PB/hNIS) and CMV promoter (Ad-CMV/hNIS) were developed in collaboration with Vira Quest (18). In brief, VQ5k-ARR2PB/hNIS and VQ-Ad-CMV/hNIS were each cotransfected into 293 cells with near full-length adenoviral DNA previously restricted to remove a portion of the E1 region. After the transfection, plaques were harvested, and lysates were prepared by three rounds of freeze/thawing. Lysates were then used to infect 100-mm plates of 293 cells. After complete cytopathic effect, cells were harvested and lysed by freeze/thawing, and functional analysis was performed by measurement of iodide uptake in LNCaP cells. After two more rounds of plaque purification, each resulting recombinant adenovirus was expanded in 293 cells and purified by banding on CsCl density gradients, followed by dialysis.

Cell Lines and Virus Infection. Androgen-dependent (LNCaP) and independent (PC-3 and DU145) prostate cancer cell lines, a liver cancer cell line (SNU449), a breast cancer cell line (MCF-7), and a colon cancer cell line (HCT116), an ovary cancer cell line (OVCAR-3), and a pancreas cancer cell line (Panc-1) were used to examine the specificity of the adenoviral constructs. Adenovirus infection was carried out by 3-h coinoculation with no serum medium containing virus constructs (Ad-ARR2PB/hNIS and Ad-CMV/hNIS). Medium was replaced by fresh culture medium after infection. Virus-infected cells were further maintained for 72 h in 10% charcoal-stripped fetal bovine serum containing growth medium with or without 3.2 mM mibolerone, a synthetic androgen. All groups of cells were prepared in triplicate for each iodide uptake study, which was repeated at least three times.

Iodide Uptake Studies. Iodide uptake study is the method for detection of the functional NIS protein, which is expressed in cytoplasmic membrane. Uptake of 125I by virus-infected cells was determined at steady-state conditions as described by Weiss et al. (19). In brief, cells were plated on twelve-well plates (2.5 × 10^5 cells/well), and 72 h after infection, 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. KClO4 (100 μM) was added to control wells. Trapped iodide was removed from cells by 20-min incubation in 1 N NaOH and measured by gamma-counting.

Membrane Preparation. Seventy-two h after infection with Ad-ARR2PB/hNIS and Ad-CMV/hNIS, cell membranes were prepared from LNCaP cells by a modification of previously described procedure (20). 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 centrifugation, 100 μl of 1 M Na2CO3/ml of buffer A were 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 concentration was determined by a protein assay (Bio-Rad DC protein assay; Bio-Rad, Hercules, CA).

Western Blot Analysis. For Western blot analysis, the NuPAGE electrophoresis system (NOVEX, San Diego, CA) was used. Aliquots of membranes (6 μg) prepared from infected LNCaP cells were reduced by incubation with 0.5 mM DTT for 10 min at 70°C and loaded on 12% bis-Tris-HCl-buffered polyacrylamide gels. After gel electrophoresis for 1 h, proteins were transferred to nitrocellulose membranes. Membranes were preincubated for 1 h in 5% low-fat dried milk in TBS-T (20 mM Tris, 137 mM NaCl, and 0.05% Tween-20) to block nonspecific binding sites, then they were incubated with a mouse monoclonal antibody directed against amino acid residues 468–643 of hNIS (dilution, 1:20,000) for 1.5 h at room temperature (21). After washing with TBS-T, horseradish peroxidase-labeled goat-antimouse antibody was applied (dilution, 1:50,000) for 1.5 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 3 min using Kodak BIOMAX MR films (Eastman Kodak, Rochester, NY). Prestained protein molecular weight standards (Life Technologies, Inc.) were run in the same gels for comparison of molecular weight and estimation of transfer efficiency.

Immunocytochemical Staining. Immunocytochemical staining was performed using the peroxidase substrate kit (Vector Laboratories, Burlingame, CA). Cells were grown onto two-chamber slides and infected with virus constructs. Seventy-two h after incubation, monolayers were washed and fixed in 100% methanol for 15 min at −20°C. Air-dried slides were rehydrated in PBS and preincubated for 20 min with blocking serum to inhibit nonspecific binding. Cell monolayers were then incubated with the mouse monoclonal antibody mentioned above at a dilution of 1:2,400 for 90 min at room temperature, then washed and incubated with biotin-conjugated antimuscle 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. Slides were counterstained with malachite green 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-reactions between the antibodies and conjugates used.

RESULTS

Iodide Uptake Assay. Seventy-two h after adenovirus infection, iodide uptake was measured. Ad-CMV/hNIS- and Ad-ARR2PB/hNIS-infected LNCaP cells showed androgen-dependent and perchlorate-sensitive iodide uptake (Fig. 1). Iodide accumulation in LNCaP cells infected with Ad-ARR2PB/hNIS was 5.2-fold increased compared with those co-incubated with perchlorate (15,184 ± 1,173.5 cpm versus 2,837 ± 187 cpm). The impact of androgen in Ad-ARR2PB/hNIS-infected LNCaP cells was much stronger than that seen in Ad-CMV/hNIS-infected cells, showing a greater reduction of iodide uptake by androgen deprivation [Ad-ARR2PB/hNIS (95%) versus Ad-CMV/hNIS (47%)]. Because of the resistance to adenoviral infection, androgen-independent prostate cell lines (PC-3 and DU145) showed no significant iodide uptake (data not shown). In comparison with Ad-CMV/hNIS, Ad-ARR2PB/hNIS was able to induce higher levels of iodide uptake in LNCaP cells in the presence of 3.2 mM mibolerone (Fig. 2). Iodide accumulation in LNCaP cells infected with Ad-ARR2PB/hNIS was 3.2-fold above cells infected with Ad-
CMV/hNIS (MOI, 30). Furthermore, in contrast to Ad-CMV/hNIS, Ad-ARR2 PB/hNIS was able to induce cell-specific iodide accumulation (Fig. 3). Iodide uptake counts in non-prostate tumor cell lines infected with Ad-ARR2 PB/hNIS were relatively low when compared with LNCaP cells: 2,423 ± 69 cpm (SNU449), 957 ± 53 cpm (MCF-7), 1,930 ± 10 cpm (HCT116), 210 ± 9 cpm (OVCAR-3), and 164 ± 20 cpm (Panc-1), respectively (Fig. 3A). Ad-CMV/hNIS, however, induced relatively high levels of iodide uptake in non-prostate cell lines (Fig. 3B).

**Western Blot Analysis.** NIS protein expression in virus-infected, androgen-dependent LNCaP cells was evaluated by Western blot analysis (Fig. 4). Western blotting of the membranes infected with Ad-CMV/hNIS and Ad-ARR2 PB/hNIS, using a mouse monoclonal antibody that recognizes the COOH terminus of hNIS, revealed a major band with a molecular weight of ~100,000 and several minor bands similar to that of native NIS (21). NIS protein expression caused by Ad-ARR2 PB/hNIS was undetectable in an androgen-deprived state.

**Immunocytochemical Staining.** h-NIS-specific immunoreactivity was detected in infected LNCaP cells using a mouse monoclonal

---

Fig. 2. Comparison of Ad-ARR2 PB/h-NIS and Ad-CMV/h-NIS. Iodide uptake counts at different MOIs were compared. Iodide accumulation in Ad-ARR2 PB/h-NIS-infected LNCaP cells was higher than in those infected with Ad-CMV/h-NIS (P < 0.0001). Bars, SD.

**Fig. 3.** Iodide uptake counts in non-prostate tumor cell lines infected with Ad-ARR2 PB/h-NIS (A) and Ad-CMV/h-NIS (B). Iodide uptake counts in non-prostate tumor cell lines infected with Ad-ARR2 PB/h-NIS were relatively low when compared with that of the LNCaP cell line. Conversely, Ad-CMV/hNIS caused higher iodide uptake in those cell lines than Ad-ARR2 PB/hNIS (B; P < 0.001). Bars, SD.

**Fig. 4.** Western blot analysis. Western blot analysis of membrane from virus-infected LNCaP cells [Ad-CMV/hNIS (Lanes 1 and 2) and Ad-ARR2 PB/hNIS (Lanes 3 and 4)] was performed by using a mouse monoclonal anti-hNIS antibody. hNIS protein was detected as a major band with a molecular weight of ~100,000 and several minor bands similar to that of native NIS. NIS protein expression caused by Ad-ARR2 PB/hNIS was as high as that caused by Ad-CMV/hNIS in the presence of androgen (Lanes 1 and 3) and clearly disappeared in the androgen-deprived state (Lane 4).
h-NIS-specific antibody, Ad-ARR2PB/hNIS-infected LNCaP cells showed generalized h-NIS-specific immunoreactivity that was concentrated at the cytoplasmic membrane, whereas <10% of Ad-CMV/hNIS-infected cells showed predominant cell membrane staining by h-NIS-specific antibody (B), whereas <10% of Ad-CMV/hNIS-infected cells demonstrated membrane staining (E). In contrast, non-prostate cell lines [SNU449 (C and F), Panc-1 (G and I), and OVCAR-3 (H and J)] infected with Ad-ARR2PB/hNIS were negative (<5%; C, G, and H), whereas Ad-CMV/hNIS-infected cell lines showed 50 to ~60% of h-NIS-specific immunoreactivity in the intracellular compartment as well as the cell membrane (F, I, and J).

**DISCUSSION**

For the development of targeted radioactive iodine therapy of extrathyroidal tumors using h-NIS as a therapeutic gene, tissue-specific expression of the transgene offers the ability of limiting radiation exposure, thereby reducing potential adverse effects to other organs. A number of proteins, promoters for which may serve as targeting mechanisms for gene expression, have been described that demonstrate prostate tissue-specific expression, including PSA, prostate-specific membrane antigen, human kallikreins 2, relaxin H2, and PB (12, 17, 22–25). A 6-kb fragment of the 5′-flanking region of the PSA gene has been shown to target cytotoxic genes to the prostate in an androgen-sensitive and highly tissue-specific manner (26). Spitzweg et al. (12) used this large PSA promoter fragment to establish a stably transfected prostate cancer cell line with h-NIS (NP-1). NP-1 revealed therapeutically relevant levels of prostate-specific NIS expression, which was sufficient to allow a cytocidal response to accumulated 131I in vitro and in vivo (13). The development of viral vectors was the next crucial step toward clinical application of h-NIS transfer, followed by radioiodine therapy to allow in vivo NIS gene delivery. Because of the limited packaging capacity of adenoviral vectors, the 6-kb PSA promoter fragment could not be used, and shorter fragments of the PSA promoter or other prostate-specific proteins needed to be developed and characterized.

Similar to the 6-kb PSA promoter, the ARR2PB construct resulted in reliably high and prostate-specific transgene expression that was developmentally and hormonally regulated during the development of transgenic mouse models of prostate cancer (17, 26). To date, two investigators have developed replication-deficient adenoviruses carrying the Bax gene linked to ARR2PB and reported successful induction of androgen-dependent therapeutic apoptosis in prostate cancer (27, 28). These data support the application of ARR2PB for targeting cytotoxic therapies to prostate cancer.

Zhang et al. originally reported that the ARR2PB promoter demonstrated specificity, by preferential expression in prostate tissue, in demonstrating relatively higher basal CAT activity among prostate tumor cell lines (LNCaP, PC3, and DU145) than non-prostate cell lines (17). In contrast, our preliminary data from plasmid transfection revealed that ARR2PB promoter-driven h-NIS-mediated iodide uptake was observed exclusively in an androgen-dependent prostate cell line. Although we cannot compare directly our results with those original data, we suspect that the sensitivity of the CAT assay, as compared with the 125I uptake assay (the method for the detection of the functional NIS protein), is different and explains this discrepancy.
Furthermore, in the Zang study, AR induction by cotransfection may have induced changes in CAT levels in the prostate cell lines, regardless of their similar basal CAT activity levels (17). In the current study, the Ad-ARR2PB/hNIS construct was able to cause androgen-sensitive LNCaP cells to concentrate 131I >25-fold (Ad-ARR2PB/hNIS) over noninfected cells. This iodide concentrating ability, exceeding that seen in normal thyroid cells, suggests that radioactive iodide (131I) therapy will result in a cytotoxic effect in prostate tumors [and these studies are currently being pursued (29)].

The likelihood of response to therapeutic doses of 131I appears to be enhanced by Ad-ARR2PB/hNIS, as compared with Ad-CMV/hNIS. In androgen-replete conditions, adenovirus-mediated ARR2PB-driven h-NIS transfer induced comparable and somewhat higher amounts of NIS protein expression in LNCaP cells than seen with CMV promoter, resulting in 3.2 times higher iodide uptake activity. In agreement with those observations, Ad-ARR2PB/hNIS-infected LNCaP cells were characterized by predominant cytoplasmic membrane staining by NIS antibody (nearly 90%), whereas Ad-CMV/hNIS-infected cells demonstrated low-level cytoplasmic membrane staining (10%). Conversely, in an androgen-deprived state, the ARR2PB promoter did not yield detectable NIS protein expression, whereas the CMV promoter triggered NIS protein expression, although somewhat lower than that observed in an androgen-replete state. This result may be partly explained by the androgen dependency of LNCaP growth and metabolic activity. Furthermore, the tissue specificity of Ad-ARR2PB/hNIS was evaluated by both immunohistochemical analysis and by iodide uptake assay.

In conclusion, the results of our current study suggest that Ad-ARR2PB/hNIS can be used to achieve high levels of hNIS expression in prostate tissue. Because of its tissue specificity, it is capable of limiting radioactive iodine accumulation to prostate tissue, thereby minimizing extratumoral cytotoxicity. Application of this virus construct for in vivo h-NIS delivery in prostate cancer xenografts and examination of its capacity to induce accumulation of therapeutically effective doses of radioactive iodine are the next steps to further examine its potential use for patients with prostate cancer.

REFERENCES


Probasin Promoter (ARR2PB)-Driven, Prostate-Specific Expression of the Human Sodium Iodide Symporter (h-NIS) for Targeted Radioiodine Therapy of Prostate Cancer

Hideaki Kakinuma, Elizabeth R. Bergert, Christine Spitzweg, et al.


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/63/22/7840

Cited articles
This article cites 29 articles, 7 of which you can access for free at:
http://cancerres.aacrjournals.org/content/63/22/7840.full.html#ref-list-1

Citing articles
This article has been cited by 7 HighWire-hosted articles. Access the articles at:
/content/63/22/7840.full.html#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.