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 radiiodine 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 radioiodide 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 $^{125}$I 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 vs. 2,837 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 potentially effective new therapeutic modality in the anticancer armamentarium against malignancy (3, 4).

Recently, the mechanism mediating active iodide transport across the basolateral membrane of thyroid follicular cells has been clarified by cloning and characterization of the NIS (5–10). NIS, which is an intrinsic membrane protein, is responsible for the ability of the thyroid gland to transport and concentrate iodide, approximately 20–40-fold above plasma concentration (9, 10). Clinically, radioactive iodine treatment for thyroid cancer, the most effective form of this systemic radiotherapy available, is well-established and improves the prognosis of metastatic thyroid cancer patients significantly (11). However, because of the lack of the endogenous h-NIS gene expression in prostate tissue, prostate cancer patients cannot benefit from this therapy without first transferring h-NIS into the tumor.

In previous work, we have demonstrated the potential of h-NIS as a novel therapeutic gene for prostate cancer (12–14). Those studies revealed that prostate tumors in vivo were successfully treated with systemic administration of $^{131}$I after h-NIS transfer using an adenovirus vector in which NIS expression was driven by the CMV promoter (14). However, a more ideal vector for clinical trials would provide tissue-specific delivery of h-NIS to prostate, thereby reducing extratumoral toxicity.

PB, isolated as an abundant protein from rat prostate nuclei, is a well-characterized, prostate-specific gene (15). An 11.5-kb fragment of the PB promoter achieved high levels of transgene expression in prostate tissue attributable to its length but was not suitable for incorporation into the expression cassettes of gene transfer vectors (16). The ARR2 PB 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 ARR2 PB for targeted radioiodide 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'-TATGGATCCGCTCGCCGACCACCCTCAT; Reverse, 5'-ATAGAATTCTCCATCCCAGGGTGTCAGGTGCAGTGCAGTGCA; 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 10x 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/5k and VQ/5k-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 ARR2B (17). ARR2B was subcloned into the VQ5k-hNIS immediately upstream of h-NIS to give VQ5k-ARR2B/hNIS. Replication-deficient human recombinant type 5 adenovirus carrying h-NIS linked to ARR2B (Ad-ARR2B/hNIS) and CMV promoter (Ad-CMV/hNIS) were developed in collaboration with Vira Quest (18). In brief, VQ5k-ARR2B/hNIS and VQ-Ad-CMV/hNIS were each co-transfected 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), 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 coincubation with no serum containing growth medium with or without 3.2 nM 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 × 10⁵ 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. KCIO₄ (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), 10 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 Technol-

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**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-

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**Fig. 1.** Iodide uptake was measured in LNCaP cells infected with Ad-ARR2PB/hNIS (Lanes 1–5) and Ad-CMV/hNIS (Lanes 6–8). Bar, SD.
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 ± 49 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 antibody.
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 LNCaP 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 cytoselective 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 125I >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 androgen receptor activity and specificity of promoters from prostate-expressed genes. Prostate, 26, 1998.


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

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