Probebrain 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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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 131I. 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 ± 1,137 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 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 131I 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 ARR2PB for targeted radioiodine 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, S'TATGGAAGCCTCGGCTC- CGCACCCCAT; Reverse, S'TATAGAATTCTCCATCCCAGGGTGTTATGATCCGCCTC-

Received 2/13/03; revised 9/4/03; accepted 9/9/03.

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1 The abbreviations used are: PSA, prostate-specific antigen; NIS, sodium iodide symporter; CMV, cytomegalovirus; PB, probasin; MOI, multiplicity of infection; CAT, chloramphenicol acetyltransferase; AR, androgen receptor.

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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 ARR-PB (17), ARR-PB was subcloned into the VQ5k-hNIS immediately upstream of h-NIS to give VQ5k-ARR-PB/hNIS. Replication-deficient human recombinant type 5 adenovirus carrying h-NIS linked to ARR-PB (Ad-ARR-PB/hNIS) and CMV promoter (Ad-CMV/hNIS) were developed in collaboration with Vira Quest (18). In brief, VQ5k-ARR-PB/hNIS and VQ5k-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), 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-ARR-PB/hNIS and Ad-CMV/hNIS). Medium was replaced by fresh culture medium after infection. Virus-infected cells were further maintained for 72 h at 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 $^{125}$I 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 $\times 10^5$ cells/well, and 72 h after infection, iodide uptake studies were performed in HBSS supplemented with $10\mu$M NaI, $0.1\mu$Ci of Na$^{125}$I/ml, and 10 mM HEPES at pH 7.3. KClO$_4$ (100 mM) was added to control wells. Trapped iodide was removed from cells by 20-min incubation in 1N NaOH and measured by gamma counter.

**Membrane Preparation.** Seventy-two h after infection with Ad-ARR-PB/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 $\mu$g/ml leupeptin, 10 $\mu$g/ml aprotinin, and 1 mM phenylmethylsulfonyl fluoride]. The homogenate was centrifuged twice at 500g for 15 min at 4°C. After centrifugation, 100 $\mu$l of 1M Na$_2$CO$_3$/ml of buffer A were added to the supernatant and incubated at 4°C for 45 min with continuous shaking. An additional centrifugation at 100,000g for 3 min using Kodak BIOMAX MR films (Eastman Kodak, Rochester, NY). Pre-stained 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 $\sim$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 antiuminoglutamin antibody 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 counter-stained 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-reactivities 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-ARR-PB/hNIS-infected LNCaP cells showed androgen-dependent and perchlorate-sensitive iodide uptake (Fig. 1). Iodide accumulation in LNCaP cells infected with Ad-ARR-PB/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-ARR-PB/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-ARR-PB/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-ARR-PB/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-ARR-PB/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-ARR-PB/h-NIS (Lanes 1–5) and Ad-CMV/h-NIS (Lanes 4–6). Bars, 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. NIS 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).
**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 cytoreductive response to accumulated $^{131}$I 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 $^{125}$I 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 $^{131}I$ greater than 25-fold (Ad-ARR2PB/hNIS) over noninfected cells. This iodide concentrating ability, exceeding that seen in normal thyroid cells, suggests that radioactive iodide ($^{131}I$) 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 $^{131}I$ 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


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