Retinoic Acid-Induced Stimulation of Sodium Iodide Symporter Expression and Cytotoxicity of Radioiodine in Prostate Cancer Cells


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We reported recently the induction of androgen-dependent iodide uptake activity in the human prostate adenocarcinoma cell line LNCaP using a prostate-specific antigen (PSA) promoter-directed expression of the sodium iodide symporter (NIS) gene. This offers the potential to treat prostate cancer with radioiodine. In the current study, we examined the regulation of PSA promoter-directed NIS expression and therapeutic effectiveness of 131I in LNCaP cells by all-trans-retinoic acid (atRA). For this purpose, NIS mRNA and protein expression levels in the NIS-transfected LNCaP cell line NP-1 were examined by Northern and Western blot analysis following incubation with atRA (10^-9 to 10^-6 M) in the presence of 10^-5 M mibolerone (mib). In addition, NIS functional activity was measured by iodide uptake assay, and in vitro cytotoxicity of 131I was examined by in vitro clonogenic assay. Following incubation with atRA, NIS mRNA levels in NP-1 cells were stimulated 3-fold in a concentration-dependent manner, whereas NIS protein levels increased 2.3-fold and iodide accumulation was stimulated 1.45-fold. This stimulatory effect of atRA, which has been shown to be retinoic acid receptor mediated, was completely blocked by the pure androgen receptor antagonist casodex (10^-8 M), indicating that it is androgen receptor dependent. The selective killing effect of 131I in NP-1 cells was 50% in NP-1 cells incubated with 10^-9 M mib. This was increased to 90% in NP-1 cells treated with atRA (10^-7 M) plus 10^-9 M mib. In conclusion, treatment with atRA increases NIS expression levels and selective killing effect of 131I in prostate cancer cells stably expressing NIS under the control of the PSA promoter. Therefore atRA may be used to enhance the therapeutic response to radioiodine in prostate cancer cells following PSA promoter-directed NIS gene delivery.

(Diagram: Clonogenic assay and radioiodine therapy)

The unique property of thyroid follicular cells to trap and concentrate iodide because of expression of the sodium iodide symporter (NIS) allows imaging as well as effective therapy of differentiated thyroid cancers and their metastases by administration of radioiodine. Thus, the prognosis and treatment of thyroid cancer is improved significantly (1). Cloning of the human and rat NIS genes in 1996 and their extensive characterization since then have paved the way for the development of a novel cytoreductive gene therapy strategy for the treatment of extrathyroidal malignancies based on targeted NIS gene transfer followed by radioiodine therapy (2-5). NIS gene transfer using tissuespecific promoters provides a way of selectively targeting the NIS gene to tumor cells, thereby maximizing tissue-specific cytotoxicity with minimal toxic side effects in other organs (6).

In contrast to thyroid cancer, despite a variety of treatment options including androgen ablation, surgery, external radiotherapy, interstitial brachytherapy, and systemic cytotoxic chemotherapy, no curative therapy for metastatic prostate cancer exists, which still represents the second leading cause of cancer death in men (7). Therefore, the development and evaluation of novel treatment strategies, including prostate-specific antigen (PSA) promoter-targeted NIS gene transfer followed by radioiodine therapy, are urgently needed. Gene therapeutic approaches for the treatment of prostate cancer are attractive because of the possibility of selective targeting of therapeutic genes to tumor cells by application of tissue-specific promoters, thereby reducing extratumoral toxicities associated with treatments such as cytotoxic chemotherapy. We therefore chose prostate cancer as a tumor model to investigate the feasibility of radioiodine therapy of nonthyroidal tumors following NIS gene transfer.

The PSA gene is regulated strictly in a tissue-specific manner. Thus, the PSA promoter represents an important tool for prostate cell-specific gene delivery (8-10). A 6-kb PSA promoter fragment (11) has therefore been used in our studies to induce tissue-specific androgen-dependent iodide uptake activity in the androgen-sensitive human prostate adenocarcinoma cell line LNCaP in vitro by liposome-mediated NIS gene delivery (12). LNCaP cells stably transfected with the human NIS gene under the control of the PSA promoter revealed androgen-dependent and prostate-specific iodide uptake activity that was high enough to allow a therapeutic effect of accumulated 131I in an in vitro clonogenic assay and in vivo in xenografts in nude mice (12-14). These studies clearly showed the potential of NIS as a novel therapeutic gene allowing radioiodine therapy of nonthyroidal cancers, in particular prostate cancer.

Retinoids, synthetic and natural analogs of vitamin A, play a well-characterized role in cancer development, cell differ-

Abbreviations: atRA, All-trans-retinoic acid; 9-cis-RA, 9-cis-retinoic acid; HBSS, Hanks’ balanced salt solution; mib, mibolerone; NIS, sodium iodide symporter; PSA, prostate-specific antigen; RAR, retinoic acid receptor; SSC, saline sodium citrate.
entiation, and cell growth (15). Induction of NIS expression and radiiodine uptake has been demonstrated in breast cancer cells following treatment with all-trans-retinoic acid (atRA), allowing a therapeutic effect of 131I in vitro (16). In addition, atRA has been shown to suppress iodide uptake and NIS mRNA levels in normal, nontransformed FRTL-5 cells, whereas NIS mRNA expression levels were up-regulated in human follicular thyroid carcinoma cell lines in vitro (17). A clinical study in 20 patients with advanced thyroid cancer (8 follicular, 7 papillary, 5 oxyphilic) showed that 13-cis-retinoic acid treatment (1.5 mg/kg/d for 5 wk) was capable of reinducing iodine uptake in 50% of tumors (18). Treatment with retinoid acid may therefore provide a means of reestablishing the therapeutic efficacy of radiodiode therapy by targeted up-regulation of iodide transport in thyroid cancer cells and down-regulating iodide accumulation in surrounding normal thyroid tissue. Furthermore, atRA has been reported to inhibit cell cycle progression and induce apoptosis in many tumor cell lines. Also, it has been used in a number of clinical studies to investigate the therapeutic effect of retinoids in a variety of tumors, including prostate cancer (15, 19, 20). Retinoids have been reported to increase PSA secretion in LNCaP cells and inhibit their cell growth by an androgen receptor-dependent mechanism preceded by the stimulation of androgen receptor gene expression (21–23).

In the current study, we therefore used atRA to examine the effect of retinoids on androgen-dependent NIS expression and therapeutic response to 125I in LNCaP cells stably expressing the human NIS gene under the control of the PSA promoter.

Materials and Methods
Plasmid constructs and NIS gene transfer

The expression and control vectors have been generated as previously described (12). We used the pEGFP-1 vector (CLONTECH Laboratories, Inc., San Diego, CA) that had been precut with HindIII and Not restriction enzymes, thereby removing the 800-bp EGFP fragment. The resulting expression plasmid construct contained full-length human NIS cDNA coupled to the 6-kb PSA promoter fragment (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).

Transient and stable transfection of prostate cancer cells was performed as previously described (12). In brief, the androgen-sensitive human prostatic adenocarcinoma cell line LNCaP and androgen-independent prostate cancer control cell lines without PSA expression (PC-3, DU-145) were 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 for stable transfection of LNCaP cells was performed with geneticin and surviving clones were isolated and subjected to screening for androgen-dependent iodide uptake activity. NP-1, the LNCaP cell clone with the highest androgen-dependent iodide uptake activity was chosen for the studies as well as the stably transfected LNCaP control cell lines P-1 (PSA-pEGFP-1) and N-1 (NIS-pEGFP-1).

Cell culture and treatments

LNCaP, PC-3, and DU-145 cells were grown in RPMI 1640 medium (Life Technologies, Inc., Karlsruhe, Germany) supplemented with 10% fetal bovine serum (Life Technologies, Inc.) at 37°C and 5% CO₂. Following incubation in serum-free RPMI 1640 medium for 24 h or transient transfection, respectively, cells were incubated with mibolerone (mib; synthetic androgen, which is not metabolized by LNCaP cells and has similar affinity to the androgen receptor as dihydrotestosterone) (10⁻¹¹ to 10⁻⁷ M), atRA (10⁻⁸ to 10⁻⁶ M) (Sigma, Taufkirchen, Germany), 9-cis-retinoic acid (9-cis-RA), TTNPB (4-[E2-5,6,7,8-tetrahydro-5,6,8-triamethyl-2-naphthylenyl-1-propeny]I) (Sigma), and the antiandrogen casodex (bicalutamide, 10⁻⁶ M, a generous gift from Pharmacia, Macclesfield, UK) in the presence of 10% charcoal-treated fetal bovine serum for 24–72 h.

Cell proliferation assay

Cell proliferation was measured using the commercially available MTS-assay (Promega Corp., Mannheim, Germany) according to the manufacturer’s recommendations. Cells were incubated with freshly prepared MTS [3-(4,5-dimethylthiazol-2)-yl-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium]/phenazine methosulfate solution (ratio 1:1 by volume) for 1.5 h at 37°C in a humidified 5% CO₂ atmosphere. The absorbance of the formazan product was read at 490 nm, which is directly proportional to the number of living cells in culture.

Iodide uptake studies

Uptake of 125I by untreated and treated prostate cancer cells was determined at steady-state conditions as described by Weiss et al. (24). In brief, cells were plated on 6-well plates (2 × 10⁶ cells/well) and following incubation with mib (10⁻¹¹ to 10⁻⁷ M) and atRA (10⁻⁸ to 10⁻⁶ M), respectively, iodide uptake studies were performed in Hanks’ balanced salt solution (HBSS) supplemented with 10 μM NaI, 0.1 μCi Na 125I/ml, and 10 mM HEPES (pH 7.3). A 100-μM concentration of KClO₄ 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. Results were normalized to cell survival measured by cell proliferation assay (see above) and expressed as cpm/A490 nm.

Measurement of iodide organification

Cells, grown in 6-well plates, were incubated for 2 h at 37°C with HBSS supplemented with 10 μM NaI, 0.1 μCi Na 125I/ml, and 10 mM HEPES (pH 7.3). Contents of organified iodide in the cells were determined by trichloroacetic acid precipitation as described by Urabe et al. (25).

Iodide efflux studies

Efflux of 125I was determined as described by Weiss et al. (24). In brief, cells were plated on 6-well plates (2 × 10⁶ cells/well), following incubation with mib (10⁻⁷ M) and atRA (10⁻⁷ M), respectively, cells were incubated with HBSS supplemented with 10 μM NaI, 0.1 μCi Na 125I/ml and 10 mM HEPES (pH 7.3) at 37°C for 1 h. Medium was then replaced every 5 min with fresh HBSS. The content of 125I in the collected supernatant was measured by γ-counter. After the last time point, trapped 125I was removed from cells by a 20-min incubation in 1 N NaOH and measured by γ-counting.

Membrane preparation

Cell membranes were prepared from treated and untreated LNCaP cells by a modification of a previously described procedure (26). In brief, cells plated on 100-mm dishes were washed with PBS, harvested, and resuspended in buffer A, consisting of 250 mM sucrose, 10 mM HEPES (pH 7.5), 1 mM EDTA, 10 μg/ml leupeptin, 10 μg/ml aprotinin, and 1 mM phenylmethanesulfonyl fluoride. The homogenate was centrifuged twice at 5000 × g for 15 min at 4°C. Following centrifugations, 100 μl 1 N Na₄CO₃/ml buffer A was added to the supernatant and incubated at 4°C for 45 min with continuous shaking. Then a further centrifugation at 100,000 × g was performed for 15 min, and the pellet was resuspended in an appropriate volume of buffer B consisting of 250 mM sucrose, 10 mM HEPES (pH 7.5), and 1 mM MgCl₂. Protein concentrations were determined by a protein assay (DC protein assay, Bio-Rad Laboratories, Inc., Munich, Germany).

Western blot analysis

For Western blot analysis, the NuPAGE electrophoresis system (Invitrogen, Karlsruhe, Germany) was used. Equal amounts of membrane
protein as determined by DC protein assay (10 μg) (Bio-Rad Laboratories, Inc.) were reduced by incubation with 0.5 M dithiothreitol for 10 min at 70°C and loaded on 4–12% Bis-Tris–HCl buffered polyacrylamide gels. Following gel electrophoresis for 1 h, proteins were transferred to nitrocellulose membranes using electroblothing, and transfer of equal amounts of membrane protein was confirmed by colloidal gold staining (Bio-Rad Laboratories, Inc.). Following blotting, membranes were preincubated for 1 h in 5% low-fat dried milk in TBS-T (20 mM Tris, 137 mM NaCl, and 0.1% Tween-20) to block nonspecific binding sites. Membranes were then incubated with a mouse monoclonal antibody directed against amino acid residues 468–643 of human NIS (dilution 1:3000) (27) for 2 h at room temperature. After washing with TBS-T, horseradish peroxidase-labeled goat-antimouse antibody was applied (dilution 1:5000) for 1 h at room temperature before incubation with enhanced chemiluminescence Western blotting detection reagents (Amersham, Braunschweig, Germany) for 1 min. Exposures were made at room temperature for approximately 1 min using BIOMAX MR films (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. For quantitative analysis computer-assisted densitometric analysis of band intensities was performed.

RNA preparation and Northern blot analysis

Total RNA was isolated from untreated and treated LNCaP cells, respectively, by the modified acid guanidinium thiocyanate-phenol-chloroform method according to Chomczynski and Sacchi (28) using the Rneasy Mini kit (QIAGEN, Hilden, Germany). Aliquots (15 μg) of RNA were electrophoresed on a 1% agarose gel containing 2 M formaldehyde and transferred overnight in 20× saline sodium citrate (SSC) to a positively charged nylon membrane (QIAGEN). The human NIS-gene specific cDNA-fragment (nucleotides 1184–1667), which was generated as described previously (29), was radiolabeled with [α-32P]dATP by random priming (Amersham), and used as a hybridization probe. Blots were prehybridized at 68°C in hybridization solution (Express Hyb solution, CLONTECH Laboratories, Inc., Heidelberg, Germany) for 30 min, followed by hybridization at 68°C for 1 h. Blots were then rinsed four times in 2× SSC/0.1% SDS at room temperature for 10 min and twice in 0.1× SSC/0.1% SDS at 50°C for 20 min, respectively. Exposures were made at ~80°C for 48 h using X-OMAT AR films (Kodak). To strip off the NIS cDNA probe, blots were treated in 0.5% SDS at 95°C for 10 min and reprobed with a rat β-actin cDNA probe to monitor RNA integrity and quantity. Computer-assisted densitometric analysis of band intensities was performed, and NIS measurements were normalized for β-actin signal intensity.

In vitro clonogenic assay

The in vitro clonogenic assay was performed as described by Mandell et al. (30). In brief, untreated and treated LNCaP cells were grown to 50% confluency, washed with HBSS, and incubated for 7 h with 10 ml of 80 μCi/ml Na125I in HBSS supplemented with 10 μM Nai and 10 mM HEPES at pH 7.3. Following incubation with radioidine, cells were washed with HBSS, trypsinized using 0.05% trypsin-EDTA, counted, 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, stained with crystal violet, and colonies containing more than 50 cells were counted. Parallel experiments were performed for each cell line using HBSS without 125I, and all values were adjusted for plating efficiency. The percentage of survival represents the percentage of cell colonies after 125I treatment, compared with mock treatment with HBSS. Results are expressed as means ± SEM of quadruplicates. Statistical significance was tested using t test.

Statistical methods

All experiments were carried out in triplicates. Results are presented as mean ± SEM of triplicates. Statistical significance was tested using t test.

Results

Iodide uptake studies in vitro

The effect of atRA on iodide accumulation was examined in LNCaP cells following PSA promoter-directed NIS gene transfer (NP-1) as well as in control cell lines P-1, N-1, and in androgen-independent control cell lines PC-3 and DU-145. For this purpose cells were incubated with or without mib (10−9 M) and atRA (10−9 to 10−6 M), respectively, and iodide accumulation was measured using an iodide uptake assay (Fig. 1, A–E). A 48-h treatment with atRA (10−7 to 10−7 M) increased androgen-dependent and perchlorate-sensitive iodide accumulation in NP-1 cells approximately up to 1.45-fold in a concentration-dependent and time-dependent manner. Maximal stimulation of iodide accumulation was seen after 48 h at 10−7 M atRA (Fig. 1, A and C). The iodide uptake following incubation with mib and atRA was completely blocked by the pure androgen receptor antagonist casodex (10−6 M), indicating that the effect of atRA is androgen receptor dependent (Fig. 1A). No iodide accumulation above background level was observed in androgen-deprived NP-1 cells when incubated in the presence or in the absence of atRA (10−9 to 10−6 M) (Fig. 1A). Iodide accumulation in NP-1 cells was stimulated by mib (10−11 to 10−7 M) in a concentration-dependent manner with maximal stimulation at 10−8 M mib (Fig. 1B). To evaluate whether the stimulation of iodide uptake is retinoic acid receptor (RAR) mediated, we examined the effect of several retinoid compounds on iodide accumulation in NP-1 cells (Fig. 1D). The RAR-specific ligand TTPNB increased iodide uptake in NP-1 cells in the presence of mib (10−9 M) to the same level as atRA, suggesting that RAR mediates the effect. 9-cis-RA, which activates RAR as well as retinoid X receptor, also had a similar effect as atRA on iodide accumulation in NP-1 cells. Furthermore, in contrast to NP-1 cells, no perchlorate-sensitive iodide uptake was observed in P-1 and N-1 cells incubated in the presence or absence of mib or atRA, respectively (Fig. 1E). No iodide accumulation was detected in control cell lines PC-3 and DU-145 following transient transfection with NIS/PSA-pEGFP-1 in the presence or absence of mib and atRA, respectively (Fig. 1E). Taken together, treatment with atRA significantly increased androgen-induced iodide accumulation in NP-1 cells in a concentration- and time-dependent manner. This effect has been shown to be RAR mediated and androgen receptor dependent.

Measurement of iodide organification

To investigate whether trapped iodide is organified in LNCaP cells following PSA promoter-directed NIS gene transfer (NP-1) and determine the effect of atRA on iodide organification, cells were incubated with or without atRA (10−7 M) in the presence of mib (10−9 M) and iodide organification was measured by trichloroacetic acid precipitation of cell lysate after the incubation of the cells with 0.1 μCi Na125I/ml (Fig. 2A). No organification was observed in NP-1 cells incubated with mib alone, which was not changed by additional treatment with atRA.

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FIG. 1. A, Effect of atRA on iodide accumulation in LNCaP cells following PSA promoter-directed NIS gene transfer. LNCaP cells stably expressing NIS under the control of the PSA promoter (NP-1) were incubated with or without mib (10^{-9} M) and atRA (10^{-9} to 10^{-6} M), respectively, and iodide accumulation was measured as described by Weiss et al. (24). A 48-h incubation with atRA in the presence of mib increased androgen-dependent and perchlorate-sensitive iodide accumulation in NP-1 cells up to 1.45-fold in a concentration-dependent manner. No perchlorate-sensitive iodide uptake was observed in androgen-deprived cells in the presence or absence of atRA. The iodide uptake following incubation with mib and atRA was completely blocked by the androgen receptor antagonist casodex. Results have been normalized to cell proliferation measured by cell proliferation assay and are expressed as percent of control. Values are mean ± SEM of triplicate experiments. *, P < 0.01. B, Concentration-dependent stimulation of iodide uptake in LNCaP cells following PSA promoter-directed NIS gene transfer by Spitzweg et al. - Stimulation of NIS Expression by Retinoids.
Iodide efflux studies

To determine the effect of atRA on iodide efflux in LNCaP cells following PSA promoter-directed NIS gene transfer (NP-1), cells were incubated with or without atRA (10⁻⁹ M) in the presence of mib (10⁻⁹ M) and an iodide efflux assay was performed (Fig. 2B). Iodide efflux was rapid in NP-1 cells, more than 70% of the accumulated ¹²⁵I was released into the medium during the initial 5 min, and treatment with atRA had no effect on the kinetics of iodide efflux.

Northern blot analysis

The effect of atRA on NIS mRNA expression was examined in LNCaP cells following PSA promoter-directed NIS gene transfer (NP-1). For this purpose cells were incubated with or without mib (10⁻⁹ M) and atRA (10⁻⁹ to 10⁻⁶ M), respectively, and NIS RNA steady-state levels were examined by high-stringency Northern blot analysis using a 32-P-labeled human NIS-specific cDNA probe (Fig. 3). Following a 48-h incubation with atRA (10⁻⁹ to 10⁻⁶ M) in the presence or absence of mib (10⁻⁹ M), NIS mRNA was detected as a single species of approximately 4 kb (Fig. 3, top). When normalized for β-actin mRNA signal intensities (Fig. 3, middle), treatment with atRA (10⁻⁹ to 10⁻⁷ M) increased NIS mRNA levels up to 3-fold in a concentration-dependent manner (Fig. 3, bottom). No NIS mRNA expression was detected in androgen-deprived NP-1 cells when incubated in the presence (data not shown) or absence of atRA (10⁻⁹ to 10⁻⁶ M).

Western blot analysis

The effect of atRA on NIS protein expression was examined in LNCaP cells following PSA promoter-directed NIS gene transfer (NP-1). For this purpose cells were incubated with or without mib (10⁻⁹ M) and atRA (10⁻⁹ to 10⁻⁶ M), respectively, and NIS protein expression levels were examined by Western blot analysis using a mouse monoclonal human NIS-specific antibody. NIS protein was detected as a band of approximately 90 kDa (Fig. 4, top). Treatment with atRA (10⁻⁹ to 10⁻⁷ M) increased NIS protein levels up to 2.3-fold in a concentration-dependent manner (Fig. 4, bottom). No NIS protein expression was detected in androgen-deprived NP-1 cells when incubated in the presence (data not shown) or absence of atRA (10⁻⁹ to 10⁻⁶ M).

In vitro clonogenic assay

To determine the effect of atRA treatment on cytotoxicity of ¹³¹I in vitro in NP-1 and P-1 cells incubated in the presence or in the absence of androgen a clonogenic assay was performed (Fig. 5). Whereas only about 15% of P-1 cells were killed by exposure to ¹³¹I, approximately 50% of NP-1 cells incubated for 48 h with 10⁻⁷ M mib were killed in an in vitro clonogenic assay. Following treatment with 10⁻⁷ M atRA for 48 h in the presence of 10⁻⁹ M mib, approximately 90% of NP-1 cells were selectively killed by ¹³¹I. In androgen-deprived NP-1 cells incubated in the presence or absence of atRA, the cytotoxic effect of ¹³¹I was similar to the one seen in P-1 cells (data not shown). No cytotoxic effect of atRA (10⁻⁷ M) was detected in NP-1 cells incubated in the presence or absence of androgens without ¹³¹I.

Discussion

The findings in the present study demonstrate the effect of retinoids on androgen-dependent expression of the NIS and therapeutic response to ¹³¹I in LNCaP cells stably expressing the human NIS gene under the control of the PSA promoter. NIS mRNA and protein levels as well as iodide accumulating activity were stimulated following treatment with atRA plus androgen. This resulted in a significant increase of the efficacy of radioiodine therapy in prostate cancer cells following PSA promoter-directed NIS gene transfer.

NIS is an intrinsic membrane protein that mediates the active iodide uptake across the basolateral membrane of benign and malignant thyroidal cells (2–4, 31). Diagnostic imaging and effective therapy of differentiated thyroid carcinomas and their metastases by administration of radioiodine are based on thyroidal expression of functionally active NIS protein. Since cloning of the NIS gene in 1996, several investigators explored the capacity of NIS gene transfer into nonthyroidal tumor cells to induce radioiodine accumulation, thereby allowing radioiodine therapy of extrathyroidal malignancies. Using different gene delivery techniques, including electroporation, liposomes, and adenoviral and retroviral.
roviral vectors, radioiodine accumulation was induced in vitro and in vivo in a variety of cancer cell lines (dedifferentiated thyroid cancer cells; glioma and neuroblastoma cells; melanoma, cervix, breast, lung, liver, colon, and ovarian carcinoma cells) by NIS gene delivery (30, 32–40). In an earlier study, we explored the efficacy of NIS gene transfer to induce accumulation as well as a therapeutic effect of $^{131}$I in stably transfected prostate cancer cells (12, 13) using the PSA promoter to target NIS gene expression to prostate cells, thereby minimizing extratumoral cytotoxicity (6, 8, 9, 11). 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 and in vivo. Furthermore, the amount of accumulated $^{131}$I was shown to be sufficiently high to selectively kill NIS-transfected LNCaP cells in vitro as well as in vivo in LNCaP cell xenografts (12–14). These studies clearly showed for the first time that tissue-specific NIS gene delivery into nonthyroidal tumors is capable of inducing the accumulation of therapeutically effective radioiodine doses in vitro and in vivo and might therefore represent an effective and potentially curative therapy for extrathyroidal tumors, in particular prostate cancer.

In search for new therapeutic strategies for prostate cancer, it has been found that prostate cancer cell proliferation is modulated by androgens and a number of other factors including retinoids. Retinoids are known to play a crucial role in the control of normal epithelial cell proliferation and differentiation. They induce apoptosis and inhibit carcinogenesis, growth, invasion, angiogenesis, and metastasis of a variety of tumor types, including prostatic carcinoma cells (15, 21–23, 41, 42). Epidemiologic studies have presented evidence that retinoid deficiency plays a role in the development of prostate cancer (43, 44). Therefore, treatment with retinoids might offer a new avenue for therapy of androgen-dependent as well as androgen-independent prostate cancer. To examine the response of prostate cancer cells to retinoids, in particular regarding their proliferation, differentiation...
and androgen responsiveness, many investigators have used the LNCaP cell model because the growth of these cells remains androgen responsive in vitro, and many of their differentiated functions such as production of acid phosphatase and PSA are androgen regulated (23, 45–48). In addition, LNCaP cells express not only high concentrations of an androgen receptor but they also have receptors for other members of the nuclear receptor family, such as retinoids (41). The action of retinoids is mediated through two families of nuclear receptors. RARα, RARβ, and RARγ are activated by both atRA and 9-cis-RA, and the retinoid X receptors α, β, and γ are activated by 9-cis-RA only (49, 50). All of these six forms of retinoid receptors are expressed in LNCaP cells (41).

In the current study, we examined the effect of retinoids on androgen-dependent NIS expression and therapeutic response to 131I in LNCaP cells (NP-1) that stably express the human NIS gene under the control of the PSA promoter. NIS mRNA as well as protein levels in NP-1 cells were stimulated in a concentration- and time-dependent manner following incubation with atRA (10^{-9} to 10^{-7} M). Hybridization with β-actin probe served as a control (middle). Computer-assisted densitometric analysis of band intensities was performed, and NIS measurements were normalized for β-actin signal intensity (bottom). *P < 0.01; **P < 0.001.

In the control experiments without 131I no cytotoxic effect of atRA was detected in NP-1 cells treated with atRA in the presence or absence of androgens suggesting that the increased therapeutic effect of 131I was due to enhanced NIS
protein expression and iodide accumulation. The fact that androgen-independent prostate cancer cells without PSA expression (PC-3, DU-145) did not reveal iodide uptake activity following PSA promoter-directed NIS gene transfer in the presence or absence of atRA demonstrates that atRA-induced stimulation of functional PSA promoter-directed NIS expression is selective for PSA-expressing cells and is therefore not expected to cause toxic side effects in other tissues. These data further suggest that the stimulatory effect of atRA on PSA promoter-directed NIS expression in LNCaP cells is mediated through the PSA promoter.

The growth inhibitory effects of retinoids on LNCaP cells appear to depend on a number of factors, including the passage number of the cells, concentration of RA, and presence or absence of androgens (22, 23, 28, 41–53). The growth inhibition of LNCaP cells by retinoids was shown to be mediated by an androgen receptor-dependent mechanism preceded by the induction of androgen receptor gene expression (21). Also, differentiated function of prostate cells, as measured by the secretion of PSA, is enhanced by retinoids (22, 23). Other studies showed stimulation of RARα and -γ gene expression in LNCaP cells by androgens, suggesting that RAR-mediated processes might be involved in androgen regulation of prostate cells (54). In our study the stimulatory effect of atRA on androgen-induced iodide accumulation in LNCaP cells stably expressing NIS under the control of the PSA promoter was completely blocked by the androgen receptor antagonist casodex, indicating that the effect is androgen receptor mediated. AtRA-mediated stimulation of NIS gene and protein expression in LNCaP cells following PSA promoter-directed NIS gene transfer might be caused by an increase in androgen-receptor expression, thereby enhancing androgen-mediated activation of PSA promoter activity. Future studies assessing the exact interactions of androgen- and retinoid-regulated processes in prostate cancer cells are needed to fully understand the mechanisms by which atRA treatment enhances PSA promoter-directed NIS expression.

Decreased intercellular adhesion following treatment with atRA might result in enhanced NIS expression and iodide accumulation in our LNCaP cell model by increasing cell surface available for NIS protein expression and by an increased number of NIS molecules being in contact with the radioiodine-containing cell medium. Retinoic acid has been reported to affect the morphology of prostatic epithelial cells that form cell colonies containing widely spaced cells lacking intercellular adhesion when incubated in the presence of RA (42). Similar morphological changes were seen by Young et al. (51) in LNCaP cells following treatment with atRA in vitro. Further experiments are required to examine the effects of RA treatment-associated morphological changes of LNCaP cells on NIS gene and protein expression as well as iodide accumulation activity.

Potential adverse effects of androgen application in prostate cancer patients have to be considered before NIS gene therapy using the androgen-dependent PSA promoter (55). Although long-term application of androgen in these patients is certainly not recommended, exposure to androgen for only a short time period (24–48 h), which would be sufficient for PSA promoter-directed NIS gene transfer, seems to be acceptable, in particular because it serves as a prerequisite for the application of 131I, a potentially curative
therapeutic approach as our previously reported data have clearly shown (12–14). In addition, in our current study, we show that treatment with atRA increases androgen-induced functional NIS expression, which not only enhances the killing effect of $^{131}$I in LNCaP cells stably expressing NIS under the control of the PSA promoter but also allows reduction of the applied androgen dose.

In conclusion, treatment with atRA increases androgen-induced functional NIS expression levels, thereby significantly enhancing the selective killing effect of $^{131}$I in LNCaP cells stably expressing NIS under the control of the PSA promoter. Thus, systemic retinoid treatment might be considered as a potent adjunct to radioiodine therapy following tissue-specific NIS gene transfer, a novel and promising cytokereductive gene therapy strategy for nonthyroidal tumors.

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