Decoy Androgen-Responsive Element DNA Can Inhibit Androgen Receptor Transactivation of the PSA Promoter Gene

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Abstract. Chemotherapy, hormonal therapy, or surgery may cause devastating toxic or other side effects. Androgen receptors (ARs) in the cytoplasm are activated by binding with androgen. Androgen-activated ARs bind to a specific genomic DNA sequence, the androgen-responsive element (ARE), and initiate gene expression at the transcriptional level. Even without androgen activation, ARs may have a role in androgen-refractory prostate cancer. Thus, inhibition of AR activity may have therapeutic value. We applied a genetic reporter of the Dual-Luciferase Assay System to test whether a short double-stranded genomic DNA containing prostate-specific antigen (PSA) ARE sequence as decoy DNA would inhibit the function of activated AR. A 21-mer phosphorothioated PSA ARE decoy DNA was synthesized, with a plasmid vector containing the PSA promoter upstream from a luciferase gene, the reporter gene. The promoter and reporter were co-transfected into a human prostate cancer cell line PC3-M with the aid of Lipofectamin 2000. After 24 hr exposure to androgens, the cells were lysed and luciferase activity measured to determine the ARE decoy inhibitory effect on the function of ARs. Luciferase activity was reduced significantly in the ARE decoy transfected cells but not with inactive control decoy. The results demonstrate that ARE decoy DNA can effectively suppress androgen-activated ARs in prostate cancer cells and indicate the potential utility of decoy DNA for developing a novel therapy for prostate cancer. (received 10 December 2004; accepted 19 March 2005)

Keywords: androgen receptor, androgen-responsive element, prostate cancer, reporter gene

Introduction

Androgens are essential for the development, differentiation, and normal function of the prostate gland [1]. They exert their function through the intracellular androgen receptor (AR) [1]. AR is a ligand-dependent transcription factor that requires activation by androgen. After activation, AR moves from the cytoplasm into the nucleus and binds to a specific DNA sequence, the androgen-responsive element (ARE), at the promoters of androgen target genes, including the prostate-specific antigen (PSA) gene [2]. The binding of AR to ARE initiates the transcription of these genes [1-4].

ARs are believed to have a major role in the development and progression of prostate cancer [1,5-8]. By repressing AR function, hormonal therapy is a major option for managing advanced prostate cancer. Although most patients initially respond well to treatment, >80% eventually become refractory to hormonal therapy. Strong evidence suggests that ARs are still active in prostate cancer cells in these patients [8]. In fact, ARs are activated by several peptide growth factors or cytokines and stimulate the growth of prostate cancer cells independently of androgens [9]. Therefore, finding a novel way to
block AR activity would be useful for treating hormonal-refractory prostate cancer.

PSA is expressed specifically in epithelial cells of the prostate [10,11] and is regulated by androgens [4]. PSA is a biomarker for detecting prostate cancer and monitoring androgen action [1-3]. The proximal region of the PSA gene promoter contains 1 ARE (AGAACAgcaAGTGTC) at position -170 from its transcription start site [1,2]. Activated ARs bind this responsive element [1,2], causing up-regulation of PSA expression.

A transcriptional factor decoy strategy is the use of short double-stranded oligodeoxynucleotides containing a high-affinity binding site for specific transcription factors as a decoy DNA to be transfected into target cells [12-16]. Inside the cells, the decoy DNA competes with the endogenous high-affinity binding site of the target genes for binding to specific transcription factors, and consequently inhibits activated AR function [16]. Decoy DNA has potential for treatment of cardiovascular disease [12]. It also induces apoptosis in certain cell lines [13].

Our study combined the decoy strategy with a genetic reporter system of a luciferase gene to observe the effect of ARE decoy DNA on the function of ARs in human prostate cancer cells by measuring the light generated by luciferase.

Materials and Methods

Construction of pGL3-PSA plasmid from pGL3-basic plasmid.

To prepare the PSA promoter fragment for insertion, a 6-kb human PSA promoter DNA was used as a template with a pair of a 5’-primer and a 3’-primer for the polymerase chain reaction (PCR) to generate a 640-bp fragment spanning the proximal PSA promoter region (from -607 to +33), which contains 2 endonucleases, SacI and Kpn1, restriction sites at the 5’ and 3’ ends, respectively. The PCR product was digested with these 2 restriction enzymes, purified with agarose gel, and inserted upstream from a luciferase reporter gene (Luc+) into a pGL3-basic vector containing 2 adjacent endonuclease cutting enzymes and gel electrophoresis. It was verified further by DNA sequencing. The recombinant constructed plasmid was named pGL3-PSA (Fig. 1). In addition to the pGL3-basic plasmid vector, the other 2 plasmid vectors used in our study were pGL3-control and pRL-TK. Both are available commercially (Promega, Madison, WI). The pGL3-control plasmid can express luciferase as a reporter constitutively by its SV-40 promoter. It was used to monitor the maximum transfected luciferase activity. The vector pRL-TK expresses renilla luciferase constitutively by its thymidine kinase promoter. When co-transfected with other experimental vectors, it was used to monitor and normalize the transfection efficiency of the experimental vector, pGL3-PSA vector, or the pGL3-control vector.

Synthesis of oligodeoxynucleotides of ARE and control decoy.

The ARE decoy was designed on the basis of the DNA sequence (-173 to -153) of the ARE in the proximal promoter region of the PSA gene. An unrelated sequence was also synthesized and used in the control studies. In both ARE and control decoys, phosphothioated nucleotides were used to prevent the decoy DNA from enzyme degradation.

ARE decoy: 5’-TGC AGA ACA GCA AGT GCT AGC-3’;
3’-AGG TCT TGT GCT TCA CGA TCG-5’
Control decoy: 5’-GTC TGA TAA AGG GTG TTC TTT TT-3’;
3’-CAT ACT ATT TCC CAC AAG AAA AA-5’

To obtain a perfectly matched double-stranded DNA decoy, each pair of complement oligodeoxynucleotides, either ARE decoy or control decoy, was dissolved in TEN buffer (10 mmol/L Tris-HCl, 0.1 mmol/L EDTA, 0.1 mol/L NaCl, pH 8.0) at equal molar concentrations, heated to 95°C for 10 min to completely dissociate the pair, and then slowly cooled to room temperature for annealing to a perfectly matched pair.

Cell culture.

Human prostate cancer cell line PC3-M, an immortalized cell line that lacks PSA expression, was maintained in RPMI 1640 medium supplemented with 5% calf bovine serum, 100 units/ml penicillin, and 0.1 mg/ml streptomycin at 37°C in a 5% CO₂ incubator.

Transfection.

PC3-M cells (from Shanghai Institutes for Biological Science [SIBS], Chinese Academy of Science, Shanghai, China) at a density of 2 x 10⁵ cells per well were seeded in a 12-well plate and incubated for 18 to 24 hr. When cells reached 90% to 95% confluency, double-stranded decoy DNA and plasmids were cotransfected into PC3-M cells by Lipofectamin 2000, according to the manufacturer’s protocol (Invitrogen, Carlsbad, CA). Briefly, 0.4 to 1.6 μg of decoy DNA and double-stranded decoy DNA and 1-4 μl of lipofectamin were used per well. The cells were incubated with 0.8 ml of serum-free medium during the 5-hr transfection. Plasmid pRL-TK (7.6 ng/ml), expressing renilla luciferase, was used as an internal control. The ratio of pRL-TK and experimental vector was 1:25 (7.6 ng/ml:190 ng/ml, respectively).

Luciferase assay.

At 24 hr after transfection and exposure to testosterone, the cells were washed once with PBS and lysed with 1x passive lysis buffer (Promega) without actively scraping.
Fig. 1. pGL3-PSA plasmid map. A prostate-specific antigen (PSA) promoter DNA fragment containing an androgen-response element (ARE) was inserted immediately upstream from the reporter gene, luciferase.

Fig. 2. The requirements of both androgen receptors and testosterone for prostate-specific antigen (PSA) promoter-mediated transcription of the luciferase reporter. Luciferase activity was measured in PC3-M cells after co-transfections with increasing amounts of pGL3-PSA and a fixed amount of androgen-receptor expression plasmid in the presence of testosterone. The relative light intensities of luciferase were determined.

Fig. 3. Determination of optimal concentrations of pGL3-PSA in transfections: Luciferase activity was measured in PC3-M cells after co-transfections with increasing amounts of pGL3-PSA and a fixed amount of androgen-receptor expression plasmid in the presence of testosterone. The relative light intensities of luciferase were determined.

Fig. 4. Determination of optimal concentrations of androgen-receptor (AR) expression vector in transfections. Luciferase activity was measured in PC3-M cells after co-transfection with increasing amounts of AR expression plasmid and a fixed amount (190 ng/ml) of pGL3-PSA in the presence of testosterone.
The Dual-Luciferase Reporter Assay System (Promega) was used to automatically measure the 2 reporter enzyme activities of firefly and renilla luciferases sequentially. The luminometer was programmed to perform a 2-sec premeasurement delay, followed by a 10-sec measurement for each reporter luciferase activity. Then 100 µl of Luciferase Assay Reagent II (Promega) and 20 µl of cell lysate were mixed in the luminometer tube in the luminometer for measuring firefly luciferase activity of the pGL3-PSA plasmid. In the same luminometer tube, 100 µl of Stop & Glo Reagent (Promega) was added for measuring renilla luciferase activity from the pRL-TK plasmid. The firefly luciferase requires ATP and oxygen, whereas the renilla luciferase requires only oxygen; the function of the Stop & Glo reagent is to quench firefly luciferase activity and start renilla luciferase activity. Both firefly and renilla luciferase activities were measured automatically with the Dual-Luciferase Reporter Assay System (Promega). To eliminate variation in transfection efficiency, the firefly luciferase activity was divided by the renilla luciferase activity of each tube and expressed as relative light units. Each experiment was repeated at least 3 times.

Statistical analyses. All luciferase measurements were analyzed and expressed as the mean ± SE. Results were considered significant if p < .05 was obtained by an appropriate ANOVA procedure and Student’s t-test.

Results

Androgen and AR regulation of luciferase by the PSA promoter. To demonstrate that the PSA promoter can regulate the expression of the reporter luciferase in the presence of androgens and AR, 190 ng/ml of pGL3-basic, pGL3-control, or pGL3-PSA vector was co-transfected with pRL-TK vector into PC3-M cells with or without AR expression vector and then treated with or without 100 nM of testosterone for 24 hr (Fig. 1). An excess amount of testosterone, 100 nM, was used because testosterone may be easily destroyed in the cell culture system during 24-hr incubation.

Luciferase activity is shown in Fig. 2. Because pGL3-basic plasmid vector has no promoter, it showed no luciferase activity, even in the presence of testosterone (lane 1). The pGL3-control vector containing its own SV-40 promoter expressed relatively high luciferase activity constitutively (lane 2). It required no ARE promoter to express luciferase activity. The pGL3-PSA showed low basal levels of luciferase activity with either androgens or AR alone (lanes 3 and 4) because the system required both androgen and AR to generate full activity. It could produce a high reporter activity similar to that of pGL3-control vector only in the presence of both androgens and AR (lane 5).

Testing concentrations of pGL3-PSA and AR vectors for transfection. To determine the suitable ratio of pGL3-PSA and AR expression vectors for transfection, the AR expression vector was tested at a temporarily fixed concentration of 1.5 µg/ml to co-transfect with increasing amounts of pGL3-PSA into PC3-M cells. At a concentration of 375 ng/ml, pGL3-PSA produced maximal reporter activity (Fig. 3). Next, pGL3-PSA at a concentration of 190 ng/ml was co-transfected with increasing amounts of AR expression vector into PC3-M cells (Fig. 4). We found that AR at 380 ng/ml generated maximal reporter activity.

Comparisons of control and ARE decoy at increasing concentrations. From the results shown in Figs. 3 and 4, we chose 190 ng/ml of AR expression vector and 190 ng/ml of pGL3-PSA vector for the following decoy co-transfection experiments. We chose both 190 ng/ml for AR expression vector and 190 ng/ml for pGL3-PSA vector because they were the next lower concentration of maximum response in order to obtain the most sensitive reporter measurements for monitoring decoy activity. We tested the efficacy of the ARE decoy DNA (0 to 2.64 µg/ml) and compared it with that of control decoy (0 to 1.98 µg/ml) for the inhibition of AR function on the activation of PSA transcription (Fig. 5). The control decoy showed no inhibition of the reporting activity. The ARE decoy demonstrated dose-dependent inhibitory activity. At the concentration of 1.98 µg/ml of ARE decoy, the inhibition rate could reach 95% (Fig. 5), which almost completely diminished the function of ARs.

Duration of inhibitory effect. Next, we determined the duration of the effect of the ARE decoy DNA on AR function by measuring reporter activity at 24, 48, and 72 hr. As shown in Fig. 6, one dose of ARE decoy DNA of 1.98 µg/ml could inhibit luciferase activity induced by androgens up to 72 hr without significantly reducing its efficacy.
Discussion

Androgens and ARs are risk factors for the development of prostate cancer [1,6,7,17-21]. Considerable information supports the roles of androgens and ARs in the development and progression of prostate cancer, although the evidence is mainly indirect. The removal of androgens or blocking their synthesis can induce apoptosis of both normal and cancerous prostate epithelia in vivo [1,5,6,8,22]. Males who have 5α-reductase deficiency or who were castrated at an early age do not develop benign or malignant prostate tumors [1,5,6]. This evidence provides strong support for androgen deprivation-based chemoprevention and chemotherapy for prostate neoplastic disease. Recently, finasteride, a competitive inhibitor of 5α-reductase, exhibited only a certain degree of effectiveness in a clinical trial for prostate cancer prevention [22]. AR may also be a factor in the development of androgen-refractory prostate cancer [8]. Therefore, blocking AR activity may be a key therapeutic target in prostate cancer.

AR is the mediator of androgen action [1,8]. Without androgens, ARs remain in the cytoplasm, bind to heat shock proteins, and have an inactive form. When activated by androgen, ARs move from the cytoplasm into the nucleus and bind to a specific DNA sequence ARE in the promoter of target genes to initiate gene transcription. The expression of PSA is induced mainly by androgens at the transcriptional level through the interaction of AR with ARE in the PSA promoter [2], making this an ideal model to test our hypothesis. ARE decoy DNA applied exogenously could effectively inhibit the function of ARs that, in turn, blocked androgen and AR function completely. Transfection of cis-element double-stranded oligodeoxynucleotides, the decoy, or decoy DNA, has been reported to be a powerful tool in a new class of anti-gene strategies [12-14]. Transfection of double-stranded oligodeoxynucleotides corresponding to the cis sequence results in the attenuation of authentic cis-trans interaction, leading to the removal of transcription factors from the endogenous cis element and suppression of the expression of regulated genes. Note, the above cis
and trans are referred to as DNA motifs [12-14] and transcription factors that bind specific DNA motifs, respectively. In this experiment, we constructed luciferase gene eukaryotic expression vector by linking the PSA promoter to a luciferase gene, which is a reporter gene. The system required both androgen and AR to generate full activity, as shown in Fig. 2. The effect of ARE decoy on transcription factor AR was studied after co-transfection with the PSA promoter vector into PC3-M cells. The results showed that the ARE decoy could inhibit the transcriptional activity of AR by blocking its DNA binding site to the endogenous ARE sequence, resulting in the inhibition of PSA promoter-mediated luciferase expression. Our studies verified the critical role of ARE for AR-regulated gene expression on androgen activation.

Different cis-element decoy DNA can inhibit the corresponding transcription factor after it is transfected into cells, so it can be used for studying the mechanisms of the cis-element and transcription factor in transcriptional regulation as well as the interaction of cis-element and transcription factor [12]. In addition, transfection of decoy DNA can induce the suppression of cell growth and cell apoptosis by induction of activation of caspase 3 [13]. Decoy oligodeoxynucleotide-induced apoptosis in human primary osteoclasts is being studied as a possible nonviral gene therapy for severe osteoporosis [13]. The goal of the US National Cancer Institute is to eliminate, by 2015, the suffering and death caused by cancer [23]. With regard to prostate cancer, investigations of its molecular basis may lead to the development of treatments that would replace current chemotherapy, hormonal therapy, and surgical therapy and their side effects. To achieve the challenging goal requires successful molecular investigations to build the foundations for clinical studies.

Our results suggest that the strategy of decoy DNA may lead to a novel approach for treating prostate cancer and other neoplasms. This possible therapeutic strategy warrants further study.

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References