ORIGINAL ARTICLE

Prostate Cancer Cells Increase Androgen Sensitivity by Increase in Nuclear Androgen Receptor and Androgen Receptor Coactivators; A Possible Mechanism of Hormone-Resistance of Prostate Cancer Cells

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ABSTRACT

Although androgen-hypersensitivity is one of the possible pathways of hormone-resistance in prostate cancer, the mechanisms of androgen-hypersensitivity are still largely unknown. Using androgen-hypersensitive prostate cancer cells LN-TR2, established from androgen-sensitive LNCaP cells by the long term treatment with tumor necrosis factor α, we explored the mechanisms of androgen-hypersensitivity in prostate cancer cells which may thus play a role in hormone-resistance. We examined the androgen receptor (AR) DNA sequence and the expression levels of AR and 8 AR cofactors in LNCaP and LN-TR2 cells. As a result, no novel mutation was developed in AR DNA in LN-TR2 cells. We observed higher expressions of nuclear AR upon androgen-treatment and 2 AR coactivators, ARA55 and TIF2, in LN-TR2 compared to LNCaP cells. An overexpression of ARA55 or TIF2 enhanced androgen-induced AR transcriptional activity in LNCaP cell. In the presence of those AR coactivators, AR activity was observed even at low concentrations of androgen. In 2 of 6 patients, the expression level of ARA55 was higher in cancer cells in hormone-resistant tumor than those in hormone-sensitive tumor. Taken together, our results suggest that prostate cancer cells change androgen-sensitivity by an overexpression of nuclear AR and AR coactivators, thus, resulting in transition from androgen-dependent to androgen-independent prostate cancer cells. An increase in nuclear AR and AR coactivators may cause androgen-hypersensitivity of prostate cancer cells and thus play a role in hormone-resistance, at least in some patients with prostate cancer.

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Keywords: Prostate cancer, Hormone-resistance, Androgen receptor, Cofactor, TNFα.

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INTRODUCTION

Although more than 80 percent of all patients with prostate cancer initially respond to androgen-deprivation, prostate cancer cells progress from hormone-dependent to hormone-independent under androgen-deprivation therapy and irreversible resistance to androgen-deprivation therapy occurs in the vast majority of patients. Most therapeutic modalities cannot significantly prolong the survival of patients with hormone-refractory prostate cancer. Recent clinical studies of docetaxel-based chemotherapy demonstrated a survival benefit for hormone-refractory prostate cancer for the first time.
(1, 2). In those studies, however, the median survival times were 17.5 months and 18.9 months, which are still unsatisfactory. To explore the effective treatment for patients with hormone-refractory prostate cancer, it is important to understand the mechanisms by which prostate cancer cells become androgen-independent. Androgen-hypersensitivity is one of the possible mechanisms of hormone resistance. When prostate cancer cells become androgen-hypersensitive, they can survive and grow under low concentrations of androgen. We previously observed androgen hypersensitivity after a long term treatment with TNFα.

**METHODS**

**Cell culture**

Androgen-hypersensitive LN-TR2 cells were established from androgen-sensitive LNCaP cells by culturing in the presence of 50 ng/ml of TNFα for more than 3 months (3). LNCaP and LN-TR2 cells were maintained in Dulbecco’s modified Eagle medium (Sigma Chemical Co., St. Louis, MO, USA) containing 5 percent fetal calf serum and 1 percent penicillin/streptomycin (Gibco, Scotland, UK).

**Patients**

A total of 6 patients with pathologically proven prostate cancer (12 matched tumors) were selected for analysis, because tumor samples of hormone-sensitive and hormone-resistant state were available in them. Initial hormone sensitive tumor samples were obtained by needle biopsy or prostatectomy, and the samples after the progression to hormone-resistant cancers were by needle biopsy, transurethral resection, or autopsy.

**RNA extraction, preparation of cDNA, and DNA sequencing**

The total RNA extraction and preparation of cDNA were performed as previously described (4). To identify potential structural mutation at a hinge region (exon 2 and 3) and a ligand-binding domain (LBD, exon 4-8) of the AR gene in LNCaP and LN-TR2 cells, the following primers were employed for the PCR reaction. The PCR primers for amplifying those co-activators included SRC1, ARA54, TIF2, ARA70, RAC3, and FHL2) and a corepressor (SMRT). The PCR primers for amplifying those co-activators were designed according to a published sequence of ARA70 (DNA 613–918 plus 63 bp of the 3′ untranslated region. cDNA (1 µl) was amplified by PCR with AmpliTaq Gold (Perkin-Elmer, Boston, MA, USA) at 94°C for 1 min, annealing at 54°C for 1 minute, and extension at 72°C for 1 min) were followed by a terminal extension at 72°C for 1 min. The PCR products were purified using QIA quick PCR purification kit (Qiagen, Valencia, CA, USA) according to the manufacturer’s instruction. Aliquots of the purified products were sequenced directly using the same primers employed for the initial PCR reaction and a Big Dye terminator V 1.1 Cycle Sequencing kit (Applied Biosystems, Foster City, CA, USA).

**RT-PCR of AR cofactors**

We investigated the expression of 7 coactivators (ARA55, SRC1, ARA54, TIF2, ARA70, RAC3, and FHL2) and a corepressor (SMRT). The PCR primers for amplifying those co-activators were designed according to a published sequence of each target gene. The primers and PCR conditions used in the present study and expected product sizes are shown in Table 1. CDNA (1 µl) was amplified by PCR with AmpliTaq Gold (Perkin-Elmer, Boston, MA, USA) as described above. The 600 bp fragment of GAPDH, amplified with 28 cycles of PCR using a primer set for human GAPDH (Stratagene Japan, Japan), was used to demonstrate comparable RNA amount and quality among samples. Each PCR product was subjected to electrophoresis in 1.5 percent agarose gels containing ethidium bromide and then photographed.

**Preparation of anti-ARA55 polyclonal antibody**

Anti-ARA55 polyclonal antibody was generated with the synthetic peptide SGSEGSSGSDKDH+Cys (codon 28-45 of ARA55) prepared by Funakoshi Co. (Tokyo, Japan). Polyclonal antibody to ARA55, N-99-F1, was generated by multiple immunization of rabbits with the synthetic peptide and purified by affinity column chromatography with the synthetic peptide that was used for immunization. This antibody could detect in vitro transcripted/translated ARA55 protein (data not shown).
Western blot analysis

1 x 10^6 cells were treated with or without 10^{-9} M DHT for 12 hours and harvested. For the detection of AR protein, 50 µg of total protein and 15 µg of nuclear protein extracted from LNCaP and LN-TR2 cells were resolved on 8 percent sodium dodecyl sulfate (SDS)-polyacrylamide gel, then electrotransferred to a P-membrane (Amersham Pharmacia Biotech, Piscataway, NJ, USA). The immobilized proteins were incubated with anti-human AR antibody, NH-27 (5), (1:5000 dilution), anti-ARA55 antibody N-99-F1 (1:500 dilution), and anti-TIF2 antibody GRIP1 (Santa Cruz Biotechnology, CA, USA, 1:500 dilution). The presence of the protein was identified by a 500-fold diluted anti-rabbit antibody conjugated with alkaline phosphatase and stained by Western blot kit (Kirkegaard Perry Lab, MD, USA). The intensity of AR protein bands was quantitated by NIH image 1.61 after an image of the membrane was scanned into a computer.

Transfection and reporter gene assa

Transfections and luciferase assays were performed as previously described (6). Briefly, cells were transected by SuperFect (Qiagen, Valencia, CA < USA) with 1.5 µg of DNA (1.0 µg of pSG5-ARA55, or pSG5-TIF2 0.4 µg of MMTV-LUC and 0.1 µg of pRL-TK) according to SuperFect transfection instructions. After 2–3 hours incubation, cells were treated with medium supplemented with charcoal-stripped FBS containing either ethanol or DHT (10^{-11}–10^{-8} M) for 24 hours. The cells were then harvested and whole cell extracts were used for luciferase assay. The luciferase activity was determined using a Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA) and luminometer. pRL-TK was used for an internal control. At least 3 independent experiments were carried out.

Immunohistochemistry

The prostate cancer tissues were formalin-fixed and paraffin-embedded by standard methods. The 4-µm-thick sections were deparaffinized with xylene and rehydrated through a series of graded ethanol. The sections were incubated in 0.1 percent hydrogen peroxidase in phosphate buffer saline (PBS pH 7.4) for 20 minutes at room temperature to block endogenous peroxidase activity. After being washed in PBS, the sections were preincubated with 10 percent BSA for 20 minutes at 37°C, and subsequently incubated overnight at 4°C with anti-ARA55 polyclonal antibody (N-99-F1) or anti-TIF2 antibody (GRIP1, Affinity BioReagents Inc, CO, USA) at 1:000 or 1:500 dilution in 0.1 M PBS containing 0.5 percent Triton X-100 and 2 percent normal rabbit serum, respectively. Intervening washes in PBS were followed by incubation with a 1:200 dilution of the biotinylated anti-rabbit secondary antibody (Vector Lab., Buringame, CA, USA) in PBS containing 0.5 percent Triton X-100 and 2 percent normal rabbit serum for 120 minutes at room temperature. The sections were finally incubated with avidin-biotin peroxidase complex (Vectastain ABC Elite Kit, Vector Lab., Buringame, CA, USA) in PBA for 90 minutes. Following several washes in PBS, the sections were developed with 0.03 percent 3,3′-diaminobenzidine (DAB) containing 0.15 percent nickel ammonium sulfate and 0.03 percent hydrogen peroxidase in PBS for 3 minutes. The sections were lightly counterstained with Meyer hematoxylin. Substitution of rabbit IgG for the primary antibody was used as a negative control.

RESULTS

No novel mutation developed in AR gene in androgen-hypersensitive cells

We sequenced the region from the hinge to the LBD of AR, because these regions are critical for nuclear translocation and ligand-induced transactivation of AR (7–9). AR DNA sequence in LN-TR2 cells was completely identical to that in LNCaP cells and no mutation was identified except the well-known LNCaP mutation at codon 877: Thr (ACT) to Ala (GCT). Therefore, androgen-hypersensitivity of LN-TR2 cells was not due to mutation in AR gene.

Nuclear AR protein increased in androgen-hypersensitive cell

To determine if increased AR protein contributed to androgen-hypersensitivity of LN-TR2 cells, we compared AR protein level in LN-TR2 and LNCaP cells. The total AR protein level in LN-TR2 cells was almost identical to that in LNCaP cells (Figure 1A). However, nuclear AR protein after DHT treatment was much increased in LN-TR2 compared to LNCaP cells. DHT induced a 4-fold increase in nuclear AR protein level in LN-TR2 cells compared to only a 2-fold increase in LNCaP cells at 12 hours (Figure 1B). These results indicate that
androgen-induced increase in nuclear AR protein may contribute to androgen-hypersensitivity of LN-TR2 cells.

Two AR coactivators, ARA55 and TIF2, increased in androgen-hypersensitive cells

Among cofactors examined, the expression levels of mRNA of SRC1, ARA54, ARA70, RAC3, FHL2, and SMRT were almost identical between LNCaP and LN-TR2. Interestingly, ARA55 and TIF2 were well expressed in LN-TR2 cells, but the expression levels of these coactivators were none or little in LNCaP cells (Figure 2A). In Western blotting, ARA55 and TIF2 protein was also well expressed in LN-TR2, but none or a little in LNCaP cells (Figure 2B).

ARA55 and TIF2 can function as an AR coactivator in LNCaP cells

We next examined whether ARA55 and TIF2 can function as an AR coactivator in LNCaP cells, because cofactors’ activities are different among cell types used. As shown in Fig. 3A, the expression level of ARA55 and TIF2 protein in transfected LNCaP cells were almost identical with that in LN-TR2. ARA55 and TIF2 enhanced DHT-induced AR transactivation at every concentration of DHT by about 3-fold and 2-fold, respectively. In the absence of ARA55 or TIF2, AR transactivation was low at lower concentrations of DHT ($10^{-11}$ to $10^{-10}$ M). In the presence of ARA55 or TIF2, however, AR transactivation was much increased even at lower concentrations of DHT and was almost identical to that at higher concentrations of DHT ($10^{-9}$ to $10^{-8}$ M) without those cofactors (Figure 3B). Those observations are suggesting that those coactivators can act as an AR coactivator in LNCaP cells and the cells expressing those coactivators can respond well to low concentrations of androgen, resulting in hypersensitive to androgen.

Expression of ARA55 and TIF2 in hormone-sensitive and hormone–resistant prostate cancer tissues

Because the data described above suggested that ARA55 and TIF2 might play a role in the hormone-resistance of prostate cancer cells, we investigated the expression of those AR coactivators in clinical samples. We examined the expression of ARA55 and TIF2 in paired hormone-sensitive and hormone-resistant tumors from the same patient by immunohistochemistry. In 4 patients, ARA55 was well expressed in stromal cells and little in epithelial cells, either malignant or benign cells and immunoreactivity in cancer cells was identical at hormone-sensitive and hormone-resistant state. In 2 patients, however, immunoreactivity of ARA55 was much increased in cancer cells after progression to hormone-resistant tumor (Figure 4B) compared to that in hormone-sensitive tumor (Figure 4A). These results suggest that expression of ARA55 increases after hormone-relapse at least in some patients with prostate cancer. Regarding TIF2,
no obvious difference was observed between hormone-sensitive and hormone-resistant cancer cells in our limited number of cases (data not shown). Those results suggest that expression of ARA55 increases after the progression to hormone-resistant cancer and overexpression of ARA55 may cause androgen-hypersensitivity of prostate cancer cells, resulting in hormone-resistance in a subgroup of prostate cancer cases.

**DISCUSSION**

Multistep and various mechanisms should be involved in the process of prostate cancer progression from androgen-dependent to androgen-independent cancer. The possible mechanisms of androgen-independence are an overexpression of AR protein, mutation(s) of AR, a change in the expression of AR cofactors, the activation of phosphorylation and growth factor pathways, and upregulation in the number of anti-apoptotic genes (10). In the present study, we demonstrated that long term TNFα treatment caused an increase in nuclear AR protein after androgen treatment and an overexpression of AR coactivators that can enhance AR transactivation.

Several reports demonstrated that AR was overexpressed in hormone-resistant prostate cancer and AR overexpression provided prostate cancer cells with androgen-hypersensitivity. Edwards et al. (11) demonstrated that AR gene amplification and an overexpression of AR were noted in hormone-resistant tumors in comparison to matched hormone-sensitive tumors from the same patient. Chen et al. (12) demonstrated that AR-overexpressed prostate cancer cells become androgen-hypersensitive and that a modest increase in the AR concentration permitted the AR to function despite the presence of lower levels of androgen. Gregory et al. (13) also reported a high level expression, increased stability, and nuclear localization of AR in recurrent tumor cells to be associated with an increased sensitivity to the growth-promoting effects of DHT. Recently, Hu et al. (14) found that AR coactivator ARA70 increases the amount of nuclear localized AR by increasing AR protein stability and nuclear translocation upon ligand-treatment. Because the expression levels of AR coactivators, ARA55 and TIF2, were higher in LN-TR2 than LNCaP cells, these coactivators may also contribute to an overexpression of the nuclear AR protein in LN-TR2 cells.

Many mutations in AR DNA in hormone-resistant prostate cancer cells have been identified. In the present study, the AR DNA sequence was identical between androgen-hypersensitive LN-TR2 and LNCaP cells. However, mutations at the boundary of the hinge and LBD of AR have been reported to increase androgen-induced AR transactivation (15). In some cases, therefore, an AR mutation may confer androgen-hypersensitivity.

After the cloning of steroid receptor cofactors, either coactivator or corepressor, it is noted that steroid receptors including AR need cofactors for efficient transcriptional regulation (16). If the coactivators increase or corepressors decrease in the cells, then the androgen-induced AR activity should increase. Out of several cofactors, we examined the expression levels of 8 AR cofactors, because they were well characterized and demonstrated to function as AR cofactors (17–24). Among the cofactors tested, the expression levels of ARA55 and TIF2 were significantly higher in androgen-hypersensitive LN-TR2 in comparison to the parental LNCaP cells. These data suggest that the prostate cancer cells increase the expression of AR coactivators and can thus become androgen-hypersensitive during the process of progression. An immunohistochemical analysis of paired hormone-sensitive and hormone-resistant cancer specimens from the same patient demonstrated the ARA55 expression to be much higher in hormone-resistant than hormone-sensitive cancer in 2 of 6 patients. In these patients, ARA55 might contribute to the hormone-resistance of prostate cancer cells. In the present study, we could not show an increase in TIF2 in hormone-resistant cancer cells. Linja et al. (25) reported the expression level of TIF2 to be identical in untreated and hormone-resistant cancer. Gregory et al. (26), however, reported the expression level of AR coactivators, such as SRC1 and TIF2, to be much higher in hormone-relapsed cancer than hormone-sensitive cancer. Nishimura et al. (27) also demonstrated an overexpression of AR coactivator gelsolin in hormone refractory prostate cancer. The limited sample number or the different methods used (immunohistochemistry, RT-PCR, or immunoblot) may explain, in part, the discrepancy among the results by different authors. In the CWR22 prostate xenograft system, in which the CWR22 tumors progress from androgen-dependent to androgen-independent after castration, the expression levels of AR coactivator ARA70 (28) and ARA55 mRNA (personal communication with H. Tin at University of Rochester, New York, USA) increased in the androgen-independent tumors. These observations indicate that a subgroup of prostate cancers may progress from a hormone-sensitive to hormone-resistant state by an overexpression of AR coactivators. Further work investigating many other AR cofactors in many clinical samples and interfering with function of AR cofactors remains to be done to further confirm the role of AR cofactors in the process of prostate cancer progression.

As described above, an overexpression of AR and AR coactivators may thus contribute to the hormone-resistance of prostate cancer.
after a long term treatment with TNFα. Androgen-hypersensitive LN-TR2 cells were established prostate cancer and it was also associated with a poor prognosis (29). Androgen-hypersensitive LN-TR2 cells were established after a long term treatment with TNFα. Therefore, an elevated TNFα level in advanced prostate cancer may cause androgen-hypersensitivity by an overexpression of nuclear AR protein and AR coactivators such as ARA55 and TIF2, thereby resulting in the progression to hormone resistant prostate cancer.

In conclusion, the present study demonstrated that long term TNFα treatment could thus make prostate cancer cells androgen-hypersensitive by increasing the expression of nuclear AR and AR coactivators. Since androgen-hypersensitivity is one of the mechanisms of hormone-resistance in some cases, developing novel hormone therapies such as more potent antiandrogens, inhibitors of adrenal androgens, or modifying the AR coactivator function, thus, may improve the prognosis of patients with advanced prostate cancer.

REFERENCES