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Androgen Receptor and TGFbeta1/Smad Signaling are Mutually Inhibitory in Prostate Cancer

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Abstract

Purpose: The androgen receptor (AR) has been reported to modulate TGFbeta1/Smad signaling and is, like transforming growth factor beta 1 (TGFbeta1) often overexpressed in hormone refractory prostate cancer (HRPC). In human prostate cancer cell lines the role of AR in the response to TGFbeta1 is evaluated.

Material and methods: PC3 cells grow hormone independently, lack AR expression, and have a functioning TGFbeta1/Smad signaling cascade whereas LNCaP cells express (a mutated) AR, and lack TGFbeta receptor (TGFbetaR2) expression. Luciferase reporter assays for AR signaling, TGFbeta1/Smad signaling, and E2F transcriptional activity were performed. PC3 cells and TGFbetaR2 stably-transfected LNCaP cells (LNCaP-R2) were incubated with dihydrotestosterone (DHT), or TGFbeta1. Wst-1 assay and flowcytometric evaluation of annexin-V staining were applied to quantify cell growth and apoptosis. Immunoblot analysis was performed to evaluate c-Myc expression.

Results: Luciferase reporter assays showed mutual transcriptional inhibition of AR and TGFbeta/Smad signaling in AR transfected PC3 and LNCaP-R2 cells. AR expression reduced the TGFbeta1/Smad transcriptional activity and the growth inhibitory effects of TGFbeta1 also in the absence of DHT in PC3 cells. TGFbeta1 reduced the E2F transcriptional activity of AR activation by DHT. This was associated with a reduced c-Myc expression in PC3 cells. AR expression in PC3 cells prevented TGFbeta1 induced growth inhibition and apoptosis.

Conclusion: AR overexpression is an effective way of hormone refractory prostate cancer cells to overcome the growth inhibitory effects of elevated serum TGFbeta1 levels even in the absence of DHT. These findings provide an explanation for how AR overexpression favors growth in HRPC.

Keywords: Androgen receptor; Prostate cancer; Smad; TGFbeta1

1. Introduction

Prostate cancer is the most common cancer in men in the western world. Both (chemical) castration and antiandrogens postpone clinical symptoms of disease. Unfortunately, the majority of metastasized tumors progresses despite hormonal treatment. Mutations in the androgen receptor (AR) reverting androgen suppression into activation, do occur but only in a minority of hormone refractory prostate cancers (HRPC) [1]. However, in one-third of patients with HRPC levels of AR were found elevated e.g. through gene amplification [2]. The role of AR overexpression in HRPC progression is yet unclear but recent analysis of xenograft models showed that modest increase in AR mRNA resulted in hormone resistance [3].

Other growth signaling cascades are active in prostate cancer. The PI3-kinase/Akt signaling cascade was shown to be essential in prostate cancer growth

Abbreviations: AR, androgen receptor; DHT, dihydrotestosterone; EGF, epidermal growth factor; HRPC, hormone refractory prostate cancer; mTOR, mammalian target of rapamycin; ONPG, o-nitrophenylgalactoside; PI3kinase, 3-phosphatidylinositol 3-kinase; TGFbeta1, transforming growth factor beta 1.

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transferring for example epidermal growth factor (EGF) signaling. A more controversial function has been reported for TGFbeta1 [4]. TGFbeta1 is a member of the TGFbeta growth factor family and is known to be involved in differentiation and growth inhibition in normal epithelium whereas it is associated with tumor progression in more advanced tumor stages. TGFbeta1 serum levels were shown to be predictive of tumor recurrence after surgery and metastasized prostate cancer [5]. Interestingly, functional cross-talk between PI3-kinase signaling and TGFbeta1 was reported [6,7]: inhibition of PI3kinase/mTOR signaling increased the growth inhibitory response of prostate cancer cells to TGFbeta1. Like PI3-kinase/mTOR signaling, AR was shown to bind and inhibit Smad3 signaling in a mutual fashion [8]. In other words, AR amplification in HRPC may provide protection against the growth inhibitory signals of TGFbeta1/Smad signaling. Simultaneously, TGFbeta1 signaling may inhibit AR signaling through binding of members of the Smad protein family to AR. This provides the tumor with a growth advantage through stromal activation and neoangiogenesis without inhibition of the epithelial tumor compartments.

c-Myc was found upregulated upon androgen stimulation and confers androgen independent growth in a mouse model [9]. Since it was recently shown that TGFbeta1 downregulates c-Myc expression through upregulation of the inhibitory p107-E2F4 complex [10] there is reason to hypothesize that E2F transcriptional activity by AR is regulated by TGFbeta1. The human prostate cancer cell line PC3 does not express the androgen receptor but has an intact TGFbeta1/Smad signaling cascade, whereas LNCaP cells lack the TGFbeta1 R2 receptor (TGFbetaR2) [11] and express the androgen receptor, albeit in a mutated form. Hence these two prostate cancer cell lines provide an opportunity to study the role of AR and TGFbeta1/Smad interaction.

In the current analysis we studied the interaction of TGFbeta1/Smad and AR signaling with respect to E2F transcriptional activity, c-Myc expression, and cell growth in two prostate cancer cell lines.

2. Material and methods

2.1. Cell lines

Human prostate cancer cell lines LNCaP and PC3 were cultured in DMEM containing 10% fetal bovine serum and 100 IU/ml penicillin, and 100 ug/ml streptomycin at 5% CO2 and 20% oxygen. The PC3 cell line was chosen for it lacks AR expression, yet is responsive to TGFbeta1. Contrary to PC3 the LNCaP cell line lacks a receptor for TGFbeta1 (R2) and expresses (a mutated) AR. LNCaP cells stably transfected with the TGFbeta-R2 receptor were kindly provided by Dr. Kyprianou's laboratory, Kentucky State University, USA [11]. For cytotoxicity assays, 10e4 cells were plated in 96-well plates and grown for 2 days in DMEM containing 10% charcoal treated serum. Subsequently, medium was replaced with medium containing different concentrations of agents and 10% charcoal treated serum.

2.2. Growth assays

Growth inhibition was assessed using the wst-1 assay (Cell Proliferation assay, Roche, Indianapolis, USA) and by plating an equal number of cells (1 × 10e4) and count number of cells after 10 days of treatment. After drug exposure, the medium containing the agents was removed and replaced with wst-1 reagent-containing medium according to the manufacturers protocol (10%). Cells were thus incubated at 37C for 20 minutes and the plates were read at 450 nm and optical density values were normalized to baseline values. Cell count assays were conducted plating 10e4 cells in a 6 well plate and treatment for 5 days. Dihydrotestosterone (DHT, 5α-androstan-17β-ol-3-one, Sigma-Aldrich, Zwijndrecht, Netherlands)was dissolved in 100% ethanol to a final stock concentration of 1 mM, 3.25 mM, 10 uM, and 10 mM, respectively. Recombinant human TGFbeta1 (R&D systems, Minneapolis, MN, USA) was dissolved in 4 mM HCl containing 1% BSA (1 mg/ml) as a 2 ug/ml stock solution. All growth assays were performed in phenol red-free DMEM medium, containing 10% charcoal treated serum and penicillin/streptomycin unless stated differently. For growth assay 10e4 LNCaP-R2 cells were cultured under different conditions for 7 days, fixed in a 6 well plate and stained with crystal violet.

2.3. Smad-reporter, E2F-reporter, and AR-reporter assays

TGFbeta1/Smad transactivational activity was assessed using a Smad-binding element containing *firefly*-luciferase reporter (pGL3-SBE4-luc) (provided by Dr. P. ten Dijke) [6]. E2F transcriptional activitity was assessed using pE2F-luc (Clontech, Franklin Lakes, NJ, USA) a 5.0 kB luciferase reporter plasmid that contains four copies of the E2F enhancer element, located upstream of the TATA-like promoter region from the herpes simplex virus thymidine kinase. AR transcriptional activity upon DHT activation was assessed using the luciferase reporter plasmid pAR-E1b-luc.

To control for transfection efficacy a co-transfection with a CMV-beta galactosidase containing plasmid was used and beta-galactosidase activity assessed using densitometry at 425 nm after incubation of lysed cells with ONPG-$MgCl_2$ sodium phosphate solution. Of all reporter plasmids 100 ng of DNA was added to 10e4 cells cultured overnight in a 96-well plate after incubation of the plasmid DNA in PEI (polyethylenimine) for 10 minutes at room temperature. For reporter assays, cells were incubated with specific agents for 72 hours at different doses. Supernatant was removed and cells were lysed with reporter lysis buffer (Promega, Madison, USA) using two freeze-thaw cycles. Subsequently, the luciferase Assay System (Promega, Madison, USA) was used according to the manufacturers protocol: 20 um of lysed cells solution was added to 50 um of the luciferin containing assay solution. Immediately, illuminator reading was obtained. Expression levels were normalized for background and no-agent reactions. All experiments were conducted in triplicate and repeated twice.

2.4. AR transfection

To analyse the effect of different AR concentrations in PC3 cells, transient transfection with different AR containing plasmid concentrations was performed. The wild-type full androgen receptor containing plasmid pAR0 [12] in a double transfection with