Introduction

The first recognition that advanced prostate cancer (PCa) could be treated systemically by lowering circulating levels of androgen was over 60 years ago [1]. Regardless of the protocol used [2-5], hormone ablation is effective only temporarily and results ultimately in the development of castrate-resistant (CR) PCa and, almost invariably, death. Therefore, a thorough understanding of the changes in gene expression that result in the development of CR PCa is critical.

The IGF axis consists of two ligands (IGF-I and IGF-II), two receptors (IGF1R and IGF2R), as well as a family of six closely related, high affinity IGF binding proteins (IGFBP 1-6) [6]. It is generally considered that altered expression in IGF axis members is associated with the development of several cancers [7,8]; in particular, increased expression of IGFBP-2 is associated with the advanced nature of several cancers [9]. Increased IGFBP-2 expression is correlated with the expansion of PCa cells post castration [10] that lead to development of the CR phenotype [11], and the lethal phenotype attributed to CR PCa cells [9,12,13]. Despite these studies, the role of IGF family members in bone metastasis remains unclear [14]. It has been proposed that IGFBP-2 may be a suitable marker for stratifying patient survival [15], as well as a tar-

Original Article

Androgen mediated translational and postranslational regulation of IGFBP-2 in androgen-sensitive LNCaP human prostate cancer cells

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Abstract: The insulin-like growth factor (IGF) axis is associated intimately with prostate cancer (PCa) development, growth, survival and metastasis. In particular, increased levels of IGFBP-2 expression are associated with advanced PCa, bone metastasis, and the development of castrate resistant PCa. Previously, we reported that androgen treatment decreased intracellular and extracellular IGFBP-2 in the androgen sensitive (AS) PCa cell line, LNCaP. Nonetheless, the mechanism by which androgen treatment decreases expression of IGFBP-2 is not clear. Since elevated IGFBP-2 is associated with a variety of advanced cancers, including PCa, coupled with the fact that hormone ablation is the customary treatment modality for advanced PCa, a complete understanding of the influence of androgens on IGFBP-2 expression is essential. Androgen treatment initially increased steady state IGFBP-2 mRNA levels in LNCaP cells. Extended androgen treatment on LNCaP resulted in a time-dependent decrease in both steady state IGFBP-2 mRNA and protein. Polysomal mRNA analysis showed no difference in IGFBP-2 association with a given fraction; however, Q-PCR revealed less IGFBP-2 mRNA in each androgen-treated fraction. In addition, there was an overall decrease in polysome mRNA after androgen treatment. Extracellular proteolysis of IGFBP-2 was prevented in the presence of serine protease inhibitors. These data indicate that androgen acts via multiple levels to down-regulate IGFBP-2 in LNCaP PCa cells.

Keywords: Serine protease, growth factor, prostate cancer, regulation, high-density culture, androgen, insulin-like growth factor binding protein
get for the prevention of progression to CR disease [16]. Therefore, IGFBP-2 and other IGF family members are correlated with metastatic disease and appear to be involved directly in the metastatic process. Despite the compelling evidence that IGFBP-2 is associated with the transition to castrate resistance, the influence of androgen on the regulation of IGFBP-2 in PCa has not been explored fully. Therefore, we analyzed of the effects of DHT and the non-metabolizable androgen R1881 on the most commonly used androgen-sensitive cell line in PCa research, LNCaP, in which we and others have shown a hormonal effect on IGFBP-2 levels [11,17].

Materials and methods

Cell culture

The androgen receptor and PSA positive human PCa cell line, LNCaP [18] was maintained continuously in T-medium (Invitrogen/Gibco Carlsbad, CA) according to the original specifications [19] and supplemented with 5% (v/v) FBS and 1% penicillin (10,000 I.U./mL)/streptomycin (10,000 mg/mL), “complete T-medium” as described previously [20] and used at passage numbers less than 40.

Q-PCR

Three million cells were seeded in 60 mm dishes in complete T-medium and allowed to attach overnight as described previously [21]. Attached cells were washed twice with PBS and serum free, phenol red free (SPF) RPMI 1640 (Invitrogen/Gibco) was added. Cells were allowed to adjust to these conditions for 24 hours, whereupon LNCaP cells were washed twice with PBS and fresh SPF RPMI 1640 replaced. R1881 (methyltrienolone, Perkin Elmer Wellesley, MA), 1 nM final concentration, or EtOH vehicle control (0.1% v/v), for various time points, was added to culture medium. Total cellular RNA was purified via the Trizol method (Invitrogen). cDNA reactions were performed using 0.5 mg of cytoplasmic RNA (SuperScript III First-Strand Synthesis System for RT-PCR, Invitrogen). Amplicons for both RT-PCR and Q-PCR were produced with the use of IGFBP-2 specific primers (forward 5'-GCAGGTTGCAGACAATGGCG-3', reverse 5'-GTGGTCGCAGCTTCTTGGGC-3') from IDT (Integrated DNA Technologies, Coralville, IA). For RT-PCR, the resulting amplicons were purified by QIAquick PCR Purification Kit (Qiagen) and sequenced in the University of Delaware Sequencing Core (http://www.delawarehsa.org/genomics.php) to confirm identity. α-tubulin, a housekeeping gene whose expression level does not change with the androgen level under SPF conditions, was utilized as the internal control. Amplicons were visualized on a 1% (w/v) agarose tris-acetate buffered EDTA gel to compare and estimate the fold change in IGFBP-2 expression levels both in the presence and absence of R1881 treatment. For Q-PCR, the Quanti-tech SYBR Green kit (Qiagen) and the BioRad iCycler iQ real-time detection system were used as described by the manufacturer. Cycle threshold levels were used to calculate the fold change in IGFBP-2 expression levels, both in the presence and absence of R1881 treatment relative to obtained cycle threshold values of α-tubulin from the same cDNA samples as reported previously by us [21].

Polyribosome analysis

Briefly, LNCaP cells were plated at high density and treated with R1881 as described above. Cells were collected by trypsinization followed by low speed centrifugation. Cell pellets were then washed in cold PBS and lysed in ice-cold NP-40 lysis buffer (10 mM Tris HCl pH 8.0, 150 mM NaCl, 1.5 mM MgCl2, and 0.5% Nonidet P-40) supplemented with 12.5 ml RNasin (40 U/ml). Following lysis, samples were centrifuged to pellet nuclei. Supernatants were transferred to new microcentrifuge tubes containing 13.3 ml heparin (50 mg/ml), 15 ml cyclohexamide (10 mg/ml), 20 ml DTT (1 M), and 10 ml PMSF (0.1 M) and centrifuged at 15,000 x g for 5 min at 4°C to remove mitochondria and membrane debris. The supernatant was then layered onto a continuous (15-45%) sucrose gradient. Samples were centrifuged in a Beckman L8-70 ultracentrifuge using an SW41Ti rotor at 38,000 rpm for 2 h at 4°C, under vacuum. Following centrifugation, gradients were immediately harvested in 24, 0.5-mL fractions into tubes containing 25 ml 20% (w/v) sucrose gradient. Samples were centrifuged in a Beckman L8-70 ultracentrifuge using an SW41Ti rotor at 38,000 rpm for 2 h at 4°C, under vacuum. Following centrifugation, gradients were immediately harvested in 24, 0.5-mL fractions into tubes containing 25 ml 20% (w/v) SDS. To these tubes 10 ml EDTA (0.5 M, pH 8.0) and 10 ml proteinase K (10 mg/ml in 50 mM Tris HCl with 1 mM CaCl2) were added, followed by incubation for 30 minutes at 37°C. Each fraction then was incubated briefly with 0.6 ml P/C/I alcohol followed by phase separation via centrifugation for 5 min at room temperature, after which 0.45 ml
of the upper aqueous phase was collected. 1 ml of glycogen (20 mg/mL, Fermentas) and 50 ml sodium acetate (3 M, pH 5.2) were added to collected aqueous phase and the RNA was precipitated with 1 ml ethanol overnight at minus 20°C. The next day, samples were centrifuged for 30 min at full speed in a microcentrifuge; pellets were washed with 70% ethanol and resuspended in dH2O. cDNA was prepared from 3 µg RNA from each fraction (Applied Biosystems). Q-PCR was performed on the ABI 7900ht machine using iTaq SYBR green supermix with ROX (BioRad). The IGFBP-2 primers, as previously described, were utilized. The GAPDH primers used were 5’-AAGGTCGGAGTCAACGGATTTGGT-3’ (forward) and 5’-ATGGCATGGACTGTGGTCATGAGT-3’ (reverse). All primers were from IDT.

Western blotting

For the analysis of intracellular IGFBP-2 expression, cells (3 X 10^6 cells/60 mm diameter dish) treated in the presence or absence of R1881 (1 nM) for various times were lysed in MES buffer (12 mM 2-(N-morpholino)ethanesulfonic acid, 75 mM NaCl, 1% (v/v) Triton-x-100). Protein concentrations were determined via standard BCA protocol (Pierce, Rockford, IL). Electrophoresis of 25 mg whole cell lysate was performed on either a 4-12% (w/v) or 12% (w/v) acrylamide Bis-Tris gel (Invitrogen-Novex) for 50 minutes at 200V. Electrophoretic transfer to nylon reinforced nitrocellulose membranes (Osmonics, Minnetonka, MN) was performed for 50 minutes at 30V, followed by staining with 0.5% (w/v) Ponceau-S in 0.1% (v/v) acetic acid (Sigma, St. Louis, MO) as reported previously [12] in order to verify equal protein loading and transfer. Membranes were blocked overnight at 4°C in 5% (w/v) bovine serum albumin (BSA) in 1X PBS. The following day, nitrocellulose membranes were incubated with bovine anti-IGFBP-2 (Cat#06-107, Upstate Biotechnology, Lake Placid, NY) at a dilution of 1/2000 for 2 hours followed by incubation with donkey anti-rabbit horseradish peroxidase (HRP) (Cat#NA934V, Jackson Labs, Bar Harbor, ME) at a dilution of 1/5000 for 30 minutes. For analysis of R1881 treatment on secreted levels of IGFBP-2, lyophilized concentrated conditioned medias derived from high density plated cells treated with either R1881 (1 nM) or vehicle control from various time points were resuspended in equal volumes of water and prepared for electrophoresis. All blotting conditions were identical to whole cell lysate (WCL) samples.

Protease inhibition studies

Serum-starved LNCaP cells (3 X 10^6 cells/60 mm diameter dish) were treated with either R1881 or vehicle control for a period of 24 hours. Subsequently, the protease inhibitors (Sigma) pepstatin (0.1 mg/ml), phosphoramidon (150 mg/ml), EDTA (0.3 mg/ml), E64 (5 mg/ml), antipain (300 mM), leupeptin (20 mM), or pefablock (200 mM) were added for an additional 24 hours. Conditioned media was processed for Western blotting of IGFBP-2 as described above.

Statistical analysis

In Western blot experiments, protein bands were measured using densitometry and pixel density qualified using NIH Image J. The average, derived from 3 independent samples, was compared using Student t-test. p-values less than 0.05 were deemed significant.

Differences between samples in the Q-PCR analysis of IGFBP-2 expression in androgen treated LNCaP cells, were determined using 95% confidence intervals (CI) to indicate the significance between samples. Using this method, the graphical display indicates significance if error bars (95% CI) do not overlap.

Results

Extended R1881 treatment reduces steady state levels of LNCaP IGFBP-2 mRNA

Conventional RT-PCR analysis revealed little, if any, influence of 1 nM R1881 treatment on steady state levels of IGFBP-2 mRNA (Data not shown). However, the greater sensitivity of Q-PCR confirmed that 1 nM R1881 treatment resulted in an initial increase in IGFBP-2 mRNA levels at 6h as compared to controls. This was followed by a time-dependent decrease in steady state levels of IGFBP-2 mRNA (Figure 1) with an apparent half-life of about 12 hrs. IGFBP-2 mRNA levels were decreased to 16.7% of 3h R1881 treated levels and 20% of control levels, which occurred after 24 hrs and persisted at 48 hours of R1881 treatment. Accordingly, steady state levels of IGFBP-2 mRNA decreased upon prolonged R1881 treatment.
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Polysome analysis indicates possible translational control mechanism following androgen treatment.

The magnitude of change in the mRNA with R1881 treatment in LNCaP cells did not match our previously reported decrease in cytosolic and secreted IGFBP-2 [12]. Therefore, we speculated that a difference in translation of the mRNA present could result in the changes observed. Polysome analysis was used to determine the possibility of enhanced translational efficiency of the IGFBP-2 message with and without R1881 treatment. Q-PCR analysis was performed on RNA isolated from each polysome fraction for IGFBP-2 and GAPDH. Figure 2A shows the Ct values for a representative experiment. While we did not observe a shift in IGFBP-2 distribution amongst polysomes; we did find a decrease in IGFBP-2 mRNA levels in each of the heavy polysome fractions. GAPDH showed a marked shift in both polysomal fraction and the amount of mRNA in heavy polysomes following R1881 treatment. Therefore, we conclude that less IGFBP-2 mRNA is loaded onto heavy polysomes for translation. This corresponds well to the overall decrease in IGFBP-2 mRNA as shown in Figure 1. Curiously, while androgens are anabolic steroids known for their action to increase protein synthesis, the translation of global RNA was actually decreased with R1881 treatment (Figure 2B), with the slope of the polysomal curve shifting from positive to negative.

R1881 treatment decreases both extracellular and intracellular IGFBP-2 in LNCaP cells

We previously reported that 48 hours of R1881 (1 nM) treatment induced the limited proteolysis of extracellular IGFBP-2 [12]. In order to determine the kinetics of proteolysis, we isolated conditioned media from LNCaP cells treated in the presence and absence of R1881 for various time points. Initial decreases in levels of intact extracellular IGFBP-2 (Figure 3A), as well as the formation of low molecular weight proteolytic fragments were detected at 24 hours of R1881 treatment (data not shown). Forty eight hours of R1881 treatment induced marked degradation of extracellular IGFBP-2 (Figure 3B). In or-
order to determine the influence of R1881 treatment on intracellular levels of IGFBP-2, and if androgen-induced IGFBP-2 proteolysis was occurring intracellularly, cell lysates of LNCaP cells, treated in the presence and absence of R1881, were subjected to analysis by western blotting for IGFBP-2. Marked decreases in intact levels of intracellular IGFBP-2 were observed at 24 hours, with almost a complete absence of intact intracellular IGFBP-2 after 48 hours of R1881 treatment (Figure 3C). Long exposures of autoradiographs failed to reveal the presence of any proteolytic fragments within the intracellular pool (Figure 3C, middle panel).

**Mechanistic class of IGFBP-2 protease**

In an effort to determine the mechanistic class of the proteolytic pathway responsible for the limited degradation of intact, extracellular IGFBP-2 in response to androgen treatment, we treated LNCaP cells for 24 hours in the presence of R1881 (1 nM) followed by co-treatment with a variety of protease inhibitors (Figure 4). Treatment with bestatin (aminopeptidase inhibitor, 40 mM), chymostatin (serine/cysteine proteinase inhibitor, 50 mM), pepstatin (aspartyl proteinase inhibitor, 0.1 mM), phosphoramidon (metalloproteinase inhibitor, 150
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mg/ml), EDTA (metalloproteinase inhibitor, 0.3 mg/ml) (data not shown), and E64 (cysteine proteinase inhibitor, 5 mg/ml), all failed to inhibit R1881-induced IGFBP-2 proteolysis (Figure 4). However, treatment with antipain (300 mM, serine/cysteine inhibitor), leupeptin (20 mM, serine/thiol inhibitor), and pefablock (200 mM, cysteine proteinase inhibitor) inhibited R1881-induced extracellular IGFBP-2 proteolysis (Figure 4). These data indicate that the proteolysis of secreted, extracellular IGFBP-2 directly or indirectly requires the activity of a serine protease.
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Discussion:

Surgical/chemical castration remains the "gold standard" treatment modality for advanced PCa [22]. However, androgen ablative therapy represents one of the last weapons used to combat advanced and metastatic PCa. Almost uniformly, hormonal intervention fails, resulting in PCa progression to a castrate resistant (CR) state. The fact that surgical/chemical castration fails to cure advanced PCa underscores the importance in the identification of molecular targets for 1) the treatment of AS metastatic PCa; 2) monitoring for post-castration progression to CR PCa; and, 3) the development of targeted therapies for the prevention of CR PCa and metastatic PCa. It has been suggested that IGFBP-2 may be one such target as increased or forced overexpression of IGFBP-2 is correlated with both the presence of several advanced cancers and appears to promote a CR state [5,9]. Thus, understanding the androgenic regulation of IGFBP-2 in PCa will provide insight into how this important growth factor regulator contributes to CR progression.

Several previous studies have shown the influence of manipulating androgen levels on IGFBP-2 expression both in vivo and in vitro. Castration of nude mice harboring LNCaP xenografts results in a time dependent increase in IGFBP-2 mRNA levels, and ectopic overexpression of IGFBP-2 decreases the time required for LNCaP xenografts to attain CR status in a castrated host [11]. Because IGFBP-2 proteolysis depends on the presence of androgens, these effects are presumably due to intact IGFBP-2 and not protease-generated fragments. In addition, while the exogenous addition of IGFBP-2 to primary cultures of normal prostate epithelial cells results in decreased proliferation, identical treatment of AS LAPC-4 and CR DU145 cells results in increased proliferation to levels above that of IGF-1 alone, as well as increased expression of the telomerase catalytic subunit [10]. Furthermore, LNCaP conditioned media contains greater levels of IGFBP-2 in an androgen-depleted environment. When compared to AS LNCaP, the lineage derived CR and metastatic C4-2B4 has significantly elevated levels of IGFBP-2 [12,13]. In short, these findings indicate a significant role for IGFBP-2 during post-castration progression to CR. However, published reports regarding the degree and manner in which androgen modulates IGFBP-2 expression differ. For example, three separate studies indicated that 24 and 48 hours of androgen treatment increases LNCaP IGFBP-2 mRNA [23,24] and protein [25], while other studies indicate that androgen actually represses IGFBP-2 mRNA and protein levels in LNCaP [11,12]. Therefore, we undertook studies to determine the influence of androgen treatment on IGFBP-2 in the androgen-sensitive cell line, LNCaP.

Our data supports previous studies by Kiyama et. al., that indicate androgen treatment decreases IGFBP-2 mRNA levels in LNCaP, and that castration results in the observed increase in IGFBP-2 mRNA in LNCaP xenografts [11], but refute the findings of Goosens et. al. that indicated an increase in IGFBP-2 levels with extended exposure to androgens [11]. Additionally, the finding that 3 hours of R1881 treatment results in an increase in IGFBP-2 mRNA has not been reported previously. Perhaps due to the ubiquitous over expression of the IGFBP-2 gene, previous studies regarding alterations in IGFBP-2 expression in the presence/absence of androgen have not focused on the molecular biology of the IGFBP-2 promoter. It was shown previously that the immediate ~600 base pairs upstream of the transcription start site increased reporter activity in chloramphenicol acetyltransferase assays by seven fold in Jurkat k16 cells [26], identifying this area as an important cis-regulatory region in the IGFBP-2 gene. Transcription factor binding site analysis (http://www.cbil.upenn.edu/cgi-bin/tess/tess) of the immediate ~2000 bp upstream of the transcription start site in the IGFBP-2 gene revealed the presence of two AR binding sites, which are approximately 100 bp apart (data not shown). Therefore, it is possible that decreases in the steady state level of IGFBP-2 mRNA levels following androgen treatment of LNCaP cells may result in response to AR binding to these regulatory sequences within the IGFBP-2 promoter, and subsequent interactions with as yet unidentified co-repressor proteins. Nonetheless, it appears that androgen represses levels of steady state IGFBP-2 mRNA in LNCaP human PCa cells.

In order to understand further the androgen-induced expression pattern of IGFBP-2, we sought to establish the active translation of IGFBP-2 during androgen treatment. Messenger RNAs that are being translated actively are loaded with a higher density of ribosomes so
that they appear “heavier” in differential centrifugation over a sucrose gradient. In cells treated with R1881 for 48 hours, overall protein synthesis decreases as evidenced by the overall decrease in the peak heights associated with the “heavy” fractions collected from the gradient (Figure 2B). When the fractions were probed for specific localization of IGFBP-2, however, there was no change in the heavy polysome fractions containing the IGFBP-2 mRNA. (compare Figure 2A). There was, however, a notable decrease in the IGFBP-2 mRNA associated with each polysomal fraction indicating that less IGFBP-2 mRNA was now available for translation. By contrast, GAPDH is dramatically up regulated by androgen as indicated by increased polysomal loading (Figure 2A) and consistent with previous reports of GAPDH regulation by androgen [27]. Specific translation of IGFBP-2 resulting from prolonged R1881 treatment may induce a stress response to increased proteasome-dependent degradation that ultimately cannot be overcome. This may also account for the differences in the reports in the literature that have measured levels at slightly different points in time. We demonstrate in this study that androgen treatment induces a complex system controlling time-dependent processes of RNA and protein synthesis, in addition to RNA and protein degradation that ultimately results in loss of IGFBP-2 expression.

We previously reported that treatment of AS LNCaP cells with androgen results in IGFBP-2 proteolysis in a manner that requires a functional AR, resulting in decreased binding of IGF-1 and IGF-2. Furthermore, we showed that proteolysis of extracellular IGFBP-2 was attenuated with progression to CR [12]. Data presented in this report indicate that androgen-induced IGFBP-2 proteolysis is abrogated through the inhibition of serine proteases. LNCaP cells express and secrete bioactive PSA, which in addition to its utility as a biomarker for early detection of PCa as well as progression to castrate resistance, is a well-studied, androgen-responsive serine protease. However, the fact that C4-2 and C4-2B4 cells fail to proteolyze IGFBP-2 following 48 hours of R1881 treatment [12], coupled with the fact that high levels of bioactive PSA, and its upstream zymogen, hK2, can be purified from the conditioned medium of these cell lines [28] makes it highly unlikely that either hK2 or PSA is the IGFBP-2 protease. Accordingly, the protease responsible for the degradation of secreted IGFBP-2 remains to be identified.

In conclusion, IGFBP-2 is apparently regulated by androgens on multiple levels [11,16,17]. Detection of such androgen-induced shifts in IGFBP-2 may facilitate surveillance of post-castration progression to CR PCa. Shedding light on the mechanisms by which androgens regulate IGFBP-2 may dictate the routes to developing future therapeutics against AS, metastatic PCa. As such, if castrate resistance is mediated via an IGFBP-2-dependent mechanism, the development of targeted therapies to such pathways could hold promise in reverting advanced, CR disease back to an AS state. Thus, this and other detailed studies regarding androgen regulation of IGFBP-2 in PCa will provide insight into how this important growth factor regulator contributes to CR progression.

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