Proteomic analysis of androgen-independent growth in low and high passage human LNCaP prostatic adenocarcinoma cells

Yun Hee Youm1,2,#, Seyoon Kim3,#, Young Yil Bahk4,* & Tag Keun Yoo1,2,*

1Department of Urology and 2Life Science Institute, Eulji Medical Center, Eulji University School of Medicine, Seoul, 3Department of Biochemistry and 4Protein Network Research Center, Yonsei University, Seoul, Korea

The present study compared the proteomic characteristics of a low passage number (L-33) and high passage number (H-81) LNCaP cell clone. Marked differences in protein expression were noted in the response of L-33 and H-81 cells to androgens. To investigate if regulation of these proteins was androgen-dependent, expression of the androgen receptor was silenced via small interfering RNA. Consistent with the proteomic data, abrogation of androgen receptor production in H-81 cells resulted in the reversed expression level into L-33 cells compared with non-treated H-81 LNCaP cells. The results clarify the progression into an androgen-independent phenotype. [BMB reports 2008; 41(10): 722-727]

INTRODUCTION

Prostate cancer (PCa) is one of the formidable forms of cancer (1). Men with PCa diagnosed at an early stage have an excellent chance of cure by means of surgery or other modalities. However, a significant fraction of men are diagnosed with later stage PCa despite early curative therapeutic attempts. This is ominous, since metastasis to other organs and acquisition of androgen-independency can frequently prelude death (2, 3). Androgens play a pivotal role in the regulation of the growth and differentiation of normal and tumorigenic prostate cells (4). Androgen-deprivation therapy is employed in the treatment of recurrent and metastatic PCa. Surgical or pharmacological castration, with the intention of decreasing androgen action, remains a pivotal treatment for advanced PCa (5, 6). This form of endocrine therapy elicits dramatic growth arrest and apoptotic and nonapoptotic cell death in sensitive prostate cells (7-9). The combined effects lead to prostatic involution and a dramatic response in PCa tumors. Although 75% of PCa tumors initially respond to this form of treatment, the success of hormonal manipulation can be cut short by the emergence of an androgen-independent form of the disease (8, 9). The mechanism of conversion of the hormone-sensitive tumor into a hormone-independent (or refractory) one is unknown (10).

The LNCaP cell line is a PCa cell line that expresses a functional androgen receptor (AR) (11). Through passage and hormonal maintenance of the cell in vivo, lineage-related LNCaP cell clones generate a series of cells that mimic the progression of PCa from androgen sensitive to androgen-independent phenotypes (12-14). The LNCaP model of PCa progression mimics the clinical features of human PCa (13).

We used 2-dimensional electrophoresis (2-DE), image analysis, and candidate picking followed by matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF MS) to investigate changes in the proteome profile occurring during continuous subculture of LNCaP cells. Proteomic comparison of the biochemical and molecular characteristics of two clones of LNCaP human prostate cells originating from the same source; a low passage number (L-33) and high passage number (H-81) identified proteins involved in the progression of an androgen-dependent PCa to the androgen-independent phenotype. Reverse transcription-polymerase chain reaction (RT-PCR), Western blot, and small interfering RNA (siRNA) technologies were used to corroborate the proteomic results. There was strong agreement between the results obtained by differential expression on 2-DE gels and those obtained by traditional analyses of mRNA and protein. The present observations indicate that the LNCaP cell line is a good model system for the study of the protein network for the activated androgen signal transduction and the discrimination of PCa progression from its androgen sensitive PCa to androgen independent phenotypes.

RESULTS

Growth response to androgens of LNCaP cell clones

We used LNCaP cells as a model system for tumorigenic progression because it the most investigated PCa cell because of its ability to model multiple features of PCa (13, 14) and because it exhibits most features of PCa cells (15). Clonal expansion under the selective pressure of androgen deprivation maintained and
generated androgen-independent clones as has been previously described (16). A major concern was the assertion that changes or responses triggered in the cells by our experimental manipulation were specifically due to the ablation of androgen action. The original morphology of the early passage cell became round shaped at the later passage. The nucleus size gradually became smaller (Fig. 1A). Cell growth in the normal and steroid reduced culture condition was determined by the growth mediated reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). There was a significant decrease in the viability of the L-33 cell clones, but an increase in viability of the H-81 cell clones in the steroid depleted medium condition (Fig. 1B). The apoptotic nature of this effect was documented using the Hoechst stain in benign prostate epithelial cells exposed to doxazosin (17-19). To examine the effect on cell viability of doxazosin, cells were treated with doxazosin for 24 h, and cell viability was determined using MTT. Viability of L-33 and H-81 were 34.8 ± 0.11% and 63.4 ± 0.28%, respectively, after doxazosin treatment, consistent with increased doxazosin resistance with cell passage (Fig. 1C). The apoptotic effect of this drug treatment was assessed by the terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay in both LNCaP cell clones. As expected, the H-81 cell clone was much more refractory to doxazosin treatment compared to L-33 cell clone. Doxazosin significantly inhibited L-33 cell survival but not that of H-81 cells 24 h post treatment (Fig. 1A).

**Proteome analysis**

Representative 2-DE gels of the LNCaP cell line are displayed in Fig. 2. Over 1,400 silver stained spots were evident. At least three gels were analyzed for each LNCaP cell to select those whose normalized volumes were similar for the gels. These 2-DE gels exhibited highly reproducible protein profiles between the two different LNCaP cell clones. Proteomics tools were utilized to identify global protein expression profile changes between the L-33 and H-81 cell clones. The pro-

---

Fig. 1. Effect of doxazosin on apoptosis of early and late passage LNCaP cells (A), growth rates of both passage LNCaP cells in normal culture (B. a) and steroid reduced conditions (B. b), and cell viability of LNCaP cells cultured with doxazosin for 24 h in vitro (C). (A) Cultured cells were treated with doxazosin for 24 h. Merge of TUNEL (green) and Hoechst 33258 (blue). (B) Cell growth in normal (a) and steroid reduced (b) culture conditions were determined by the MTT assay and are expressed as percentage of the values of day 1 cells. (C) Cell viability of LNCaP cells cultured with doxazosin for 24 h in vitro were determined by the MTT assay and are expressed as percentage of the values of untreated controls. Data are the mean ± SEM of four different determinations performed in triplicate. P < 0.05 versus control.

Fig. 2. 2-DE maps for total cell extracts from LNCaP. For these maps, protein amounts of 350 μg (A), 450 μg (B), and 450 μg (C) were used in the second dimension, and 9-16% gradient sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was used. The gels were visualized by silver staining. The pH ranges were 4-7 (A), 4.5-5.5 (B), and 5.5-6.7 (C).
teomes from total cell lysates of two types of cell clones were compared by silver staining of the gels and identification of target proteins by MALDI-TOF MS. At the individual protein level, we analyzed proteome with various molecular masses and their pl's from 4-7. Melanie III image analysis of the approximately more than 5,000 protein spots revealed that the vast majority of these apparently were not subject to differential regulation of protein expression.

**Differential protein expression between LNCaP L-33 and H-81 cell clones**

Comparative software-guided analyses of the 2-DE profiles revealed consistent changes in their expression level on the gels. The relative staining volumes of 147 protein spots on the gels for the androgen-independent H-81 cell clone differed from those of the androgen-sensitive L-33 cell clone. Ninety-five protein spots, which were identified by MALDI-TOF MS and which consisted of 45 up-regulated and 50 down-regulated species, were responsible for direct and/or indirect changes in the tumorigenic progression of these LNCaP cells. Supplementary data summarizes the identified proteins with quantitative alterations between the LNCaP clones. The identified proteins constituted a heterogeneous group that included cytoskeletal proteins (e.g. tropomyosin, actin related protein), metabolic enzymes (e.g. isocitrate dehydrogenase, phosphoglycerate dehydrogenase, triosephosphate isomerase, translation elongation factor), proteolytic enzymes (e.g. cathepsin D), cell signaling proteins (e.g. MEK5c), molecular chaperones (e.g. heat shock protein (HSP) 27, GRP 78), and the products of previously described androgen responsive genes (e.g. selenium binding protein) (20). Five sub-sets of the identified proteins (Tim, cathepsin D, CKB, GRP78, and HSP27) that exhibited significantly different changes between the L-33 and H-81 clones and whose specific antibodies were commercially available were selected for further analysis by Western blot. The levels of each of these proteins conformed to the staining volumes on the 2-DE gels.

**Effects of silencing androgen receptor on the expression of the differential proteome profile**

We investigated whether AR affected the protein expression pattern in androgen-independent tumorigenic LNCaP cells. AR is normally expressed in LNCaP cells (Fig. 3A), thus, we decided to silence AR expression to determine whether it could induce the change in protein expression (Figs. 3B and 3C), especially the above-identified proteins whose differential expression was evident on the gels from two LNCaP clones (Fig. 4). In cells transfected with siRNA for AR silencing, AR mRNA was significantly reduced (Fig. 3B). Although a comprehensive gel-based proteomic study of the androgen response in LNCaP cells had been performed previously (20, 21), our concern was the assertion that changes or responses triggered in the cells by our experimental manipulation were specifically due to the ablation of androgen action and/or the expression of AR. We previously tested five kinds of the differentially expressed proteins (Tim, cathepsin D, CKB, GRP78, and HSP27) by Western blot analysis. The cultures were harvested 72 h post-siRNA treatment for Western blot analysis. Cells treated with androgen receptor siRNA showed the reverted expression pattern for cathepsin D, CKB, GRP78, and HSP27 (Fig. 4). However, silencing AR did not induce the reverted changes in protein expression of Tim (Fig. 4C). The proteins presently reverted in their expression by silencing AR may be direct targets for the protein network through the AR and/or the progression of androgen-sensitive LNCaP cell clone to the tumorigenic androgen-independent LNCaP cell clone.

**DISCUSSION**

Androgen response is mediated by occupation of the AR by androgens. The complex translocates to the nucleus, binds to the promoters of target genes, and activates transcription resulting in the development and maintenance of PCa as well as the promotion of the growth of prostate epithelial cells. Therefore, androgen and AR play important roles in the normal develop-
A proteomic analysis of androgen independent growth in LNCaP cells
Yun Hee Youm, et al.

Fig. 4. Magnified regions of the differentially regulated five proteins on each 2-DE gels (A) and immunoblot determined confirmation for L-33 and H-81 cells (B), and H-81 before and after treatment of siRNA specific for AR (C). Equal amounts of total cell lysates were analyzed with various specific antibodies (Tim, cathepsin D, CKB, GRP78, and HSP27), which were used as primary antibodies detected by HRP-conjugated secondary antibody. The quantity of the applied protein was normalized with anti-GAPDH antibody.

In the androgen-dependent stage of PCa, androgen deprivation therapy inhibits cancer cell growth, suggesting that androgen still controls functions of AR signaling at this stage. However, androgen deprivation therapy fails, even though AR expression is still maintained in most cases (23). This conversion of androgen-sensitive tumors to a hormone-refractory state after treatment with anti-androgen therapy is still a principal clinical problem in PCa. There are strong indications that AR amplification directly contributes to therapy failure by allowing cells to resume hormone dependent growth in the presence of low concentrations of androgens, indicating that the AR signaling pathway is malfunctioning in hormone-refractory PCa (23, 24). Moreover, studies on the progression of PCa have indicated that increases in AR mRNA and protein are both necessary and sufficient to convert PCa from an androgen-sensitive to an androgen-independent stage (25, 26). These studies suggest that AR signaling is fundamental to hormonal therapy of PCa and also implicates AR signaling in treatment failure. Therefore, AR is a key target for the treatment of both early stage PCa, and the inactivation of AR expression should be an important approach for the successful treatment of hormone-refractory PCa. However, alteration of ARs might not fully explain the conversion to the hormone refractory state in PCa (27). Indeed, reports have described an androgen response in LNCaP cells, although these have resulted in few protein identifications responsible for the progression of LNCaP cell line to hormone-insensitive stage (20, 21). Moreover, one study sought to identify differentially expressed proteins in human prostate tissue with particular interest in the proteins lost in malignancy (27). As an initial step towards discriminating the candidate proteins responsible for tumorigenic progression in LNCaP cells, we used a proteomic approach based on 2-DE separation followed by identification with MALDI-TOF MS to study changes in the global expression pattern. Our data highlights the responsiveness of some proteins for which commercially available antibodies are available (Tim, cathepsin D, CKB, GRP78, and HSP27) for the progression of PCa. We next evaluated these proteins in PCa progression by employing AR RNA interference. RNA mediated silencing Although Tim expression remained unchanged in cells subjected to RNA mediated silencing, four proteins were significantly reverted in their expression into the androgen-dependent cell clone. These results suggest that these affected proteins play direct roles at some step in the progression to the more tumorigenic androgen-independent cells. However, their specific contribution to the mechanisms underlying the tumorigenicity must be solved further. HSP27 expression is highly up-regulated in PCa cells after androgen withdrawal or chemotherapy, becoming highly expressed in androgen-independent PCa (28). Furthermore, increased HSP27 after androgen ablation is an adaptive response induced by castration to enhance cell survival, thereby promoting androgen independent tumor growth (29). In addition, increased expression of GRP78 may be associated with prostate cancer progression and the development of castration resistance (30). Its expression was also increased in the castration-resistant LNCaP-derived cell line C42B and LNCaP cells grown in androgen-deprived conditions compared with LNCaP cells grown in androgen-rich media. The collective observations suggest that these proteins are linked in a functional signaling with AR and that such a network may be organized in an ordered frame. They may serve as a potential prognostic biomarker and therapeutic targets for prostate cancer.
MATERIALS AND METHODS

Cell culture
LNCaP human prostatic adenocarcinoma cells (CRL-1740) obtained from American Type Culture Collection (Bethesda, MD) were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS; Invitrogen, Carlsbad, CA), 2 mM L-glutamine, 1.5 g/l sodium bicarbonate, 4.5 g/l glucose, 10 mM HEPES, 1 mM sodium pyruvate, and antibiotics in a humidified incubator at 37°C with 5% CO₂. Two LNCaP cell clones were used in this study: LNCaP cells that had passage numbers less than 33 were designated as L-33 and numbers over 81 as H-81. L-33 cells are androgen-sensitive while H-81 cells are androgen-independent. This cell model recapitulates the progression of human prostate tumor to the advanced hormone-refractory stage.

Determination of cell growth rate and androgen independency
Cell viability was determined by a previously described MTT assay (31). For androgen independence growth of two LNCaP cell clones, the LNCaP L-33 and H-81 cell clones were cultured in hormone-deprived media. Androgen-deprived medium was routinely prepared by adding 10% charcoal-stripped serum (CSS) instead of untreated FBS and antibiotics as described above. The androgen deprivation protocol was initiated by plating LNCaP cells at 30% confluence in serum-free medium for 2 days to control for interference from androgens remaining from the prior culture in whole serum. To prepare CSS, a solution containing 5% (w/v) activated charcoal (Sigma-Aldrich, St. Louis, MO) and 0.5% (w/v) dextran T70 (GE Healthcare, Upsala, Sweden) was prepared in 1 mM HEPES buffer (pH 7.4). The mixture was stirred gently at 4°C for 1 h and centrifuged at 2500 × g for 10 min. At room temperature, 5 g dextran-treated charcoal was added to 500 ml FBS, mixed gently for 1 h, and centrifuged at 2500 × g for 10 min. The collected supernatant was subjected to another cycle of dextran-treated charcoal treatment, filtered through a 0.2 μm pore sized membrane (Nalgene, Rochester, NY), aliquoted, and maintained at -20°C until use. Cell viability was assessed using MTT.

Western blot analysis and antibodies
Preparation of total cell lysate and the procedures for Western blot analyses were performed essentially as described previously (32). The antibodies against triosephosphate isomerase, creatine kinase B, glucose related protein 78, HSP27, and AR were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The quantity of the applied protein was normalized with an AR specific antibody and RT-PCR. All experiments were done at least in triplicate.

In-gel digestion
In gel-digestion of protein spots on gels was performed essentially as described previously (32). The resulting mixtures of tryptic peptides were analyzed directly by MALDI-TOF MS.

MALDI-TOF-MS analysis and database search for identifying proteins
Mass analysis was performed on a PerSeptive Biosystem Voyager-DE STR™ MALDI-TOF-MS (Applied Biosystems, Foster City, CA) in reflector mode for positive ion detection as described previously (32).

Statistical methods
Results were analyzed using a two-tailed Student’s t test to assess statistical significance. Values of P < 0.05 were considered statistically significant.

Acknowledgement
This work was supported by Eulji Research Grant (EJRG 05-008-11E24, T.K.Y.) and by the KOSEF grant through PNRC of Yonsei University.

REFERENCES


