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Soluble factors derived from stroma activated androgen receptor phosphorylation in human prostate LNCaP cells: Roles of ERK/MAP kinase

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Abstract

Background—Accumulated evidence suggests stromal-epithelial interactions are critical to the progression of prostate cancer. In this study, we characterized AR phosphorylation in LNCaP cells co-cultured with the conditioned medium (CM) from human prostate stromal fibroblasts.

Methods—CM harvested from prostate stromal fibroblasts was added to LNCaP cells, and both anchorage-dependent and -independent growth was determined. Status of AR phosphorylation at Ser-81 and Ser-213 was assessed by immunoblot analysis. ERK kinase activity was measured using MBP-2 protein as the substrate.

Results—The growth of LNCaP cells on plastic dishes increased by 1.7-fold upon exposure to stromal CM or androgen, and their combination resulted in additive growth (2.4-fold). Anchorage-independent growth of LNCaP cells in soft agar, however, was induced synergistically at 80-fold by both stromal CM and androgen. Stromal CM or androgen alone induced LNCaP cell growth by 10- and 26-fold, respectively. We observed ERK kinase inhibitor, U0126, but not phosphatidylinositol 3-kinase (PI-3K), LY294002, or protein kinase A (PKA) inhibitor, H-89, inhibited stromal CM or androgen-induced PSA promoter luciferase activities and anchorage-independent growth of LNCaP cells. Our results demonstrated for the first time how stromal CM acts in synergy with androgen by activation of ERK kinase and AR phosphorylation at Ser-81 but not Ser-213, for AR-regulated PSA promoter and anchorage-independent growth of human prostate cancer cells.

Conclusions—A stromal factor-activated ERK pathway mediated by AR phosphorylation at Ser-81 could be responsible for stimulating the growth of human prostate cancer cells.

Keywords

Prostate cancer; Stromal-epithelial interaction; MAP kinase; Androgen receptor; Protein tyrosine phosphorylation; Signaling transduction

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Introduction

Stromal-epithelial interaction is an important mechanism controlling organ morphogenesis, cell homeostasis, and tissue remodeling during embryonic development and adulthood.(1-3) The stromal compartment directs epithelial growth and differentiation, and epithelial cell signaling also directs stromal growth and differentiation in a reciprocal manner.(4)

AR activity is subjected to posttranslational protein modifications such as acetylation (5) and phosphorylation (6) at specific amino acid residues of the AR protein. AR-mediated signaling converges with other cytokine and growth factor-mediated signaling, including KGF (7), IL-6 (8), and EGF (9), which could synergize with androgens to enhance the transcriptional activity of genes consisting of androgen responsive elements.(9) IL-6 was reported to mediate phosphorylation of both the endogenous AR protein and its steroid receptor co-activator 1, via MAPK signaling, in prostate cancer cells to account for optimal ligand-dependent and -independent AR activation in prostate cancer cells,(10,11)consistent with “crosstalk” among cytokine, growth factor and/or AR-mediated signaling in the regulation of human prostate cancer growth (12). Likewise, EGF-induced increase in AR transcriptional activity is dependent on AR phosphorylation at Ser-515 and Ser-578, which regulates nuclear-cytoplasmic shuttling of AR through interaction with the Ku-70/80 regulatory complex (13). In response to soluble growth factors, AR was also shown to be phosphorylated by *Src* tyrosine kinase, at multiple tyrosine residues in human prostate tumors (14). Among them, substitution of Tyrosine-534 with phenylalanine inhibited transcriptional activity of AR induced by EGF, and knockdown of *Src* tyrosine kinase significantly attenuated the growth of human prostate tumors in androgen-depleted conditions (14). It has been proposed that AR remains functional but signals in a ligand-independent and growth factor-dependent manner in androgen refractory human prostate cancer.(15) Elucidating how AR signaling and stromal factors contribute to androgen-independent prostate cancer growth, progression and metastasis is critical for understanding the molecular basis of androgen-independence.(16-18)

Since AR phosphorylation has been identified at numerous serine residues, we investigated two sites of serine phosphorylation at the transactivating domain of AR, Ser-81 and Ser-213, which represent respectively an androgen-sensitive and -insensitive site of AR phosphorylation in human prostate cancer cells (6). We determine here the relationship between stromal CM and androgen in inducing AR phosphorylation in LNCaP cells, an androgen-responsive human prostate cancer cell line. Results showed a positive correlation with ERK/MAP kinase which controls prostate cancer cell growth by phosphorylating AR in an androgen and stromal factor co-dependent manner.

Materials and Methods

Cell cultures and chemical reagents

LNCaP cells were routinely cultured.(19,20) Methyltrienolone (R1881), a synthetic androgen, was purchased from New England Nuclear (Boston, MA). The androgen antagonist bicalutamide (Casodex) was kindly provided by AstraZeneca (Cheshire, UK). The ERK inhibitor U0126 and PI-3K inhibitor LY294002 were purchased from Promega (Madison, WI). PKA inhibitor H89 was purchased from Upstate (Chicago, IL).

Tissues for primary prostate stromal culture were obtained from consenting patients with prostate cancer who underwent radical prostatectomy at the Emory University Hospital. The protocol for using human prostate specimens for research was approved by the Emory IRB Committee. Stromal CM was prepared according to our previous work.(21) A pair of prostate stromal cells, C1 and C2 (harvested from the pathologically confirmed normal/

benign areas of the prostate specimens of two different patients), were used as sources of the stromal CM.

Cell proliferation assay

Cell proliferation was determined by MTS assay with the CellTiter 96 Aqueous One Solution Cell Proliferation Assay kit (Promega, Madison, WI) according to manufacturer's protocol. LNCaP cells were seeded at 5×10^3 cells/well. To determine the possible downstream signaling pathways induced by R1881 either with or without the stromal CM, cells were treated with U0126, a MAPK inhibitor, LY294002, a PI-3K inhibitor, or H89, a PKA inhibitor, for 2 hrs prior to the addition of R1881 and/or stromal CM.

Luciferase assay

A 6.1 kilobase pair upstream sequence of the PSA gene was used to construct a luciferase reporter plasmid (PSA6.1-luc) as previously described.(22) Cells were plated at a density of 1×10^5 cells/well. Plasmid DNA (1 μ g) was introduced into cells by complexing with the DOTAP reagent (Roche, Indianapolis, IN) according to the manufacturer's protocol and luciferase activities were measured as described.(22) All cell growth and luciferase assays were carried out in triplicate and repeated twice. Data were expressed as mean $\pm$ S.D., and evaluated for statistical significance.

Soft-agar colony formation assay

A standard soft agar (Difco, Detroit, MI) colony formation assay was used to measure anchorage-independent growth.(23) Tumor cells mixed with 0.4% agar were overlaid at a density of 1×10^4 cells/well above the supporting 0.6% agar, and cultured for up to 21 days with the medium replenished on alternate days. Images of colonies were captured with an inverted microscope (Axiovert 200, Carl Zeiss MicroImaging, Inc., Thornwood, NY). The total number of colonies and their diameters were counted and measured using Openlab 3.0.8 imaging software (Improvision, Inc., Waltham, MA).

Immunoblotting assays and reagents

Cells were rinsed twice with ice-cold PBS and lysed in a lysis buffer.(24) The antibodies used were: anti-AR polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA), anti-phospho-myelin basic protein (MBP) antibody (Upstate, Chicago, IL), anti-ERK polyclonal antibody (Upstate, Chicago, IL), anti-phospho ERK polyclonal antibody (Upstate, Chicago, IL), and epitope-specific anti-phospho AR antibodies (Cell Signaling, Danvers, MA). Specific protein was detected with the ECL Plus kit (Amersham Bioscience, Piscataway, NJ).

Statistical Analysis

The data derived from MTS assay and soft agar colony formation assay were analyzed with Student's *t*-test with the JSTAT - Java Virtual Machine Statistics Monitoring Tool (Sun Microsystems, Inc., Santa Clara, CA). P values ≤ 0.05 were considered as statistically significant.

Results

Stromal CM induced growth, PSA luciferase reporter activity, and colony formation in human LNCaP cells

Proliferation of the LNCaP cells was significantly enhanced at 1.68 and 1.72 fold respectively by stromal CM (C1 and C2) compared to controls. The synthetic androgen agonist R1881 enhanced the proliferation of the LNCaP cells by an average of 1.87 fold.

Moreover, the combination of R1881 with the stromal CM induced 2.39 and 2.45 fold enhanced growth compared to R1881 alone ($p=0.0493$, C1 and $p=0.0269$, C2) (Fig. 1).

Next we examined the effects of stromal CM on the transcriptional activity of an AR target gene, PSA, in LNCaP cells. LNCaP cells, transfected with a 6.1 kb PSA promoter luciferase plasmid DNA and exposed to the stromal CM, exhibited a 20-fold increase of PSA promoter luciferase activity, and this enhanced luciferase activity was diminished significantly by an ERK kinase inhibitor U0126 ($p=0.0018$), but not PI3K inhibitor LY294002 or PKA inhibitor H89. Also, LNCaP cells treated with R1881 alone markedly increased luciferase activity by 121 fold. This activated luciferase activity, as expected, can be inhibited by anti-androgen flutamide ($p=0.0279$). Combined treatment with R1881 and stromal CM further increased activation of PSA promoter luciferase activity by 189 fold compared to controls (Fig. 2A). Importantly, these enhanced activities were abrogated by flutamide ($p=0.0051$) or U0126 ($p=0.0145$) (Fig. 2A).

The combined activity of R1881 and stromal CM on LNCaP cell growth was further confirmed in an anchorage-independent soft agar growth assay. While R1881 increased the average number of colonies to 27, combining R1881 and C1 stromal CM markedly increased colony numbers to an average of 79. In support of the PSA promoter luciferase data, soluble stromal factors and R1881 synergistically promoted LNCaP cell growth in soft agar to form more abundant and larger colonies than either agent alone. Note that while bicalutamide is more effective than U0126 in inhibiting R1881-induced LNCaP cell growth in soft agar, the reverse is true when comparing bicalutamide (which is completely ineffective) and U0126, which is highly active in inhibiting stromal soluble factor-induced LNCaP growth in soft agar (Fig. 2B).

Stromal CM modulated AR activity through ERK signaling

We examined the functional status of the ERK proteins of both total and phosphorylated ERK by directly measuring the activity of the ERK kinase in the presence of its target substrate, MBP-2, in LNCaP cells. CM soluble factors induced a transient increase of ERK phosphorylation in LNCaP cells upon equal loading of cellular proteins ($7.5 \mu\text{g} / \text{track}$) (Fig. 3A). ERK phosphorylation occurred at 30 min ($2.5\times$ over the basal activity) and peaked at 90 min ($6.8\times$) after the addition of stromal CM. The increased ERK phosphorylation corresponded with increased ERK kinase activity, which was assayed by the *in vitro* phosphorylation of an ERK substrate, MBP-2 (pMBP). Concomitant with the increased pMBP, AR phosphorylation at serine 81, pAR^{S81}, was increased by 1.5-1.6 fold beginning at 60 min and lasting for at least 120 min. The same amount of protein was loaded in each lane. Data were normalized as the relative ratios of pAR^{S81} / AR (Fig. 3A).

Stromal conditioned media activated AR phosphorylation at Ser81 through the ERK-MAPK pathway

To confirm if the phosphorylation of AR is a direct target of ERK, we next performed an *in vitro* phosphorylation assay in which the endogenous AR and ERK proteins were isolated from LNCaP. We showed that endogenous ERK, as assessed by its ability to autophosphorylate ERK (pERK) and also phosphorylate directly the MBP substrate *in vitro*, catalyzed the phosphorylation of the AR at Ser81 (Fig. 3A). Specifically, based on the kinase inhibitor studies, ERK appears to phosphorylate AR at the Ser81 but not Ser213 residue (Fig. 3B). In comparison to the untreated controls (Lane 1, Fig. 3B), stromal CM plus androgen stimulated pAR Ser81 by 23.6-fold (Lane 2, Fig. 3B). Stromal CM and androgen alone stimulated pAR Ser81 by 11.2- and 10.9-fold, respectively. This induction can be partially antagonized by U0126 (Fig. 3B; lanes 2 and 6 show decreased induction of pAR Ser81 by stromal CM plus androgen from 23.6- to 7.98-fold). Under these assay

conditions, the androgen antagonist bicalutamide, was completely inactive against either stromal CM and/or androgen induction of pAR Ser81 phosphorylation in LNCaP cells (Lanes 5 and 7, Fig. 3B). Unlike pAR Ser81, we observed no consistent induction of pAR Ser213 by stromal CM either in the presence or absence of androgen (Fig. 3B).

Discussion

While cancers can be caused by successive mutations within the epithelial cell compartment, aberrant stroma responses to cancer epithelium, and proteins secreted by the “reactive stroma”, could have considerable effects facilitating cancer epithelial growth, survival and metastasis.(4,11,25) Previous studies firmly established the concept that tissue-specific stromal cells can specify the growth, survival, and differentiation of responsive normal glandular epithelial cells, (4,26)and that stromal-epithelial interaction contributes to the oncogenesis, progression, and metastasis of human solid tumors, including prostate cancer. (27) Numerous publications from our laboratory (19) and others (28,29) support the idea that the AR in LNCaP cells is functionally modulated by soluble growth factors and cytokines. These factors, mediated via cell surface receptors, could trigger a downstream signaling cascade affecting the growth and survival of cancer cells. In this report, we used an *in vitro* model to study the contribution of stromal CM soluble factors to the cell growth and androgen target gene responsiveness of prostate epithelial cells, and focused on defining specific sites of AR phosphorylation in response to stroma-derived soluble factors and the consequences for human prostate cancer cell proliferation.

Our studies showed that stromal soluble factors and a synthetic androgen, R1881, utilized different intracellular signaling pathways to promote the growth, differentiation and responsiveness of target genes in the localized tumor microenvironment. Stromal CM soluble factor had a similar effect on LNCaP cell proliferation as R1881 but the latter is superior to stromal factors for activating PSA promoter luciferase activity in LNCaP cells. This activation can be blocked by the anti-androgen flutamide or bicalutamide in R1881 but not stromal factor-induced activation.

Conversely, stromal factors, but not R1881-induced cell growth and PSA promoter-luciferase gene expression, in human LNCaP cells were sensitive to the MEK (MAP/ERK kinase) inhibitor U0126, suggesting separate and distinct signaling networks activated by androgen and stromal soluble factors. Consistent with this concept, R1881 and stromal soluble factors, either applied alone or in combination, consistently activated AR phosphorylation at serine 81 but not serine 213, blocked in part by U0126, but not bicalutamide, LY-294002 or H89. Our data suggest a threshold of pAR^{S81} is associated with prostate cancer growth and PSA expression since despite an incomplete blockade of pAR^{S81} is sufficient for inhibiting prostate cancer growth in soft agar and PSA promoter activity. This suggests that MAPK/ERK is a key signaling intermediate conferring stromal factors and/or androgen-induced prostate cancer cell growth, survival and target gene expression. Whereas U0126 was highly effective against stromal soluble factor-induced cell growth and AR phosphorylation, this inhibitor was marginally active in antagonizing similar growth and gene expression effects exhibited by the combination of both stromal soluble factors plus the synthetic androgen R1881. These results suggest the importance of considering the pleiotropic rather than single or limited downstream molecular-based targeting of hormone refractory prostate cancer. While stromal CM induced pERK, pMBP, and pAR^{S81} within the same time frame as R1881, only the former was abrogated by the ERK inhibitor U0126 (Fig. 3A). Together these data suggest that stromal soluble factor-induced AR phosphorylation at Ser-81 goes through the MAPK-ERK kinase pathway (Fig. 3B).

We suggest that MAPK/ERK activation is closely linked to ERK kinase activity which is associated with AR (Ser-81) phosphorylation enhanced by stromal soluble factors harvested from CM alone or in combination with R1881. Recent data showing EGF-induced AR phosphorylation in castration-recurrent prostate cancer resides at Ser-515 (N-terminal domain of AR) and Ser-578 (DNA binding domain) (13) and the effect of MAPK/ERK kinases on AR phosphorylation which mapped to the N-terminal half of the AR protein (such as pAR at Ser81)(11) are examples in support of the concept that AR phosphorylation plays a role in regulating androgen- and growth factor-induced prostate growth. To our knowledge, the results reported here are the first to define the MAPK/ERK signaling responsible for the phosphorylation of AR at Ser-81, and further illustrate the roles of this signaling network in support of human prostate cancer growth.(30) Understanding the molecular mechanism of AR phosphorylation in response to induction by stromal soluble factors and androgen could help improve the design of therapeutic agents targeting androgen-independent but soluble stromal factor-facilitated prostate cancer growth.

Conclusions

We demonstrated here that prostate stromal soluble factors recovered from conditioned medium stimulated prostate cancer LNCaP cell growth and PSA promoter reporter activity. Stromal soluble factors were found to exert an additive action with a synthetic androgen, R1881. MAPK/ERK was demonstrated to be the dominant signaling pathway controlling AR phosphorylation at serine 81. We found that ERK kinase, mediating its action through pAR^{S81}, regulates prostate cancer growth and PSA expression. Targeting AR phosphorylation via ERK kinase blockade could be the basis for a new approach to the management of patients with hormone-refractory prostate cancer.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

AR	androgen receptor
Ser	Serine
MAP	mitogen-activated protein
ERK	extracellular signal-regulated kinase
CM	conditioned medium
PKA	protein kinase A
MBP-2	myelin basic protein-2
PSA	prostate specific antigen
FBS	fetal bovine serum
DCC	dextran-coated charcoal
PI-3K	phosphatidylinositol 3-kinase

MEK mitogen-activated protein kinase kinase

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Figure 1. Effects of prostate stromal CM and a synthetic androgen, R-1881, on LNCaP cell proliferation

C1 and C2, two independent isolates of stromal cells, were used as described in Materials and Methods. Effects of stromal CM on growth and PSA promoter reporter activity by LNCaP cells were evaluated after 72 hrs treatment. Effects of stromal CM and R1881 (10 nM) on the proliferation of LNCaP cells were assayed by MTS assay (Materials and Methods). R1881, stromal CM (C1) and stromal CM (C2) induced significantly more cell proliferation than control ($p=0.005$, 0.0012 , and 0.0007 , respectively). In addition, combining R1881 with stromal CM (C1) and stromal CM (C2) induced more cell proliferation than R1881 alone ($p=0.0493$, and 0.0269 , respectively). Data are presented as fold of induction relative to control, mean $\pm$ SD of a triplicate assay. Asterisks denote statistically significant changes compared with controls.

Figure 2. Effects of prostate stromal CM and a synthetic androgen, R-1881, on AR transcriptional activity of PSA promoter luciferase in LNCaP cells

Panel A, effects of stromal CM and R1881 (10 nM) on AR transcriptional activity measured by 6.1 kb PSA promoter luciferase assay with relative luciferase activity/million cell number (see Materials and Methods). LNCaP cells transiently transfected with a PSA reporter luciferase gene were subjected to androgen starvation before treatment with stromal CM and/or R1881. The specificity of PSA promoter reporter activation by androgen was determined by the simultaneous addition of R1881 (1 nM), an androgen agonist, and an androgen antagonist, bicalutamide (100 nM). Luciferase activity was determined 72 hrs after the initiation of treatment with R1881 (1 nM), MEK inhibitor U0126 (20 μ M), PI-3K inhibitor LY294002 (20 μ M), and/or PKA inhibitor H89 (10 μ M). Data represent fold of induction of relative luciferase activity, mean $\pm$ SD of three separate assays. Asterisks denote statistically significant changes compared with controls.

Panel B, effects of stromal CM on anchorage-independent LNCaP soft agar colony formation. LNCaP cells suspended in agar were treated with stromal CM for 21 days. Photographic results were analyzed with computer imaging software. Data are presented with bars representing different sizes of the colony stacked to give the total number of colonies. Effects of R1881 (10 nM), antiandrogen, bicalutamide (100 nM), and MEK inhibitor U0126 (10 nM) on LNCaP soft agar formation were assessed. In each treatment, the standard deviation from 3 independent experiments was less than 10% of the mean. The insets show representative colony formation from control and treated groups ($\times 40$).

Figure 3. Stromal CM induced concomitant ERK activation and AR phosphorylation

Panel A, androgen-deprived LNCaP cells were treated with stromal CM (C1). The cells were harvested at different time points for ERK kinase and western blotting analyses. Top panels, phosphorylated ERK (pERK), phosphorylated myelin basic protein (pMBP), and total ERK proteins were detected by specific antibodies. Bottom panels, AR status in these samples was determined with specific antibodies to phosphorylated AR at the site Ser-81 (pAR^{S81}) and total AR (AR). We used the relative ratio of phosphorylated ARS81/AR, pERK1/ERK1, and pERK2/ERK2 to represent the relative extent of phosphorylation of pAR^{S81}, pERK1, and pERK2, respectively. The same amount of protein was loaded in each lane. The value of the ratios of the 1st lane was set as 1.0.

Panel B, stromal CM-activated ERK-catalyzed phosphorylation of AR at Ser-81 was assayed. LNCaP cells were treated as indicated and immunoprecipitated with anti-ERK antibodies purified and bound to G-Sepharose (G-S) beads. For endogenous AR protein, androgen-deprived LNCaP cells were used in immunoprecipitation with the anti-AR

antibody (PG21) and bound to protein G-S. The ERK-G-S complex was mixed with the substrate AR-G-S complex in the kinase reaction, then fractionated on SDS-PAGE. Specific antibodies to phosphorylated Ser-81 (pAR^{S81}) and Ser-213 (pAR^{S213}) were used. Total AR and ERK proteins were detected to determine the efficiency of the immunoprecipitation. Synthetic androgen R1881 (10 nM), bicalutamide (100 nM) and MEK inhibitor U0126 (20 μ M) were used. The relative ratios of phosphorylated ARS81/AR and phosphorylated ARS213/AR represented the relative intensities of pARS81 and pARS213. The value of these ratios of the 1st lane was set as 1.0. The ERK inhibitor U0126 successfully inhibited the pARS81 expression enhanced by combined treatment with CM (C1) and R1881 even though the anti-androgen bicalutamide did not). The same amount of protein was loaded in each lane. All experiments were conducted independently three times.