Increased Insulin-Like Growth Factor I Receptor Expression and Signaling Are Components of Androgen-Independent Progression in a Lineage-Derived Prostate Cancer Progression Model

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ABSTRACT

Apoptosis and inhibition of mitosis are primary mechanisms mediating androgen ablation therapy-induced regression of prostate cancer (PCa). However, PCa readily becomes androgen independent, leading to fatal disease. Up-regulated growth and survival signaling is implicated in development of resistance to androgen ablation therapy. We are testing the hypothesis that insulin-like growth factor (IGF) responsiveness is required for androgen-independent (AI) progression. Using the LNCaP human PCa progression model, we have determined that IGF-I-mediated protection from apoptotic stress and enhanced mitotic activity is androgen dependent in LNCaP cells but is androgen independent in lineage-derived C4-2 cells. Both cell lines exhibit androgen-responsive patterns of IGF-I receptor (IGF-IR) expression, activation, and signaling to insulin receptor substrate-2 and AKT. However, C4-2 cells express higher levels of IGF-IR mRNA and protein and exhibit enhanced IGF-I-mediated phosphorylation and downstream signaling under androgen-deprived conditions. In comparisons of naive and AI metastatic human PCa specimens, we have confirmed that IGF-IR levels are elevated in advanced disease. Together with our LNCaP/C4-2 AI progression model data, these results indicate that increased IGF-IR expression is associated with AI antiapoptotic and promitotic IGF signaling in PCa disease progression.

INTRODUCTION

Prostate cancer (PCa) is the most commonly diagnosed cancer and the second leading cause of cancer-related death in North American men (1, 2). Androgen ablation therapy is one of the few options available to patients with advanced disease. However, in a majority of cases, disease progresses to an androgen-independent (AI) phenotype, for which there is no curative option.

Mounting clinical and experimental evidence suggests that perturbations in insulin-like growth factor (IGF) axis components are associated with a variety of cancers and specifically contribute to susceptibility and progression of PCa (3, 4). Links between increased IGF ligand and receptor/substrate availability in PCa (4–10) are supported by studies in rodent models that have correlated altered IGF-I or IGF-II receptor (IGF-IR) levels with prostatic dysplasia (11), tumor growth, or metastasis (12–14). Additionally, antisense-mediated reduction of IGF-IR levels in AI DU145 cells resulted in increased susceptibility to apoptosis and inhibition of mitosis (12–14). Furthermore, using the transgenic adenocarcinoma of mouse prostate (TRAMP) and lineage-derived prostate epithelial cells [P69 and M12 (16–19)], we have shown that PCa is a heterogeneous disease, both hypotheses may be relevant to specific subsets of PCa.

The biological activity of the primary IGF-IR ligands, IGF-I and IGF-II, is modulated in part by six IGF-binding proteins that either inhibit or enhance IGF-IR-mediated signaling (6, 20–22). Ligand activation of the transmembrane tyrosine kinase, IGF-IR, results in phosphorylation and membrane recruitment of insulin receptor substrate (IRS) proteins and activation of intracellular signaling pathways including Ras/mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3'-kinase (PI3K)/AKT. These IGF-mediated signaling events control a variety of biological effects including mitogenesis, survival, and cellular transformation (17, 23).

This study examines whether maintenance of IGF-I responsiveness is important for survival and growth of PCa and whether this responsiveness is achieved through modulation of IGF axis components. Results from an in vitro LNCaP progression model are compared with changes in IGF-I expression in naïve and AI metastatic PCa from a human PCa tissue microarray (TMA). LNCaP is a human PCa cell line derived from a lymph node metastasis, and C4-2 is an AI cell line derived from LNCaP cells (24, 25). Both LNCaP and C4-2 cells express the prostate marker prostate-specific antigen, respond to androgen, and retain expression of a functional androgen receptor (AR). However, C4-2 cells are androgen independent in that they form prostate-specific antigen–secreting tumors when inoculated into intact or castrated athymic male mice (25). Additionally, C4-2 cells metastasize to the lymph node and bone and thus exhibit aspects of natural human PCa progression (26, 27). Our results demonstrate that whereas IGF responsiveness of LNCaP cells is androgen dependent, C4-2 cells maintain IGF responsiveness under androgen-deprived culture conditions. The difference in IGF responsiveness is proportional to IGF-IR expression levels in these cell lines under androgen-deprived conditions. This is the first report demonstrating that development of androgen independence is linked to enhanced AI IGF responsiveness in a lineage-derived human PCa model. Consistent with this model, we predict and show herein that IGF-IR levels are increased in AI metastatic PCa specimens.

MATERIALS AND METHODS

Cell Culture and Treatments. LNCaP and C4-2 cells were obtained from Dr. L. W. Chung (Emory University, Atlanta, GA) and maintained in T-media (Invitrogen Life Technologies, Inc., Carlsbad, CA) containing 5% fetal bovine serum (FBS; Invitrogen Life Technologies, Inc.; ref. 27). DU145 cells were obtained from the American Type Culture Collection (Manassas, VA) and maintained in Dulbecco’s modified Eagle’s medium (Invitrogen Life Technologies, Inc.) containing 10% FBS. The cells were incubated with IGF-I (Research Diagnostics Inc., Flanders, NJ), the synthetic androgen R1881 (Perkin-Elmer, Boston, MA), and the PI3K inhibitor LY294002 (Calbiochem, San...
Diego, CA) in serum-free, phenol red-free RPMI 1640 (SF-RPMI 1640; Invitrogen Life Technologies, Inc.) at the time points and concentrations indicated in the figure legends.

**Apoptosis Immunostains.** LNCaP and C4-2 cells were treated with the indicated agents in SF-RPMI 1640. Cleared cell lysates were prepared from pooled attached and suspended cells in radioimmunoprecipitation assay (RIPA) buffer (20 mmol/L Tris-HCl [pH 7.4], 1% Triton X-100, 0.1% deoxycholic acid, 1 mmol/L EDTA, 1 μg/mL leupeptin, 2 μg/mL aprotinin, 0.5 mmol/L sodium vanadate, and 2 μmol/L microcystin). Detection of nucleosome formation was performed using a Cell Death Detection ELISA PLUS kit (Roche Diagnostics, Mannheim, Germany) and 1 μg of whole cell lysate as determined using the BCA kit (Pierce Biotechnology, Rockford, IL).

**Mitotic Activity Measurements.** Mitotic activity was assessed by [3H]thymidine incorporation. After plating cells in T-media overnight, 2.5 × 10^5 LNCaP cells were cultured in SF-RPMI 1640 ± 0.1 and 1 mmol/L R1881 for 2 days. IGF-I (100 ng/mL) and [3H]thymidine (10 μCi/mL; 20 Ci/mmol; New England Nuclear, Boston, MA) were added to the media for the final 21 hours before harvesting the cells for mitotic activity analysis as described previously (28). The mitotic activity of each treatment population was calculated as the mean acid-insoluble [3H] (cpm/mg total cell protein) ± SD.

**Fluorescence-Activated Cell Sorting.** Fluorescence-activated-cell-sorting (FACS) analysis was performed on live LNCaP and C4-2 cells cultured for 48 hours in SF-RPMI 1640 ± 1 mmol/L R1881. Cells were detached in 2 mmol/L EDTA in PBS (50 mmol/L sodium phosphate, 150 mmol/L NaCl [pH 7.4]), washed, resuspended in 3% bovine serum albumin/PBS, and incubated with 1 μg/mL anti-IGF-IR monoclonal antibody (MAb) 1H7 or normal mouse IgG (Sigma, St. Louis, MO) and 0.1 μg/mL fluorescein isothiocyanate-conjugated goat antimouse IgG (Jackson ImmunoResearch, West Grove, PA). Data are expressed as the average mean fluorescence intensity per cell over background ± SD for each cell line under both treatment conditions.

**Immunoprecipitation.** After the indicated treatments, IGF-IR was immunoprecipitated from 1 mg of RIPA cell lysate using the IGF-IR Mab 3B7 and antiserum IgG-agarose (Sigma). IRS-2 was co-immunoprecipitated from 50 μg of cell lysate using a p85 subunit of PI3K polyclonal antibody (Upstate Biotechnology, Lake Placid, NY) and protein A-agarose (Invitrogen Life Technologies, Inc.). The immunoprecipitates were separated by SDS-PAGE and transferred to nitrocellulose membranes (Schleicher and Schuell, Keene, NH) for immunoblot analysis.

**Western Blot Analysis.** Whole cell lysates were prepared as described above, and 50 μg of each sample were processed for SDS-PAGE and electrophoretic transfer to nitrocellulose. IGF-IR activation was determined by immunoblotting with the phospho-tyrosine Mab antibody PY99 (Santa Cruz Biotechnology, Santa Cruz, CA). Total IGF-IR was determined using a phosphorylation-independent IGF-IR β-subunit polyclonal antibody (Santa Cruz Biotechnology). IRS-2 was determined by blotting with anti-IRS-2 polyclonal antibody (Upstate Biotechnology), and tyrosine-phosphorylated IRS-2 was detected with PY99. The p85 subunit of PI3K polyclonal antibody was from Upstate Biotechnology. AKT was detected with polyclonal antibodies directed against the p85 subunit of PI3K (Upstate Biotechnology, Santa Cruz, CA). The IGF-IR primers IGF-IRfp (5'-GAAAGTGACGTCCTGCATTTCA) and IGF-IRrp (5'-CCGGTGCCAGGTTAGTGATG) and probe (5'-VIC-CACCACGTCCAAGATCTCCGAT-TAMRA) were selected using the Primer Express Software version 1.5 (Applied Biosystems). The probe was labeled with a VIC/TAMRA quencher/reporter. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA was unaffected by the treatments and therefore deemed a suitable control for normalizing the results to total mRNA levels.

**Real-Time Polymerase Chain Reaction.** RNA was isolated using the Superscript First-Strand Synthesis System (Invitrogen Life Technologies, Inc.) at the time points and concentrations indicated in the figure legends. The IGF-IR primers IGF-IRfp (5'-TAACGGAAGTGACGTCCTGCATTTCA) and IGF-IRrp (5'-CCGGTGCCAGGTTAGTGATG) and probe (5'-VIC-CACCACGTCCAAGATCTCCGAT-TAMRA) were selected using the Primer Express Software version 1.5 (Applied Biosystems). The probe was labeled with a VIC/TAMRA quencher/reporter. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA was unaffected by the treatments and therefore deemed a suitable control for normalizing the results to total mRNA levels.

**Human Prostate Tissue Microarray Preparation.** A TMA was constructed using archival, paraffin-embedded human prostate tumor specimens. Patient characteristics are outlined in Table 1. For analysis, specimens on the array were segregated into two groups (three cores per disease site), naive and metastatic PCAs. Naïve specimens were obtained from 21 radical prostatectomies. Metastatic lesions were obtained from warm autopsies of 13 men who succumbed to PCAs. All resected naïve PCAs specimens in the array were of Gleason grade ≤ 4 and clinical stage T1 or T2. One metastatic PCA lesion was obtained per patient from seven bone lesions, three lymph node lesions, two liver lesions, and one adrenal lesion.

**Immunohistochemical Studies.** Deparaffinized and rehydrated TMA sections were steamed in citrate buffer for 30 minutes to enhance antigen retrieval. Immunohistochemical labeling with IGF-IR β antibody (1:150; Santa Cruz Biotechnology) was performed overnight. Slides were washed with PBS and incubated with streptavidin-horseradish peroxidase–conjugated goat antirabbit IgG secondary antibody (DAKO) for 10 minutes at room temperature, developed with Nova-red, and counterstained with hematoxylin. Two negative control sections were processed as described above, with the following substitutions. One section was incubated with normal goat nonimmune serum in place of the primary antibody; the other was incubated with preabsorbed primary antibody produced by incubating the IGF-IR antibody for 2 hours with IGF-IR ligand (Santa Cruz Biotechnology). The TMA slides were imaged digitally and evaluated by two independent means, visual scoring by a pathologist and Image Pro Plus (IPP), a digitalized immunohistochemistry scoring program (Media Cybernetics, San Diego, CA).

**Data Analysis.** Excluding the TMA immunohistochemical studies, all results are depicted as the mean ± SD of at least three independent experiments. Representative immunoblots are provided to demonstrate the primary data. Statistical significance of the described treatments was assessed by paired t tests from within analysis of variance analysis (P < 0.05). A t test and the Wilcoxon rank test were used to assess differences in IGF-IR immunostaining of TMA sections between PCa groups. Rank test results were confirmed by the median test. A χ2 analysis was used to assess changes in frequency of IGF-IR–negative cores between groups. For these tests, a P of at least <0.001 was considered statistically significant.

**Table 1** PCa TMA characteristics

<table>
<thead>
<tr>
<th>Tumor characteristics</th>
<th>Naïve PCa (n = 21 patients)</th>
<th>Metastatic lesions (n = 13 patients)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clinical stage (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>T1</td>
<td>12 (57)</td>
<td>0</td>
</tr>
<tr>
<td>T2</td>
<td>6 (29)</td>
<td>0</td>
</tr>
<tr>
<td>T3</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Undelinef</td>
<td>3 (14)</td>
<td>0</td>
</tr>
<tr>
<td>Distant metastasis</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Bone</td>
<td>7 (54)</td>
<td></td>
</tr>
<tr>
<td>Lymph</td>
<td>3 (23)</td>
<td></td>
</tr>
<tr>
<td>Liver</td>
<td>2 (15)</td>
<td></td>
</tr>
<tr>
<td>Adrenal gland</td>
<td>1 (8)</td>
<td></td>
</tr>
<tr>
<td>Median PSA before surgery [ng/mL (range)]</td>
<td>6.0 (1.2–12)</td>
<td>N/A</td>
</tr>
<tr>
<td>Gleason grade (%)</td>
<td>3</td>
<td>20 (95)</td>
</tr>
<tr>
<td>4</td>
<td>1 (5)</td>
<td></td>
</tr>
<tr>
<td>Visual scoring</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean score/patient ± SE</td>
<td>1.43 ± 0.122</td>
<td>2.667 ± 0.026</td>
</tr>
<tr>
<td>t test (mean score/patient naïve versus AI)</td>
<td>1.395 ± 0.122</td>
<td>2.692 ± 0.026</td>
</tr>
<tr>
<td>Median score/patient ± SE</td>
<td>1.831 ± 1.146</td>
<td>9.064 ± 2.727</td>
</tr>
<tr>
<td>Wilcoxon rank (mean score/patient naïve versus AI)</td>
<td>0.00003146</td>
<td></td>
</tr>
<tr>
<td>IGF-IR–negative cores (n = cores)</td>
<td>25.58% (43)</td>
<td>0% (36)</td>
</tr>
</tbody>
</table>

Abbreviations: PSA, prostate-specific antigen; N/A, not available.
RESULTS

IGF-I Protects C4-2 Cells but not Parental LNCaP Cells from Cytotoxic Stress in the Absence of Androgens. Previous reports suggest that increased IGF responsiveness plays a role in PCA progression (3, 5, 29). However, it has also been proposed that loss of IGF responsiveness is necessary to promote proliferation and metastasis (16–18). We have used the LNCaP/C4-2 lineage-derived AI progression PCA model to examine this discrepancy. Serum-deprived LNCaP and C4-2 cells treated with LY294002 exhibited a 6- and 12-fold increase in apoptosis respectively, as measured by nucleosome formation (Fig. 1). IGF-I treatment (10–100 ng/mL) did not diminish LY294002-induced nucleosome formation in LNCaP cells. In contrast, C4-2 cells exhibited a basal apoptotic index that was approximately half that observed in LNCaP cells and an IGF-I dose-dependent protection from LY294002-induced apoptosis. LY294002-induced apoptosis of C4-2 cells was suppressed approximately 40% when cells were treated with as little as 10 ng/mL IGF-I and increased in a dose-dependent manner up to approximately 65% at 100 ng/mL IGF-I. These results indicate that C4-2 cells have the ability to use IGF-I to protect against an apoptotic stress, under conditions in which parental LNCaP cells do not.

To determine whether androgen modified the LY294002-induced apoptotic response of either cell line to IGF-I, the cells were grown in SF-RPMI 1640 ± 10⁻¹⁰ mol/L R1881 and 100 ng/mL IGF-I (Fig. 2). Whereas R1881 provided modest (~23%) protection of LY294002-treated LNCaP cells, treatment with both IGF-I and R1881 substantially suppressed apoptosis of LNCaP cells (approximately 80% over the LY294002-treated LNCaP control). The same degree of protection was observed in LY294002-treated C4-2 cells in the presence of IGF-I alone, and LY294002-mediated apoptosis was almost completely suppressed in C4-2 cells treated with both R1881 and IGF-I. The ability of IGF-I and R1881 to protect against LY294002-induced apoptosis in LNCaP and C4-2 cells was confirmed by comparable changes in cleaved poly(ADP-ribose) polymerase levels from whole cell lysates of appropriately treated cells (data not shown). These results indicate that the antia apoptotic response of LNCaP cells to IGF-I is androgen dependent and suggest that part of the acquisition of androgen independence by C4-2 cells includes increased antiapoptotic responsiveness to IGF-I under androgen-deprived conditions.

IGF-I Differentially Promotes Proliferation of LNCaP and C4-2 Cells. LNCaP cells were shown previously to be marginally responsive to IGF-I as a mitogen under androgen-deprived conditions (30). To determine whether the differential responsiveness of LNCaP and C4-2 cells to IGF-I was also apparent in a mitogenic assay, [³H]thymidine incorporation was measured in LNCaP and C4-2 cells treated with or without R1881 (0.1 and 1 nmol/L) and with or without IGF-I (100 ng/mL) in SF-RPMI 1640 (Fig. 3). Under serum-free conditions, the mitotic index of R1881-stimulated LNCaP cells increased ~2.9-fold. IGF-I stimulation did not alter the mitotic index of LNCaP cells that were androgen deprived or stimulated with 1 nmol/L R1881; however, IGF-I treatment did suppress the mitotic index of cells treated with 0.1 nmol/L R1881 by ~50%. C4-2 cells exhibited a 2-fold higher mitotic index than LNCaP cells under serum-starved culture conditions, and R1881 stimulation further enhanced this mitotic activity 2.2- to 2.4-fold (0.1 and 1 nmol/L R1881, respectively). In contrast, stimulation with IGF-I significantly increased (2.4-fold) the mitotic index of serum-starved C4-2 cells. Additionally, IGF-I treatment further enhanced the R1881-stimulated mitotic activity of C4-2 cells 25% to 57% (1 and 0.1 nmol/L R1881, respectively). These results indicate that increased IGF-I responsiveness of C4-2 cells is also apparent as a mitotic response in either the presence or absence of androgen costimulation.

Cell Surface IGF-IR Expression is Androgen Dependent in LNCaP and C4-2 Cells. We tested whether altered surface expression segregated with differential IGF responsiveness of LNCaP and C4-2 cells by comparing the effect of androgen deprivation on cell surface IGF-I expression by FACS analysis (Fig. 4). Whereas treatment with R1881 significantly increased cell surface IGF-I expression in LNCaP cells (4.4-fold) and C4-2 cells (3.2-fold), surface expression was indistinguishable between LNCaP and C4-2 cells after 48 hours in serum-free media in the presence or absence of androgens. These results indicate that IGF-I expression in both cell lines is subject to regulation by androgens, but that surface presentation of IGF-I does not appear to account for the difference in IGF-I responsiveness observed in LNCaP and C4-2 cells under androgen-deprived conditions.

IGF-IR–Mediated Signaling Is Androgen Dependent in LNCaP Cells and Androgen Independent in C4-2 Cells. In light of the differential androgen dependence of LNCaP and C4-2 cells to IGF-I–mediated proliferative and antiapoptotic responses, we investigated whether these cells also exhibited differential IGF-I–mediated intracellular signaling. IGF-I activation was assessed from R1881-, IGF-, and LY294002-treated LNCaP and C4-2 cells by immunoprecipitation and anti–phospho-tyrosine immunoblotting of the IGF-IR β-subunit (Fig. 5A). DU145 cells were used as a positive control for IGF-IR β-subunit immunoprecipitation and IGF-I–mediated phosphorylation. In the absence of androgen, IGF-I/β-subunit tyrosine phosphorylation was 2- to 3-fold higher in C4-2 cells than in LNCaP cells. However, C4-2 cells expressed a higher basal level of total cellular IGF-IR than LNCaP cells, and this difference in receptor phosphorylation was proportional to the increased amount of total IGF-IR β-subunit immunoprecipitated from cell-equivalent lysates (C4-2/LNCaP = 2.48 ± 0.93; n = 4). Treatment with R1881 increased, to equivalent levels, the amount of IGF-IR immunoprecipitated from both LNCaP and C4-2 cells. Because LNCaP cells have a lower basal IGF-IR level, their response to androgen appears to be more robust, such that IGF-IR levels are raised to an amount comparable with that observed in C4-2 cells. After IGF-I stimulation, receptor tyrosine phosphorylation was equally enhanced, indicating that increased IGF-IR availability may translate into increased IGF-I signaling po-

Fig. 1. IGF-I mediates protection from apoptosis in C4-2 cells but not LNCaP cells. LNCaP and C4-2 cells were cultured in SF-RPMI 1640 for 24 hours with or without IGF-I (0, 10, 25, and 100 ng/mL) and with or without 40 μmol/L LY294002 (LY). LY294002-induced apoptosis was measured from total cell RIPA lysates using Roche Diagnostics nucleosome release apoptosis enzyme-linked immunosorbent assay (ELISA) and expressed as a representative assay of the relative nucleosome release normalized to LNCaP cells cultured in RPMI 1640 in the absence of IGF-I or LY294002 (±SD).
potential. These immunoprecipitation results, performed using MAb 3B7, were confirmed using the MAbs 1H7 and GR11 (EMD Biosciences, San Diego, CA).

A previous report suggested that PI3K signaling could differentially affect AR phosphorylation and activity, depending on the LNCaP passage number (31). We therefore assessed whether IGF-I–mediated changes in survival and proliferation were due to changes in AR transcriptional activity in LNCaP and C4-2 cells. We found that IGF-I treatment had no effect on the androgen dose-dependent induction of luciferase expression in pARR3-luciferase–transfected cells (data not shown) and conclude that IGF stimulation has no effect on androgen responsiveness in this lineage-derived AI progression model.

Downstream IGF-IR signaling was assessed by IGF-I–induced formation of stable immune complexes between the PI3K p85 subunit and tyrosine-phosphorylated IRS-2 (Fig. 5B). We found that in the absence of androgens, IGF-I stimulation of C4-2 cells readily induces IRS-2/PI3K complex formation and IRS-2 tyrosine phosphorylation. In contrast, LNCaP cells fail to induce a significant IRS-2/PI3K complex formation and IRS-2 tyrosine phosphorylation unless cultured with androgen. The amount of IRS-2 immunoprecipitated with p85 PI3K in C4-2 cells cultured with or without R1881 was indistinguishable. When normalized to the amount of immunoprecipitated IRS-2, the stoichiometry of IRS-2 tyrosine phosphorylation seen in LNCaP and C4-2 cells was a third of that seen in serum-starved DU145 cells.

Intriguingly, in both LNCaP and C4-2 cell lines, IGF-I–induced p85 PI3K/IRS-2 complex formation was substantially enhanced by short-term LY294002 treatment without obvious effect on IGF-IR phosphorylation. This is likely due to the suppression of PI3K-mediated negative feedback that initiates IRS degradation, allowing accumulation of the activated p85/IRS complex (32, 33). The 5-minute IGF stimulation used in these experiments was chosen based on a 30-minute time course, indicating that the magnitude of IGF-IR and p85 PI3K/IRS-2 complex formation was maximal at 5 to 10 minutes (data not shown).

To measure signaling events downstream of PI3K/IRS-2 complex formation, lysates prepared from cells treated as described in Fig. 5 were immunoblotted for total and phospho-Ser473AKT (Fig. 6A). This also provided a measure of potency for the inhibition of PI3K by LY294002. IGF-I–induced MAPK phosphorylation was found to be unaffected by LY294002 treatment (Fig. 6B). As expected, AKT exhibited constitutively elevated phospho-Ser473 levels in the PTEN-null LNCaP and C4-2 cells with or without 5 minutes of IGF-I stimulation, whereas phospho-Ser473 AKT was undetected in lysates from LY294002-treated cells. However, by 10 minutes after IGF-I stimulation, phospho-Ser473 AKT was readily detected, even in LY294002-treated LNCaP and C4-2 cells. Our results indicate that IGF-I can drive PI3K signaling even in the presence of LY294002 and that the presence or absence of the inhibitor has no effect on the marginal ability of IGF-I to activate MAPK. We conclude that LNCaP and C4-2 cells exhibit androgen-responsive IGF-IR activation. The increase in IGF-IR immunoprecipitated from C4-2 cells suggests that increased expression of activatable total cellular receptor and increased coupling to downstream signaling partners such as IRS-2,
PI3K, and AKT are possible mechanisms by which C4-2 cells have acquired enhanced IGF-I responsiveness in the absence of androgens.

**Androgens Regulate IGF-IR Messenger RNA Levels in LNCaP and C4-2 Cells.** Having observed that androgen availability regulates the amount of immunoprecipitable and activable IGF-IR in LNCaP and C4-2 cells (Fig. 5), we next investigated the molecular basis for the androgen responsiveness of IGF-IR availability by assessing changes in steady-state IGF-IR mRNA levels in LNCaP and C4-2 cells. Cells were cultured in T-media under serum-starved conditions with or without R1881 (10^(-10) mol/L) for 2 days and analyzed by real-time PCR (Fig. 7). When grown in T-media, C4-2 cells expressed marginally increased IGF-IR mRNA compared with LNCaP cells under normal culture conditions. IGF-IR mRNA expression decreased ~50% when LNCaP cells were cultured under serum-starved conditions for 2 days and increased 5.5-fold when LNCaP cells were cultured in the presence of androgens for 2 days. C4-2 cells exhibited parallel androgen responsiveness for IGF-IR mRNA expression, with levels decreasing ~50% under serum-starved conditions and increasing almost 2-fold in the presence of R1881. Interestingly, IGF-IR mRNA levels in LNCaP cells cultured in the presence of R1881 for 2 days were consistently higher than those seen in identically treated C4-2 cells. Under serum-starved conditions, LNCaP and C4-2 IGF-IR mRNA decreased to approximately the same level, with a trend toward higher levels in C4-2 cells. These results are consistent with immunoprecipitation data and suggest that steady-state IGF-IR mRNA expression in C4-2 cells is less responsive to regulation by androgens than that in LNCaP cells.

**Androgens Differentially Regulate Total Cellular IGF-IR Protein Levels in LNCaP and C4-2 cells.** As described above, androgen availability regulates the amount of immunoprecipitable IGF-IR in LNCaP and C4-2 cells. These differences correlate with the relative steady-state mRNA levels in the respectively treated cells. To more closely examine the time course of androgen stimulation or withdrawal, we characterized total cellular IGF-IR protein levels relative to expression of MAPK by immunoblotting whole cell lysates from cells cultured in serum-free media in the presence or absence of androgens over a 48-hour time course (Fig. 8A and B). Total cellular IGF-IR protein levels in LNCaP and C4-2 cells cultured in T-media were essentially identical (LNCaP/C4-2 = 0.96 ± 0.30; n = 3). This contrasts with the pattern observed for immunoprecipitable IGF-IR.
and steady-state mRNA levels. However, like previous observations, over the androgen withdrawal time course, LNCaP cells showed a steady decline in IGF-IR levels, resulting in a 2.5-fold decrease by 48 hours compared with T-media conditions. In contrast to immunoprecipitation and mRNA data, C4-2 cells exhibited no distinguishable change in total IGF-IR protein level after androgen withdrawal. However, taken together, total Western blot, immunoprecipitation, and mRNA data reveal that the IGF-IR decline in C4-2 cells is less drastic in response to androgen withdrawal compared with that in LNCaP cells. Conversely, when cultured in SF-RPMI 1640 in the presence of 10−9 mol/L R1881, LNCaP cells exhibited a time-dependent increase in IGF-IR expression (3-fold higher by 48 hours) compared with T-media conditions, whereas C4-2 cells exhibited a 2-fold change over the same time frame. In data not shown, treatment with the AR antagonist bicalutamide (10 μmol/L) did not alter basal expression of IGF-IR in either cell line cultured in SF-RPMI 1640, indicating that basal expression of IGF-IR in both LNCaP and C4-2 cells is AR independent and that the elevated basal expression of IGF-IR seen in C4-2 cells is not due to basal AI AR activity. These results complement the steady-state mRNA and immunoprecipitation results, confirming that total IGF-IR expression is less responsive to regulation by androgens in C4-2 cells compared with LNCaP cells. Furthermore, fluctuations in IGF-IR expression in C4-2 cells are much less pronounced at the protein level than at the steady-state mRNA level, suggesting that the loss of androgen-responsive regulation of IGF-IR expression in these cells may be controlled at both a posttranscriptional and a posttranslational level.

**IGF-IR Is Up-Regulated during Prostate Cancer Progression in Human Prostate Cancer Specimens.** To compare our findings from the LNCaP/C4-2 cell lines with human PCA, we examined IGF-IR expression by immunohistochemical staining of a TMA to compare hormone-responsive and AI human PCA specimens. Prostate epithelial IGF-IR levels were determined by two methods: (a) visual scoring using the traditional system of 0–3 (0 represents a negative result for IGF-IR immunohistochemical staining, and 3 represents maximal staining); and (b) IPP image analysis software using a digitized scoring range of 0 to 100. Both methods for comparison of IGF-IR levels between naïve PCA and AI metastatic lesions revealed the same trends (Table 1; Fig. 9). Median IGF-IR levels were relatively low in naïve PCA specimens. The median IGF-IR level score was 2-fold higher in the AI tumor specimens compared with naïve specimens by visual scoring and 5-fold higher by IPP analysis. A t test comparing the mean score/patient between naïve and AI samples showed a significance of P < 0.0001. Because the distribution of scores for the naïve specimens scored by IPP was not normal, a standard t test could not be used to compare the means. Using the Wilcoxon rank test to compare the ranked mean score/patient of the two groups, IGF-IR expression was determined to be significantly different (P < 0.001; Table 1). These results were confirmed using a median test (data not shown).

Furthermore, 26 of 43 naïve PCA cores scored ≤1 by visual scoring, and 11 of 43 scored negative for IGF-IR expression by IPP analysis, whereas in AI metastatic lesions, 3 of 36 cores scored ≤1 by visual scoring, and none scored 0 by IPP analysis. The difference in frequency of cores negative for IGF-IR expression by IPP was determined to be significant by χ2 analysis (0.005 < P > 0.001; Table 1). These results from human tumor specimens indicate that the proportion of IGF-IR-negative cores is elevated in
androgen-dependent tumors and that IGF-IR levels are increased in AI metastatic disease.

**DISCUSSION**

Whereas studies implicating the IGF axis as a regulator of PCa cell proliferation and survival are emerging (4–8, 11–13, 15), little is known regarding the role of IGF axis molecules in progression to AI disease. This report confirms previous studies indicating that increased IGF-IR expression is correlated with AI progression of human PCa (13, 29) and is the first to demonstrate that development of androgen independence is linked to enhanced AI IGF responsiveness in a lineage-derived human PCa model. This establishes the LNCaP/C4-2 lineage-derived PCa cell lines as an *in vitro* PCa model for investigating the role of IGF signaling in disease progression.

LNCaP and C4-2 cells are PTEN-null and exhibit constitutively activated PI3K, phosphotidylinositol-3,4,5-triphosphate [PI(3,4,5)P3] accumulation, and activated AKT. Using the PI3K inhibitor LY294002 to induce apoptosis in LNCaP and C4-2 cells as described previously (34), we demonstrate that under androgen-deprived conditions in which LNCaP cells are unresponsive, C4-2 cells can use IGF-I as an ant apoptotic growth factor. We also demonstrate that IGF-I–mediated mitogenesis is androgen dependent in LNCaP cells and androgen independent in C4-2 cells, confirming that the enhanced IGF-I responsiveness of C4-2 cells is part of their AI phenotype.
Studies suggest that IGFs may have a role as autocrine factors in disease progression (35). Although increased IGF-II mRNA and protein have been observed in PCa (35–37), IGF-II is at least an order of magnitude less effective in stimulating growth in primary epithelial cell cultures, due to its lower affinity for the IGF-IR when compared with IGF-I (38, 39). Recent reports indicate that LNCaP and C4-2 cells produce no measurable IGF-I (40) and that IGF-II mRNA can be detected in LNCaP and C4-2 cells (41). Whereas IGF-II mRNA levels were 3-fold higher in C4-2 cells than in LNCaP cells, the level of detected in LNCaP and C4-2 cells (41). Whereas IGF-II mRNA levels in LNCaP and C4-2 cells, the level of mRNA expression in C4-2 cells was ~100-fold less than that in the IGF-II autocrine-responsive neuroblastoma cell line SK-N-AS and 1,000-fold less than that of the metastatic SV40 T antigen-transformed PCa model, M12. Consistent with these findings, only trace amounts of IGF-II have been detected in conditioned media of LNCaP cells (~0.2 ng/mL; ref. 42). Furthermore, under the culture conditions used in our study, we found no evidence for autocrine activation of the endogenous IGF-IR in either cell line (Fig. 5). We therefore conclude that autocrine IGF-IR stimulation is not likely to be a major contributor to proliferation and survival signaling in LNCaP and C4-2 cells; therefore, we focus on IGF-1 as a paracrine factor for IGF-IR activation in these studies.

To investigate cell line-specific mechanisms involved in IGF-I-mediated survival and mitogenesis, we examined IGF-IR activation and downstream signaling. By FACS analysis, no difference in cell surface IGF-IR level was detected in LNCaP and C4-2 cells cultured with or without androgen. However, biochemically, differential androgen dependence for IGF-IR responsiveness is reflected by increased ability to immunoprecipitate total and activated IGF-IR from androgen-deprived C4-2 cells as compared with LNCaP cells. Activated IGF-IR directs downstream signaling through tyrosine phosphorylation of IRS molecules. We observed that phosphorylation of IRS-2 is indistinguishable in C4-2 cells with or without androgen stimulation but is androgen dependent in LNCaP cells. The difference in IGF-IR level may equip C4-2 cells with the ability to reach a threshold of IGF signaling potential that would otherwise require the contribution of androgen to achieve, effectively making C4-2 cells androgen independent in terms of IGF-IR responsiveness.

Previous reports have indicated that P38K inhibition can be overcome by AKT-independent growth factor-mediated protection from apoptosis (34, 43). However, our ability to detect AKT activation even in the presence of LY294002 suggests that AKT activation may still be a component of survival signaling even in the presence of a potent P38K inhibitor. Additionally, P38K inhibition had no effect on the marginal ability of IGF-I to activate MAPK, suggesting that increased MAPK activation does not mediate growth and survival responses to IGF-I. This contrasts conclusions by Murillo et al. (44) stating that enhanced MAPK activity correlates with AI progression in response to HER2/neu signaling, but our conclusions are in agreement with those made by Kulik and Weber (43) indicating that IGF-I-mediated survival signaling is MAPK independent.

The ability of C4-2 cells to respond to IGF-I by inducing IRS-2 phosphorylation and association with p85 under androgen-deprived conditions correlates with the AI proproliferative and antiaipoptotic IGF-I effects of these cells. IRS molecules are important mediators of mitogenesis and survival signaling and have significant overlap in function. Whereas the specific roles of the IRSs remain unclear, studies do suggest that they are not completely redundant. IRS-1 may be a more potent mitogenic factor than IRS-2 (45, 46), and IRS-2 may be more critical in promoting survival signaling (46). Because both LNCaP and C4-2 cells are IRS-1–null but do express IRS-2, perhaps the loss of IRS-1 preferentially drives the biological response toward survival rather than mitosis. Additionally, loss of IRS-1 is suggested to be important for the gain of metastatic potential by impairing the function of integrins and E-cadherin, thereby favoring cell detachment (47); however, this contrasts with recent findings indicating that in a majority of cases, IRS-1 is up-regulated in metastatic disease compared with primary PCa (29). It is important to note that some reports indicate that other metastatic PCa cell lines, such as DU145, express IRS-1, albeit at low levels (47); in addition, DU145 cells are positive for PTEN (48). It has been reported that down-regulation of IRS-1 may be correlated with loss of PTEN expression (29). PTEN is lost in many cancers, including half of lethal PCa cases (29); therefore, loss of IRS-1 and signaling through IRS-2 may be a reflection of the PTEN-null status of the cells used in these experiments and/or the differences between subtypes of PCa. Nonetheless, it is significant that up-regulation of IGF-IR translates into enhanced signaling potential for downstream targets such as the IRSs.

In terms of clinical relevance, acquiring the ability to sustain IGF-IR expression under androgen-deprived conditions may be a key adaptive response as tumors progress to androgen independence (49). Immunocytochemical data obtained from the TMA human PCa tumor specimens indicate that IGF-IR levels are significantly increased in metastatic lesions of human AI PCa. This observation supports conclusions from previous reports that IGF-IR levels increase with PCa disease progression (13, 29) and is in agreement with a reported decrease of IGF-I and IGF-IR gene expression in rodents undergoing finasteride-induced ventral prostate regression (50). These comparisons confirm the suitability of the LNCaP/C4-2 model to recapitulate potentially important aspects of PCa progression.

Interestingly, our studies in LNCaP/C4-2 cells show that androgen deprivation decreases steady-state IGF-I and IGF-II mRNA and protein levels in both cell lines and that androgen stimulation increases steady-state IGF-I and IGF-II mRNA and protein levels in both cell lines. Furthermore, C4-2 cells exhibit elevated steady-state IGF-IR mRNA and protein levels in T-media and after androgen deprivation, when compared with identically treated LNCaP cells. Androgens have been shown to regulate AR expression posttranscriptionally by increasing mRNA stability through sequestering the mRNA in polyribosomes (51). This increases both AR mRNA abundance and translation. Perhaps a similar mechanism is involved in IGF-I and IGF-II and protein accumulation in response to androgens.

The changes we observed in IGF-I level were more readily revealed in the immunoprecipitation experiments but were not as detectable by standard Western blots of whole cell lysates. The increase in activated immunoprecipitable IGF-IR from C4-2 cells compared with LNCaP cells. Activated IGF-IR directs downstream signaling through tyrosine phosphorylation of IRS molecules. We observed that phosphorylation of IRS-2 is indistinguishable in C4-2 cells with or without androgen stimulation but is androgen dependent in LNCaP cells. The difference in IGF-IR level may equip C4-2 cells with the ability to reach a threshold of IGF signaling potential that would otherwise require the contribution of androgen to achieve, effectively making C4-2 cells androgen independent in terms of IGF-IR responsiveness.

In summary, we propose that the LNCaP/C4-2 in vitro system is an excellent model for the study of IGF signaling in AI progression of PCa. First, these cell lines mimic events that occur during the natural progression of PCa. They represent two different states of PCa, androgen dependence (LNCaP) and androgen independence (C4-2). Previously, C4-2 cells have been shown to metastasize to lymph and bone, common sites for PCa metastasis in humans (27). Also, we have demonstrated that the increase in IGF-IR seen in other in vivo PCa models and in human metastatic disease is mimicked in the androgen-dependent LNCaP/androgen-independent C4-2 model. Second, LNCaP cells provide a system for detailed study of the link between IGF-IR and androgen in a naturally occurring androgen-dependent cell line. We found that IGF-I levels are responsive to androgen and decrease in response to androgen withdrawal. We propose this is one mechanism for the success of androgen ablation therapy in humans.
Third, C4-2 cells provide a system for detailed study of the uncoupling of IGF signaling from androgenic control. Although C4-2 cells are responsive to androgen, they do not respond to androgen as robustly as LNCaP cells, nor do they demonstrate as significant a decrease in IGF-IR levels and downstream activation under androgen-deprived conditions. This suggests that enhanced AI IGF-IR–expressing cells may be selected for during progression to androgen independence. If this is the case, our findings may have exciting implications for the use of IGF-IR tyrosine kinase inhibitors as drug candidates (52). Fourth, our theory regarding increased IGF responsiveness and uncoupling from androgenic control complements the observations of others (13, 15, 21). In conclusion, the up-regulation of IGF-IR in AI C4-2 cells and on metastasis, coupled with the enhanced IGF-I responsiveness in C4-2 cells as measured by survival and analysis of signaling activity, agrees with an IGF-I–mediated mechanism for acquisition of androgen independence.

ACKNOWLEDGMENTS

Metastatic PCa specimens were kindly provided by Dr. Robert Vessella (University of Washington, Department of Medicine, Seattle, WA) and the National Institutes of Health Pacific Northwest Prostate Cancer Specialized Programs of Research Excellence. We thank Dr. Zhihong Wen and Elaine Vickers (The Prostate Centre, Vancouver, British Columbia, Canada) for technical assistance, and Drs. Christopher Ong (The Prostate Centre, Vancouver, British Columbia, Canada), Alice Mui (University of British Columbia, Department of Surgery, Vancouver, British Columbia, Canada), and Torsten Nielson (University of British Columbia, Department of Pathology, Vancouver, British Columbia, Canada) for helpful discussions and insights.

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Increased Insulin-Like Growth Factor I Receptor Expression and Signaling Are Components of Androgen-Independent Progression in a Lineage-Derived Prostate Cancer Progression Model

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