In Vivo Progression of LAPC-9 and LNCaP Prostate Cancer Models to Androgen Independence Is Associated with Increased Expression of Insulin-like Growth Factor I (IGF-I) and IGF-I Receptor (IGF-IR)

Tara Nickerson, Francis Chang, Donald Lorimer, Steven P. Smeekens, Charles L. Sawyer, and Michael Pollak

ABSTRACT

Androgen deprivation therapies for metastatic prostate cancer are useful initially, but progression to androgen independence usually results in relapse within 2 years. The molecular mechanisms underlying the clinically important transition from androgen dependence to androgen independence are poorly described. Several lines of investigation have suggested that insulin-like growth factors (IGFs) are involved in the biology of prostate cancer, but little is known about their relevance to progression to androgen independence. We used three in vivo models of androgen-dependent (AD) human prostate cancer to study this issue. Progression to androgen-independent (AI) growth was associated with a 60-fold increase in expression of IGF-I mRNA in LAPC-9 xenografts and a 28-fold increase in IGF-1 expression in LNCaP xenografts, relative to the initial AD neoplasms. IGF type I receptor (IGF-IR) mRNA levels were 2-5-fold and 5-10-fold higher, respectively, in AI LNCaP-9 and LNCaP tumors compared with the original AD neoplasms. AI growth of these xenografts was also associated with significant reductions in IGF binding protein-3 expression. LAPC-4 xenografts, which previously have been shown to exhibit molecular pathology related to HER-2/neu expression with progression to AI, showed relatively minor changes in expression of the genes investigated, but we nevertheless found evidence of increased IGF-IR phosphorylation with progression to androgen independence in this model. Taken together with prior observations, our results suggest that deregulation of expression of genes related to any one of several critical receptor tyrosine kinase regulatory systems, including IGF signaling, may confer androgen independence.

INTRODUCTION

AD prostate cancer can be treated with androgen deprivation strategies such as castration or antiandrogens, but progression to AI cancer, for which there are no satisfactory treatments, usually occurs. There is a clear need to identify molecular targets for novel therapeutic approaches to either prevent the progression of prostate cancer to androgen independence or to treat AI cancers.

Study of the molecular mechanisms associated with progression to androgen independence has been limited by the availability of suitable models. The work we report here is based on three human prostate cancer models of in vivo progression to androgen independence, LAPC-9, LNCaP, and LAPC-4. The recently described LAPC-9 model is a human prostate cancer xenograft that requires androgens for growth in SCID mice, secretes PSA, and expresses a normal androgen receptor. LAPC-9 tumor cells enter a dormant state in response to castration of the host, and a subset of tumors resumes growth with an AI phenotype after prolonged androgen deprivation (1). The LNCaP model is an androgen-sensitive, PSA-secreting, immortalized prostate cancer cell line (2). A mutation in the hormone-binding domain of the androgen receptor has been demonstrated in LNCaP cells (3). LNCaP cells readily form tumors in SCID mice when co-injected with Matrigel and are associated with serum PSA levels that are directly proportional to tumor volume in intact hosts (4). After castration, the growth of LNCaP tumors is arrested, and serum PSA levels decrease significantly. Although this aspect of the model has been studied in some detail, less attention has been given to the finding that prolonged androgen deprivation leads to AI growth of LNCaP tumors, which is accompanied by PSA production similar to precastrate levels (5). The LAPC-4 human prostate cancer xenograft model has been used previously to study molecular changes associated with transition from AD to AI growth (6, 7).

IGF-I has well-characterized mitogenic and antiapoptotic effects that are mediated through the IGF-IR (8, 9). Ligand-receptor interactions are modulated by a family of high-affinity IGFBPs (reviewed in Ref. 10). There is considerable evidence from both laboratory and population studies that IGF physiology is relevant to prostate cancer. For example, it has been shown that both normal prostate epithelial cells and prostate cancer cells exhibit IGF responsiveness in vitro (11, 12), that IGF-IR inhibition inhibits prostate cancer cell proliferation (13), and that overexpression of IGF-I in prostate epithelial cells in a transgenic model leads to transformation (14). We (15) and others (16, 17) have demonstrated in prospective studies that a positive correlation exists between circulating IGF-I concentration in healthy men and risk of subsequent prostate cancer. This finding is consistent with results from many (but not all) case-control studies (reviewed in Refs. 18, 19).

A precedent for the importance of peptide growth factors in progression of prostate cancer to androgen independence is provided by the association of androgen independence in LAPC-4 human tumor xenografts with overexpression of HER-2/neu, a tyrosine kinase receptor activated by ligands in the epidermal growth factor family (6, 7). The evidence that both epithelial growth factor and IGF-I can directly activate the androgen receptor in the absence of androgens (20, 21) raises the possibility that IGF receptor signaling may also be involved in progression of prostate cancer to androgen independence. We undertook experiments to determine whether progression to AI growth in vivo is related to changes in expression of genes encoding key molecules involved in IGF signaling.

MATERIALS AND METHODS

Tumor Xenografts. The LAPC-4 and LNCaP-9 xenografts were derived as described in (1, 6). The clinical material was minced into 2-mm chunks and implanted with 200 μl of Matrigel (Collaborative Research, Bedford, MA) s.c. into male SCID mice. After initial tumor formation, tumors were harvested, minced, and reimplanted into SCID mice. The LNCaP xenograft was derived from the LNCAP cell line obtained from American Type Culture Collection.
which was injected (1 × 10^6 cells) with Matrigel into flanks of male SCID mice. Once tumors formed, the tumors were serially passaged into male mice as described for the LAPC-9 xenograft.

AD xenografts were removed from mice either before or after castration or after acquiring an AI phenotype, and mRNA was extracted for quantification of mRNA abundance by quantitative RT-PCR and Northern blotting as described previously (22).

**Quantitative RT-PCR.** Quantitative RT-PCR was performed using TaqMan technology. First-strand cDNA was synthesized from 5 μg of total RNA. PCR reactions (50 μl) were performed in a buffer containing 50 mM KCl, 10 mM Tris-HCl, 5 mM MgCl₂, 500 μM each of dATP, dCTP and dGTP, 400 μM of each forward and reverse primers, 200 μM probe, 1.25 units of AmpliTaq Gold, 0.5 unit of uracil-N-glycosylase and ~150 ng of cDNA. Primers and probes were designed using Primer Express (ABI-Perkin-Elmer) and are listed in Table 1. Thermal cycling was performed using an ABI-7700 under the following reaction conditions: 50°C for 2 min, 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 60 s.

Data were normalized to RNA and calculated as described (23). Changes in gene expression patterns were confirmed by Northern blot analysis. Control studies were carried out to demonstrate assay reproducibility by showing similar results from multiple RNA samples derived from a single tissue sample.

**Western Blotting.** Cell culture and tumor lysates were prepared with 1% Triton buffer (12.5 mM EDTA (pH 8.0), 25 mM HEPES (pH 7.5), 150 mM NaCl, 1% Triton X-100, and 1% glycerol) with protease and phosphatase inhibitors. The antibodies used were rabbit anti-IGF-IR (1:1000; Santa Cruz Biotechnology, Inc., Santa Cruz, CA), rabbit anti-IRS-1 (1:100; Santa Cruz Biotechnology), and mouse anti-β-actin (1:2000; Promega Corp., Madison, WI). Phosphoryrosine was detected with mouse antibody 4G10 (1:2000; Upstate Biotechnologies, Inc., Lake Placid, NY). RIG cells (Rat-1 fibroblasts overexpressing IGF-IR) were a kind gift of Michael Weber (University of Virginia Health Sciences Center, Charlottesville, VA).

**RESULTS**

**In Vivo Progression to Androgen Independence.** We confirmed that LAPC-9 tumor xenografts (1) grow readily in intact SCID mice, remain static after castration, and in some cases eventually develop an AI phenotype associated with growth in castrated mice (Fig. 1). Similar observations have been reported for the LAPC-4 and LNCaP models (5–7).

**Changes in Expression of IGF-I, IGF-II, and IGF-IR Associated with Progression to Androgen Independence.** Castration resulted in decreased IGF-1 gene expression in AD LAPC-9 tumors, with mRNA levels dropping to ~10% of control (Fig. 2A). Emergence of AI growth of LAPC-9 tumors was associated with a dramatic increase in IGF-I gene expression. IGF-1 mRNA in AI LAPC-9 tumors is ~60-fold higher than the level in AD tumors, as determined by quantitative RT-PCR (Fig. 2A). Northern blot analysis of the same RNA confirmed a significant increase in IGF-I mRNA abundance. Progression to androgen independence in LAPC-9 tumors was also accompanied by a ~2.5-fold increase in IGF-IR mRNA abundance relative to that in AI tumors (Fig. 2B).

The effects of castration on expression of IGF-I and its receptor in LNCaP tumor xenografts were remarkably similar to those observed in the LAPC-9 model. IGF-I mRNA decreased to <40% of control values in LNCaP tumors within 2 weeks after castration, whereas AI LNCaP tumors were found to have ~28-fold higher IGF-I mRNA levels than AD control tumors (Fig. 2A). IGF-IR gene expression increased ~5-fold in AI LNCaP tumors compared with AD tumors (Fig. 2B).

In contrast, progression to androgen independence in the LAPC-4 model was not associated with major increases in expression of IGF-I (Fig. 2A). IGF-IR mRNA levels decreased to ~15% of control after castration and remained low in AI tumors (Fig. 2B). IGF-II mRNA levels in LAPC-9, LNCaP, and LAPC-4 xenografts were also quantitated by RT-PCR. AI growth of LNCaP tumors was associated with up-regulation of IGF-II mRNA, with levels ~5-fold higher than control. No difference in IGF-II mRNA level was observed between AD and AI tumors in the LAPC-9 or LAPC-4 models (data not shown).

Fig. 3A shows that the ~5-fold increase in IGF-IR mRNA abundance associated with acquisition of AI in the LNCaP model is associated with an increase in IGF-IR expression at the protein level. Neither the ~2.5-fold increase in IGF-IR mRNA abundance in the LAPC-9 model nor the decrease in IGF-IR mRNA in the LAPC-4 model were associated with a detectable change in IGF-IR protein level, as estimated by Western blot (Fig. 3A). Interestingly, however, phosphotyrosine immunoblotting of LAPC-4 AI xenografts showed an increase in tyrosine phosphorylation of IGF-IR relative to AD LAPC-4 (Fig. 3B). Lysates immunoprecipitated with IGF-IR and blotted with anti-phosphotyrosine 4G10 antibody confirmed this observation. Upon ligand binding and activation, IGF-IR auto-phosphorylates cytoplasmic tyrosine residues. The observation that LAPC-4 AI tumors have increased phosphorylation but decreased expression of IGF-IR suggests that IGF signaling pathway may be up-regulated in LAPC-4 AI tumors relative to the AD LAPC-4 tumors.

One of the main signaling effectors of IGF-IR is IRS-1. LNCaP has been shown recently to lack IRS-1 expression along with lack of PTEN expression (24). Surprisingly, IRS-1 could not be detected in

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<tr>
<th>Table 1 Sequences of primers/probes used for quantitative RT-PCR</th>
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<td>The primers and probes were designed with Primer Express (ABI-Perkin-Elmer) using the Genbank Accession number, which appears in parentheses.</td>
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![Fig. 1. Growth of AD and AI LAPC-9 tumor xenografts. LAPC-9 xenografts were established as described in “Materials and Methods.” The growth of AD LAPC-9 tumors in intact mice is shown plotted as means; bars, SE. Some animals were castrated 5 weeks after tumor implantation (arrow), and the growth of AD and AI tumors after castration is shown.](image-url)
LAPC-4 and LAPC-9 xenografts either (Fig. 3C). In addition, IRS-1 mRNA was undetectable in these xenografts by oligonucleotide microarray analysis (data not shown). This suggests that IGF-IR substrates other than IRS-1 may be important in prostate cancer.

**Changes in Expression of IGFBPs Associated with Progression to Androgen Independence.** IGFBP-3 mRNA levels in LAPC-9 tumors increased ~15-fold by 14 days after castration, consistent with our prior results (22, 25). With emergence of androgen independence, however, IGFBP-3 mRNA abundance decreased to ~20% of levels present in AD tumors (Fig. 2C). In contrast, AI LAPC-9 tumors expressed ~18-fold higher IGFBP-5 mRNA than AD tumors (Fig. 2D).

The findings concerning IGFBP expression in the LNCaP model were different in some respects to those seen in the LAPC-9 system. In the LNCaP model, IGFBP-3 mRNA was relatively abundant in AD tumors and decreased to ~15% of control by 14 days after castration (Fig. 2C). At the time of progression to an AI phenotype, a reduction in IGFBP-3 expression compared with control was seen (as in the LAPC-9 model). Relatively minor changes in IGFBP-5 gene expression with progression to AI were observed in the LNCaP model (Fig. 2D).

In contrast to the decrease in IGFBP-3 expression observed during progression to androgen independence in the LAPC-9 and LNCaP models, AI LAPC-4 tumors expressed ~8-fold higher levels of IGFBP-3 mRNA than AD tumors (Fig. 2C). Androgen independence in the LAPC-4 model was not associated with significant changes in IGFBP-5 expression (Fig. 2D). IGFBP-2 mRNA levels were ~10-fold higher in AI LNCaP tumors compared with AD LNCaP tumors, and no changes in IGFBP-2 were observed in either the LAPC-9 or LAPC-4 model (data not shown). We also measured mRNA levels of IGFBPs 1, 4, and 6 in AI LAPC-9, LNCaP, and LAPC-4 xenografts but found no significant differences in gene expression compared with AD tumors (data not shown).

**DISCUSSION**

Our major finding is that progression of both LAPC-9 and LNCaP tumors to AI growth after castration is associated with a major increase in IGF-I gene expression in the neoplastic tissue. AI growth in these tumor models is also associated with up-regulation of IGF-IR gene expression and decreased expression of IGFBP-3. These changes could contribute to increased IGF bioactivity in the tumor microenvironment. This in turn could contribute to androgen independence by androgen receptor-independent changes related to hyperstimulation of the signaling pathways distal to the IGF-IR and/or by mechanisms related to IGF-mediated activation of the androgen receptor (20).

It seems plausible that increased IGF signaling, which is associated with mitogenic and antiapoptotic effects in most experimental systems (8, 9), could be a mechanism that would enable prostate cells to...
Less is known about the role of IGFBP-5 in prostate cancer. Castration-induced apoptotic regression of AD Shionogi tumors is associated with up-regulation of IGFBP-5 (38). In the Shionogi model, both induction of IGFBP-5 and apoptosis can be inhibited by treating mice with calcium channel blockers prior to castration. Although this suggests the possibility that IGFBP-5 may be involved in mediating castration-induced apoptosis, recent reports demonstrating that IGFBP-5 actually confers protection from apoptosis (39, 40) suggest the possibility that up-regulation of expression of IGFBP-5 may represent an attempt to survive despite the presence of other signals favoring apoptosis. Our observation that IGFBP-5 is up-regulated during AI growth of LAPC-9 also suggests the possibility that in certain physiological contexts, IGFBP-5 expression enhances cell survival.

The hypothesis that any one of several sets of molecular derangements is sufficient to confer an AI phenotype is consistent with the clinical observation that progression to androgen independence is a common rather than a rare event. Our data provide evidence that changes in expression of genes in the IGF regulatory system within prostate cancers are associated with acquisition of androgen independence. The results are in keeping with the general hypothesis (41) that

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**Fig. 3.** Western blots for IGF-IR and IRS-1 expression in human prostate xenografts. A, IGF-IR Western blots for positive control RAG cells (Rat-1 fibroblasts engineered to overexpress IGF-IR) and lysates from different xenografts. β-actin staining of each lane was used as loading control. B, IGF-IR and phosphotyrosine Western blots for LAPC-4 xenografts. 293T cell cultures were serum starved and then exposed to vehicle or to 500 ng/ml IGF-I and served as negative and positive controls, respectively. C, IRS-1 Western blots for xenografts. LNCaP and MCF-7 cell cultures were used as negative and positive controls, respectively. β-actin staining of each lane was used as loading control. Lysates and immunoblots were prepared as detailed in “Materials and Methods.”
receptor kinases are important determinants of neoplastic behavior and provide a rationale for studies concerning molecular pathology of IGF signaling in paired clinical AD and AI prostate cancer specimens. Finally, the data raise the possibility that novel pharmacological approaches that target IGF signaling may be of therapeutic value for at least a subset of AI prostate cancers.

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REFERENCES
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