An Antibody Targeting the Type I Insulin-like Growth Factor Receptor Enhances the Castration-Induced Response in Androgen-Dependent Prostate Cancer

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Abstract Purpose: To determine the effect of inhibition of insulin-like growth factor-IR (IGF-IR) signaling with an antibody to the IGF-IR, A12, in conjunction with androgen withdrawal on prostate cancer progression in a human prostate xenograft model, LuCaP 35.

Experimental Design: LuCaP 35 was implanted s.c. in severe combined immunodeficient (SCID) mice. At the time of castration, mice were randomized to one of three groups. Group 1 was castrate only; group 2 received A12 40 mg/kg i.p. for 2 weeks beginning 1 week after castration; and group 3 received A12 40 mg/kg i.p. for 2 weeks beginning 2 weeks after castration.

Results: In group 1, tumor volume decreased to 60% of the starting volume 4 weeks post-castration. In groups 2 and 3, tumor volumes nadired 6 weeks after castration at <10% of the volume at time of castration (P < 0.01). Tumor regrowth was not seen in groups 2 or 3 until 15 weeks after castration. Androgen receptor (AR) localization in tumors showed a decrease in nuclear staining in groups 2 and 3 compared with group 1 (P < 0.001). Tumor volume correlated with nuclear AR intensity. AR-regulated genes increased early in group 1, but did not increase in groups 2 and 3. Thus, tumor-specific survival was prolonged by the addition of A12 to castration.

Conclusions: This study shows that the inhibition of IGF-IR enhances the effects of castration in prostate cancer. These effects are associated with a decrease in AR signaling and nuclear AR localization, and recurrence is associated with an increase in AR-regulated gene expression.

Castration is one of the most effective therapies available for metastatic prostate cancer, with >80% response as measured by a decline in prostate-specific antigen (1, 2). However, recurrence following castration is inevitable. Recently, studies from several groups have shown that following castration, significant amounts of androgens are still detected in the prostate (3). Moreover, androgen receptor (AR) is still detected in the nucleus, and increased AR expression is correlated with prostate cancer progression following androgen withdrawal (3–6). These data suggest that further targeting of the AR and mechanisms of cell survival that occur following castration could significantly enhance castration effects and potentially prolong survival.

Following castration, signaling through the mitogenic and antiapoptotic insulin-like growth factor (IGF) system is increased by several mechanisms (7–10). Within 24 to 48 h post-castration, enhancement of ligand-induced signaling through the type I IGF tyrosine kinase receptor (IGF-IR) occurs by an increase in IGF binding proteins 2 and 5 (7–14). Although IGF-IR expression is decreased immediately after castration, clinical studies show that the receptor increases as time after castration increases (15). These data suggest that signaling through the IGF-IR may be a pathway contributing to prostate cancer cell survival and the emergence of androgen-insensitive disease (16). Zhang et al. (17) have shown that an increase in survivin, an inhibitor of apoptosis, via signaling through the IGF-IR/AKT pathway is a mechanism for the development of resistance to anti-androgen therapy. Other laboratories have also shown that growth factor stimulation of AKT enhances AR signaling, such that the AR is sensitized to transactivation by low levels of androgen (18–22). Taken together, these data suggest that in prostate cancer, signaling through the IGF-IR leads to the development of resistance to androgen deprivation. We have previously reported that A12 as a single agent in LuCaP 35 androgen-dependent (AD) and LuCaP 35v androgen-independent (AI; ref. 16) human prostate cancer xenografts results in a significant decline in the rate of tumor growth, but does not halt or reverse tumor growth (23). We have reported that the inhibition of IGF-IR signaling in AD and AI human prostate xenograft models results in decreased nuclear distribution of the AR (24).
Based on the data from our published studies (23, 25) as well as data from other groups, we hypothesize that treatment of AD prostate tumors with A12 at the time of castration would generate a synergy to enhance the effects of castration and prolong the time for AD tumors to progress to the AI phenotype. In this study, we show that targeting the IGF-IR with the fully human monoclonal antibody A12 following castration significantly enhances the effect of castration and time to occurrence of AI disease in the LuCaP 35 human xenograft model of AD prostate cancer.

**Materials and Methods**

**Xenograft cell line.** The LuCaP 35 human prostate cancer xenograft model is an androgen-dependent human xenograft maintained in severe combined immunodeficient (SCID) mice (23, 26). The xenograft regresses following androgen withdrawal, and tumor regrowth recurs ≈5 weeks after castration. The LuCaP 35 human prostate xenograft was selected for these studies because it has a wild-type AR, secretes PSA, and is representative of the prostate cancer xenografts that develop resistance to androgen deprivation by increasing AR expression (5). LuCaP 35 is pTEN negative (27).

**A12 antibody.** A12 is a fully human antibody antagonist to the human IGF-IR, generated by screening a naïve bacteriophage Fab library (28). A12 does not cross-react with the insulin receptor (28).

**In vivo study.** To study the in vivo effect of A12 on castration, tumor bits (20-30 mm³) of the LuCaP 35 AD human prostate cancer xenograft were implanted s.c. into 6- to 8-week-old intact SCID mice as previously described (23, 26). Tumors were allowed to grow to ≈400 mm³, at which time, the animal is surgically castrated (23, 26). At the time of castration, animals were randomized into one of three groups of 20 animals each: group 1 (castration), castration plus vehicle i.p. thrice a week beginning 1 week after castration; group 2 (early A12), castration plus A12 antibody i.p. at a dose of 40 mg/kg body weight thrice a week for 2 weeks beginning 1 week after castration; and group 3 (late A12), castration plus A12 antibody 40 mg/kg body weight i.p. thrice a week for 2 weeks beginning 2 weeks after castration (Fig. 1). After the single 2-week administration of A12, no further A12 treatments were given. We have shown detectable levels of antibody with the A12 dosing schedule used in this study for up to 4 weeks following cessation of therapy (25). Group 1 was considered the castration control group. The timing of A12 administration for 2 weeks beginning either 1 or 2 weeks after castration was based on published data with the LuCaP 35 cell line.

![Graphs showing tumor volume, PSA levels, and survival curves](attachment:figures.png)
line, indicating that maximum castration-induced apoptosis occurs within 4 days of castration (26). Because the inhibition of IGF-IR signaling could cause cell cycle arrest and prevent cells from undergoing apoptosis, we decided to start A12 when apoptosis was complete following castration (26, 29). In preliminary studies using docetaxel and the M12 human prostate cancer cell line, we noted that the administration of A12 before taxanes decreased taxane-induced apoptosis by arresting the cells in the G1 phase of the cell cycle (25). A12 was administered for 2 weeks and then stopped to determine the efficacy with castration and a prolonged effect of A12 as a single agent following castration. Animals were weighed twice weekly. Blood samples were collected from orbital sinus weekly. The serum was separated, and PSA levels were determined using the IMx Total PSA Assay (Abbott Laboratories). Tumors were measured twice weekly, and tumor volume was estimated by the formula: volume = length × width²/2. Following our University of Washington–approved animal protocol, animals were euthanized when the tumor reached a volume of 1,000 mm³ or when animal weight loss exceeded 20% of the initial body weight.

After euthanization, tumors were collected and treated as previously described (23, 25). A portion of the tumors were fixed in 10% neutral buffer formalin (NBF) and embedded in paraffin. Sections of 5 μm were prepared for immunohistochemistry (IHC) staining. One quarter of the tumor was separated into single cells mechanically. Total RNA and cell lysates for protein analysis were prepared at previously described (23, 25).

During the study, we sacrificed three animals from each group to represent two arbitrary time periods post-castration (period 1 was 17-70 days post-castration, and period 2 was 70-140 days post-castration). Due to the size of the tumors, adequate RNA was collected from group 1 to construct three arrays for each of the two intervals; however, due to the small size of the tumors in groups 2 and 3, two arrays could be constructed at each interval for group 2 and one array for group 3. Because the response and time to recurrence was very similar, we combined the results from the arrays in groups 2 and 3 for analysis. All animal studies and procedures were approved by the University of Washington Institutional Animal Care and Use Committee (IACUC).

Flow cytometry. To measure tumor IGF-IR expression, 5 × 10⁶ cells were incubated with anti–IGF-IR antibody SC-461 (Santa Cruz Biotechnology) and phycoerythrin-conjugated goat anti-mouse antibody and analyzed using a BD FACScan. Data were analyzed using CellQuest® software (BD BioScience; ref. 23).

Apoptosis. Apoptosis was determined by terminal deoxynucleotidyl transferase–mediated nick end labeling (TUNEL) assay and propidium iodide staining using the Apop-Tag kit (Millipore Co.) following the manufacturer’s recommendations. Apoptotic cells were determined per 300 cells per tissue slide.

Immunohistochemistry. Tumor samples were fixed in 10% NBF, embedded in paraffin, and sectioned at 5 μm onto slides. After blocking with 1.5% normal goat serum in PBS containing 0.05% Tween 20 (PBST) for 1 h, slides were incubated with mouse anti-bromodeoxyuridine (BrdUrd) antibody (1 μg/mL) for 1 h, followed by sequential incubation with biotinylated goat anti-mouse immunoglobulin G (IgG) for 30 min, peroxidase-labeled avidin for 30 min (Santa Cruz Biotechnology), and 3,3′-diaminobenzidine (DAB)/hydrogen peroxide chromogen substrate (Vector Laboratories). All incubation steps were done at room temperature. For negative control, mouse IgG (Vector Laboratories) was used instead of the primary anti-BrdUrd antibody. Numbers of BrdUrd-labeled nuclei and total nuclei were collected from 10 random views of each section. Proliferation index was calculated by the number of BrdUrd-positive nuclei divided by the total number of nuclei. Ten fields were counted per slide. AR was detected using an AR-human monoclonal antibody (F56.4.1, Biogenex). IGF-IR was detected using a monoclonal antibody from Santa Cruz Biotechnology against the α-subunit of the IGF-IR (23).

Cytosol and nuclear fractionation. LNCaP cells were grown in T-Medium with 5% fetal bovine serum (FBS) until 40% confluent. Media was replaced with RPMI T&S with 2% charcoal-stripped serum (CSS) for 24 h. Nuclear extracts were collected using the Nuclear/Cytosol Fractionation Kit (BioVision K266-100) according to the manufacturer’s protocol. Purity of fractionation was validated by Western blot using a specific antibody to Golgi, sc20587 or histone 2B, sc-8650 (Santa Cruz Biotechnology).

Deconvolution microscopy. LNCaP cells were plated in T-Medium supplemented with 5% FBS until 40% confluent. Media was replaced with RPMI T&S with 2% CSS for 24 h and/or treated with A12 antibody overnight. Another sample received A12 antibody for 1 h before adding 10⁻⁸ mol/L of dihydrotestosterone, and 20 ng/mL of IGF-I in RPMI T&S with 2% CSS. After treatment, cells were fixed in cold acetone/methanol (1:1) for 10 min and stained with an AR-specific antibody sc-7305 (Santa Cruz Biotechnology) followed by a biotin–SP–conjugated goat anti-mouse IgG (Fab'), (Santa Cruz Biotechnology) and a Streptavidin-Alexa594 (Molecular Probes). About 5 μg of 4′,6-diamidino-2-phenylindole (DAPI) was used to stain the nucleus. Cells were mounted with Prolong Anti-Fade reagent (Molecular Probes) and examined with Deltavision SA3.1 Wide-field Deconvolution Microscope. Three-dimensional results of ROI z-stacks were analyzed using the Image J Analysis software (NIH).

Western blotting. Western blotting was done as previously described and probed with α-tubulin III (23).

Prostate and serum testosterone and dihydrotestosterone. Measurements of serum testosterone and dihydrotestosterone measurements were done as previously described (6). Prostate tissue testosterone and dihydrotestosterone measurements were done in the laboratory of Dr. David Hess (Oregon Primate Center) and have also been previously described (8). Briefly, the tissue was flash frozen at the time of collection and kept at -70°C until assayed. At the time of assay, the tissue was thawed to 4°C, homogenized, and extracted with diethyl ether. Extracts were dried under NO₂ and then stored in ethanol until the time of assay. Separation of steroids was done on Sephadex LH-20 columns as described, and appropriate fractions were assayed by RIA.

cDNA microarray analysis. Custom cDNA microarrays were constructed using clones derived from the Prostate Expression Database (PEDB), a sequence repository of human prostate expressed sequence tag (EST) data available to the public (30). Methods of labeling with Cy3 and Cy5 fluorescent dyes, hybridization to the microarray slides, and array processing were as described (31).

Three tumors were pooled in each experimental group. To provide a reference standard DNA for use on cDNA microarrays, we isolated and pooled equal amounts of total RNA from LNCaP, DU145, PC3, and C4-2B cells. For one sample, reference standard RNA for use on cDNA microarrays, we isolated and pooled equal amounts of total RNA from LNCaP, DU145, PC3, and C4-2B. mRNA was amplified one round using the Ambion MessageAmp II Amplification Kit (Ambion Inc.). Hybridization probes were labeled, and quality control of the array experiments was done as described previously (31). Differences in gene expression associated with treatment groups were determined using the SAM procedure, with a false discovery rate (FDR) of <10% considered significant (32). Similarities between samples were assessed by unsupervised, hierarchical clustering of genes and samples using Cluster 3.0 software and viewed by TreeView.

Real-time reverse transcription-PCR. Survivin and β-tubulin III were assayed by PCR using primers and methods previously described (25). A standard PCR fragment of the target cDNA was purified. About 1 μg of total RNA from each group of pooled tumor was used for first-strand

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cDNA synthesis using Superscript First Strand Synthesis System (Invitrogen). Real-time reverse transcription-PCR (RT-PCR) was done in 20 μL of reaction mixture consisting of 1 μL of first strand of cDNA, specific primers sets, and Lightcycler FastStart DNA Master Plus SYBR Green using a Roche Lightcycler following the manufacturer’s protocol (Roche). RT-PCR products were subjected to melting curve analysis on Lightcycler software v3.5. The amplicon sizes were confirmed by agarose gel electrophoresis. Each sample was assayed in duplicate.

Results

Inhibition of IGF-IR with A12 significantly enhances tumor regression induced by castration and delays time to tumor recurrence. Figure 1A shows a decrease in LuCaP 35 tumor volume following castration (P ≤ 0.05) at 5 weeks when compared with maximum tumor volume at the time of castration. The nadir in PSA occurred at week 3 and subsequently increased throughout the remainder of the study (Fig. 1B). The addition of A12 at either the early or late time points resulted in a rapid decrease in tumor volume that was significantly different from group 1 (Fig. 1A). The slopes of the decrease in tumor volumes were similar between both A12 groups. The apparent difference between groups 2 and 3 during the first 9 weeks of the study is accounted for by the 1-week difference in time between the initiation of A12 treatment. The initial decrease in PSA was similar in each group, and the subsequent increase in PSA began with the regrowth of the tumors. Group 1 tumor volume decreased to 60% of the starting volume 4 weeks after castration (P < 0.01). By 17 weeks, all animals in group 1 had to be sacrificed because tumor volumes had reached 1,000 mm³. In contrast, in the A12 plus castration groups, only two animals had to be sacrificed because of tumor volume by the end of the study, and these events occurred at 14 weeks after the castration.

![Fig. 2. A](image1.png)

**A**, a representative tumor sections from groups 1, 2, and 3 with IHC for the AR. Arrows and numbers, relative values given to nuclear AR staining. Magnification, 40×; no counterstain. Note the marked number of tumor cells with nuclear AR in castrate only group 1 in spite of the fact that the tumor was removed >10 wk after castration.

**B**, correlation between nuclear AR intensity and tumor volume. r = 0.66, P < 0.01. ○, group 1 values; ●, castrate + A12 early and late values, groups 2 and 3. Values are the mean value for 100 nuclei graded per tumor.
In groups 2 and 3, tumor volumes reached their nadir 6 weeks after castration at <10% of the volume at castration ($P < 0.01$). Figure 1C presents survival data on the three groups using tumor death as an end point. Tumor death is based on the tumor volume reaching 1,000 mm$^3$ because this is the tumor volume at which the animal must be euthanized according to the University of Washington IACUC protocol. The study was terminated when all of the group 1 animals met the criteria for tumor volume.

To investigate whether the change in AR nuclear translocation with A12 treatment was a potential mechanism for the interaction of A12 and castration in this study, we did AR IHC on tumors from each of the three groups. We assigned a nuclear AR staining score to 100 nuclei from each tumor (Fig. 2A).

Fig. 3. Significant gene expression changes between the two time periods of A12-treated tumors. A, out of 3,170 unique genes on the array with sufficient data to test, there were 21 up-regulated (including many androgen-regulated ($\uparrow$)) and 41 down-regulated with $\leq 0.10$ $q$ value in the late time period when tumors began to recur compared with the early time period.

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Nuclei were scored blindly by two individuals, and the mean of the two scores was counted as the score for the respective tissue. There was a significant positive correlation between tumor volume and nuclear AR intensity \( (r = 0.66; P \leq 0.01; \text{Fig. 2B}) \).

**Enhanced effect of castration plus A12 treatment is associated with a decrease in AR-regulated gene expression.** cDNA microarrays were done on RNA samples from tumors in each group at the time frames indicated in Materials and Methods. The arrays done before day 73 were included into the “early” time period, and those after that point were termed the “late” time period. No genes were found to be significantly altered between the time periods for group 1 (castration alone) when tested by two-sample t-test in SAM \( (q \text{ value} \geq 100\%) \). In addition, unsupervised, hierarchical clustering of known androgen-regulated genes did not segregate the two time periods. This may not be surprising because the animals in group 1 had PSA and growth recurrence and increased nuclear AR scores in the tumors removed for array analysis at early and late time points. In contrast, there were significant changes in gene expression between the two time periods of A12-treated tumors in groups 2 and 3. Out of 3,170 unique genes on the array, 21 were up-regulated (including many androgen regulated), and 41 were down-regulated with \( \leq 10\% \) q value in the late time period when tumors began to recur compared with the early time period (Fig. 3A). Unsupervised, hierarchical clustering of known androgen-regulated genes clearly differentiated the A12-treated, two time periods into two separate clusters (Fig. 3B). Changes in the expression of representative AR-regulated genes, such as KLK3, FKBP5, and PART1 were further validated by qRT-PCR (Fig. 3C). These data indicate that with progression to AI disease...
after A12 treatment plus castration, greater localization of AR to the nucleus is associated with AR transcriptional activity as a probable mechanism of progression.

Survivin and β-tubulin (TUBB) expression are significantly decreased by A12. Survivin was selected for evaluation because it is an antiapoptotic protein regulated by IGFs and associated with the resistance to castration and progression in prostate cancer (17, 33, 34). As shown in Fig. 4A, qRT-PCR shows a significant positive correlation between survivin copy number and tumor volume ($r = 0.66; P \leq 0.01$). A second gene that correlates with IGF-IR–induced tumor formation is β-tubulin, TUBB (35). In Fig. 4B, TUBB expression was decreased significantly in groups 2 and 3 compared with group 1. The copy numbers of TUBB correlate positively with tumor volume ($r = 0.59; P \leq 0.01$). A third gene that was not differentially expressed over on the microarrays in group 1 but was decreased in the two early time periods in the groups 2 and 3 was PSA. The change in PSA expression was confirmed by a similar pattern in the serum PSA levels (Fig. 1B).

BrdUrd and TUNEL staining show A12 plus castration results in decreased proliferation. As shown in Table 1A, proliferation was significantly greater in the group 1 tumors compared with groups 2 and 3 ($P \leq 0.01$). In contrast, apoptosis as determined by TUNEL staining was higher in group 1 compared with groups 2 and 3 (Table 1A).

Changes in tumor cell surface IGF-IR expression. In group 1, IGF-IR increased with time following castration ($r = 0.32; P \leq 0.02$). No changes in IGF-IR expression over time were seen in either group 2 or 3 and seemed to remain low throughout the course of the study (Fig. 5).

Tumor androgens are not modulated by A12 treatment. We assayed tumor tissue androgens to explore whether A12 might mediate its effect by further suppressing tissue androgen levels. Testosterone and dihydrotestosterone levels were readily detectable in the xenografts when tumors were removed at the time of sacrifice at least 5 weeks after castration (Table 1B; ref. 5). There was no significant difference in tissue androgens in the tumors treated with castration alone versus those treated with castration plus A12 at the time of sacrifice in all three groups. Reduced serum testosterone levels as well as the decrease in PSA confirmed that the animals were castrated, and that modulation of tissue androgen levels is not a mechanism by which A12 mediates its effects on AR translocation.

IGF-IR signaling modulates AR nuclear translocation in LnCaP cells. In this study, we propose that the inhibition of IGF-IR signaling affects the translocation of the endogenous AR. Because the LuCaP xenograft does not grow in vitro, we have elected to use the LnCaP line because it is one of the few human prostate cancer cell lines with an endogenous AR. As seen in Fig. 6A to D, addition of dihydrotestosterone to the medium induces the nuclear translocation of the AR, with a further increase in nuclear AR density when IGF-I and dihydrotestosterone are added in combination, consistent with previous studies (24). When cells were treated with A12 before the addition of IGF-I, the level of nuclear AR was similar to the level seen with dihydrotestosterone alone (Fig. 6E and F). To further assess the effect of the IGF-IR on nuclear AR localization, we isolated the nuclear and cytoplasmic fractions of LnCaP cells treated as indicated with dihydrotestosterone and IGF (Fig. 6G). The purity of fractions was determined by Golgi and histone 2B Western blots for cytoplasmic or nuclear contamination, respectively. The Western blots were done in triplicate experiments, and AR bands were quantitated and controlled for

Fig. 3 Continued. C, RT-PCR of three androgen-regulated genes that had decreased expression in the A12 + castration treatment versus castration alone. Note that the changes on the gel are consistent with changes noted on the array. Gene identification compares to HUGO definition in Fig. 4A.
loading by histone 2B protein. The nuclear/cytoplasmic AR (Nu/Cy) ratio increased significantly ($P< 0.01$) when cells were treated with IGF-I and returned to the level of dihydrotestosterone treatment alone when cells were treated with A12 before the treatment with IGF-I (Fig. 6I). These results were similar to those obtained by deconvolution microscopy, further confirming the effects of the IGF-IR on AR nuclear translocation. Associated with the decrease in nuclear AR, we have seen a decrease in PSA mRNA and protein (data not shown). The Western blots also indicate that in the absence of androgen, there is no effect of IGF or A12 because IGF-1R is not expressed in LnCaP cells without androgen (36).

**Discussion**

This study provides preclinical evidence that treatment with an IGF-IR inhibitory monoclonal antibody, A12, following castration significantly prolongs and accentuates the effect of androgen deprivation on prostate cancer response and time to recurrence in a human xenograft model of prostate cancer (23). A12 inhibits IGF-IR activity by interfering with IGF-ligand binding to the receptor, and A12 significantly decreases IGF-IR cell surface expression by lysosomal degradation (23, 28). In this study, a 2-week period of administration of A12 soon after castration was associated with a greater than 4-fold lengthening of the time of tumor regression in these mice. Because the study was stopped at 24 weeks, the entire length of time until tumor-related sacrifice in the A12-treated animals is not known. However, because SCID mice have a life span of approximate 52 weeks, the 2-week period of A12 treatment extended the tumor regression period by 40% of the animal’s life span (The Jackson Laboratory).

Recent data in men as well as human prostate cancer xenografts in mice have shown that current methods of androgen ablation fail to decrease prostatic androgens below that expected to activate the AR (3, 6, 37, 38). Furthermore, it has been shown that AR expression is the most consistent factor associated with the progression of prostate cancer following androgen withdrawal (5, 39). AR expression increases in the LuCaP 35 human prostate cancer xenograft when regrowth occurred after castration (5). Despite these data indicating that current methods of androgen withdrawal fail to completely abrogate AR-driven tumor progression, castration remains the mainstay of treatment for recurrent prostate cancer with progression-free survival times between 12 and 36 months and time to death of 24 to 72 months.

The addition of A12 to androgen withdrawal has the potential to enhance the effects of castration through various pathways. One pathway would be blocking IGF-mediated recovery from apoptosis. The mechanisms by which the IGF-IR can abrogate apoptosis have been reviewed in several recent papers and are beyond the scope of this discussion, but may involve the Ras–extracellular signal-regulated kinase (Ras-ERK) and the phosphoinositide-3-kinase pathways (40, 41). This pathway is not unique to the prostate, but is one of the most common

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**Table 1. Analysis of proliferation, apoptosis, and steroid levels in tumors**

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>Apoptosis (TUNEL; mean ± SE)</th>
<th>BrdUrd (mean ± SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Castrate</td>
<td>6.58 ± 1.41</td>
<td>27.74 ± 1.93</td>
</tr>
<tr>
<td>Castrate + A12 early</td>
<td>1.29 ± 0.49*</td>
<td>17.78 ± 2.74*</td>
</tr>
<tr>
<td>Castrate + A12 late</td>
<td>1.16 ± 0.37*</td>
<td>12.36 ± 1.75*</td>
</tr>
</tbody>
</table>

**B. Serum testosterone levels and prostate xenograft levels of testosterone (60) and dihydrotestosterone (DHT)**

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Serum testosterone (nmol ± SE)</th>
<th>Prostate testosterone (pg/mg ± SE)</th>
<th>Prostate DHT (pg/mg ± SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-castrate (n = 5)</td>
<td>15.00 ± 6.00</td>
<td>0.60 ± 0.21*</td>
<td>1.07 ± 0.13*</td>
</tr>
<tr>
<td>Castrate (n = 5)</td>
<td>0.70 ± 0.60</td>
<td>1.72 ± 1.41</td>
<td>2.18 ± 0.81</td>
</tr>
<tr>
<td>Castrate + A12 (n = 5)</td>
<td>nd</td>
<td>1.16 ± 0.15</td>
<td>3.18 ± 1.68</td>
</tr>
</tbody>
</table>

NOTE: Note the decreased castrate levels of testosterone in the serum but increased levels of testosterone and dihydrotestosterone in prostate tissue compared with non-castrate tumors. Samples from groups 2 and 3 are included in the castrate + A12 category.

* $P < 0.001$ compared with castrate group.

† $P < 0.001$ versus serum testosterone.

![Fig. 5. IHC of IGF-IR. A, IgG control. B, castrate only, group 1. Note the well-defined IGF-IR expression on cell surface and in cytoplasm. C and D, representative tumors from groups 2 and 3, respectively. Note the decrease in overall IGF-IR staining in tumors from groups 2 and 3 compared with castrate only group 1.](image-url)
Mechanisms involved in recovering from an apoptotic insult in multiple tissues (40, 42–51). In prostate cancer, there is clear clinical evidence that activation of IGF signaling occurs soon after castration, and IGF-IR expression increases in human prostate cancer as the time post-castration increases and with the development of androgen-independent disease (36). A second mechanism by which IGF-IR inhibition could prolong the effects of androgen withdrawal is by maintaining the tumor in cell cycle arrest following the initial apoptosis of androgen withdrawal. After the initial cell death induced by castration, the remaining prostate epithelial cells remain in a state of cell quiescence until tumor growth recurs. We and others have shown that IGF-IR inhibition can cause prostate cancer cells to undergo cell cycle arrest as well as enhance apoptosis (23). The BrdUrd uptake done on tumors harvested at the end of the study would suggest that this is one mechanism by which A12 has prolonged the effects of castration. A third potential mechanism for the activity of A12 in conjunction with castration would be to enhance the inhibition of signaling through the AR. Such a mechanism would be of interest because it would pose a specific reason for use of IGF-IR inhibition in prostate cancer.

We have looked at the expression of two genes that are regulated by the IGF-IR and associated with the development of resistance to castration, tubulin-β peptide (TUBB), and survivin (17, 34, 52). In this study, we noted that survivin and TUBB were suppressed by A12 in the castration-plus-A12 groups compared with the castrate-alone group. Because survivin has been shown as a pathway by which androgen-insensitive disease may arise following androgen deprivation and its expression is increased by IGF-IR activation, a decrease in survivin by the inhibition of IGF-IR may be an important mechanism and marker of A12 activity in prostate cancer. Likewise, TUBB is specifically increased in IGF-IR-mediated cell transformation, and its suppression by A12 may also be a specific biomarker and pathway for inhibition of prostate cancer by A12 (53).

Because the LuCaP 35 tumors used in this study are pTEN negative and because we have shown that A12 decreases AKT phosphorylation in these tumors, we would conclude that the loss of pTEN activity is not a mechanism of resistance to IGF-IR inhibition (27). The effectiveness of targeting the IGF-IR in the absence of pTEN has also been shown in other studies in the prostate as well as other tumors (54, 55).
Persistent signaling through the AR is a common feature of prostate cancer progression in all circumstances where it was examined. In fact, recent data from several groups suggest that not only is the AR continually expressed and active following androgen deprivation, but the prostate may be able to synthesize dihydrotestosterone from several precursor steroids and possibly acetate (3, 4, 39, 56, 57). If these data are correct, the activation of the AR in androgen-deprived prostate cancer patients has become an autocrine rather than endocrine function, and therapy needs to be directed at the AR. In this study, we present evidence that signaling through the IGF-IR enhances classic AR signaling by increasing the translocation to the nucleus. Furthermore, when IGF-IR signaling is blocked soon after castration, the effects of castration are augmented. Associated with the enhanced effects on tumor growth is a marked decrease in nuclear AR. Although marked decreases in serum PSA were noted with castration alone or castration plus A12, PSA began to increase within 4 weeks as tumor size increased in the animals treated with castration alone and was consistent with the regrowth of the tumor. In contrast, in the castration-plus-A12–treated animals, PSA remained suppressed significantly longer than those that received castration as the only treatment. When PSA did subsequently increase, tumor volume also began to increase. This suggests that in spite of being castrated, there was a return of androgen signaling. These differences between groups were confirmed by the cDNA array analysis. In the castration-only group, there were no differences in gene expression between tumors assayed at any of the time points noted. Although in the castration-plus-A12–treated animals, a significant increase in androgen-regulated genes was noted when the tumors recurred, suggesting a return to AR-driven tumor progression. When cDNA arrays were compared between those tumors that had recurred in the castrate group and those that had recurred in the castrate-plus-A12–treated groups, there were no differences, indicating that once the tumors had recurred, the same forces were driving tumor progression as determined by gene expression. We have also effectively ruled out the possibility that these changes in androgen-regulated genes are mediated by the modulation of tissue androgens. Therefore, other means by which AR-mediated signaling is abrogated must be invoked.

The finding most relevant to prostate cancer was the correlation between decreased nuclear AR and tumor volume. This suggests that the inhibition of IGF-IR signaling, in addition to its effects on antiapoptotic and proliferation pathways, may have a specific effect in prostate cancer by altering AR nuclear translocation and subsequent AR signaling. The mechanism by which A12 accomplishes this activity has yet to be defined. However, we do show that in vitro A12 decreases the nuclear/cytoplasmic ratio of AR, and that this change results in a decrease in androgen-regulated gene expression in LNCaP and M12 AR cells (27). We have previously reported that IGF treatment decreases AR phosphorylation (27), and Gioeli et al. have shown that phosphorylation of the AR at Ser<sup>650</sup> is necessary for nuclear export of the AR (57). Therefore, a potential mechanism for the effect of A12 is to enhance AR phosphorylation at Ser<sup>650</sup> and facilitate nuclear export. Lin has reported that signaling via the IGF-IR results in the phosphorylation of the AR in contrast with our studies (58). However, Gioeli has shown that there is no direct phosphorylation of the AR by IGF (59). Regardless, the decrease in phosphorylation of AR and increase in nuclear AR localization is consistent with the current study. The mechanism by which IGF induces dephosphorylation has not been defined.

Finally, as shown in the cDNA array data, the resumption of tumor growth in the castration-plus-A12 groups is associated with an increase in AR-regulated gene expression, consistent with the recurrence of AR-driven progression and the demonstration that A12 functions, at least in part, by the suppression of AR function.

In summary, we show in a preclinical study that the combination of castration plus IGF-IR inhibition significantly prolongs the time to the appearance of AI prostate cancer when compared with castration alone. If these data were confirmed in clinical trials, the addition of an IGF-IR monoclonal antibody in conjunction with castration would significantly increase the survival and symptom-free period of men with recurrent prostate cancer.

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References


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