In vivo Effects of the Human Type I Insulin-Like Growth Factor Receptor Antibody A12 on Androgen-Dependent and Androgen-Independent Xenograft Human Prostate Tumors

Jennifer D. Wu,1 Austin Odman,2 Lily M. Higgins,3 Kathy Haugk,3 Robert Vessella,2 Dale L. Ludwig,4 and Stephen R. Plymate1,3

Abstract

Purpose: The type I insulin-like growth factor receptor (IGF-IR) and its ligands have been shown to play a critical role in prostate carcinoma development, growth, and metastasis. Targeting the IGF-IR may be a potential treatment for prostate cancer. A fully human monoclonal antibody, A12, specific to IGF-IR, has shown potent antitumor effects in breast, colon, and pancreatic cancers in vitro and in vivo. In this study, we tested the in vivo effects of A12 on androgen-dependent and androgen-independent prostate tumor growth.

Experimental Design: Androgen-dependent LuCaP 35 and androgen-independent LuCaP 35V prostate tumors were implanted s.c. into intact and castrated severe combined immunodeficient mice, respectively. When tumor volume reached about 150 to 200 mm3, A12 was injected at 40 mg/kg body weight thrice a week for up to 5 weeks.

Results: We find that A12 significantly inhibits growth of androgen-dependent LuCaP 35 and androgen-independent LuCaP 35V prostate xenografts, however, by different mechanisms. In LuCaP 35 xenografts, A12 treatment induces tumor cell apoptosis or G1 cycle arrest. In LuCaP 35V xenografts, A12 treatment induces tumor cell G2-M cycle arrest. Moreover, we find that blocking the function of IGF-IR down-regulates androgen-regulated gene expression in androgen-independent LuCaP 35V tumor cells.

Conclusions: Our findings suggest that A12 is a therapeutic candidate for both androgen-dependent and androgen-independent prostate cancer. Our findings also suggest an IGF-IR–dependent activity of the androgen receptor in androgen-independent prostate cancer cells.

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Conclusions: Our findings suggest that A12 is a therapeutic candidate for both androgen-dependent and androgen-independent prostate cancer. Our findings also suggest an IGF-IR–dependent activity of the androgen receptor in androgen-independent prostate cancer cells.

Prostate carcinoma is the most common malignancy in men (1). The current available therapies for primary tumors are surgical resection or radiotherapy. However, tumors frequently recur, and although the recurrent disease initially responds to androgen deprivation, in most cases, the disease eventually becomes hormone independent (2, 3). Therefore, there is an urgent need for new and effective treatment modalities for hormone-independent disease.

The type I insulin-like growth factor receptor (IGF-IR) and its ligands, IGF-I and IGF-II, are potent mitogens and are critical in maintenance of the transformed phenotype for a variety of cancer cells (4–7). Malignancy associated modifications in the IGF-IR system have been reported in a broad range of human cancers (8–13), including prostate cancer (14–16). Reagents that impair the function of IGF-IR have been shown to inhibit tumor cell growth effectively in vitro and in vivo (17–20).

Using an antagonistic antibody to target IGF-IR has become a potential attractive therapy for cancers. Several anti-IGF-IR antibodies have been developed (21–25). However, not all the antibodies show inhibitory affects on tumor growth in vitro or are immunologically ideal for future clinical implications. The recently developed fully human monoclonal antibody, A12 specific to IGF-IR, has exhibited potent antitumor effects in breast, colon, and pancreatic cancers in vitro and in vivo (25). A12 was shown to induce apoptosis and inhibit tumor growth by two mechanisms (25). One is mediated by blocking ligands binding to IGF-IR. The other is mediated by rapid induction of IGF-IR internalization and degradation.

Increasing evidence has shown that IGF-IR and its ligands are important in the development and maintenance of prostate cancer. Elevated levels of plasma IGF-I and reduced levels of the main serum binding protein, IGF-BP3, have been shown to be associated with an increased risk of prostate cancer (26–29). Although the level of IGF-IR expression during progression of prostate cancer has been shown to be variable, its expression is not lost at any stage of the disease (14, 15). Blocking the interaction of IGF ligands with IGF-IR using a small...
IGF-like peptide has exhibited inhibition of growth of the PC-3, DU-145, and LNCaP prostate cell lines (30). Several studies have shown that IGF-IR antisense oligonucleotides inhibit human prostate cancer cell line growth and migration in vitro (31) and murine prostate tumor growth in vivo (32). These studies suggest that targeting IGF-IR is an important therapeutic alternative for prostate cancer.

In this study, we test the in vivo effect of the human IGF-IR antibody A12 on xenograft prostate tumor growth. The availability of the androgen-dependent xenograft model, LuCaP 35 (33) and its variant androgen-independent xenograft model, LuCaP 35V (33) enables us to examine the effects of the human IGF-IR on both androgen-dependent and androgen-independent prostate cancers. The LuCaP 35 and LuCaP 35V tumors exhibit many in vivo properties analogous to those of prostate cancer in man and represent an excellent model system to evaluate new treatment modalities (33). For the first time, we show that the human IGF-IR antibody, A12, causes growth inhibition of both androgen-dependent and androgen-independent prostate tumors in vivo. Most significantly, we show differential mechanisms of A12 effects in androgen-dependent and androgen-independent prostate tumors. In androgen-dependent tumors, A12 induces apoptosis or G1 cell cycle arrest. In androgen-independent tumors, A12 induces G2/M cell cycle arrest. The results suggest that the human IGF-IR antibody A12 has therapeutic potential for both androgen-dependent and androgen-independent prostate cancer.

Materials and Methods

A12 antibody. A12 is a fully human antibody antagonist to the human IGF-IR, generated by screening a naive (nonimmunized) bacteriophage Fab library (25). The characterization of A12 has been previously described (25).

Cell culture. The human prostate tumor cell line M12 and its parental cell line P69 were cultured in RPMI 1640 supplemented with 5% FCS, 10 ng/mL epidermal growth factor, 0.02 mmol/L dexamethasone, 5 μg/mL insulin, 5 μg/mL transferrin, 5 ng/mL selenium, fungizone, and gentamicin at 37°C with 5% CO2. The derivation of these cell lines has been previously described (34).

In vivo study. To study the in vivo effect of A12 on androgen-dependent and androgen-independent prostate tumor growth, tumor bits (20-30 mm³) of LuCaP 35 and LuCaP 35V were implanted subcutaneously into 6- to 8-week-old intact or castrated severe combined immunodeficient mice respectively as previously described (33). The tumors express IGF-I and IGF-II.5 Twenty-six intact and castrated mice were used in this study. When the implanted tumor was observed to reach a volume of 150 to 200 mm³, half of the animals in each xenograft group were administrated A12 antibody interperitonally at a dose of 40 mg/kg body weight thrice a week for up to 5 weeks. The remaining animals in each xenograft group were given with the vehicle saline buffer as the control reagent. Animals were weighed twice a week. Blood samples were collected from orbital sinus weekly. Serum was separated and prostate-specific antigen (PSA) level was determined using the IMx Total PSA Assay (Abbott Laboratories, Abbott Park, IL). Tumors were measured twice weekly and tumor volume was estimated by the formula, volume = L × W²/2. Following our University of Washington-approved animal protocol, some animals were euthanized at an earlier time when tumor reached a volume of 1,000 mm³ or when animal weight loss exceeded 20% of initial body weight. Bromodeoxyuridine (BrdU)rd) was injected into the tumors 1 hour before the animals were euthanized for evaluation of in vivo tumor cell proliferation rate. To study the toxicity of A12, tumor-free 6- to 8-week-old severe combined immunodeficient mice were injected with A12 (n = 12) or control saline buffer (n = 8) as described above.

After euthanization, tumors were collected and quartered. A portion of the tumors were fixed in 10% neutral buffer formalin and embedded in paraffin. Five-micrometer sections were prepared for immunohistochemistry staining. The remaining portion of the tumors were separated into single cells mechanically by mincing and filtering through 70-μm nylon sieves.

Flow cytometry. To measure tumor IGF-IR expression, 5 × 10⁵ cells were incubated with anti-IGF-IRα antibody SC-461 (Santa Cruz Biotechnology, Santa Cruz, CA) at 4°C for 30 minutes. After two washes, cells were incubated with phycoerythrin-conjugated goat anti-mouse antibody and analyzed using a BD FACscan. Data was analyzed using CellQuest™ software (BD BioScience, San Jose, CA).

Apoptosis and cell cycle assay. Apoptosis and cell cycle were measured by terminal deoxynucleotidyl transferase-mediated nick-end labeling assay and propidium iodine staining using the Apop-Direct kit.

Fig. 1. Effect of A12 on prostate cancer cell models. A, A12 inhibits IGF-IR signaling in M12 and P69 cells. After 24 hours of serum starvation, cells were stimulated with rhIGF-1 (10 ng/mL) for 15 minutes in the absence or presence of A12 antibody (10 μg/mL) and lysed. Total protein (25 μg) from each cell lysate was used for Western blot analysis. Antibody against phospho-IGF-IRβ, phospho-Akt, and GAPDH was used. B, A12 down-modulates IGF-IR level in M12 and P69 cells. After addition of A12 (10 ng/mL) to the growth medium for indicated time (12 and 36 hours), cells were harvested and lysed. Total cellular IGF-IR was analyzed by Western blot using anti-IGF-IRβ antibody. C, relative levels of surface IGF-IR in LuCaP 35 and LuCaP 35V tumor cells. Cells were incubated with anti-IGFIRα monoclonal antibody followed by phycoerythrin-conjugated goat antimouse IgG and analyzed by flow cytometry. Relative levels of surface IGF-IR were measured by mean fluorescence intensity of the antibody staining.

5 Plymate, unpublished data.
A
incubation with 0.3% H2O2 in methanol for 15 minutes. After
with PBS. Endogenous peroxidase activity was quenched by an
were allowed to cool for 30 minutes, followed by sequential rinsing
with 0.01 mol/L citric acid (pH 6.0) at 95
several washes, cells were permeabilized with 0.1% Triton X-100 and
slides. After deparaffinization and rehydration, antigens were retrieved
buffer formalin, embedded in paraffin, and sectioned at 5
A
method using the Cell Titer 96 AQueous kit (Promega, Madison, WI)
for 48 hours. Number of viable cells was quantified by a colorimetric
was added to the culture at a concentration of 20
Following overnight incubation, A12 antibody or control human IgG
incubation with biotinylated goat antimouse IgG for 30 minutes,
incubation with peroxidase-labeled avidin for 30 minutes (Santa Cruz Biotechnology),
incubation with biotinylated dUTP and terminal deoxynucleotidyl
were fixed with 10% neutral buffer formalin
j
nuclei divided by the total number of nuclei. Ten fields were counted
incubation steps were done at room temperature. Slides were
were stained by using H&E (Richard Allen, Roche, Indianapolis, IN). For assaying in vivo effect of A12, freshly prepared xenograft tumor cells were washed with PBS and lysed as described above. Protein (25 μg) was resolved on 4% to 15% SDS-PAGE, transferred onto a nitrocellulose membrane, and probed with anti-IGF-IR (BioSource International, Inc., Camarillo, CA), anti-phospho-IGF-IR (pY1158/1161/1163, Cell Signaling Technology), anti-phospho-Akt (Ser473, Cell Signaling Technology), or anti-phospho-ERK (Santa Cruz Biotechnology) antibodies overnight at 4°C. The blot was washed and incubated with a horseradish peroxidase-conjugated secondary antibody (Pierce, Rockford, IL) and re-probed with anti-GAPDH antibody (Chemicon, Temecula, CA) as described above. Independent experiments validated that this stripping procedure did not lead to loss of signal.

**Statistical tests.** Statistical significance between means of control and A12-treated animals was assayed using Student’s t test; 95% confidence interval (P < 0.05) was considered significant.

### Results

**In vitro effect of A12 on prostate cancer cell lines.** Previous studies have shown that A12 significantly blocks IGF-IR signaling in human breast, pancreatic, and colon cancer cell lines in vitro and inhibits their xenograft tumor growth in vivo (25). To investigate whether A12 may also be an effective therapeutic candidate for the treatment of prostate cancer, we first examined the effect of A12 on IGF-IR signaling in SV40T immortalized prostate epithelial cell line P69 and its metastatic subline M12 (34). The M12 cells are more tumorigenic and express ~4-fold fewer IGF-IR than P69 (34). The growth of both P69 and M12 cell lines are shown to be regulated by IGF (34). As shown on Fig. 1A, A12 inhibits phosphorylation of IGF-IR and its downstream signaling molecules Akt and extracellular signal–related kinase (ERK) in both P69 and M12 cell lines. The inhibitory effect may be in part due to A12-induced degradation of IGF-IR (Fig. 1B) as shown previously (25).

We investigated the in vitro effect of A12 on the growth of the androgen-dependent cell line LNCaP (36), the LNCaP subline androgen-independent C4-2 (36), and the androgen

### Table 1. The in vitro effects of A12 on prostate cancer cell survival and growth

<table>
<thead>
<tr>
<th>Cell line</th>
<th>G1-G2 control</th>
<th>G2-G1 A12</th>
<th>% Apoptosis control</th>
<th>% Apoptosis A12</th>
<th>Cell growth (MTT) control</th>
<th>Cell growth (MTT) A12</th>
</tr>
</thead>
<tbody>
<tr>
<td>LnCaP</td>
<td>1.9 ± 0.3</td>
<td>12.5 ± 0.5*</td>
<td>1.6 ± 0.2</td>
<td>8.4 ± 0.6*</td>
<td>1.21 ± 0.02</td>
<td>0.87 ± 0.03*</td>
</tr>
<tr>
<td>C4-2</td>
<td>7.6 ± 1.2</td>
<td>1.5 ± 0.2*</td>
<td>2.4 ± 0.3</td>
<td>2.7 ± 0.2</td>
<td>1.19 ± 0.03</td>
<td>0.91 ± 0.02*</td>
</tr>
<tr>
<td>M12</td>
<td>8.4 ± 2.2</td>
<td>2.0 ± 0.3*</td>
<td>8.2 ± 1.1</td>
<td>24.6 ± 3.2*</td>
<td>1.06 ± 0.05</td>
<td>0.68 ± 0.02*</td>
</tr>
</tbody>
</table>

**NOTE:** All studies were performed at 48 hours after addition of A12 or human IgG as a control. Cell cycle was measured by propidium iodine staining. Apoptosis was assayed by terminal deoxynucleotidyl transferase-mediated nick-end labeling analysis. For measuring cell growth, cells were plated on a 96-well plate at 2,500 per well.

Viable cell numbers were measured by MTT assay after 48 hours of A12 or control IgG treatment.

*P < 0.05, significantly different from control IgG treatment. Each assay was done in triplicate.

(35). Briefly, 1 × 106 cells from the single-cell suspension were fixed with 10% neutral buffer formalin followed by 70% ethanol alcohol at −20°C for 30 minutes. After several washes, cells were permeabilized with 0.1% Triton X-100 and incubated with FITC-conjugated dUTP and terminal deoxynucleotidyl transferase enzyme at 37°C for 1 hour followed by an incubation with propidium iodine/RNase buffer (100 μg/mL of propidium iodine, 50 μg/mL RNase) at room temperature for 60 minutes. Samples were analyzed by flow cytometry using a BD FACscan. Data were analyzed with CellQuest software.

### Immunohistochemistry

Tumor samples were fixed in 10% neutral buffer formalin, embedded in paraffin, and sectioned at 5 μm onto slides. After deparaffinization and rehydration, antigens were retrieved with 0.01 mol/L citric acid (pH 6.0) at 95°C for 2 × 5 minutes. Slides were allowed to cool for 30 minutes, followed by sequential rinsing with PBS. Endogenous peroxidase activity was quenched by an incubation with 0.3% H2O2 in methanol for 15 minutes. After blocking with 1.5% normal goat serum in PBS containing 0.05% Tween 20 (PBST) for 1 hour, slides were incubated with mouse anti-BrdUrd antibody (1 μg/mL) for 1 hour followed by sequential incubation with biotinylated goat antimouse IgG for 30 minutes, peroxidase-labeled avidin for 30 minutes (Santa Cruz Biotechnology), and diaminobenzidine/hydrogen peroxide chromogen substrate (Vector Laboratories, Burlingame, CA) for 5 to 10 minutes. All incubation steps were done at room temperature. Slides were counterstained with hematoxylin (Sigma, St Louis, MO), and mounted with permount (Fisher Scientific, Fair Lawn, NJ). For negative control, mouse IgG (Vector Laboratories) was used instead of the primary anti-BrdUrd antibody. Slides were examined under a Zeiss Microscope and digital images were obtained. Nuclei 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. H&E staining was done by using H&E (Richard Allen, Kalamazoo, MI).

### Western blotting

For assaying in vitro effect of A12 on A12 and M69 cell lines, cells were serum starved in T and S medium (Invitrogen, San Diego, CA) for 24 hours followed by addition of 50 ng/mL of recombinant human IGF-I (rhIGF-I; R&D Systems, Minneapolis, MN) alone or combined with 10 μg/mL of A12 to the medium for 15 minutes before harvesting. Cells were washed with PBS and lysed with cold lysis buffer [50 mmol/L HEPES (pH 7.5), 150 mmol/L NaCl, 1.5 mmol/L MgCl2, 1 mmol/L EGTA, 10% Triton X-100] containing phosphatase Inhibitor Cocktail II (Sigma) and protease inhibitors (Complete Mini Tablets, Roche, Indianapolis, IN).

**Targeting IGF-IR in Prostate Cancer**

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in all three cell lines, cell growth as measured by MTT assay was significantly inhibited by A12 treatment. Further analyses showed that A12 posed different effects on cell cycle and survival of these cell lines (Table 1). In androgen-dependent LNCaP cells, A12 induced a combination of apoptosis and the proapoptotic cell cycle G1 arrest. In androgen-independent C4-2 cells, A12 induced cell cycle G2 arrest but not apoptosis. In the AR-negative M12 cells, the growth of which is dependent of IGF-IR function (35), A12 induced cell cycle G2 arrest and apoptosis.

The growth characteristics of LuCaP 35 and LuCaP 35V tumor cells do not permit us to assay the in vitro effect of A12 on these xenograft models (33). However, to assure the suitability of using LuCaP 35 and LuCaP 35V xenograft models for testing the in vivo effect of A12, we examined the surface IGF-IR expression level of freshly isolated LuCaP 35 and LuCaP 35V tumor cells by flow cytometry. As shown in Fig. 1C, LuCaP 35 cells express a similar level of surface IGF-IR to the M12 cell lines although fewer IGF-IR is expressed on LuCaP 35V cells. Inhibition of A12 on androgen-dependent and androgen-independent prostate tumor growth. We first assessed the in vivo effect of A12 on androgen-dependent prostate tumor growth using the LuCaP 35 xenograft model. As shown in Fig. 2A and B, by end of the study, 54% (7 of 13) of animals in the saline-treated control group had tumors reaching the volume of 1,000 mm$^3$ (Fig. 2A), whereas none of the animals in A12-treated group had tumors reaching a volume of 1,000 mm$^3$ (Fig. 2B). It has to be noted that four animals in the control group had to be sacrificed due to large tumor growth.

![A12 inhibits androgen-dependent and androgen-independent prostate tumor growth](image-url)
volumes (exceeding 1,000 mm$^3$) 4 weeks after A12 treatment initiation. At this time point, the mean tumor volume in A12-treated animals was reduced ~60% compared with that in the control saline–treated animals (Fig. 2C, $t$ test, $P < 0.05$).

The data show that A12 significantly inhibited androgen-dependent xenograft prostate tumor growth. We next sought to assess the effect of A12 on androgen-independent prostate tumor growth. As presented in Fig. 2D, the androgen-independent LuCaP 35V tumors grew more aggressively than the androgen-dependent LuCaP 35 tumors. Ten days after A12 treatment initiation, tumor volume in the control saline–treated animals started reaching 1,000 mm$^3$. At the experimental end point (5 weeks after A12 treatment initiation), 15 of 16 of the animals in the control group had tumors reaching a volume of 1,000 mm$^3$ (Fig. 2D), whereas in the A12-treated group, only 5 of 16 animals had tumors reaching a volume of 1,000 mm$^3$ (Fig. 2E). Two and half weeks after A12 treatment initiation, 50% of the animals in the control group had to be sacrificed due to a tumor volume exceeding 1,000 mm$^3$. At this time point, the average tumor volume in A12-treated animals was reduced about 40% (Fig. 2F, $t$ test, $P < 0.05$). Although the degree of response to A12 treatment is different between LuCaP 35 and LuCaP 35V tumors, these results have shown that A12 is a powerful candidate reagent for inhibiting both androgen-dependent and androgen-independent prostate tumor growth.

A12 induces apoptosis and/or cell cycle arrest in xenograft prostate tumors. To elucidate the mechanisms by which A12 inhibits androgen-dependent LuCaP 35 and androgen-independent LuCaP 35V prostate tumor growth in vivo, we first examined the events of apoptosis and/or cell cycle progression in these tumors. As represented in Fig. 3A, A12 treatment exhibits two typical effects on LuCaP 35 tumors: cell cycle G1 arrest and/or apoptosis. In 7 of 13 LuCaP 35 xenograft animals, A12 treatment predominantly induced an increase in cell cycle G1 index from an average of 77.5 ± 3.5% to 91.3 ± 4.8% ($t$ test, $P < 0.05$) with minor apoptotic events.

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**Fig. 3.** Analyses of apoptosis, cell cycle progression, and IGF-IR signaling in A12-treated xenograft tumors. A, A12-treated LuCaP 35 tumor cells were shown to undergo predominantly G1 cell cycle arrest or apoptosis and A12-treated LuCaP 35V tumor cells were shown to undergo G2-M cell cycle arrest but not apoptosis. Single-cell suspension ($1 \times 10^6$) of the tumor cells were fixed and permeabilized for terminal deoxynucleotidyl transferase-mediated nick-end labeling assay and propidium iodine staining described in Material and Methods. Apoptosis by FITC-conjugated dUTP incorporation based on terminal deoxynucleotidyl transferase-mediated nick-end labeling assay. Cell cycle by PI staining. B, A12-treated LuCaP 35, but not LuCaP 35V, tumor cells were shown to possess pyknotic nuclei in H&E-stained sections ($\times 400$).
(apoptotic index, <5%). Some tumor cells were shown to possess pyknotic nuclei in H&E-stained sections, indicative of programmed cell death (Fig. 3B). In 6 of 13 LuCaP 35 xenograft animals, A12 treatment predominantly induced apoptosis with an index of 62.26 ± 14%. Tumor cells from these animals possess a noticeable level of pyknotic nuclei in comparison with those from control saline–treated animals (Fig. 3B). A greater inhibition of tumor growth (74% reduction in average tumor volume) was seen in the A12-treated animals where induction of apoptosis was a predominant effect (Fig. 2C). A12 had a different effect on the LuCaP 35 xenograft animals. In 11 of 16 animals, A12 treatment resulted in cell cycle G2-M arrest (Fig. 3A), an increase in G2-M index from 13.81 ± 1.0% to 90.4 ± 1.5% (t test, P < 0.001). An average of 57% reduction in tumor growth was seen in these 11 animals 2 weeks after A12 treatment initiation (Fig. 2E). In 5 of 16 LuCaP 35 xenograft animals, A12 treatment did not show an effect on cell cycle progression (data not shown) or inhibition of tumor growth (Fig. 2E and F). No apoptotic event was observed in A12-treated LuCaP 35 xenograft tumor cells. No noticeable level of pyknotic nuclei were seen in H&E-stained sections of these tumors (Fig. 3B). These results suggest possible differential roles of IGF-IR in the maintenance of androgen-dependent and androgen-independent prostate tumor growth.

Studies have shown that the effect of A12 on tumor cell growth is mediated by two mechanisms: (a) block ligands binding to IGF-IR and (b) induce rapid IGF-IR internalization and degradation (25). To investigate whether changes in IGF-IR expression in cells contribute to the different effects of A12 on tumors in vivo, we examined IGF-IR expression on freshly isolated A12-treated xenograft tumor cells using Western blot analysis. As shown representatively in Fig. 3C, A12-treated LuCaP 35 xenograft tumors showed two different levels of IGF-IR expression: as high as in control tumors or significantly reduced. Further analysis has elucidated a correlation between IGF-IR level and whether tumor cells are undergoing apoptosis or G1 arrest. In A12-treated LuCaP 35 tumors, when cells maintained IGF-IR expression levels, G1 cell cycle arrest was observed; when IGF-IR expression was significantly reduced, induction of apoptosis was observed. In LuCaP 35V xenografts, a reduction in IGF-IR expression was found in some, but not all, A12-treated tumors (Fig. 3C); however, no correlation between levels of IGF-IR expression and induction of cell cycle G2 arrest was found.

To further investigate whether the discriminating effect of A12 on LuCaP 35 and LuCaP 35V tumors is due to different degrees of inhibition of principle IGF-IR signaling pathways, we analyzed phosphorylation status of Akt and ERK in tumor cell lysates with and without A12 treatment. As shown in Fig. 3C, A12 treatment resulted in a remarkable inhibition of Akt phosphorylation in both LuCaP 35 and LuCaP 35V tumors in which G2-M arrest is induced. No noticeable change in ERK phosphorylation in LuCaP 35 or LuCaP 35V tumors with A12 treatment was observed in Western blots (data not shown). These data suggest that other alternative pathways may regulate the responses of LuCaP 35 and LuCaP 35V tumors to A12 treatment. A12 inhibits cell proliferation in xenograft prostate tumors. To assess cell proliferation between experimental groups, paraffin sections of A12-treated and control saline–treated tumors were immunohistochemically stained with anti-BrdUrd antibody. In the LuCaP 35 model, a noticeable reduction in BrdUrd uptake was seen in all A12-treated tumors as representative shown in Fig. 4A and cell proliferation was significantly inhibited with A12 treatment (Fig. 4C, t test, P < 0.05). However, no significant difference was observed between A12-treated tumors that are apoptotic and undergoing cell cycle G1 arrest (Fig. 4C). In the LuCaP 35V model, with A12 treatment, a noticeable reduction in BrdUrd uptake and a significant inhibition in cell proliferation was only seen in those undergoing G2-M cell cycle arrest (Fig. 4B and D, t test, P < 0.05). Because cell cycle arrest, in particular G1 arrest, has been proposed to be a prerequisite step for induction of apoptosis (37, 38), these results suggest that A12 inhibits cell proliferation in LuCaP 35 and LuCaP 35V tumors by induction of G1 or G2-M cell cycle arrest. Effect of A12 on serum prostate-specific antigen level. PSA is a product of AR transcriptional activity in prostate tumor cells and is commonly used as a variable for prostate tumor growth (39). Serum was taken from animals weekly and levels of PSA were measured. In androgen-dependent LuCaP 35 xenograft intact mice, although A12 treatment did not significantly affect average PSA level from all animals, interestingly, an increase in PSA level was seen in animals where tumor cells were under G1 cell cycle arrest (Fig. 5A). In contrast, in androgen-independent LuCaP 35V xenograft castrated mice, a reduction of serum PSA was observed in A12-treated animals where tumor cells were under cell cycle G2 arrest (Fig. 5B).

Cytotoxicity. A12 has been found to cross react with mouse IGF-IR. Thus, we assayed the cytotoxicity of A12 treatment in these animals. No abnormal daily activity was observed in A12-treated animals. No significant effect of A12 treatment on kidney cells was observed by both cell cycle and apoptosis assay (data not shown). No significant change in body weight was

Fig. 3 continued. C, Western blot analyses of IGF-IR expression and Akt phosphorylation in A12-treated xenograft tumors. A portion of the xenograft tumors were lysed and 25 μg total protein of the tumor lysates was analyzed using specific antibody for IGF-IR, phospho-Akt, or GAPDH. IGF-IR expression was reduced in some but not all A12-treated xenograft tumors and that Akt phosphorylation was reduced in all A12-treated xenograft tumors.
observed between A12-treated and nontreated tumor-free animals (Fig. 6). Although the stress of tumor bearing resulted in animal weight loss, A12 treatment did not have a significant effect on the body weight of these animals (Fig. 6).

**Discussion**

The current study examines the effect of a fully human anti-IGF-IR antibody, A12, on androgen-dependent and androgen-independent human prostate tumors. The results of this study showed two significant findings. First, our results have shown that using A12 to target IGF-IR results in a significant inhibition in both androgen-dependent and androgen-independent human prostate tumor growth *in vivo* with no significant toxicity. Clinically, this study suggests that the fully human anti-IGF-IR antibody A12 is an important candidate for therapy in both androgen-dependent and androgen-independent prostate cancer. Second, the effect of A12 treatment on tumor growth as previously reported in breast and colon cancer xenograft models was mediated by a combination of apoptosis and inhibition of cell proliferation. Differing from these studies, we show differential effects of A12 on cell cycle in androgen-dependent and androgen-independent prostate tumors. Our results indicate that A12 induces G1 cell cycle arrest in androgen-dependent prostate tumors. In contrast, in androgen-independent tumors, A12 inhibits tumor growth by induction of G2-M cell cycle. Mechanistically, this is the first study that describes disruption of the function of IGF-IR inducing cell cycle G2-M arrest.

Various strategies, including antisense technology (31, 32), inhibitory antibodies (21), and dominant-negative IGF-IR (35), have been used to inhibit prostate cancer cell growth *in vivo* or *in vitro* by disrupting IGF-IR function. These studies suggested that inhibition of IGF-IR function is a potent strategy for inhibition of prostate cancer growth. However, relevant clinical implications of these strategies have not been defined. The current study defines the effect of a human anti-IGF-IR antibody, A12, on prostate tumor growth in androgen-dependent and androgen-independent xenograft models, which reflect the primary and advanced hormone-refractory human prostate cancers, respectively (33). Although a considerable cross-reactivity of A12 with mouse IGF-IR has been observed (34), no significant toxicity of A12 was observed in this study. Together, our results suggest that the human IGF-IR antibody A12 has...
potential direct clinical therapeutic implication for human prostate cancer.

IGF-IR signals through two principle pathways: the phosphoinositide 3-kinase/pAkt anti-apoptotic pathway and the mitogen-activated kinase/ERK pathway (40). Activation of phosphoinositide 3-kinase protects cells from apoptosis via activation of Akt and downstream phosphorylation of many proapoptotic proteins (4–6). Our in vitro data have clearly showed that A12 treatment down-modulates IGF-IR levels and inhibits Akt and ERK phosphorylation in prostate tumor cell lines. In LuCaP 35 and LuCaP 35V xenograft tumors, we were not able to show evident changes in ERK phosphorylation with A12 treatment, suggesting that the in vivo effects of A12 are predominantly mediated through the phosphoinositide 3-kinase/Akt pathway. The ERK pathway is maintained possibly by other growth factors in vivo such as epithelial growth factor.

Our data suggest that IGF-IR may function differently in androgen-dependent and androgen-independent prostate cancers. Our in vivo studies have shown that A12 down-modulates surface IGF-IR expression and inhibits Akt phosphorylation in androgen-dependent and androgen-independent prostate tumors; however, apoptosis was only induced in androgen-dependent prostate tumors. The results are consistent with our in vitro observation with androgen-dependent LNCaP and androgen-independent C4-2 cell lines. The discrepancy of A12 effect did not correlate with the level of surface IGF-IR expression albeit down-regulation of IGF-IR expression has been shown important for obtaining the full biological response of targeting IGF-IR in vitro (41). Our results suggest that IGF-IR signaling is a pivotal survival pathway for androgen-dependent prostate cancer cells, consistent with the up-regulation of IGF-IR in primary prostate cancers (14) and up-regulation of IGF-IR expression by androgen (34). Available literature suggests that
IGF-IR may be down-regulated in androgen-independent prostate cancer (15); however, current understanding on the function of IGF-IR in androgen-independent prostate cancer is limited. Our data suggests that IGF-IR plays an important role in the growth of androgen-independent prostate cancer and that a pAkt-independent antiapoptotic pathway may play a role in the survival of androgen-independent prostate cancer cells. Li et al. have recently shown that inactivation of the proapoptotic protein FKHR by AR via a complex formation may play a role in protection of androgen-independent prostate cancer cells from apoptosis (42). Thus, one might explain the resistance of the A12-treated androgen-independent LuCaP 35V tumors and C4-2 cell lines to apoptosis as a consequence of decreased phosphorylation of Akt and the downstream target FKHR, which would result in an increase the antiapoptotic AR-FKHR complex formation.

There is evidence that, in androgen-dependent LNCaP prostate cancer cells, activation of IGF-IR down-regulates AR function through pAkt phosphorylation of AR (43). Consistent with these findings, here we show that blocking IGF-IR function in androgen-dependent LuCaP 35 tumor cells resulted in an increase in PSA expression. In androgen-independent LuCaP 35V xenografts, disruption of IGF-IR function with A12 resulted in a decrease in PSA expression, suggesting that the activity of AR in androgen-independent prostate cancer cells is also regulated by IGF-IR signaling but via different pathways. It has to be noted that, although androgen was depleted by castration in androgen-independent LuCaP 35V xenograft animals, androgen receptor transcriptional activity remained, which was evidenced in a previous study by nuclear localization of AR and secretion of the androgen-regulated protein, PSA (33). Hypotheses exist on transactivation of AR in the absence of ligand (44–46); however, the mechanism for this activity of AR remains unknown. How the activity of AR in androgen-independent prostate cancer cells is regulated by IGF-IR signaling remains to be explored as well.

This study shows that the human IGF-IR antibody A12 inhibits growth of androgen-dependent and androgen-independent xenograft prostate tumor growth, although via different mechanisms. Activation of IGF-IR signaling has been shown to play a critical role in the development and maintenance of prostate cancer. Therefore, this study has established a preclinical basis for A12 as a potential effective therapeutic reagent for treatment of both androgen-dependent and androgen-independent human prostate cancers.

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References

In vivo Effects of the Human Type I Insulin-Like Growth Factor Receptor Antibody A12 on Androgen-Dependent and Androgen-Independent Xenograft Human Prostate Tumors

Jennifer D. Wu, Austin Odman, Lily M. Higgins, et al.


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