Inhibition of Interleukin-6 with CNTO328, an Anti-Interleukin-6 Monoclonal Antibody, Inhibits Conversion of Androgen-Dependent Prostate Cancer to an Androgen-Independent Phenotype in Orchiectomized Mice

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Abstract

Initially, prostate cancer is androgen dependent. However, most cases progress to an androgen-independent state through unknown mechanisms. Interleukin-6 (IL-6) has been associated with prostate cancer progression including activation of the androgen receptor (AR). To determine if IL-6 plays a role in the conversion of prostate cancer from androgen dependent to androgen independent, we established androgen-dependent LuCaP 35 human prostate cancer xenografts in nude mice, castrated the mice, and blocked IL-6 activity using a neutralizing antibody (CNT0328) for a period of 18 weeks. IL-6 inhibition increased survival of mice and inhibited tumor growth, as reflected by decreased tumor volume and prostate-specific antigen levels, compared with that in mice receiving isotype control antibody. To test the effect of IL-6 inhibition on the conversion from androgen dependent to androgen independent, tumor cells from the treated mice were assessed for their androgen dependence both *in vitro* and by implanting them into sham-operated or orchiectomized mice. Tumor cells derived from the isotype-treated animals converted to androgen-independent state, whereas tumor cells from the anti-IL-6 antibody-treated mice were still androgen dependent *in vitro* and *in vivo*. Although there was no difference in AR levels between the androgen-independent and androgen-dependent tumors, IL-6 inhibition promoted both apoptosis and inhibited cell proliferation in tumors and blocked the orchiectomy-induced expression of histone acetylases, p300 and CBP, which are AR cofactors. These data show that IL-6 contributes to the development of androgen independence in prostate cancer and suggest that it mediates this effect, in part, through modulation of p300 and CBP. (Cancer Res 2006; 66(6): 3087-95)

Introduction

Prostate cancer is the second leading cause of cancer death and the most common cancer diagnosis in men in the United States. In 1999, it was estimated that 179,300 men were diagnosed with prostate cancer, and 37,000 men died from the disease (1). At diagnosis, many patients have an advanced form of the disease and require systemic androgen ablation therapy. Initially, the cancer is responsive to this type of treatment because it exhibits an androgen-sensitive phenotype (2). Unfortunately, the cancer typically recurs in a form that does not respond to further androgen deprivation and has been termed androgen-independent cancer. Androgen-independent prostate cancer is resistant to therapeutic interventions and ultimately leads to demise of the patients. The mechanisms through which prostate cancer converts to androgen independence is unknown, but understanding this biology may help identify targets to prevent this therapy resistant form of prostate cancer.

Interleukin-6 (IL-6), a proinflammatory cytokine, has been associated with progression of prostate cancer in both clinical prostate cancer patients and murine models (reviewed in ref. 3). IL-6 is shown to be elevated in serum levels of men with advanced prostate cancer and associated with morbidity in prostate cancer patients. In addition, IL-6 has the ability to activate the androgen receptor (AR) in the absence of androgen. Inhibition of IL-6 using an IL-6 monoclonal antibody has been shown to inhibit prostate cancer cell growth both *in vitro* (4) and in a murine model (5). These findings suggest that IL-6 may play an important role in the transition of prostate cancer from an androgen-dependent to an androgen-independent state. Accordingly, to determine if IL-6 is involved in the progression of prostate cancer to an androgen-independent state, we examined the effect of inhibiting IL-6 activity on the conversion to androgen independence of a human androgen-dependent prostate cancer LuCaP 35 xenograft implanted mice.

Materials and Methods

Anti-IL-6 Antibody

CNTO328 was engineered from CLB8, a murine anti-human IL-6 antibody (6), to express a human Fc fragment at Centocor, Inc. (Radnor, PA; ref. 7).

Preparation of Single-Cell Suspension

LuCaP 35, kindly provided by Dr. Robert Vessella (Department of Urology, University of Washington, Seattle, WA), is an androgen-sensitive, prostate-specific antigen (PSA)–producing human prostate cancer xenograft derived from the lymph nodes of a patient that had failed androgen deprivation therapy (8, 9). Single-cell suspensions of LuCaP 35 were prepared by resecting the s.c. xenografts and cutting them into small pieces in HBSS with 1% fetal bovine serum (FBS). The small pieces were then gently rubbed between frosted glass slides to obtain single-cell suspensions in HBSS containing 1% FBS. RBC were lysed with ammonium chloride solution (StemCell Technologies, Inc., Vancouver, British Columbia, Canada) and centrifuged at 300 × *g* for 10 minutes in HBSS with 1% FBS, and the cell pellet was resuspended in DMEM media with 10% FBS. Cell viability was determined by trypan blue counting, and only preparations with >90% viability were used for *in vivo* injection. The suspension contained >90%

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epithelial cells with an admixture of stromal appearing cells. All cell preparations for *in vitro* and *in vivo* use were diluted to the required cell number based on the epithelial cell count.

Cell Growth *In vitro*

Cell growth *in vitro* was measured using the CellTiter 96 AQ Nonradioactive cell proliferation assay (Promega, Madison, WI). Briefly, single suspension of LuCaP cells derived from tumors were added to the wells of a 96-well plate at 5,000 per well in triplicates in DMEM plus 5% charcoal-stripped FBS with the addition of vehicle or 10 nmol/L dihydrotestosterone. Cells were allowed to grow for 48 hours, then 20 μL/well of combined 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium/phenazine methosulfate solution was added. After incubation of 1 hour at 37°C in a humidified 5% CO_2 atmosphere, the absorbance at 490 nm was recorded by using an ELISA plate reader.

Mice

Eight-week-old nude (*nu/nu*) mice (Charles River Laboratories, Wilmington, MA) were kept in a specific pathogen-free environment in microisolator cages. They were fed sterile rodent chow and water *ad libitum*. The animal protocol was approved by the University of Michigan Animal Care and Use Committee.

In vivo Experiments

Effect of CNTO328 on tumor growth *in vivo*. LuCaP 35 tumor cell suspension (10^6) was made using a previously established xenograft and diluting the cells in RPMI. The mice were injected twice s.c. with 100 μL of this suspension. The mice were then monitored for tumor growth, and measurements using microcalipers began when the tumors became palpable. Tumor volumes were calculated using [(minimum measurement)^2 × (maximum measurement)] / 2. When tumors were ~200 mm^3, the mice were orchiectomized. The mice were then randomly assigned to one of two treatment groups, anti-IL-6 monoclonal antibody and isotype control mouse IgG. Weekly i.p injections of 500 μg/mouse/wk were given in both groups for a period of 18 weeks. The tumors were monitored and measured twice weekly. Upon the completion of this study, mice were sacrificed, and tumor, lung, and liver tissues as well as blood serum were collected for further analysis.

Growth of post-treatment tumors in orchiectomized animals. To determine the androgen responsiveness of tumors present at the end of the CNTO328 treatment study, a portion of the tumors from each group (n = 5/group from each of the isotype or CNTO328 groups) or from LuCaP 35 tumors obtained from untreated, sham-orchiectomized mice was resected at the time of sacrifice and made into single-cell suspensions. The suspension contained >90% epithelial cells with an admixture of stromal-appearing cells. Two weeks before sacrifice of mice of the CNTO138-treated mice, a separate group of mice were either orchiectomized (ORX) or sham (Sham) orchiectomized. At the time of sacrifice of the CNTO328-treated animals, single-cell suspensions of a portion of the LuCaP 35 tumors were made and injected s.c. at 1×10^6 in 100 μL of DMEM. Tumors were measured every 5 days using calipers for a total of 45 days, at which time animals were sacrificed.

PSA Measurement

Total PSA levels in serum were determined using the Accucyte Human PSA assay (Cytimmune Sciences, Inc., College Park, MA) as recommended by the manufacturer. The sensitivity of this assay is 0.488 mg/mL.

IL-6 Bioassay

Bioactive IL-6 was measured using the B9 cell bioassay as previously reported (10). Briefly, 2×10^3 B9 cells/100 mL were placed in wells of a flat-bottomed, 96-well microtiter plate (Corning, Corning, NY). Test supernatant (50 mL) or serum (diluted 1:10 in RPMI) was also placed in each well. Additionally, standard curves were developed for each plate using recombinant murine IL-6 (PharMingen, San Diego, CA). Both standards and samples were measured in duplicates. The plate was incubated for 68 hours at 37°, 5% CO_2. Then 25 mL of 5 mg/mL 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide was placed in each well and incubated for 4 hours at 37° followed by the addition of 100 mL of extraction buffer (25), which was incubated with the sample overnight at 37°, 5% CO_2. The plates were read at 550 nm on a microplate reader (Dynatech Laboratories, Inc., Chantilly, VA). A standard curve generated by linear regression was used to calculate IL-6 levels.

Real-time Reverse Transcription-PCR

Total RNA was extracted from tumors using TRIzol method as directed by the manufacturer (Life Technologies, Gaithersburg, MD); 200 ng of total RNA was subjected to real-time reverse transcription-PCR (RT-PCR; LightCycler, Roche Diagnostics, Indianapolis, IN) using the SYBR Green I RNA amplification kit (Roche Diagnostics). RT-PCR reactions were mixed and then subjected to 40 cycles of 94°C for 5 seconds, 55°C for 10 seconds, and 72°C for 1 minute. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control. PCR primers used were IL-6 upstream, 5′-ATGAACTCCTTCTCCACAAGCGC-3′ and downstream, 5′-GAAGAGCCCT-CAGGCTGGACTG-3′ to give a 628-bp product; IL-6R upstream, 5′-CATTGCCATTGTTCTGAGGTTC-3′ and downstream 5′-AGTAGTCTG-TATTGCTGATGTC-3′ to give a 251-bp product; cyclic AMP response element binding protein (CREB) binding protein (CBP) upstream, 5′-ACAGCCCTT-TAGTCAAGCTGG-3′ and downstream 5′-TGCTTGTGTGGGTACAATTCC-3′ to give a 183-bp product; p300 upstream, 5′-ACCCCTCCCAATAGCATGC-CACC-3′ and downstream 5′-CTGGAAGGGGTGGCTGAGCAGT-3′ to give a 128-bp product; and GAPDH upstream, 5′-TGAAGGTCGGAGTCAACG-GATTTGGT-3′ and downstream, 5′-CATGTGGGCCATGAGGTCCACCAC-3′ to give a 983-bp product. The size of the PCR products was confirmed using gel electrophoresis before using the primers for real-time PCR (data not shown).

Histopathology and Detection of Apoptosis

Excised tumors were placed in 10% formalin, embedded in paraffin, and sectioned at 10-mm thickness. Sections were examined using standard H&E staining for routine histopathology. To evaluate apoptosis, sections were deparaffinized, rehydrated, and subjected to terminal deoxynucleotidyl transferase–mediated nick-end labeling analysis using ApopTag Plus Peroxidase kit (Intergen, Purchase, NY) according to the manufacturer's directions. The number of apoptotic nuclei per ×200 field (averaged from three random ×200 fields) was determined for each section by an investigator that was blinded to the samples as we have previously described (5).

Immunohistochemistry

Formalin-fixed tumor tissue was subjected to immunohistochemistry using standard methods. Briefly, antibodies against AR, PSA, p300, and CBP were used as the primary antibodies. The bound antibody was visualized by the streptavidin-biotin method, including horseradish peroxidase and diaminobenzidine chromogen (Histostain kit, Zymed, San Francisco, CA). Percentage of cells staining and staining intensity were scored as a semiquantitative evaluation of overall staining of the tissue section independently by two investigators. To score each slide, six ×200 fields per a section were used to determine both percentage of cells staining and overall staining intensity. The six fields were designed to represent the upper right end, middle, and left end and the lower right end, middle, and left end for each slide. The overall staining intensity was then determined as the average of the six scores for each slide. The overall scores of each investigator were then averaged to give a final score for the slide. There was >90% interobserver agreement in staining intensity among the two investigators. To provide scores for staining intensity, each investigator looked at all the slides to develop an appreciation for the range of staining intensity. Then based on this, a staining intensity grading scale was developed that was composed as no detectable signal (score 0), weak signal (score 1), moderate signal (score 2), and strong signal (score 3).

Statistical Analysis

Statistical analysis was done using Statview Software (Abacus Concepts, Berkley, CA). ANOVA was used for initial analyses of continuous data followed by Fisher's protected least significant difference for post hoc analyses. χ^2 was used for nominal data. Student's t test was used for comparisons between two groups. Differences with a $P < 0.05$ were determined as statistically significant.

Results

To determine if IL-6 contributes to the conversion of prostate cancer from androgen dependent to androgen independent, the androgen-dependent human prostate cancer xenograft LuCaP 35 was injected s.c. into mice and allowed to grow to a size of

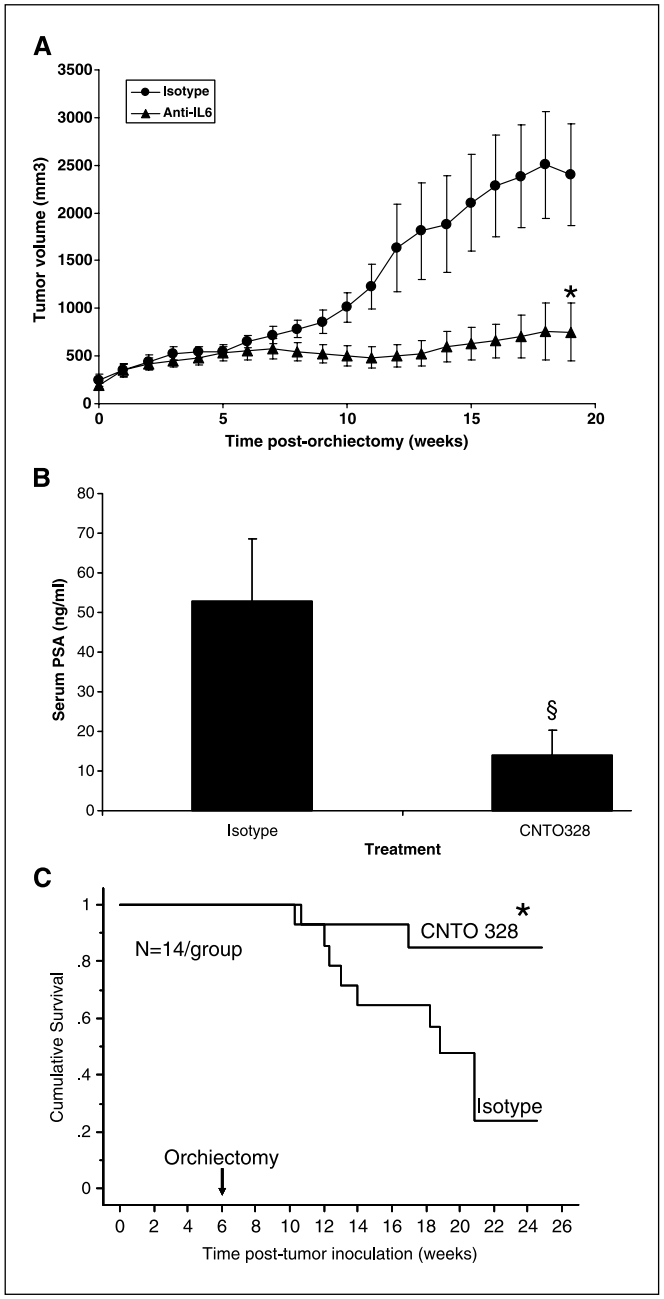

Figure 1. CNTO328 decreased progression of LuCaP 35 tumors in orchiectomized mice. LuCaP 35 tumor cells were implanted s.c. into mice. The tumors were allowed to develop until they reached ~200 mm^3. At this point, the mice were orchiectomized and randomized to receive either isotype control antibody or CNTO328 by i.p. injection weekly at 500 μg/mouse/wk. Some mice died or were euthanized due to tumor burden during the study. Otherwise, mice were maintained for a period of up to 19 weeks after orchiectomy (total of 25 weeks with tumor). *A,* tumor volumes were measured weekly. *, $P < 0.01$ versus isotype (two-way ANOVA). *B,* serum obtained from mice that died during or at the end of the study was subjected to ELISA for PSA protein expression. §, $P < 0.01$ versus isotype (Student's *t* test). *C,* Kaplan-Meier survival curve. *, $P = 0.02$ (log-rank).

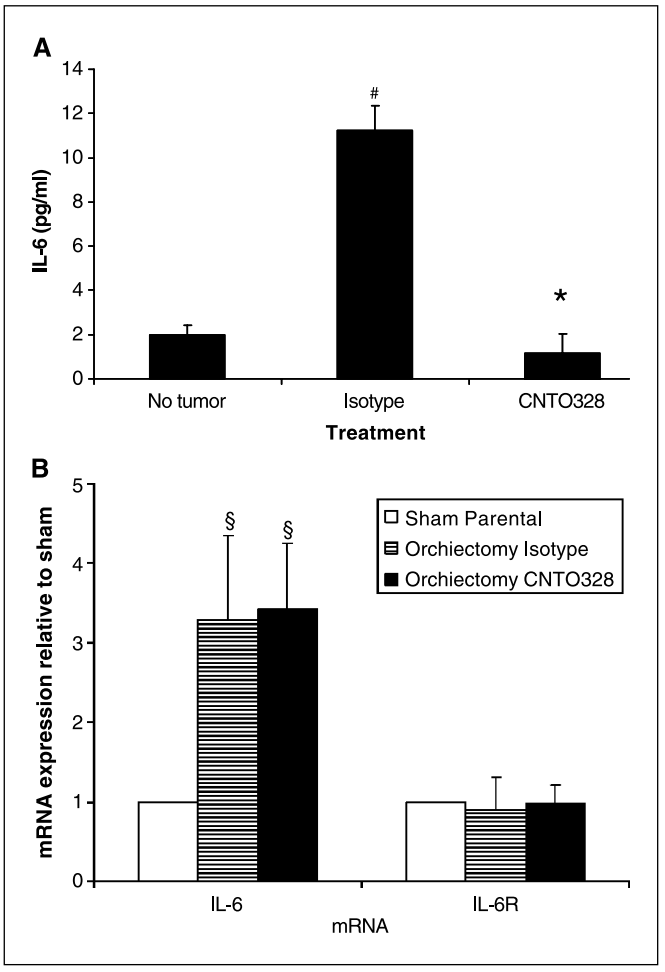

Figure 2. CNTO328 blocks orchiectomy-induced expression of IL-6 in LucaP 35 tumors growing in mice. *A,* serum was collected from either healthy mice without tumors (*no tumor*) or orchiectomized mice with LuCaP 35 tumors growing and treated with either isotype antibody or CNTO328, as described in Fig. 1. The serum was subjected to bioassay for IL-6 activity ($n = 5$ per group). #, $P < 0.01$ versus no tumor; *, $P < 0.01$ versus isotype (ANOVA and Fisher's protected least significant difference for post hoc analysis). *B,* total RNA from tumors tissues growing in either sham orchiectomized mice (*Sham Parental*) or orchiectomized mice with LuCaP 35 tumors and treated with either isotype antibody or CNTO328, as described in Fig. 1, was subjected to real-time PCR for IL-6 and IL-6 receptor (*IL-6R*; $n = 5$ per group). §, $P < 0.01$ versus Sham Parental (ANOVA and Fisher's protected least significant difference for post hoc analysis).

~200 mm^3. At this time, mice were orchiectomized, and weekly administration of either isotype control antibody or CNTO328, a murine anti-human IL-6 antibody engineered to express a human Fc region, was initiated and continued until the end of the study, which occurred 19 weeks after orchiectomy. Although tumors continued to increase in volume in the isotype-treated group, CNTO328 administration inhibited tumor growth (Fig. 1*A*). Evaluation of tumor prevalence at the end of the study revealed that 7 of 14 (50.0%) isotype-treated mice had evidence of tumor compared with only 2 of 14 (14.2%)of the CNTO328-treated mice, which shows that CNTO328 decreased tumor prevalence by 35.8% ($P = 0.043$, χ^2 test). Consistent with decreased tumor volume, CNTO328 administration was associated with decreased serum PSA levels (Fig. 1*B*).

In addition to evaluating tumor burden, we assessed if administration of CNTO328 had an effect on survival of mice with LuCaP 35 tumor implants. Mice either died spontaneously or were

euthanized due to tumor necrosis or cachexia. All mice that died spontaneously or were euthanized had necropsy done on them to ensure there was no pathology, other than tumor-related pathology. At 19 weeks after initiation of CNTO328 administration (25 weeks total time with tumors), all remaining mice were euthanized to end the study. Administration of CNTO328 prolonged survival time of mice (Fig. 1*C*), which is consistent with its ability to decrease tumor burden.

Orchiectomy has been associated with increasing systemic IL-6 levels. To determine if this occurred in the orchiectomized mice implanted with LuCaP 35 tumors and to ensure that CNTO328 effectively blocked IL-6 activity *in vivo*, we measured bioactive IL-6 present in the sera of orchiectomized mice without tumors and the mice from the two treatment groups. The presence of tumor increased the amount of bioactive IL-6 by >5-fold, and CNTO328 administration completely blocked this increase (Fig. 2*A*). Because CNTO328 blocks human and not murine IL-6 and it completely blocked the increased serum IL-6 activity, this indicated that the increased activity was due primarily to IL-6 produced from the LuCaP 35 cells (which are human) as opposed to from the mice. To determine if IL-6 expression was increased in the LuCaP 35 tumors of orchiectomized mice, we did real-time PCR on the tumors for both IL-6 and IL-6 receptor mRNA. LuCaP 35 tumors from sham-orchiectomized mice had very low levels of IL-6, and orchiectomy increased IL-6 expression in the tumors from both the isotype-treated and CNTO328-treated mice (Fig. 2*B*). IL-6 receptor was expressed in LuCaP 35 cells and did not change expression upon orchiectomy (Fig. 2*B*).

Histology of the tumors derived from both treatment groups revealed no cellular morphologic difference on H&E-stained sections (Fig. 3*A*). To determine the cellular events that accounted for differences in tumor volumes between the different treatment groups, we evaluated the tumors for indices of apoptosis and proliferation. CNTO328 treatment induced apoptosis (Fig. 3*A* and *B*) and decreased proliferation (Fig. 3*A* and *C*) of the tumor cells compared with isotype administration.

Androgen deprivation therapy for prostate cancer typically results in the eventual recurrence of the cancer in an androgen-independent state. Therefore, we assessed if there was an effect on the androgen responsiveness of the tumors from the isotype-treated and CNTO328-treated mice. To determine the androgen responsiveness of the tumors that were present at the end of the study, we did both *in vitro* and *in vivo* evaluations using single-cell suspensions of cells derived from portions of the tumors harvested at the end of the study. Initially, we examined the *in vitro* androgen responsiveness of cells. To accomplish this, a portion of the tumors from each group (n = 5/group from each of the isotype or CNTO328 groups) or from LuCaP 35 tumors obtained from untreated, sham-orchiectomized mice was resected at the time of sacrifice and made into single-cell suspensions. The suspension contained >90% epithelial cells with an admixture of stromal-appearing cells. The cells were plated phenol red–free DMEM containing charcoal-stripped FBS, and either dihydrotestosterone or ethanol vehicle was added to the wells; the cells were grown for 48 hours, and cell number was quantified. Dihydrotestosterone induced an increase in cell number compared with vehicle-treated cells in the LuCaP 35

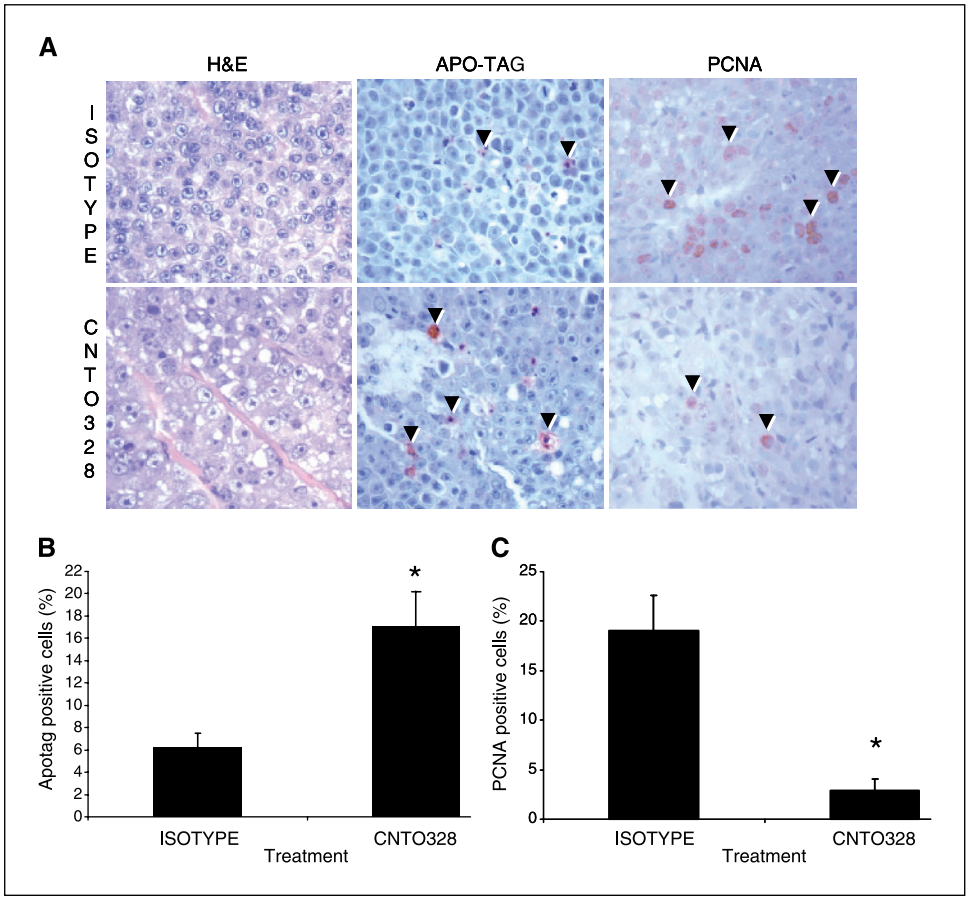

Figure 3. CNTO328 promotes apoptosis and inhibits proliferation of LuCaP 35 cells. Tumors were collected from mice at the end of the study as described in Fig. 1. *A,* tumors were subjected to H&E staining, terminal deoxynucleotidyl transferase in nick end-labeling (TUNEL) using ApoTag kits, and immunohistochemistry for proliferating cell nuclear antigen (*PCNA*). *Arrows,* sample apoptotic cells or proliferating cells in the ApoTag and PCNA, respectively. Original magnification, ×200. *B,* % ApoTag-positive cells was quantified (n = 5). *, P < 0.01 compared with isotype (Student's t test). *C,* % PCNA-positive cells was quantified (n = 5). *, P < 0.01 compared with Isotype (Student's t test).

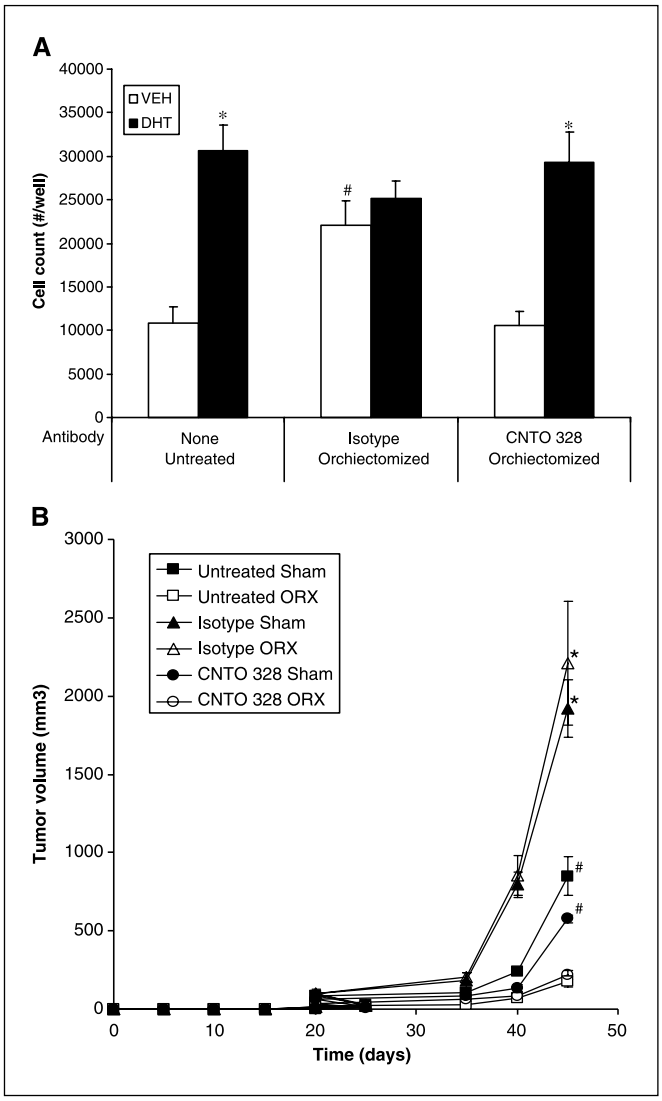

Figure 4. CNTO328 prevents loss of androgen responsiveness in LuCaP 35 tumors growing in orchiectomized mice. Mice implanted with LuCaP 35 tumors were orchiectomized and treated with either CNTO328 or isotype antibody. A parallel group of mice with LuCaP 35 tumors implanted were sham orchiectomized and did not receive treatment (*Untreated*). Nineteen weeks after orchiectomy, tumors were harvested and made into single-cell suspensions ($n = 5$ per group). *A,* cells were plated and treated with dihydrotestosterone (*DHT*; 10 nmol/L) or vehicle (*VEH*) for 48 hours, then cell numbers were counted. LuCaP 35 tumors grown in sham-operated mice with no antibody treatment were used as controls. *, $P < 0.01$ versus vehicle treatment for the respective antibody. #, $P < 0.01$ versus vehicle for None or CNTO328. *B,* two weeks before sacrifice of mice treated with CNTO328, a separate group of mice were either orchiectomized (*ORX*) or sham (*Sham*) orchiectomized. Tumor cells as described above were then injected s.c. into the orchiectomized or sham-orchiectomized mice, and tumor volumes were measured every 5 days. *, $P < 0.01$ versus Untreated Sham, Intact ORX, CNTO328 Sham, and CNTO328 ORX groups. #, $P < 0.01$ versus CNTO328 ORX and Untreated ORX groups. ANOVA and Fisher's protected least significant difference for post hoc analysis.

parental cells that were grown in sham operated mice (Fig. 4*A*), which shows they are androgen responsive. The cell number of LuCaP 35 cells from the isotype-treated mice and incubated with vehicle *in vitro* was increased compared with the cell numbers seen in vehicle-containing cultures from both the untreated sham-operated mice and the CNTO328-treated mice. This indicates that orchiectomy increased the ability of LuCaP 35 tumor cells to grow. Dihydrotestosterone did not increase cell numbers compared with vehicle in the LuCaP 35 from the isotype-treated mice, which indicates that these cells are androgen nonresponsive. In contrast, dihydrotestosterone induced an increase in cell number compared with vehicle-treated cells in the LuCaP 35 cells isolated from the CNTO328-treated mice, which indicates that these cells, similar to the parental LuCaP 35 cells, are androgen responsive. Taken together, these data show that orchiectomy induced an androgen-nonresponsive state (isotype orchiectomized versus untreated sham mice), and that CNTO328 abrogated the orchiectomy-induced conversion of LuCaP 35 to an androgen nonresponsive tumor (CNTO328 orchiectomized versus isotype orchiectomized mice).

To provide further evaluation of the androgen responsiveness of the tumors derived from the CNTO328 study, we evaluated the growth of these tumors in either orchiectomized or sham-orchiectomized mice. Two weeks before sacrifice of mice in the CNTO328 study, a separate group of mice were either orchiectomized or sham orchiectomized in preparation for implants of tumors from the CNTO328 study. At the time of sacrifice of mice from the CNTO328 study, single-cell suspensions of a portion of the LuCaP 35 tumors were made. Additionally, LuCaP 35 tumor cells were derived from mice that were sham-operated and did not receive any treatments (untreated group). The cells from the three groups (untreated mice, isotype-treated orchiectomized mice, and CNTO328-treated orchiectomized mice) were injected s.c. into the orchiectomized or sham orchiectomized mice and tumors were measured every 5 days up to 45 days at which time animals were sacrificed. The LuCaP 35 cells derived from the untreated mice grew more rapidly in the sham versus orchiectomized mice (Fig. 4*B*), indicating they were androgen dependent. The cells from the isotype-treated mice grew at the same rate in both the sham and orchiectomized mice, which indicated they were androgen independent. In contrast, the cells from the CNTO328-treated mice grew more rapidly in the sham versus orchiectomized mice, which indicated they were androgen dependent. Taken together, these data indicate that the orchiectomy converts androgen-dependent LuCaP 35 to an androgen-independent state, and that blocking IL-6 activity with CNTO328 blocks the conversion to androgen independence, resulting in maintaining LuCaP 35 as an androgen-dependent tumor.

The conversion to androgen-independent is associated with heterogeneous expression of the AR in men with advance prostate cancer. Accordingly, we determined the AR expression in the tumors from the isotype-treated and CNTO328-treated mice. A subset of tumor tissue was subjected to immunoblot for AR. We could not identify any difference of AR expression among untreated LuCaP 35 and tumors from either treatment group (Fig. 5*A*). Although total AR protein may not be different between tumor groups, as detected by immunoblot, it is possible that areas within tumors have heterogeneous expression. To evaluate for differences among the proportion of cells expressing AR, we did immunohistochemistry on tumors derived from the CNTO328 study. Immunohistochemistry for AR revealed heterogeneous expression of AR within and among tumors in untreated LuCaP 35 tumors and both treatment groups (Fig. 2*B* and *C*), resulting in no detectable difference in the percent of cells staining or the overall stain intensity among the groups. PSA is often used as an index of androgen activation in prostate tissues. Accordingly, we determined PSA expression within the tumors. Immunohistochemistry for PSA revealed heterogeneous PSA expression, including percentage of cells staining and stain intensity, among the untreated LuCaP 35 tumors and both treatment groups (Fig. 2*B* and *C*).

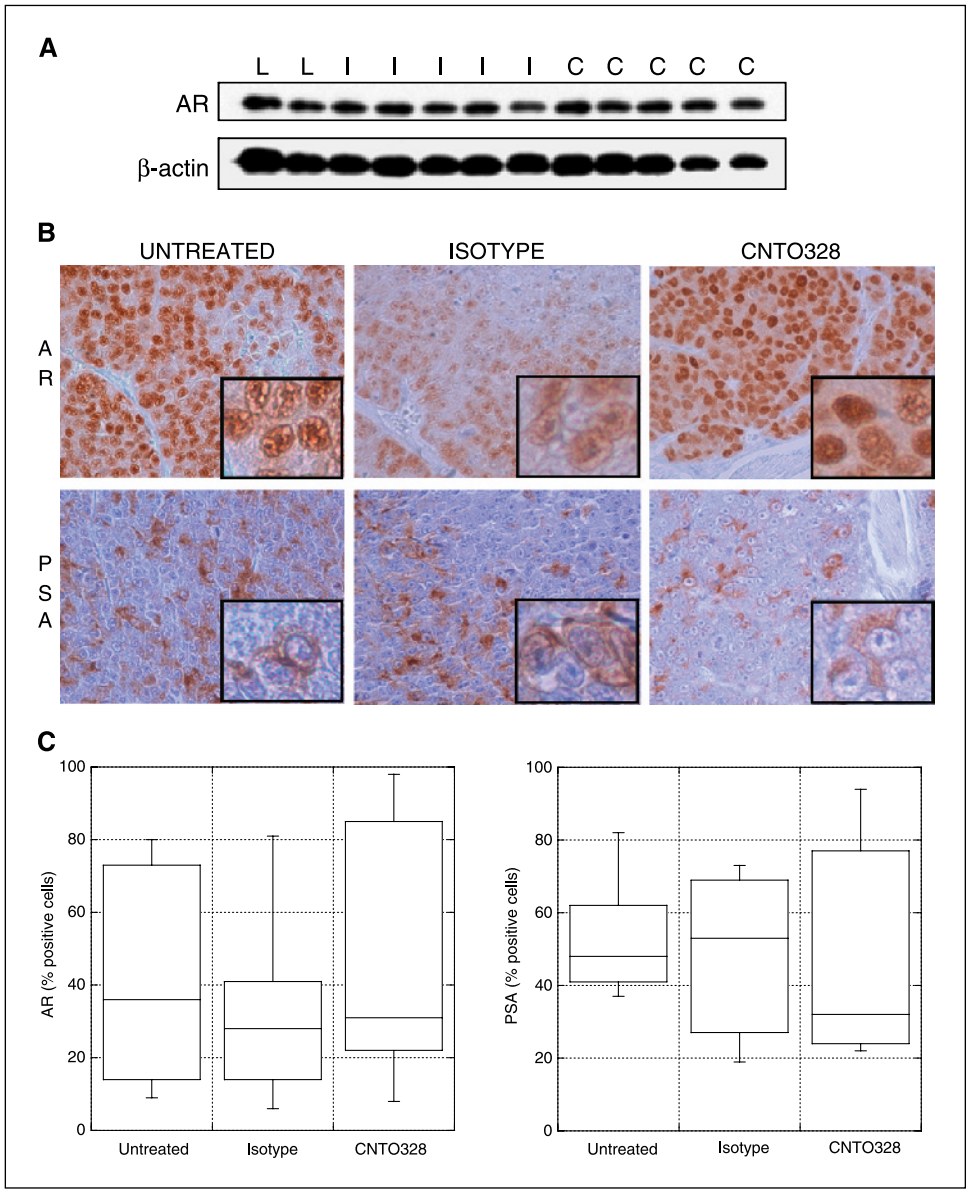

Figure 5. CNTO 328 did not affect AR expression in LuCaP 35 tumors. Tumors were collected as described in Fig. 4. *A,* immunoblot for AR. *L,* LuCaP 35 untreated cells; *I,* LuCaP 35 cells from orchiectomized mice treated with isotype antibody for 19 weeks; *C,* LuCaP 35 cells from orchiectomized mice treated with CNTO328 for 19 weeks. *B,* immunohistochemistry was done for AR and PSA. Original magnification, ×200 and ×400 (*inset*). Note nuclear staining in AR-stained cells and cytoplasmic staining in PSA-stained cells. *C,* % either AR- or PSA-positive cells was quantified in tumors by counting 100 cells in each of five random fields (n = 5 per group). Box plots in which the end of the lower tail indicates the lowest value: the box represents the mid-50% of the data, the bar within the box indicates the median value, and the end of the upper tail indicates the maximum value. There was no statistical significance among these groups.

The mechanisms through which IL-6 promotes androgen-independent activity have not been defined; however, recent evidence suggests that IL-6 activates AR activity through up-regulation of several AR cofactors. Specifically, CBP (11) and the CBP-related protein p300 (12), which have both been shown to be up-regulated in androgen-independent prostate cancer. Therefore, we evaluated if CBP/p300 protein expression was altered in the tumors from the mice treated with CNTO328. More than 80% of the cells were positive for CBP and p300 among both the untreated tumors and those from the different treatment groups, thus showing no difference in the number of cells staining for CBP/p300 (Fig. 6*A*). However, staining intensity for both CBP and p300 were increased in the tumors from orchiectomized mice treated with isotype compared with untreated tumors from intact mice (Fig. 6*B* and *C*), indicating that orchiectomy induces both CBP and p300 expression. Treatment with CNT0328 diminished the orchiectomy-induced increase of staining intensity of both CBP (Fig. 6*B*) and p300 (Fig. 6*C*), indicating that IL-6 contributes to orchiectomy-induced expression of CBP and p300. To accurately quantify changes in CBP/p300 mRNA expression among the treatment groups, we did real-time PCR. Consistent with the immunohistochemistry analysis both CBP and p300 were increased in the tumors from isotype-treated orchiectomized mice compared with tumors from untreated mice (Fig. 6*D* and *E*). Treatment with CNT0328 diminished the orchiectomy-induced increase of CBP (Fig. 6*D*) and p300 (Fig. 6*E*) to baseline levels. These data indicate that p300/CBP expression is increased in androgen-independent prostate cancer, and that this increase is mediated, in part, by IL-6.

Discussion

The development of androgen-independent prostate cancer creates a major challenge for treatment of prostate cancer patients because androgen-independent prostate cancer is minimally responsive to most current therapies. Accordingly, understanding the biology of how prostate cancer converts from an androgen-dependent to an androgen-independent phenotype may help identify new important therapeutic strategies. Based on extensive

published evidence indicating that IL-6 is associated with prostate cancer progression and that it activates the AR, we explored if IL-6 contributes to the development of androgen-independent prostate cancer. Our data show that blocking IL-6 prevents the conversion of androgen-dependent LuCaP 35 cells to an androgen-independent phenotype in orchiectomized mice. These results suggest that IL-6 promotes the development of androgen-independent cancer in men undergoing androgen deprivation therapy.

There are multiple lines of clinical and experimental evidence that suggest IL-6 promotes prostate cancer progression (reviewed in ref. 3). Several studies have shown that IL-6 is elevated in the sera of patients with metastatic prostate cancer (13–17). In addition, these clinical observations, *in vitro* cellular studies, have provided the suggestion that IL-6 modulates prostate cancer cell growth. Chung et al. showed that IL-6 promoted cell growth of hormone-refractory cells but had no effect on the growth of hormone-dependent cell lines (4). Addition of exogenous IL-6 to the culture media of LNCaP cells by several groups has resulted in a dose-dependent inhibition of cell growth with development of a neuroendocrine cell phenotype (4, 18–22). On the other hand, some researchers observed a stimulatory response after treatment with IL-6 (23, 24). The reasons for these differences have not been clarified to date, but it seems that IL-6 is only inhibitory to LNCaP cells but not other prostate cancer cell lines (3). The observation in our *in vivo* model that inhibition of IL-6 decreases proliferating cell nuclear antigen expression suggests that that IL-6 promotes prostate cancer cell proliferation *in vivo*. This observation is consistent with previous reports that show IL-6 stimulates prostate cancer cell proliferation (23, 24), and inhibition of IL-6 decreases prostate cancer proliferation (4).

In addition to increased cell proliferation contributing to increased tumor volume, decreased apoptosis can also promote increased tumor volume. A previous report showed that orchiectomy induced conversion of LuCaP 35 tumors to an androgen-independent phenotype (8). However, in that study, the mechanism of how androgen-independent developed was not identified. Our results that CNTO328 promoted apoptosis in the LuCaP 35 tumors indicate that IL-6 has an antiapoptotic effect in these tumors.

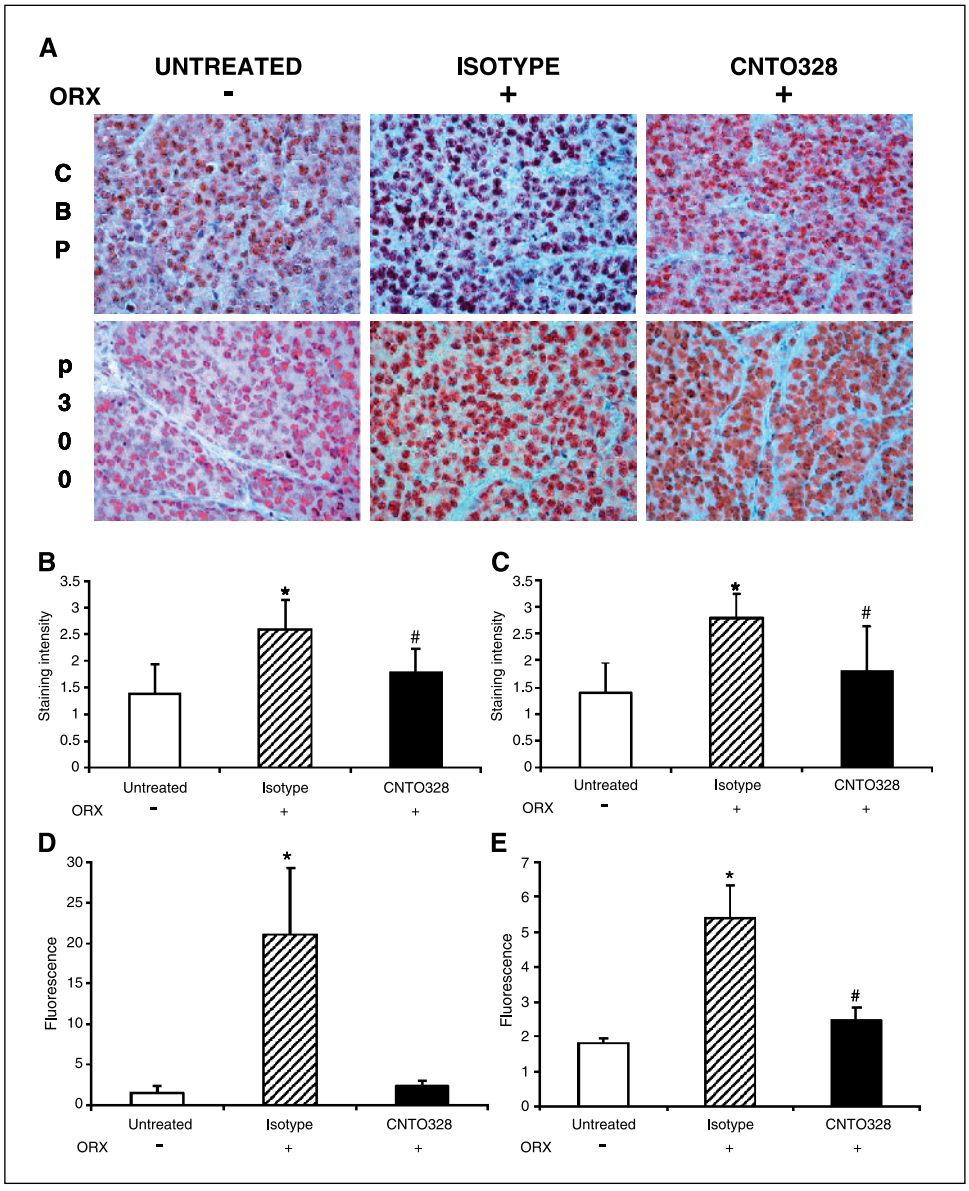

Figure 6. CNTO 328 decreases orchiectomy-induced increase of p300 and CBP expression in LuCaP 35 tumors. LuCaP 35 tumor cells were implanted s.c. into mice. The tumors were allowed to develop until they reached ~200 mm^3. At this point, the mice were orchiectomized (*ORX*) and randomized to receive either isotype control antibody or CNTO328 by i.p. injection weekly at 500 μg/mouse/wk. Mice were maintained for a period of up to 19 weeks after orchiectomy. Another parallel group of mice had LuCaP 35 tumors implanted and were maintained intact without treatment (*Untreated*). Tumors were collected and processed for standard immunohistochemistry for p300 and CBP or for real-time PCR as described in Materials and Methods. *A,* original magnification, ×100. Note nuclear staining. *B,* CBP staining intensity was graded as no detectable signal (score 0), weak signal (score 1), moderate signal (score 2), and strong signal (score 3; $n = 5$ per group). $P = 0.01$ among all groups (ANOVA); *, $P = 0.003$ versus Untreated; #, $P = 0.031$ versus isotype (Fisher's protected least significant difference). *C,* p300 staining intensity was graded as in (*B*). $P = 0.012$ among all groups (ANOVA); *, $P = 0.004$ versus Untreated; #, $P = 0.028$ versus isotype (Fisher's protected least significant difference). *D,* real-time PCR was done for CBP mRNA levels ($n = 7$ per group). *, $P < 0.001$ versus Untreated and CNTO328 (ANOVA and Fisher's protected least significant difference). *E,* real-time PCR was done for p300 mRNA levels ($n = 7$ per group). *, $P < 0.001$ versus Untreated and CNTO328 (ANOVA and Fisher's protected least significant difference).

These results are consistent with a previous *in vivo* study in which the anti-IL6 antibody CLB8 (6), which was used to derive CNTO328, promoted apoptosis in androgen-independent PC-3 prostate cancer cells growing in mice (5). IL-6 has been recognized in many different cell systems to have an antiapoptotic effect, including prostate cancer cells (25, 26). In prostate cancer, IL-6 protects against apoptosis through activation of signal transducers and activators of transcription 3 (25) and phosphatidylinositol-3 kinase (26). Taken together, the observations that inhibition of IL-6 decreased cell proliferation and increased apoptosis suggests that both IL-6 stimulates both of these processes, resulting in an additive effect to promote increased tumor volume in androgen-independent tumors.

IL-6 has been shown to have an autocrine effect in several different tumor types, including prostate cancer. The combination of presence of the IL-6 receptor in the LuCaP 35 cells in combination with the increased IL-6 expression observed in the LuCaP 35 tumors suggests that IL-6 has the ability to produce an autocrine effect on prostate cancer cells. It has been previously shown that orchiectomy induces IL-6 expression in mice (10), and androgens inhibit IL-6 expression in prostate cancer cells (27). Thus, it follows that the androgen decline associated with orchiectomy accounts for the increased IL-6 mRNA expression we observed in the LuCaP 35 tumors. The IL-6-induced activity (i.e., proliferative and antiapoptotic effects) in the LuCaP 35 cells is most likely due to autocrine production of IL-6, as although murine IL-6 levels increase in orchiectomized mice, murine IL-6 does not interact with the human IL-6 receptor. (28).

Changes in the *AR* gene are associated with androgen-independent prostate cancer, including AR gene amplification, and in a subset of patients treated with anti-androgen therapy the AR develops point mutations (reviewed in ref. 29). LuCaP 35 has been shown to have amplification of the AR (30) and wild-type AR sequence (8). Thus, LuCaP 35 mimics aspects of androgen-independent prostate cancer but yet is androgen dependent. It has been identified that AR expression is not only heterogeneous among tumors within the same patient (31) but also within tumors of the same patient (32). Consistent with these reports, we found that immunohistochemistry revealed that AR expression was heterogeneous within each treatment groups, and that this heterogeneity was present among the different groups. However, our observation that we could not detect changes in AR protein expression on immunoblot suggests that overall AR protein expression did not change in the tumors as the protein was isolated from pieces of tumor. This suggests that although individually evaluated histologic sections of different tumors within each group expressed different proportion of cells that expressed AR, as previously reported in clinical samples (32), the overall AR expression per cell in the entire tumor was not different among groups. Regardless of the overall expression of AR, the observation that AR is present suggests that it may play an important role in either the development or maintenance of androgen-independent prostate cancer.

The mechanisms through which IL-6 activates the AR are not currently identified; however, several pathways may play a role (33, 34). Evidence is accumulating that increased expression of the AR coactivator p300/CBP may contribute to the development of androgen-independent prostate cancer, including that p300/CBP is up-regulated by androgen withdrawal and increased in advanced prostate cancer (11). Our results, showing that expression of p300 and CBP were increased in tumors from orchiectomized compared with intact mice agrees with these findings. Our observation that p300 and CBP expression were decreased by CNTO328 suggests that IL-6 promotes their expression. It has been shown that p300/CBP are downstream of the mitogen-activated protein kinase pathway during trans-activation of the AR by IL-6, and that p300 mediates AR activation by IL-6 (12). Accordingly, the increased expression of p300/CBP in the androgen-independent tumors suggests that they may activate the AR in these tumors, even with the low levels of androgen present in the orchiectomized mice. Our observation that IL-6 increases p300 taken together with the report that p300 activates the PSA promoter independent of androgen (35) suggests that IL-6 activates androgen-responsive genes in androgen-independent prostate cancer. The ability of IL-6 to activate androgen-responsive genes may promote the development of prostate cancer growth in the absence of androgen, resulting in the development of androgen-independent prostate cancer.

We observed that parental LuCaP 35 cells grew when implanted into orchiectomized mice, and also that established LuCaP 35 tumors continued to grow in orchiectomized mice when treated with CNTO328, albeit the growth rate was slower than the androgen-independent LuCaP 35 cells. This indicates that there are cells within the tumors that do not require normal levels of androgen or IL-6 to grow. The mechanism for this continued growth is not know, but it is possible the LuCaP 35 contains a mixture of both androgen-dependent and androgen-independent cells, and the androgen-independent cells are being selected for as the tumors grow in the androgen/IL-6–diminished environment. This possibility reflects the concept that androgen-independent cells are already present in the prostate cancer at the time of diagnosis as opposed to genetic changes occur over long-term androgen deprivation (reviewed in ref. 36). Along these lines, it should be noted that tumors continued to be viable, albeit did not grow, in the group of mice treated with CNTO328, which indicates that inhibition of IL-6 would most likely have limited use as a monotherapy for prostate cancer but suggests it would be a useful adjunct to a cytotoxic therapy.

In summary, our results show that IL-6 contributes to the conversion of LuCaP 35 tumors to an androgen-independent state. It is unclear from these data if the ability of IL-6 to affect androgen dependence is due to an effect of tumor growth itself, which may then decrease the possibility for an evolutionary event to occur that confers an androgen-independent phenotype or if IL-6 is required to maintain the androgen-independent phenotype. Regardless, our data indicate that IL-6 most likely promotes androgen-independent through a combination of increasing prostate cancer cell proliferation and decreasing prostate cancer cell apoptosis. Inhibition of IL-6 activity with CNTO328 prevented the conversion to an androgen-independent state. These results suggest that targeting IL-6 would be a useful adjunct therapy for prostate cancer in conjunction with androgen deprivation therapy.

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