Anti-Interleukin-6 Monoclonal Antibody Induces Regression of Human Prostate Cancer Xenografts in Nude Mice

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BACKGROUND. Despite clinical associations and in vitro data suggesting that autocrine interleukin-6 (IL-6) production contributes to prostate cancer progression or chemotherapy resistance, there have been no reports that explore the role of IL-6 on prostate tumors in vivo. In the present study, we investigated the effect of IL-6 inhibition on the growth of human prostate cancer xenografts in nude mice.
METHODS. To determine if autocrine IL-6 production contributes to prostate cancer growth and chemotherapy resistance in vivo, xenografts of a human prostate cancer cell line that produces IL-6 (PC-3) were established in nude mice. The mice were randomly divided into four treatment groups: (1) saline (vehicle control) + murine IgG (isotype control); (2) etoposide + murine IgG; (3) saline + anti-IL-6 monoclonal antibody; and (4) etoposide + anti-IL-6 monoclonal antibody. Tumors were measured twice weekly during a 4-week treatment period. At the conclusion of the study, all mice were sacrificed, and in addition to final volume, tumors were evaluated for the degree of apoptosis by TUNEL analysis.
RESULTS. Anti-IL-6 Ab (with saline or etoposide) induced tumor apoptosis and regression (~60% compared to initial tumor size). Etoposide alone did not induce tumor regression or apoptosis in this animal model, and there was no synergy between anti-IL-6 Ab and etoposide.
CONCLUSIONS. These studies suggest that IL-6 contributes to prostate cancer growth in vivo, and that targeting IL-6 may contribute to prostate cancer therapy. *Prostate 48:47–53, 2001*

KEY WORDS: **cytokine; interleukin-6; antibody; prostate cancer; chemotherapy**

INTRODUCTION

Prostate cancer is the most common cancer diagnosed in men and the second leading cause of cancer death among men in the United States. In 1999, it was estimated that 179,300 patients were diagnosed with prostate cancer, and 37,000 patients died from the disease [1]. Radical prostatectomy can be curative in patients with localized prostate cancer. Unfortunately, many patients have an advanced form of the disease at the time of diagnosis, and require systemic androgen-ablation therapy. Initially the cancer appears as an androgen-sensitive phenotype and is responsive to this treatment. However, after a median time of 12–18 months, it commonly recurs as a hormone-refractory phenotype that is also resistant to other therapeutic

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modalities including chemotherapy [2]. The precise mechanism of drug resistance in prostate cancer is not fully understood, but the secretion of protective factors by these tumors may play a role.

The cytokine interleukin-6 (IL-6) has been implicated in a number of pathophysiologic processes including stimulation of tumor proliferation [3]. In the past few years, evidence has been accumulating that IL-6 may contribute to the progression of prostate cancer [4]. For example, IL-6 serum levels are correlated with morbidity and tumor burden of prostate cancer patients [5]. Furthermore, the addition of anti-IL-6 antibody to the growth medium of androgen-independent prostate cancer cell lines has been shown to inhibit cell growth [6,7]. Additionally, inhibition of IL-6 activity enhances the cytotoxic activity of certain chemotherapeutic agents in prostate cancer cell lines that are resistant to the drugs [8]. In spite of the many studies demonstrating that IL-6 promotes prostate cancer proliferation and survival in cell culture, there has been no in vivo evidence to confirm that IL-6 contributes to prostate cancer growth. Accordingly, to determine if IL-6 contributes to prostate cancer progression, we examined the effect of inhibiting IL-6 activity on prostate cancer progression in mice implanted with a human prostate cancer xenograft.

MATERIALS AND METHODS

Cell Lines

The hormone-independent prostatic carcinoma cell lines PC-3 and DU145, and the hormone-dependent cell line, LNCaP (ATCC) were cultured in complete medium (RPMI 1640 with L-glutamine supplemented with 10% heat-inactivated fetal bovine serum, and 1% penicillin/streptomycin solution containing 10,000 units/ml penicillin G and 10,000 μg/ml streptomycin). All three cell lines were grown in a humidified incubator at 37°C in 5% CO_2. Cells were treated with trypsin-EDTA, washed, and resuspended in complete medium prior to their use in cytotoxicity assays.

Antibodies

Anti-hIL-6 (CLB, Amsterdam, The Netherlands) antibody is a mouse monoclonal (subtype IgG1) specific for human IL-6 [9]. Mouse $IgG1_k$ (Sigma, St. Louis, MO) was used as an isotype control antibody.

Etopsoide

Etoposide (Sigma) was dissolved in 1 ml DMSO to a final stock concentration of 25 mg/ml and stored at 4°C. The stock solution was diluted to the indicated concentrations in complete medium (in vitro experiments) or normal saline (in vivo experiments) immediately prior to use.

IL-6 Measurements in Cell Culture

Cells were grown in 10-cm polystyrene tissue culture dishes. Confluent cells were washed once with phosphate-buffered saline and then incubated for 48-hr in complete medium, at which time the supernatant was collected and stored at −20°C until assayed. IL-6 concentration was measured using a commercial ELISA kit (Quantikine Human IL-6 ELISA Kit; R&D, Systems Inc., Minneapolis, MN) as directed by the manufacturer.

In some cases, IL-6 levels were measured following the addition of anti-IL6 antibody. In these instances, cells were plated in 6-well polystyrene tissue culture plates at a density of 5×10^5/well in 2.5-ml complete medium. Anti-IL6 monoclonal antibody, isotype control (final concentration of 500 ng/ml), or complete medium was added to appropriate wells and cells were incubated at 37°C. Supernatant samples were collected from each well at 24, 48, and 72 hr and stored at −20°C until ELISA was performed.

Serum IL-6 Levels

At the time of sacrifice, blood samples were collected via cardiac puncture and centrifuged at 2,700 rpm for 10 min. The serum was removed from each sample and stored at −80°C until assayed for IL-6 using the B9 cell IL-6 bioassay as previously described [10].

In Vitro Cytotoxicity Experiments

Cell lines were seeded in 96-well plates at a density of 2×10^3 cell/well in 100-μl complete medium. Either anti-IL6 or isotype control antibody was added at a concentration of 2 μg/ml (final concentration to be 500 ng/ml) in 50 μl of complete medium, and etoposide was added in a 50-μl/volume to reach a final concentration of 0.1 or 10 μg/ml (approximate ID_{25} and ID_{50}, respectively; data not shown). Saline vehicle was added to cells that did not receive etoposide. The cells were then incubated for 48 hr. Cell viability was then determined using an MTS assay (Promega, Madison, WI) as directed by the manufacturer. Cytotoxicity was calculated as follows:

$$\%\,\text{cytotoxicity} = [1 - (\text{absorbance of experimental wells}/\text{absorbance of control wells})] \times 100.$$

Mice

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

In Vivo Experiments

Confluent PC-3 cells were harvested by trypsinzation, washed twice with PBS and resuspended at a density of 1×10^7 cells/ml. The mice were injected subcutaneously with 100 μl of the tumor cell suspension (10^6 cells) combined with 100 μl of Matrige® (Bectin-Dickson, Bedford, MA). The mice were monitored for tumor growth, and when tumors were detected by palpation, measurement of the tumors began. Tumor volumes were calculated by the formula: Volume = [(minimum measurement)^2 (maximum measurement)] ÷ 2 [as described in Ref. [11]]. Tumors were measured every other day, and when tumors reached a volume of 126 mm^3 the mice were randomly assigned to one of four treatment groups (n = 10/group). Treatment groups included isotype + saline, isotype + etoposide, anti-IL-6 + saline, and anti-IL-6 + etoposide. The treatment regimen consisted of weekly i.p. injection of anti-IL6 or isotype antibody at 500 μg/mouse/week as previously described [12] and daily i.p. injections of etoposide at 50 mg/m^2/day, which is the human-equivalent dose [13], or an equal volume of saline. Mouse IL-6 does not react with human IL-6 receptor [14]. Thus, using an anti-human-IL-6 alone will inhibit the IL-6 specifically produced by the human prostate cancer cells. Treatment continued for 4 weeks, during which time the tumors were measured on a twice-weekly basis. At the conclusion of the study all mice were sacrificed, and blood and tissue samples were collected for further analysis.

Tumor Histopathology and Detection of Apoptosis

Excised tumors were placed in 10% formalin, embedded in paraffin and sectioned at 10 μM thickness. Sections were examined utilizing standard hematoxylin and eosin (H&E) staining for routine histopathology. To evaluate apoptosis, sections were deparaffinized, rehydrated, and subjected to terminal deoxytransferase UTP end-labeling (TUNEL) analysis using ApopTag® Plus Peroxidase Kit (Intergen, Purchase, NY) according to the manufacturer's directions. The number of apoptotic nuclei per 200X field (averaged from three random 200X fields) was determined for each section by an investigator that was blinded to the samples as previously described [15].

Statistical Analysis

To determine differences among treatment groups for tumor size, two-way analysis of variance (ANOVA) was used followed by Fisher's least significant difference for post-hoc analysis. Statistical significance was determined at $P < 0.05$.

RESULTS

IL-6 Secretion by Prostate Cancer Cell Lines

The detection of IL-6 secretion by prostate cancer cell lines is fairly inconsistent between laboratories [6,7,16]. Thus, it was critical to determine the IL-6 expression of various cell lines in our laboratory prior to proceeding with an in vivo challenge. Accordingly, we measured the amount of IL-6 secreted into the culture supernatant of various prostate cancer cell lines. PC-3 cells secreted the most IL-6 followed by DU-145 cells (Fig. 1A). IL-6 was undetectable in LNCaP cell culture supernatant (Fig. 1A).

Effect of IL-6 Antibody Treatment on IL-6 Levels in PC-3 Cell Culture Supernatant

In order to provide a maximum challenge to our ability to inhibit IL-6, we performed the remaining experiments with the PC-3 cells, which secreted the highest levels of IL-6. To confirm that the anti-IL-6 antibody we were using effectively inhibited IL-6 levels over a length of time, we incubated PC-3 cells with 500 ng/ml of either anti-IL-6 or isotype control antibody for 24, 48, and 72 hr, then measured IL-6 levels using ELISA. Anti-IL-6 antibody decreased the detection of IL-6 by ≥ 50% at all three time points compared to the isotype antibody (Fig. 1B).

Effect of Anti-IL-6 and Etoposide on Cell Proliferation of PC-3 Cells

To determine if anti-IL-6 antibody enhances the etoposide-mediated cytotoxicity of prostate cancer cells, we incubated prostate cancer cells with antibody and etoposide, then measured viable cell number. Anti-IL-6 antibody alone decreased the number of viable cells by approximately 10% (Fig. 2). Etoposide alone at low (0.1 μM) and high (10 μM) doses induced approximately 5% cytotoxicity. Anti-IL-6 antibody combined with the high dose of etoposide induced approximately 25% cytotoxicity, thus demonstrating a synergistic effect between etoposide and anti-IL-6 antibody in vivo. These in vitro data provided the rationale to pursue the ability of IL-6 antibody to modulate prostate cancer cell growth in vivo.

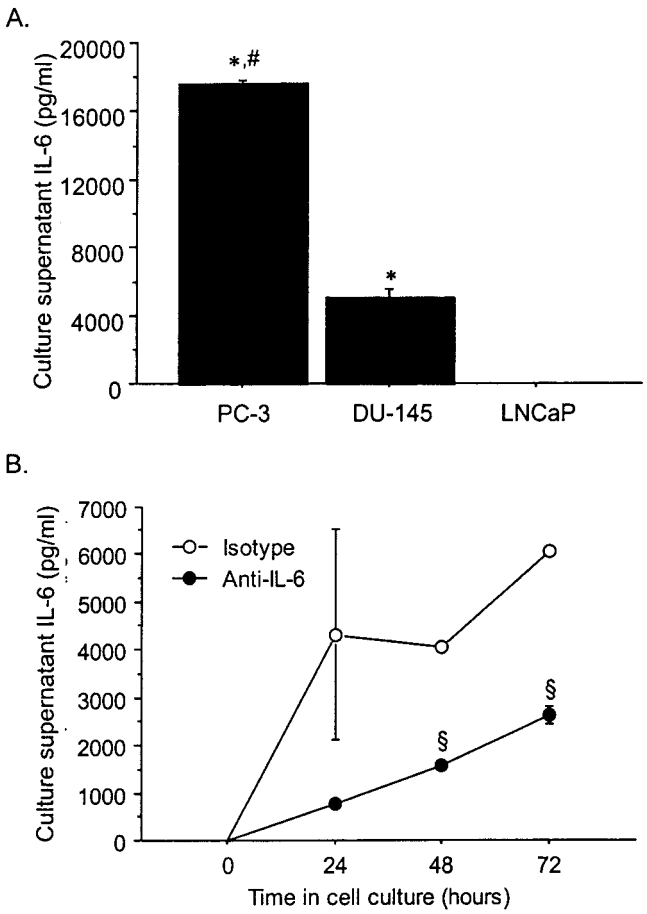

Fig. 1. Anti IL-6 antibody diminished ELISA detectable IL-6 from PC-3 cells. **A:** The indicated prostate cancer cell lines were plated at a density of 5×10^6 cells/10 ml in 10 cm tissue culture plates and cultured for 48 hr. Supernatant was then collected and subjected to ELISA for IL-6. **B:** PC-3 cells were plated in 6-well polystyrene tissue culture plates at a density of 5×10^5/well in 2.5 ml complete medium. Anti-IL-6 monoclonal antibody or isotype control (final concentration of 500 ng/ml) was added and cells were incubated at 37°C. Supernatant samples were collected from each well at 24, 48, and 72 hr and subjected to ELISA for IL-6. All time points were run on the same plate. Both assays were performed in triplicate. $^*P < 0.01$ vs. LNCaP, $^{\#}P < 0.01$ vs. DU-145, $^{\S}P < 0.05$ vs. isotype.

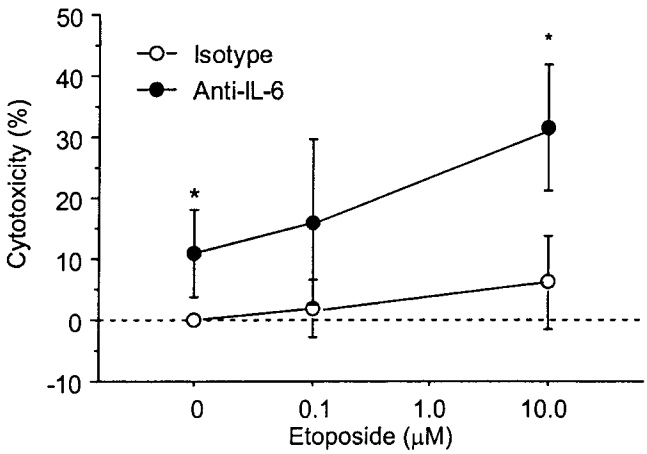

Fig. 2. Inhibition of IL-6 induces cytotoxicity of PC-3 cells in vitro. Cell lines were seeded in 96-well plates at a density of 2×10^3 cells/well in 100 μl complete medium. Either anti-IL6 or isotype control antibody was added at a concentration of 2 μg/ml (final concentration to be 500 ng/ml) in 50 μl of complete medium. Etoposide was added in a 50 μl/volume to reach a final concentration of 0.1 μg/ml or 10 μg/ml (approximate ID_{25} and ID_{50}, respectively; data not shown). Saline vehicle was added to wells not receiving etoposide. The cells were then incubated for 48 hr. Cell viability was then determined using an MTS assay and cytotoxicity was determined as described in the Methods section. The assay was performed in triplicate $^*P < 0.05$ vs. isotype.

Tumor Response

Based on our observation that inhibition of IL-6 alone inhibited PC-3 survival in addition to enhancing-mediated cytotoxicity in vivo, we next explored if inhibiting IL-6 would mimic these effects in vivo on established prostate cancer tumors. To accomplish this, PC-3 cells were subcutaneously injected into nude mice. The tumors were allowed to develop until they were approximately 126 mm^3, at which time anti-IL-6 or isotype antibody and etoposide administration was initiated. Antibody was administered at a level that inhibited IL-6 bioactivity by approximately 20% (based on B9 bioassay; data not shown). Treatment was continued for a 4-week period. Using an ELISA specific for human IL-6 (and non-cross-reactive with murine IL-6), we determined that serum human IL-6 levels were 30.1 ± 10.4 pg/ml in tumor-implanted saline + isotype control mice, compared to undetectable levels in mice not implanted with tumor, demonstrating that the tumors produced IL-6 in vivo. The tumors in the isotype-treated mice had a continuous, albeit slow, tumor growth rate, whereas the tumors in the isotype + etoposide-treated mice did not grow (Fig. 3). In contrast, the final tumor volumes were reduced by approximately 60% compared to their initial size in the mice receiving anti-IL-6 or anti-IL6 + etoposide. Furthermore, they were approximately 75% smaller than the tumors in the mice receiving isotype alone (Fig. 3). There was no significant difference between the tumor volumes in the mice treated with isotype alone compared to the mice treated with etoposide alone.

Apoptosis

We evaluated the effect of anti-IL-6 antibody and etoposide on the amount of apoptosis present in the PC-3 tumors. Routine histological evaluation of the tumors did not demonstrate any differences among the treatment groups. However, administration of anti-IL-6 antibody was associated with marked apop-

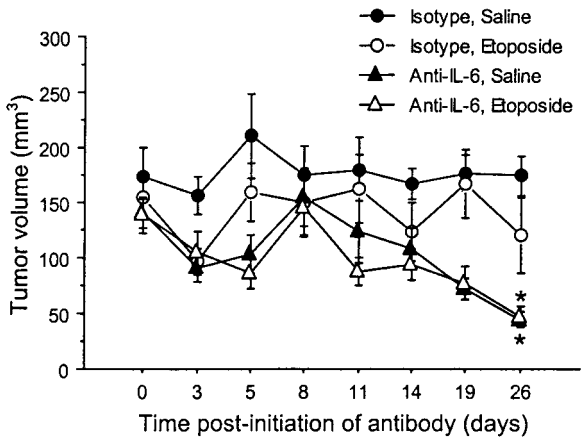

Fig. 3. Inhibition of IL-6 induces PC-3 xenograft regression in mice. PC-3 cells (10^6) in a Matrigel slurry were subcutaneously implanted in male nu/nu mice. When tumors reached 126 cm^3, weekly i.p. injections of anti-IL6 or isotype antibody (500 μg/mouse) and daily i.p. injections of etoposide (50 mg/m^2) or saline vehicle were initiated. Tumors were measured twice-weekly for 4 weeks. There were 10 animals/group. Results are shown as mean ± SD. $^*P < 0.01$.

tosis in the tumors from both the saline vehicle and etoposide treated mice, compared to the moderate level of apoptosis in the tumors from the isotype and isotype + etoposide-treated mice (Fig. 4). These data demonstrate that IL-6 has an anti-apoptotic action in PC-3 cells in vivo.

DISCUSSION

A large body of evidence has accumulated that suggests IL-6 contributes to prostate cancer progression. The evidence includes both clinical observations that increased levels of IL-6 are associated with increasing grade of prostate cancer in patients [5,17,18] and in vitro experiments that have demonstrated that IL-6 promotes prostate cancer cell growth and prevents chemotherapeutic-mediated cytotoxicity [7,19]. In the current study, we provide the first demonstration that IL-6 activity promotes prostate cancer growth in vivo.

Our data demonstrate that high levels of IL-6 are secreted by PC-3 and DU-145 cells, whereas IL-6 levels were not detectable using ELISA methodology in LNCaP cells. These findings are consistent with previous reports on IL-6 secretion by prostate cancer cell lines [6,8]. PC-3 and DU-145 cells are androgen non-responsive, whereas LNCaP cells are androgen-responsive. Thus, these results suggest that loss of androgen responsiveness is associated with increased IL-6 expression. This postulation is consistent with the observations that elevation of serum IL-6 levels is associated with increasing grade of prostate cancer [5,18,20]. A cause and effect cannot be determined based on the current data. However, it has been previously reported that the androgen dihydrotestosterone inhibits IL-6 expression in prostate cancer cells [16] and that orchiectomy increased IL-6 expression in murine bone marrow [10]. Thus, it is plausible that loss of androgen-response promotes IL-6 expression.

Prostate cancer is poorly responsive to chemotherapy. Therefore, a mechanism to enhance chemotherapeutic killing of tumors would be a boon for prostate cancer patients. Borsellino et al. [7] have reported that inhibition of IL-6 activity enhances chemotherapeutic killing of prostate cancer cell in vitro. However, this effect has not been reported in vivo. In the current study, the cell type and therapeutic agent were chosen based on in vitro studies, and the human equivalent dose of etoposide was used to calculate the dosage administered to the mice. However, we did not observe an effect of etoposide on the PC-3 in vivo. In contrast, tumors responded to anti-IL-6 antibody, although the combination of anti-IL-6 and etoposide did not significantly enhance this inhibitory effect compared to anti-6 alone. Taken together, these data suggest that inhibition of IL-6 does not enhance etoposide-mediated killing in vivo. However, they clearly demonstrate that inhibition of IL-6 alone, or in the presence of etoposide, induces regression of PC-3 tumors. This observation provides in vivo evidence that IL-6 contributes to prostate cancer cell growth, and that inhibition of its activity promotes tumor regression.

This ability to inhibit IL-6 activity in humans has been previously demonstrated in several clinical trials using murine monoclonal antibodies in patients with multiple myeloma [21,22]. Early trials demonstrated the feasibility of blocking IL-6 activity in this manner, and that such therapy had beneficial effects [21]. These trials also revealed certain limitations to anti-IL-6 therapy using murine monoclonal antibodies. One such limitation is that in some patients with advanced disease, IL-6 levels were so high that the antibody was unable to neutralize them [22]. Another limitation is that development of antibodies to mouse immunoglobulin may result in rapid clearance of the murine monoclonal antibody and diminished efficacy of treatment [23]. This problem has been addressed through the chimerization [23,24] and humanization [24] of murine anti-IL-6 antibodies. Tsunenari et al. [24] demonstrated reduced antigenicity of chimeric antibodies and even lower antigenicity of humanized murine antibodies (24) while a later study by van Zaanen et al. [23] showed no induction of human anti-chimeric antibodies in multiple myeloma patients receiving chimeric anti-IL-6 antibodies. Overall, these studies suggest that inhibition of IL-6 activity in prostate cancer patients is achievable.

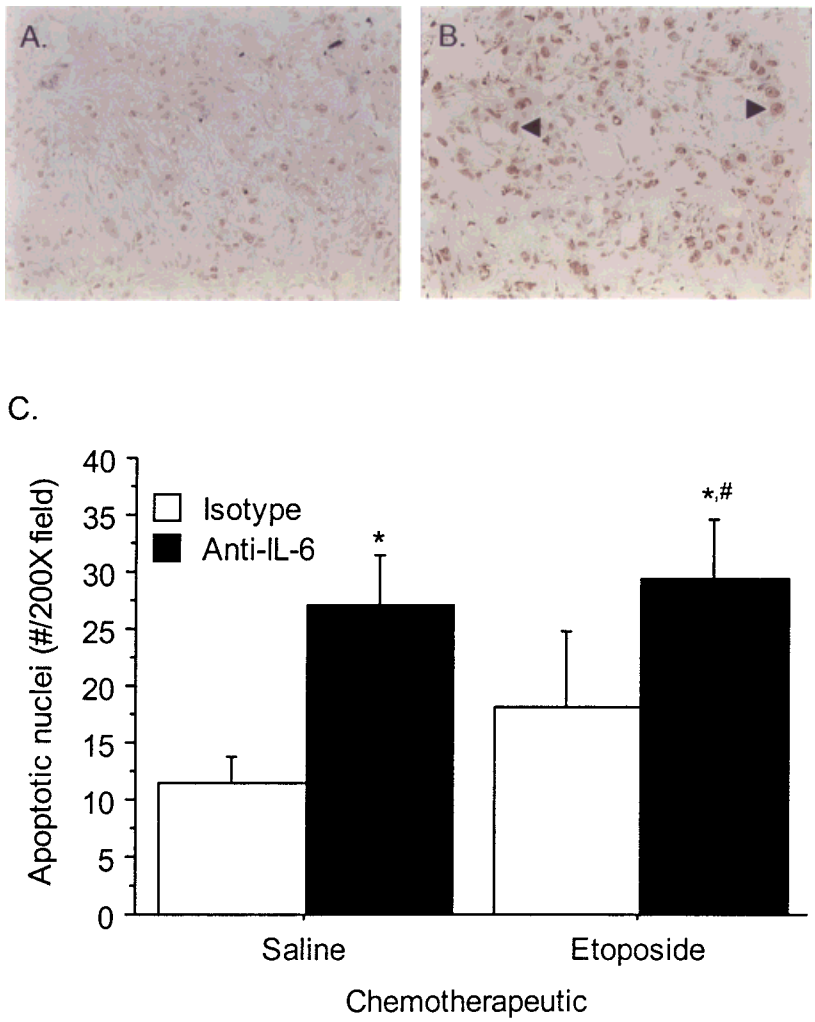

Fig. 4. Inhibition of IL-6 induces apoptosis in PC-3 tumors in mice. Tumors were excised from mice 4 weeks after initiation of anti-IL6 and etoposide as described in Fig. 3. Breaks in DNA were determined by labeling 3′OH termini using terminal deoxytransferase and staining with peroxidase. Tumor sections are shown from (**A**) isotype-treated mice and (**B**) anti-IL-6 treated mice. Apoptotic nuclei are dark brown (arrowheads). Original magnification 100 ×. **C**: To determine the degree of apoptosis, the number of apoptotic nuclei/200 × field (average of triplicate) were determined by an investigator blinded to the samples. *$P < 0.01$ compared to the saline + isotype mice; *$P < 0.05$ compared to the etoposide + isotype mice. Data are shown as mean ± SD of 4 mice/group.

The mechanism through which IL-6 contributes to overall prostate tumor growth is not clear. There are conflicting reports regarding the effect of IL-6 on prostate cancer cell proliferation in vitro [4]. Thus, it is unclear if IL-6 directly contribute to tumor growth through stimulation of cell proliferation. In addition to increased cell proliferation, a tumor may enlarge due to decreased apoptotic death of cell. The observation that IL-6 has been demonstrated to have anti-apoptotic action in several cell types [19,25,26] including the prostate cancer cell lines LNCaP and PC-3 (27) led us to evaluate IL-6's effect on apoptosis in the prostate cancer xenografts. Our observation that the level of apoptosis in tumors of mice that received anti-IL-6 compared to those who received isotype control antibody demonstrates that IL-6 protects prostate cancer cells from apoptosis in vivo. These findings are consistent with the in vitro results of Chung et al. [27] who demonstrated the antiapoptotic effects of IL-6 in PC-3 and LNCaP cell lines, and showed that this effect is the result of IL-6 activation of phosphatidylinositol (PI)-3 kinase. These previous reports, taken together with the currently reported murine studies, suggest that inhibition of apoptosis is one mechanism through which IL-6 contributes to prostate cancer progression.

CONCLUSION

In summary, the current study demonstrates that anti-IL-6 antibody induces apoptosis and regression of established PC-3 tumors in mice. However, the in vivo

data do not support the in vitro observations that IL-6 enhances etoposide-mediated killing. These data, combined with the clinical reports that IL-6 is associated with prostate cancer stage [17,18,28], provide compelling evidence that IL-6 contributes to prostate cancer progression and suggests that targeting IL-6 may induce prostate cancer regression.

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