Androgens Regulate Vascular Endothelial Growth Factor Content in Normal and Malignant Prostatic Tissue


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ABSTRACT

In previous studies, we have demonstrated that androgen ablation-induced growth inhibition of androgen-responsive PC-82 and A-2 human prostate cancer xenografts involves not only direct activation of programmed (apoptotic) death of these cells but also indirect activation of this death process via a decrease in tumor angiogenesis secondary to a reduction in tumor vascular endothelial growth factor (VEGF) levels. To determine whether androgens consistently regulate angiogenesis via control of VEGF levels, an additional human (i.e., LnCaP) and two rodent (i.e., Dunning G and H) androgen-sensitive prostate cancer sublines were tested. Androgen ablation causes a decrease in the subsequent growth rate of each of these three additional prostate cancer sublines, and this growth inhibition is consistently associated with a >60% reduction in tumor VEGF levels. To examine whether androgens regulate VEGF levels not only in malignant but also in normal prostatic tissue, male rats were castrated, and the temporal changes in the VEGF content of ventral prostate tissue were determined. One week after castration, VEGF content decreased to <20% within the ventral prostate. Subsequent replacement with exogenous androgen to long-term castrated rats simulated an 8-fold rise in ventral prostate VEGF content within 1 week. To evaluate whether androgen regulation of VEGF is due to a direct effect of androgen on prostatic cells, the dose-response ability of androgens to increase VEGF levels in media of LnCaP cells grown in vitro was tested. These studies demonstrate that androgens directly stimulate VEGF secretion in these cells. The presence of 4-5-fold higher levels of VEGF in prostatic fluid versus seminal vesicle fluid obtained from benign prostatic hyperplasia and clinically localized prostate cancer patients suggests that elevated levels of VEGF may contribute to the progression of these prostatic conditions by promoting angiogenesis. In summary, one of the mechanisms for androgen sensitivity for the control of the growth of both normal and malignant prostatic tissue is via its stimulation of VEGF levels.

INTRODUCTION

Angiogenesis is a prerequisite for the expansion of solid tumors beyond 1–3 mm³ (1, 2). It has been demonstrated that the majority of latent prostate cancers detectable in autopsy material from men who died with no clinical indication of prostate cancer have very low blood capillary density ratios compared to prostate cancers that produced clinical symptoms and metastasized (3, 4). Because the acquisition of an angiogenic phenotype seems critical for the development of aggressive prostate cancers, inhibition of angiogenesis should be a highly effective method to inhibit the growth and metastasis of these cancers.

A number of studies from this laboratory have demonstrated that Linomide, a quinoline-3-carboxamide, has antitumor effects in vivo against a series of androgen-dependent and -independent rodent prostate cancers grown in rats and human prostate cancers grown as xenografts through its ability to inhibit tumor angiogenesis (5–7). Linomide inhibits several steps in the angiogenic process including endothelial cell proliferation, chemotactic migration, and invasion (7). Linomide also inhibits the number of tumor-associated macrophages and the ability of tumor-associated macrophages to synthesize and secrete the angiogenic molecule tumor necrosis factor α (8, 9). Additionally, Linomide can also inhibit the angiogenic response induced by VEGF, acidic fibroblast growth factor, bFGF, and tumor necrosis factor α (10).

It is well established that androgen ablation induces programmed death of androgen-dependent normal (11) and malignant prostatic cells (12). Recent studies from this laboratory have demonstrated that androgen ablation-induced growth inhibition of androgen-responsive Dunning G and PAP rat prostate cancer sublines is associated with a reduction in blood vessel density within these tumors (13). When androgen ablation is combined with the well-documented antiangiogenic agent Linomide, a further reduction in tumor growth as well as tumor blood vessel density is observed (13). In addition, we have demonstrated that androgen ablation inhibits the growth of the androgen-responsive PC-82 and A-2 human tumors grown as xenografts in SCID mice indirectly via inhibition of angiogenesis in addition to directly inducing programmed cell death (14). In both the PC-82 and the A-2 human tumors, androgen abla-
tion-induced inhibition of tumor angiogenesis is due to the inhibition of tumor VEGF without an effect on bFGF levels (14). When given in combination, androgen ablation also potentiated the inhibition of PC-32B and A-2 human tumor growth induced by Linomide alone (14).

To test whether androgens consistently regulate angiogenesis in androgen-responsive prostate cancers via control of VEGF levels, tumor tissues from androgen-sensitive LnCaP human and Dunning G and H rat prostate cancers were obtained from intact and surgically castrated hosts, and the tissues were assayed for their VEGF content. To address the issue of whether androgens regulate VEGF levels not only in malignant but also in normal prostatic tissue, the temporal effects of castration alone and androgen replacement to long-term castrated hosts on the VEGF content of rat ventral prostate tissue was tested. In addition, in vitro cultures of LnCaP cells were tested to evaluate whether androgen regulation of VEGF is due to a direct effect of androgen on these cells. Finally, to validate the relevance of these studies to human prostatic disease, the VEGF content in expressed prostatic secretion fluid versus seminal vesicle fluid obtained from men with BPH and prostate cancer was also determined.

MATERIALS AND METHODS

Animals. Specific pathogen-free SCID mice used in these studies were obtained from Charles River Breeding Laboratories, Inc. (Wilmington, MA). Inbred Copenhagen male rats were obtained from Harlan Sprague Dawley (Indianapolis, IN). All animals were housed in groups of four per individual microisolator cage placed in well-ventilated rooms under controlled light (12-h light/12-h dark cycle, lights set at 7 a.m.), temperature (21°C–22°C), and humidity (50%). Food (Picolab Rodent Diet 20 (PMI Feeds, Inc., St. Louis, MO) for mice and Rat Chow (Purina Mills, Inc., Richmond, IN) for rats) and water were available ad libitum.

Reagents. Insulin, transferrin, and selenium + culture supplement were obtained from Collaborative Biomedical Products (Two Oak Park, Bedford, MA). DHT and testosterone were from Steraloids, Inc. (Wilton, NH).

Tumors. The origin and the characteristics of the androgen-sensitive LnCaP (15) and Dunning G and H tumors have been described elsewhere (16). To produce tumors, either cells or trocar pieces of tumors were injected into the flank region of animals under Metofane (Mallinkrodt Veterinary, Inc., Mundelein, IL) anesthesia. To produce LnCaP tumors, SCID mice were injected with 1 × 10^6 LnCaP cells in 0.2 ml of Matrigel (Collaborative Research, Bethesda, MD) as described previously (17). To produce Dunning G and H tumors, solid trocar pieces of 5-mg tumor tissues were placed in Copenhagen rats. After the development of palpable tumors, individual tumor dimensions were serially measured using calibrated microlithers. Tumor volumes in cm^3 were calculated using the formula \( V = \frac{1}{2} \times w \times h \times 0.5236 \), and then volume doubling times were calculated as described previously (16). When tumors were 1–2 cm^3, animals were randomized into control and castrate groups. Castration was performed via a scrotal route under Metofane anesthesia.

Analysis of VEGF in Prostatic and Seminal Vesicle Fluid from Patient Samples. Expressed prostatic fluid was obtained by digital massage from 32 men with clinical and/or biopsy-proven BPH and from 16 men with biopsy-proven prostate cancer. Seminal vesicle fluid was obtained intraoperatively from seven men undergoing radical retropubic prostatectomy for localized prostate cancer. The samples were centrifuged at 1000 × g for 20 min to remove cellular debris and stored at −70°C until assayed for VEGF.

Cell Culture. The origins and characteristics of the human LnCaP prostate cancer cell line have been described previously (15). These cells were maintained by in vitro culture in RPMI 1640 (Life Technologies, Inc., Grand Island, NY) supplemented with 10% fetal bovine serum (HyClone, Logan, UT) and 100 IU/ml potassium penicillin G/streptomycin sulfate (Sigma Chemical Co., St. Louis, MO) at 37°C in a humidified atmosphere containing 5% CO_2 in air.

Sample Preparation. LnCaP cells were plated at a density of 8 × 10^4 cells/35-mm dish in phenol red-free RPMI 1640 medium (Life Technologies, Inc.) containing 10% fetal bovine serum. After 48 h, the media were removed, cells were washed, and fresh phenol red-free RPMI containing insulin, transferrin, and selenium + culture supplement was added to the control dishes. The treatment dishes were similarly washed, and medium containing 0–100 ng/ml DHT was added. After 96 h, the conditioned medium was collected, centrifuged at 1000 × g for 10 min to remove cellular debris, aliquoted, and stored at −70°C until assayed for VEGF and PSA. On removal of the conditioned medium, the cells were trypsinized, and the total number of cells present was counted using a Coulter electronic particle counter (Coulter Electronics, Inc., Hialeah, FL).

To determine tissue VEGF or bFGF levels, tumors and ventral prostate glands harvested at various times after castration were homogenized in normal saline, centrifuged at 1000 × g for 20 min to remove cellular debris, aliquoted, and stored at −70°C until assayed for VEGF.

VEGF, bFGF, and PSA Assays. Samples stored at −70°C were thawed and diluted appropriately, and VEGF and bFGF concentrations were determined by ELISA following the protocol provided by the manufacturer (R&D Systems, Inc., Minneapolis, MN). The ELISA assay (catalogue number DVE00) used to measure prostatic and seminal vesicle fluid, LnCaP cell culture media, and tumor tissue VEGF levels has been demonstrated by the manufacturer to be specific for humans and does not recognize the murine homologue. The ELISA assay (catalogue number MM00) used to measure G and H tumor tissue VEGF levels has been demonstrated by the manufacturer to be specific for humans and does not recognize the murine homologue. The PSA levels in these samples were measured using the TANDEM-R PSA kit (Hybritech, Inc., San Diego, CA) following the manufacturer's instructions. Results of the conditioned media were expressed as the amount of VEGF, bFGF, or PSA/10^6 cells/24 h, whereas those of the tumors and ventral prostate glands were expressed as the amount of VEGF per gram of tissue.
Table 1  Effect of castration on the growth rate, weight, VEGF, and bFGF content of LnCaP human prostate cancers grown in SCID mice

<table>
<thead>
<tr>
<th>LnCaP tumor</th>
<th>21 days of treatment</th>
<th>Growth rate (volume doubling time in days)</th>
<th>Weight (mg)</th>
<th>Content of VEGF (ng/g tissue)</th>
<th>Content of bFGF (ng/g tissue)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None (control)</td>
<td>7.44 ± 0.73</td>
<td>509 ± 140</td>
<td>15.18 ± 3.26</td>
<td>3.02 ± 0.66</td>
<td></td>
</tr>
<tr>
<td>Castration</td>
<td>33.61 ± 11.88</td>
<td>121 ± 70</td>
<td>6.41 ± 3.41</td>
<td>3.44 ± 0.05</td>
<td></td>
</tr>
</tbody>
</table>

* Values are mean ± SE, five to nine observations/treatment group.

* *P* < 0.05 compared with the parameters in the appropriate control group.

Table 2  Effect of castration on the growth rate, weight, and VEGF content of Dunning G and H rat prostate cancers grown in Copenhagen rats

<table>
<thead>
<tr>
<th>G tumor</th>
<th>Treatment</th>
<th>Growth rate (volume doubling time in days)</th>
<th>Weight (gm)</th>
<th>Content of VEGF (ng/g tissue)</th>
<th>H tumor</th>
<th>Growth rate (volume doubling time in days)</th>
<th>Weight (gm)</th>
<th>Content of VEGF (ng/g tissue)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None (control)</td>
<td>4.0 ± 0.2</td>
<td>5.1 ± 0.4</td>
<td>58.5 ± 22.0</td>
<td>21 ± 2</td>
<td>2.4 ± 0.3</td>
<td>4.9 ± 2.9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Castration</td>
<td>11.0 ± 0.3</td>
<td>3.4 ± 0.3b</td>
<td>0.6 ± 0.2a</td>
<td>1.1 ± 0.2a</td>
<td>0.6 ± 0.2a</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Values are mean ± SE; three to four observations/treatment group.

* P < 0.05 compared with the value for the appropriate control group.

* One month postcastration, no net growth occurred in the Dunning H tumors.

Statistical Analysis. Data are presented as mean ± SE. The experimental data for statistical significance were analyzed using one-way ANOVA followed by the Newman-Keuls multiple comparison. Comparisons between two experimental groups were performed using the unpaired t test. All statistics were calculated using the True Epistat 3.0 statistical program (Epistat Services, Richardson, TX).

RESULTS AND DISCUSSION

Effect of Castration on Growth and VEGF and bFGF Contents of Androgen-sensitive LnCaP Human Prostate Cancer Xenografts. We have demonstrated that in both the androgen-responsive PC-82 and the A-2 human prostate cancer xenografts, castration induces inhibition of tumor growth that is associated with a reduction in tumor VEGF levels (14). To determine whether the androgen-sensitive LnCaP human prostate cancers respond to androgen in a similar manner, SCID mice were inoculated with LnCaP cells. When the tumors were 0.15 cm³ (i.e., 18 days after inoculation), animals were randomized into two groups, one untreated and the other castrated. The tumor volumes were determined over 21 days. On day 21 after castration, the animals were sacrificed, the tumors were harvested and weighed, and VEGF concentrations were measured. These studies demonstrated that castration did not cause a regression of the LnCaP tumor, only a decrease in its subsequent growth rate, as demonstrated by the 4-fold increase in tumor volume doubling time (Table 1). The growth inhibition resulted in a 75% lower tumor weight by 21 days postcastration (Table 1). Castration induced a >60% reduction in the tumor VEGF levels without any inhibition on bFGF levels (Table 1). Because the antibody used in these assays is specific for human VEGF (i.e., it does not cross-react with murine VEGF), this demonstrates that the inhibition of VEGF content is due to an effect on the LnCaP tumor cells themselves and is not due to the murine stromal cells in the tumors. This is consistent with our previous observation with the androgen-responsive PC-82 and the A-2 human tumors (14).

Effect of Androgen Ablation on VEGF Content of Androgen-responsive Dunning G and H Rat Prostate Cancers. Our previous studies have demonstrated that castration decreases the tumor growth and the blood vessel density in both Dunning PAP and G rat prostatic cancers (13). Because androgen ablation-induced inhibition of tumor growth rate is associated with a reduction in tumor VEGF content in all of the human prostate tumors tested, we examined whether the androgen ablation-induced inhibition of Dunning G and H rat prostate cancer growth is also associated with a reduction in tumor VEGF content. To accomplish this, Dunning G and H tumors were transplanted into Copenhagen rats. When the tumors reached 1–2 cm² in size (i.e., after 2 months for the G sublines and 6 months for the H sublines), half of the tumor-bearing animals were castrated. As reported previously (16), after castration, G tumors did not regress but decreased their subsequent growth rate, whereas the H tumors completely stopped growing for 4–6 weeks postcastration (Table 2). After 2 weeks postcastration for G tumors and 1 month postcastration for H tumors, tumors were harvested from both the intact and the castrated rats, and VEGF levels were determined (Table 2). Dunning G tumors produced 10-fold more VEGF than did the Dunning H tumors (Table 2). Castration induced a >10-fold reduction of VEGF levels in H tumors and a 100-fold reduction in G tumors (Table 2). These observations are consistent with androgen stimulation of angiogenesis via regulating VEGF levels in both of these Dunning androgen-responsive rat prostate cancers.

Effect of Androgen Ablation and Restoration on VEGF Content of Rat Ventral Prostate Glands. Because androgens regulate VEGF levels in both human and rodent prostate cancers in vivo, we examined whether this is unique to cancers or whether androgens regulate VEGF levels in normal prostate tissue. To accomplish this, a series of Copenhagen rats were
castrated, and their ventral prostates were harvested on days 3, 7, and 30 after castration. In addition, testosterone (2 mg/day in propylene glycol, s.c.) was administered daily to rats castrated 30 days previously, and the prostate glands were harvested 1, 3, and 7 days after testosterone replacement. These studies demonstrated that VEGF content of the ventral prostate gland is regulated by androgens (Table 3). Interestingly, neither the reduction of prostatic VEGF after castration nor its restoration after androgen replacement is rapid (Table 3). It requires more than 3 days of androgen ablation before prostatic VEGF levels decrease significantly. This is significant because previous studies have demonstrated that in the rat, the maximal daily rate of ventral prostatic glandular cell death (i.e., ~50% of glandular cells dying/day) is reached between 2 and 3 days postcastration (11). Thus, this initial induction-programmed death of prostatic glandular cells is not the direct result of a decrease of prostatic angiogenesis. Likewise, the restoration of VEGF levels after androgen replacement requires more than 3 days (Table 3). This is again significant because previous studies have demonstrated that the maximal daily rate of prostatic glandular cell proliferation (i.e., ~50% of the glandular cells proliferating/day) occurs between days 2 and 3 of androgen replacement to castrated rats (18, 19). Thus, this initial induction of prostatic glandular cell proliferation is not simply related to enhanced angiogenesis.

**In Vitro Effect of DHT on Growth and Secretion of PSA and VEGF by LnCaP Cells.** Because androgens can regulate VEGF levels in normal and malignant prostatic tissue *in vivo*, we evaluated whether androgens regulate VEGF levels directly or require the involvement of host stromal cells using *in vitro* cell culture of the androgen-sensitive LnCaP cells. Because previous studies have reported that androgens stimulate LnCaP cell growth and PSA levels *in vitro* (20), these parameters were also measured. To accomplish this, LnCaP cells were plated, and after 48 h, the cells were washed and fed with serum-free medium containing 0–100 nM DHT. After 4 additional days, the medium was collected, and the cell number was determined. The growth effect of DHT on these cells was very minimal (Table 4). At doses above 1 nM, DHT stimulated PSA secretion, however, in a dose-dependent manner (Table 4). This suggests that these cells respond to androgens *in vitro* by up-regulating the synthesis and secretion of PSA. We previously demonstrated that LnCaP cells secrete appreciable amounts of VEGF *in vitro* (14). In the present study, this secretion was demonstrated to be sensitive to DHT stimulation in a dose-dependent manner (Table 4). Although 1–100 nM DHT stimulated PSA secretion, only 100 nM DHT was able to stimulate VEGF secretion (*P < 0.05*). This demonstrates that androgens can regulate VEGF levels in androgen-sensitive LnCaP cells, but at a higher concentration than that needed to stimulate PSA secretion.

**Levels of VEGF Protein in Prostate Versus Seminal Vesicle Fluid from Patients with Prostate Cancer and BPH.** To validate the relevance of these *in vitro* and *in vivo* model systems to the establishment and progression of human prostate cancer, we determined the amount of VEGF protein secreted by the prostate gland *versus* the seminal vesicles from patients diagnosed with prostate cancer or BPH. This comparison was performed because, unlike the prostate, seminal vesicles do not undergo either benign or malignant transformation. Expressed prostatic fluid obtained from men with BPH or prostate cancer contained large amounts of VEGF. The mean concentration was 1.26 ± 0.22 (range, 0.07–7.20 μg/ml) and 1.10 ± 0.49 μg/ml (range, 0.05–7.75 μg/ml) in men with BPH and prostate cancer, respectively. In comparison, the mean concentration of VEGF in seminal vesicle fluid (0.26 ± 0.07 μg/ml; range, 0.04–0.61 μg/ml) obtained from clinically localized prostate cancer patients was 4–5-fold lower (*P < 0.05*) than that in expressed prostatic fluid from men with BPH or prostate cancer. Consistent with these observations, Brown et al. (21) have also demonstrated that human prostatic fluid strongly expresses both VEGF mRNA and protein.

While this manuscript was being submitted, Moon et al. (22) demonstrated that after surgical or medical androgen ablation therapy, there is a profound decrease in endothelial cell proliferative index in human prostate cancers that was associated with a significant reduction in VEGF expression in the cancer tissue. These observations suggest that VEGF contributes to the establishment and progression of malignant prostatic disease and that androgen ablation can curtail the progress of the disease.
disease both by inhibiting angiogenesis via decreasing VEGF levels within these cancers and by activating programmed cell death of androgen-dependent prostate cancer cells (12). Unfortunately, androgen-dependent prostate cancer cells eventually progress to become androgen independent. These androgen-independent prostate cancer cells can be of two distinct subtypes (23). One subtype retains expression of the androgen receptor and a degree of growth stimulation by androgen, although the cells are able to grow progressively after androgen ablation (23). Thus, these cancer cells are androgen independent because they are not cured by androgen ablation, but they are still androgen-sensitive because replacement with androgen stimulates their growth (23). The Dunning R-3327 AT- and -G rat adenocarcinomas (13). These latter cells, VEGF is constitutively expressed and up-regulated not by androgens but by cellular hypoxia (14). Thus, there seems to be a coordinated loss of androgen regulation of VEGF expression associated with the progression of prostate cancer to an androgen-independent insensitive state.

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REFERENCES

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