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Androgen Withdrawal Inhibits Tumor Growth and Is Associated with Decrease in Angiogenesis and VEGF Expression in Androgen-Independent CWR22Rv1 Human Prostate Cancer Model

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Abstract. *Recent evidence suggests that androgens stimulate growth of human prostate cancer partly by regulating expression of growth factors such as vascular endothelial growth factor (VEGF) in vitro and in vivo. In this study, we used CWR22Rv1, a novel androgen-responsive but androgen-independent human prostate cancer model, to evaluate the effect of androgen withdrawal on tumor growth, expression of VEGF and the cell proliferation marker Ki-67, and angiogenesis. A time-release testosterone pellet was implanted three days before inoculation of CWR22Rv1 cells in the mice. The tumor volumes were measured every three days. Serum PSA was measured on days 1, 12, 20, 27 and 34 post inoculation. Castration was performed on the 20th day post inoculation. Immunohistochemical assays were used to evaluate cell proliferation and microvessel density. Enzyme-linked immunosorbent assay (ELISA) was used to quantify VEGF expression. The average tumor volumes in the castration group on the 27th and 34th days were 122 and 168 mm^3, respectively, compared to 156 and 210 mm^3 in the non-castration group ($p<0.01$). Serum PSA level in the castration group decreased to about 41% of the level of the non-castration group ($p<0.01$). The VEGF protein levels in the tumors of castrated and non-castrated mice on day 34 were 0.62 pg and 1.36 pg/100 μg total protein, respectively ($p<0.001$). The mean percentage of Ki-67-positive tumor cells in the castrated and non-castrated groups were 1.8% and 2.8%, respectively ($p=0.015$). The mean microvessel densities in the castrated and non-castrated groups were 15 and 22 vessels/field, respectively ($p<0.01$) We conclude androgen withdrawal reduced both VEGF and microvessel density, and this was associated with decreased cellular proliferation in androgen-independent CWR22Rv1 human prostate cancer tumor in vivo .*

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Based on the evidence that the growth of prostate cancer is androgen-dependent and that prostate tumors regress after androgen deprivation, androgen antagonists and the ablation of testicular and/or adrenal androgen sources have become mainstays of therapy for metastatic prostatic carcinoma. Though hormonal therapy provides symptomatic relief and probably a prolongation in survival(1-4), it is not curative. In time, the metastatic prostatic carcinoma becomes androgen-independent. Thus, elucidation of cellular and molecular mechanisms underlying the response of prostate cancer to androgen or androgen withdrawal is essential for the development of effective adjuvant treatment modalities for androgen-independent prostate cancer.

Angiogenesis is required for many physiological and pathological processes including solid tumor growth (5-7). The regulation of tumor angiogenesis involves the release of soluble factors produced by tumor and/or stromal cells (8). Vascular endothelial growth factor (VEGF) has been found to be an important promoter of angiogenesis in a variety of tumors (9, 10). Control of the angiogenic switch and regulation by these soluble factors is of great importance for the rational design of cancer treatment strategies.

Although angiogenesis is known to correlate with tumor growth and metastasis in prostate cancer (9, 11), the regulation of the expression of angiogenic mediators is not well understood. Recently the expression of VEGF mRNA, protein and VEGF receptor has been localized in prostate cancer cells (12-14). Furthermore, reduction of angiogenesis and VEGF expression has been observed in tissue

specimens of human prostate cancer and in animal models after androgen ablation (11, 13, 15, 16). Taken together, these results indicate that regulation of angiogenesis and VEGF expression is essential in the response of prostate cancer to androgen.

CWR22Rv1 is an androgen-responsive but androgen-independent human prostate carcinoma cell line developed from the relapsed CWR22 xenograft after castration (17). The relapsed CWR22 xenograft has been shown to mimic the androgen-independent state of prostate cancer with androgen receptor expression (18-20). In this study, we characterize the growth behavior of CWR22Rv1 cells *in vivo* and examine the effect of androgen ablation on angiogenesis and expression of VEGF and Ki-67, a cell proliferation marker in prostate cancer.

Materials and Methods

Human prostate cancer cell line CWR22Rv1 and the culture conditions. The CWR22Rv1 cell line was kindly provided by Dr. Jacobberger (17). The cells were grown in 300 cm^2 flasks in DMEM medium containing 10% fetal calf serum and 50 U/ml penicillin/streptomycin at 37˚C with 5% CO_2.

Animals and CWR22Rv1 tumor transplantation. Six- to 8-week-old BALB/c athymic nude male mice were obtained from Harlan Sprague Dawley, Inc. (Indianapolis, IN, USA). The mice were randomly divided into two different groups. A time-release testosterone pellet was implanted underneath the skin of the mice three days before inoculation of CWR22Rv1 cells. The use of the testosterone pellet eliminated the wide variation of serum testosterone levels and maintained a serum testosterone level of approximately 4.0 ng/ml. Before cell inoculation, CWR22Rv1 cells were washed 3 times with 50 ml of phosphate-buffered saline (PBS). The cells were resuspended in EMEM medium (Biofluid, Rockville, MD, USA). Trypan blue stain was used to count the cells and to check the viability of the cells. The viability of cells used in inoculation was over 90%. The cell density was adjusted with an equal volume of Matrigel (Sigma, St. Louis, MO, USA). An inoculum of $4x10^6$ cells in 0.2 ml cell mixture was injected subcutaneously into the flank regions of each mouse (21, 22). The tumors were measured every 3 days with a caliper. The tumor volume was calculated according to the literature [sagittal dimension (mm) x cross dimension (mm) x 0.52], and expressed as mm^3 (23, 24) Castration was performed on the 20th day post inoculation and the testosterone pellet was removed on the same day.

Quantitation of VEGF. VEGF levels were measured by an enzyme-linked immunosorbent assay (ELISA) (R&D System, Minneapolis, MN, USA). Briefly, the detecting antibody to VEGF was coated onto 96-wells. Tumor tissue was washed in cold PBS and was cut into small pieces. The tissue was transferred to prechilled Ependorff tubes containing 1 ml of PBS and homogenized with a PT 300 politrone (Kinematica, Switzerland) on ice. The homogenized tumor tissue was then centrifuged and the supernatants were collected.

The ELISA was done according to the manufacturer's protocol. Briefly, the standard VEGF was serially diluted into the concentrations suggested by the provider and used as antigen for the standard curve. The detecting antibody labelled with horseradish peroxidase was added to the wells and incubated for 1 hour at room temperature. Excess antibody was removed by washing. The enzyme cleaved the substrate to generate the color, which was read by a plate reader at a wavelength of 450 nm. The O.D. values were converted to the VEGF concentration by the method according to the manufacturer's protocol.

Immunohistochemistry detection of Factor VIII and Ki-67. Immunostains for Factor VIII and Ki-67 were performed on formalin-fixed paraffin-embedded tissue with the Histostain-plus kit (Zymed, San Francisco, CA, USA) according to the manufacturer's protocol. Sections were incubated with primary antibodies against Factor VIII or Ki-67 (DAKO, Carpinteria, CA, USA) at 4˚C overnight in a humid chamber. The slides were developed with 3,3-diaminobenzidine (DAB) and counterstained with hematoxylin or light green. Positive and negative controls were run in parallel for each staining procedure and were evaluated by pathologists.

Microvessel density determination. The microvessel density assay was assessed by immunostaining for Factor VIII-related antigen (DAKO). Microvessel density determination was performed as previously described (25). The stained slide was counted under a 20x power objective using an Olympus AH3 microscope (Olympus Optical, Tokyo, Japan) and any Factor VIII-related antigen-positive endothelial cell was considered to represent a single microvessel. Six fields were counted for each case and the average number per field was recorded as microvessel density. Field size was 0.50 mm^2 for the x200 field (objective 20x and ocular 10x).

Cell proliferation index. Counting of the Ki-67-stained tumor nuclei was performed under a 40x objective. At least 1000 cells were counted on each slide. The cell proliferation index was the percentage of Ki-67-positive cells in the total number of cells counted (26).

Quantitation of PSA from serum of the tumor-bearing animals. Blood samples were collected from the tail veins on days 1, 12, 20, 27 and 34 post inoculation. Serums were isolated and frozen at -80˚C. An automatic PSA assay system (Roche Elecsys System 2010, Roche, Basel, Switzerland) was used to measure the PSA concentration according to the manufacturer's protocol.

Statistics. Statistical analysis was performed by analysis of variance followed by Student's *t*-test. A p value < 0.05 was considered statistically significant.

Results

Androgen withdrawal reduced the growth of CWR22Rv1 tumor in vivo. The tumors were palpable about 2 weeks after inoculation. The dimensions of the tumors were measured every three days with a caliper and the tumor volumes were calculated as described in Materials and Methods. The average tumor volumes on the 27th and 34th day in the castration group were 122 and 168 mm^3, respectively, compared to 156 and 210 mm^3 in the non-castration group

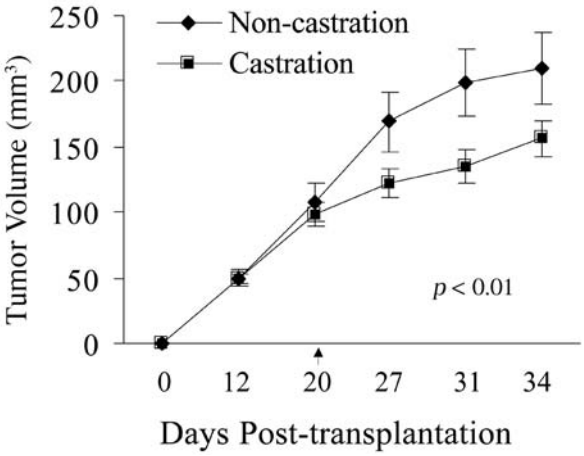


Figure 1. *In vivo tumor growth of CWR22Rv1 human prostate cancer in the castrated and non-castrated nude mice. Castrated group (■) showed a significantly slower tumor growth compared with the non-castrated mice (◆). Arrow (↑) indicated the castration date post inoculation. Error bars represent the standard error of the mean.*

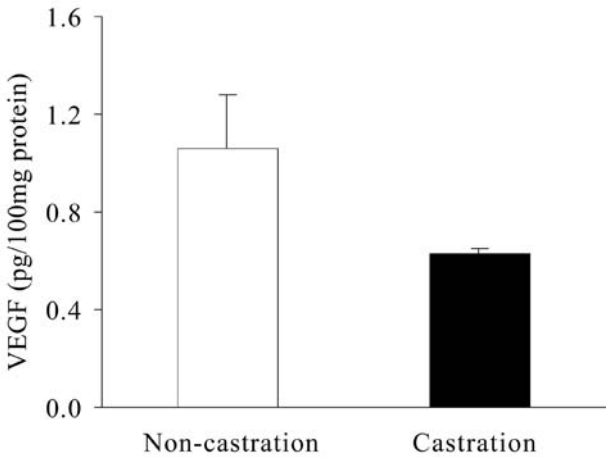


Figure 2. *The VEGF expression in CWR22Rv1 tumor. Castrated mice (■) expressed about 40% less VEGF compared with non-castrated mice (□) (0.62 vs 1.06 pg/100μg total protein). Error bars represent the standard error of the mean.*

(n=10, $p<0.01$) (Figure 1). Tumor volumes increased 57% in the non-castration group from day 20 to day 34 post inoculation, whereas tumor volume increased only 35% in the castration group in the same period.

VEGF expression in the tumors. There was a statistically significant difference in the levels of VEGF expression between the non-castration group and the castration group ($p<0.001$) (Figure 2). The average VEGF levels in the tumors of the non-castration and the castration groups were 1.36 and 0.62 pg/100μg total protein, respectively.

Quantitation of microvessel density. The sections were immunostained with antibody to Factor VIII-related antigen (Figure 3A and 3B). The positive endothelial cells within the tumor area were counted. The average microvessel density was 22 vessels/field in the non-castration group compared with 15 vessels/field in the castration group ($p<0.001$).

Cell proliferation activity in the tumor. An immunohistochemical assay of Ki-67 was used to evaluate the proliferation activity of the CWR22Rv1 tumors. Positive immunostaining of Ki-67 was detected within the tumor nuclei (Figure 3C and 3D). The mean percentage of Ki-67-positive tumor cells in the non-castration and castration groups on day 34 were 2.8% and 1.8%, respectively ($p=0.015$).

Serum PSA. The serum PSA level in the non-castrated animals showed stepwise increase and reached 23.5 ng/ml by day 34; the serum PSA concentration in the castrated animals decreased by 38% in the first week after castration and by 59% two weeks after castration (Figure 4).

Histopathology. The non-castrated tumors showed diffuse sheets of tumor cells with high nuclear cytoplasmic ratios, hyperchromatic nuclei and nuclear pleomorphism (Figure 5). Numerous mitotic figures are present. The tumor cells in the castrated group were arranged loosely in an edematous stroma. The tumor cells showed condensed nuclei and acidophilic cytoplasm. At autopsy, no metastasis was seen in either castrated or non-castrated groups.

Discusson

In this study, we examined the effects of androgen withdrawal on tumor growth, expression of VEGF and Ki-67 cell proliferation marker, and angiogenesis in the CWR22Rv1 prostate tumor model. We found that androgen withdrawal reduces tumor growth and that this is associated with reduced serum PSA secretion and decreases in expression of VEGF, percentage of cells positive for Ki-67 and microvessel density in the tumors.

Androgen ablation is a standard treatment for patients with advanced prostate cancer. Prostate cancer typically becomes androgen-independent two to three years after androgen ablation therapy. Recent data indicate that growth of androgen ablation therapy-resistant prostate cancer is still stimulated by androgen. Some reports have shown that, in the state of androgen deprivation, some androgen-regulated genes show similar or even higher expression levels compared with their expression prior to androgen deprivation (18-20). For example, prostate-specific antigen and human kallikrein-2 mRNA levels in recurrent tumors were higher than the levels in regressing tumors from castrated mice and similar to those in androgen-stimulated

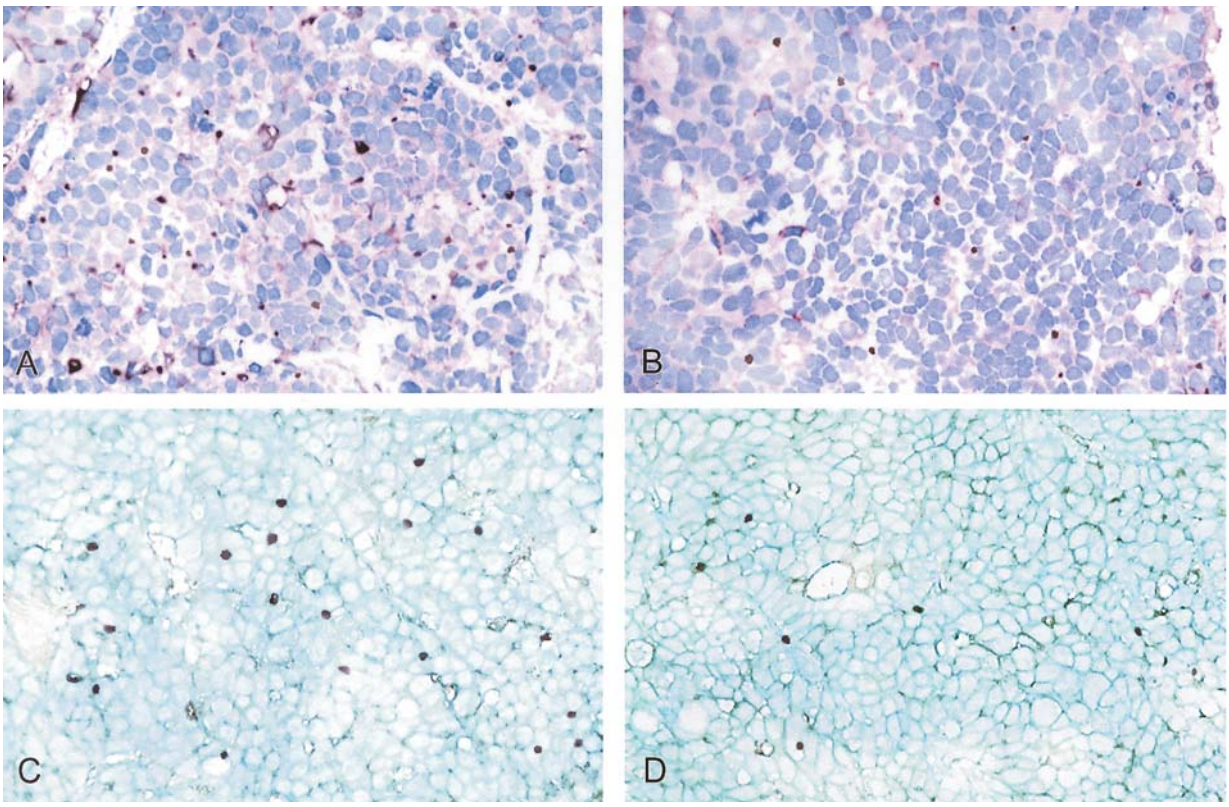
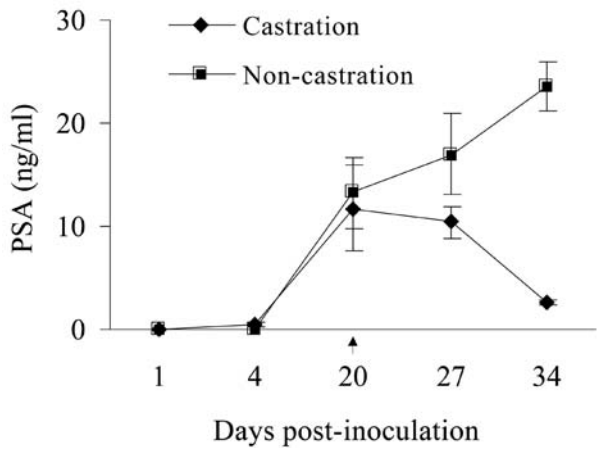


Figure 3. *Immunohistochemstry of Factor VIII related antigen (A and B) and Ki-67 cell proliferation marker (C and D). A and C: tumors without castration; B and D: tumors with castration.*



↑ Figure 4. *Serum PSA concentrations in the nude mice with or without castration. Blood samples were collected from tail veins and serum PSA concentrations were measured by an automated PSA assay system (see Materials and Methods). The chart showed the average PSA levels of each time point for the castrated mice (◆) and non-castrated mice (■). Error bars show the standard error of the mean. Arrow (↑) indicates the castration date post inoculation.*

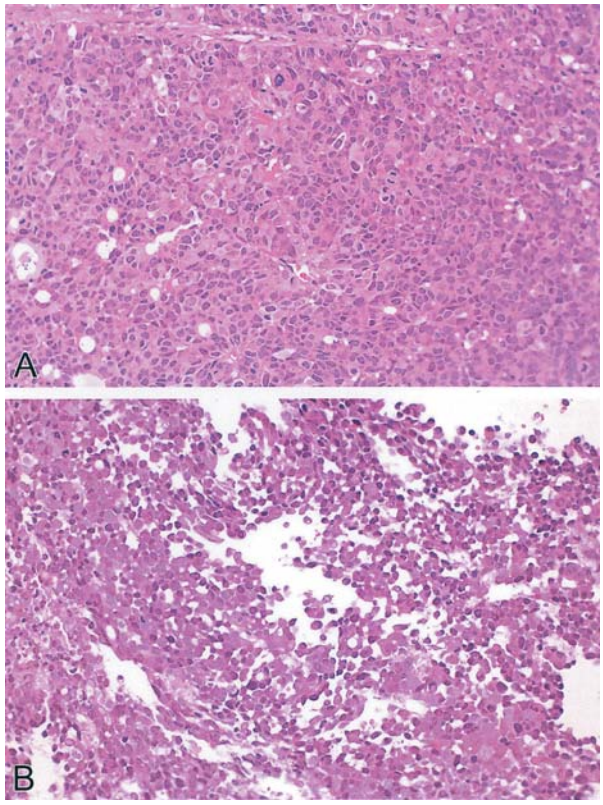


Figure 5. *Histopathology of the CWR22Rv1 tumor. The non-castrated tumor (A) showed diffuse sheets of poorly-differentiated tumor cells in active growth. Numerous mitotic figures are seen. The tumor cells from the castrated group (B) showed fewer mitotic figures. Cystic degeneration is noted. Some tumor cells showed condensed nuclei with acidophilic cytoplasm.* →

mice. In addition, after androgen deprivation, the recurrent cancer expresses higher levels of androgen receptor. These results suggest that the androgen receptors are still active in androgen-independent prostate cancer.

Neovascularization is a critical process in tumor growth and metastasis of various tumors including prostate cancer. It is important to understand the mechanisms responsible for regulating the angiogenic mediators in prostate cancer since anti-angiogenic therapy is emerging as a new anticancer treatment for prostate cancer. In this study, we examined the effects of androgen withdrawal on expression of VEGF and associated tumor growth and angiogenesis in androgen-independent prostate cancer. Our results demonstrate that androgen withdrawal inhibits the expression of VEGF and this is associated with decreases in CWR22Rv1 prostate cancer volume, microvessel density and the *in vivo* growth rate. Recent findings from studies of other animal models or patients' prostate cancer tissues demonstrate that VEGF is an important angiogenic mediator in androgen-dependent prostate cancer (9). Androgen ablation resulted in a rapid decrease in the blood vessel density in rat prostate cancer (27).

It is unlikely that VEGF is the only growth factor regulating angiogenesis and growth in androgen-independent prostate cancer. A large number of angiogenic and anti-angiogenic mediators have been found in various tumors (8). Recent reports demonstrate that other mediators such as basic fibroblast growth factor, angiopoietin-1, angiopoietin-2 and insulin-like growth factor are regulated by androgen. These factors may also be involved in angiogenesis in androgen-dependent prostate cancer (10, 19, 20, 28). Recent data indicate that androgen modulates the balance between VEGF and other angiogenic factors in prostate epithelial and smooth muscle cells (28).

The exact molecular mechanisms responsible for VEGF gene expression regulated by androgen and how VEGF is regulated in prostate cancer after androgen withdrawal remain unclear. The findings reported by others that, after androgen deprivation, androgen receptors and androgen-regulated genes are expressed at levels similar to those prior to androgen deprivation are interesting (19, 20). Taken together, these data indicate that the androgen-signaling pathway is still intact and functional in androgen-independent prostate cancers (19). In addition, these results suggest that other growth factors and cytokines may regulate tumor growth and angiogenesis through an androgen-dependent mechanism.

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