Androgen deprivation induces rapid involution and recovery of human prostate vasculature

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Androgen deprivation induces rapid involution and recovery of human prostate vasculature. Am J Physiol Endocrinol Metab 300: E263–E275, 2011. First published August 10, 2010; doi:10.1152/ajpendo.00210.2010.—The response of the prostate tissue microenvironment to androgen deprivation (AD) represents a critical component in the treatment of benign prostatic hyperplasia and prostate cancer (CaP). Primary xenografts of human benign and CaP tissue transplanted to immunocompromized SCID mice were used to characterize the response of the prostate vasculature. Leakage of vascular contents in the interstitial space was apparent between days 1 and 3 after AD; however, the vascular permeability barrier reestablished between days 7 and 14. Expression of vascular endothelial growth factor (VEGF)-A, VEGF receptor-2, and basic fibroblast growth factor protein increased in endothelial cells between days 2 and 4 after AD, which preceded vascular recovery and appeared to be a direct and specific response of the endothelial cells to AD. Lack of comparable upregulation of these genes in primary cultures of human prostate endothelial cells in response to AD suggests a role for paracrine signaling mediated through stromal or epithelial cells. VEGF-A expression by prostate endothelial cells appears to represent a key facilitator of the vascular rebound in human prostate tissue induced by removal of circulating testicular androgens.

human prostate vasculature; prostate; primary xenograft; vascular endothelial growth factor-A

THE REGULATORY FUNCTION of androgens in prostate physiology has been capitalized upon for over 60 years for the treatment of benign prostatic hyperplasia (BPH) and prostate cancer (CaP) and provides the rationale for androgen deprivation therapy (ADT) (26, 44, 51, 59). The clinical effect of ADT is thought to be mediated predominantly through the apoptotic death of androgen-sensitive luminal epithelial or CaP epithelial cells, which produces dramatic amelioration of symptomology for BPH or advanced CaP (6, 7). However, the response of BPH to ADT, and the extent and duration of the induced effect, varies by patient and by treatment modality (21, 29, 46, 47, 62), and, while ADT for CaP initially induces a response in most patients, recurrence of CaP occurs in 80% of cases within 12–18 mo of treatment (28, 30–32, 44, 52). The lack of durable remission, or cure, by ADT indicates that a greater understanding of the integrated androgen responsiveness of the multiple cellular compartments within the prostate tissue microenvironment is required to make ADT a more effective treatment for BPH or CaP. Androgen deprivation differentially affects stromal, myostromal, neuroendocrine, basal epithelial, smooth muscle, and vascular endothelial cells in the prostate tissue microenvironment, and each/all of these cellular compartments potentially contribute to the androgen-mediated regulation of prostate epithelial cells and cancer cells, and the transition to castration-recurrent CaP (9). Specifically, prostate endothelial cells play a key role in determining androgen responses in the prostate, since they are the portal for entrance of circulating androgens in the prostate tissue microenvironment, expresses AR and are androgen sensitive (20), and provide paracrine signaling and vascular support that facilitates recurrent growth during ADT. Furthermore, while endothelial cells in the microvascular network or multiple organs, and tumors of those organs, express androgen receptor (AR), the role of androgen in regulation of vascular homeostasis and angiogenesis remains unclear (16, 39, 66). Significantly, AR-expressing endothelial cells of other organs do not respond to ADT as do prostate endothelial cells, suggesting the importance of other cellular compartments of the prostate tissue microenvironment in mediation of prostate endothelial cell homeostasis (56).

In the adult rat ventral prostate, castration reduced endothelial cell numbers and endothelial cell proliferation, and both effects were normalized by testosterone treatment (13). The laboratories of Buttyan and Bergh (36, 55, 56) demonstrated independently that the first physiological effect of androgen deprivation on the rat prostate gland was a drastic reduction in blood flow. Perturbation of prostatic blood flow was evident as early as 18 h after castration and coincided with the appearance of apoptotic endothelial cells (56, 58). The reduction of blood flow was associated with the induction of an ischemic/hypoxic environment in the prostate, and hypoxia inducible factor (HIF)-1α protein levels increased 20-fold by 48 h after castration (57). By day 7 after castration, major reductions were observed in epithelial weight, stromal weight, blood vessel lumina weight, and endothelial cell number (15). Prostate epithelial cell loss was thought to result from an indirect effect of hypoxic/ischemic conditions within the prostate gland that resulted from the castration-induced endothelial cell death. The

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endothelial cells were the first cells in the rat prostate to die in response to castration but also were the first cells to proliferate after restimulation with androgen (15, 58). However, rat prostate endothelial cells were reported to lack expression of AR (50). Hence, an androgen-regulated intermediary paracrine signaling molecule synthesized by AR-expressing prostate epithelial or stromal cells was proposed to regulate survival of endothelial cells.

In humans, the acute effects of androgen deprivation on prostate vasculature have not been characterized. However, inhibition of androgenic signaling by AR antagonists (e.g., flutamide or bicalutamide) and steroid metabolism inhibitors (e.g., finasteride or dutasteride) was demonstrated to inhibit hematuria due to BPH or after prostate surgery (12, 21, 40, 46, 47, 62). A recent study showed that 6 wk of dutasteride treatment (inhibitor of 5-a-reductase isoenzymes types 1 and 2) reduced prostate tissue vascularity in the periurethral area in patients (33). We provided a mechanistic explanation for vascular involution by demonstration of AR expression and functionality in primary cultures of human prostate endothelial cells (HPEC) established from both benign prostate tissue and CaP tissue. The presence of AR in endothelial cells suggested a direct role of androgen in regulation of HPEC biology and proliferation in normal and pathological conditions (20).

Our laboratory developed a preclinical model of primary xenografts of fresh surgical specimens of human prostate tissue transplanted to immunocompromized mouse hosts that had been preimplanted with sustained-release testosterone pellets to provide human serum levels of circulating androgen (22, 49). This model recapitulates the tissue architecture and androgen-regulated homeostasis of the intact human prostate, with the majority of the vasculature in the primary xenografts of human prostate tissue being of human origin (22). Therefore, the model provides a unique opportunity for characterization of the acute effect of androgen deprivation on human prostate vasculature in an intact human prostate tissue microenvironment.

MATERIALS AND METHODS

Human prostate primary xenografts. Prostate tissue was collected in accordance with National Institutes of Health guidelines on the use of human subjects, with approval by the Institutional Review Boards at The University of North Carolina at Chapel Hill (UNC) and Roswell Park Cancer Institute (RPCI). Human prostate tissue samples were obtained by radical prostatectomy from at least eight different patients. Prostate tissue was dissected out by a pathologist and designated as benign (noninvolved) and tumor tissue using a recently reported protocol (45). The majority of these patients were adult men over 55 years old. Gleason grade in these patients varied between 3+3 to 4+3. None of the patients had undergone treatment of BPH or hormonal therapy before surgery. Tissue specimens were submerged immediately in ice-cold ViaSpan solution (Burr Laboratories, Pomona, NY) and transported on ice for transplantation. An initial tissue (IT) specimen of at least 8 mm³ was removed from each surgical tissue sample before transplantation, fixed in 10% formalin, and paraffin-embedded for histological confirmation of the tissue as benign or malignant.

Primary xenografts of freshly harvested human prostate tissues were established in SCID mice as described previously (22, 49). All experimental protocols that involved laboratory animals were performed in accordance with the National Institutes of Health guidelines and were approved by the Institutional Animal Care and Use Committee at UNC and RPCI. In brief, the tissue specimen was cut into wedge-shaped pieces 2–3 mm in length and 1–2 mm in width at the broadest end, and the wedges were transplanted into male SCID mice, 3 mo of age, that previously had been castrated and implanted subcutaneously with a 12.5-mg sustained-release testosterone pellet (Innovative Research of America, Sarasota, FL) to maintain serum testosterone levels at ~4.0 ng/ml throughout the study. For implantation of prostate tissue, small (~3-mm) incisions were made in the skin on the right and left flanks of immunocompromized mouse hosts anesthetized with Domitor (1 mg/kg ip) (Pfizer, New York, NY), tissue wedges to be implanted were dipped in Matrigel (BD Biosciences, Bedford, MA), and the coated tissue wedges were inserted in the subcutaneous space through a 10-gauge trocar device (Popper & Sons, Lincoln, RI). Between 3 and 5 wedges from each patient were implanted along each flank through individual incisions, with up to 10 fragments transplanted per animal. Incision sites were closed with Nexband tissue glue (Veterinary Products Laboratories, Phoenix, AZ).

ADT was initiated by removal of the testosterone pellet 1 mo after transplantation of prostate tissue. Xenografts from control hosts (day 0) were harvested from animals killed before removal of the testosterone pellet. For analysis of the effects of androgen deprivation, animals were killed, and xenograft tissues were collected on days 1, 2, 4, 7, 10, and 14 after androgen deprivation. Harvested xenografts were fixed in 10% formalin for a minimum of 24 h, after which the fixed tissues were paraffin-embedded. Paraffin blocks were sectioned (5.0 μm) onto ProbeOn Plus slides (Fisher Scientific, International, Suwanee, GA).

Prostate tissue digestion and primary cell culture. Primary cultures of HPEC were prepared using a Dynalbead-based methodology (DYNAL Biotech ASA, Oslo, Norway) as described previously (20, 61). Fresh surgical specimens of prostate tissue were minced into 8-mm³ pieces and digested with dispase (2.4 U/ml; Invitrogen, Carlsbad, CA) for 16 h at 4°C. The heterogeneous tissue hydrolyzate was cultured for ~1 wk in endothelial growth media (Endothelial cell growth medium MV; Promocell, Heidelberg, Germany) supplemented with 5% FCS at 37°C in 5% CO₂. After 1 wk in culture, HPEC were isolated using anti-CD31-conjugated magnetic beads and cultured in endothelial growth media for no more than six passages before experimental studies were performed.

Immunostaining analysis. Histological specimens were prepared from each IT specimen harvested from the tissue specimen before implantation, and from all corresponding xenograft specimens, and one section from each block was stained with AMACR (racemase) to assess the presence of CaP. Complete specimen sets were available for eight patients that contained IT specimens and xenograft specimens of matched benign and CaP tissue harvested at all the Short Time Points After Castration (STP-ACX) (days 1, 2, 4, and 7). Each specimen set included an AMACR-stained IT specimen and AMACR-stained specimen from xenografts harvested at all time points. Individual sections from each specimen were characterized using immunohistochemistry (IHC) to establish the pattern of expression of an apoptotic marker (cleaved caspase-3), endothelial cell markers (CD31 and CD34), and angiogenesis-related proteins. Species specificity of the anti-human CD31 antibody was confirmed by IHC using formalin-fixed, paraffin-embedded human or mouse prostate tissue [Supplemental Fig. 1 (Supplemental data for this article may be found on the American Journal of Physiology: Endocrinology and Metabolism website)].

Tissue sections were deparaffinized using CitraLyse (Fisher), hydrated through graded washes of ethanol and deionized water, and equilibrated in automation buffer (Biogenex, San Ramon, CA). Antigens were retrieved by boiling the slides in 10 mM citric acid buffer (pH 6.0). Endogenous peroxidase activity and background avidin-biotin reactivity were blocked according to the manufacturer’s protocol (Vector Laboratories, Burlingame, CA) before quenching of nonspecific antibody binding with Power Block (Biogenex, Jackson, WY). Blocked sections were incubated with antigen-specific primary antibodies at optimized dilutions in common antibody diluent (Bio-
genex) for 30 min at 37°C, followed by incubation for 20 min at 37°C with a biotinylated secondary antibody matched to the host species of the primary antibodies. Immunoreactive target antigens were visualized using a Vectastain Elite ABC Immunoperoxidase Kit (Vector) with DAB (Vector or Sigma, St. Louis, MO), NovaRed (Vector), Histomark Black (KPL, Gaithersburg, MD), or TrueBlue (KPL) peroxidase chromogen kits according to each manufacturer’s protocol. Slides were counterstained with methyl green (Sigma) to identify nuclei, dehydrated with ethanol, and mounted with Vectamount (Vector). Sections of BPH tissue were included in all staining procedures as a positive control, and primary antibody was omitted as a negative control.

Analysis of expression of angiogenic factors. STD-ACX human prostate primary xenograft specimens were immunostained for expression of selected angiogenesis-related factors [vascular endothelial growth factor (VEGF)-A, basic fibroblast growth factor (bFGF), platelet-derived growth factor (PDGF)-AA, PDGF-BB, and ANG-1], their cognate receptors [VEGF receptor (VEGFR)-2, FGF receptor (FGFR)-1, PDGF receptor (PDGFR)-α, and Tie-2], and HIF-1α (Supplementary Table 1). Expression of the individual proteins in the epithelial, endothelial, and stromal compartments was scored independently, with the levels of expression evaluated manually in blinded specimens. The scoring system was based upon the intensity of staining: 0 (no stain), 1 (weak stain), 2 (moderate stain), and 3 (strong stain). The assigned score represented the level of expression in the majority of the cells in each prostate cellular compartment. Levels of expression were scored at each time point after androgen deprivation for multiple microscopic fields from each xenograft for each patient; and scores were averaged across all samples from all patients at each time point. Two investigators, blinded to the origin of the specimen, scored all slides independently, and the scores were averaged for a composite staining intensity. Interobserver scoring of the levels of protein expression demonstrated significant concordance (r = 0.90).

Reverse transcription, PCR, and quantitative real-time PCR. Total RNA from primary cultures of HPEC was prepared using the RNAeasy mini-kit (Qiagen, Valencia, CA). Reverse transcription from mRNA was performed using the SuperScript III First-Strand kit (Invitrogen) (19). Approximately 1.0 μl of the reverse-transcribed cDNA product was used as template in the Platinum PCR Supermix (Invitrogen) reaction mixture that contained 200 nM of each primer. PCR products were separated by electrophoresis in 2% agarose gels and visualized with ethidium bromide. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a loading control in analytical gels. Quantitative real-time PCR was performed using SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA) according to the manufacturer’s instructions in the ABI PRISM 7300 system. For each sample, triplicates of 25-μl reactions were prepared using 2X SYBR Green PCR Master Mix (Applied Biosystems) along with 1.5 μl of cDNA and 200 nM of each primer. Primer sequences are as follows: VEGF-A, forward 5'-AGAAGGAGGAGGCGCCA-GAAC-3' and reverse 5'-GATCTCACATTGTTGCTG-3'; VEGF2, forward 5'-GATCTTCACTCTGTTTACG-3' and reverse 5'-CTTTACATTCCCTTTATCC-3'; bFGF, forward 5'-CGTTACCCTCGCTATAGAGGA-3' and reverse 5'-TTTCTGCCAGGTCCTGTGTT-3'; AR, forward 5'-TACCAGCTCCACAAGCTCT-3' and reverse 5'-GCTTTGCTGCTGCTGTTTAC-3'; and GAPDH, forward 5'-ACCAACATGCTGCTGCTGCTGCT-3' and reverse 5'-TCACTGAGGAGGAGGAGGAG-3'. The reactions were performed in 96-well ABI Prism (Applied Biosystems) optical reaction plates capped with optical caps. PCR product levels were measured continuously using the ABI7300 system during 40 cycles.

Digital image collection and analysis. Digital images of sections immunohistochemically stained for cleaved caspase-3 and angiogenesis-related proteins were collected using a Kontron ProgRes 3012 camera (TriPath Imaging, Burlington, NC) mounted on an Axioskop microscope (Carl Zeiss, Thornwood, NY) at ×100, ×200, ×400, and ×630 magnification, depending on the intended use of the images. Image analysis for manual counts of objects was performed using Imaged software with the Cell Counter Plug-In (Research Services Branch, National Institute of Mental Health, Bethesda, MD). Three ×100 (0.480 mm²), ×200 (0.120 mm²), or ×400 (0.030 mm²) fields were collected and analyzed. Investigators were blinded to the origin of the specimen for all manual scoring of histological specimens. Digital images of immunohistochemically stained sections for the endothelial cell markers CD31/CD34 were collected using a Hamamatsu Color Chilled 3CCD camera (Hamamatsu, Bridgewater, NJ) mounted on an Axioskop microscope (Carl Zeiss) at a total magnification of ×400. Five images (0.4 mm²) were analyzed to determine the average number of vessels per field (microvessel density, MVD).

Analyses of vascular integrity. Measurements of vessel lumen diameter were made using Image Pro Plus 5.0 (Media Cybernetics, Bethesda, MD). Lumen diameter was measured by drawing a line from the abluminal side of one end of the immunostained CD31/CD34 vessels to the opposite abluminal side. Lumen diameter at each time point after androgen deprivation (day 1, day 2, day 4, day 7, day 10, and day 14) was compared with the control lumen diameter (day 0) measurement using Student’s t-tests. Vascular leakage after androgen deprivation was evaluated in primary xenografts using intravital binding of lectin administered systemically, as described previously (42). Briefly, mice bearing xenografts were anesthetized with ketamine (100 mg/kg ip) plus xylazine (10 mg/kg ip). Biotin-labeled Lycopersicon esculentum lectin (100 μl of 1.0 mg/ml in 0.9% NaCl; Vector Laboratories) was injected in the tail vein and allowed to circulate for 5 min. At the conclusion of the incubation, the chest was opened, and the vasculature was perfused with a fixative solution (1.0% paraformaldehyde in PBS, pH 7.4) through an 18-gauge cannula inserted in the aorta via an incision in the left ventricle for 2 min at a pressure of 120 mmHg. Blood and fixative exited through an opening cut in the right atrium. Fixed xenograft tissues were removed, frozen in optimum cutting temperature compound, and processed for immunohistochemistry. Anti-biotin and anti-fibrinogen (fragments D and E) antibodies were used to evaluate vascular permeability using standard immunohistochemical procedures, as described above.

VEGF-A measurement. The measurement of VEGF-A protein production and secretion was carried out using enzyme-linked immunosorbent assay (ELISA) (R&D Systems, Minneapolis, MN) with affinity-purify antibodies directed against VEGF-A. HPEC were seeded in 24-well plates and grown to confluence using endothelial cell medium (Endothelial cell basal medium supplemented with 5% FBS; Promocell). After plating (24 h), androgen deprivation was initiated by transfer to endothelial cell medium supplemented with charcoal-stripped 5% FBS. Measurement of VEGF-A production and secretion by HPEC was performed as follows: a 1.0% aliquot was taken from the supernatant of cell cultures at time 0 (control) and at 24 and 48 h after androgen deprivation. As a functional control, cultures also were exposed to 48 h of androgen deprivation and were refed with endothelial cell media with charcoal-stripped 5% FBS supplemented with 1 nM dihydrotestosterone (DHT) for an additional 24-h incubation.

Analysis of serum testosterone levels. Serum testosterone levels in host mice were measured using a testosterone ELISA (Immunobio- logical Laboratories, Minneapolis, MN) according to the manufacturer’s instructions. Briefly, serum was collected from blood obtained by eye puncture, and 25 μl of each serum sample was combined with 200 μl of antibody-conjugate and incubated for 60 min at room temperature. The samples were read at 450 ± 10 nm with a microtiter reader (Bio-Tek Instrument EL800). Testosterone concentrations were determined using a standard curve.

Statistical analysis. Data sets were prepared for statistical evaluation by analyzing the reason for missing values and applying appropriate standard data adjustments to provide values in place of blanks.
Temporal analyses of the effect of androgen deprivation on xenografts from a single patient used Student’s *t*-tests and Wilcoxon Signed Rank tests. Additionally, descriptive statistical analysis, including median, mean, count, standard deviation, and normality testing (Shapiro-Wilk and Kolmogorov-Smirnov with Dallal-Wilkinson-Lilliefors P value), were calculated to confirm or reject the assumption of normality for mean comparison tests (Student’s *t*-test or Wilcoxon Signed Rank test). Both power analysis and sample size estimation were performed to design this study.

Analyses of data sets from castrate hosts in studies with five time points per patient (day 0, and days 1, 2, 4, and 7 after castration) used a paired two-tailed Student’s *t*-test or a Wilcoxon Signed Rank test (95% confidence) between the 10 possible comparison pairs (day 0 to day 1, day 0 to day 2, day 0 to day 4, day 0 to day 7, day 1 to day 2, day 1 to day 4, day 1 to day 7, day 2 to day 4, day 2 to day 7, and day 4 to day 7). Each pairwise test was performed at 95% confidence, and each familywise test was performed at 99.5% confidence.

Analysis of the expression of HIF-1α and angiogenesis-related factors based upon the intensity scores was performed using a Mack-Wolfe Unimodal Umbrella Test with Unknown Peak, adapted for two-sided analysis of *P* values (25). The described critical values were used to interpret *P* values between 0.02 and 0.2 (25).

Descriptive statistics, normality testing, Student’s paired *t*-tests, Signed Rank tests, and data correlations were performed using Prism 4 (GraphPad Software, San Diego, CA). Power and sample size analysis was performed using StatMate 2 (GraphPad). Standard error of the mean calculations was performed with Microsoft Excel 2003 (Microsoft, Redmond, WA). All data, with the exception of the staining intensity scores, were graphed ± SE. Additional information concerning the application of missing value substitution, the testing of temporal data, and the results of temporal data *t*-tests, Wilcoxon Signed Rank tests, and Mack-Wolfe Unimodal Umbrella testing is provided in the Supplementary Data (Supplemental Tables 2 and 3).

**RESULTS**

Androgen deprivation induces acute vascular damage in human prostate xenografts. The initial goal of this study was to determine if androgen deprivation (castration of the host and removal of the supplementary testosterone pellet) would induce an acute response in the AR-expressing human prostate microvascular endothelial cells in primary xenografts of human prostate tissue, as was demonstrated in rat prostate in which the endothelial cells are AR-negative (36, 56). Confocal laser scanning microscopy imaging was performed on CD31 immunostained thick sections (100 μm) obtained from primary xenografts of prostate tissue harvested on days 0 (control), 1, and 2 after androgen deprivation (Fig. 1, Ai-iii). Three-dimensional reconstruction of the fluorescent staining in serial optical sections was achieved using Imaris software (Bitplane, St. Paul, MN) (Fig. 1, Aiv-vi). Marked perturbation of the prostatic vasculature was apparent on day 2 after androgen deprivation (Fig. 1A). Alteration of the structure of the prostate vasculature correlated with leakage of fibrin/fibrinogen in the interstitial space.

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Fig. 1. Acute vascular effect of androgen deprivation (AD) in short-term primary xenografts of human prostate tissue. A: confocal laser scanning micrographs of blood vessels in primary xenografts of prostate tissue visualized using CD31 immunolabeling on day 0 (control, i), and days 1 (ii) and 2 (iii) after androgen deprivation, and 3-dimensional (3D) reconstruction (iv-vi) of the fluorescent immunostaining in serial optical sections (i-iii). Bar: 50 μm. B: the time of maximal alteration of vascular morphology [day 2 (d2)] was concurrent with the time of maximal level of fibrinogen leakage (fragments D and E) in the interstitial tissue space. Bar: 50 μm. C: vessel apoptotic index indicates the percentage of vessels/field that contained at least one endothelial cell nucleus positive for activated caspase-3 in human prostate primary xenografts on day 0 (C, control) and days 1, 2, 4, and 7 after androgen deprivation. ++ Significant compared with control, P < 0.005. *Significant compared with day 2, P < 0.005. **Significant compared with day 2, P < 0.005.
tissue space visualized by IHC, which suggested compromise of the endothelial cell vascular barrier and induction of vascular leakage (Fig. 1B). To confirm that vascular damage induced by androgen deprivation was a direct result of perturbation of endothelial cells, endothelial cell apoptosis was analyzed by quantitation of vessels containing caspase-3-positive human endothelial cells vs. the total number of vessels (as determined by a combination of CD31 and CD34 immunostaining). The vessel apoptotic index (VeAI) demonstrated a significant trend that was the inverse of the changes observed in MVD, with a nearly twofold increase in VeAI between day 0 to day 2, followed by a significant decrease from day 2 to day 4, and from day 2 to day 7 (Fig. 1C and Supplemental Table 2). Taken together, these results confirmed reports in rat prostate that androgen deprivation induced rapid vascular damage associated with apoptotic death of endothelial cells. However, the decrease of the VeAI from day 2 to day 7 suggested that vascular damage induced by androgen deprivation was transient, and possibly was followed by vascular recovery.

Androgen deprivation-induced prostate vascular damage is followed by a complete recovery of the vasculature. Normalization of the prostate vasculature within the rat prostate after androgen deprivation required introduction of exogenous androgen in castrated animals. The ability of the human prostate vasculature damaged by androgen deprivation to recover in the absence of androgen was analyzed to determine if revascularization required androgen stimulation, as in rat prostate. Rodents are an ideal model to analyze the effect of androgen deprivation, since, unlike humans, they produce very low levels of circulating adrenal androgen that can become an alternative source of DHT after removal of testicular androgen by castration (64). An extended morphological and immunohistochemical analysis of the effect of castration on MVD was conducted in primary xenografts of human prostate tissue at day 0 and days 1, 2, 4, 7, 10, and 14 after androgen deprivation. Apoptosis in the prostate epithelial compartment increased progressively after androgen deprivation, peaking at day 7. During this interval, hematoxylin and eosin staining demonstrated that tissue architecture of the human prostate glandular and stromal compartments was maintained unaffected in xenografts throughout the 14 days of the experiment (Fig. 2A). The MVD in prostate xenografts fell to a nadir on day 2 after androgen deprivation; however, the MVD began to recover on days 4–5 after androgen deprivation and continued to increase through days 10–14 after androgen deprivation (Fig. 2A and B). The effect of androgen deprivation on prostate endothelial cells was characterized by quantitation of vessels labeled by CD31 or CD34 individually (Fig. 2A) and by total MVD (CD31 + CD34; Fig. 2B) in primary xenografts of human prostate tissue. Pairwise analysis of MVD between time points failed to demonstrate statistical significance; however, the data suggested a nadir of MVD occurred between days 1 and 2 after androgen deprivation and a rebound by days 5–7 to nearly the precastration MVD. Furthermore, MVD increased beyond precastration levels, and by day 14 attained levels almost two times the level present on day 0 (Fig. 2, A and B). In contrast, while pairwise analysis of the epithelial cell apoptotic index failed to demonstrate statistical significance, the data suggest a progressive increase in epithelial cell apoptosis from day 0 to day 7 (Supplemental Table 2). Consequently, recovery of the microvasculature was established before the time apoptotic death of the epithelial cells reached maximal levels. Quantitation of circulating testosterone levels on day 0, and days 1, 2, 4, 7, 10, and 14 after removal of the testosterone pellet, confirmed that castration and removal of the supplemental testosterone pellet induced a state of permanent androgen deprivation (Fig. 2C). ELISA analysis demonstrated a rapid reduction of circulating testosterone to 0.01 nM, and that this level was maintained throughout the 14 days of the experiment.

To characterize further the effect of androgen deprivation on human prostate vascular integrity, changes in the diameter of vascular lumens and the leakage of serum constituents in the interstitial tissue space were evaluated in the human prostate xenograft model after androgen deprivation. Consistent with the reports in rat prostate (56), androgen deprivation was associated with a rapid and significant decrease (P < 0.05) in lumen diameter of vessels, with the maximal decrease in vascular lumen diameter observed on days 2–4 after androgen deprivation (Fig. 3A). However, vascular diameter started to increase by days 4–5 after androgen deprivation and reached precastration levels between days 10 and 14 after androgen deprivation. The androgen deprivation-induced perturbation of vascular integrity was correlated temporally with leakage of serum proteins in the interstitial tissue space. The presence of fibrin/fibrinogen in the interstitial tissue space (Fig. 1B), and the leakage of systemically administered lectin through the compromised vasculature, peaked on day 1 after androgen deprivation (Fig. 3B), started to decrease on day 3 after androgen deprivation, and ceased between days 7 and 14 after androgen deprivation (Fig. 3B).

Increased VEGF-A expression in HPEC precedes vascular recovery after androgen deprivation. The rapid involution of human vascular endothelial cells in the prostate xenografts in response to androgen deprivation appeared to result from a loss of AR-mediated vascular signaling within vascular endothelial cells. However, the source of signaling that drove the rapid recovery of the vascular endothelial cell compartment in the absence of androgen signaling following androgen deprivation was unknown. The signals that induced endothelial cell proliferation could be autocrine, of endothelial cell origin, or be paracrine, originating from prostate stromal and/or epithelial cells within the tissue microenvironment.

A semiquantitative, IHC-based analysis of expression of signaling proteins and growth factors associated with vascular stabilization or angiogenesis was developed to characterize changes induced by androgen deprivation in the endothelial, epithelial, and stromal cell compartments of the prostate xenografts. Immunohistochemical staining protocols were optimized for detection and localization of HIF-1α, VEGF-A, VEGFR-2, bFGF, FGFR-1, PDGF-AA, PDGF-BB, PDGFR-α, ANG-1, and Tie-2 protein. Several distinct temporal patterns of change in protein levels for genes associated with vascular homeostasis and angiogenesis were observed in response to androgen deprivation. HIF-1α was expressed at comparable levels in all three cellular compartments in prostate xenografts, and temporal changes in the protein levels after androgen deprivation were not observed (Table 1, Fig. 4A, and Supplemental Table 3). In contrast, VEGF-A, VEGFR-2, and bFGF protein levels demonstrated distinct temporal differences after androgen deprivation (Table 1, Fig. 4, A and B, and Supplemental Table 3). Endothelial cell upregulation of expression of the angiogenesis-related proteins VEGF-A, and one of its
cognate receptors, VEGFR-2, was the most marked response of the prostate microenvironment to androgen deprivation. Expression of both VEGF-A and VEGFR-2 in endothelial cells peaked on day 2 after androgen deprivation and returned to baseline levels on day 7 (Table 1, Fig. 4, A and B, and Supplemental Table 3). The trend for the upregulation of VEGF-A and VEGFR-2 was significant, with a 99% confidence level. A similar temporal pattern for VEGF-A expres-
sion, with a peak on day 2, was observed in the stromal compartment; however, the magnitude of the upregulation was smaller than that observed in the endothelial cell compartment. Confirmation of VEGF-A expression in HPEC in primary xenografts was demonstrated by colocalization of VEGF-A and CD31 protein (Fig. 4Biv). In contrast to VEGF-A, bFGF protein levels increased from day 0 to day 2, and levels remained elevated through day 7 (Table 1, Fig. 4A, and Supplemental Table 3). The trend observed for bFGF expression was significant at a 90% confidence level. In contrast, expression of VEGF-A, VEGFR-2, and bFGF in the epithelial compartments did not demonstrate temporal trends. FGFR-1 was expressed in all three cellular compartments in prostate xenografts but demonstrated little temporal difference in expression in endothelium and epithelium. However, a significant temporal trend was observed in stromal cells, where staining intensity decreased after day 2 (Supplemental Table 3). In contrast, androgen deprivation did not induce significant temporal differences in expression of PDGF-AA, PDGF-BB, PDGFR-α, ANG-I, and Tie-2 in any of the prostate cellular compartments (Supplemental Table 3). Taken together, these results demonstrate that androgen deprivation induced a temporally limited increase in the protein levels of VEGF-A, VEGFR-2, and bFGF in HPEC, which suggests an autocrine mechanism(s) for vascular stabilization and regrowth after removal of testicular androgen.

Androgen deprivation failed to induce VEGF-A expression in primary cultures of HPEC. The presence of functional AR in HPEC in vivo and in vitro (20), and the rapid upregulation of VEGF-A in response to androgen deprivation, suggested AR could have a direct regulatory effect on VEGF-A expression in HPEC. To investigate if AR negatively regulated VEGF-A expression in prostate endothelial cells, primary cultures of HPEC established from fresh surgical specimens of human prostate using a Dynabead-based methodology (20, 61) were used to evaluate in vitro whether removal of testosterone stimulated upregulated VEGF-A expression. HPEC in primary culture showed endothelial cell morphology, functionality, and marker expression profiles comparable to human umbilical vein endothelial cells (20). Initially, HPEC in primary culture demonstrated an elongated appearance, which after 1 wk in culture changed to a more typical cobblestone morphology of flattened cells as cultures became confluent (Fig. 5A). Immunocytochemical analyses using a specific antibody against von Willebrand Factor confirmed the purity of primary endothelial cell cultures, with >95% of the cells expressing von Willebrand Factor (Fig. 5B) with a granulated or punctate cytoplasmic pattern of localization, consistent with specific inclusion in Weibel-Palade bodies, a characteristic of endothelial cells (67).

Primary cultures of HPEC were subjected to androgen deprivation for 24 and 48 h, and to 24 h of androgen deprivation (charcoal-stripped FBS) followed by 24 h of replenishment of androgen (charcoal-stripped FBS supplemented with 1 nM DHT) to confirm that the increased VEGF-A expression in HPEC in response to androgen deprivation was a direct effect. Androgen deprivation induced a slight increase in VEGF-A mRNA expression in primary cultures of HPEC (Fig. 5C). However, no effect was observed in VEGFR-2 or bFGF...
Table 1. Immunohistochemistry analysis of angiogenesis factor and growth factor protein levels in the three prostate cellular compartments in primary prostate tissue xenografts

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mRNA (Fig. 5C). AR mRNA was slightly decreased after androgen deprivation, which confirmed the presence of AR in these cells and that androgen regulated AR expression mostly at the protein level through increasing AR protein stabilization. Real-time PCR was performed to validate the upregulation in expression of VEGF-A mRNA induced by androgen deprivation. No changes in the levels of expression of VEGF-A, VEGFR-2, or bFGF mRNAs were apparent when normalized to GAPDH mRNA level (Fig. 5D). VEGF-A protein levels in HPEC were analyzed using ELISA to confirm that expression of VEGF-A was not affected in primary cultures of HPEC by androgen deprivation. No significant differences in the level of VEGF-A protein expression/secretion were apparent after androgen deprivation (Fig. 5E).

DISCUSSION

Short-term primary xenografts of human prostate tissue provide a unique model for characterization of the response of the individual cellular compartments of human prostate to androgen deprivation because of the ability to study xenografts from one individual across multiple time points, or to simultaneously study xenografts from multiple patients transplanted to a single host. Understanding the androgen dependence of the cell-cell interactions within an intact prostate microenvironment, and the consequences of androgen deprivation on these interactions, is essential for modeling benign and malignant prostatic disease, particularly the progression to castration-recurrent CaP.

The temporal pattern for induction of endothelial cell apoptosis and vascular damage in human prostate tissue in primary xenografts correlated closely with the pattern of castration-induced apoptosis of endothelial cells in the rat ventral prostate in vivo reported by Shabsigh et al. (55, 56). However, the baseline level of apoptosis in human prostate xenografts was greater than in the rat prostate. The reduction in MVD and significant reduction in microvascular lumen diameter observed during the first 2 days of androgen deprivation in our study suggested acausal relationship could be a factor in reduction of vascular flow, as reported by Kevrick et al. (33). Consequently, rat and human prostate tissues may share common responses to androgen deprivation that could result in tissue hypoxia and cell death (5, 24, 33, 55–58). However, in the reports of Lekas et al. (36) and Shabsigh et al. (55, 56) that described the effects of castration on rat prostate vasculature, rat prostate endothelial cells were reported to lack expression of AR (50). Consequently, the vascular effects in rat prostate elicited by androgen deprivation were proposed to result from loss of androgen-regulated paracrine signaling from the tissue microenvironment (14, 27). This hypothesis was supported by the demonstration that castration-induced involution of tumor vessels in the androgen-dependent Shionogi carcinoma was concurrent with decreased expression of VEGF in tumor epithelial cells (27). However, extrapolation of observations with a mouse mammary tumor cell line, vascularized by host vessels, to intact human prostate tissue in primary xenografts is of unknown predictive value. In humans, AR expression in endothelial cells has been observed in several tissues, including skin (4, 38), salivary gland (35), bone (1), bone marrow (43), corpus cavernosum from the penis (53), and skeletal muscle (60). Recently, we reported expression of functional AR in HPEC in vivo and in vitro and demonstrated that AR modulated prostate endothelial cell proliferation in vitro and directed transactivation of gene expression in vivo (20). Consequently, a major component of the response of HPEC to androgen deprivation could represent a direct effect of the loss of AR-mediated signaling within human endothelial cells, and could represent a plausible mechanism for the acute vascular involution. A second major difference between the primary xenografts of intact human prostate tissue and the rat model is the return of the MVD and VeAI to preandrogen deprivation levels by days 5–7 after androgen deprivation, and to even higher MVDs on days 10–14 after androgen deprivation, without introduction of exogenous androgen. The rapid revascularization of the human prostate xenografts suggests an innate response that reverses the anti-vascular effect of castration, rapidly switching the androgen-deprived prostate from an anti-angiogenic to a proangiogenic microenvironment.

Androgen deprivation reduces clinical symptoms for the majority of patients with advanced CaP; however, most cancers recur within two years as castration-recurrent CaP, the
lethal phenotype. The mechanisms involved in the transition from androgen-stimulated to castration-recurrent CaP are not understood, and the contribution of angiogenesis to the development and maintenance of CaP and the transition to castration-recurrent CaP (37, 41) and the importance of increased MVD as a mechanistic marker of progression to the castration-recurrent phenotype are unknown. Gustavsson et al. (23) demonstrated that tumors of the castration-recurrent subline (LNCaP-19) had abnormal vessel morphology and increased MVD compared with the androgen-stimulated human CaP cell line (LNCaP) (23). Similar differences between LNCaP and PC3 tumors were described by Wilson and Sinha (68). However, no studies have investigated the in vivo effect of androgen deprivation on human prostate vasculature or the time frame required for changes in the tumor microvasculature during CaP progression. Therefore, no data are available to suggest whether the increase in tumor MVD occurs as an early/acute event, occurs continuously throughout progression, or is a late event associated with the transition to the castration-recurrent phenotype. Therefore, it is unknown whether angiogenesis represents a cause or consequence of this process.

Our study observed androgen deprivation induced an increase in MVD within the initial 2 wk after androgen deprivation in the primary xenografts, and that the increased MVD was maintained for at least 30 days after androgen deprivation. However, the time frame of the studies was not of sufficient duration to determine whether the increased number of vessels was pruned back to a level comparable to the initial MVD, as is characteristic of the angiogenic response in wound healing (63). The rapid revascularization response was observed in xenografts of both benign prostate and CaP tissue specimens but was not observed in xenografts of human renal cell carcinoma (data not shown), which suggests the responses observed in prostate may be innate and peculiar to prostate tissue. Our observation is consistent with the report of Benjamin et al. (3) that the endothelial cells of the benign human prostate demonstrated among the highest proliferation rates of any vascular bed in the human, suggesting high levels of remodeling. In accordance with previous studies, our results showed that the neovasculature that developed in the postandrogen deprivation prostate was characterized by thinner vessels with tortuous morphology, which suggests these new vessels probably are unstable and more permeable, a phenotype consistent with that of the angiogenic vessels in the human prostate xenografts observed during the initial 14 days after transplantation. Differences in vessel morphology in benign vs. tumor tissues have been reported, with tumor vessels less stable, more permeable, more dilated, and more sensitive to proliferative signals (48). The “remodeling” phenotype characteristic of tumor vasculature was associated with a lack of association of the endothelial

Fig. 4. Vascular endothelial growth factor (VEGF)-A expression after androgen deprivation in the short-term human prostate primary xenografts. A: immunostaining of hypoxia-inducible factor (HIF)-1α, VEGF-A, VEGF receptor (VEGFR)-2, basic fibroblast growth factor (bFGF), and FGF receptor (FGFR)-1 in endothelial cells of human prostate xenografts was performed, and staining intensities were scored 0–3 using manual microscopy at ×100–400. Data are averaged scores of two blinded investigators; changes in expression were evaluated for significance at 90% confidence, or greater. B: immunostaining for VEGF-A on day 0 (i), day 2 (ii), and day 7 (iii) after androgen deprivation in prostate xenografts. Arrows indicate endothelial cells. iv: VEGF-A and CD31 colocalization (arrow) confirmed the presence of VEGF-A in human prostate endothelial cells. Bars: 30 μm.
cells with pericytes (3) and a reduced expression of adhesion molecules (2). However, in our study, the microvasculature in xenografts of benign prostate tissue and CaP tissue responded identically to androgen deprivation; microvascular integrity was lost rapidly, as demonstrated both by three-dimensional reconstruction studies and leakage of fibrin/fibrinogen and exogenously added lectin in the interstitial tissue spaces. Furthermore, the acute perturbation of the microvasculature in xenografts of both benign and CaP tissue was repaired rapidly, with reestablishment of vascular architecture and integrity within 10–14 days of androgen deprivation. These studies did not determine, however, if the new vasculature became mature, if it maintained an immature phenotype that was more sensitive to angiogenic signals emanating from the CaP microenvironment, or if the endothelial cells initiated paracrine effects over CaP epithelial cells that can drive recurrent growth.

Transplantation of human prostate tissue to mouse hosts implanted with testosterone pellets that maintain human levels of circulating testosterone resulted in a dramatic increase in MVD due to angiogenesis by the endogenous human vessels (22). In the androgen-stimulated microenvironment, VEGF-A and bFGF production by stromal cells, and to a lesser extent VEGF-A production by epithelial cells, provided the angiogenic signals that drove the increase in MVD during xenograft engraftment. However, the androgenic environment ultimately stabilized the nascent microvessels, decreased endothelial cell proliferation and apoptosis to baseline levels, and determined endothelial cell maturation (data not shown). In this study, the novel application of a bimodal form of the Mack-Wolfe order-restricted statistical test to analysis of scoring of immunostaining intensity was employed with the goal of providing an evaluation of statistical significance of discrete descriptors of staining intensities measured over time after androgen deprivation. The increased bFGF expression in the epithelium and increased PDGF-AA expression in the stroma on days 1–4 after androgen deprivation appeared to have no angiogenic effect because the MVD was decreasing as endothelial cell apoptosis increased. In contrast, androgen deprivation increased expression of both VEGF-A and VEGFR-2 in endothelial cells, which suggested the initiation of an autocrine signaling loop capable of enhancing survival, and inducing proliferation (8, 17, 65). In this model, AR appears to negatively

Fig. 5. Effect of androgen deprivation on VEGF-A expression in primary cultures of human prostate endothelial cells (HPEC). A: HPEC in primary culture showed a typical cobblestone morphology as the cultures became confluent. Black bar: 25 μm. B: immunofluorescence analysis demonstrated that >95% of cells expressed von Willebrand Factor. White bar: 25 μm. C: primary cultures of HPEC were exposed to androgen deprivation by culturing the cells for 24 h (d1) and 48 h (d2) in endothelial cell growth media containing charcoal-stripped 5.0% FBS. Standard PCR analyses indicated that androgen deprivation induced a slight increase in VEGF-A mRNA; however, no effect was observed on VEGFR-2 and bFGF mRNA expression in primary cultures of HPEC. Androgen receptor (AR) mRNA was slightly decreased after androgen deprivation (D). Standard PCR results were confirmed using quantitative real-time PCR. E: quantitative measurement by enzyme-linked immunosorbent assay of VEGF-A protein levels in primary culture of human prostate endothelial cells showed no significant increase in VEGF-A after androgen deprivation (day 1 and day 2). GAPDH, glyceraldehyde-3-phosphate dehydrogenase.
regulate VEGF-A expression in endothelial cells. Differential regulation of gene expression by AR in endothelial cells vs. in other androgen-sensitive cell types is consistent with our demonstration that HPEC demonstrate different capabilities to transactivate AR-regulated reporter constructs compared with prostate epithelial cells (20). However, the data do not exclude the possibility that increased expression of VEGF and PDGF-AA by epithelial cells, and bFGF expression by stromal cells, also contributes to the vascular rebound by surviving microvascular endothelial cells in the absence of androgen.

Androgen deprivation of primary cultures of HPEC did not induce upregulation of VEGF-A, VEGFR-2, and bFGF expression, as observed in prostate endothelial cells in situ, even though AR was previously demonstrated to be active in prostate endothelial cells in vivo and in vitro (20). Therefore, upregulation of these genes in endothelial cells in prostate xenografts may be affected by paracrine signaling mechanisms present in the microenvironment of the androgen-deprived prostate. If so, these paracrine signals from stromal or epithelial cells must be negatively regulated by AR, because AR expression was not detected in the nuclei of epithelial/stromal cells on day 2 after androgen deprivation, the interval of maximal expression (Supplemental Fig. 2). Unlike in stromal cells or epithelial cells, VEGF-A signaling in endothelial cells may not depend on the presence of a functional AR or, as discussed above, may be negatively regulated by AR. Alternatively, data obtained by other groups (10), and confirmed in our laboratory, indicated that some genes were downregulated rapidly when endothelial cells were cultured in vitro. For example, CD34, an endothelial cell marker that is expressed highly in vivo, was barely detected on endothelial cells in primary cultures. In addition, analyses in postcapillary high endothelial venule endothelial cells freshly isolated from human tonsil revealed striking changes in blood vessel type-specific, and tissue-specific, characteristics of endothelial cells occurred as early as 48 h after isolation and culture, suggesting that specialized characteristics of endothelial cells are under the control of their tissue microenvironment (34). Therefore, more experiments are necessary to define clearly the biological role of androgens and AR in human endothelial cell homeostasis in vitro and in vivo.

Hypoxia-mediated upregulation of expression of the proangiogenic HIF-1α-VEGF signaling cascade in epithelial and stromal cells has been demonstrated to induce angiogenesis (11, 18, 54). Our characterization of the androgen-deprived prostate microenvironment demonstrated that upregulation of VEGF-A, VEGFR-2, and bFGF expression in response to androgen deprivation was correlated with the acute reparative angiogenic response. However, there was no evidence for an increase in HIF-1α protein levels in any of the prostate cellular compartments during the initial week after androgen deprivation. Upregulation of GLUT1, another marker of hypoxia, also was absent in all cellular compartments, which suggests that hypoxia is not involved in the upregulation of VEGF-A in prostate endothelial cells. However, HIF-1α protein levels are regulated posttranslationally, and the lack of an apparent increase in HIF-1α protein levels 24 h after androgen deprivation could suggest a transient hypoxic microenvironment immediately after androgen deprivation that is resolved before the initial experimental time point. Analyses of HIF-1α expression at earlier times after androgen deprivation would be necessary to discard a possible contribution of hypoxia in the upregulation of endothelial VEGF-A and recovery of prostate vasculature. However, the small size of the xenografts may preclude the hypoxia in the xenografts due to efficient perfusion of oxygen, nutrients, and steroids from the host tissue.

In conclusion, the human prostate primary xenografts represent a valuable model for translational research because the tissue retains all of the components of the human prostate microenvironment, the in vivo tissue architecture and human pathophysiology, and androgen responsiveness. This unique preclinical model has provided three important new observations of the human prostate tissue microenvironment: 1) there is a transient, short window (4–7 days) immediately following androgen deprivation in which the integrity of the prostate microvasculature is compromised; 2) androgen-sensitive human endothelial cells rapidly develop an alternative mechanism(s) for maintaining homeostasis and regenerating the microvascular network in the absence of androgen; and 3) androgen deprivation results in upregulation of VEGF-A by specifically the prostate endothelial cells, potentially creating autocrine-mediated growth that would be insensitive to anti-VEGF therapy. A comparable androgen-independent revascularization was not observed in rat prostate. The difference in the response of rat prostate and the human prostate tissue xenografts could result in missed opportunities for identification of more effective treatments for BPH or advanced CaP.

ACKNOWLEDGMENTS

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DISCLOSURES

Gary J. Smith and James L. Mohler have financial interest in Androbyosis, Inc.

REFERENCES


