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Androgen Withdrawal Fails to Induce Detectable Tissue Hypoxia in the Rat Prostate

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Abstract

BACKGROUND—It has been reported that significant hypoxia may occur in the rat prostate following androgen deprivation (AD). It is well known that hypoxia substantially reduces radiation sensitivity of cells both in vitro and in vivo. Given that contemporary management of men with intermediate and high-risk prostate cancer includes the use of neoadjuvant androgen suppression and radiation, AD-induced hypoxia in the prostate could result in suboptimal therapeutic results. Given this concern, we fully investigate possible AD-induced hypoxia in the ventral prostate (VP) of adult rats by two independent methods.

METHODS—Tissue pO₂ levels in the VP of adult Sprague-Dawley rats were evaluated prior to and at various time points following castration by two independent techniques. First, an Oxylab tissue oxygen monitor with a 240 μm probe was used for quantitative monitoring of global VP oxygenation. Second, fluorescence immunohistochemistry using the hypoxia marker EF5, known to be metabolically activated by hypoxic cells, was used to evaluate cell-to-cell variation in hypoxia at various days post-castration.

RESULTS—Neither the oxygen probe nor EF5 method demonstrate any substantive change in pO₂ levels in the rat VP at any time point post-castration.

CONCLUSIONS—We find no evidence that the rat VP becomes hypoxic at any point following castration using an animal model that closely mimics the human prostate. These data are in contrast to previous reports suggesting prostatic hypoxia occurs following AD and provide assurance that our present therapeutic strategy of neoadjuvant AD followed by radiation is not compromised by AD-induced tissue hypoxia.

Keywords

hypoxia; ventral prostate; androgen withdrawal

INTRODUCTION

Contemporary management of men with intermediate and high-risk localized prostate cancer includes treatment with androgen deprivation therapy (ADT) and radiation therapy. The addition of ADT to radiation has resulted in improved biochemical recurrence free survival and, for men with high-risk disease, improved overall survival, compared to men treated with radiation alone or ADT alone [1,2]. Despite these advances, some 50% of men with high-risk disease will experience a recurrence within 10 years. Ensuring maximal therapeutic effectiveness of our treatment and the development of newer treatment strategies is, therefore, in order.

An important consequence of neoadjuvant androgen deprivation (AD) is prostate involution. This involution is thought to improve outcomes with radiation because of a reduction in the number of tumor clonogens that radiation must treat and because the reduced prostate size may decrease the volume of irradiated tissue resulting in less toxicity [3].

Interestingly, it has been proposed that a reduction in circulating androgen levels results in hypoxia within the prostate and that prostate cell death and subsequent prostate involution is indirectly induced by this hypoxic state. The hypothesis proposes that androgens are essential for production of critical vascular regulatory factors in the prostate and without these factors the capillary network regresses, leading to decreased blood flow, resulting in hypoxia-induced death of prostatic epithelium [4,5]. If true, this could have a substantive negative impact on radiation mediated cell killing.

Radiation-induced cell death is negatively impacted by low tissue oxygen levels [6,7]. In fact, tissue hypoxia has been shown to increase tumor resistance by a factor of 3. We hypothesized that increasing tissue hypoxia in the prostate before radiation could potentially have deleterious effects on many of our present day therapeutic strategies that combine radiation and ADT. Most obviously, increased hypoxia and subsequent increase in radioresistance would compromise the therapeutic effects of radiation. In addition, the prostate cells that do not die secondary to AD [8,9] might be more likely to accumulate pro-mutagenic DNA damage following radiation, enhancing the carcinogenic process. Given these significant concerns, more information as to these possible ADT-induced effects is necessary.

Several studies have investigated the hypothesis of prostate tissue hypoxia after AD but none has incorporated multiple methods to comprehensively evaluate and confirm it. Here, we detail a set of experiments designed to thoroughly investigate possible ADT-induced hypoxia in the ventral prostate (VP) of rats. We employ two independent methods of analysis and find no evidence for prostate hypoxia at any time following castration.

MATERIALS AND METHODS

In order to evaluate prostate hypoxia, two methods were used to determine if and to what degree tissue hypoxia was present in the VP of Sprague-Dawley rats before and following castration.

Oxygen Probe Measurements

For quantitative monitoring of oxygen in prostate tissue we used an Oxylab tissue oxygen monitor with a 240 μm probe (Oxford Optronix Ltd, Oxford, UK). Oxygen levels are reported in millimeters of mercury (mmHg). Mature (2–3 month old) male Sprague-Dawley rats (250 g) were randomly divided in seven different groups on an animal protocol approved by the JHU IACUC. Each group contained at least four animals. The animals were castrated (scrotal incision) as previously described [10], then anesthetized with Nembutal and their VP exposed and analyzed for evidence of hypoxia at 1, 2, 3, and 7 days following castration. The oxygen probe was placed into the VP ($n=7$) and the pO_2 value determined for each condition. The rectus abdominis muscle (RA) was exposed and tissue measurements were taken for comparison as a non-androgen dependent organ. Non-castrated rats were used as a baseline control. As a positive control, we employed two different methods. First, pO_2 was measured after ligatures were surgically placed on the vasculature of the prostate for 30 min. We also measured the pO_2 in the VP before and 10 min after death by overdose with Nembutal.

EF5 Immunohistochemical Measurements

The second method to assay for prostate hypoxia employed a fluorescence immunohistochemical technique for measuring levels of EF5 binding. EF5 is a 2-nitroimidazole, metabolically activated by cells at a rate that is inversely dependent on oxygen concentration. This method has been validated for quantitative studies of both neoplastic and non-neoplastic tissue pO_2 and allows for evaluation of hypoxia at a cell-to-cell resolution [11].

To determine if the hypoxic state of prostate tissue changes during involution, we used Sprague-Dawley rats. The animals were injected with EF5 at Day 0, 2, 4, 5, or 21 after castration. Intravenous injections were delivered followed by an intra-peritoneal injection of EF5, 3 hr later as previously described [12,13]. The VP was harvested from sedated (Nembutal) animals and frozen at -80°C . Every VP was cut in multiple 10 μm slices at two or three levels separated by approximately one millimeter using a Microm HM 505 cryostat (Carl Zeiss, MICROM laborgeräte GmbH, Waldorf, Germany) and treated independently. For sections labeled Regular Staining, samples were fixed, blocked and stained with Cy-3 labeled anti-EF5 antibodies (75 $\mu\text{g}/\text{ml}$). One slice received no anti-EF5 antibody treatment and the fluorescence was measured to determine the level of endogenous fluorescence—none was found (Fig. 1). From the same VP, a section received treatment with anti-EF5 antibody in a solution containing free EF5 to determine the extent of non-specific binding; this sample is referred to as Competed Stain (CS). The values of fluorescence with CS were subtracted from the values of RS though all three staining methods gave readings close to background. Slides were stored at 4°C in phosphate-buffered saline containing 1% paraformaldehyde and imaging was done using a cooled CCD camera (Photometrics 'Quantix) and Nikon Labphot fluorescence microscope (Nikon Instrument group, Inc., Melville, NY). The cut-off point for hypoxia is an absolute value of 100. The absolute fluorescence of 100 is equivalent to 2% oxygen [12]. In order to confirm that the tissues were equally capable of high EF5 binding at low pO_2 , "Cube Reference Binding" experiments were performed in vitro. In these experiments, tissue cubes of VP (roughly 1–2

mm per side) were prepared by dissecting fresh tissue from non-EF5 treated animals at various days post-castration. The cubes were incubated at 37° in glass dishes with EF5-containing medium. The dishes were sealed in aluminum chambers under hypoxic conditions (0.2% O₂) while gently shaken [14]. A detailed review of the image analysis of EF5 employed has been previously published [15]. Statistical analysis was performed with commercially available GraphPad Prism® (v6.03). An unpaired *t*-test with Welch's correction was used to compare two groups.

RESULTS

Oxygen Probe Measurements

We first employed oxygen probe measurements (pO₂ in mmHg) at various days post-castration to determine if and to what degree tissue hypoxia occurs. These experiments revealed that the average pO₂ measured in prostate tissue in intact (uncastrated) rats was 41.6 ± 4.0 mmHg (n = 7) (Fig. 2). After androgen depletion a significant involution of the prostates was observed but no evidence of hypoxia was found. The pO₂ measured in any of the prostates from the castrated animals was essentially identical to the pO₂ measured in the prostates of intact animals on multiple days following castration (Fig. 2). In the castrated rats the lowest average pO₂ values measured were 40.1 ± 3.4 mmHg (n = 4) at 2 days post castration (*P* > 0.3 as compared to the average uncastrated values). The average pO₂ values at 1, 3, and 7 days post castration averaged 44.5 ± 4.8 mmHg (n = 4), 42.8 ± 2.3 (n = 6) mmHg, and 44.5 ± 2.6 mmHg (n = 4), respectively (Graph 1). The pO₂ was also measured in the RA of these rats to serve as a normal tissue control for any effect of anesthesia. The overall average of measured pO₂ values of RA in intact rats was 37.5 ± 2.1 mmHg (n = 7) (data not shown). Even though the pO₂ in the RA shows some variance in the animals after castration, it does not become hypoxic. In contrast, placing ligatures on the vasculature of the prostate caused a significant decline in pO₂ levels to an average of 3.2 ± 1.0 mmHg (n = 4). (*P* < 0.0001 ligatures as compared to the lowest observed pO₂ values in the castrated group) assuring the ability of the oxygen probe to detect low values of pO₂ in tissue if present.

The pO₂ was also measured before and 10 min after death as another means to mimic tissue hypoxia. These experiments revealed average pO₂ values of 40.3 ± 2.4 mmHg (n = 4) before death and 2.8 ± 0.4 mmHg (n = 4) 10 min after death (Fig. 2) again documenting that the prostate was metabolically active and consuming oxygen to become hypoxic after cessation of blood flow.

EF5 Immunohistochemistry Measurements

The next series of experiments employed EF5 binding to investigate for evidence of prostate tissue hypoxia. The mean ± SD absolute fluorescence in the prostate slices in an intact (uncastrated) rat was 13.5 ± 3.3 (n = 2). At 2 days following castration, 4 slices were stained from 3 different rats and found to have an average fluorescence of 10.5 ± 4.0 (n = 4) (Fig. 2). For the Day 4, Day 5, and Day 21 post-castration end points, fluorescence measurements did not exceed 18.5 with an average of 13.3 ± 3.7 (n = 4) (Fig. 2). Comparing uncastrated mean ± SD 13.5 ± 3.3 (n = 2) to castrated mean ± SD 11.9 ± 3.9 (n = 8) (*P* = 0.63), shows no

significant difference. The absolute fluorescence of the castrated animals never approached 100 ($P < 0.0001$), a level that would be consistent with tissue hypoxia ($\sim 2\% \text{ O}_2$) (Fig. 3).

In dramatic contrast, EF5 binding for the cube reference binding tests (performed as positive controls for hypoxia in tissues from control and Day 4, Day 5, and Day 21 post castration) averaged 501.5 ± 154.4 ($n = 3$) (Fig. 3) documenting the consistent ability of prostate tissue to metabolize EF5 under hypoxic conditions.

DISCUSSION

The precise sequence of events leading to prostate involution following castration is unclear. Some studies demonstrate that the prostatic blood supply decreases during ADT. This was observed after treatment with anti-androgens like bicalutamide, as well as with a 5α -reductase inhibitor [16], which is used for treatments of less severe diseases like benign prostatic hyperplasia or alopecia androgenetica. It is stated that the decrease in blood flow is followed by decreased cell proliferation and an increase in apoptosis of prostate cells, especially epithelial cells [4]. Other reports suggest that the reduced blood flow causes hypoxia which leads to apoptosis and eventually involution of the prostate [4,5]. If hypoxia in prostate tissue is confirmed, such information would suggest the present strategy of combining neoadjuvant ADT with radiation may not be ideal if, in fact, a hypoxic pool of cells is created that would be more radiation resistant. It would also suggest that the surviving prostate stem cells are hypoxic and might similarly create a condition of relative radioresistance. Given the importance of these questions, we believe the analysis detailed in this report is important to help inform the everyday management of men with prostate cancer.

Following castration, we find no evidence that the rat VP becomes hypoxic at any point by oxygen probe analyses in an animal model that closely mimics the human prostate. Our analysis included several time points between pre-castration to near total prostate involution. To confirm that our oxygen probe technique would detect hypoxia in the prostate if it were present, we conducted two, independent experiments to induce hypoxia in the prostate. Both ligation of the arteries feeding the VP as well as death of the animal resulted in substantial hypoxia in the prostate that was detectable via the oxygen probe technique. Finally, to ensure that anesthesia of the rats did not in some way result in inadvertent tissue re-oxygenation in the rats, we measured tissue pO_2 in the RA at the same time points as the prostate measurements. We never detected any substantive change in pO_2 of the RA confirming the lack of significant anesthesia-induced oxygen tissue changes.

In order to more completely evaluate for hypoxia in the prostate following castration, we also employed an independent immunohistochemical analytic technique using the 2-nitroimidazole, EF5. Unlike the oxygen probe measurements, which provide a more global evaluation of pO_2 , EF5 provides an analysis of hypoxia on a cellular level and would be capable of demonstrating localized hypoxia niches. The EF5 studies confirm the results of the oxygen probe revealing that at no point before or following castration was there evidence of EF5 binding consistent with hypoxic levels of oxygen. Similar to studies done using the oxygen probe, we also incorporated experimental conditions that allowed us to

verify that EF5 would detect hypoxic conditions in the prostate if present. Under hypoxic conditions, EF5 binding in prostate tissue cubes incubated in hypoxia in vitro was more than 30-fold higher than was found in vivo. Based on the semi-quantitative scale of tissue pO₂ levels derived previously [13] these data suggest only physiological conditions of oxygenation in the prostate before and following castration, with no hint of even mild hypoxia.

We acknowledge that our results address oxygen levels in the normal rat prostate before and after AD and specifically do not evaluate these endpoints in prostate cancer. While an important distinction, our data are still clinically relevant in that the entirety of the prostate, not just the cancerous portion, is irradiated with routinely applied radiation therapy techniques like external beam radiation or brachytherapy. Others have investigated oxygen levels in prostate cancer pre- and post-androgen suppression. It has been reported that prostate cancer tends to be relative hypoxic in men with the disease when analyzed by oxygen probe techniques [17]. In those same patients, it has been reported that the administration of neo-adjuvant AD results in an increase in tumor oxygenation in the prostate. Such a change would tend to enhance radiation response [18,19]. This androgen suppression induced increase in prostate cancer oxygenation has been confirmed in another report by measuring hypoxia inducible factor-1 α (HIF-1 α) expression before and after ADT. A significant decline of HIF-1 α levels was observed after initiation of treatment, which was thought to correlate to an increase in oxygenation [20]. While data like these tend to evaluate only one point in time following androgen withdrawal (the earliest time point being 30 days after initiation of treatment and not multiple points in time as done in our experiments), these data support the notion that AD does not result in more hypoxia in prostate cancer and, therefore, should not be considered detrimental to subsequent radiation therapy.

Recently, it has been reported that androgen withdrawal induces hypoxia in androgen-sensitive tissue, suggesting that this tissue oxygenation change may provide a mechanism for driving the malignant progression seen in locally advanced prostate cancer after treatment with ADT [21]. The authors found that oxygen levels in tumor xenografts fall for a period of roughly 2 weeks after initiation of treatment and subsequently increase again. While interesting and seemingly contradictory to previous reports in humans, it should be noted that the authors used human metastatic prostate cancer cells (LNCaP) in subcutaneous xenografts. This is probably not an ideal model given that LNCaP cells have been adapted to growth on plastic, at the relatively hyperoxic levels typically used in tissue culture. In addition, the pO₂ levels of the LNCaP xenografts in untreated animals was found to be low, at about 6% O₂, even prior to AD.

Together, our two, independent techniques for measuring tissue oxygen levels, oxygen probe and EF5 immunohistochemistry, confirm that castration of the male rat does not result in hypoxic conditions in the prostate at any time point analyzed. We believe these data are extremely important in that they solidify that the contemporary use of neo-adjuvant androgen suppression does not result in an oxygen state that would be deleterious to subsequent curative radiotherapy. In addition, we find no evidence that a hypoxic condition is created in the prostate at the time of full prostate involution that would allow for enhanced

DNA injury in the remaining prostate stem cells which might otherwise provide a promutagenic environment that would increase cancer risk. In sum, we believe these experiments represent the most complete analysis to date of prostate oxygenation status following androgen withdrawal in the rat prostate and we believe concerns of an induced hypoxic state that might alter subsequent care are unfounded.

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Fig. 1.

Immunohistochemical detection of EF5 in ventral prostate tissues from rats. **A1, B1, C1** is Flooded Hoechst staining showing all nuclei (camera exposure time 0.11 sec). **A2**: Prostate tissue from cube; 4 days post castration incubated in 95% oxygen (RS) (camera exposure time 4 sec) showing no fluorescence. **B2**: Prostate tissue from cube; 21 days post castration incubated in 0.2% oxygen (RS) (camera exposure time 0.11 sec) showing fluorescence. **C2**: Prostate tissue; 5 days post castration (RS) (camera exposure time 4 sec) showing no fluorescence.

Fig. 2.

Values of PO₂ measured at different days following castration in ventral prostate of Sprague-Dawley rats with an Oxylab tissue oxygen monitor with a 240 µm probe.

Fig. 3.

Values of absolute fluorescence (log-scale) in EF5 stained tissue from VP of Sprague-Dawley rats at various days after castration. For the positive control the prostate cubes where incubated in 0.2% O₂. Absolute fluorescence >100 suggests Hypoxia (marked as dotted line).