Concurrent Androgen and Estrogen Ablation and Inhibition of Steroid Biosynthetic Enzyme Treatment for Castration-resistant Prostate Cancer

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Abstract. *Background/Aim: About 80 to 90% of prostate cancer (PCa) is androgen-dependent at diagnosis, but patients ultimately develop castration-resistant prostate cancer (CRPC), which is usually not amenable to androgen deprivation (ablation) therapy (ADT). Patients with CRPC usually succumb to death in less than 5 years and there is no cure. Here, we investigated reasons for ADT failure. Materials and Methods: Biopsy specimens from untreated and diethylstilbestrol (DES)-treated patients were assessed for localization of antibody IgGs against androgen (AR) and estrogen (ER) receptors. Results: In untreated and DES-treated sections, methylene blue stained basic proteins in dark basal (undifferentiated) PCa cells, whereas light basal cells were not stained. AR localized to light basal cells which showed widespread degeneration in sections from DES-treated patients, indicating their dependence on androgen. In contrast, dark basal cells did not show widespread degeneration in DES-treated patients; ER was usually localized in dark cells. The number of dark cells progressively increased in DES-treated patients indicating their androgen-independence. The localization of AR and ER in some light and dark basal cells indicated that the supply of androgen/estrogen was not inhibited during ADT. Dark basal cells had emerged prior to treatment and proliferated during DES treatment, that also indicated their androgen-independence. Conclusion: PCa has at least two populations of cells: androgen-dependent light basal and estrogen-dependent dark basal cells. ADT did not destroy estrogen-dependent cells which may have given rise to CRPC tumors. Therefore, ADT is an incomplete treatment. For a more complete treatment of PCa, we recommend concurrent androgen and estrogen ablation, together with the inhibition of selected steroid biosynthetic enzymes.*

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Adenocarcinoma of the prostate is the second most common solid organ tumor diagnosed in U. S. men, with an estimated 220,800 new cases and 27,540 deaths in 2015 (1). Prostate cancer (PCa) is a complex disease and its complexity is further increased by intra-tumoral heterogeneity. Steroid hormones play a key role in regulating the functions of benign prostate and its diseases. About 80 to 90% cases are androgen-dependent at the initial diagnosis (2) and androgen deprivation (ablation) therapy (ADT) is given as the primary treatment (3). In spite of the beneficial clinical effects of androgen, ADT is also associated with several adverse effects in men (4). Furthermore, many patients ultimately develop castration-resistant prostate cancer (CRPC) and typically succumb to death in less than 5 years (5, 6). The expression of androgen (AR), estrogen (ER) and progesterone receptors has been demonstrated in PCa (7, 8). The long-standing history of ADT, as first-line of treatment, has essentially precluded the utilization of estrogen/ progesterone ablation and inhibitors of enzymes of steroid biosynthesis.

Bilateral castration treatment was first utilized in 1895 by White (9). This was followed with chemical castration by diethylstilbestrol (DES) in the early 1940s by Charles Huggins and associates; this therapy continued until the 1970s (10, 11). At this point in time, the Veterans

Administration Cooperative Urological Research Group (VACURG) showed that DES treatment increased the survival of patients (12, 13). The study actually found that DES treatment (specifically at 5 mg) was associated with significant cardiotoxicity, and if administered, should be given at a lower dose (1 mg or 3 mg). A later analysis found there was a survival benefit when DES was given early to men with high-grade disease (12, 13). Subsequently, a variety of drugs (such as luteinizing hormone-releasing hormone agonists, anti-androgens, abiraterone acetate, and estrogen) were introduced and they were considered advanced ADT for PCa (14, 15). Here, we showed that patients have received incomplete treatment for over 125 years. At the present time, there is no cure for CRPC (16).

Men below the age of 30 years, when testosterone/dihydrotestosterone (T/-DHT) production is at its peak (11, 17-19) and estrogen and progesterone production are at relatively low levels (20-22), rarely develop PCa (23, 24). However, the incidence of PCa increases with each decade of aging, but varies depending on race and ethnicity. Starting at about 30 years of age, testosterone gradually declines (about 1 to 1.5%) each year and reaches about 50% of the starting level in men over 50 years of age (20, 25-27). This alters the relationship between androgen and estrogen levels, creating a hormonal imbalance at about 50 years of age, the purported age of PCa origin (23, 24). Hormonal imbalance is also associated with an increase in aromatase, which converts testosterone to estrogen resulting in a further decline in testosterone, and an elevation in sex hormone-binding globulin. In addition, hormonal imbalance is also created by an exogenous supply of estrogen (or phytoestrogen) in men of meat-eating and affluent countries (19, 28). The role of exogenous estrogen in the development of PCa is essentially unknown. Thus, the cellular microenvironment undoubtedly varies in men of different ages and races (25, 29). Finally, although the initial malignant transformation in genes and stem cells occurs in the microenvironment (milieu) of hormonal imbalance, other factors, such as mutated genes, dietary, and lifestyle-related factors, contribute to prostate carcinogenesis (28, 30).

We previously identified light and dark basal cells in methylene blue-stained biopsy sections from untreated and DES-treated patients with PCa using light and electron microscopy (31). We showed that light cells, but not dark cells, were androgen-sensitive and degenerated in response to DES treatment. We designated dark cells as androgen-independent (androgen-insensitive) and concluded that dark cells are unresponsive to ADT treatment (31). In this study, we hypothesized that the localization of AR and ER could characterize light and dark basal cells and also provide clues to the emergence of CRPC in untreated and DES-treated patients. We further hypothesized that androgen-dependent columnar/cuboidal and light basal cells would show degeneration in DES-treated cases, while dark cells would not.

Materials and Methods

Former VAMC urology surgeon Dr. Clyde E. Blackard and his associates selected 25 patients and biopsied areas suspected of cancer. Prostate specimens were submitted to the Pathology Service at the Minneapolis VAMC. Biopsy specimens not used for diagnosis of cancer were provided for research according to the institutional review board (IRB) guidelines in place for the VA and the University of Minnesota. Specimens were collected between 1972 and 1975 at the VAMC. Specimens not needed by the Pathology Department were fixed in 3% glutaraldehyde in 0.1 M phosphate buffer at pH 7.3 for 2 hours, washed several times, post-fixed in 1% to 2% buffered osmium-tetroxide, washed and dehydrated in graded ethanol, and then embedded in Epon 812 as previously described (31).

The specimens of four patients had benign prostatic hyperplasia (BPH); 13 patients were not treated with any hormone prior to biopsy and eight patients were treated with DES alone or DES plus Provera for 37 days to 18 years and 9 days. Clinical details of all DES-treated patients were previously published (31). Since sections from previously untreated patients, as reported in our paper (31) were not suitable for AR/ER studies, we selected similar cases for the current studies. The age of patients ranged from 53 to 86 years with a mean±standard error of the mean of 70.12±1.88 years. Sections were graded by Drs. Donald F. Gleason and Nancy A. Staley, former staff pathologists of the Minneapolis VAMC. Patients had PCa with pathological grades III and IV tumors which are comparable to Gleason histological scores 6 to 10 (32). Clinical stages were B, C and D (33).

Epon-embedded blocks were re-sectioned using microtome (Reichert-Jung) to produce 1 to 2 μm-thick sections and were stained with methylene blue for morphological analysis and immunogold localization of AR and ER receptors. Methylene blue stains basic protein (34). Localization of antibody IgGs against AR showed specificity to human prostate and prostate cancer cell line (AR N-20; Santa Cruz Biotechnology, Santa Cruz, CA, USA). AR variant 7, lacking the hormone binding domain (AR-V-7), does not cross react with intact AR as per the manufacturer (Precision antibody; A& G Pharmaceutical, Inc., Columbia, MD, USA). We also obtained rabbit antibody IgGs against ERβ (H-150: sc-8974; Santa Cruz Biotechnology). Initially, small amounts of anti-AR-N-20 antibody and anti-AR variant-V7 antibody were kindly provided by Dr. Scott Dehm of the University of Minnesota, Minneapolis, MN and Dr. Jun Luo of Johns Hopkins University, Baltimore, MD, respectively. After initial testing, we obtained commercial antibodies for the current study.

For immunogold localization, methylene blue-stained sections were etched for 10 minutes with 10% H_2O_2, washed with distilled water for 10 minutes (four changes) subjected to antigen retrieval for 10 minutes with citrate buffer (pH 6.0; Invitrogen Corp., Fredrick, MD, USA), and washed with phosphate-buffered saline (PBS). After blocking of non-specific localization by a mixture of 1 to 2% bovine serum albumin and 1 to 2% normal goat or sheep serum, sections were incubated with diluted primary antibody for 1 hour at room temperature (or overnight at 0˚C). Localization of AR, AR-V-7 and ERβ antibodies was achieved by using rabbit or mouse secondary antibody IgGs or protein A conjugated with 15 to 25 nm Aurion gold particles (Aurion Immuno Gold Reagents, Wageningen, The Netherlands). The reaction product was enhanced using an Aurion R-Gent silver enhancement kit (Electron Microscopy Sciences, Hatfield, PA, USA).

Results

Benign prostatic hyperplasia (BPH). The untreated specimens yielded benign prostate, prostatic intraepithelial neoplasia (PIN) and BPH areas. Benign prostate had tall columnar/cuboidal luminal cells and light basal cells that expressed AR. Columnar and cuboidal cells were differentiated with secretory granules and their nuclei were positioned at various levels, but mostly towards the basement membrane (Figure 1a). We observed the localization of both full-size AR (AR-N-20) and the variant AR (AR-V-7, not illustrated) in benign prostate and ERβ in PIN (Figure 1a and b). The localization of AR -N-20 and the variant AR-V-7 produced similar patterns in non-cancerous tissues, indicating that there was overlap between staining by the two antibodies.

Untreated prostate cancer. Specimens from untreated patients had mostly differentiated columnar/cuboidal cells and some undifferentiated basal cells (Figure 1c and d). Methylene blue staining revealed that most untreated PCa specimens had light basal cells interspersed with dark basal cells (Figure 1c and d). AR (AR-N-20) and ERβ (H-150) antibody were usually localized to light and dark basal cancer cells, respectively, as shown by immunogold particles (Figure 1c and 2a). AR variant V-7 was localized to some light and some dark basal cells (not illustrated). ERβ was predominantly localized to dark basal cells and occasionally to light cells (Figure 2a).

Androgen deprivation by DES in PCa. In DES-treated patients, the luminal columnar/cuboidal cells had sloughed leaving scant cytoplasm surrounding prominent undifferentiated light or dark basal cells. The number of dark cells progressively increased in patients treated with DES for 37 days and 18 years and 9 days (Figure 1e and f, 2b-f and 3a-c). In a patient treated with DES and provera for 1 year, 3 months and 10 days, many light cells localized ERβ (Figure 2b). Some light and dark cells formed invading prong in the stroma (Figure 2b). Occasional invasive cells that localized ERβ were found in a capillary (Figure 2b). AR/ER did not localize to fibroblasts and muscle fibers (Figure 2b-d). In this case, numerous light basal cells surrounded by dark cells formed an invading edge (Figure 2d). The invading edge was surrounded by fibrous stroma that was probably formed due to lytic enzyme(s) activity (Figure 2d). AR-N-20 localized in some dark cells (Figure 2d). Light and dark basal cells showed localization of AR/ER receptors (Figure, 2b-e). Figure 2f shows the distribution of light and dark cells in acini A and acini B and localization of AR-V-7 primarily in dark cells. There were many dark cells and some light cells that were surrounded by degenerating cancer and stromal cells (Figure 3a). Some dark cells showed localization of AR (AR-N-20). In a patient-treated with DES for 18 years and 9 days there was widespread degeneration of light basal cells, but not of dark basal cells (Figure 3b). Stroma had also degenerated (Figure

3b). In this patient, many cancerous acini degenerated as shown by the outline of the acinar pattern (Figure 3c). Light cells progressively degenerated, whereas dark cells survived, as shown by their increased number (Figures 3 a-c).

Localization of ER receptor in untreated and DES-treated PCa. In untreated and treated PCa, nuclei of dark cells localized ER (Figure 1f, 2a, b). In a specimen from a patient treated with DES for 37 days, dark cells localized ER. In one patient treated for 67 days with DES, numerous dark cells showed ER localization (Figure 1f). Localizattion of ER in treated patients is illustrated (Figure 2b).

Discussion

Previous studies on endocrine-treated PCa had shown the presence of cancer cells in paraffin-embedded sections (35-37). Prout *et al.* identified androgen-dependent and androgen-independent PCa cells (37). Periodic acid Schiff (PAS) staining did not identify light and dark basal cells (38) as did the methylene blue staining of Epon-embedded sections (31). In response to 37-day treatment with DES, the cytoplasm of differentiated (columnar/cuboidal) cells had already sloughed resulting in undifferentiated cells. This loss of cytoplasm in columnar/cuboidal cells is an early event that probably occurred soon after the start of DES treatment. This observation is also supported by our study of castrated and testosterone-treated mice (39, 40). We found that sloughing of luminal columnar/cuboidal cells occurred in less than 72 h in castrated mice (39) and these cells could regenerate after 72 h of testosterone treatment (40).

Androgen deprivation, undoubtedly, reduces tumor burden by inducing degeneration of androgen-dependent cancer cells. Localization data indicate that AR and ER progressively declined in DES-treated cases. Localization of AR and ER in light and dark basal cells indicated continued biosynthesis of steroidal hormones during ADT which does not inhibit steroid biosynthesis. To inhibit steroid biosynthesis, multiple enzymes would be needed. In addition, ADT treatment does not induce degeneration of estrogen-dependent dark cells. This may be one of the main reasons that ADT does not cure CRPC.

The degeneration of light basal cells in DES-treated cases is indicative of their androgen dependence, whereas the lack of such degeneration in dark cells is indicative of their estrogen dependence. As DES treatment progressed from 37 days to 18 years and 9 days, there was an increased degeneration of light basal cells and a concurrent increase in the number of dark cells. The presence of dark cells in untreated PCa (prior to any hormonal therapy) (31) suggests that their origin is also independent of androgen, allowing them to survive ADT. Since dark basal cells are dependent on estrogen, but not androgen, they are the likely drivers of CRPC. An assessment of dark cells in patients with PCa prior
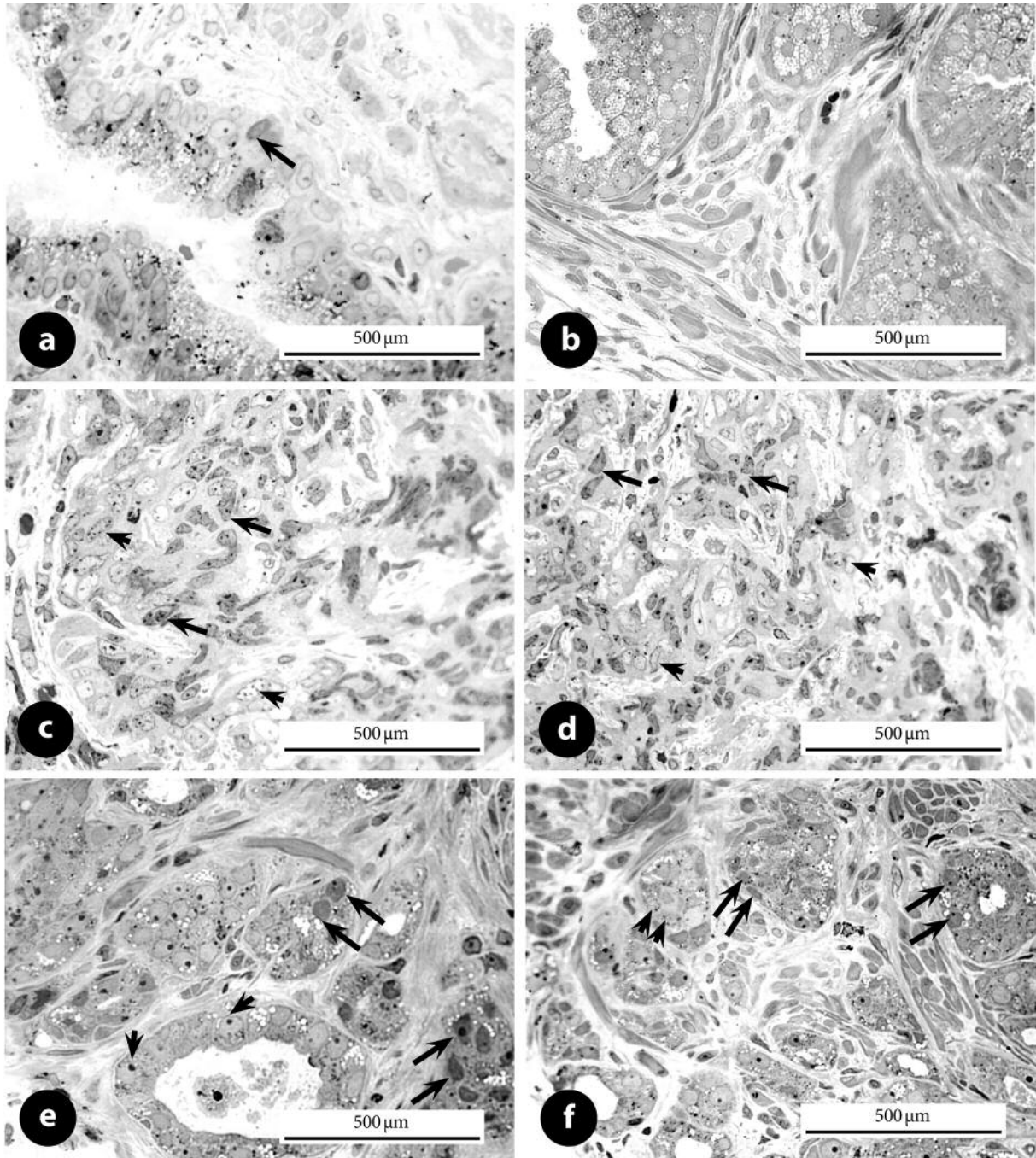
Figure 1. *Androgen and estrogen receptors in benign prostate and prostate cancer. a) Localization of AR (N-20) in nuclei of light basal and columnar/cuboidal cells in untreated benign prostate. The arrow shows an unidentified dark cell. There is no localization in the stroma and lumen, except for isolated dark particles. Columnar cells show secretory granules. b) Localization of estrogen receptor (H-150) is shown in light basal and columnar/cuboidal cell nuclei in untreated prostatic intraepithelial neoplasia. c) Untreated prostate scancer shows localization of AR (N-20 in light and dark cells by immunogold particles. Many dark cell nuclei are pleomorphic. Arrows show localization of immunogold particles in light and dark cells. d) Localization of ERβ (H-150) in untreated prostate is in nuclei of light (arrow heads) and dark (arrows) basal cells. Many dark cell nuclei are pleomorphic. e) In a DES-treated patient, the localization of AR (N-20) by immunogold particles is shown in the nuclei of light basal cells (small arrows) and dark cells (large arrows). Columnar/cuboidal cell cytoplasm was greatly reduced. Methylene blue stained nuclei of dark cell nuclei (large arrows). Some acini have more light cells and others have more dark cells. f) Immunogold particles localized ERβ (H-150) in nuclei of light basal (small arrows) and some dark basal cells (arrows) in DES-treated patient. The cytoplasm of the columnar/cuboidal cells was greatly reduced and secretory granules were essentially absent. Isolated invasive cells in stroma showed localization of ER by immunogold particles. Androgen ablation did not prevent localization of estrogen receptors. Bar indicates magnification.*
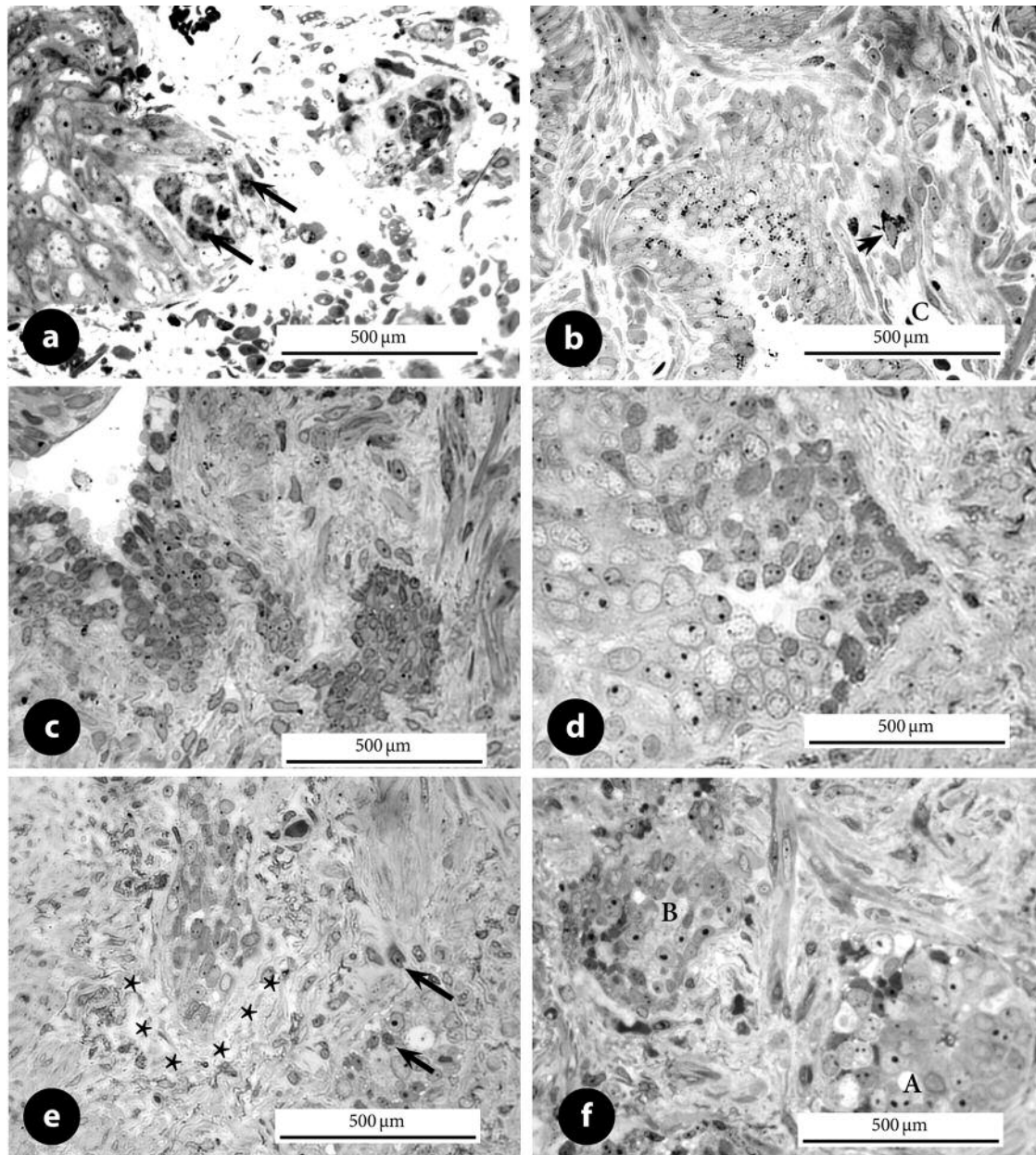
Figure 2. *Estrogen and androgen receptors in an untreated and treated prostate cancer. a) Immunogold particles localized ERβ (H-150) in nuclei of dark basal (large arrows) cells from untreated PCa. Light basal cells did not localize ER. b) In a DES-treated section localization of ERβ (H-150) is shown in nuclei and cytoplasm of light and dark basal cells by immunogold particles. A group of light and dark basal cells appear to be invading the stroma. An occasional invasive cell (arrowhead) is shown by localization of gold particles, has invaded capillary (C). Androgen ablation did not prevent localization of ER. Treated PCa. c) In a patient treated with DES for 5 years and 6 months localization of AR (N-20) was found in relatively few dark basal cells by immunogold particles. The prostatic duct had light and dark basal cells. A cluster of dark cells had invaded the stroma, whereas others were still connected to the duct. Androgen ablation did not induce the degeneration of dark cells. Connective tissue and muscle fibers appear relatively intact. d) In a patient treated with DES and Provera for 1 year, 3 months and 10 days, there are mostly dark basal cells and some light basal cells. Dark basal cells are at the leading edge and the light basal cells are more internal to the dark cells. A few dark and light cells show localization of AR (N-20). Stromal connective tissue has degenerated around the leading invasive edge of dark cells, probably due to some unknown enzyme(s) activity. The micrograph also shows degeneration of stromal cells and muscle fibers. Androgen ablation did not induce the degeneration of dark cells. e) Another section from the prostate of the above patient shows a column of dark cells and a few light basal cells that appear to be invading the stroma at the leading edge. Stromal connective tissue degenerated at the leading invasive edge (see asterisks). Occasional dark basal cells show localization of AR (N-20). Near the bar, there is an acinus showing both light and dark cells, but localization is sparse. Androgen ablation did not induce the degeneration of dark cells (arrow). Invasive dark cells are shown by arrows. f) Immunolocalization of AR-V7 in nuclei of dark and light basal cells. In acinus A, many light cells have degenerated, but some have not. In acinus B, numerous dark cells and light cells have degenerated. This patient was treated with DES for 67 days before biopsy. Bar indicates magnification.*
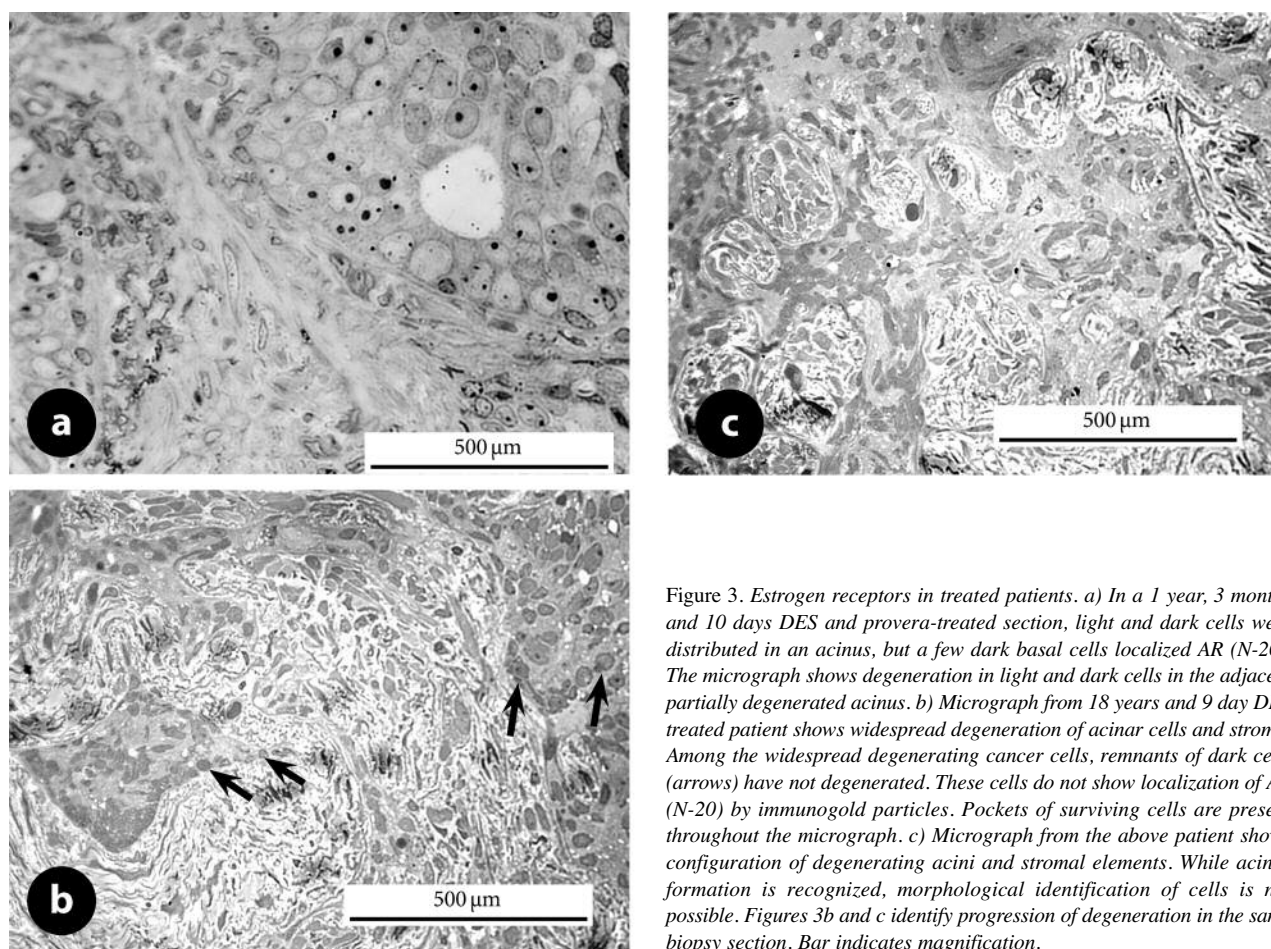
Figure 3. *Estrogen receptors in treated patients. a) In a 1 year, 3 months and 10 days DES and provera-treated section, light and dark cells were distributed in an acinus, but a few dark basal cells localized AR (N-20). The micrograph shows degeneration in light and dark cells in the adjacent partially degenerated acinus. b) Micrograph from 18 years and 9 day DES treated patient shows widespread degeneration of acinar cells and stroma. Among the widespread degenerating cancer cells, remnants of dark cells (arrows) have not degenerated. These cells do not show localization of AR (N-20) by immunogold particles. Pockets of surviving cells are present throughout the micrograph. c) Micrograph from the above patient shows configuration of degenerating acini and stromal elements. While acinar formation is recognized, morphological identification of cells is not possible. Figures 3b and c identify progression of degeneration in the same biopsy section. Bar indicates magnification.*

to treatment may provide some clues regarding those who will develop androgen-independent (or CRPC) tumors. This warrants further investigation.

The emergence of light and dark basal PCa cells is likely due to the malignant transformation of genes. Malignant transformation produces two types of cancer stem cells: one under the influence of androgen and the other under the influence of estrogen/progesterone. Support for this comes from the fact that ADT induced degeneration in androgen-dependent cells but not in androgen-independent cells. Thus, ADT alone has been an incomplete treatment for about 125 years. Some recent drugs, *e.g.* abiraterone acetate, an inhibitor of androgen synthesis (5), and trilostane (5, 41) used in the treatment of CRPC can interfere with steroid biosynthesis. They do not inhibit specific enzyme in the steroid biosynthetic pathway. Furthermore, our conclusion should be validated in a larger number of patients and a clinical trial before this approach could be used for treatment of PCa.

Approach for treating other types of steroid-dependent cancer. The current treatments of female cancers (namely, breast cancer, uterine and cervical) are on the 'hit and miss' principle. Each type of cancer is regulated by specific organ-related steroid hormone and their receptors, which in turn are modulated by menstrual cycle and menopause. The genes and their products involved in these cancers are, undoubtedly, modulated as well. For example, steroid hormones in breast cancer are modulated differently in nulliparous females than in pregnant (parous) and menopausal females. The hormone dependency of breast cancer also varies with age and menopausal status. The underlying biological principles may provide clues into a selection of successful treatment for breast cancer. With a predicted significant increase in breast cancer in Western countries, our idea needs to be explored without delay in the above cancers.

Conclusion

We have shown the significance of dark cells in the development of CRPC tumor that is not amenable to ADT. We suggest that concurrent androgen and estrogen ablation

together with inhibition of certain biosynthetic enzymes are required for more complete treatment of PCa, including CRPC. This treatment needs to start early after clinical trial.

Conflicts of Interest

The Authors have no conflict of interest in regard to the publication of this article.

Disclaimer

The opinion expressed in this article is that of the Authors and not of the U.S. Government, Department of Veterans Affairs or the University of Minnesota.

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