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MiniReview

Perinatal Exposure to Oestradiol and Bisphenol A Alters the Prostate Epigenome and Increases Susceptibility to Carcinogenesis

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Abstract: An important and controversial health concern is whether low-dose exposures to hormonally active environmental oestrogens such as bisphenol A can promote human diseases including prostate cancer. Our studies in rats have shown that pharmacological doses of oestradiol administered during the critical window of prostate development result in marked prostate pathology in adulthood that progress to neoplastic lesions with ageing. Our recent studies have also demonstrated that transient developmental exposure of rats to low, environmentally relevant doses of bisphenol A or oestradiol increases prostate gland susceptibility to adult-onset precancerous lesions and hormonal carcinogenesis. These findings indicate that a wide range of oestrogenic exposures during development can predispose to prostatic neoplasia that suggests a potential developmental basis for this adult disease. To identify a molecular basis for oestrogen imprinting, we screened for DNA methylation changes over time in the exposed prostate glands. We found permanent alterations in DNA methylation patterns of multiple cell signalling genes suggesting an epigenetic mechanism of action. For phosphodiesterase type 4 variant 4 (PDE4D4), an enzyme responsible for intracellular cyclic adenosine monophosphate breakdown, a specific methylation cluster was identified in the 5′-flanking CpG island that was gradually hypermethylated with ageing in normal prostates resulting in loss of gene expression. However, in prostates exposed to neonatal oestradiol or bisphenol A, this region became hypomethylated with ageing resulting in persistent and elevated PDE4D4 expression. In total, these findings indicate that low-dose exposures to ubiquitous environmental oestrogens impact the prostate epigenome during development and in so doing, promote prostate disease with ageing.

Prostate cancer is the most common solid cancer in males and is the second leading cause of cancer deaths in American men. The most recent cancer statistics for 2007 indicate that prostate cancer incidence continues to rise in the USA [1]. The reason for this high propensity to develop cancer within the prostate is not well understood and is an area of intense investigation. It has been suggested that the unique embryological origin of the prostate gland – from the endodermal urogenital sinus, as opposed to the mesodermal Wolffian duct structures that form the other male accessory sex glands – may play a fundamental role in the high rates of abnormal growth as men age. During embryonic development, the prostate gland is highly dependent on steroid hormones and it is notable that imbalances in steroid levels during early life can result in aberrant prostate growth [2,3]. The present review will highlight data from several studies that support a hypothesis that early life exposures to oestrogenic compounds, including the environmental oestrogen bisphenol A, may predispose the prostate gland towards abnormal growth and carcinogenesis later in life.

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Influence of oestrogen exposures during prostate development

In human beings, prostate development initiates towards the end of the first trimester in response to rising foetal androgen levels and glandular morphogenesis is largely completed during the second trimester as circulating androgen levels peak. During the third trimester of *in utero* development, foetal androgen production declines while maternal oestrogen levels rise resulting in an increased oestrogen/testosterone (E/T) ratio. This increased E/T ratio has been shown to directly stimulate extensive epithelial squamous metaplasia that regresses after birth as oestrogen levels rapidly decline [4]. Although the natural role for oestrogens during prostatic development is unclear, it has been proposed that excessive oestrogen exposures during development may contribute to the high incidence of prostate disease currently observed in the ageing male population [5,6]. The sons of women who took diethylstilboestrol during pregnancy where shown to have persistent abnormalities in prostate structure shortly after birth [7]. Furthermore, indicators of pregnancy oestrogen levels such as length of gestation, pre-eclampsia and jaundice have shown a high correlation between elevated oestrogen levels and prostate cancer risk [8,9]. Interestingly, African-American mothers have elevated

levels of maternal oestrogens and androgens during early gestation as compared to their Caucasian counterparts and it has been postulated that these elevated steroids may contribute to the 2-fold increased risk of prostatic carcinoma in African-American men [10,11].

Unlike human beings, the rodent prostate gland is rudimentary at birth and undergoes morphogenesis and differentiation during the first 2 weeks of life [12]. Thus, the neonatal rodent prostate gland is a useful model for foetal prostate development in human beings. Our laboratory and others have shown that brief perinatal exposure of rats to high doses of natural or synthetic oestrogens alters the prostate gland in a permanent manner resulting in reduced growth, differentiation defects, aberrant gene expression and perturbations in cell signalling mechanisms [13,14]. This process, referred to as developmental oestrogenization or oestrogen imprinting, leads to prostatic lesions as the animals age including chronic immune cell infiltration, epithelial hyperplasia and prostatic intraepithelial neoplasia, the precursor lesion of prostate cancer [15]. We thus propose that excessive oestrogen exposure during the developmental critical period may be a predisposing factor for prostatic disease later in life. It is noteworthy that a comparable rodent model for female perinatal diethylstilboestrol exposures accurately predicted the uterine and vaginal lesions found in daughters of diethylstilboestrol-exposed pregnant women [16] who provide credibility for the rodent model system in assessing similar pathologies in males. Whether similar effects may be induced by low-dose oestrogenic exposures has until recently remained unclear. This is currently a critical issue because hormonally active xenooestrogens are ubiquitous in the environment and have potential for adverse health outcomes in both human beings and animals [17].

Bisphenol A

Bisphenol A, a synthetic polymer, is a prevalent environmental oestrogen that was initially tested for efficacy as a synthetic oestrogen in 1936 [18]. Shortly thereafter, Dodds synthesized diethylstilboestrol that possessed much greater oestrogenic potency and the use of bisphenol A as a synthetic oestrogen was set aside. Today, bisphenol A is used as a cross-linking chemical in the manufacture of polycarbonate plastics, epoxy resins and several other common household products, and is one of the highest volume chemicals produced worldwide ($>6 \times 10^9$ lbs/year). Unfortunately, bisphenol A monomers leach from plastics and epoxy resins when heated or after repeated washings and bisphenol A is now found at significant levels in environments throughout the world [19,20]. As a result, unconjugated bisphenol A is found in the serum of 95% of human beings at levels ranging from 0.2–20 ng/ml [21,22]. Importantly, bisphenol A is found in 3–4-fold higher concentrations in amniotic fluid as compared to maternal serum [23] and placental and foetal tissue concentrations can exceed 100 ng/g with the highest levels found in foetal males [24,25]. While bisphenol A binds to classical oestrogen receptors with reduced affinity relative to 17β-oestradiol [26], it possesses equivalent activational capacity of the non-classical membrane oestrogen receptor [27]. Thus, there is potential for this compound as a toxicant for developing human tissues, particularly the oestrogen-sensitive reproductive end organs. In this regard, foetal exposures to environmentally relevant doses of bisphenol A in animal studies have been shown to advance puberty [28], increase prostatic growth [29], alter pubertal mammary gland development [30] and permanently change the morphology and functionality of female reproductive tract organs in mice [31].

Developmental bisphenol A exposure increases prostate gland susceptibility to hormonal carcinogenesis

It has been shown that that developmental exposure to low doses of oestrogen augments the responsiveness of female reproductive end-organs to elevated oestrogens at puberty and beyond. In this context, we asked whether low-dose oestrogens during development might shift the sensitivity of the prostate gland to adult oestrogenic exposures. This is highly relevant because prolonged adult exposure to oestradiol is capable of driving prostatic carcinogenesis in the Noble rat model [32] and oestrogens are associated with an increased prostate cancer risk in men [33]. Furthermore, the serum E/T ratio increases in ageing men, in part, due to increased body fat and aromatase activity [34], and this coincides with the increased propensity of ageing men to develop prostate cancer.

We chose to work with Sprague–Dawley rats as an animal model because this strain is less sensitive than the Noble rat to adult oestrogen-induced carcinogenesis [35]. A 'two-hit' model for carcinogenesis was established. The 'first hit' consisted of a brief exposure to a low dose of oestradiol (0.1 μg/kg body weight/day) or an environmentally relevant dose of bisphenol A (10 μg/kg body weight/day) on neonatal days 1, 3 and 5 when the prostate undergoes branching morphogenesis and differentiation. This bisphenol A dosage was chosen because it provides serum bisphenol A concentrations that are similar to those measured in the blood of human foetuses at term (i.e. 0.2–9.2 ng unconjugated bisphenol A/ml) [25,36]. To avoid intake variability between pups associated with lactation, precise doses of oestradiol and bisphenol A were delivered via subcutaneous injections using oil as the vehicle that provides slow release of the compound over several hours. While this non-oral route avoids first-pass liver metabolism, it is noteworthy that neonatal rat pups have limited metabolic capacity for bisphenol A [37]. When the neonatal-exposed rats in our study reached adulthood, they were given either oil (control group) or a 'second hit' 4-month exposure to ~75 pg/ml oestradiol that is by itself able to drive carcinogenesis in 100% of Noble rats but only 33% of Sprague–Dawley rats [32,35]. Our goal was to determine if neonatal low-dose estradiol or bisphenol A exposure could increase the susceptibility of the prostate to adult-induced carcinogenesis.

Individual prostate lobes were histologically assessed at 7 months of age for proliferation, apoptosis and pathologic lesions including prostatic intraepithelial neoplasia or

PIN, the precursor lesion for human prostate cancer [38,39]. While neonatal high-dose oestradiol exposure alone and to a lesser degree, early low-dose oestradiol exposure increased the incidence of PIN lesions, early exposure to bisphenol A alone had no effect on prostate pathology, proliferation or cell death as the animals aged. However, when rats were exposed to an environmentally relevant dose of bisphenol A (10 μg/kg body weight/day) early in life followed by adult oestradiol exposure for 4 months, the incidence of PIN lesions significantly increased to 100% as compared to 40% in control Sprague–Dawley rats that received oil neonatally and oestradiol in adulthood. Importantly, the lesions were mostly classified as high-grade PIN and the severity and incidence was similar to that found in rats exposed neonatally to pharmacological levels of oestradiol. As compared to controls, the PIN lesions in rats exposed to neonatal bisphenol A and adult oestradiol also exhibited significantly higher rates of epithelial cell proliferation and apoptosis that is considered key evidence that these are relevant precancerous lesions with similarity to human high-grade PIN, the precursor lesion to prostate cancer [40]. Taken together, these published findings suggest that early oestradiol exposures predispose the prostate to PIN lesions with ageing and that an environmentally relevant dose of bisphenol A is capable of increasing susceptibility of the prostate gland to carcinogenesis brought on by elevated oestradiol in the ageing male animals.

Developmental oestradiol and bisphenol A exposures alter the prostatic epigenome

DNA methylation is one of three epigenetic systems that regulate mitotically heritable changes in gene expression that are not coded in the DNA sequence. DNA methylation occurs at the C^5 position of cytosine in cytosine-guanine (CG) dinucleotides (CpG). In mammalian cells, CpGs are often found as aggregates, or *CpG islands* (CGI), in the promoter or 5′-coding region of genes and methylation status at these sites can regulate gene transcription [41]. Simplistically, hypermethylation of CGIs will cause stable heritable transcriptional silencing while hypomethylation permits transcription. Once established in somatic cells, CpG methylation patterns within the genome remain relatively stable and are heritable through cell divisions except during early embryonic development and tumourigenesis when drastic alterations in DNA methylation occur. Alterations in DNA methylation have been shown to contribute to both cancer initiation and promotion [42,43] including prostate cancers [44,45]. Furthermore, there is some evidence that early hormonal exposures can alter DNA methylation in reproductive tract tissues [46–48].

In this context, we asked whether the molecular underpinning whereby brief exposure to oestradiol or bisphenol A during development could permanently affect prostate carcinogenic susceptibility might be a result of epigenomic alterations in DNA methylation of specific genes. To identify potential methylation-regulated genes in prostates exposed neonatally to oestradiol and bisphenol A, we used methylation-sensitive restriction fingerprinting that screens for novel CpG-rich sequences whose methylation status undergoes alterations following treatments [47,49]. This approach allowed us to monitor epigenetic alterations over time as well as with different hormonal treatments. Importantly, this global screening method does not require the identities of the genes whose methylation status changes thus in addition to the suspected genes involved, one can identify novel genes that may play a role in developmental oestrogenization of the prostate gland. Our preliminary screens identified over 50 DNA candidate sequences with repeatable methylation alterations across multiple samples and prostate lobes [38].

The candidate sequences were subsequently cloned and 28 unique candidate clones were identified (complete table in Ho et al. [38]) Sixteen candidates showed no homology with known rat genes while the remaining eight genes were identified as PLCβ3, NVP3, CARK, GPCR14, PDE4D4, PDGFRα, CAR-X1 and SLC12A2. Several of these genes are involved in signal transduction pathways including Na-K-Cl cotransport (SLC12A2), MAPK/ERK pathway (PDGFRα), phosphokinase C pathway (PLCβ3), cAMP pathways (PDE4D4 and HPCAL1) and neural/cardiac development (CARXI, CARK). Because these signalling pathways play a role in cell cycle and/or apoptosis pathways within cells and tissues, it is intriguing to speculate that developmental oestrogenic exposures may perturb proliferation/apoptosis equilibrium in the prostate gland through an epigenetic gene (de)regulation mechanism. These findings may also provide clues to previously unrecognized participants in prostate carcinogenesis.

We observed overlapping as well as unique methylation alterations for high-dose oestrogen, low-dose oestrogen and bisphenol A. This suggests two important points. First, common prostatic genes may be epigenetically modified by different oestrogenic compounds and doses suggesting common pathways that predispose to prostate carcinogenesis with ageing. These identified candidates could be applicable not only to developmental oestrogenic exposures but may provide clues to new participants in prostate cancer. Second, unique candidate genes specific to an oestrogenic compound or dose may allow us to formulate specific epigenomic signatures that could serve as useful molecular markers for specific developmental exposures.

Prostatic PDE4D4 expression is methylation-regulated by oestradiol and bisphenol A exposure

We have initiated studies to determine whether altered DNA methylation due to neonatal oestrogenic exposures results in altered gene expression. PDE4D4 was chosen for further characterization because the differentially methylated DNA fragment identified by methylation sensitive restriction fingerprinting corresponded to the 5′-flanking region, the PDE4D4 fragment was consistently hypomethylated by all neonatal oestrogenic exposures and the changes were

identified as early as day 10 of life. PDE4D4 is an intracellular enzyme that specifically degrades cAMP [50]. Downstream cAMP signalling pathways include PKA activation and phosphorylation of cAMP-responsive element binding protein that regulates transcription of genes involved in cell growth and differentiation [51]. Thus, persistent activation of cAMP pathways may contribute to neoplastic transformation. In this regard, recent studies have shown a tight association between PDE4D4 expression and cancer cell proliferation, including gliomas [52], osteosacromas [53] and chronic lymphocytic leukaemia [54].

As previously detailed [38], PDE4D4 contains a 700-bp CpG island with 60 CpG sites in the 5′-regulatory region that encompasses the gene transcription and translation start sites. Methylation site mapping was performed by bisulfite genomic sequencing and a cluster was noted between 49–56 CG sites that became increasingly methylated in the normal control rat prostates with ageing, reaching 100% methylation by 7 months of age. In contrast, these 49–56 CG sites remained hypomethylated in ageing prostates exposed neonatally to high- or low-dose oestrogen or BPA. Importantly, these differential methylation patterns were inversely correlated to PDE4D4 gene expression as determined by real-time RT-PCR. Thus, while normal aged rats contained low prostatic expression of PDE4D4, this gene was expressed at high levels in rats exposed to oestradiol or bisphenol A during development. Importantly, this differential gene expression pattern was observed prior to adult exposure to oestradiol that indicates that molecular changes had occurred in the prostates of neonatal bisphenol A-exposed rats that may have contributed to its increased predisposition to hormonal carcinogenesis as an adult. This later observation suggests that PDE4D4 methylation and/or gene expression may be a useful early marker of adult-onset disease initiated by developmental oestrogen exposures in the prostate gland.

Conclusions

In summary, we have shown that a range of oestrogenic exposures during the early period of prostate development, from low-dose oestradiol and environmentally relevant doses of bisphenol A to pharmacological doses of oestrogens, results in an increased susceptibility to pre-neoplastic lesions of the prostate gland with ageing. Based on these findings, we propose that oestrogenic exposures during critical developmental periods may provide a foetal basis for adult prostatic diseases. Furthermore, we have obtained evidence that early exposures to oestradiol or bisphenol A can alter DNA methylation in a gene-specific manner that implicates epigenetic alterations as an underlying mechanism of action in developmental oestrogenization. Because several of these genes are interconnected through similar signalling pathways, we predict that oestrogen-induced alterations may produce complex changes within the prostatic cell that ultimately predispose the gland to carcinogenesis as the animal ages.

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