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Comparative effects of neonatal diethylstilbestrol on external genitalia development in adult males of two mouse strains with differential estrogen sensitivity

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ABSTRACT

The effect of neonatal exposure to diethylstilbestrol (DES), a potent synthetic estrogen, was examined to evaluate whether the CD-1 (estrogen insensitive, outbred) and C57 (estrogen sensitive, inbred) mouse strains differ in their response to estrogen disruption of male ExG differentiation. CD-1 and C57BL/6 litters were injected with sesame oil or DES (200 ng/g/5 μl in sesame oil vehicle) every other day from birth to day 10. Animals were sacrificed at the following time points: birth, 5, 10 and 60 days postnatal. Neonatally DES-treated mice from both strains had many ExG abnormalities that included the following: (a) severe truncation of the prepuce and glans penis, (b) an abnormal urethral meatus, (c) ventral tethering of the penis, (d) reduced os penis length and glans width, (e) impaired differentiation of cartilage, (f) absence of urethral flaps, and (g) impaired differentiation of erectile bodies. Adverse effects of DES correlated with the expression of estrogen receptors within the affected tissues. While the effects of DES were similar in the more estrogen-sensitive C57BL/6 mice versus the less estrogen-sensitive CD-1 mice, the severity of DES effects was consistently greater in C57BL/6 mice. We suggest that many of the effects of DES, including the induction of hypospadias, are due to impaired growth and tissue fusion events during development.

1. Introduction

Differences amongst mouse strains in response to estrogen was demonstrated decades ago when Gardner and Argyris (1957) Gardner and Argyris identified variability in response of vaginal mucosa to estrogen in inbred mice strains. Since then evidence has demonstrated strain-specific variability in response to estrogen for a variety of endpoints in both female and male mice (Spearow et al., 1999, 2001; Spearow and Barkley, 1999). Given the essential role of external genitalia (ExG) in reproduction and the known teratogenesis of estrogen on ExG development (Baskin et al., 2001b; Goyal et al., 2007), in the accompanying paper we examined strain-specific differences on ExG development following prenatal treatment with diethylstilbestrol (DES). These studies demonstrated an enhanced incidence and severity of male ExG malformations in inbred C57BL/6 versus outbred CD-1 mice (Mahawong et al., in press). Given that the neonatal mouse model best represents the developmental stages of in utero human estrogenic exposure (Ma, 2009), in this paper we extend our analysis of strain differences in estrogenic susceptibility to mice treated neonatally with DES.

Outbred CD-1 and inbred C57Bl6 strains are the most commonly used mouse models for investigating the effects of estrogens on development. CD-1 is popular because of its vigor, ease of breeding, and large litter size even though CD-1 mice are less sensitive to estrogen than inbred mouse strains (Spearow et al., 1999). Another commonly used mouse strain is C57BL/6, which represents the genetic background of many mutant mouse models, including steroid hormone receptor knockout mice. C57BL/6 mice are particularly sensitive to estrogens (Spearow et al., 1999). Thus, use of CD-1 mice may under-estimate the effects of estrogen, and use of C57BL/6 mice over-estimate the effects estrogen.

Whereas the presence (males) or relative absence (females) of androgens plays a central role in sex differentiation of the ExG

Abbreviations: ExG, external genitalia; DES, diethylstilbestrol; MUMP, male urogenital mating protuberance; SEM, scanning electron micrograph; PFA, paraformaldehyde; DMSO, dimethyl sulfoxide; ERα, estrogen receptor alpha; ERβ, estrogen receptor beta; gbw, gram body weight; OPT, optical projection tomography

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(Rodriguez et al., 2012), exogenous estrogenic compounds can derail ExG development by impairing differentiation and eliciting abnormal morphogenetic patterns including induction of hypospadias in mice and rats (Cunha et al., submitted for publication; Mahawong et al., in press; Goyal et al., 2007; Vorherr et al., 1979). Exogenous estrogens have been shown to perturb patterning of the penile urethral meatus, to reduce overall size of the external genitalia, to impair bone and cartilage differentiation and growth, to induce abnormal differentiation of fat cells within cavernous spaces, to impair smooth muscle differentiation and to induce hypospadias (Goyal et al., 2005, 2007; Blaschko et al., 2013; Rodriguez et al., 2012; Cunha et al., submitted for publication).

The field of mouse hypospadias has suffered for many years due to uncritical evaluation of developmental defects and resultant ambiguity in the literature. For example, many studies report "hypospadias" as an open urethral/preputial groove observed at the end of gestation in mice treated prenatally with estrogens (Kim et al., 2004). The tacit (but unproven) assumption is that such estrogen-induced malformations observed in late gestation fetuses are irreversible and will result in enduring malformations in adulthood. In this regard, to date there are 22 reports of murine "hypospadias" based upon an open urethral/preputial groove in late gestation mice without verification that the observed embryonic malformations persist as adult hypospadias (Cunha et al., submitted for publication). Such embryonic malformations may represent retardation of development capable of resolving to normality with time as discussed previously (Cunha et al., submitted for publication; Mahawong et al., in press). It is for this reason that we have proposed objective criteria of mouse hypospadias and emphasize that to avoid ambiguity, the best time to diagnose mouse hypospadias is at puberty or better yet in adulthood when penile development is complete and malformations (if present) are irreversible and enduring (Cunha et al., submitted for publication).

Hypospadias in male mice involves two structures: (a) the prepuce and (b) the penile urethra. Preputial hypospadias is an abnormality in the form of the external prepuce and typically is manifest as exaggerated clefting of the external prepuce and generalized preputial hypoplasia (Mahawong et al., in press). (It should be recognized that the mouse has two prepuces, external and internal (Blaschko et al., 2013)). Fundamentally, mouse penile hypospadias is an abnormality in the urethral meatus either in shape, position or both that departs significantly from normal morphology. The mouse urethral meatus is formed by the fusion of the male urogenital mating protuberance (MUMP) with the MUMP ridge (Weiss et al., 2012; Yang et al., 2010; Blaschko et al., 2013; Rodriguez et al., 2011). Accordingly, we define mouse penile hypospadias as an abnormality in the morphological patterning of the elements that form the urethral meatus (the MUMP and MUMP ridge) (Blaschko et al., 2013; Mahawong et al., in press; Cunha et al., submitted for publication), which can be readily detected in end-on photographs, scanning electron micrographs (SEMs) or histologic sections (Blaschko et al., 2013; Mahawong et al., in press). Proximal to the urethral meatus are two structures, the urethral flaps and the os penis (Rodriguez et al., 2011), which accordingly are rarely observed in transverse sections containing an open ventral urethral cleft as described previously (see Fig. 2 in (Mahawong et al., in press)). Thus, "exposed urethral flaps" and "exposed os penis" are additional objective criteria of mouse hypospadias usually associated with an elongated ventral cleft in the MUMP ridge (Mahawong et al., in press).

Based upon morphology of human hypospadias, the ideal expectation for an animal model of hypospadias is an abnormal urethral meatus at "mid-shaft". We have recently questioned whether such an abnormality is possible in mice given that the mechanism of penile development is different in mouse versus human (Mahawong et al., in press). The human penile urethra forms as a result of midline fusion of the edges of the urethral groove (Yamada et al., 2003). In contrast, formation of most of the mouse urethra has been suggested to occur via extension and canalization of the embryonic urethral plate (Seifert et al., 2008), even though the mouse urethral meatus appears to develop via fusion of the elements (MUMP and MUMP ridge) constituting the urethral opening (Blaschko et al., 2013; Mahawong et al., in press; Rodriguez et al., 2011). Whereas the developing human genital tubercle (penile rudiment) always has a free surface exposed to amniotic fluid (Baskin, 2000; Jirasek, 1971), the mouse genital tubercle is over-grown by the developing external prepuce (Perriton et al., 2002; Petiot et al., 2005; Baskin et al., 2004), and consequently the epidermal surface of the mouse penis is represented from birth onward by the solid preputial epithelial lamina, the precursor of the inner preputial epithelium and penile surface epithelium. In fact, the mouse penile surface epithelium is not manifest until puberty (days 24–30 postnatal) when penile development is complete (Mahawong et al., in press). Thus, for several weeks before development of the mouse penile surface, the mouse penile urethra (with the exception of the meatal region) is fully formed and appropriately incorporated into the substance of the penis. Thus, fusion events may only be involved in development of the mouse penile urethral meatus.

Hypospadias of the human penile shaft involves the absence/defect of 3 elements: (a) ventral skin, (b) epithelium of the ventral urethral wall, and (c) associated stroma and the corpus spongiosum forming the stroma of the ventral urethral wall (See Fig. 15 of the accompanying paper) (Mahawong et al., in press). Even though hypospadias of the mouse penile shaft may be unlikely as discussed above, it is imperative to recognize estrogen-induced defects in the mouse penis that are homologous or related to the 3 defective elements constituting human mid-shaft hypospadias. Accordingly, in neonatally DES-treated mice we have observed defects in penile skin, urethral epithelium and the corpus cavernosum urethrae, the mouse homolog to the human corpus spongiosum.

The purpose of this paper is to explore teratogenic strain differences in ExG in neonatally DES-treated mice including hypospadias, to summarize differences in prenatal (accompanying paper) versus neonatal DES-treatment, and to provide evidence for the developmental basis of estrogen-induced ExG malformations. It is worth noting that exposure to exogenous estrogens such as DES during the perinatal period elicits a variety of abnormalities in both male and female reproductive tracts and mammary gland (Bern and Talamantes, 1981; Mori et al., 1979; McLachlan et al., 1975, 1980). Our results suggest that developmental defects elicited by DES are profoundly different for prenatal versus neonatal DES treatment and that estrogen-induced teratogenesis is similar but consistently more severe in C57BL/6 versus CD-1 mice.

2. Materials and methods

2.1. Animal and housing

The University of California, San Francisco (UCSF) Institutional Animal Care and Use Committee approved all animal protocols. Adult wild-type CD-1 and C57BL/6 mice (Charles River Breeding Laboratories, Wilmington, MA, USA) were housed in polycarbonate cages ($20 \times 25 \times 47$ cm^3) with laboratory-grade pellet bedding in the UCSF Pathogen Specific Barrier housing facility. The mice were given Purina lab diet and tap water ad libitum. They were acclimated to 20–23 °C and 40–50% humidity on a schedule of 14 h light and 10 h dark. After mating within the same strains,