Activated Vitamin D₃ and Pro-activated Vitamin D₃ Attenuate Induction of Permanent Changes Caused by Neonatal Estrogen Exposure in the Mouse Vagina

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Abstract. Exposure of mice to a high dose of estrogens including diethylstilbestrol (DES) during the neonatal period modifies the developmental plan of the genital tract, which leads to various permanent changes in physiology, morphology and gene expression. These changes include development of an abnormal vaginal epithelium lined with hyperplastic mucinous cells accompanied by Tff1 gene expression in mice. Here, the influence of vitamin D on the direct effect of estrogen on the developing mouse vagina was examined. The mid-vagina of neonatal mice was cultured in a serum-free medium containing estradiol-17β (E₂) and various concentrations of 1,25-dihydroxyvitamin D₃ (1,25(OH)₂D) ex vivo and then was transplanted under the renal capsule of ovariectomized host mice for 35 days. Exposure to E₂ alone caused the vaginal tissue to develop estrogen-independent epithelial hyperplasia and to express TFF1 mRNA, while addition of a low nanomolar amount of 1,25(OH)₂D added at the same time as E₂ to the culture medium attenuated the effects of estrogen. Expression of vitamin D receptor was also evident in the neonatal mouse vagina. Interestingly, addition of 25-hydroxyvitamin D₃, a pro-activated form of 1,25(OH)₂D successfully attenuated DES-induced ovary-independent hyperplasia in the vagina in neonatal mice in vivo. Thus, manipulation of vitamin D influenced the harmful effects of estrogens on mouse vaginal development.

Key words: Cyp27B1, Estrogens, Trefoil factor 1, Vaginal development, Vitamin D

E xposure to exogenous estrogen during the early stages of life causes endocrine disruption and organogenetic abnormalities, which sometimes lead to severe results such as infertility, deformity and carcinogenesis, in laboratory animals and humans [1–4]. Prenatal exposure to diethylstilbestrol (DES), a potent synthetic ligand for estrogen receptors, resulted in various reproductive tract abnormalities including cervix cancer, the so-called DES syndrome or developmental estrogenization syndrome, in humans [e.g., 5, 6]. Similar genital abnormalities have been shown in experimental animals exposed perinatally to estrogens. Neonatal treatment of female mice with physiologically overdoses of estrogenic substances such as estradiol-17β (E₂) and DES, induces both ovary-dependent (via its effects on brain development that leads to continuous secretion of follicle-stimulating hormone and corresponding secretion of estrogen from the ovary) and ovary-independent proliferation and cornification of the vaginal epithelium [7–9]. The latter vaginal changes, induced via direct action of estrogen on the developing vagina, are irreversible and frequently lead to the development of cancerous lesions when the mice become adults. The toxic effects of estrogen appear to be a result of disrupted epithelial-mesenchymal interaction, the cellular and molecular mechanisms of which are largely unknown. Previous experiments, through investigation of recombination of the epithelium and stroma, have suggested that the initial permanent change induced by neonatal estrogens in the perinatal mouse vagina is that on the epithelium but not the stroma [10, 11] and that the permanent changes are linked to the unusual expression of the Tff1 gene on the vaginal epithelium [11].

The developmental effect of neonatal exposure to overdosed estrogens can be influenced by other bioactive factors such as vitamins and growth factors. In fact, vitamin A (retinol) and a FGF receptor 2 (IIIb) blocker attenuated the estrogen effects on the neonatal mouse vagina in our previous studies, which provided insights on the mechanisms by which estrogens misleads the developmental processes of the genital tracts [12, 13]. In the present study, the influence of vitamin D and its active derivates on the estrogen effects was explored for the first time. Vitamin D is one of hydrophobic vitamins, and can be ingested from a dietary source and/or synthesized by means of UV exposure in the skin from the pro-vitamins synthesized from cholesterol in the liver. Vitamin D₃ (VD₃), also designated cholecalciferol, is the major type of dietary and/or biosynthetic vitamin D in animals. In general, it is readily converted to 25-hydroxyvitamin D₃ (25(OH)D₃), a pro-active form of vitamin D, by a hepatic enzyme cytochrome
Animals and chemicals

C3H strain mice purchased from Clea Japan (Tokyo, Japan) were kept at the Laboratory Animal Resource Center of the University of Tsukuba. They were housed in a plastic cage in standard laboratory animal facilities with controlled lighting (14 h/day), the temperature controlled to 25 ± 1°C and food and water provided ad libitum. All experimental protocols involving animals complied with the guidelines of the University of Tsukuba Animal Care Committee. The C3H strain of mice was used in the present study instead of SHN mice, which used in our previous studies, as the latter mice, which were infected with murine mammary tumor virus (MTV), were difficult to keep in our present facility. So, we started by examining effects of exposure to estrogen, E₂, in this case, on the neonatal C3H-MTV mouse vagina in vivo and ex vivo. First, newborn pups were injected with E₂, and the vagina was then examined in adulthood after the ovariectomy. As expected, the vaginal epithelium was a thick stratified squamous one that consisted of several layers of cells and a keratinized luminal surface in neonatally estrogenized mice, while 2-3 layers of cuboidal cells were found in vehicle-treated controls. Thus, a typical permanent effect of neonatal exposure to estrogen on the vaginal development was confirmed in female C3H mice in vivo (data not shown).

Next, effects of E₂ on the developing vagina were examined in vivo. After transplantation in the ovariecotomized host for 35 days, the

VITAMIN D ATTENUATES DES SYNDROME

Permanent neonatal E₂ exposure-induced changes in the vaginal epithelium in C3H mice in vivo and ex vivo

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luminal surface of the vagina was mostly lined with tall mucinous cells (Fig. 1B) and/or a multilayered stratified squamous epithelium (Fig. 1C) in all (7/7; in 7 out 7 cases) of the E2-exposed vaginal tissues, while a thin epithelium consisted from 2-3 layers of cuboidal cells was observed in the control transplants (4/4) (Fig. 1A). The mucinous cells were also observed preceding estrogen-independent hyperplasia in our previous study on SHN mice [11] and considered to be an earlier symptom of the permanent changes induced by developmental estrogenization in the mouse vagina. In fact, after 70 days of ectopic culture under the renal capsule, a thick stratified squamous epithelium was observed in all specimens examined (3/3).

**Influence of VD$_3$ on the estrogen effect on the neonatal vagina ex vivo**

First, 1,25(OH)$_2$D was added together with E$_2$ to the culture media to see if the activated form of the vitamin has an influence on vaginal development. Interestingly, in the presence of 1 nM or a higher concentration of 1,25(OH)$_2$D, E$_2$ failed to induce any symptom of permanent changes in the vagina, and the vaginal epithelium appeared just like that in the vagina without E$_2$-exposure (Fig. 2A). At 0.1 nM, on the other hand, 1,25(OH)$_2$D did not affect the E$_2$ effects, and a thick mucinous epithelium was observed in the ectopic vaginal implant (Fig. 2B). Addition of 1,25(OH)$_2$D alone even at a high dose (100 nM) did not appear to influence vaginal development, and the transplants had a thin layer of epithelium (Fig. 2C). The activated vitamin D-exposed epithelium became thick in response to the exogenous estrogen injected into the host mice just 2 days before excision of the transplanted tissue (Fig. 2D), and therefore responsivity to estrogen in adulthood was not influenced by neonatal exposure to 1,25(OH)$_2$D.

Whether or not cholecalciferol and 25(OH)D affect the E$_2$ action that leads to a permanent change in the developing vagina was also investigated. In the presence of cholecalciferol, at least at the dose examined (up to 10 µM), E$_2$ altered the fate of the developing vagina and induced ovary-independent hyperplasia of the epithelium (Fig. 2F). Interestingly, on the other hand, a high dose (1 µM) of 25(OH)D did attenuate development of an abnormal vaginal epithelium induced by E$_2$ (Fig. 2E).
Expression of 25(OH)D 1α-hydroxylase in the neonatal mouse vagina

The fact that 25(OH)D acted like 1,25(OH)₂D in the isolated neonatal vagina suggested that activation of the pro-activated vitamin into the activated form occurred in the organ. The 1α-hydroxylation of 25(OH)D is catalyzed by Cyp27b1 which is known as a renal-specific enzyme in mice. So, amplification of Cyp27b1 gene mRNA by RT-PCR was performed in the neonatal vagina to clarify if the tissue expresses the gene. The results shown in Fig. 3 indicate that the neonatal vagina expresses Cyp27b1 mRNA.

Tff1 expression in the E₂- and/or 1,25(OH)₂D-exposed vaginal transplants

Tff1 is a molecular marker for the permanently modified epithelium in the neonatally E₂-exposed mouse vagina [11]. To confirm from the viewpoint of gene expression that 1,25(OH)₂D attenuated E₂-action in the developing vagina, expression of Tff1 gene mRNA was examined in the E₂- and/or 1,25(OH)₂D-exposed transplants by RT-PCR. As shown in Fig. 4, Tff1 mRNA was detectable in the E₂-exposed vaginal transplants after 35 days of incubation under the kidney capsule in the host mice but not in tissue exposed to both E₂ and 1,25(OH)₂D. The Tff1 gene was not induced in the latter tissue even when the host mice were injected with E₂ so that the vaginal epithelium would proliferate and form thick stratified squamous cell layers.

Distribution of VDR in the neonatal mouse vagina

The results above indicated that vaginal tissue from neonatal mice responded to activated vitamin D₃, which suggests existence of the receptor for the vitamin, VDR. To clarify which type(s) of vaginal cells is responsible for the vitamin D₃ action, the distribution of VDR in the neonatal mouse vagina was examined by immunohistochemistry (Fig. 5). As a result, almost all cell nuclei were found to be immunoreactive for VDR. The signaling in the epithelium appeared to be enhanced by E₂ exposure (Fig. 5C).

Attenuation of the permanent estrogen effect on neonatal vagina in vivo by the activated form of VD₃

The influence of 1,25(OH)₂D on the development of DES syndrome was examined in neonatal mice in vivo. When 1 pmole of 1,25(OH)₂D was injected simultaneously with DES daily for 4 days in the neonatal mice, many (5/8) of the pups died for unidentified reasons within several days after the first injection. The rest of the pups, however, grew up to be young adults without any abnormalities as far as we knew based on our daily conventional observations, such as monitoring of weight and suckling behavior. Their vaginas showed no symptoms of the permanent changes, and the epithelium consisted of 2-3 layers of cuboidal epithelial cells after ovariectomy (Fig. 6C). The lower dose (0.1 pmole) of 1,25(OH)₂D showed neither lethal effects on the neonates nor an inhibitory influence on the neonatal E₂ effects (Fig. 6D). Thus, a sublethal level of activated vitamin D₃ attenuated the direct and permanent effects of estrogen on the neonatal mouse vagina.

Discussion

The main finding of the present study is that activated vitamin D is an effective modulator of estrogen action and attenuates the E₂ effects that make the neonatal mouse vagina prone to the permanent alterations in the later developmental program of the epithelium. Our preliminary experiments in vivo failed to find an appropriate dose of vitamin D to explore its effects on the neonates, and we gave up exploring further the influence of vitamin D on mouse vaginal development (data not shown). The present study suggests that our previous frustration came partly from the narrow range for an effective dose of activated vitamin D between a lethally toxic overdose and a noneffective underdose. Establishing an ex vivo
The mechanisms by which activated vitamin D influences the developing mouse vagina might be beyond the scope of the present study. Nevertheless, the distribution of VDR shown in Fig. 5 suggests that vitamin D could act through VDR upon both the epithelium and stroma in the developing mouse vagina. Considering the distribution of estrogen receptors (ERs) in the developing vagina [24] as well, vitamin D/VDR signaling may crossstalk and interfere with estrogen/ERs signaling within a cell or those of different cells through other peripheral cell signaling factors that mediate and/or influence on the estrogen effects in the genital tracts [25–27]. For example, vitamin D/VDR may inhibit KGF/KGFR signaling that is essential for estrogen to evoke permanent changes in the developing vagina [13, 19, 28]. It is an interesting fact that both vitamin A and D attenuated estrogen effects. Both VDR and the retinoic acid receptor (RAR) belong to a subgroup of nuclear receptors that bind with RXR to form “nonpermissive” heterodimers [29]. Specific intranuclear environments governed by these transcriptional factors might be required for overdosed estrogens to act on the developing vaginal epithelium. In any case, further studies are required to explore mechanisms by which VDR signaling influences estrogen receptor (ER) signaling in the developing mouse vagina.

Another interesting finding of the present study is the action of 25(OH)D on the developing vagina and corresponding expression of Cyp27b1 in the organ. This result was surprising because expression of the Cyp27b1 gene is usually restricted to the kidney and brain in mice, although it is more broadly expressed in humans [30]. Cyp27b1 has been identified as the sole 25(OH)D 1α-hydroxylase in many species, including the mouse and human. Taken together, vagina-borne Cyp27b1 catalyzed 1α-hydroxylation of 25(OH)D to produce 1,25(OH)2D, which appeared to be responsible for the attenuation of the estrogen effects in the neonatal mouse vagina. On the other hand, cholecalciferol did not have any influence on the E2 effect, and consistent expression of the Cyp2r1 gene, a liver- and bone-specific enzyme in mice, was not detectable in the vagina. Although recent analysis of Cyp2r1 gene-deficient mice has revealed that Cyp2r1 is not an exclusive enzyme responsible for 25(OH)D production [31], it seems clear that the developing vagina does not produce enough 25(OH)VD3 to interfere with the exogenous overdosed E2 effects.

![Fig. 5. Distribution of VDR in the neonatal mouse vagina. The localization of VDR protein was examined by immunohistochemistry in the neonatal mouse vagina. The vagina tissue sections from an intact mouse (A, B) and those from an estrogen-treated newborn (C, D) at day 3 postpartum were immunostained for anti-VDR antibody (A, C) or control rat Ig (B, D). Note that cell nuclei in the epithelium and stroma were specifically immunostained. The arrows indicate the epithelium. The bar indicates 50 µm.](image)

![Fig. 6. Influence of 1,25(OH)2D on the neonatal DES-induced changes in vaginal development in vivo. 1,25(OH)2D was injected simultaneously with DES daily for 4 days in the neonatal mice, and the vagina was examined at young adulthood after ovariectomy. (A) vehicle-treated control, (B) neonatally DES-treated control, (C) DES with 1 pmole of 1,25(OH)2D, (D) DES with 0.1 pmole of 1,25(OH)2D. The bar indicates 50 µm.](image)
Estrogen exposure during the neonatal period leads to ovary-independent persistent proliferation and cornification in the vaginal epithelium in mice. In the *ex vivo* system used in the present study, development of a hyperplastic stratified squamous epithelium was delayed when compared with the case *in vivo*. Instead, unusual development of mucinous cells accompanied by Tgf1 gene expression was consistently observed, which has previously been identified as a definitive sign of the permanently modified vaginal epithelium induced by estrogen. The delay of the development might come from absence of the ovary *in situ* (as *ex vivo* cultured tissue was transplanted into an ovariectomized host) and therefore absence of estrogen that stimulates vaginal hyperplasia. In neonatal estrogenized mice in particular, changes in the brain lead to ovary-dependent estrogenic stimulation of the vagina but may contribute to the accelerated integration of development of an abnormally hyperplastic vaginal epithelium.

In conclusion, activated vitamin D directly acted on the neonatal mouse vagina and attenuated estrogen-induced permanent changes in the developmental program of the organ.

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