p21 and Notch Signalings in the Persistently Altered Vagina Induced by Neonatal Diethylstilbestrol Exposure in Mice

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ABSTRACT. Female reproductive organs show organ-specific morphological changes during estrous cycles. Perinatal exposure to natural and synthetic estrogens including diethylstilbestrol (DES) or estrogenic chemicals induces estrogen-independent persistent proliferation of vaginal epithelium in mice. To understand the underlying mechanism of the estrogen-independent persistent vaginal changes induced by perinatal DES exposure, we examined global gene expressions in the vaginae of ovariectomized adult mice exposed neonatally to DES using a microarray. The cell cycle-related gene, p21, a cyclin-dependent kinase inhibitor, showed upregulation in the vagina, and p21 protein was localized in the basal layer of the vaginal epithelium in mice exposed neonatally to DES and an estrogen receptor α agonist, propyl pyrazole triol (PPT). The expressions of Notch receptors and Notch ligands were unchanged; however, the mRNAs of hairy-related basic helix-loop-helix (bHLH) transcription factor genes, Hes1, Hey1 and Hey2, were persistently downregulated in the vagina, indicating estrogen-independent epithelial cell proliferation in mice exposed neonatally to DES and PPT.

KEY WORDS: diethylstilbestrol, neonatal exposure, p21 and Notch signalings, persistent vaginal changes, vagina.


Long-term estrogenic stimulation is a known risk factor for carcinogenesis in laboratory animals and humans [25, 26]. In humans, transplacental exposure to a synthetic estrogen, diethylstilbestrol (DES), induced vaginal clear-cell adenocarcinoma in young women [16]. In mice, developmental exposure to estrogens within a critical developmental period elicits various permanent alternations in female reproductive tracts [5, 7, 29, 30, 32–35, 47]. For example, neonatal estrogen administration induces persistent vaginal epithelial cell proliferation and keratinization even after ovariectomy, resulting in hyperplastic lesions and vaginal cancers later in life [8, 27, 48].

Previously, we characterized the mRNA expression patterns in the neonatal mouse vagina exposed to DES at different ages and the persistently altered vagina resulting from neonatal DES exposure using a DNA microarray and real-time quantitative RT-PCR [30, 46]. In the vagina of mice exposed neonatally to DES, expressions of various genes were modulated, and interleukin-I (IL-I) and insulin-like growth factor-I (IGF-I) signalings were activated without estrogen stimulation [30]. In particular, IGF-I receptor (IGF-IR) and its downstream factor, Akt, were phosphorylated, which may lead to persistent cell proliferation and differentiation in the mouse vagina [29, 30]. The vaginae of mice exposed to DES at different ages showed that genes related to keratinocyte differentiation and cell cycle-related genes, such as Gadd45a, 14-3-3 sigma, Spry2 (small proline-rich protein 2), Klf4 (Kruppel-like factor 4) and p21, were induced by DES [46].

p21 (also called WAF1, CAP20, Cip1 and Sdi1) [6, 10, 38, 56], a founding member of the Cip/Kip family of CKIs including p27 [41, 52] and p57 [21, 52], can bind and inhibit a broad range of cyclin/Cdk complexes, with a preference for those containing Cdk2 [11, 56]. It plays an essential role in growth arrest after DNA damage [1, 2, 4], and its overexpression leads to G1 and G2 arrest [37] or S-phase arrest [39]. Moreover, the anti-oncogenic effect of Notch family genes, one of the fundamental signaling pathways that regulate metazoan development and adult tissue homeostasis, appear to be mediated by p21 and by repression of Shh and Wnt signalings [3, 36, 50]. Wnt signaling is suppressed by Notch1 activation in keratinocytes, showing that Notch1 activation downregulates this pathway by suppressing Wnt-4 expression [3]. p21 mediates this negative regulation; Notch1 activation increases p21 protein levels, which is subsequently associated with E2F1 transcription factors at
the Wnt4 promoter, downregulating Wnt4 expression [3]. On the other hand, p21 is often responsible for stress-induced p53-dependent and p53-independent cell cycle arrest, which permits cells to pause and to repair damage and then to continue cell division [9], and p21 expression has been shown to be regulated largely at the transcriptional level by both p53-dependent and p53-independent mechanisms [9].

We therefore focused on the p21, p53 and Notch signaling in order to understand the molecular mechanisms underlying the persistently altered vagina resulting from neonatal DES exposure in mice.

MATERIALS AND METHODS

Reagents: Diethylstilbestrol (DES) was obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). An estrogen receptor α (ERα) specific ligand, 4,4',4''-(4-propyl-[1H]-pyrazole-1,3,5-triyl)trisphenol (propyl pyrazole triol, PPT), and ERβ specific ligand, 2,3-bis(4-hydroxyphenyl)-propionitrile (diarylpropionitrile, DPN), were obtained from Tocris Bioscience (Ellisville, MO, U.S.A.). Sesame oil and dimethyl sulfoxide (DMSO) were obtained from Kanto Pharmaceutical Co., Ltd. (Tokyo, Japan).

Animals and treatments: C57BL/6J mice purchased from CLEA Japan (Tokyo, Japan) were maintained under a 12 hr light/12 hr dark cycle at 23–25°C and fed laboratory chow (CE-2, CLEA) and tap water ad libitum. All procedures and protocols were approved by the Institutional Animal Care and Use Committee at the National Institute for Basic Biology, National Institutes of Natural Sciences.

The middle part of the vagina, Müllerian duct origin, was used for the current study. Newborn female mice were given 5 daily subcutaneous (s.c.) injections of 2.5 µg DES/g body weight (bw) (n=6) dissolved in sesame oil or the oil vehicle alone (n=6) beginning from day 0 (the day of birth). These mice were ovariectomized at 8 weeks of age and sacrificed at 10 weeks of age (n=6, in each experimental group). These mice were used for DNA microarray analysis.

Newborn females were given 5 daily s.c. injections of 2.5 µg DES/g bw (n=6), 25 µg/g bw PPT (n=4) or DPN (n=4) dissolved in 5.6% DMSO or the vehicle alone (n=4) beginning from day 0. These mice, ovariectomized at 13 weeks of age, were sacrificed at 15 weeks of age, and their vaginas were used for real-time quantitative RT-PCR and immunohistochemistry. Since we have already demonstrated that there is no age difference in histology and gene expression at least between 2–4 months of age in neonatally DES-exposed mice [29, 30, 32, 33], we used preserved mouse vaginas treated with the abovementioned chemicals to reduce animal use.

DNA microarray analysis: Total RNA from vaginas exposed neonatally to 2.5 µg DES/g bw or oil vehicle alone was extracted using TRIzol (Invitrogen, Carlsbad, CA, U.S.A.) and purified using an RNeasy mini kit (Qiagen, Chatsworth, CA, U.S.A.). Quality and quantity of total RNA were confirmed with an Agilent 2100 Bioanalyzer (Agilent, Palo Alto, CA, U.S.A.). cRNA probes were prepared from the purified RNA using an Affymetrix cRNA probe kit (Affymetrix, Santa Clara, CA, U.S.A.) according to the manufacturer’s protocol. All preparations met the recommended criteria of Affymetrix for application to their expression array. The amplified cRNA was hybridized to high-density oligonucleotide arrays (Mouse U74A; Affymetrix), and the scanned data were analyzed with GeneChip software (Affymetrix) and processed as described previously [55]. To confirm the estrogen-related changes in gene expression revealed by DNA microarray analysis, we independently repeated the same experiment twice. The expression data were analyzed with GeneSpring software (Agilent) as described previously [53–55].

Real-time quantitative RT-PCR: Total RNA, isolated with an RNeasy kit (Qiagen) from vaginas of each group, was used for real-time quantitative RT-PCR reactions carried out with SuperScript III reverse transcriptase (Invitrogen).

Changes in gene expression were confirmed and quantified using an ABI Prism 5700 Sequence Detection System (Applied Biosystems, Foster City, CA, U.S.A.) and SYBR Green Master Mix (Applied Biosystems) according to the manufacturer’s instructions. The PCR conditions were as follows: 50°C for 2 min, 95°C for 10 min, and 36 cycles of 95°C for 15 sec and 60°C for 1 min with a 15 µl reaction mixture. Relative RNA equivalents for each sample were obtained by standardization of ribosomal protein L8 levels. The sequences of the gene primer sets are given in Supplementary File. More than three pools of samples per group were run in triplicate to determine sample reproducibility, and the average relative RNA equivalents per sample were used for further analysis.

Immunohistochemistry: Tissues were fixed in 10% neutral buffered formalin, embedded in paraffin and sectioned at 4 µm. Deparaffinized sections were incubated with 0.3% H2O2 in methanol for 15 min to eliminate endogenous peroxidase. After washing with PBS, the sections were stained with a Universal LSAB™ 2 kit (Dako, Carpinteria, CA, U.S.A.) according to the manufacturer-supplied protocol. Anti-p21 monoclonal antibody was obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, U.S.A.). The sections were incubated at 1:50 dilution in PBS containing 1% BSA (Sigma) overnight at 4°C. For negative controls, murine IgG2b (Dako) was used at the same dilution.

Statistical analysis: Statistical analyses were performed using the Student’s t-test or Welch’s t-test followed by the F-test as appropriate. Differences with P<0.05 were considered significant.

RESULTS

DNA microarray analysis: The DNA microarray analysis revealed 11,219 of 22,689 Genechip probes in the vaginas of 10-week-old control mice or 2.5 µg DES-exposed mice ovariectomized at 8 weeks of age. Compared with the controls, 423 probes were upregulated and 351 probes were downregulated in the vagina exposed neonatally to DES (data not shown). We further analyzed genes related to the cell cycle. Compared with the oil controls, 26 genes (31 probes) were upregulated (Table 1), and 8 genes (10 probes)
were downregulated (Table 2) in the DES-exposed vagina.

**p21 and p53 mRNA expression:** Since the expression of p21 was specifically upregulated in the cell cycle-related genes compared with the controls, we analyzed p21 mRNA expression in vaginae of 15-week-old ovariectomized mice exposed neonatally to 25 µg DPN, 25 µg PPT and 2.5 µg DES. This was because we previously showed persistent proliferation and keratinization of epithelial cells in the vagina exposed neonatally to DES and PPT [33, 34]. The expression of p21 was very low in the vaginae of control mice, while p21 was upregulated in the vaginae of mice exposed neonatally to 25 µg DES and 25 µg PPT (Fig. 1). The mRNA of p53, unlike that of p21, was unaltered in the DES-, PPT- and DPN-exposed vagina (Fig. 1).

**p21 protein localization:** We performed immunohisto-
chemistry to investigate the localization of p21. Fifteen-week-old, ovariectomized mice neonatally exposed to 2.5 µg DES or the oil vehicle alone were stained with p21 antibody (Fig. 2A and 2C) or murine IgG2b as a negative control (Fig. 2B and 2D). p21 was localized in the basal layer of vaginal epithelial cells in mice exposed neonatally to DES (Fig. 2A), but no p21 staining was observed in the oil-treated controls (Fig. 2C), indicating that p21 expression was correlated with proliferation of the vaginal epithelial cells.

**Expression of Notch signaling pathway-related genes in the vagina of mice exposed neonatally to DES, PPT or DPN:**

We analyzed the expression of genes related to the Notch signaling pathway; however, all Notch receptors mRNA (Notch 1, Notch 2, Notch 3 and Notch 4) (Fig. 3A) and Notch ligands mRNA (Dil 1, Dil 4, Jagged 1 and Jagged 2) (Fig. 3B) were unaltered in the DES-exposed vagina. On the other hand, the expression of Notch target genes, Hes1, Hey1 and Hey2, was significantly decreased in the vagina exposed neonatally to DES or PPT compared with the control and neonatally DPN-treated mice (Fig. 3C).

**DISCUSSION**

Estrogens tightly regulate cell proliferation and differentiation in the female reproductive tracts [17, 48]. However, perinatal exposure to estrogens, including synthetic estrogen, DES and other estrogenic chemicals, induces persistent anovulation caused by alteration of the hypothalamo-pituitary-gonadal axis, polyovular follicles, uterine abnormalities and persistent vaginal changes in mice [5, 7, 16–18, 34, 35, 45–48]. In particular, transplacental exposure to DES, which was routinely prescribed to pregnant women for preventing miscarriages from the 1940s to 1970s, reportedly induced vaginal clear-cell adenocarcinoma in young women [16]. It has been hypothesized that in utero DES exposure influences the incidence of breast cancer, squamous neoplasia of the cervix and vagina and vaginal clear-cell adenocarcinoma later in life [13, 15, 16, 40].

DNA microarrays have been successfully used to analyze estrogen-responsive genes in the mouse vagina and genes possibly related to persistent vaginal epithelial cell proliferation induced by neonatal DES exposure [30, 33, 46, 55]. In the present study, we found upregulation of cell cycle-relat-
ed genes in the persistently proliferating vaginal epithelial cells of mice neonatally exposed to DES. In particular, p21 mRNA showed persistent upregulation in the vagina with irreversible proliferation, and p21 was localized in the basal layer of vaginal epithelial cells. However, the expression of p53 mRNA did not change in the DES- and PPT-exposed mouse vagina. A number of previous studies suggested that p21 was regulated by p53-independent mechanisms. Notably, many of these studies suggested that serum and other growth factors, e.g., epidermal growth factor (EGF), might be involved in the upregulation of p21 in various cell types [23, 28]. Moreover, Akt phosphorylated by PI3K leads to the stabilization of p21 and enhanced tumor cell survival [22]. Previously, we found the phosphorylation of Akt and persistent expression of some growth factors in the vagina neonatally exposed to DES [29, 30]. In the present study, p21 might be regulated by p53-independent mechanisms, and the expression of p21 might be involved in the persistent epithelial proliferation in the mouse vagina exposed neonatally to DES or PPT.

Notch family genes are evolutionarily conserved and participate in a variety of cellular processes, for example, cell fate decision (including proliferation, differentiation and apoptosis), cardiovascular development, endocrine development and cancer [19, 20, 42, 51]. The anti-oncogenic effect of Notch1 in murine skin appears to be mediated by p21 induction and by repression of Wnt signaling [3]. In the present study, the mRNA expressions of Notch receptors (Notch 1, Notch 2, Notch 3 and Notch 4) and Notch ligands (Dll 1, Dll 4, Jagged 1 and Jagged 2) were not changed in the vagina exposed neonatally to DES and PPT, suggesting that these signalings are not regulated by p21-dependent mechanisms and not involved in the persistent vaginal changes induced by neonatal DES or PPT exposure. Further studies are needed to clarify this phenomenon.

On the other hand, the expressions of other Notch target genes, i.e., Hes1 [43], Hey1 [24] and Heyl, were permanently downregulated in the vagina neonatally exposed to DES or PPT. Hes/Hey gene dosage is essential not only for generation of appropriate numbers of hair cells and supporting cells by controlling cell proliferation but also for hearing ability by regulating cell migration, cell alignment and polarity [49]. Moreover, overexpression of HEY-1 inhibits migration and proliferation, whereas, inhibition of HEY-1 expression disrupts the processes of alignment and tube formation and re-establishment of the mature vessel phenotype [14]. Irreversible epithelial proliferation in the vagina exposed neonatally to DES might be affected by downregulation of Hes1, Hey1 and Heyl. Ström et al. [12, 44] have previously shown that the expression of HES-1 is downregulated by 17β-estradiol (E2) and that forced expression of HES-1 inhibits an E2-mediated proliferation of breast cancer cells. Moreover, Müller et al. [31] have revealed a novel negative estrogen responsive element (ERE) associating with the HES-1 promoter, recruiting nuclear receptor co-regulators to the ERE in response to E2 and then docking in the HES-1 promoter region. In the present study, downregulation of Hes family genes in the vagina exposed neonatally to DES or PPT might be responsible, at least in part, for the irreversible epithelial proliferation caused by permanent activation of ERα [29, 30].

In conclusion, we demonstrated that the expressions of
both p21 mRNA and p21 protein are permanently upregulated and the Hey1 and Heyl mRNAs are permanently downregulated in the vagina exposed neonatally to DES. Additional elucidation of the molecular mechanism of the irreversible proliferation in vaginal epithelial cells is essential in the near future.

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