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REGULAR ARTICLE

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Prevention of neonatal estrogen imprinting by vitamin A as indicated by estrogen receptor expression in the mouse vagina

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Abstract Treatment of female mice with estrogen during the neonatal period induces estrogen-independent persistent proliferation and cornification of the vaginal epithelium when the animals become adults. However, the occurrence of such irreversible vaginal changes is blocked by concurrent retinol acetate (RA) treatment. This study aimed to determine the expression pattern of estrogen receptor (ER) α and β in the vaginas of ovariectomized 35-day-old mice treated neonatally with 17β-estradiol (E_2) and/or RA. The amounts of ERα and ERβ mRNA molecules in the vaginal RNA samples were determined by competitive reverse transcription/polymerase chain reaction. The levels of both mRNAs were lower in ovariectomized mice that had been treated neonatally with E_2 but not in those treated with E_2 plus RA. Neonatal E_2 treatment caused a decrease in the percentage of ERα-immunoreactive cells in the vaginal stroma during adulthood, and concurrent RA treatment inhibited the decrease. The amount of each ER mRNA was also measured in the vaginas of mature mice treated with E_2 and RA; no inhibitory activity of RA was seen in the mature mice. Our studies indicate that, in mouse vagina, the irreversible effects of neonatal imprinting by estrogen might be prevented by the simultaneous administration of vitamin A through the inhibition of a decrease of the number of ER-expressing cells.

Keywords Estrogen receptor · Vitamin A · Vagina · Competitive RT-PCR · Mouse (*Mus musculus*, SHN strain)

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Introduction

Disruption of the endocrine system during the early stages of animal life causes irreparable disorder of the developmental plan in many organs and leads to infertility, deformity, carcinogenesis, etc. (Herbst and Bern 1981; Arai et al. 1983; Mori and Nagasawa 1988; Safe 2000). Intrauterine exposure of female human fetuses to diethylstilbestrol (DES) is associated with a wide range of reproductive tract abnormalities, including vaginal cancer (Herbst et al. 1971). Prior to these findings, it had been well established that exposure of neonatal mice to physiological overdoses of estrogen or estrogenic substances resulted in abnormalities of the genital tract, such as ovary-independent proliferation and cornification of the vaginal epithelium (Takasugi et al. 1962; Takasugi 1976, 1979; McLachlan et al. 1982; Iguchi 1992; Boyd et al. 1996). The vaginal changes are irreversible and frequently develop into precancerous or cancerous lesions when the mouse becomes adult. In normal adult mice, ovary-derived estrogens are responsible for the periodic cornification of the vaginal epithelium during the estrous cycle. However, estrogen effects on the vaginal epithelial cells appear to be mediated by epithelial growth factor, which the stromal tissue secretes, through estrogen receptor (ER), although the epithelial response to estrogen involves both stromal and epithelial ERs (Buchanan et al. 1998; Hom et al. 1998). In addition, vaginal disorders after neonatal estrogen imprinting are caused by an alteration of the vaginal stroma rather than by an alteration of the epithelium or ovarian function under neuroendocrine control (Cooke et al. 1997). Thus, the irreversible proliferation of the vaginal epithelium in neonatally estrogenized mice is probably attributable to an estrogen-independent persistent activation downstream of estrogen signaling in the stromal cells. Indeed, a high level of expression of epidermal growth factor (EGF) is maintained in the vaginas of neonatally estrogenized mice even after ovariectomy (Falck and Forsberg 1996).

Recent studies of the changes induced by neonatal exposure to estrogenic substances have focused on the mo-

lecular changes in the target cells. Nevertheless, the way in which endocrine disruption by estrogenic substances during neonatal life leads to irreversible changes in the vaginal stroma remains unknown. As the first step to clarifying the way in which estrogen induces the irreversible proliferation of vaginal epithelial cells, the modulation of estrogen-specific receptors and the estrogen ligand-receptor complex, which activate or inactivate various target genes through interactions with DNA and other transcription-regulating factors, should be elucidated. Here, we have investigated the expression of ER α and β mRNAs in the vaginas of neonatally estrogenized adult mice and discuss the relationship between ER expression and estrogen-independent cornification of the vaginal epithelium.

On the other hand, we and others have previously found that concurrent injection of vitamin A inhibits estrogen-independent cornification of the vaginal epithelium in mice exposed neonatally to estrogens (Mori 1968, 1969; Takasugi and Tomooka 1976); the vaginal epithelium of the mice receiving neonatal estrogen administration in combination with retinol acetate (RA) appears normal. In the present study, the effect of neonatal treatment with both estrogen and vitamin A on the expression of ERα and β in the vagina has therefore also been examined and compared with the effect of estrogen alone.

Materials and methods

Animals

Newborn female mice of the SHN strain (Nagasawa et al. 1976) were divided into four groups within 24 h of birth. Two groups of mice were given subcutaneous injections of 20 µg 17β-estradiol (E_2) or E_2 together with 222 µg RA for 5 consecutive days from the day of birth (NE_2 or NE_2+NRA group), the daily doses being dissolved in 20 µl sesame oil. E_2 and RA was obtained from Sigma (St. Louis, Mo., USA) and was dissolved in dimethylsulfoxide (DMSO); it was used after dilution to 4% DMSO in 20 µl sesame oil. Mice of the remaining two groups received similar injections of 222 µg RA or 20 µl sesame oil containing 4% DMSO (NRA or NC group). All groups were ovariectomized at 25 days of age and killed at 35 days. Vaginal smears were checked daily from 26 to 35 days of age. Immediately after autopsy, the vaginas were dissected out and stored in liquid nitrogen until used.

In addition to the experiment on neonatal mice, intact adult SHN mice that showed a normal estrous cycle between 30 and 45 days of age were used as normal adults. They were divided into four groups and were ovariectomized at 45 days of age. Three groups of mice were treated daily with E_2 (AE_2 group), both E_2 and RA (AE_2+ARA group), or the vehicle only (AC group) from 50 to 54 days of age. The remaining group was treated with E_2 for 2 days only from 53 days of age: the AE_2(2d) group. The administration of E_2 and RA to the adults was carried out by the same method as used for the administration to neonates. After mice were killed at 55 days of age, the vaginal tissues were used for histological and biochemical analyses.

All mice were kept in a standard laboratory animal facility with controlled lighting (12 h/day) and temperature (25±0.5°C). All experiments were in accordance with the principles outlined in the Guide for Animal Care and Use Committee of the University of Tokyo and the regulations described in the NIH Guide for the Care and Use of Laboratory Animals.

Table 1 Primers for reverse transcription and PCR. The primers were designed to amplify a fragment corresponding to the mouse estrogen receptor α or β (*a* primers for ERα, *b* primers for ERβ, *RT* primers for reverse transcription, *F* sense primers, *R* antisense primers, *QF* and *QR* sense and antisense primers for competitive RT-PCR assay, respectively, *small letters* point-mutated nucleotides)

Primers	Sequence
a-1F	5′-TATGATCCTTCTAGACCCTTC -3′
a-1R	5′-TCCAgCATGCCTTCCACACA -3′
a-2F	5′-GGCATGcTGGAGATCTTTGACA -3′
a-2R	5′-CTcCCGGGGGTATGTAGTAGG -3′
a-QF	5′-GATGGGCTTATTGACCAACCTAGC -3′
a-QR	5′-TTGGCCATCAGGTGGATCAAAGTG -3′
a-RT	5′-GTTACTCATGTGCC -3′
b-1F	5′-GTaCTAGTGAGCCGTCCCAG -3′
b-1R	5′-ATaAGCTTGCCGGGGTGGTCG -3′
b-2F	5′-GCAAGCTtATCTTTGCTCCAG -3′
b-2R	5′-CTcTCGAGCAGCACTCAGACCC -3′
b-QF	5′-GATGTCCCTCACGAAGCTGGCTGAC-3′
b-QR	5′-CTTCGAAATCACCCAGACCAGGGCA-3′
b-RT	5′-GTTACTGATGTGCC -3′

Competitive reverse transcription/polymerase chain reaction

Competitor RNAs for the quantification of the amount of mouse ERα and β mRNAs by competitive reverse transcription/polymerase chain reaction (RT-PCR) were prepared by means of the procedure previously developed for prolactin receptor (Matsuda and Mori 1997; Imaoka et al. 1998). Additional *Sph*I and *Hin*dIII sites were introduced into the cDNA fragment of ERα and β, respectively, and the resultant cDNA fragments were used as the DNA templates for in vitro transcription of competitor RNAs. The competitor templates were made by joining two cDNA fragments corresponding to the ligand-binding domains of ERα and β amplified by PCR with specific primer sets: a-1F/a-1R or a-2F/a-2R, and b-1F/b-1R or b-2F/b-2R (Table 1), from the ovary and prostate, respectively. They were subcloned into pT7blue-2 vector (Novagen, Madison, Wis.), and the recombinant plasmids were transcribed in vitro with T7 RNA polymerase (Stratagene, La Jolla, Calif.). The synthetic RNAs were used as the competitors for quantitative RT-PCR after removal of the template DNA by DNase I (Stratagene) treatment. The RNA concentration was estimated by measuring the absorbance at 260 nm. Sequences of the primers used are listed in Table 1. The primary structure of the template plasmid DNA for ER competitor RNAs was confirmed by sequencing with an autosequencer: ABI-PRISM 310 (PE Applied Biosystems, Foster City, Calif.).

Total RNA was extracted from the tissues using Isogen (Nippongene, Tokyo), and its concentration was estimated from the absorbance at 260 nm. Total RNA (10 ng for ERα or 100 ng for ERβ) was mixed with the corresponding competitor RNAs, respectively, and reverse-transcribed with Superscript (Gibco-BRL, Gaithersburg, Mo.) and 10 µM each of a gene-specific reverse-transcription (RT) primer (a-RT or b-RT) in a 10-µl reaction mixture containing 0.5 mM dNTP mixture (Takara), 10 mM dithiothreitol (Gibco-BRL), and 20 U RNase inhibitor (Stratagene). ER cDNA was then amplified by 30 cycles of manually hot-started PCR with ExTaq DNA polymerase (Takara) in a 10-µl reaction mixture containing 10 kBq [α-^{32}P] dCTP (DuPont, Wilmington, Del.), 1 µl of the cDNA sample, and 50 pmol of primers (a-QF and a-QR, or b-QF and b-QR; Table 1). Each PCR cycle for ERα or β consisted of 94°C for 60 s, 55°C for 30 s, and 72°C for 60 s, or 94°C for 60 s, 62°C for 30 s, and 72°C for 60 s, respectively. Subsequently, the PCR product was completely digested with *Sph*I or *Hin*dIII and resolved by 5% polyacrylamide gel electrophoresis (PAGE). The radioactivities of non-digested (target mRNA-derived) product and cleaved (competitor-derived) product were

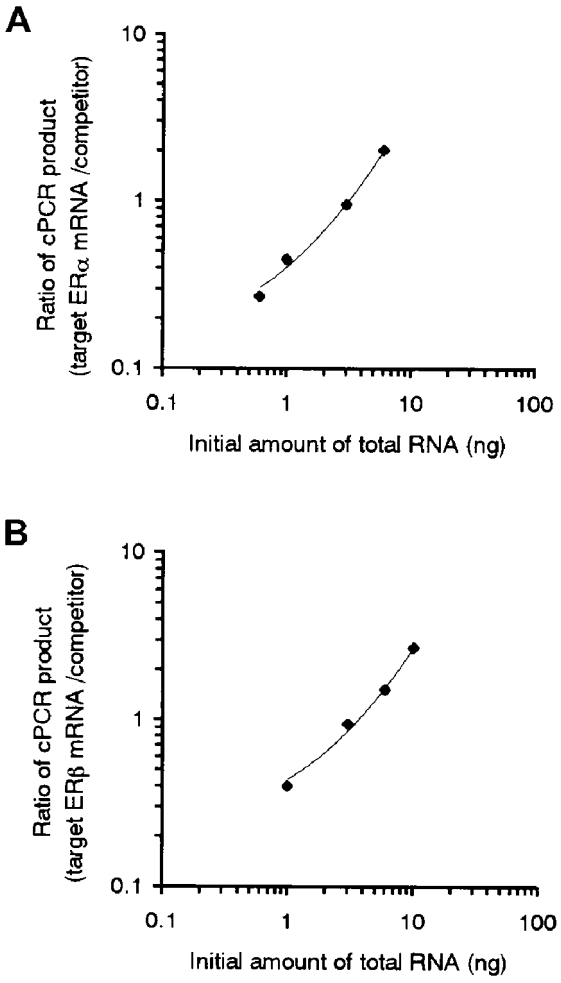

Fig. 1A, B Standard curves of the relationship between the initial amount of total RNA and the PCR ratio of target/competitor. The logarithms of the PCR product ratio were plotted against the initial amount of the total RNA from the vagina of a 35-day-old mouse. **A** ERα mRNA, **B** ERβ mRNA

measured on a photo-image-analyzing system (BAS-2500, Fuji Photo Film, Tokyo), and the initial amount of the target mRNA in total RNA samples was estimated from the ratio of these two PCR products. These estimated numbers of ERs were converted into numbers per 1 µg total RNA sample. All RT and PCR primers used in this study were obtained from Greiner Japan (Tokyo; Table 1). To examine the relationship between the initial amount of total RNA and the PCR ratio of target/competitor, the PCR product ratio was estimated by the competitive RT-PCR method by using a serially diluted total RNA sample from the vagina of a 35-day-old mouse. The logarithms of the PCR product ratio were plotted against the initial amount of the total RNA (Fig. 1). The amounts of ERα and β mRNAs were estimated within the linear range of this standard curve.

Immunohistochemistry

Detection of ERα was performed immunohistochemically in formalin-fixed paraffin-embedded vaginal tissue sections. ER was visualized with the ABC method (Vectastain Elite ABC Kit; Vector Laboratories, Burlingame, Calif.) by using a polyclonal anti-ERα antibody (SC542; Santa Cruz Biotechnology, Santa Cruz, Calif.). Briefly, 4-µm-thick tissue sections were rehydrated, heated in 10 mM sodium citrate pH 6.0, at 95°C for 5 min, treated with 0.3% hydrogen peroxide in methanol and then with 10% blocking serum, and incubated in the primary antibody solution diluted 1:200 for 30 min at room temperature. The primary antibody bound to antigens in the tissue section was detected with horseradish-peroxidase-labeled secondary antibody and 3,3'-diaminobenzidine tetrahydrochloride. Sections were very weakly counterstained with diluted Mayer's hematoxylin for 5 s. The proportion of cells with ERα-immunoreactive signals relative to the total number of stromal cells examined was determined in a 1-µm^2 area in three sections selected from every ten sections in the complete set of serial sections of upper part of each vagina.

Statistical analysis

Densitometric analysis of the intensity of the signals in the competitive RT-PCR was analyzed by using the MacBAS v2.52 program (Fuji Photo Film). All parameters were expressed as mean ± SEM. Statistical analysis was performed by analysis of variance (ANOVA), and $P<0.05$ was considered statistically significant.

Results

ERα mRNA expression in the vagina exposed neonatally to E_2 and/or RA

The amount of ERα mRNA was measured in 1-µg total RNA samples of the vaginas of 35-day-old mice treated neonatally with E_2 and/or RA by using competitive RT-PCR. There were no significant differences in the tissue weight or the amount of total RNA in the vaginas among all the groups of mice (data not shown). Figure 2A shows an example of a PAGE image of the competitive RT-PCR products digested with *Sph*I. The *Sph*I-cleaved (238 and 212 bp) and non-cleaved (450 bp) products originated from competitor RNA and target ERα mRNA, respectively, and the former signal was much stronger than the latter signal in the NE_2 group. Further quantitative densitometric analysis demonstrated that exposure to E_2 in the neonatal period (NE_2 group) caused a significant decrease (about 30% of the NC value) in ERα mRNA expression in the vagina when the mice became adult (Fig. 2B). However, the expression of vaginal ERα mRNA was affected only slightly by neonatal exposure to E_2 together with RA.

ERβ mRNA expression in the vagina exposed neonatally to E_2 and/or RA

The expression of ERβ mRNA in the vagina was also examined by competitive RT-PCR (Fig. 3). The amount of ERβ mRNA in the total RNA samples of the vagina was approximately 10^5 times lower than the amount of ERα mRNA. As in the case of ERα, however, neonatal E_2 injections significantly decreased ERβ mRNA content in the vaginas of 35-day-old ovariectomized mice, and this effect of neonatal E_2 was prevented by simultaneous injection of RA.

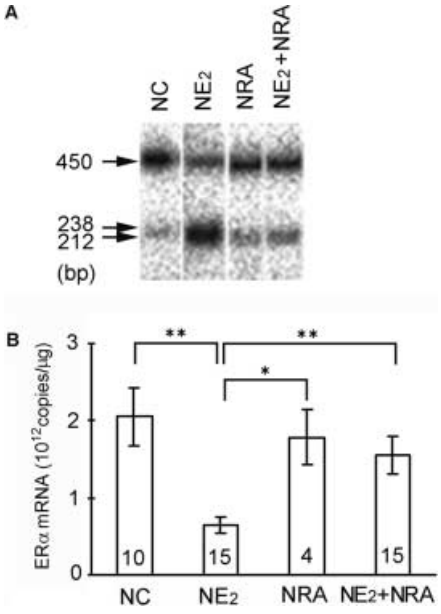

Fig. 2 **A** Representative results of competitive RT-PCR for ERα mRNA. The amount of ER mRNA in the vaginas of mice treated neonatally with E_2 and/or RA was normalized on the basis of the competitor mRNA concentration in the same strip. The 450-bp PCR product was derived from ERα mRNA, whereas the 238-bp and 212-bp cleaved products were from competitor RNAs. Four groups of female mice were used (*NC* a group of mice neonatally treated with vehicle only, *NE_2* treated with E_2, *NRA* treated with RA, *NE_2+NRA* treated with E_2 and RA). **B** Densitometric analysis of ERα mRNA expression in the vaginas of ovariectomized 35-day-old mice assessed by competitive RT-PCR. The number of mice examined is shown in each *column* (**P<0.01, *P<0.05)

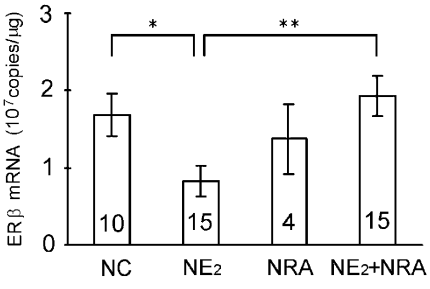

Fig. 3 Densitometric analysis of ERβ mRNA expression in the vaginas of ovariectomized 35-day-old mice assessed by competitive RT-PCR. Four groups of female mice were used (*NC* a group of mice neonatally treated with vehicle only, *NE_2* treated with E_2, *NRA* treated with RA, *NE_2+NRA* treated with E_2 and RA). The number of mice examined is shown in each *column* (**P<0.01, *P<0.05)

Effect of E_2 and RA on the expression of ERα and β mRNAs during adulthood

To examine the estrogen effect on ER mRNA expression and direct interaction of E_2 with vitamin A, the tissue content of ER mRNAs in the vagina was measured in ovariectomized adult mice treated with E_2 for a relatively short (2 days) or long (5 days) time (Fig. 4). The short-term treatment with E_2 significantly increased the

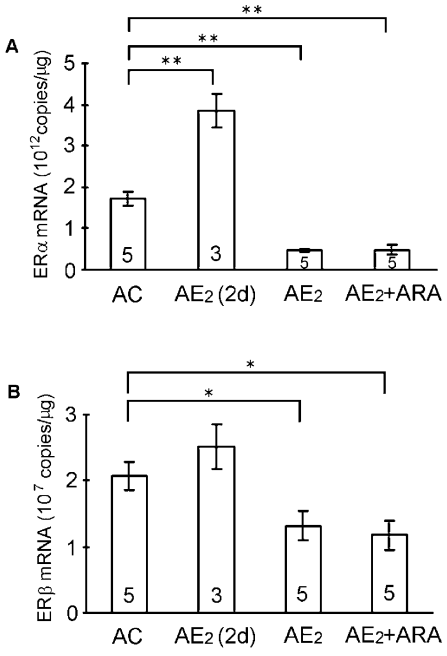

Fig. 4 Densitometric analysis of ERα (**A**) and ERβ (**B**) mRNA expression assessed by competitive RT-PCR in the vaginas of female mice ovariectomized at 45 days of age and exposed to vehicle only for 5 days from day 50 (*AC*), exposed to E_2 for 2 days from day 53 (*AE_2(2d)*), exposed to E_2 for 5 days from day 50 (*AE_2*), or exposed to E_2 and RA for 5 days from day 50 (*AE_2+ARA*). The number of mice examined is shown in each *column* (**P<0.01, *P<0.05)

expression of ERα mRNA. However, ERβ mRNA expression showed no significant increase after the short-term estrogenization because of a large standard error. On the other hand, the long-term treatment with E_2 resulted in the down-regulation of both ERα and β expression. In adult mice, concurrent treatment of RA with E_2 resulted in a significant decrease of ERα and β mRNA expression, similar to the effect of E_2 alone, suggesting that RA did not nullify the estrogen effect directly (Fig. 4).

Distribution of ERα-immunoreactive signals in the vaginal stroma

The tissue distribution of ERα was examined by immunohistochemistry in the vaginas of mice treated with E_2 and/or RA during the neonatal period or adulthood. Neonatal administration of E_2 resulted in the cornification of the vaginal epithelium, resulting in 5–9 layers of cells in all mice even after ovariectomy (NE_2 group; Fig. 5). These histological characteristics were similar to those of the vaginas of ovariectomized adult mice treated with E_2 before being killed. However, neonatal E_2 treatment in combination with RA failed to cause the ovary-independent cornification of the vaginal epithelium (NE_2+NRA group). Histological features of the vaginas of the NE_2+NRA group were identical to those of

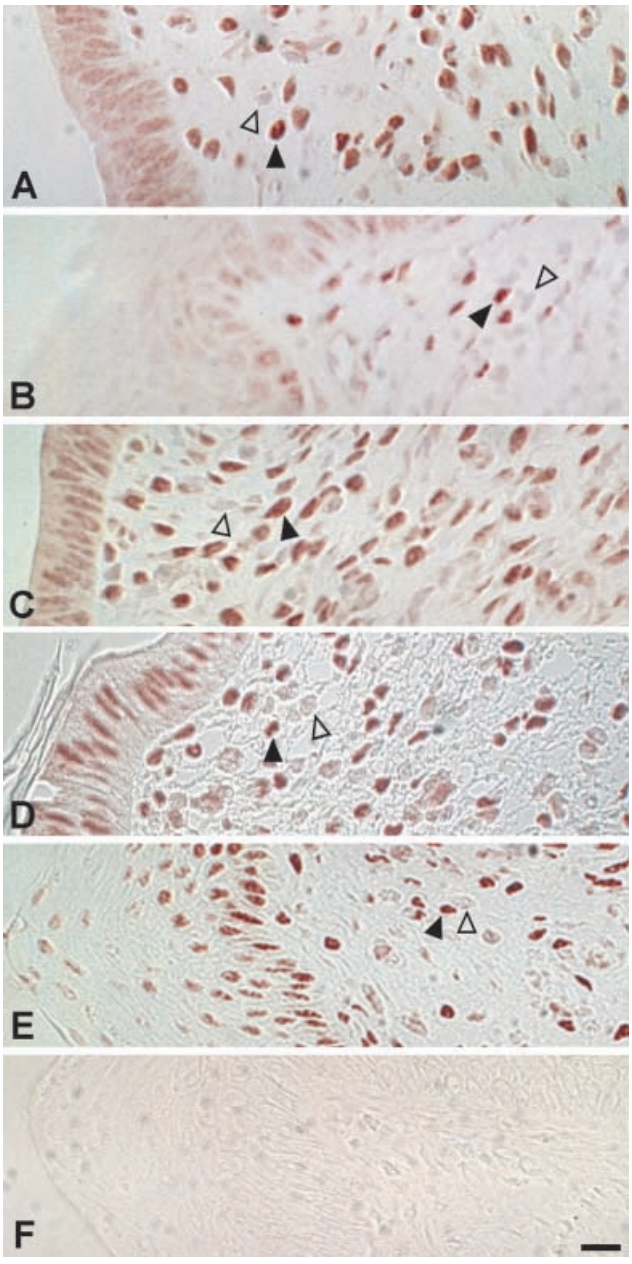

Fig. 5A–F Immunohistochemical localization of estrogen receptor (ER) α in the vaginas of mice. **A** Vagina from an ovariectomized 35-day-old mouse neonatally treated with vehicle only was processed for ERα immunohistochemistry. **B** Vagina from an ovariectomized 35-day-old mouse neonatally treated with E_2 for ERα. **C** Vagina from an ovariectomized 35-day-old mouse neonatally treated with E_2 and RA for ERα. **D** Vagina from an ovariectomized 55-day-old mouse given five daily injections of vehicle as an adult: ERα immunohistochemistry. **E** Vagina from an ovariectomized 55-day-old mouse given five daily injections of E_2 as an adult for ERα. **F** Vagina from an ovariectomized 55-day-old mouse given five daily injections of E_2 as adult: treatment with normal goat serum instead of ERα antibody. The sections were weakly counterstained with a diluted hematoxylin for 5 s; no ERα-positive signals were observed. Cornification of the vaginal epithelium consisting of 5–7 layers of cells is visible in **B** and **E**, but the epithelium is composed of 1–2 layers of cells without cornification in **A**, **C**, and **D** (*black arrowheads* ERα-positive cells, *open arrowheads* ERβ-negative cells). *Bar* 1 μm

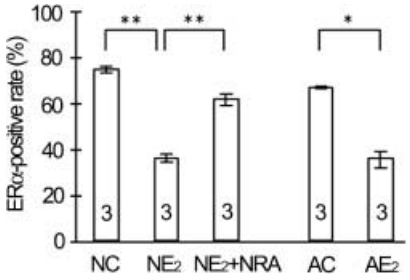

Fig. 6 The rate of ERα-positive cells per unit area in the vaginal stroma of ovariectomized 35-day-old mice neonatally treated with vehicle only (*NC*), treated with E_2 (*NE_2*), and treated with E_2 and RA (*NE_2+NRA*). Ovariectomized 55-day-old mice treated with vehicle only for 5 days from day 50 (*AC*) and with E_2 for 5 days (*AE_2*). The number of mice examined is shown in each *column* (**P<0.01)

the NC and NRA groups, the epithelium of which was composed of 1–2 layers of cells without cornification. Immunoreactivity against ERα was exclusively localized in some of the stromal cell nuclei in the vagina, except that weak immunoreactive-signals were observed in the nuclei of epithelial cells (Fig. 5). The majority of the stromal cells (about 75%) appeared to be ERα-immunoreactive in the vagina, with non-cornified epithelium in the NC, NE_2+NRA, NRA, and AC groups. In contrast, a significantly lower percentage (about 40%) of ERα-immunoreactive stromal cells was found in the vaginas with estrogen-independently cornified epithelium in the NE_2 group and estrogen-dependent cornified epithelium in AE_2 mice than in the comparable control groups (Fig. 6). These changes in the number of ERα-immunoreactive cells in the NE_2 and AE_2 groups paralleled the changes in the amount of ERα mRNA in the respective groups.

Discussion

Female rodents exposed neonatally to estradiol or DES show anovulatory syndrome associated with the development of estrogen-independent persistent vaginal hyperplasia and cornification (Takasugi 1976, 1979; McLachlan et al. 1982). The occurrence of those abnormalities is attributable to permanent perturbation of the hypothalamic-pituitary-gonadal axis and/or direct effects of estrogen on the developing organs (McLachlan et al. 1982). The direct effect of neonatal estrogen on the vagina leads to an irreversible activation of the estrogen signaling cascade without estrogenic stimulation. Persistent transcriptional activation of the stromal ER could be an immediate explanation for the vaginal change induced by neonatal estrogen treatment. However, our present results show that the expression of both ERα and β mRNAs in the vagina exposed neonatally to E_2 is not stimulated but rather decreased in mice at maturity. This result is consistent with previous reports demonstrating that neonatal DES treatment results in a consistent decrease in the

vaginal cytosolic ER measured by a binding assay (Bern et al. 1987) and in vaginal ERα mRNA assessed by Northern blot hybridization (Kamiya et al. 1996). The present results also indicate that ERα-immunoreactive nuclear signals are present in a relatively small number of stromal cells. Although ERβ-immunoreactive signals have not been examined in this study, it is unlikely that an extremely low level of ERβ mRNA expression would provide a biologically relevant level of receptor in the vagina. In addition, the decrease in the ER mRNA levels might not be attributable to a dilution of the ER mRNA by the RNA from the proliferating epithelial cells present in the samples of neonatally estrogenized vagina, since the epithelial cells show very weak immunoreactive signals for ER. Therefore, the overall decrease in ER mRNA expression may not be attributable to a decrease of the amount of ER expression in each stromal cell, but rather to a decrease of the number of ERα-expressing cells in the vaginal stroma. It has been reported that truncated ER, which is derived from alternative splicing and lacks the ligand-binding domain, is related to ligand-independent activation of estrogen signaling in human cancers (Fuqua et al. 1991; Fujimoto et al. 1997). In the mouse vagina, however, such a splicing variant of ER mRNA has not been detected by RT-PCR analysis with several sets of primers tested (unpublished data). These results suggest that the irreversible changes in the vagina caused by neonatal estrogen treatment may not be driven by the changes in ER expression per se but by a ligand-independent activation of the estrogen-signaling cascade in the stroma at one or more of the other steps.

In ovariectomized adult mice, relatively long-term (5-day) treatment with E_2 resulted in decreases in the expression of ERα and β mRNA in the vagina and ER-immunoreactive signals in vaginal stromal cells, whereas short-term (2-day) treatment up-regulated the vaginal ERα mRNA expression only. The vaginas of neonatally estrogenized and ovariectomized adult mice showed cornification of the epithelium and a decrease in the number of ER-immunoreactive cells similar to that of ovariectomized normal mice treated with estrogen for 5 days before killing. These observations also imply that estrogen-independent activation downstream of estrogen signaling persists in the stroma of neonatally estrogenized mice.

We and others have demonstrated that the histological changes induced in the vagina by neonatal exposure to estrogen are prevented by the concurrent injection of RA in mice (Mori 1968, 1969; Takasugi and Tomooka 1976). In this study, we have confirmed the inhibitory effect of RA on the action of neonatal estrogen treatment at the histological level. Furthermore, RA has been found to prevent the decrease of ER mRNA expression in the vaginas of mice exposed neonatally to estrogen. Studies of the molecular mechanism by which neonatal estrogenic substances cause irreversible changes in the vaginal stroma and of how vitamin A inhibits the estrogen effects have just been started. RA fails to inhibit the estrogen effects on ER mRNA expression in adult mice, at least at the dose we used. This may exclude the possibility that vitamin A simply counteracts the effects of estrogens. Disorder in vaginal histology induced by neonatal exposure to estrogen is not prevented by vitamin A if the vitamin A is not injected during the critical period (Mori 1969). Thus, the effects of estrogen and vitamin A on the developing vagina appear to be different from those on the mature vagina. According to Hom et al. (1998), EGF mediates estrogen's actions, leading to irreversible vaginal disorders in neonatal mice. Thus, it is possible that vitamin A inhibits the persistent expression of the EGF gene in the developing vagina exposed to estrogen.

Pretreatment with all-*trans*-retinoic acid before E_2 administration diminishes DNA synthesis and cell division in the uterine stromal and myometrial cells in immature rats (Boettger-Tong and Stancel 1995). Recently, we have found that, in neonatal male mice, vitamin A lowers the reactivity for estrogenic endocrine disrupters, leading to an irreversible malfunction of the reproductive organs (Nakahashi et al. 2001). The effects of estrogens and vitamin A on the reproductive organs of perinatal male and female mice may share an identical molecular mechanism, and development of the reproductive organs may proceed under a delicate balance of estrogens and vitamin A, since both of these are important morphogenetic inducers in developing organs (Van der Burg et al. 1999).

In conclusion, we have examined the ERα and β expression in vaginas of ovariectomized adult mice treated neonatally with E_2 and RA by using competitive RT-PCR and immunohistochemistry. The results show that neonatal injection of E_2 alone results in a decrease of ERα and β mRNA expression in the vagina, associated with persistent proliferation and cornification of the epithelium. However, concurrent administration of RA nullifies the neonatal imprinting and disorder brought about by E_2. Thus, the influence of endocrine disruption by estrogens and estrogenic substances immediately after birth is abolished by simultaneous exposure to vitamin A, at least in some developing organs.

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