Induction of Abnormal Epithelial Changes by Estrogen in Neonatal Mouse Vaginal Transplants

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ABSTRACT

Adenosis occurred in transplanted C57BL and BALB/c mice Mullerian-derived reproductive tract regions, cervix, and/or fornix (FX), and middle vagina but never in the urogenital sinus-derived portion of the vagina, after a 1-mo exposure to endogenous ovarian hormones or exogenous estradiol (E2). Grafts in ovariec-tomized hosts did not exhibit adenosis, confirming its dependence on estrogen. C57BL FX and midvaginal transplants from 1-, 3-, and 5-day-old donors but not from 7- or 10-day-old donors developed adenosis, indicating a critical period before day 6. Prolonged E2 exposure (to 2 mo) decreased the adenosis incidence observed in the C57BL FX group but not in midvaginal transplants. Progesterone added during the second half of transplantation to continuing exogenous E2 prevented this reduction in the FX group; however, adenosis incidence in the similarity treated middle vagina group was less than that observed after 1 or 2 mo of E2 treatment alone. Progesterone present throughout the 2-mo transplantation period did not significantly affect adenosis incidence induced by 2-mo exposure of midvaginal or FX grafts to E2 alone. Changes suggestive of squamous cell carcinoma were found in a few BALB/c midvaginal grafts after E2 exposure for 1 mo and in some C57BL midvaginal and FX grafts after E2 and progesterone exposure for 2 mo.

INTRODUCTION

The association between human fetal exposure to DES4 and the subsequent development of vaginal abnormalities including the presence of glandular columnar epithelium in the vagina (vaginal adenosis) and portio vaginalis of the cervix (cervical ectropion) has been well documented (1, 2). Several investigators have speculated that vaginal adenosis could be a premalignant stage in the development of adenocarcinoma (3–7). A possible association between human fetal exposure to DES and adenocarcinoma of the cervix and vagina has been suggested (8). However, most studies examining the effects of sex steroids on the reproductive tract separated neonatally and transplanted. A critical period was determined for the induction of abnormal epithelial changes in areas derived from the Mullerian ducts.

MATERIALS AND METHODS

Series 1. The reproductive tracts from 1-day-old (within 24 h of birth) female BALB/cCrgl and C57BL/Crgl mice were freed from the urethra and separated into 3 regions under a dissecting microscope (Chart 1), CX/FX (endo- and ectocervical and fornicai tissues combined), middle vagina, and urogenital sinus-derived vagina. These regions were transplanted for 1 mo into syngeneic hosts, into cleared inguinal mammary fat pads, or s.c. Hosts were left intact, ovariectomized, and given a Silastic implant of cholesterol (as a control), or ovariectomized and given a Silastic implant of E2. Mice were housed in plastic cages under a 12-h light/12-h dark schedule in a temperature (72°F)-controlled room and fed Purina rat chow and water ad libitum.

Series 2. Reproductive tracts from 1-, 3-, 5-, 7- and 10-day-old neonatal female C57BL mice were dissected as in Series 1 and separated into CX (endocervical tissue), FX and midvaginal regions (Chart 1) and miduterine segments. These tissues were transplanted for 1 mo under the renal capsule of syngeneic hosts. Hosts were ovariectomized and given a Silastic implant of cholesterol or an E2 silastic implant.

Series 3. CX, FX, and midvaginal regions and miduterine segments from 1- and 7-day-old female C57BL mice were transplanted under the renal capsule of syngeneic hosts. Hosts were ovariectomized and given one of the following treatments: (a) cholesterol Silastic implant; (b) E2 Silastic implant; or (c) E2 and PG Silastic implants at the time of transplantation. After 1 mo, the hormonal environment was altered by (a) removal of E2, (b) removal of E2 and addition of PG or (c) addition of PG to a group given E2 alone for the first mo of transplantation. Grafts were recovered 2 mo after initiation of transplantation.

Implants. The Silastic (inside diameter, 0.058 in.; outside diameter, 0.077 in.; Dow Corning, Midland, MI) implants were packed with a 2-mm length of crystalline E2 (Calbiochem-Behring, La Jolla, CA) or cholesterol (Sigma, St. Louis, MO) or a 5-mm length of crystalline PG (Calbiochem-Behring) and sealed with 1-mm plugs of Silastic medical grade adhesive (Dow Corning). All implants were s.c. Previous observations (9) and our initial pilot studies indicated that in mice bearing similar E2 or PG implants, regularly monitored vaginal smears were characteristically cornified or mucified, respectively, for the duration of these experiments.

All grafts were dissected, fixed in Bouin’s fluid, embedded in Paraplast (Sherwood Medical, St. Louis, MO), and serially sectioned at 7 μm. Sections were stained with Harris’s hematoxylin and eosin. Data were analyzed by Fisher’s exact probability test.

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4 The abbreviations used are: DES, diethylstilbestrol; E2, 17β-estradiol; PG, progesterone; CX, cervix; FX, fornix.

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EPITHELIAL CHANGES IN MOUSE VAGINAL TRANSPLANTS

Table 1
Incidence of adenosis in transplanted regions of mouse cervicovaginal tract recovered 1 mo after transplantation into the inguinal mammary fat pad s.c. or under the renal capsule (data combined) of syngeneic mice ovariectomized, left intact, or ovariectomized and given estradiol

<table>
<thead>
<tr>
<th>Region transplanted (1-day-old donor)</th>
<th>C57BL host</th>
<th>BALB/c host</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ovariectomized</td>
<td>Intact</td>
</tr>
<tr>
<td></td>
<td>+ cholesterol</td>
<td></td>
</tr>
<tr>
<td>Fomix/cervix</td>
<td>0/24</td>
<td>20/30 (67)</td>
</tr>
<tr>
<td>Middle vagina</td>
<td>0/21</td>
<td>1/25 (4)</td>
</tr>
<tr>
<td>Sinus</td>
<td>0/11</td>
<td>0/19</td>
</tr>
</tbody>
</table>

* Numbers in parentheses, percentage of incidence.

b Three of 18 showed squamous cell carcinoma-like lesions.

RESULTS

Series 1. Transplants of Müllerian duct origin (CX/FX and middle vagina) from both BALB/c and C57BL mice survived and proliferated for 1 mo in all sites, under the renal capsule, in cleared inguinal mammary fat pads, or in the subcutis of syngeneic hosts. Transplants of urogenital sinus origin survived and grew poorly in all host sites, even in the presence of the host's ovaries or of E₂ from a Silastic implant. The kidney capsule proved to be the most favorable site based on transplant size and percentage survival (data not shown). In later series, only the renal capsule was utilized as a transplant site.

Recovered grafts of vaginal tissues of Müllerian duct and urogenital sinus origin consisted of closed spheres lined by 1–2 layers of cuboidal cells after transplantation in nonhormonally treated ovariectomized hosts (given cholesterol) (Fig. 1). However, transplants from ovariectomized hosts given E₂ were lined by stratified squamous epithelium with superficially keratinized layers. Sloughed cells in the lumen of CX/FX and midvaginal grafts from intact hosts exhibited a layered pattern of leukocytes, keratinized and nucleated epithelial cells indicating responsiveness of the grafts to the cyclic release of hormones from host ovaries.

Adenosis, defined as a cluster of isolated columnar cells juxtaposed to the basement membrane and found abnormally lining the lumen or as glandular downgrowths in the stroma (Figs. 2 and 3), appeared in both regions of Müllerian origin (CX/FX and middle vagina) transplanted into ovariectomized hosts bearing a Silastic E₂ implant in both strains of mice (Table 1). Adenosis, often with associated cuboidal cell nodules, was found in transplanted midvaginal and CX/FX regions from C57BL donors but only in CX/FX from BALB/c donors transplanted into intact hosts (Table 1). The host's endogenous ovarian hormones (presumably estrogen) induced adenosis in midvaginal and/or CX/FX regions but the incidence was lower than that encountered after treatment of ovariectomized hosts with a higher dose of continuous E₂ (from a Silastic implant). Adenosis and nodules were never found in the sinus-derived transplants recovered from any host or in Müllerian-derived tissue recovered from ovariectomized hosts given cholesterol implants.

Squamous cell carcinoma-like lesions occasionally exhibiting glandular elements were found in 3 of 18 BALB/c midvaginal transplants from E₂-treated ovariectomized hosts (Fig. 4). Since columnar epithelium, indistinguishable from adenosis, is normally encountered in the upper common cervical canal, in the next experiment the CX (containing ecto- and endocervical epithelium) was transplanted separately from the upper vagina FX to study the effect of E₂ on these regions independently.

Series 2. One mo after transplantation in an E₂-treated host, columnar cells were found in the epithelial lining of C57BL FX and midvaginal transplants from 1-, 3- and 5-day-old donors and CX transplants from donors of all ages (Table 2). No adenosis was found in FX and midvaginal transplants from 7- or 10-day-old donors. The incidence of adenosis decreased with increasing donor age.

Uterine transplants were examined for the presence of squamous epithelium and/or nodules of cuboidal cells. All transplants from ovariectomized hosts exhibited low columnar epithelium.

Table 2
Incidence of adenosis in transplanted (under the renal capsule of syngeneic mice) vaginal tract regions from 1- to 10-day-old C57BL mouse donors 1 mo after different host treatments

<table>
<thead>
<tr>
<th>Host treatment Age of donor (days)</th>
<th>Adenosis incidence in region transplanted</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cervix</td>
</tr>
<tr>
<td>Ovariectomized</td>
<td>1</td>
</tr>
<tr>
<td>+ E₂</td>
<td>19/21 (90)</td>
</tr>
<tr>
<td></td>
<td>12/12 (100)</td>
</tr>
<tr>
<td></td>
<td>4/6 (67)</td>
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<tr>
<td></td>
<td>4/6 (67)</td>
</tr>
<tr>
<td></td>
<td>3/13 (23)</td>
</tr>
<tr>
<td>Ovariectomized + cholesterol</td>
<td>1–10</td>
</tr>
</tbody>
</table>

* Numbers in parentheses, percentage of incidence.
characteristic of the unstimulated uterus. The uterine transplants from E2-treated ovariectomized hosts had tall irregular columnar epithelium after 1 mo. Most uterine transplants from donors of all ages exposed to E2 were cystic. No squamous metaplasia was found; however, 1 of 6 transplants from a 1-day-old donor had a squamous cell nodule.

**Series 3.** Adenosis and nodules were observed in C57BL FX and midvaginal transplants from 1-day-old donors and columnar epithelium was encountered in CX transplants from 1- and 7-day-old donors transplanted under the renal capsule of syngeneic mice (Table 3). At 2 mo, CX and FX grafts exhibited 1–2 layers of epithelial cells after removal of E2 (1 mo after transplantation). The presence of cornified cells within the lumen indicated the action of previously administered E2. However, 6 of 7 similarly treated midvaginal transplants exhibited 3–7 layers of stratified squamous/cuboidal cells without superficial cornified layers.

The effect of prolonging E2 treatment to 2 mo (Series 3) appeared to decrease the incidence of adenosis in C57BL FX grafts (compared with Series 2, the FX group exposed to E2 for 1 mo only); however, there was no change in the incidence of columnar epithelium/adenosis in similarly treated midvaginal or CX transplant groups. The addition of PG during the second half of the transplantation period did not significantly decrease the adenosis incidence seen in the FX group after continued treatment with E2 alone for 2 mo; however, the sample size was small. The addition of PG to continued E2 during the second half of the transplantation period led to a significant decrease in the adenosis incidence in the midvaginal transplants (P < 0.02 compared with the group exposed to E2 alone for 2 mo). Exposure to PG during both halves of the transplantation period led to adenosis incidences similar to those encountered in the FX and middle vagina groups exposed solely to E2 for 2 mo. The incidence of columnar cells in the CX transplant group was not significantly altered by the presence of PG during any part of the transplantation period.

Squamous cell carcinoma-like lesions occasionally exhibiting glandular elements were found in 1 of 8 midvaginal transplants (1-day-old donors) and in 2 of 11 midvaginal transplants (7-day-old donors), and in 4 of 10 FX transplants (7-day-old donors) from ovariectomized hosts given E2 and PG for 2 mo. Two of 21 uterine transplants from 1-day-old donors in E2-treated hosts had squamous cell nodules. Most uterine grafts from E2-treated hosts formed cysts.

**DISCUSSION**

The predominant changes in vaginal epithelium of female mice exposed neonatally to estrogenic hormones, including DES, are epidermoid: persistent proliferation and cornification; hyperplastic downgrowths into the underlying stroma; and transplantable squamous cell tumors (10–18). However, adenosis has also been demonstrated in the endo- and ectocervix and/or vaginal fornix after neonatal E2 or DES treatment of females from 3 inbred strains and two outbred stocks of mice (4, 5, 19, 20, 23, 28, 29), and after prenatal DES treatment of CD-1 mice (22, 24). This study elaborates upon the neonatal time period and treatment required to induce adenosis in the mouse. Vaginal adenosis is the most frequent consequence of intrauterine DES exposure of human females (cf. Ref. 7).

In newborn mice, the upper and middle regions of the vagina, derived from Müllerian duct, are lined by a pseudostratified columnar epithelium. In contrast, the lower vaginal region derived from urogenital sinus is composed of a solid cord of cuboidal epithelial cells without a lumen (30). Forsberg (4) concluded that inhibition of epithelial differentiation is the primary action of estrogen on the neonatal mouse fornix (Müllerian derived) and that the estrogen-inhibited columnar epithelium represents the site of future adenosis. Gland-like downgrowths into the stroma have been shown to occur after puberty (20) and their formation was inhibited by ovariectomy (5). In the present study, adenosis was induced by endogenous ovarian hormones or by E2 treatment in the separated Müllerian-derived lower reproductive tract regions but never in the urogenital sinus-derived region. Alternatively Plapinger (31) proposed that the primary action of estrogen...
gen on the neonatal mouse vaginal fornix might be stimulation of a uterus-type epithelial response and that certain of the epithelial cells thus stimulated might be incipient adenosis cells.

The critical period for induction of estrogen-independent persistent proliferation and cornification of the vaginal epithelium was delineated by injection of E2 for 5 days starting at different postnatal ages. In C57BL/Tw mice, the critical period was within 4 days of age. However, vaginas of mice treated with E2 from 6 days of age showed some proliferation and mucification of the epithelium (32). In BALB/cCrGl mice, this critical period is within 3 days after birth (33). The present study demonstrates that the critical period for induction of adenosis in separated vaginal regions (FX and middle vagina) of C57BL mice was before the sixth day of life. The critical period for adenosis induction has not been reported for the intact vagina. Columnar epithelium was also found in C57BL CX transplants from all aged donors examined.

The progressive development of possibly retained (heterotopic) columnar epithelium to gland-like downgrowths into the stroma has been described as a postpubertal event controlled by ovarian factors (5, 20). We were unable to measure blood levels of E2 and PG; however, based on theoretical release rates (34), vaginal smear data, and reproductive tract histology (35), these steroid levels appeared higher than endogenous levels. It was not possible to distinguish between quantity of steroid administered and continuity of delivery as the factor responsible for the increased incidence of adenosis over that seen in intact mice. It is probable that both dose and delivery are responsible. The adenosis encountered in the present study was estrogen dependent since none was found in transplants grown in ovariectomized hosts given cholesterol or after removal of the E2 implant midway into the transplantation period. In contrast to previous reports, recent studies in our laboratory indicate that ovarian-independent adenosis can be induced by neonatal treatment with high doses of DES.5

A differential sensitivity of the various regions of the vagina to neonatal and later steroid exposure was observed (Table 3). E2 treatment between the first and second mo after transplantation may be associated with a reduction in the incidence of adenosis in FX but not in midvaginal transplants (Tables 2 and 3). This reduction is possibly the result of replacement of the adenosis by squamous metaplasia, perhaps analogous to that observed in the human with age (cf. Ref. 7). Exogenous estrogen promotes normal proliferation of stratified squamous vaginal epithelium and induces abnormal squamous metaplasia of uterine columnar epithelium (36). Progesterone (administered after 1 mo of treatment with E2 alone) tended to maintain adenosis in FX transplants presumably by interfering with E2-induced squamous metaplasia but reduced adenosis in midvaginal transplants. Such a difference in response to hormones present in early adulthood may explain previous reports of the rarity of adenosis in the vagina proper (middle vagina) and its presence in the fornix (humans, cf. Ref. 7; mice, cf. Ref. 31), although both regions are derived from the Müllerian vagina. Interestingly the presence of progesterone throughout the 2 mo of transplantation did not affect the incidence of E2-induced adenosis in FX or midvaginal transplants; the incidence after E2 alone was similar to that observed after combined E2 and PG treatment. This suggests that the presence of PG during the neonatal period may alter the response of the vagina to a later PG exposure.

Differential sensitivity of various regions of the intact neonatal vagina to estrogens has been suggested previously (30). Separated midvaginal and FX transplants responded differently to E2 present neonatally. Upon cessation of E2 exposure (after 1 mo), midvaginal transplants exhibited E2-independent stratified squamous epithelium in contrast to the atrophic appearance exhibited by FX transplants. In the caudal Müllerian vagina (corresponding with the midvaginal region in the present experiment) of estrogen-treated 5-day-old mice, the basal zone becomes hyperplastic and squamous cells proliferate cranially under the original columnar epithelium (4). Perhaps physical separation of the FX region from the middle vagina in our experiment prevents invasion of these cells which later exhibit E2-independent proliferation and stratification. The differential epithelial response could also be due to effects on the stroma leading to altered inductive capacity (cf. Ref. 37).

The gradual development of gland-like epithelial downgrowths among squamous-cell downgrowths has been suggested as a source of mixed tumors, adenocarcinoma and squamous cell carcinoma (4). In the present study, changes suggestive of squamous cell carcinoma often containing glandular elements were found in BALB/c midvaginal transplants from E2-treated ovariectomized hosts. Such downgrowths at this early age (1 mo) have not been reported in situ and may be the result of continuous excess exposure to estrogen of the developing transplanted tissue. Moreover similar lesions were found in C57BL midvaginal transplants from 1-day-old donors and FX and midvaginal transplants from 7-day-old donors maintained in ovariectomized hosts given E2 and PG Silastic implants for 2 mo. Since adenosis was rare in FX and midvaginal transplants from 7-day-old donors and 6 of 10 of the transplants that developed squamous cell carcinoma-like lesions were from 7-day-old donors, a transition from adenosis to squamous cell lesions seems unlikely. A recent report describing a case of adenosquamous carcinoma of the cervix in a DES daughter who did not exhibit adenosis (38) also suggests the lack of a direct link between adenosis and squamous cell cancer development in the human. Factors which determine expression of a tumor in affected individuals have yet to be elucidated.

Stratification of the uterine epithelium with or without squamous metaplasia has been described in adult mice receiving perinatal injections of estrogenic hormones (14, 15, 21, 27, 35, 39). In the present study, stratification of the uterine epithelium was also demonstrated in transplants from 1-day-old donors only but the incidence was low. As almost all uterine grafts from ovariectomized hosts given E2 implants underwent cystic atrophy, long-term transplantation of uterine segments is not a feasible procedure for the study of neonatally estrogen-induced changes.

Recently an in vivo model has been developed for the study of human urogenital tract differentiation involving transplantation of reproductive tracts from aborted human fetuses into athymic nude mice (40). Exposure of the developing human vagina transplanted under the renal capsule of the athymic nude mouse to DES resulted in glandular epithelium (adenosis) in the vagina. The host ovary did not affect either normal development or the induction of this abnormality in transplanted human tissues. Our results demonstrate that both ovarian factors from the host and

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5 T. Iguchi and P. L. Ostrander, unpublished data.
Exogenously administered E2 can induce adenosis in transplanted mouse vaginal regions. This difference may be attributable to a difference in sensitivity between the human and mouse reproductive tracts to the nature and quantity of host ovarian factors.

The present results suggest explanations for the regional distribution of adenosis in neonatally estrogen-treated mouse vagina for the critical period in which it is induced and for the apparent lack of a direct link between adenosis and early squamous cell cancer development in the mouse. However, more information is required to elucidate the basis for the demonstrated differential sensitivity of the various regions of the Mullerian-derived vagina to hormones administered neonatally and later in life. These results may have relevance to the induction and fate of vaginal adenosis induced in humans by in utero DES exposure.

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Fig. 1. Graft of C57BL mouse FX/CX region (1-day-old donor) 1 mo after transplantation into an ovariectomized adult syngeneic host. Note the atrophic appearance of the epithelium, 1-2 layers of cuboidal cells. Bar, 50 μm; H & E, ×270.

Fig. 2. Graft of C57BL mouse FX region (1-day-old donor) 1 mo after transplantation into an ovariectomized adult syngeneic host given E₂. Note the adenosis (simple columnar epithelium) (A) and squamous cell nodules (N) lining the lumen of the graft. Bar, 100 μm; H & E, ×140.

Fig. 3. Graft of C57BL mouse CX/FX region (1-day-old donor) 1 mo after transplantation into an ovariectomized adult syngeneic host given E₂. Note adenosis both lining the lumen (A) and forming glandular elements within the stroma (G). Bar, 50 μm; H & E, ×200.

Fig. 4. Graft of BALB/c mouse midvaginal region (1-day-old donor) 1 mo after transplantation into an ovariectomized adult syngeneic host given E₂. Note squamous cell carcinoma-like lesion (squamous epithelial growths) extending into the stroma. Bar, 100 μm; H & E, ×150.
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