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Changes in the Hormone Dependency of Epithelial Cell Proliferation in the Genital Tract of Mice Following Neonatal Oestrogen Treatment

D.F.C. Gibson, S.A. Roberts and G.S. Evans

The genital tract epithelium of the female laboratory mouse has been widely studied as a model of oestrogen-dependent growth and proliferation. Perturbation of the hormonal imprinting of these tissues during neonatal development has also been used to study the development of pathological abnormalities, particularly in the cervical epithelium. This study demonstrates that mice treated neonatally from days 1–5 with supraphysiological concentrations of oestrogen are able to maintain high levels of proliferation following the removal of the ovaries later in adult life. This high level of proliferation was shown to be independent of the ovarian oestrogens and of oestrogens produced peripherally by aromatisation. These results suggest conversion of the genital tract in these mice to a fully hormonal "independent" state. However, neonatal treatment with oestrogen was not found to produce a uniform change to hormonal independence. Further challenge of the adult ovariectomised mice with oestrogen, demonstrated that a population of cells still retained the ability to respond to the mitogenic influence of this hormone.
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INTRODUCTION

In 1971, a cluster of clear cell adenocarcinoma cases was reported in young women, which was linked to the intra-uterine exposure [1] to a synthetic oestrogen, diethylstilbestrol (DES). Earlier studies had also shown the development of pathological abnormalities in the genital tract of female mice exposed neonatally to high doses of oestrogen [2]. Similar effects were seen when oestrogen and DES were given during fetal mouse development, except that DES was effective at much lower doses than oestrogen. This apparent anomaly was thought to be the result of the protective effects of the high concentration of fetal α-fetoprotein, which binds to the natural but not the synthetic oestrogen [3]. Apart from this difference, the effects of oestradiol-17β and DES during early development are thought to be very similar, since the synthetic agent binds to the oestrogen receptor [4].

The development of the human reproductive tract during the first trimester (the critical period for DES exposure) closely correlates with that of the perinatal mouse, and so the mouse represents a useful model for investigating the effects of oestrogen upon the development of the genital tract [5]. A wide range of genital tract abnormalities have also been described

following prenatal (days 9–15) oestrogen exposure. These include persistent epithelial cornification, adenosis and adenocarcinoma of the vagina, cervical enlargement and squamous metaplasia and hyperplasia of the uterine epithelium [6, 7]. In addition to such morphological alterations, differences in growth responses, cellular differentiation [8] and receptor levels in the epithelium [9] have been reported.

Whilst such studies have described "persistent proliferation and mitotic" figures in the epithelium [10], there has been no detailed examination of the proliferation within the genital tract of mice treated neonatally with oestrogen. There is evidence that the proliferation in this model system is "ovary independent" [11], although complete "oestrogen independency" has yet to be demonstrated clearly. The aim of this study was to determine if neonatal treatment of mice with oestrogen produced an ovarian or fully oestrogen-independent proliferation in the genital tract, and whether these changes were uniform.

MATERIALS AND METHODS

Animals

Successful matings of female mice (B6D2F1; cross C57B6 × DBA2) were detected by the appearance of a vaginal plug. Such pregnant mice were housed individually, and the first appearance of the litter (average size 6–7) determined as day 1. The female neonates were maintained under a 12-h light/dark cycle (light 0700–1900 h GMT), in mixed stock rooms, and given food and water *ad libitum*.

At approximately 12 weeks of age, animals were ovariectomised via the dorsal route under anaesthesia. All animals were used for further experimentation at least 2 weeks after ovariectomy.

Drug administration

The female neonates were injected subcutaneously with oestradiol-17β (Sigma) dissolved in 0.01% ethyl alcohol and administered as 1 μg/g (body weight) in 0.05 ml of Arachis oil (BDH Chemicals, Poole, UK). This was administered neonatally and on a daily basis from days 1–5. The control regimen consisted of the alcohol/oil mixture only.

To examine whether oestrogens of non-ovarian sources were produced, the animals treated neonatally with oestrogen and oil were ovariectomised at 12 weeks, left for 14 days, and then treated with aromataze inhibitors. Two inhibitors were used in this study; 1,4,6-androstatrien-3, 17-dione (ATD, Steraloids, Croydon, UK) and aminoglutethimide (AG, Ciba-Giegy, Horsham, UK). ATD was initially dissolved in benzoyl alcohol (Sigma) and subsequently administered as 0.05 mg/g (body weight) in 0.05 ml Arachis oil. AG was dissolved in 0.3% hydroxypropylmethyl cellulose (Sigma) and then administered as 0.025 mg/g (body weight) in 0.1 ml of isotonic saline (Phoenix Pharmaceuticals, Gloucester, UK). These two agents were injected subcutaneously every 12 h for up to 5 days, and the control regimen consisting of carrier only was also given twice daily for the same period of time. In order to circumvent the effect of AG upon the adrenal cortex, the animals under treatment received drinking water containing 0.1% sodium chloride (BDH).

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ATD is a steroidal type I aromataze inhibitor, similar in shape to the natural substrate, and its mode of action is by competition for the binding site of the enzyme [12]. In comparison, AG is a non-steroidal type II inhibitor that binds to the cytochrome P-450 moiety of the aromataze enzyme some distance from the steroidal binding site, but which has no agonistic qualities [12].

For the experiments examining the effects of hormonal stimulation, female neonates were treated for 5 days after birth with oestradiol-17β or oil according to the method previously described. The animals were then ovariectomised at adulthood and 2 weeks later retreated with either 100 ng/g (body weight) oestradiol-17β in 0.1 ml of Arachis oil, or an equal volume of Arachis oil only as a control. These treatments were given only twice for a 24-h period at time 0 and 12 h later.

Tissue preparation

The complete genital tract (uterus, cervix and vagina) was removed, cleaned of adherent fatty tissue and placed in cold (4°C) Carnoys fixative (absolute alcohol, chloroform and propionic acid in a 6:3:1 proportions by volume) for 3 h. The tissues were then transferred to 70% alcohol and put into phosphate buffered saline (PBS, Oxoid, UK). Under a dissecting microscope, the tissues were further dissected free of any remaining adherent fatty tissue and the uterine horns separated from the lower tract by a transverse incision at the base of the horns. The upper (uterus) and lower (cervix and vagina) halves of the genital tract were then weighed separately.

The tissues were dehydrated and embedded in 2-hydroxypropylmethacrylate resin (HPMA; BioRad). Sagittal sections of 2 μm were cut along the length of the tissue, and to check the accuracy of the sectioning, the microtome was calibrated prior to the experiments, and found to cut with a thickness error of 10–15% for the 2 μm sections.

Tritiated thymidine labelling indices

40 minutes prior to killing, all mice were injected via the peritoneum with 0.925 MBq tritiated thymidine (^{3}H-TdR) of specific activity 185 GBq/mmol (Amersham International, Bucks, UK), to produce a pulse of labelled cells in DNA synthesis.

Scratch and fold-free Feulgen stained resin sections were dipped in Ilford K5 emulsion (Ilford, Mobberley, UK) (mixed 1:2 in distilled water) and exposed for 15 days in the dark at 4°C. The autoradiographs were then developed using standard conditions using D19 developer (Kodak) and Amfix (May and Baker, Dagenham, UK) and stained in 0.1% light green (BDH) for 1 min.

A systematic stratified counting procedure [13] was used to count at least 1000 epithelial and stromal cells in each animal in order to determine the ^{3}H-TdR labelling indices. These proliferative indices were expressed as a proportion of the total cells counted. Separate counts in the uterus of luminal and glandular cells were made, and of basal and suprabasal cells along the lower cervix to vagina. Given the degree of regression in the tracts of the ovariectomised control animals, it was not possible to accurately distinguish the extent of the cervical region. A more general comparison was therefore made between the stratified epithelium of the lower tract and the simple epithelium of the upper tract.

Cell numbers per organ

Using stereological point counting methods as previously described [14], the number of epithelial and stromal cells per

Table 1. Proliferative activities in the genital tissue of mice.

Tissue	Treatment group			
	E_2-E_2	E_2-CON	CON-E_2	CON-CON
Uterus				
Luminal epithelium	NS	NS	< 0.001	NS
Glandular				
Epithelium	0.033	NS	< 0.001	NS
Stromal	0.004	NS	NS	NS
Cervix and vagina				
Basal epithelium	NS	NS	< 0.001	NS
Suprabasal				
Epithelium	0.026	NS	< 0.001	NS
Stromal	NS	0.026	0.002	NS

P values, indicating any significant stimulatory effect upon proliferation. NS = not significant.

organ was estimated. This involved measuring the volume density of each tissue (epithelial or stromal) by point counting, and the density of the nuclei within microscope eyepiece grids. The crude counts of the nuclei were corrected for the effects of sectioning [14], and the total cellularity of each tissue (epithelial or stromal) was calculated as the tissue volume/density of the nuclei (epithelial or stromal) in a given volume of that tissue.

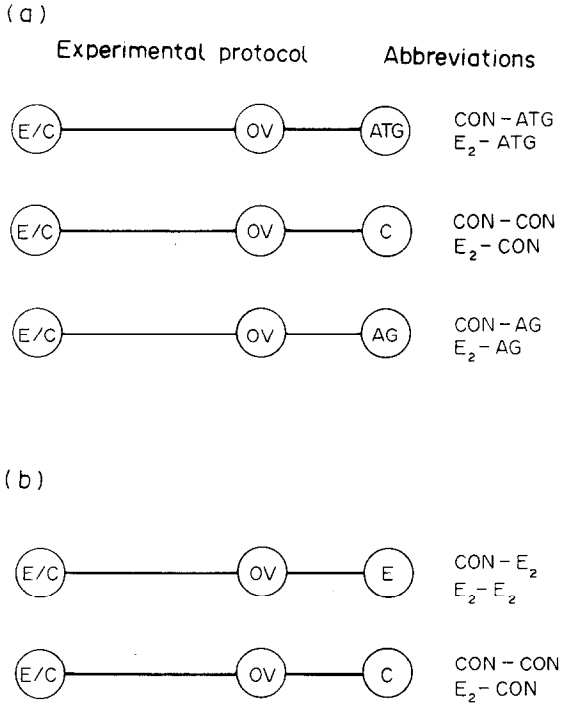

Fig. 1. Summary of experimental protocols for (a) aromatase inhibition experiments; and (b) oestrogen-17β stimulation experiments, where E = oestradiol-17β injections, C = control injections, OV = ovariectomy; ATD = 1,4,6 androstatrien-3, 17 dione; AG = aminoglutethimide. For all of the animals oestradiol-17β (1 μg/g in 0.01 ml Arachis oil) (or control oil) was injected at days 1–5, followed by ovariectomy at 12 weeks of age, and 14 days later (a) injections of ATD (0.05 mg/g in 0.05 ml of oil), AG (0.025 mg/g in 0.1 ml of carrier), or control injections, every 12 h for up to 5 days. (b) Two injections of oestradiol-17β (100 ng/g in 0.01 ml Arachis oil) or control oil, at time 0 and 12 h later.

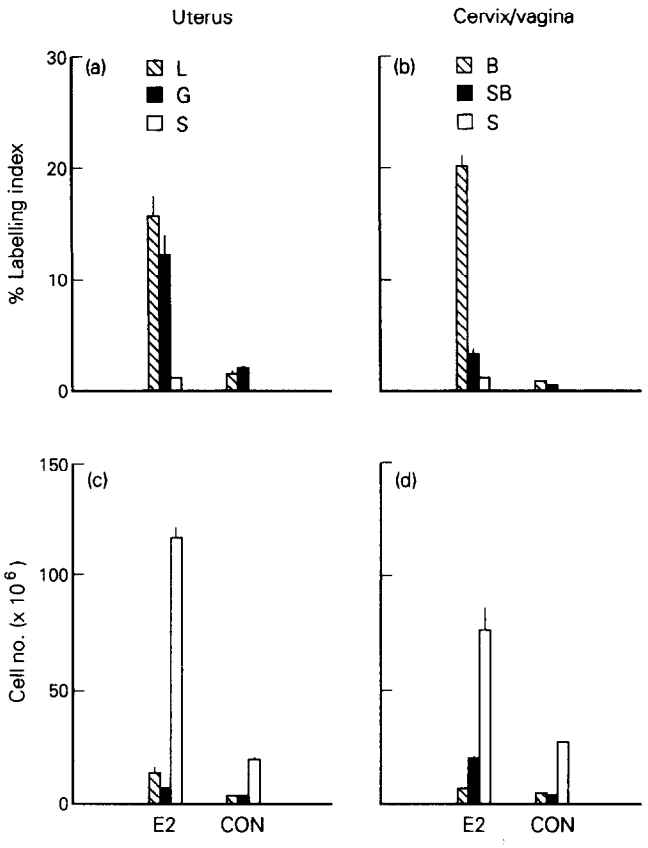

Fig. 2. (a,b) Tritiated thymidine labelling indices and (c, d) cell numbers per organ in the genital epithelium and stromal cells of mice treated with oestradiol-17β subsequently ovariectomised at adulthood (approximately 12 weeks of age) and killed 14 days later. Mean (S.E.), *n* = 12 animals per group. Kruskal-Wallis non-parametric analysis of variance was used to test for differences between the complete groups of data (where $P < 0.05$). L = uterine luminal; G = glandular epithelial cells; S = stromal cells; B = cervical and vaginal basal epithelium; SB = cervical and vaginal suprabasal epithelium.

No attempt was made to categorise all of the different constituent cell types in the stroma of these organs. Given that there are so many, this would require a quite separate study.

Data analysis

For the experiments with the aromataze inhibitors, the results were analysed using non-parametric statistical tests: Mann-Whitney *U* test for the experiments with two independent groups, and the Kruskal–Wallis one way analysis of variance by ranks for experiments with three or more independent groups. Probability values < 0.05 were taken as significant and are indicated for each test. For the experiments with the hormonal stimulation, the effects of stimulation were assessed by using linear regression on logarithmic (log [variable + 0.05]) transformed data, to test for a trend with time. *P* values < 0.05 were taken as significant and are indicated where there was a significant trend in the indices with time after treatment with oestradiol-17β.

Experimental groups

All mice were treated neonatally (from days 1–5), either with 1 μg/g oestradiol-17β in 0.05 ml Arachis oil, or equivalent volumes of oil as controls. They were then ovariectomised at 12 weeks of age and left 14 days before any additional experimental treatments.

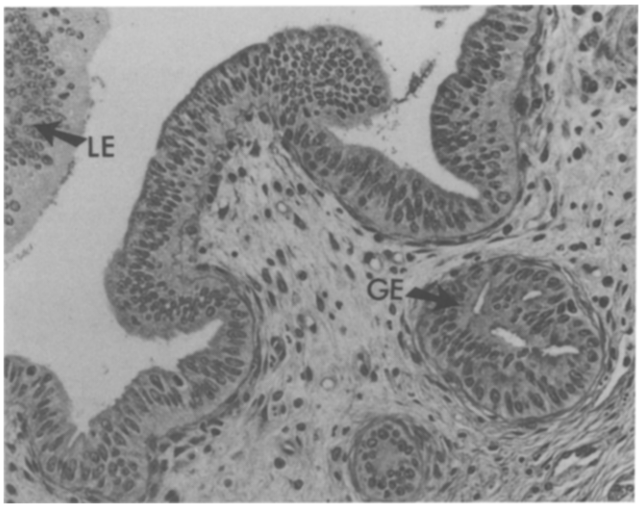

Fig. 3. Development of hyperplastic luminal (LE) and glandular (GE) epithelium of adult mice treated neonatally with oestradiol-17β and subsequently ovariectomised at adulthood. 2 μm HPMA resin sections stained with haematoxylin and phloxine B/orange G (magnification 300 ×).

To determine if non-ovarian sources of oestrogen were present, the ovariectomised mice were given injections of ATD (0.05 mg/g in 0.05 ml of oil), AG (0.025 mg/g in 0.1 ml of carrier), or control injections, every 12 h for up to 5 days.

To establish the hormonal responsiveness of these tissues, the ovariectomised mice were given two injections of oestradiol-17β (100 ng/g in 0.01 ml of Arachis oil) or control oil, at time 0 and 12 h later.

A summary of the protocols is shown in Figs. 1a, b.

RESULTS

The role of ovarian and peripheral sources of oestrogen as promoters of genital tract growth in animals treated neonatally with oestrogens

To examine what effect neonatal oestrogen treatment had upon the hormone dependency of the genital tract proliferation,

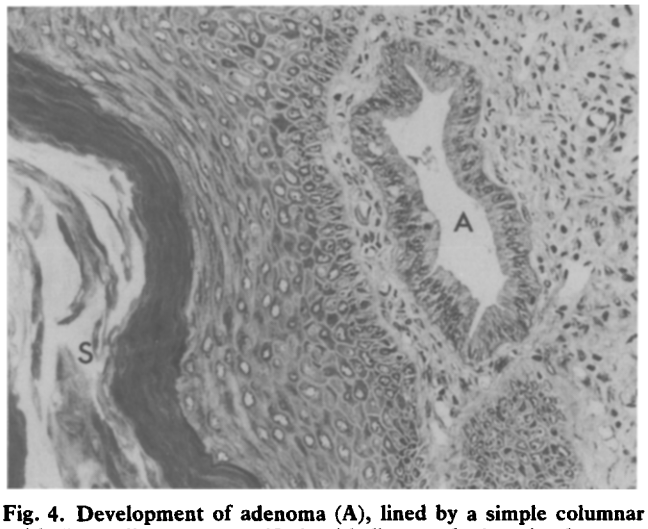

Fig. 4. Development of adenoma (A), lined by a simple columnar epithelium adjacent to stratified epithelium at the junction between the vagina and cervix. In addition to the adenoma, the stratified epithelium (S) was fully cornified in this ovariectomised adult mouse. Haematoxylin and phloxine B/orange G, magnification 300 ×.

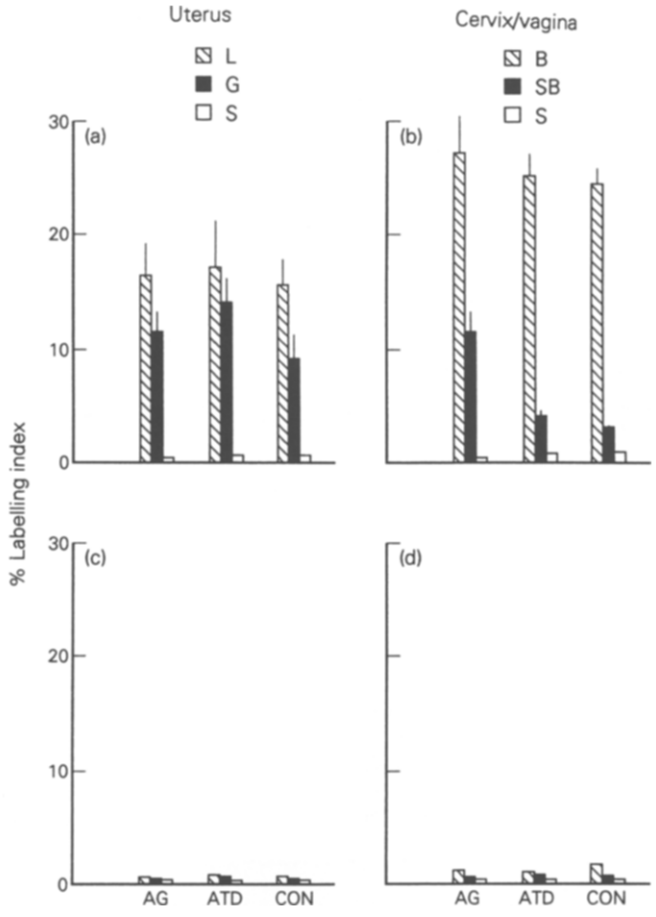

Fig. 5. Tritiated thymidine labelling indices in the genital epithelium and stromal cells of mice treated with aromataze inhibitors. Mice were treated with oestradiol-17β (a, b) or with control oil only (c, d), then ovariectomised at 12 weeks of age and after 14 days treated with either ATD (0.05 mg/g every 12 h), AG (0.025 mg/g every 12 h) or control regimes (CON). Mean (S.E.), $n = 10$ animals per group. Kruskal-Wallis non-parametric analysis of variance was used to test for differences between complete groups of data (where $P < 0.05$), and a Mann-Whitney U test ($P < 0.02$) to compare the differences between the three groups of data for any one tissue. L = uterine luminal epithelium, G = uterine glandular epithelium, S = stromal cells, B = cervix and vaginal basal epithelium, SB = cervix and vaginal suprabasal epithelium.

animals were ovariectomised at adulthood and left for 14 days. Figure 2a summarises the effect of ovariectomy on the proliferative indices in the animals treated neonatally with oestrogen or oil, and Fig. 2b the measurement of cellularity. In the neonatal oestrogen treatment group, the labelling indices and cellularity values (for epithelium and stroma) were significantly higher

Table 2. Comparisons of proliferative activities in the genital tissue of E_2–E_2 and E_2–CON mice

	E_2-E_2 vs. E_2-CON groups	
Tissue	Uterus	Cervix and vagina
Luminal epithelium	< 0.001	0.006
Glandular epithelium	NS	0.026
Stromal	NS	NS

P values indicate any significant differences between the mean labelling indices (over the 24 h of simulation) analysed by Mann–Whitney *U* test. NS = not significant.

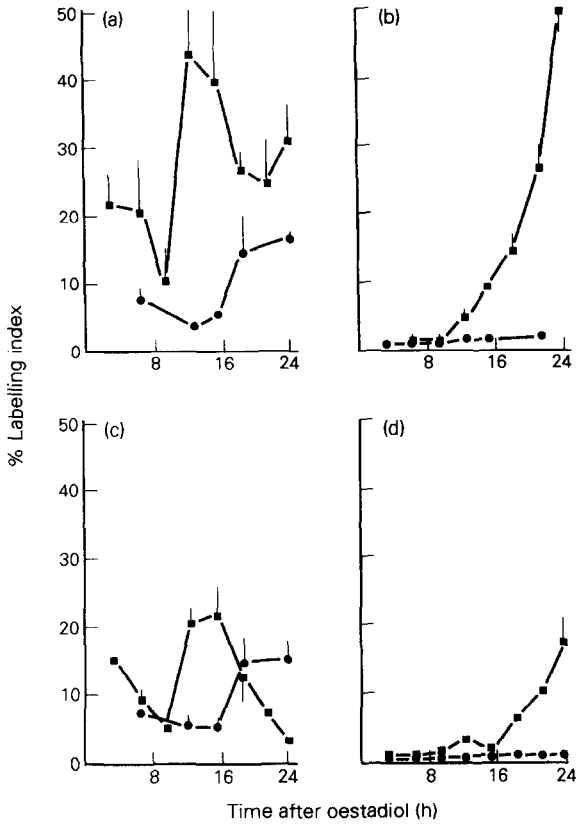

Fig. 6. Tritiated thymidine labelling indices in the uterine luminal epithelium (a, b) and glandular cells (c, d) of mice treated on days 1–5 with either oestradiol-17β (a, c) or control oil (b, d), then ovariectomised at adulthood, left 14 days, then subjected to two injections of oestradiol-17β (■) or Arachis oil only (●) as a control. The horizontal scale shows the time elapsed after the first injection of oestradiol. Mean (S.E.), $n = 3$ animals for each time point.

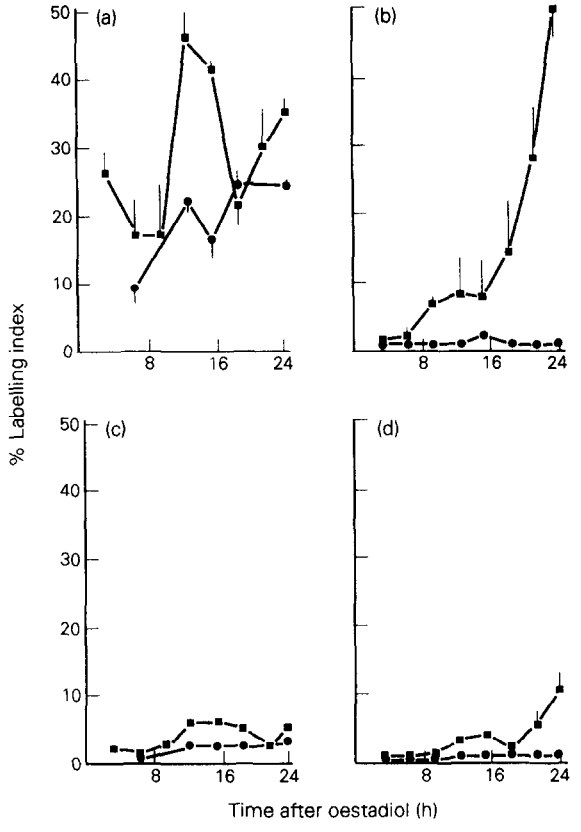

Fig. 7. Tritiated thymidine labelling indices in the cervical and vaginal basal epithelium (a, b) and suprabasal epithelium (c,d) of mice treated with (a, c) oestradiol-17β (b, d) Arachis oil as a control, ovariectomised at adulthood, left 14 days and then subjected (■) to two injections of oestradiol-17β or (●) Arachis oil as a control.

($P < 0.001$) compared to the control group treated neonatally with oil. Abnormal morphological features were also observed in the genital tracts of the ovariectomised mice, but only in those treated neonatally with oestrogen. These changes included metaplasia and hyperplasia of the uterine simple epithelium (Fig. 3), a persistent cornification of the stratified epithelium of the lower tract (cervix and vagina) and the formation of adenosis in the border between the cervix and vagina (Fig. 4). In the ovariectomised animals treated with oil neonatally, the uterine horns were reduced to thin tubes lined by cuboidal simple epithelium and rudimentary glands, and the lower tract (cervix and vagina) by a stratified bilayer of cells showing no signs of cornification.

Having observed that there were high levels of an "ovary-independent" cell proliferation that resulted from the neonatal oestradiol-17β treatment, it was important to establish that this proliferation was not being maintained by the peripheral aromatisation of oestrogens. Treatment of ovariectomised animals with two separate inhibitors of this aromatisation process, together with control regimens, was then carried out. No further changes (Fig. 5) were observed in the proliferation previously described for either the neonatal oestrogen (E_2-AG + E_2-ATD) or oil treatment groups (CON-AG + CON-ATD). This lack of response was also observed in the cell numbers and wet weights of the tissues (unpublished observations). The abnormally high levels of proliferation seen in ovariectomised adults following neonatal oestrogen treatment did not therefore appear to be dependent upon either ovarian or peripheral sources of oestrogen.

Genital tissue responses to oestrogen in ovariectomised mice treated neonatally with oestrogens

Neonatal treatment with oestrogen also affected the ability of the genital tissue to respond to a subsequent challenging dose of oestrogen (E_2-E_2). In the control group treated neonatally with oil and then ovariectomised at adulthood, further treatment with oil (CON-CON) had no effect, but administration of oestrogen (CON-E_2) resulted in a large and synchronous increase in epithelial cell proliferation (and wet weights: unpublished observations). This started after a quiescent period which lasted approximately 12 h (Figs 6b, d; Fig. 7b, d; Table 1), and is typical of the response in normal ovariectomised animals to a challenging dose of oestrogen (unpublished observations). The proliferative activity in ovariectomised mice following neonatal oestrogen treatment was already high, and a further challenging dose of oestrogen resulted in a non-synchronous increase in this activity (Fig. 6a, c; Fig. 7a, c; Table 1). The mean labelling index for this group (E_2-E_2) was significantly different than the respective control group (E_2-CON, see Table 2), suggesting that a population of cells remain responsive to oestrogen stimulation following neonatal treatment with this hormone.

DISCUSSION

Ovariectomy of the control animals (treated neonatally with oil), caused the genital tissues to regress to a basal and inactive kinetic state by 14 days (Fig. 2a, b). This contrasted with the effect of neonatal oestrogen treatment where an ovarian-independent proliferation was maintained (Fig. 2a, b). Peripheral aromatisation of oestrogens in body fat, muscle and the adrenals [12] is an additional source of oestrogens which could maintain the abnormal proliferation seen in these animals. However, application of two types of aromataze inhibitors (steroidal and non-steroidal) did not reduce the elevated level of proliferation, confirming earlier suggestions that neonatal oestrogen treatment causes the development of fully oestrogen independent proliferation [15].

A problem with this approach to blocking the peripheral production of oestroens is that the levels of this hormone cannot be measured in castrate animals with conventional radioimmunoassay, and would require far more sensitive mass spectrometry techniques to detect any oestrogens remaining after aromataze inhibitor treatment. Steroidal inhibitors may themselves act directly as weak agonists of oestrogen-dependent proliferation [16], and a small increase in the proliferation of epithelial cells in the ovariectomised control animals (CON-ATD) was seen (Fig. 5b). For this reason we also compared a non-steroidal (non-agonist) inhibitor, aminoglutethimide and this had no effect upon the proliferation in either the control (CON-AG) or oestrogen-treated animals (E_2-AG) (Fig. 5). An alternative approach to blocking the activity of residual oestrogens would be to use a "perfect" anti-oestrogen (i.e. a total antagonist) for the mouse, such as those described by Wakeling and Bowler [17].

The use of the term hormone "independence" to describe the changes in the neonatal oestrogen-treated animals must be considered carefully. Not all constituents of the genital tract responded uniformly to hormonal withdrawal after ovariectomy. Whilst labelling indices and the cellularity of the stroma remained high, the numbers of epithelial cells and wet weights of the tracts were reduced significantly compared to the pre-ovariectomy levels (unpublished observations). This heterogeneity contrasted with oil-treated neonates, where all kinetic and cell numbers reached basal levels 14 days after ovariectomy.

Further evidence that neonatal oestrogen treatment caused heterogeneity in the hormone responsiveness was demonstrated by challenging ovariectomised mice with a further dose of oestrogen. In normal animals, the genital tract epithelial cells enter a quiescent resting cell cycle state [18], and subsequent stimulation with oestrogen induces a synchronous entry of cells into DNA synthesis after a prereplicative state of approximately 12 h [10, 19]. Oestrogens are therefore necessary for the genital tract epithelium to continue in the cell cycle, and to stimulate quiescent epithelial cells back into cycle.

The objective of the next experiment was to determine if there was a change in the response to oestrogen following neonatal treatment with this hormone. In the control animals treated neonatally with oil, a challenging dose of oestrogen stimulated a typical "synchronous" proliferative response (CON-E_2, Figs 6b, d; Figs 7b, d), whereas the neonatal oestrogen-treated animals responded with a non-synchronous increase in proliferation (E_2-E_2, Figs 6a, c; 7a, c).

The means by which hormonally-unresponsive cells are produced by the neonatal oestrogen treatment is poorly understood. However, the window of exposure from late fetal development to the first 5 days after birth is known to be a critical period of development during which the "imprinting" of steroid hormone regulation occurs in the genital tissues [20]. It has been argued that the effects of oestrogen are mediated through the receptor even at this early stage of development, and a number of studies have demonstrated "nuclear" receptor in the perinatal genital epithelial and stromal cells (as early as day 4) using immunohistochemistry [21]. Whether the effect of oestrogen is mediated directly to the epithelium or indirectly via the stromal cells is still a matter of debate [22]. A possible mechanism for the long-term change in hormonal responsiveness following neonatal oestrogen treatment may be an alteration in the expression and structure of the receptor molecule [9, 23]. The expression and regulation of important genes (i.e. growth factors) could also be lost as a consequence of this treatment, leaving a population cells capable of growing independently of regulation by oestrogen.

These developmental changes occur at very high (pharmacologial) doses of oestrogen and so it has also been suggested that direct mutagenic changes may be involved, such as chromosome loss, aneuploidy, non-dysfunction [24], unscheduled DNA synthesis and even specific point mutations [25] due to the formation of DNA adducts [26].

Gross pathological changes and the development of tumours are well recognised long-term changes following oestrogen treatment neonatally. The early signs of this are an increased tendency for inappropriate cell differentiation, e.g. squamous cell appearing amongst the simple epithelium of the uterus [27]. The uterus is normally lined by this simple epithelium, but in animals treated neonatally with oestrogen, a second "reserve layer" was observed (Fig. 8), and this may represent a precursor to the development of squamous metaplasia. Before and following ovariectomy, these areas of metaplasia were infrequent and small in size, but greatly increased in frequency and size following the secondary exposure to oestrogen. This suggests that continued exposure to oestrogen may promote the expression of this and other forms of atypical pathology [28]. These sorts of pathological changes are generally regarded as benign; however, in the longer term we have noted the development of adenomas

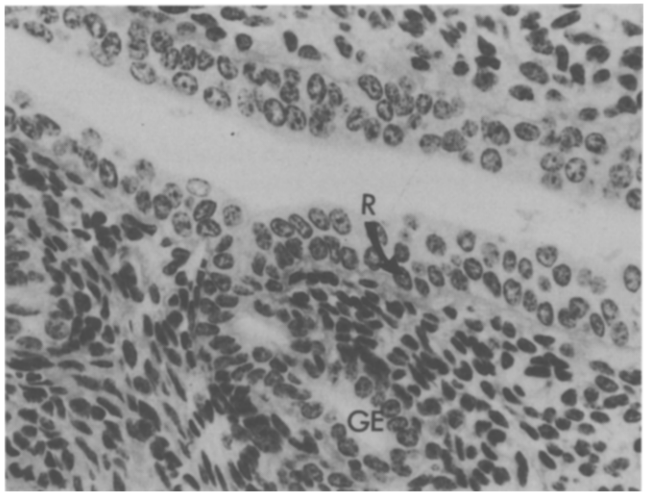

Fig. 8. Development of a "reserve cell" (R) basal layer prior to the formation of squamous metaplasia in the uterine luminal epithelium. A section uterine gland (GE) still lined by the typical simple cuboidal epithelium is shown. The mice were treated neonatally with oestradiol-17β, then ovariectomised at 12 weeks of age, and subsequently given two injections of oestradiol-17β (100 ng/g) over a 24-h period. 2 μm HPMA resin section stained with haematoxylin and phloxine/orange G (magnification × 450).

and adenocarcinomas in intact (non-ovariectomised) mice that had been treated neonatally with oestrogen. We did not observe any gross changes to the organisation of the smooth muscle and stromal cells as a result of neonatal oestrogen treatment, as has been previously reported [29]. However, the cellularity of the stroma remained high following ovariectomy compared to the control tracts (Figs 2c, d), and it is possible that there were variations in the incidence of different types of the stromal and muscle cells. This is an interesting aspect of the effect of neonatal oestrogen treatment which requires further study.

The influence of secondary oestrogen exposure on the development of malignant growth in this model is another unresolved aspect. The progression of oestrogen-independent subpopulations of cells must be considered against a background of hormonally dependent and responsive cells and the effects of the hormonal oestrous cycle. In this respect the cells altered by neonatal oestrogen exposure may have a proliferative advantage (possibly due to the unregulated expression of growth factors) over those cells which remain either dependent of responsive to oestrogens.

This model system is analogous to the development of many breast cancers where populations of hormone-dependent and independent cells can co-exist. There has been a tendency to study the clinical application of anti-oestrogens using cloned lines of hormone-dependent or independent breast cancer epithelial cells [30]. These systems cannot adequately represent the outcome of antihormone therapy in these situations. The mouse neonatal oestrogen model is therefore of potential value for studies of this kind.

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