Epidermal Growth Factor Receptor Levels in Reproductive Organs of Female Mice Exposed Neonatally to Diethylstilbestrol

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Abstract. **Binding of epidermal growth factor (EGF) to membrane preparations of vagina, uterus, ovary, oviduct, and liver was examined in mice treated neonatally with diethylstilbestrol (DES) and compared with that in untreated mice. Binding in the vagina (12.5 ± 0.73 fmol/mg protein) was somewhat higher than in the uterus (8.0 ± 0.34 fmol/mg protein). Level of specific binding was of the order: liver (18.4 ± 1.09 and 16.0 ± 1.53 fmol/mg protein) > vagina (12.5 ± 0.73 and 8.2 ± 0.57 fmol/mg protein) > uterus (8.0 ± 0.34 and 6.8 ± 0.56 fmol/mg protein) > ovary (6.8 ± 0.36 and 8.0 ± 1.05 fmol/mg protein) > oviduct (2.1 ± 0.32 and 1.7 ± 0.05 fmol/mg protein) in control and neonatally DES-exposed mice, respectively. Thus, neonatal DES exposure significantly lowered the binding site level only in the vagina, without modifying the binding affinity (K_d = 5.4 × 10^{-9} *M* in controls vs 4.6 × 10^{-9} *M* in DES-exposed mice). Reduction of EGF receptor level in the vagina correlates with ovary-independent persistent proliferation and keratinization of the vagina induced by neonatal DES exposure. EGF receptors were immunohistochemically demonstrated in epithelial cells of vagina, uterus, and oviduct and in stromal cells in uterus and oviduct using a polyclonal antibody to human EGF receptor protein.**
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Estrogens stimulate growth of the uterus, vagina, and other target organs *in vivo*; however, the mechanism by which estrogens produce these effects is not clearly understood (1). Recently, it has been proposed that estrogen-induced uterine growth may be mediated by autocrine/paracrine growth factors, such as epidermal growth factor (EGF). Both EGF and EGF receptors are present in the uterus (2–6). Endogenous EGF concentrations are raised by estrogens in the mouse uterus (7, 8) and in the rabbit endocervix (9). Estrogen-stimulated growth of uterine cells in organ culture is blocked by anti-EGF antibodies (10, 11). Estrogens upregulate autocrine or paracrine EGF signaling pathways whose effects, in turn, are partially mediated by the estrogen receptor itself, suggesting that cross-talk between EGF and estrogen receptors may be relevant to the regulation of normal growth and differentiation in female mouse reproductive tracts (12). A mitogenic effect of EGF on mouse vaginal and uterine epithelial cells has been demonstrated *in vivo* and *in vitro* (13–19). In these culture systems, estrogen itself has no mitogenic effect on the epithelium (15, 17–21).

Herbst *et al.* (22) have demonstrated that there is a close correlation between occurrence of vaginal clear cell adenocarcinoma in young women and early intrauterine exposure to diethylstilbestrol (DES). Mice exposed prenatally or neonatally to DES provide a model for exploration of the consequences of DES exposure in the human, because murine genital tract development at birth is similar to that of the human fetus at

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the end of the first trimester. The neonatal mouse model has been utilized to demonstrate the long-term effects of early sex hormone exposure on the female reproductive tract (for reviews, see [23–29]). Neonatal treatment of female mice with natural and synthetic estrogens including DES induces various abnormalities in the reproductive tract, such as ovary-independent cervicovaginal keratinization, adenosis, and tumors; uterine hypoplasia, epithelial metaplasia, and tumors; oviducal tumors; and polyovular follicles and polyfollicular ovaries. In addition, the growth response of neonatally DES-exposed reproductive organs to estrogen may be reduced (30, 31), as are estrogen receptor levels (32).

The present experiments were aimed at examining EGF receptor levels in reproductive organs of mice treated neonatally with DES.

Materials and Methods

Mice. Female BALB/cCrgl and C57BL/Tw mice were given daily subcutaneous injections of either 1 μg of diethylstilbestrol (Sigma, St. Louis, MO) in 0.02 ml of sesame oil (Hain Pure Food, Los Angeles, CA) or the vehicle alone for the first 5 days of life beginning within 18 hr after birth. The dose used is one known to induce histopathologic changes in the female mouse genital tract (32). Litters were adjusted to 4–6 pups with the addition or removal of newborn males. All mice were weaned at 1 month of age. At 40 days of age, BALB/cCrgl mice were sacrificed and vaginas, uteri, oviducts, ovaries, and livers were separately collected and pooled in containers submerged in ice. Keratinized layers were removed from the vaginas immediately after dissection. Membranes were prepared from the respective pooled organs. Control (n = 10) and neonatally DES-exposed (n = 10) C57BL/Tw mice were also sacrificed at 40 days of age, and vaginas, uteri, and oviducts were used for EGF receptor immunostaining. Another 10 mice from both C57BL groups were used for Western blot detection of EGF receptor.

Plasma Estradiol Levels. Plasma concentrations of estradiol were measured by an estradiol assay kit (Japan DPC Co., Tokyo), using 10 C57BL mice, DES-treated and controls, at 40 days of age.

Membrane Preparation. Membranes were prepared by the following method modified from Edery *et al.* (33, 34). The organs were weighed, and 4 vol (v/w) of buffer (0.3 *M* sucrose, 0.05 m*M* phenylmethylsulfonyl fluoride [Sigma], and 0.1 soybean trypsin inhibitor unit aprotinin [Sigma]) were added and homogenized by a Polytron (PT-10 ST, Brinkmann Instruments, Westbury NY) at a setting of 5 with two 10-sec bursts at 4°C. The crude homogenates were then centrifuged at 9,000*g* for 20 min at 4°C. The supernatant containing the membrane fraction was then centrifuged at 100,000*g* for 75 min at 4°C. The resulting pelleted membranes were homogenized in a Teflon-glass homogenizer in 0.5 ml of 25 m*M* Tris/10 m*M* $MgCl_2$ (pH 7.4) and stored at −20°C until further use. The protein content of the membrane fraction was determined by the method of Lowry *et al.* (35), with bovine serum albumin (BSA) as a standard.

Receptor Assay. EGF binding was determined by a method modified from Edery *et al.* (34). Briefly, membrane protein (300 μg) was incubated with 1.3×10^{-10} *M* (0.4 ng; *ca.* 60,000 cpm) [^{125}I]EGF (sp act 107–120 μCi/μg; Collaborative Research, Waltham, MA) in the presence and absence of excess (1 μg) unlabeled mEGF (Collaborative Research). In previous studies, a concentration of 60,000–100,000 cpm [^{125}I]EGF (sp act 100–200 μCi/μg) had been used by several investigators for EGF receptor assay in mouse tissues (34, 36, 37). The incubation mixture consisted of 300 μl of TMB (25 m*M* Tris, 10 m*M* $MgCl_2$, and 0.1% BSA) buffer (pH 7.4) with 0.05 m*M* phenylmethylsulfonyl fluoride and 0.1 soybean trypsin inhibitor unit aprotinin, 100 μl of [^{125}I]EGF, and 100 μl of sample. This mixture was incubated at 23°C for 16 hr, whereupon the reaction was terminated by the addition of 4 ml of cold TMB buffer, followed by immediate centrifugation at 2,000*g* at 4°C for 20 min. Previous study demonstrated that 16 hr was sufficient to reach binding equilibrium in mouse tissue (34). The supernatant was discarded, and the dried pellet was counted in a Beckman gamma-spectrometer (Beckman, Palo Alto, CA) with a counting efficiency of 40%. Nonspecific binding was the binding of [^{125}I]EGF in the presence of excess unlabeled EGF (and was usually less than 2% of the total added radioactivity). Specific binding was measured as the difference between total (in the absence of unlabeled EGF) and nonspecific binding, and expressed as fmol/mg protein.

Statistics. DES-treated and control preparations (three to eight) were assayed simultaneously to minimize variability among assays. Analysis of variance coupled with Duncan's multiple range test was used to examine differences between control and DES-exposed mice.

Immunoblot Analysis. Membrane fractions of vagina from control and neonatally DES-exposed C57BL mice were mixed with equivalent volumes of Tris buffer containing 22.2% glycerol and 2.2% sodium dodecyl sulfate (Wako, Tokyo) and incubated at 4°C for 15 min. Then the lysate was drawn repeatedly up and down by hypodermic needle (Terumo, Tokyo). Protein concentration was determined by bicinchoninic acid protein assay reagent (Pierce, Rockford, IL) using BSA as a standard. Five microliters of 1% bromphenol blue (Wako) and 50 μl of 2-mercaptoethanol were added to 500 μl of lysates. After incubation at 100°C for 4 min, the lysate was centrifuged at 9,000*g* for 7 min, and the supernatant was used for gel electrophoresis. Sodium

dodecyl sulfate-polyacrylamide gel electrophoresis (7.5%) was performed according to the method of Laemmli (38). Samples for electrophoresis were diluted to a final concentration of 650 μg/ml, and 20 μl were applied per lane. Molecular weight standards used for calibration of the gels were: myosin, 200,000; *Escherichia coli* β-galactosidase, 116,250; rabbit muscle phospholipase B, 97,400; BSA, 66,200; and hen egg white ovalbumin, 45,000 (sodium dodecyl sulfate-polyacrylamide gel electrophoresis molecular weight standards; Bio-Rad, Richmond, CA).

Proteins from unstained one-dimensional gels were transferred electrophoretically onto polyvinylidene difluoride membrane (Immobilon-P; Millipore, Bedford MA) using Semi-Dry Electroblotter (Sartorius, Bohemia, NY), as described by Kyhse-Anderson (39). After blocking of nonspecific protein-binding sites with 0.5% skim milk (DIFCO, Detroit MI) dissolved in TST (10 m*M* Tris-HCl, 150 m*M* NaCl, 0.05% polyoxyethene (20) sorbitan monolaurate [Tween 20; Wako]) buffer (pH 8.0) overnight at 4°C, the membranes were washed three times with TST and incubated in rabbit antibody against synthetic peptides corresponding to the amino acid residues of human EGF receptor (Cambridge Research Biochemicals, Cambridge, UK) for 2 hr at 35°C. The antibody was diluted 1/10 in TST containing 0.5% skim milk. The membranes were rinsed in TST, incubated in horseradish peroxidase-labeled goat anti-rabbit IgG F(ab')2 (Amersham, Arlington Heights, IL) for 2 hr at room temperature and rinsed three times with TST and then once with TS (TST without Tween 20). The peroxidase reaction was developed using 0.05% diaminobenzidine (Sigma) and 0.03% H_2O_2 dissolved in TS (40).

Immunohistochemistry. Bouin's-fixed vagina, uterus, and oviduct from 40-day-old C57BL/Tw mice were embedded in paraffin and cut at 8 μm. Deparaffinized sections were blocked with 1% BSA in phosphate-buffered saline for 30 min, then incubated in rabbit antibody to human EGF receptor (Cambridge Research Biochemicals) (1/100) overnight at 4°C. The sections were rinsed in phosphate-buffered saline, incubated in horseradish peroxidase-labeled donkey anti-rabbit IgG $F(ab')_2$ fragment (Amersham) (1/200) for 1 hr at room temperature, and rinsed again in phosphate-buffered saline. Immunoreactive EGF receptor was visualized by the method of Straus (41). Control sections were prepared as described above, except that normal rabbit serum was used instead of immune serum.

Results

The membrane receptors of both vagina and uterus showed a high binding affinity for [^{125}I]-EGF. Specific binding was observed at levels ranging from 3×10^{-12} to 18×10^{-10} *M* [^{125}I]-EGF. Nonspecific binding was less than 2% of the total radioactivity.

Scatchard analyses of equilibrium binding using membrane preparations obtained from the whole vagina in both 40-day-old control mice and 40-day-old neonatally DES-exposed mice indicate one class of receptors with apparent K_d values of 5.4×10^{-9} *M* and 4.6×10^{-9} *M* and receptor concentrations of 12.5 ± 0.73 and 8.2 ± 0.57 fmol/mg protein, respectively ($P < 0.01$) (Fig. 1A, Table I). Scatchard analyses using membrane preparations from the whole uterus of control and neonatally DES-exposed mice indicate K_d values of 13.7×10^{-9} *M* and 13.3×10^{-9} *M*, and receptor concentrations of 8.0 ± 0.34 and 6.8 ± 0.56 fmol/mg protein, respectively (Fig. 1B, Table I). When expressed on the basis of tissue weight, which is representative of cell content, the difference in receptor content between vaginas from neonatally DES-exposed mice and from controls was still significant (18.9 ± 2.73 vs 41.5 ± 3.50 fmol/g tissue); this calculation takes into account a possible antiproliferative effect of DES.

EGF binding to membrane preparations of ovary, oviduct, and liver is also shown in Table I. Receptor concentrations in these organs were not statistically different between control and neonatally DES-exposed mice. Western blot analysis revealed a major protein band of *ca.* 170 kDa, consistent with the M_r for EGF

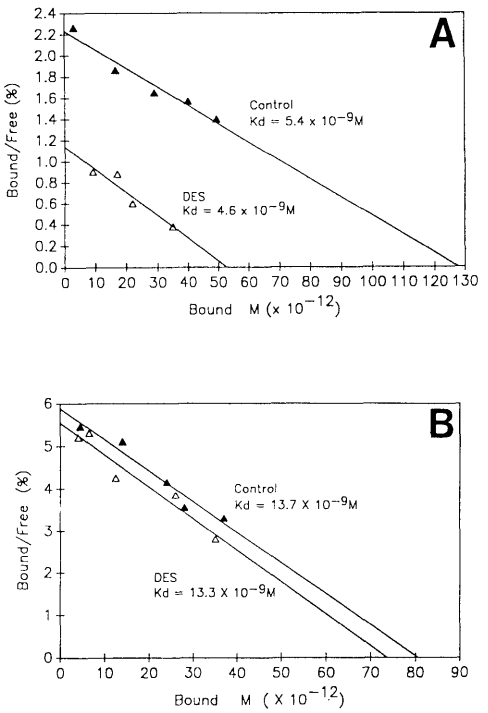

Figure 1. Scatchard plots of data using whole-organ membrane fractions from (A) vagina and (B) uterus. Aliquots of membrane fraction protein (0.3 mg) were incubated with various concentrations of [^{125}I]-mEGF (3×10^{-12}-18×10^{-10} *M*), either alone or in the presence of 1 μg of unlabeled mEGF. K_d, dissociation constant, was calculated from the slope of the plot which yields $-1/K_d$. Each point represents the average value of duplicate determinations from one of the two similar analyses.

Table I. Epidermal Growth Factor Receptor Levels in Various Organs of Neonatally Diethylstilbestrol-Exposed Mice[a]

	EGF receptor levels				
	Liver ($n = 90$)[b]	Uterus ($n = 90$)	Vagina ($n = 90$)	Ovary ($n = 120$)	Oviduct ($n = 120$)
fmol/mg protein					
Control	18.4 ± 1.09 (7)	8.0 ± 0.34 (8)	12.5 ± 0.73 (7)	6.8 ± 0.36 (3)	2.1 ± 0.32 (3)
DES	16.0 ± 1.53 (7)	6.8 ± 0.56 (7)	8.2 ± 0.57[c] (8)	8.0 ± 1.05 (3)	1.7 ± 0.05 (3)
fmol/g tissue					
Control	118.0 ± 4.50 (7)	37.2 ± 1.63 (8)	41.5 ± 3.50 (7)	29.3 ± 0.27 (3)	23.1 ± 1.71 (3)
DES	112.6 ± 5.20 (7)	36.6 ± 2.52 (7)	18.9 ± 2.73[c] (8)	32.2 ± 2.60 (3)	22.1 ± 0.18 (3)

[a] Data are expressed as mean ± SE. The number of determinations is in parentheses.
[b] Total number of animals used for tissue pools.
[c] $P < 0.01$ (Duncan's multiple range test).

receptor, in the vagina of both control and neonatally DES-exposed mice (Fig. 2). No difference was found in the band size between the two groups; as Western blot analysis is only semiquantitative at this level, the 35% reduction in receptor level in vagina reported in the present study would not be visualized.

The vaginal epithelium was composed of stratified cells with a superficial keratinized layer in both normal estrous mice and neonatally DES-exposed mice. The uterus in normal estrous mice and DES-exposed mice was composed of a layer of tall columnar cells, uterine glands, stromal cells, and muscle cells (not shown). However, the uterus in control estrous mice was larger than in neonatally DES-exposed mice. The oviduct was composed of epithelial cells, stromal cells, and muscle cells in both normal estrous and DES-exposed mice. EGF-receptor immunoreactivity was localized intracellularly in all types of cells of the vagina and in the

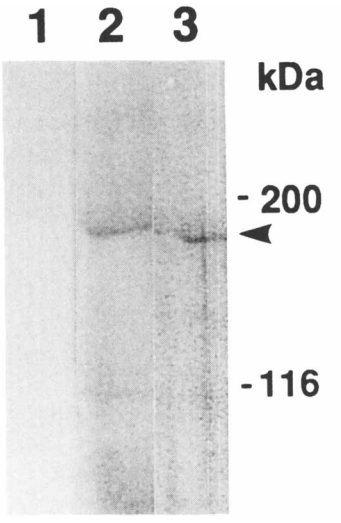

Figure 2. Western blots of EGF receptor in vagina of 40-day-old mice treated neonatally with oil or diethylstilbestrol. Lane 1, control vagina stained with nonimmune serum; Lanes 2 and 3, control and neonatally DES-exposed vaginas stained with antibody for EGF receptor.

oviduct; however, particularly intense staining was observed in the epithelial cells. In the uterus, luminal and glandular epithelial cells stained more strongly than stromal and muscle cells in both control and neonatally DES-exposed mice (Fig. 3), in agreement with previous studies (3–5, 16). No differences in histology and EGF-receptor immunoreactivity in these reproductive tracts were found between normal estrous mice and DES-exposed mice.

Plasma concentrations of estradiol in neonatally DES-exposed mice (10.3 ± 1.9 pg/ml) were not statistically different from those in controls (7.2 ± 0.6 pg/ml).

Discussion

Considerable evidence suggests a physiological role for EGF in estrogen-mediated growth of various tissues. Estrogens increase the production of immunoreactive EGF in the mouse uterus (8) and of EGF-like peptides by MCF-7 human mammary tumor cells (41). EGF antibodies block the estrogen-induced growth of uterine cells in organ culture (10–11). EGF stimulates the *in vitro* growth of MCF-7 cells (42), mouse uterine myometrial cells (43), and uterine and vaginal epithelial cells (12–19). EGF receptors have been demonstrated in the stroma, myometrium, and luminal and glandular epithelium of the adult rat uterus by autoradiography (4) and in the different cell types in neonatal and immature mouse uterus and vagina by immunohistochemistry and autoradiography (6). Also, EGF receptor levels in the rat uterus increase during the peri-implantation period (44). Receptor levels change in parallel with plasma estrogens and occupied nuclear estrogen receptors (5). Transforming growth factor-α shares structural and functional homology with EGF and binds to the EGF receptor (45). Transforming growth factor-α is secreted at high levels into mouse luminal fluid after estrogen treatment (46) and stimulates proliferation of mouse placental cells *in vivo* (47). In the present study, EGF receptors were demonstrated in all

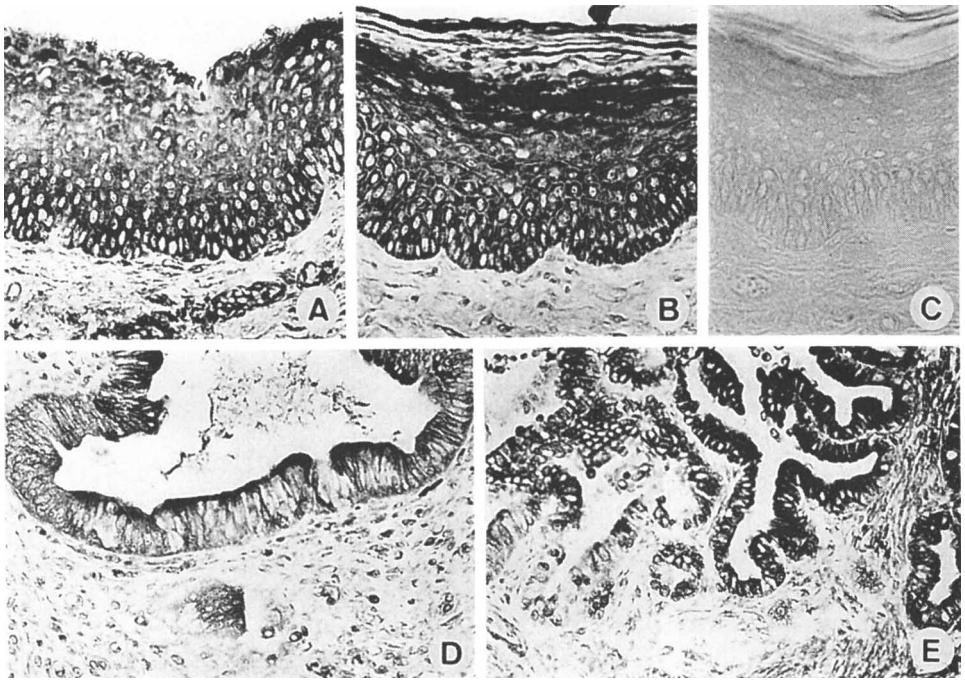

Figure 3. Immunohistochemical localization of EGF receptor in vagina, uterus, and oviduct of 40-day-old mice treated neonatally with oil or diethylstilbestrol (×200). Vagina of a (A) normal estrous mouse and (B) a neonatally DES-exposed mouse. EGF receptor is present predominantly in epithelial cells. (C) Vagina of a normal estrous mouse stained with control serum showing no staining. (D) Uterus and (E) oviduct of neonatally DES-exposed mice, showing intensely positive staining for EGF receptor in epithelial cells.

cell types in the vagina, uterus, and oviduct of adult mice by immunohistochemistry.

Perinatal treatment of female mice with estrogen including DES results in various abnormalities in both the hypothalamo-hypophysio-ovarian axis and the reproductive organs. The abnormalities are induced by DES in a dose-dependent manner (32, 48). In addition, high doses of DES (1 μg/day for 5 days from the day of birth) induce a significant increase in progestin receptors but a significant decrease in estrogen receptors in mouse vagina and uterus (32).

In the present study, EGF receptors were found to be distributed in the uterus, ovary, and liver, as shown previously in rats and mice (2–6, 49). We have further demonstrated EGF receptors in the mouse vagina and oviduct by binding assay and immunohistochemistry. In a previous study, Edery *et al.* (34) found EGF receptor levels in mouse mammary gland (3.8–23.5 fmol/mg protein) by the same method used in the present study; the levels are similar to those (1.7–18.4 fmol/mg protein) measured in mouse liver and genital organs. Neonatal DES exposure (1 μg/day for 5 days from the day of birth) significantly decreased EGF receptor levels in the vagina but not in the other organs studied. Vaginal epithelial cells from DES-exposed mice showed lower sensitivity to EGF than those from control mice *in vitro* (18, 19). The reduction of EGF receptor levels in the DES-exposed vagina correlates with this reduction of sensitivity *in vitro* (18, 19), with reduction of estrogen receptor levels (32), and with ovary-independent changes in the vaginal epithelium (proliferation and keratinization) (32). Since keratinized layers in the vagina were removed immediately after the dissection, the reduction noted is not ascribable to the presence of dead keratin. Recently, Chilton *et al.* (9) demonstrated that EGF receptors are present in all major cell types of the cervix, and mucus-secreting cells had the highest concentration of EGF receptors. Neonatal DES exposure did not change the number of EGF receptors in the rabbit endocervical epithelial cells when examined in adulthood. However, the numbers of mucus-secreting cells were decreased, thereby reducing the EGF receptor content of the epithelium. In the present study, neonatal DES exposure also did not alter uterine EGF receptor level.

Uterine EGF receptors are increased by endogenous and exogenous estrogens in both immature and mature rats (2, 3, 5) and decreased by progesterone in rabbits (9). Ovarian EGF receptors are increased by endogenous gonadotropins in the rat (50). Perinatal treatment with natural and synthetic estrogens produced nonovulatory polyfollicular ovaries owing to alteration of the hypothalamo-hypophysial axis in rats and mice. In addition, similar treatment with large doses of estrogen frequently caused permanent proliferation of uterine epithelium accompanied by endometrial and myometrial abnormalities (24, 25, 27, 28, 31). In the present study, EGF receptor levels in uterus

and ovary, and plasma estradiol levels of neonatally DES-exposed mice, were not different from those measured in the controls, suggesting that there may be no significant difference in blood levels of gonadotropins between control and DES-exposed mice at 40 days of age. In fact, no major difference was reported in gonadotropin levels between neonatally DES-exposed mice and controls at 42 days of age in NMRI mice (51). Although EGF receptor levels in mouse liver show circadian differences (49), we sacrificed all mice at 0900–1000 hr and prepared protein samples at the same time, thus excluding this source of variation. In conclusion, the presence of EGF receptors was demonstrated by binding assay of radiolabeled EGF and immunohistochemistry in vagina, uterus, and oviduct from neonatally DES-exposed mice and controls. Neonatal DES exposure significantly lowered the binding site level only in the vagina, which may correlate with the ovary-independent epithelial proliferation and cornification induced in this organ by early exposure to DES.

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