Effects of Early Exposure to Diethylstilbestrol on Cellular Protein Expression by Mouse Vaginal Epithelium and Fibromuscular Wall (43138)

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Abstract. **Epithelia and fibromuscular walls were dissociated from the vaginae of ovariectomized BALB/cCrgl mice (ca. 41 days old) exposed neonatally to diethylstilbestrol (DES) or sesame oil and purified by centrifugation through Percoll density gradients. Neonatal exposure to DES caused the vaginal epithelium to become permanently proliferated and partially keratinized; the control epithelium was low cuboidal. The major cellular proteins expressed in each tissue compartment were examined by two-dimensional gel electrophoresis of [^{35}S]methionine-labeled tissues. The epithelia and fibromuscular walls displayed distinctive two-dimensional protein patterns. In the DES-exposed vaginal epithelium, the expression of two proteins (one had a molecular size of 65 kDa and a pI of 6.0, and the other had a molecular size of 38 kDa and a pI of 6.3) was increased, while the expression of three proteins with molecular sizes of 25, 30, and 140 kDa and pIs of 5.6, 5.6 and 6.7, respectively, was reduced, relative to the control epithelium. In the DES-exposed vaginal fibromuscular walls, the expression of 9 proteins was increased whereas the levels of 21 specific proteins, distinct from those in the epithelium, were decreased. Thus, long-term tissue-specific alterations in the synthesis of a select number of cellular proteins occur in the DES-exposed vagina.**
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Estrogens, including diethylstilbestrol (DES), induce *in vivo* persistent vaginal cornification, hyperplastic lesions, and cervicovaginal cancer in female mice if administered immediately after birth, while the development of the reproductive tract is being completed (1, 2). Reproductive tract lesions due to estrogen exposure *in utero* also occur in humans. For example, transplacental exposure of the female fetus to DES results in cervical ectropion, vaginal adenosis, and low incidence of vaginal adenocarcinoma (3).

The mechanism by which early DES exposure causes structural changes in the female reproductive tract is not well understood. Correlation of the onset of these alterations with puberty suggests the direct or indirect involvement of endogenous hormones; as a result, many investigators (4–6) have focused on the ovarian steroids and their receptor systems. Biochemical studies show that the relative amount of cytosolic estrogen receptors is lowered while progestin receptor levels are elevated in the mouse female genital tract after neonatal estrogen exposure. The specific location of these steroid receptor level changes in the tissue compartments of the mouse vagina has recently been determined (7). Newbold *et al.* (8) found that the expression of a major cellular uterine protein was altered in immature female mice exposed transplacentally to DES. In general, however, little is known about the molecular consequences of neonatal DES exposure.

The mouse vagina is composed of several tissue types, and, conceivably, DES may differentially affect the expression of proteins in a tissue-specific manner. To test this notion, a collagenase dissociation technique, which we have recently optimized (9, 10), was used to separate the mouse vagina into two tissue compartments—the epithelium and fibromuscular wall. We report here the long-term effects of early DES

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exposure on the expression of the major cellular proteins specific to the vaginal epithelium and to the vaginal fibromuscular wall.

Materials and Methods

Materials. Medium 199 and Waymouth's (×10) medium were purchased from Grand Island Biological (Grand Island, NY); methionine-free Dulbecco's modified Eagle's medium was purchased from the University of California Tissue Culture Facility (San Francisco, CA); collagenase CLS III (189 units/mg) was obtained from Worthington (Freehold, NJ); and Percoll was purchased from Pharmacia Fine Chemicals (Piscataway, NJ). L-[^{35}S]methionine (1000 Ci/mmol) was obtained from Amersham Corp. (Arlington Heights, IL). Other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO), unless otherwise stated.

Animals. Newborn female BALB/cCrgl mice were obtained from the barrier breeding colony at the University of California at Berkeley. These mice were weaned at 3 weeks of age, housed in litters of five to six mice per cage in a temperature-controlled room (ca. 22°C) with 12-hr-light and 12-hr-dark periods, and provided with pine shavings for bedding, water, and Wayne Sterilizable Rodent Block Diet (Teklad/Premier Laboratory Diets, Bartonville, IL) *ad libitum*.

Injections of DES. Newborn female mice received 0.02-ml interscapular injections of DES (50 μg/ml) in sesame seed oil daily for 5 consecutive days, starting within 18 hr after birth. Control animals received 5 daily injections of 0.02 ml of sesame oil.

Tissue Dissociation. Vaginae were dissected from BALB/cCrgl mice (ca. 41 days old) 10–11 days after ovariectomy and were separated into tissue compartments by a previously described procedure (9) with the following modifications: DES-exposed vaginae were incubated with collagenase (0.12%) for 1.5 hr, while the control vaginae were incubated with collagenase (0.12%) for 2 hr. The uppermost keratin layers of epithelial cells were removed from the DES-exposed vaginal epithelium with jeweller's forceps under a dissecting microscope and discarded. The epithelia and fibromuscular walls were centrifuged on preformed Percoll density gradients in order to remove cellular debris and proteolytic enzymes (exogenous and endogenous) present during the preparation of the two vaginal tissue compartments (10). Tissues were rinsed three times with phosphate-buffered saline (pH 7.5, 4°C) before being radiolabeled.

Radiolabeling of Vaginal Tissues. Isolated vaginal tissues were radiolabeled for 16 hr at 37°C in a humidified atmosphere of air–CO_2 (95%:5%) in serum- and methionine-free Dulbecco's modified Eagle's medium containing 400 μCi/ml of [^{35}S]methionine. The tissues were pelleted by centrifugation at 600*g* for 10 min and washed with phosphate-buffered saline (pH 7.5), after which they were frozen in a dry ice-ethanol bath and stored at −70°C.

Two-Dimensional Gel Electrophoresis. The frozen tissues were thawed, suspended in two volumes of lysis buffer (9.5 *M* ultrapure urea and 2% ampholines [1.6%, pH 5 to 7 and 0.4%, pH 3 to 10; Bio-Rad Laboratories, Richmond, CA], 2% [w/v] Nonidet P-40, 5% [v/v] β-mercaptoethanol in distilled water, 0.5 m*M* phenylmethylsulfonyl fluoride), sonicated with two 3-sec pulses at 70% maximal output with a sonifier cell disrupter (Branson Sonic Power Co., Danbury, CT), and centrifuged at 23,000*g* for 10 min. The amount of [^{35}S]methionine incorporated into total protein was determined by precipitating a sample (5 μl) of the soluble fraction with 10% trichloroacetic acid. Radiolabeled proteins (ca. 200,000 cpm) from epithelium and fibromuscular walls were fractionated by two-dimensional gel electrophoresis (11, 12). Isoelectric focusing gels contained 80% (pH 5–7) ampholines and 20% (pH 3–10) ampholines. The second dimension sodium dodecyl sulfate-polyacrylamide slab gels contained 10% acrylamide (13). Protein gels were fixed, stained in 0.04% Coomassie brilliant blue in a solution of 10% acetic acid-25% isopropyl alcohol-65% distilled water, and then destained in 10% acetic acid at room temperature. Fluorography of gels was carried out by impregnation with En^{3}Hance solution (Dupont, NEN Research Products, Boston, MA). X-Omat AR films (Eastman Kodak, Rochester, NY) were combined with dried gels (60°C) and exposed at −70°C.

The relative expression of specific proteins was quantified by soft-laser densitometry of four autoradiographs representing control tissue (two epithelial and two fibromuscular) and DES-exposed tissue (two epithelial and two fibromuscular). To obtain these autoradiographs, dissociated tissues pooled from 30 animals per group were radiolabeled and the results from one of two duplicate samples from each of two separate experiments were compiled by densitometry. Only the changes consistently observed in both experiments were tabulated.

Results

Effects of Collagenase Treatment. Histologic analysis demonstrated that the changes described in the adult mouse vagina after neonatal exposure to relatively high doses of DES (5) are maintained in the epithelia and fibromuscular walls separated after a short treatment with collagenase. The appearance of the control vaginal epithelium from ovariectomized mice is one of two to three layers of cuboidal cells (Fig. 1A), similar to that of a vagina from an immature mouse. In contrast, the vaginal epithelium from DES-exposed mice exhibits several layers of epithelial cells (persistently proliferated state), which varies from a basal cuboidal

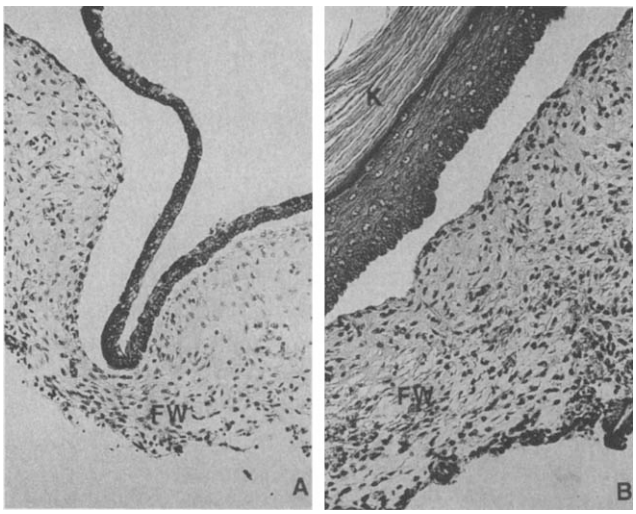

Figure 1. Cross-sections of vaginae of ovariectomized mice treated neonatally. (A) The mouse was treated with sesame oil daily for the first 5 days after birth (H & E, original magnification ×150). Note the clear separation of epithelium, consisting of two to three layers of cuboidal cells, from underlying fibromuscular wall (FW) after 2-hr treatment with 0.12% collagenase. (B) The mouse treated neonatally with 1 μg of DES daily for the first 5 days after birth (H & E, × 150). Note the clear separation of the keratinized (K) stratified squamous epithelium from underlying fibromuscular wall (FW) after 1.5-hr treatment with 0.12% collagenase.

layer to the superficial stratified squamous layers (Fig. 1B). Keratin is also present. The fibromuscular wall of both control and DES-exposed mice consists of a lamina propria of loose connective tissue with some lymphocytes and interspersed muscle cells (Fig. 1). Some pycnotic nuclei are evident at the periphery of collagenase-treated fibromuscular wall tissues. Thus, the striking effects of neonatal DES exposure on epithelial histology are maintained after separation by collagenase digestion.

Effects of Neonatal DES Exposure on Major Cellular Protein Patterns. The regulation of vaginal histology suggested that neonatal exposure to DES might cause concomitant alterations in gene expression. To examine this notion, the expression patterns of cellular proteins labeled *in vitro* with [^{35}S]methionine in the separated DES-exposed and control vaginal epithelia and fibromuscular wall tissue compartments were examined by two-dimensional gel electrophoresis. A reproducible set of three proteins (actin, α-tubulin, and β-tubulin) was used to orient the epithelial fluorographs (8, 14, 15), whereas a set of two proteins (actin and K protein) was used to orient the fibromuscular wall fluorographs. The DES-influenced changes in the synthesis of specific ^{35}S-labeled proteins were quantified by soft-laser densitometry of autoradiographs representing multiple experiments and are summarized in Table I. As shown in Figure 2B, neonatal DES treatment stimulated the steady-state production of two epithelial proteins (one with a molecular size of 65 kDa and a pI of 6.0, and the other with a molecular size of 6.0, 38 kDa and a pI of 6.3), designated IV and V, while the concentrations of three other epithelial proteins (with molecular sizes of 25, 30, and 140 kDa and pIs of 5.6, 5.6, and 6.7, respectively), designated I, II, and III, were lowered relative to control epithelium (Fig. 2A). Thus, despite the major alterations in the histology of vaginal epithelium caused by neonatal DES exposure, there was only a small number of changes in protein expres-

Table I. Physical Properties of Altered Cellular Proteins of the Vaginal Epithelium and Fibromuscular Wall from DES-Exposed Mice[a]

Protein expression in tissue compartments	Protein no.	Molecular size (kDa)	pI
Reduced in DES-exposed epithelium			
	I	25	5.6
	II	30	5.6
	III	140	6.7
Induced in DES-exposed epithelium			
	IV	65	6.0
	V	38	6.3
Reduced in DES-exposed fibromuscular wall			
	1	98	5.2
	2	16	5.4
	3	52	5.6
	4	50	5.6
	5	31	5.6
	6	160–120	5.6–6.0
	7	97	5.6
	8	28	5.9
	9	24	6.2
	10	52	6.7
	11	68	6.2
	12	65	6.3
	13	60	6.3
	14	50	6.3
	15	120	6.4
	16	165	6.7
	17	38	6.7
	18	60	6.9
	19	52	7.0
	20	34	7.0
	21	24	7.0
Induced in DES-exposed fibromuscular wall			
	22	96	5.6
	23	36	5.7–5.8
	24	39	5.8
	25	38	5.8
	26	120	5.8–6.0
	27	50	6.0
	28	20	6.4
	29	43	6.5
	30	37	7.0

[a] Proteins reduced or induced after neonatal DES treatment were identified by scanning the autoradiographs shown in Figures 2 and 3 with a soft laser densitometer. The molecular sizes were determined by comparing the two-dimensional electrophoretic migration to known standards. The pIs were calculated by their one-dimensional isoelectric focusing in comparison with the measured pHs of gel segments.

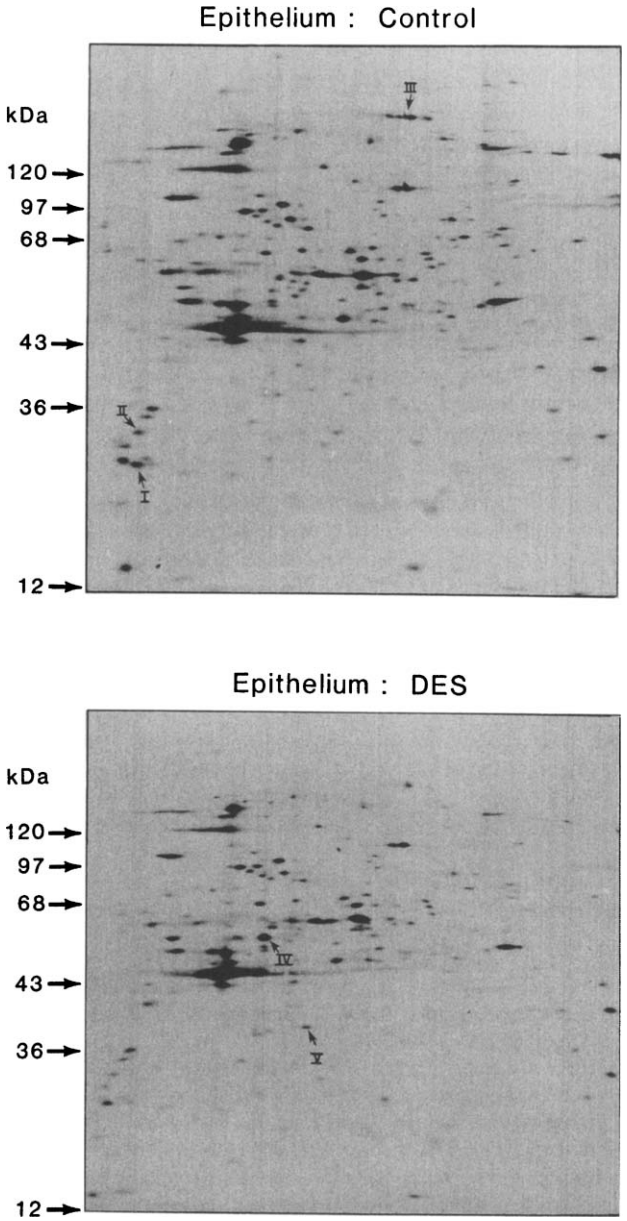

Figure 2. Full fluorograms of the region spanned by pI 5.0–7.0 on the abscissa and molecular sizes of 12 to 120 kDa on the ordinate of [^{35}S]methionine-labeled proteins from the vaginal epithelium of ovariectomized mice (ca. 41 days old); mice were control (A) and exposed neonatally to 1 μg of DES daily for 5 days (B). Arrows in a given panel identify the location of proteins whose expression is significantly increased in comparison to the other panel.

sion since synthesis of the vast majority of cellular proteins remained unaltered.

In contrast to the epithelium, in the fibromuscular wall DES treatment caused the concentrations of a large number of proteins to be either increased or decreased relative to control tissue. Analysis of fluorograms of DES-treated fibromuscular walls showed an increased expression of nine major cellular proteins, designated 22 through 30. DES exposure also lowered the expression levels of 21 other major cellular proteins, designated 1 through 21, relative to control fibromuscular wall tissue (Fig. 3). These changes are summarized in Table I. Thus, even though the fibromuscular wall compartment shows relatively minor alterations in morphology (increased cell density and more prominent nuclei), DES treatment caused major changes in protein expression.

Discussion

Our results demonstrate that early DES exposure induces long-term, permanent changes in major cellular protein expression in the separated tissue compart-

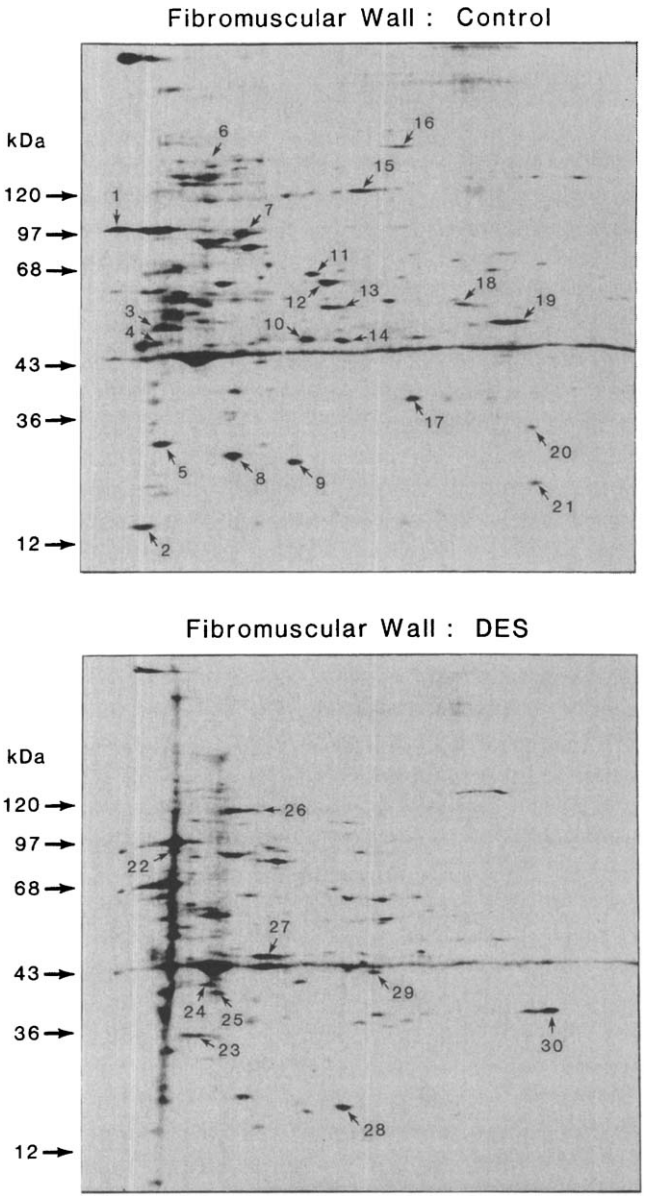

Figure 3. Full fluorograms of the region spanned by pI 5.0–7.0 on the abscissa and molecular sizes of 12 to 120 kDa on the ordinate of [^{35}S]methionine-labeled proteins from the vaginal fibromuscular wall compartments of ovariectomized mice (ca. 41 days old); mice were control (A) and exposed neonatally to 1 μg of DES daily for 5 days (B). Arrows in a given panel identify the location of proteins whose expression is significantly increased in comparison to the other panel.

ments (epithelium and fibromuscular wall) of the adult mouse vagina. Intriguingly, the tissue compartment which showed the most significant DES-mediated changes in structure (epithelium) contained only a relatively small number of alterations in the synthesis of specific protein products. For example, in the DES-exposed vaginal epithelium, increased expression of two proteins was detected while the expression of three proteins was reduced relative to the control epithelium. In contrast, although the histology of the fibromuscular wall after DES exposure was less obviously affected than was the epithelium, DES influenced the expression levels of a large number of cellular proteins. Conceivably, the small number of protein changes detected in the epithelium may account for the altered morphology, although it is tempting to consider that the regulated protein changes in the fibromuscular wall influence the structural changes observed in the epithelium. In this regard, other investigators (16) have postulated, for male genital tract tissue, that the epithelial morphology is directly regulated by the fibromuscular wall (stromal) compartment. The signalling between these tissues most likely involves stromal paracrine factors, suggesting that similar activities may be expressed by the ovariectomized fibromuscular wall as a result of neonatal DES treatment.

The ability to separate the individual epithelium and fibromuscular wall compartments by collagenase digestion allowed us to examine DES-influenced protein changes in each vaginal tissue. Relatively evenly stained tissue sections of control and DES-exposed samples showed few (fibromuscular wall) or no (epithelium) pycnotic nuclei, indicating that the brief collagenase treatment is not seriously detrimental to the vaginal tissues. A 30-min difference in the duration of collagenase treatment was, however, required to separate the tissue compartments (2 hr, control; 1.5 hr, DES exposed). The reasons for the shorter duration of enzymatic treatment required to separate the DES-exposed vagina into tissue compartments are unclear. Conceivably, DES-treated tissue is inherently more sensitive to collagenase digestion because of alterations in the extracellular matrix, thus making the organ structurally less stable.

The DES-induced alterations in epithelial and fibromuscular wall protein expression may be due to modulation of one or more cellular events. Given the transcriptional mechanism of steroid hormone action (17, 18), DES may act by the selective induction or repression of the rate of RNA synthesis encoding the epithelium- and fibromuscular wall-specific proteins. Alternatively, through secondary pathways, DES may be selectively affecting the translation, processing, and/or stability of the DES-regulated protein products. Since early neonatal exposure to DES causees long-term changes in protein expression, it is likely the protein differences described reflect persistent changes in vaginal structure and not the initial gene products under direct DES control. For example, the gene products under direct DES control may function to alter the long-term expression of a variety of other genes, such as vaginal tissue steroid receptors (7, 19). Characterization of the expression (and identification) of the DES-regulated proteins by using nucleic acid probes and antibodies will eventually be required to understand the precise cellular processes affected by early DES exposure. This approach may possibly help to determine if the altered expression of cellular proteins is a long-term consequence or a direct cause of the teratogenic changes in the female reproductive tract.

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