Effect of Certain Growth Factors on Proliferation in Serum-Free Collagen Gel Culture of Vaginal Epithelial Cells from Prepuberal Mice Exposed Neonatally to Diethylstilbestrol (43316)

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Abstract. **Neonatal treatment with diethylstilbestrol (DES) induces ovary-independent vaginal epithelial changes in mice. The response of vaginal epithelial cells from intact prepuberal BALB/cCrgl mice treated neonatally with 2 μg of DES for 5 days to growth-stimulatory and -inhibitory factors was studied using a serum-free collagen gel culture system that sustains the growth of normal vaginal epithelial cells. Cells from control and DES-exposed mice at 21 days of age showed about a 5-fold increase in number during 10 days in a serum-free medium supplemented with transferrin, bovine serum albumin fraction V, insulin, and epidermal growth factor. Epidermal growth factor and insulin stimulated dose-related proliferation of vaginal epithelial cells from both control and DES-exposed mice; however, cells from DES-exposed mice showed a reduced growth response to epidermal growth factor and an increased growth response to insulin, compared with control cells. Insulin-like growth factor I (1–100 ng/ml) tested in the absence of insulin failed to stimulate cell growth. Transforming growth factor-β (0.05–5 ng/ml) consistently inhibited cell growth in a dose-dependent manner.**
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Perinatal exposure of female mice to natural and synthetic sex hormones, including diethylstilbestrol (DES), results in vaginal abnormalities that include ovary-independent proliferation and keratinization of the vaginal epithelium (1–5). The vaginal epithelium altered by perinatal estrogen treatment responds differently to later exogenous estrogen: mitotic and alkaline phosphatase activities are decreased, whereas these activities are stimulated in control vaginal epithelium (6). The DES-exposed vagina has fewer estrogen receptors than the control (7). Using a system in which vaginal epithelial cells are separated from the stroma and cultured in collagen gel matrix with a serum-free medium, we can investigate the possible direct effect of various agents on epithelial cells (8–10). Recently, using the serum-free collagen gel culture system, Uchima *et al.* (11) have demonstrated that neonatal DES exposure resulted in alterations in vaginal epithelial cells of ovariectomized adult mice, as indicated by decreased initial rate of proliferation and by altered sensitivity to epidermal growth factor (EGF) and insulin. The present study was aimed at determining an optimal medium and comparing the sensitivity to various growth factors of normal and neonatally DES-exposed immature mouse vaginal epithelial cells in a serum-free collagen gel culture system.

Materials and Methods

Animals. Newborn female BALB/cCrgl mice were obtained from the barrier-breeding colony at the University of California at Berkeley. Newborn female mice received five daily subcutaneous injections of 2 μg of DES dissolved in 0.02 ml of sesame oil starting within 18 hr after birth. Control animals were injected daily

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with 0.02 ml of sesame oil. The mice were weaned in a temperature-controlled room (22°C) with 12:12-hr light:dark periods. Animals were provided with pine shavings for bedding, fresh water, and Wayne sterilizable rodent block diet *ad libitum*. All animals were weaned and sacrificed at 21 days of age.

Media and Supplements. Medium 199, Ham's F-12, Waymouth's (10 X), and Dulbecco's modified Eagle media were obtained from Grand Island Biological (GIBCO; Grand Island, NY); diethylstilbestrol, bovine serum albumin fraction V (BSA), human transferrin (Tr), bovine insulin, penicillin, streptomycin, HEPES buffer, and DNase were from Sigma Chemical Co. (St. Louis, MO); mouse EGF and human transforming growth factor (TGF)-β were from Collaborative Research (Lexington, MA); collagenase CLS III (189 unit/mg) was from Worthington (Freehold, NJ); Percoll was from Pharmacia Fine Chemicals (Piscataway, NJ); and recombinant bovine insulin-like growth factor IGF-I was from Monsanto (St. Louis, MO).

Cell Dissociation. Vaginae were dissected from 21-day-old BALB/cCrgl mice given five daily injections of 2 μg of DES dissolved in 0.02 ml of sesame oil or the vehicle alone from the day of birth, and dissociated by the procedure described previously (8), with the following modifications. The vaginae were incubated in Medium 199 containing 0.1% collagenase and 5 mg/ml of BSA for 90 to 120 min at 37°C in a shaking water bath. Epithelial sheets were manually separated from the fibromuscular wall; the mucus was then removed by forceps. The epithelial sheets were minced on a Teflon board with a razor blade, and the cell clumps were collected and dispersed in 0.04% DNase solution. The epithelial cells purified by a preformed Percoll density gradient (12) were washed twice in Medium 199, and the cell number was estimated by mixing 1 vol of cell suspension with 9 vol of 0.02% crystal violet in 0.1 *M* citric acid and counting stained nuclei in a hemocytometer.

Culture Procedure. Collagen solution and gels were prepared by a modified method (12). Briefly, 4 g of rat tail collagen fibers (tendons) were sterilized in 70% ethanol overnight and dissolved in 1 liter of 1/1000 acetic acid in sterile distilled water; the supernatant after centrifugation at 13300*g* for 90 min at 4°C provided the stock collagen solution. Eight volumes of stock solution were mixed with a 2 vol of Waymouth's (10 X) medium:0.34 *N* NaOH mixture (2:1) and kept on ice to prevent immediate gelation. Cells were added to the cold gelation mixture; 0.5 ml, containing 2×10^5 cells, was overlaid on a 0.3-ml base of gelled collagen in each well of a Falcon 24-multiwell plate and allowed to gel at room temperature. The cells were cultured in SF20 medium (K. T. Mills and C. Wong, unpublished observations) consisting of a 1:1 (v/v) mixture of Dulbecco's modified Eagle and Ham's F-12 (D:H) media buffered at pH 7.2 with 20 m*M* HEPES with 50 units/ml of penicillin and 50 μg/ml of streptomycin, to which Tr (10 μg/ml), BSA (5 mg/ml), insulin (10 μg/ml), and EGF (10 ng/ml) were added. This medium differs from the SFc medium used previously (e.g., Ref. 11) in the absence of cholera toxin. Cultures were incubated at 37°C in a humidified atmosphere of 95% air and 5% CO_2, and the media were changed every other day. At each time point, collagen gels were removed from the plates and stored frozen at −20°C until DNA assay. Cells were recovered by dissolution of collagen gels in 4.35 *N* acetic acid. Cell number was estimated by a fluorometric DNA assay using mammary tumor cells as a standard (12, 13). In order to normalize the difference in the initial seeding density, data were expressed as increases over the initial cell number. Cell cultures were repeated 3 times, with triplicate cultures used for each point; the data are expressed as mean ± SE. Data were analyzed by Student's *t* test.

Results

Vaginal epithelial cells of both control and DES-exposed mice grew in collagen gel matrix with SF20 medium containing Tr (10 μg/ml), BSA (5 mg/ml), insulin (10 μg/ml), and EGF (10 ng/ml), showing about a 5-fold increase during 10 days. Cells from control and DES-treated mice failed to grow in unsupplemented D:H medium (Fig. 1).

Vaginal epithelial cells from both control and DES-exposed mice did not grow in a serum-free medium without EGF, even in the presence of insulin (10 μg/ml), BSA, and transferrin in the medium. A low concentration of EGF (0.1 ng/ml) stimulated the growth of vaginal epithelial cells from control mice, but failed to stimulate the growth of cells from DES-exposed mice. A high concentration of EGF (100 ng/ml) inhibited proliferation of DES-exposed mouse vaginal epithelial cells (Fig. 1).

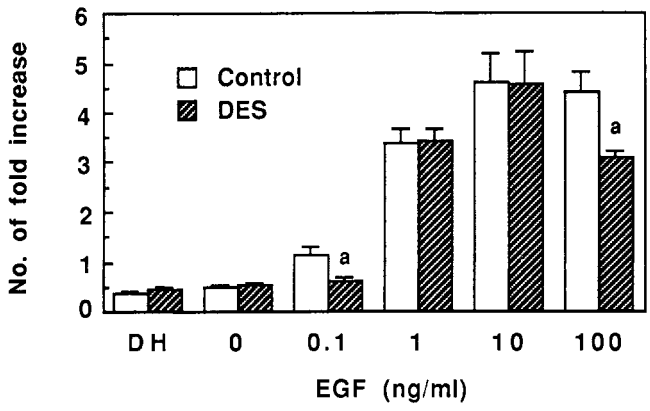

Figure 1. Growth of vaginal epithelial cells (mean ± SE, $n = 3$) cultured for 10 days in D:H medium supplemented with Tr (10 μg/ml), BSA (5 mg/ml), insulin (10 μg/ml), and various concentrations of EGF. a, $P < 0.05$ vs control.

In serum-free medium without insulin, control mouse vaginal epithelial cells increased in number about 2-fold, but cells from DES-exposed mice failed to grow. A lower concentration of insulin (0.1 μg/ml) stimulated the growth of DES-exposed mouse vaginal epithelial cells, but the same concentration of insulin had no stimulatory effect on the control mouse vaginal epithelial cells (Fig. 2). Higher concentrations of insulin (10 and 20 μg/ml) stimulated the maximum growth of both control and DES-exposed mouse vaginal epithelial cells. There was no difference in growth stimulation between the two concentrations (data not shown). IGF-I (1–100 ng/ml) had no effect on the growth of vaginal epithelial cells from either control or DES-exposed mice in the absence of insulin (Fig. 2).

TGF-β (0.05–5 ng/ml) consistently inhibited proliferation of vaginal epithelial cells from control and DES-exposed mice in a dose-dependent manner. No cell growth was found at the highest concentration (5 ng/ml) of TGF-β used (Fig. 3). Short-term exposure to TGF-β (1 ng/ml) beginning at Day 6 of culture significantly inhibited the growth of cells from both control and DES-exposed mice (Fig. 4).

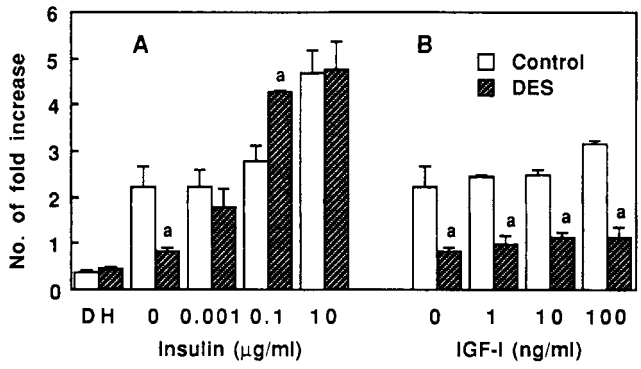

Figure 2. Growth of vaginal epithelial cells (mean ± SE, $n = 3$) cultured for 10 days in D:H medium supplemented with Tr (10 μg/ml), BSA (5 mg/ml), EGF (10 ng/ml), and various concentrations of (A) insulin or (B) IGF-I. a, $P < 0.05$ vs control.

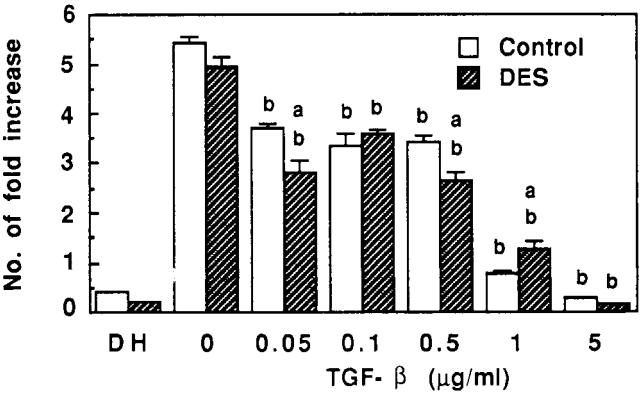

Figure 3. Growth of vaginal epithelial cells (mean ± SE, $n = 3$) cultured for 10 days in D:H medium supplemented with Tr (10 μg/ml), BSA (5 mg/ml), EGF (10 ng/ml), insulin (10 μg/ml), and various concentrations of TGF-β. a, $P < 0.05$ vs control; b, $P < 0.05$ vs 0.

Discussion

The present study demonstrates that EGF is indispensable in this system for *in vitro* growth of vaginal epithelial cells from both control and DES-exposed intact prepuberal (21-day-old) mice. However, vaginal epithelial cells from DES-exposed mice showed lower sensitivity to EGF than those from control mice. EGF receptor levels in the DES-exposed mice vaginae are lower than in the controls (T. Iguchi, M. Edery, P.-S. Tsai, S. Ozawa, H. A. Bern. Epidermal growth factor receptor levels in reproductive organs of female mice exposed neonatally to diethylstilbestrol. Submitted for publication.), and it is likely that this is at least partly responsible for the lower sensitivity to EGF.

Vaginal epithelial cells from 21-day-old DES-exposed mice, which did not grow in SF20 medium without insulin, seem to have a higher dependency on insulin for growth than cells from controls showing 2-fold growth in the insulin-depleted medium. Vaginal epithelial cells from ovariectomized, 40-day-old, DES-exposed mice showed 2-fold growth in an insulin-free, serum-free medium containing EGF, Tr, BSA, EGF,

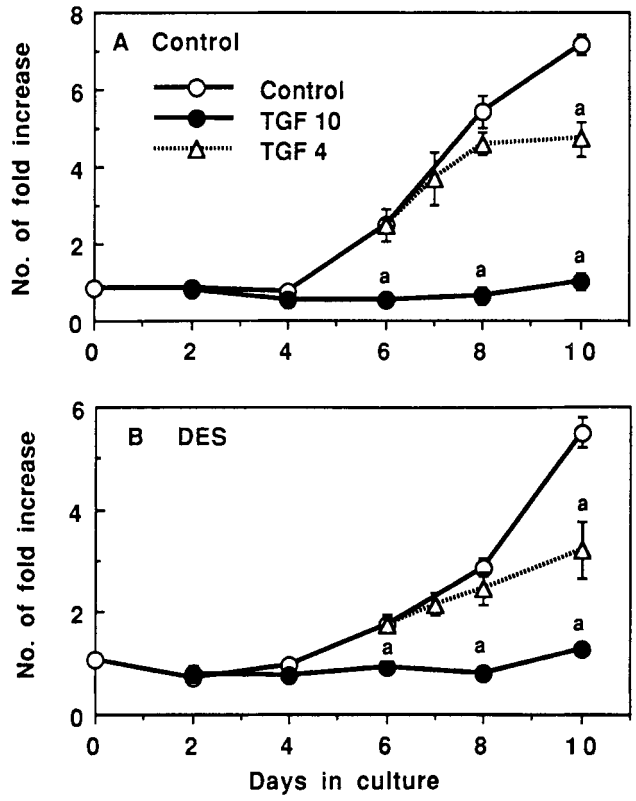

Figure 4. Growth of vaginal epithelial cells from (A) control and (B) DES-exposed mice (mean ± SE, $n = 3$) cultured for 10 days in D:H medium supplemented with Tr (10 μg/ml), BSA (5 mg/ml), EGF (10 ng/ml), insulin (10 μg/ml), and TGF-β (5 ng/ml). TGF-β was added on Day 0 (TGF 10) or on Day 6 (TGF 4) of culture. a, $P < 0.05$ vs control.

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and cholera toxin (10 ng/ml) (11). The differences in the insulin requirement for DES-exposed vaginal epithelial cell growth may arise from differences in the composition of the media used, in the age of the mice, and in the endocrine status of the mice. The mouse ovary begins to secrete estrogen after 21 days of age (14). Vaginal epithelial cells of the 40-day-old, ovariectomized mice were exposed to endogenous estrogen, but those of the 21-day-old mice were not, since the 40-day-old mice were ovariectomized at 30 days of age. IGF-I, which can replace insulin in human fibroblast (15, 16) and mouse mammary epithelial cell (17) cultures, did not support growth of vaginal epithelial cells from either control or DES-exposed mice in a medium lacking insulin. Although IGF-I receptors have been identified in the rat uterus (18, 19), there are no reports on the occurrence of IGF-I receptors in the mouse vagina.

TGF-β, a general growth inhibitor of various types of epithelial cells in culture (20), also inhibited the *in vitro* proliferation of vaginal epithelial cells from control and DES-exposed mice in a dose-dependent manner. Since TGF-β is a hormone-regulated peptide that may act in an autocrine or a paracrine fashion (21), it may prove to be the mediator of estrogen inhibition of mouse vaginal epithelial cell proliferation (8–10, 22).

DES-exposed mammary epithelial cells in serum-free collagen gel culture were less responsive to lithium ion in growth (23) and to prolactin and growth hormone in casein production (24) than were control cells. These prior results and the present observations suggest that cells isolated from the DES-exposed mouse may be generally less sensitive to hormones and growth factors than control cells.

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