Prolactin Sensitivity of Mammary Epithelial Cells from Mice Exposed Neonatally to Diethylstilbestrol (42977)

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Abstract. **We examined the responsiveness to prolactin and growth hormone of mammary epithelial cells from mice exposed neonatally to diethylstilbestrol (DES) and from control mice. The mammary epithelial cells were cultured inside collagen gels with serum-free medium containing insulin, epidermal growth factor, and linoleic acid. This produces prolactin-sensitive cells with low levels of casein production, as measured in cellular homogenates with a specific enzyme-linked immunosorbent assay for α-casein. The collagen gels containing these cells were then released and the medium supplements changed to insulin, linoleic acid, and prolactin at concentrations from 10 to 1000 ng/ml and growth hormone at 0, 10, or 100 ng/ml. This second phase of the culture, the differentiation phase, allows the cells to accumulate casein if they have this capacity. When cultured with prolactin only (no growth hormone), the cells from DES-exposed mice consistently accumulated 50–100% of the casein content of normal cells, but never more. Growth hormone, when added to prolactin-containing medium, increased casein accumulation above the levels seen with prolactin alone. Combinations of prolactin and growth hormone enhanced the difference between casein accumulation in DES-exposed and control cells, and DES-exposed cells were much less responsive to growth hormone. In our studies, the isolated mammary epithelial cells of estrogen-exposed mice are not more sensitive to prolactin than cells from normal animals as previous reports had suggested, but rather are generally less sensitive to hormonal stimulants.**

[P.S.E.B.M. 1989, Vol 192]

Prenatal exposure to diethylstilbestrol (DES) in humans causes genital tract abnormalities and increases the risk of dysplastic and neoplastic changes in the vaginal and cervical epithelium (1). In mice, neonatal exposure to DES (for the first 5 days of life) causes analogous changes in the genital tract (reviewed in 2). Neonatal exposure to estrogen, either DES or estradiol, also causes changes in the mammary gland, including increases in ductal growth (3), ductal and alveolar hyperplasia, and dysplasia and ductal dilation (4, 5), as well as increases in virus-associated tumor incidence (6, 7). Most of these changes in the responsiveness of the mammary epithelial cells are ovary dependent (6–8).

In addition to morphologic changes and changes related to neoplasia, neonatal exposure to estrogen changes the hormonal responsiveness of the exposed mammary glands. Warner and her colleagues (9, 10) reported that neonatally estrogen-exposed mammary epithelial cells in organ culture are more sensitive to prolactin (PRL) as measured by increased lobuloalveolar morphogenesis and increased casein mRNA content. Although demonstration of this altered *in vitro* sensitivity requires *in vivo* priming with estrogen and progesterone, it indicates that the epithelial cells in contact with their stroma (normal organotypy) display a hypersensitivity that might be predicted from *in vivo* observations.

In contrast to the work which demonstrated increased mammary epithelial sensitivity to PRL in organ culture, when Tomooka *et al.* (11) placed DES-exposed mammary epithelial cells in serum-free collagen gel culture they were less sensitive than control cells to at least one growth-stimulating agent, lithium ion. A continuing interest in the hormone sensitivity of isolated DES-exposed mammary epithelial cells in cell culture led us to examine this question in terms of casein accumulation in response to PRL and growth hormone (GH) in a two-stage serum-free collagen gel culture system (12).

Received March 20, 1989. [P.S.E.B.M. 1989, Vol 192]
Accepted June 27, 1989.

0037-9727/89/1922-0187$2.00/0

Materials and Methods

DES Treatment. BALB/cCrgl mice were treated for the first 5 days of life with DES dissolved in sesame oil at a daily dose of 1 μg of DES/mouse (in 50 μl of sesame oil). Control animals were injected with oil only. At this dose DES has permanent ovary-independent effects on the vagina (13). The mice were ovariectomized at 2 months of age and used for the cell isolation procedure 1 month later.

Epithelial Cell Isolation. The cell dissociation procedure was essentially as described previously (14, 15). Briefly, the mammary glands were minced and dissociated with collagenase (0.05% CLS II; Worthington, Malvern, PA), and further digested with 0.01% Pronase (Calbiochem-Behring, San Diego, CA). The epithelial cells were separated from stromal cells and cell fragments by centrifugation on preformed 42% Percoll (Pharmacia, Piscataway, NJ) density gradients and used directly for collagen gel culture.

Collagen Gel Culture. Collagen gel culture of mammary epithelial cells has been extensively described and reviewed elsewhere (14, 15). The serum-free medium, which has also been described (16, 17) was a 1:1 (v/v) mixture of Ham's F12 and Dulbecco's modified Eagle's medium. This medium was supplemented with fatty acid-free bovine serum albumin (Armour, Tarrytown, NY) conjugated to the sodium salt of linoleic acid (Sigma) (17), used with the concentrations of fatty acid at 5 μg/ml for growth or 10 μg/ml for differentiation and bovine serum albumin at 1 mg/ml. Insulin (Sigma) was added at 10 μg/ml. This medium will be referred to as the basal medium. The medium was changed every 2 days.

For growth, the cells were cultured in the basal medium with epidermal growth factor (EGF) (Collaborative Research, Bedford, MA) at 10 ng/ml. After a 6-day growth phase in the EGF-containing medium, the cultures were induced to accumulate casein by releasing the gels with a small spatula to float in the medium (12, 18). Release of the gel was followed by addition of the differentiation medium and an additional 6 days of culture, the differentiation phase. Differentiation medium was basal medium supplemented with PRL (10 to 1000 ng/ml, NIADDK ovine PRL PS-17) without or with GH (10 or 100 ng/ml, NIADDK bovine GH B-1). After the differentiation phase (10 to 12 days total in culture), the cultures were terminated by blotting the collagen gel on a stack of paper towels to remove trapped medium (19) and freezing the resulting membrane-like collagen/cell combination at −70°C for future assay.

Casein Assay Procedures. Prior to assay the frozen gels were homogenized in phosphate-buffered saline with 5 m*M* EDTA and 1% Triton X-100 at 4°C. The homogenate was centrifuged at 12,000*g* for 30 min at 4°C. The clear supernatant was used for the α-casein enzyme-linked immunosorbent assay and the pellet was used for DNA assay according to the method of Hinegardner (20).

The enzyme-linked immunosorbent assay was developed from the method of Engvall and Carlsson (20) and has been described previously (12). Briefly, purified α-casein (21) was coated onto Costar EIA plates. Dilutions of standard α-casein or the culture homogenates were added to these plates, followed by casein antiserum (21), diluted 1/100,000. The plates were incubated for 44 hr at 4°C, washed, and incubated with alkaline phosphatase-conjugated second antibody (Sigma) and then with *p*-nitrophenyl phosphate. The reaction product was measured by absorbance at 405 nm. As this assay does not distinguish between intracellular and secreted α-casein and the culture topology does not allow separation of the two, we refer to accumulated α-casein.

Statistical Analysis. The effect of all culture treatments on casein accumulation was determined on triplicate cultures in each experiment. The experiments were repeated four or five times. Data were pooled and are shown as the mean with the standard error of the mean. Data were log-transformed and analyzed by two-way or three-way ANOVA using CRISP software (Oakland, CA).

Results

Mammary epithelial cells cultured inside collagen gel in medium containing EGF and linoleic acid do not accumulate significant α-casein whether the cells were isolated from control or DES-treated animals (data not shown, see 12). After release of the collagen gels and replacement of the growth medium with PRL-containing medium, the cells accumulated α-casein dependent on the concentration of PRL (Fig. 1A). At every concentration of PRL used except one, 300 ng/ml, DES-exposed cells accumulated 50–60% of the α-casein of control cells. This point is made more clearly by Figure 1B, where the α-casein accumulation of the DES-exposed cells is normalized to the α-casein accumulation value for control cells at the same PRL concentrations. The reduced ability of DES-exposed cells to accumulate α-casein was statistically significant by two-way ANOVA ($P \leq 0.05$, $n = 5$).

When GH is added to the medium in the absence of PRL, little α-casein is accumulated over that seen in response to medium with insulin and linoleic acid alone: 4–6 ng of α-casein/10^6 cells with 100 ng/ml GH versus 1–2 ng of α-casein/10^6 cells without GH. Although this effect of GH in the absence of PRL is statistically significant for control cells ($P \leq 0.05$, *t* test), the response is minimal compared with PRL or combinations of PRL and GH. However, when GH is added to medium containing PRL, there is a GH concentration-dependent increase in the α-casein accumulation

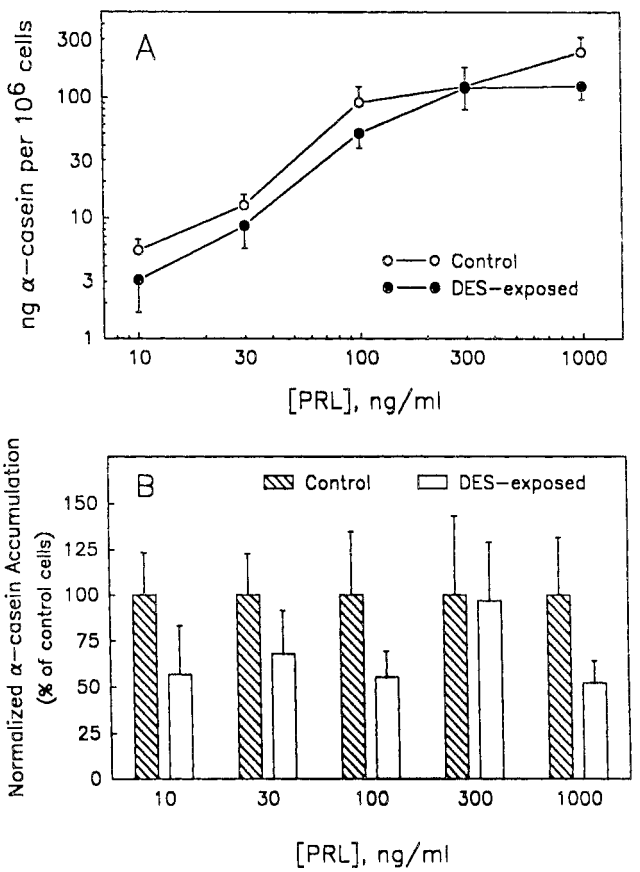

Figure 1. PRL-sensitive α-casein accumulation differs between control and neonatally DES-exposed mouse mammary epithelial cells. Mammary epithelial cells obtained from neonatally DES-treated or control mice were grown in collagen gel with serum-free basal medium supplemented with insulin, linoleic acid, and EGF for 6 days. The gels were then released and the medium supplements switched to insulin, linoleic acid, and PRL at the concentrations shown (differentiation medium). The cultures were continued for 6 more days, then terminated and assayed for α-casein. (A) Accumulation of α-casein in DES-exposed or control cells after 6 days of exposure to differentiation medium. Note the log ordinate scale. The reduction in α-casein accumulation seen in the DES-exposed cells is statistically significant by two-way ANOVA ($P \leq 0.05$). (B) The data used to plot (A) were normalized to the amount of casein made by the control cells at each PRL concentration. The amount of casein produced by the control cells at each PRL concentration was set to 100%. In A and B, each point or bar represents the mean of four or five difference experiments with standard error of the mean.

in the control cells (Fig. 2) which is statistically significant by Dunnett's test ($P \leq 0.05$ for GH at 10 ng/ml and $P \leq 0.01$ for GH at 100 ng/ml). There is no statistically significant GH-dependent increase in α-casein accumulation in DES-exposed cells. The difference between the control and DES-exposed cells is also evident in Figure 2 and is significant by three-way ANOVA ($P \leq 0.001$).

Discussion

This study clearly shows the DES-exposed mammary epithelial cells are less responsive to a mammogenic/lactogenic hormone (PRL) than unexposed cells, at least when cultured in serum-free collagen gel culture. This result parallels earlier findings using mammary epithelial cells (11) and genital epithelial cells (22 and F-D. A. Uchima, personal communication) in cell culture; DES-exposed epithelial cells of all types appear to be less responsive to a variety of stimulations in cell culture than their control or untreated counterparts.

The seat of this reduced PRL responsiveness has not been determined. It may be related to a generalized

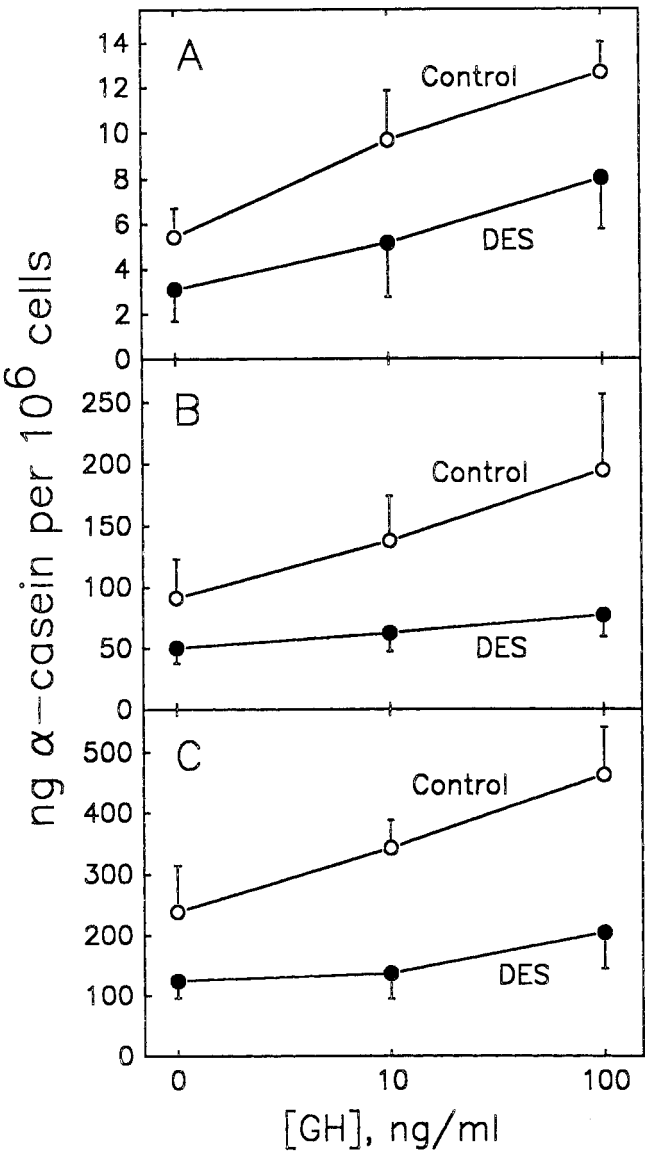

Figure 2. GH increases PRL-sensitive α-casein accumulation in control cells more than in neonatally DES-exposed cells. Mammary epithelial cells obtained from DES-treated or control mice were grown in collagen gel with serum-free basal medium supplemented with EGF for 10 days. The gels were then released and the medium supplements switched to PRL at 10 ng/ml (A), 100 ng/ml (B), or 1000 ng/ml (C) and GH at the concentrations shown. The effect of GH on α-casein accumulation in the control cells was statistically significant by Dunnett's test ($P \leq 0.05$, GH at 10 ng/ml; $P \leq 0.01$, GH at 100 ng/ml) when compared with cells cultured in the absence of GH. The difference between the DES-exposed and control cells was statistically significant by three-way ANOVA ($P \leq 0.001$). Each point represents the mean of five different experiments with standard error of the mean.

reduced cellular response of DES-exposed estrogen-sensitive tissues to any stimulus in cell culture, as noted above. Previously it has been shown that the mammary glands of DES-treated mice have a reduced estrogen receptor content and may have a reduced PRL receptor content (23). If the PRL receptor content of the mammary epithelial cells were reduced, the cellular response to PRL might also be reduced. We have not measured the PRL receptor content of the control or DES-exposed mammary epithelial cells *in vitro*. However, mammary gland PRL receptor content is known to be increased by PRL (24, 25). This is significant because some studies have shown that serum PRL is increased in neonatally DES-treated mice (26) and that the increase is ovary dependent (27). The hyperplastic/neoplastic changes seen *in vivo* in the mammary gland are ovary dependent (6, 8, 13), and thus perhaps dependent on elevated serum PRL and elevated mammary PRL receptor levels. Clearly, more investigation into the PRL receptor levels of the mammary epithelial cells from DES-treated mice is necessary to clarify this point. Finally, we cannot currently exclude the possibility that the differences we observe in cell culture are caused by the cell isolation procedure itself. Both the DES-exposed mammary gland (data not shown) and DES-exposed vagina (F-D. A. Uchima, personal communication) show differential sensitivity to collagenase digestion than the control tissues. We are currently examining this question.

In contrast to these cell culture studies, estrogen-exposed mammary explants in organ culture are more responsive to PRL than control explants (9, 10). This apparent discrepancy may reflect the importance of the mammary stroma in the organ cultures. Transplantation of mammary epithelial cells from DES-treated mice to unexposed mice, allowing the DES-exposed mammary epithelial cells to grow out into a normal stromal environment (mammary fat pad), produces entirely normal outgrowths (28). This indicates that the stimulus for the DES-induced epithelial hyperplasia normally seen *in vivo* with lower concentrations of DES (13) may not rest within the epithelial cells themselves.

Finally, the data in this article, along with the previous work cited above (11, 22, and F-D. A. Uchima, personal communication) demonstrate that *in vitro* culture of DES-exposed epithelial cells in collagen gel reveals an alteration in the hormonal responsiveness of the epithelial cells. These alterations are different from those demonstrated *in vivo* or in organ culture, both cases where the normal stroma is present, and suggest that the presence of the stromal cells masks or conceals alterations in the epithelial cells.

Although the contrast between this cell culture study and previous organ culture studies emphasizes the importance of the stromal-epithelial interaction in determining the overall responsiveness of the mammary tissue, it also demonstrates that important biologic phenomena may be masked if only one culture system is used. In this case, the interactions in organ culture apparently mask a "defect" in the responsiveness of the DES-exposed epithelial cells. This defect is only uncovered when the epithelial cells are cultured in a supportive stimulatory environment, in the absence of stromal cells. Culture of stromal cells from the DES-exposed mammary glands to determine their responsiveness and perhaps to use them in a coculture system similar to that developed by Levine and Stockdale (29, 30) might reveal other differences between normal and DES-exposed mammary tissue.

This research was aided by Public Health Service Grants CA09041 and CA05388 provided by the National Cancer Institute, Department of Health and Human Services. We thank Ms. S. Hamamoto for performing the enzyme-linked immunosorbent assays, Dr. S. McCormick for assistance with statistical analysis, and Ms. K. Tanada Mills for assistance with the DES-treated mice. Ovine PRL and bovine GH were the gifts of the NIADDK and the National Hormone and Pituitary Program.

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