Pituitary Tumors in Mice Exposed Prenatally to Diethylstilbestrol

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ABSTRACT

Hyperprolactinemia and prolactinomas are among the abnormalities reported for women exposed prenatally to diethylstilbestrol (DES). To pursue this issue in an animal model replicating the other abnormalities of prenatal DES exposure, pituitary glands were studied in the offspring of CD-1 mice receiving an i.p. injection of 1 or 2 μg DES/g body weight during late pregnancy. Among 132 mice exposed prenatally to DES and then raised to terminal illness, there were 24 pituitary tumors compared to only 1 tumor among 64 controls. The tumors consisted predominantly of cells with an eccentric nucleus and cytoplasm characterized by an acidophilic core and basophilic rim. These cells were identified as lactotrophs on the basis of prolactin immunohistochemistry and by an expected variation in frequency relative to physiological states. Evaluation of ovaries from the same mice revealed a deficiency of corpora lutea and an elevated incidence of ovarian tumors. These findings are consistent with abnormal sex differentiation of the fetal hypothalamus being the cause of most adverse effects from prenatal DES exposure.

INTRODUCTION

Anomalies and cancer of the vagina and cervix, as well as increased fetal losses during pregnancy, have been reported for women exposed prenatally to DES1 (1). These abnormalities have been replicated in a murine animal model (2-4), which provides an opportunity to test the impact of aging and to search for the mechanism of the DES effect. Increased frequencies of hyperprolactinemia and prolactinomas have also been reported for DES-exposed women (5-7). Using the murine model that has replicated the other effects of DES in prenatally exposed women, we present evidence for pituitary tumors of the prolactinoma type. Ovarian morphology, including tumors, will also be described to assess possible correlating events.

MATERIALS AND METHODS

DES Treatment. CD-1 mice purchased from Charles River Laboratories (Wilmington, MA) were maintained on a controlled light cycle with 10 h of darkness and given free access to water and food (Wayne Mouse Breeder Blox). They were mated during the dark cycle and pregnant mice were given a single i.p. injection of DES (Sigma Chemical Co., St. Louis, MO) at a dose of 1 μg/gbw at 16 days and 16 h postconception (day 16/16), or on day 17/0, or 17/16, as described previously (4). Controls were given the equivalent volume of vehicle, 1 μg/gbw of olive oil. In addition, 3 pregnant mice were given an injection of 2 μg/gbw of DES on day 17/16. Female offspring were maintained until they died naturally, or were killed with an overdose of anesthetic (methoxyflurane) if terminal illness was recognized. For comparison of cell types under varied physiological conditions, pituitaries were taken from a mouse ovariec-tomized at 6 weeks of age and killed at 16 weeks of age, from a lactating mouse, and from a young adult male. Pituitary glands were removed and fixed in 10% formalin. The glands were then weighed and embedded in paraffin or in glycol methacrylate (Polysciences, Inc., War-lington, PA). Some pituitaries were cut transversely with one half being embedded in paraffin. Paraffin sections were cut at 6 μm and stained with hematoxylin and eosin. Plastic sections were cut at 2 μm and stained with Lee’s methylene blue-basic fuchsin (8). Evaluation of mammary gland hypertrophy was made at autopsy and was based on mammary duct distension recognized as a thick, white network with the dissecting microscope.

Histometric Analysis. Cell counts were performed on plastic sections. One section near the center of each gland was chosen for histometric analysis by oil immersion light microscopy. Three equidistant transverse rows, one field wide, were studied starting one-fourth of the way through the section. Only two cell types, acidophils and dual-staining cells, were counted. They were identified by the following criteria. Cells with a distinct cell boundary, homogeneously eosinophilic cytoplasm, and a central nucleus were scored as acidophils. Cells with an eccentric nucleus and cytoplasm showing eosinophilic staining centrally and basophilic staining peripherally were scored as dual-staining cells (Fig. 1). Immunohistochemical testing was performed on paraffin sections with rabbit antiserum against ovine prolactin, using the peroxidase-antiperoxidase technique (Shandon Lipshaw Immunotags Systems, Pittsburgh, PA). Ovaries were evaluated for the presence of follicles, corpora lutea, and tumors by mounting about 60 sections from the center of each ovary and studying every fourth section.

Statistics. The χ² test was used to compare the frequency of tumors, hyperplastic glands, follicles, and corpora lutea between control and DES-exposed mice and to compare control pituitary cell counts with pituitary cell counts from lactating, ovariec-tomized, or male mice. Trends in the frequency of cell types with increasing pituitary gland weights were evaluated by Spearman’s coefficient of rank correlation. Age distributions at autopsy for mice with and without tumors were compared by t test.

RESULTS

Pituitary Tumors. Pituitary gland weights for DES-exposed mice were skewed to the right compared to control glands (Fig. 2). There was one pituitary above 9 mg in vehicle-exposed mice and 22 pituitaries above that weight in DES-exposed mice (P < 0.01). When these oversized glands were excluded, the average weight of pituitaries from vehicle-exposed mice was 3.67 mg, whereas pituitaries from DES-exposed mice averaged 4.19 mg (P < 0.01). Among glands weighing 5 to 8 mg from both DES- and vehicle-exposed mice, there was a trend towards hypertrophy of the anterior lobes. Glands above 9 mg consistently had one enlarged anterior lobe accounting for the increased weight of the gland. Microscopically, these glands typically showed increased mitotic frequency, increased vascularization with vascular lake formation, occasional cell hypertrophy, and predominantly one type of glandular cell. They were classified as large tumors (Table 1). Among 12 large tumors embedded in plastic, almost all of the well-stained cells were the dual-staining type (Fig. 1). Two glands in the weight range of 5 to 8 mg from DES-exposed mice showed microscopic characteristics similar to those of the large tumors and were listed as small tumors (Table 1). The average age at autopsy of DES-exposed mice with pituitary tumors was 21.5 ± 3.1 (SD) months, compared to 19.9 ± 4.9 months for DES-exposed mice without tumors (P > 0.1). The latter was not significantly different from the average of 20.0 ± 6.3 months for control mice without tumors (P > 0.9). The single control mouse with a pituitary tumor was 23 months of age.

Cell counts on plastic sections were limited to acidophilic cells and dual-staining cells, since these were the most common glandular cells that were clearly identified in the anterior lobes with the stain used. The proportion of these two cell types was not significantly different...
5 were nonreactive (including the only tumor from a vehicle-exposed female). Among the strongly reactive tumors, 3 showed this reaction mainly in a peripheral area of normal-sized cells; the central area with hypertrophied cells was negative. Plastic sections were available for 8 of the tumors tested immunohistochemically and all of these tumors were composed mainly of dual-staining cells, even though 4 of them did not react to prolactin antibody.

**Ovarian Tumors.** Among the 7 ovarian tumors found in DES-exposed mice (Table 3), 1 was a granulosa cell tumor, 1 was a tubular adenoma, and the other 5 were papillary cystadenomas according to published criteria for murine ovarian tumors (9). More ovaries with follicles and without corpora lutea were encountered in DES-exposed mice than in vehicle-exposed mice (Table 3). An ovarian tumor occurred only once in a mouse that also had a pituitary tumor, and this is consistent with a random event \((P > 0.5)\). The average age at autopsy of mice with ovarian tumors was 26.3 ± 2.4 months. This is significantly older than the average of 19.6 ± 5.3 months for DES-exposed mice without ovarian tumors \((P < 0.01)\).

Among 6 pituitaries and 12 ovaries from 6 mice exposed prenatally to 2 \(\mu g/gbw\), there were 3 pituitary tumors and 2 ovarian tumors. Compared to 21 pituitary tumors and 5 ovarian tumors from 126 mice exposed to 1 \(\mu g/gbw\), this is a significantly higher number of total tumors relative to total organs at risk \((P < 0.01)\). Mammary duct hypertrophy was present in 16% of mice with pituitaries within the normal weight range and in 50% of mice with tumors weighing over 9 mg, which is a significant difference \((P < 0.01)\).

**DISCUSSION**

Spontaneous pituitary tumors are rare in mice, with only one reported from a review of 11,188 mice of the Slye stock (10). In the

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**Table 1 Number of normal, hypertrophic, and adenomatous pituitary glands among the female offspring of mice given DES injections during pregnancy**

<table>
<thead>
<tr>
<th>DES injection (days/h postconception)</th>
<th>Normal</th>
<th>Hyper-</th>
<th>Small</th>
<th>Large</th>
<th>% of tumors</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle-exposed</td>
<td>27</td>
<td>7</td>
<td>1</td>
<td>3</td>
<td>10.5</td>
</tr>
<tr>
<td>17/16, 1 (\mu g/gbw)</td>
<td>23</td>
<td>7</td>
<td>1</td>
<td>4</td>
<td>14.3</td>
</tr>
<tr>
<td>17/16, 1 (\mu g/gbw)</td>
<td>34</td>
<td>7</td>
<td>0</td>
<td>12</td>
<td>22.6</td>
</tr>
<tr>
<td>17/16, 2 (\mu g/gbw)</td>
<td>3</td>
<td>0</td>
<td>0</td>
<td>3</td>
<td>50.0</td>
</tr>
</tbody>
</table>

Statistically significant difference \((P < 0.05)\) in number of large tumors compared to vehicle-exposed mice.

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**Table 2 Frequency of acidophils and dual-staining cells in pituitary glands of various sizes and functional states**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. of glands counted</th>
<th>Av. wt (mg)</th>
<th>Total cells counted</th>
<th>Av. no. of cells/field</th>
<th>Acidophils</th>
<th>Dual-staining</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle-exposed</td>
<td>3</td>
<td>3.9</td>
<td>1152</td>
<td>8.32</td>
<td>1.5</td>
<td></td>
</tr>
<tr>
<td>DES-exposed</td>
<td>3</td>
<td>3.7</td>
<td>870</td>
<td>9.05*</td>
<td>0.86*</td>
<td></td>
</tr>
<tr>
<td>DES-exposed</td>
<td>6</td>
<td>6.6</td>
<td>2738</td>
<td>8.26</td>
<td>6.78</td>
<td></td>
</tr>
<tr>
<td>DES-exposed</td>
<td>3</td>
<td>12.9</td>
<td>1566</td>
<td>1.31</td>
<td>8.20</td>
<td></td>
</tr>
<tr>
<td>DES-exposed</td>
<td>5</td>
<td>8.43</td>
<td>2401</td>
<td>0.66*</td>
<td>9.43*</td>
<td></td>
</tr>
<tr>
<td>Ovariectomized</td>
<td>1</td>
<td>4.3</td>
<td>567</td>
<td>19.0</td>
<td>0.34*</td>
<td></td>
</tr>
<tr>
<td>Lactating</td>
<td>1</td>
<td>4.9</td>
<td>525</td>
<td>14.8</td>
<td>5.98*</td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>1</td>
<td>3.7</td>
<td>520</td>
<td>18.04</td>
<td>0.54*</td>
<td></td>
</tr>
</tbody>
</table>

* No statistically significant difference \((P > 0.05)\) compared to DES-exposed mice in frequency of hypertrophic compared to normal pituitary glands.

* No statistically significant difference \((P < 0.05)\) in cell frequency compared to vehicle-exposed pituitary glands.

* No statistically significant difference \((P < 0.05)\) in frequency of acidophils with increasing weight of DES-exposed pituitary glands.

* Statistically significant increase \((P < 0.05)\) in frequency of dual-staining cells with increasing weight of DES-exposed pituitary glands.

* Statistically significant difference \((P < 0.01)\) compared to DES-exposed mice in frequency of dual-staining cells.

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**Table 3 Number of ovaries with follicles, corpora lutea, and tumors in female offspring of mice given DES injections during pregnancy**

<table>
<thead>
<tr>
<th>DES injection (days/h postconception)</th>
<th>Total no. of ovaries</th>
<th>No. (%) of ovaries with</th>
<th>Ovarian morphology</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Follicles</td>
<td>Corpora lutea</td>
</tr>
<tr>
<td>16/16, 1 (\mu g/gbw)</td>
<td>84</td>
<td>71* (85)</td>
<td>24* (29)</td>
</tr>
<tr>
<td>17/10, 1 (\mu g/gbw)</td>
<td>62</td>
<td>52* (84)</td>
<td>9* (15)</td>
</tr>
<tr>
<td>17/16, 1 (\mu g/gbw)</td>
<td>106</td>
<td>95* (90)</td>
<td>16* (15)</td>
</tr>
<tr>
<td>17/16, 2 (\mu g/gbw)</td>
<td>12</td>
<td>11* (92)</td>
<td>3* (25)</td>
</tr>
<tr>
<td>Controls</td>
<td>128</td>
<td>81 (63)</td>
<td>73 (57)</td>
</tr>
</tbody>
</table>

* Statistically significant increase \((P < 0.05)\) compared to controls.

* Statistically significant decrease \((P < 0.05)\) compared to controls.

* Statistically significant difference \((P < 0.05)\) compared to the total number of tumors for all DES treatment groups.
CD-1 strain, one pituitary tumor was encountered in 1000 autopsies, although only 144 of these mice were over 16 months of age (11). In the present experiment, one pituitary tumor occurred among 64 control mice. Thus, the 24 pituitary tumors found among 132 old female mice exposed prenatally to DES can be confidently attributed to the treatment. The treatment was not acting through an effect on longevity. Among DES-exposed mice, those with tumors were not significantly older than those without tumors. In addition, DES-exposed mice did not have a longer life span than control mice. All tumors were composed predominantly of one cell type, according to the stain used. This does not appear to be a limitation of the staining technique, since the same stain revealed pituitary tumors involving three different cell types in a previous study on the same mouse strain (12).

The predominant cell in the tumors was characterized by an eccentric nucleus and by cytoplasm with an acidophilic core and basophilic perimeter, which is consistent with the ultrastructure of a lactotroph (13). The decreased frequency of this cell in a male and an ovariec
tomized female and its increased frequency in a lactating female matches previously reported differences in lactotroph frequencies under these conditions (13-15). Also, the tendency for mammary duct hypertrophy in the mice with pituitary tumors is consistent with these tumors being prolactinomas (16). Immunohistochemical binding of prolactin to most of the tumors is consistent with the dual-staining cell being a lactotroph. The 3 tumors in which there was a peripheral mass of small, reactive cells and a central mass of large, nonreactive cells
der these conditions (13-15). Also, the tendency for mammary duct hypertrophy in the mice with pituitary tumors is consistent with these tumors being prolactinomas (16). Immunohistochemical binding of prolactin to most of the tumors is consistent with the dual-staining cell being a lactotroph. The 3 tumors in which there was a peripheral mass of small, reactive cells and a central mass of large, nonreactive cells could be interpreted as evidence that the basic tumor cell was a lactotroph, but anaplastic changes in the main tumor modified the reaction in some manner, such as causing rapid release of hormone (17). The other 5 large tumors with dual-staining cells may have been nonreactive to prolactin for the same reason. Considering the multiple sources of evidence that the dual-staining cells were lactotrophs, it is reasonable to conclude that all the pituitary tumors in this study were prolactinomas.

Ovaries of the DES-exposed females at the end of their life span were characterized by a deficiency of corpora lutea and an excess of follicles compared to control mice. This condition in transplanted ovaries has been shown to increase the number of lactotrophs in castrated male mice (18). The effect was attributed to estrogen being secreted continuously, with little progesterone to counterbalance its effects. The deficiency of corpora lutea is an indirect effect of prenatal DES exposure, since transplantation of ovaries between DES-treated and control mice led to steroid synthesis patterns and presence or absence of corpus luteum development characteristic of the host, not the donor (19).

Spontaneous ovarian tumors are generally not common in mice (9), although this varies with the strain. None were reported from 564 autopsies of female CD-1 mice (11). However, 3 ovarian tumors were found in CD-1 mice exposed prenatally to DES at 9 through 15 days postconception (20), and 2 of these overlapped histopathologically with the ovarian tumors reported in the current study. Ovarian tumors can induce prolactinomas (21), although this was not a general explanation for the prolactinomas in the present study due to the lack of a significant overlap in occurrence of pituitary and ovarian tumors in the same mouse. The average age of mice with ovarian tumors was greater than that of DES-exposed mice without tumors, but this does not explain the tumorigenic effect of DES, since DES-exposed mice did not have a longer average life span than control mice.

The significance of pituitary tumors arising from prenatal exposure to DES pertains to the cause of the tumors in relation to possible basic effects of DES on the fetus. Hypotheses about these basic effects include somatic mutation and disruption of hypothalamic function (22, 23). These are also the hypotheses offered to explain the origins of pituitary tumors (24, 25). Somatic mutation has been reported in a pituitary adenoma (26) and DES has the potential to damage DNA (27). DES can reach the fetal pituitary, but it also has been localized in the hypothalamus (28). Production of pituitary tumors by estrogenic substances may arise through its direct action on the pituitary, and/or through a suppressing effect on tubulointerstitial proliferative tumors (17, 25). The latter would explain hyperprolactinemia, as well as prolactinomas. Development of mammary cystic tumors in pituitaries grazed to areas distant from the hypothalamus is also consistent with escape from the suppressing effects of dopamine neurons (29).

Combining hypotheses about a hypothalamic cause for both the origin of a prenatal DES effect and the origin of pituitary adenomas produces the following sequence. DES alters sex differentiation of the fetal hypothalamus either by acting directly as an estrogenic substance, or by displacing natural estrogen from α-fetoprotein (22). This produces a delayed anovulatory syndrome in the adult, with loss of cyclic luteinizing hormone surges (30). The ovary then contains nonovulating follicles and lacks corpora lutea. The persistent estrogen secretion without substantial progesterone predisposes to the formation of pituitary tumors by direct action on the pituitary and/or inhibition of hypothalamic dopamine neurons. Alternatively, the latter neurons may have been affected directly by DES modification of fetal brain differentiation.

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5. Menzner, J., Dulitzky, M., Ben-Baruch, G., and Modan, M. Primary infertility in
PITUITARY TUMORS FROM PRENATAL DES


Pituitary Tumors in Mice Exposed Prenatally to Diethylstilbestrol

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