Uterine Adenocarcinoma in Mice following Developmental Treatment with Estrogens: A Model for Hormonal Carcinogenesis

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ABSTRACT

In order to study the effects of perinatal exposure to estrogens on the developing reproductive tract, outbred female mice were treated neonatally (days 1 to 5) with varying doses of diethylstilbestrol (DES) and sacrificed from 1 to 18 months of age. Uterine adenocarcinoma was observed in a time- and dose-related manner after DES treatment; at 18 months, neoplastic lesions were seen in 90% of the mice exposed neonatally to 2 µg/pup of DES/day, while none was observed in the corresponding control mice. These DES-induced uterine tumors were estrogen dependent; when DES-treated mice were ovariectomized before puberty, no uterine tumors developed. As a marker for neoplasia, uterine tumors were transplanted and carried as serial transplants in nude mice. The transplanted tissue retained some differentiated uterine gland structure and function and also required estrogen supplementation for maintenance. Additional groups of neonatal mice were treated with various DES analogues (hexestrol and tetrafluoro-diethylstilbestrol) and steroidal estrogens. The compounds were ranked according to developmental estrogenic potency (hexestrol > trifluoro-diethylstilbestrol > DES > 17β-estradiol). The combined prevalence of uterine atypical hyperplasia and adenocarcinoma follows the order of estrogenic potency. The experimental induction of these tumors will provide the basis for additional studies in mechanisms of hormonal carcinogenesis.

INTRODUCTION

Estrogens have long been implicated as important etiological agents in malignant tumors of the breast and reproductive tract. Furthermore, many tumors are dependent on estrogen for continued growth, regressing when estrogen is withdrawn or when estrogen antagonists are provided. However, the exact role of natural and synthetic estrogenic hormones in the carcinogenic transformation of target cells is not known.

Many questions on the mechanisms of hormonal carcinogenesis remain unanswered. Can hormones act as complete carcinogens, interacting directly or indirectly with the DNA of target cells to induce transformation events? Is the carcinogenic effect of hormones separable from their hormonal activities? Are hormones acting as tumor promoters after other factors have initiated the neoplastic events? Do synthetic hormones cause events similar to those by the naturally occurring hormones? Are hormones involved in carcinogenesis through their hormonal activity or through some other functional part of the molecule? Does hormonal carcinogenesis involve mechanisms other than those described for multistage chemical carcinogenesis? Are the terms initiation and promotion appropriate when describing mechanisms of hormonal carcinogenesis? Although no definitive answers exist for these questions, the association between estrogenic hormones and cancer of the reproductive system is irrefutable.

For many years, research in our laboratory has centered on the broad topic of hormonal carcinogenesis. In trying to answer the specific question of whether compounds with estrogenic activity are carcinogenic because they cause increased proliferation or are they carcinogenic due to other mechanisms including receptors or metabolism, we have compared a series of estrogenic compounds with various metabolic pathways and estrogenic potential. Thus far, we have shown that prenatal exposure of mice to DES2 results in various neoplastic lesions in the male including rete testis adenocarcinoma (1, 2), testicular tumors (3), and tumors in retained mullerian duct remnants (4) as well as neoplasms in the female including vaginal adenocarcinoma (5, 6), uterine tumors (5), and tumors of mesonephric remnants (7). The incidence of all of these tumors is low with the most frequent, rete adenocarcinoma, occurring in only 5% of the prenatal DES-exposed male mice (1, 2).

In order to study the mechanisms involving hormonal carcinogenesis, a model is needed in which tumors can be induced at a higher frequency than the prenatal treatment models previously described which use developmentally differentiating tissues, exposed to DES at relatively low doses and for a short exposure period. Believing that the developmental period is highly susceptible to perturbation with estrogens, we have expanded the exposure period to include neonatal days 1 through 5, a time which corresponds to late prenatal human development (8). Persistent changes in the vaginal epithelium have been reported following neonatal treatment with estrogen (9–12). In addition, these studies have described cellular changes resembling carcinoma in the uterus, although these tumors were not characterized as extensively as the lesions in the lower reproductive tract. As described in the hamster by Leavitt et al. (13, 14), the uterus seems especially sensitive to neoplastic transformation with estrogens. In fact, in the present report we have induced uterine adenocarcinoma in 95% of the DES-exposed mice by 18 months of age. A comparison of various estrogenic compounds reveals that neonatal estrogenicity and metabolism probably play a role in the observed long-term carcinogenic changes.

MATERIALS AND METHODS

Animals and DES Treatment

Pregnant outbred female CD-1 [Crl:CD-1(ICR)BR] mice near term were obtained from the breeding colony at the NIEHS, Research Triangle Park, NC. Mice were individually housed in plastic cages under a 12-h light and 12-h dark schedule in a temperature (21–22°C)-controlled room and fed NIH 31 mouse chow and fresh water ad libitum. All animal procedures complied with NIEHS animal care guidelines. At delivery, litters were adjusted to 8 female pups/mom. Pups were given daily s.c. injections of DES (Sigma Chemical Co., St. Louis, MO) in corn oil or corn oil alone (as a control) for days 1–5 of life. Doses of DES ranged from 0.002 to 2 µg/neonatal mouse/day. The 2-µg/pup/day dose was chosen as the highest amount since it had

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1 To whom requests for reprints should be addressed.

2 The abbreviations used are: DES, diethylstilbestrol; TF-DES, tetrafluoro-diethylstilbestrol; NIEHS, National Institute of Environmental Health Sciences.
been previously reported to result in uterine alterations (6, 10). At 1 month of age, 15 intact corn oil-treated (control) and 18 intact DES mice were sacrificed by cervical dislocation. The remaining control and DES-treated mice were weaned, housed 5/cage, and maintained until sacrifice at the following ages: 2 months (10 control and 10 DES-treated); 4 months (10 control and 10 DES-treated); 6 months (12 control and 12 DES-treated); 8 months (5 control and 5 DES-treated); 12 months (17 control and 17 DES-treated); and 18 months (10 control and 10 DES-treated) of age. Reproductive tract tissues were quickly removed, fixed in 10% neutral buffered formalin, embedded in paraffin, and sectioned at 6 µm. Tissue sections were stained with hematoxylin-eosin and evaluated using a light microscope. If an area of pathological change was observed, tissues were serially sectioned.

Effects of Ovariectomy

At 17 days of age, prior to puberty, a group of control and DES-exposed mice were ovariectomized and maintained until sacrifice at, 3, 10, 12, and 18 months of age. Reproductive tract tissues were removed and processed as previously described for the intact mice.

Tumorogenesis Assay

Nude female and male mice (nu/nu-CD-1 from Charles River Research Labs., Wilmington, MA) 6 to 10 weeks of age were gonadectomized and implanted s.c. with pellets containing 0.05 mg of cholesterol (placebo), 0.05 mg of 17β-estradiol, 0.05 mg progesterone, or 0.05 mg dihydrotestosterone. Pellets were purchased from Innovative Research of America, Toledo, OH, and supplemented every 30 days. Reproductive tract tissues were obtained for transplant from control and DES-treated mice which were sacrificed when they were over 18 months of age. The right uterine horn was removed and fixed for histological studies. The left uterine horn was removed using aseptic techniques, minced with scalpel blades in cold Basal medium (Eagle)-modified basal medium (Earle's) (Grand Island Biological Company (GIBCO), Grand Island, NY) containing penicillin and streptomycin (5000 units/ml-5000 µg/ml solution from Grand Island Biological Company). Uterine tissue fragments (1 to 2 mm in diameter) were injected s.c. in the nude mice using a 12-gauge precision trocar (Innovative Research of America). Tumor growth was monitored twice weekly and tumor measurements were determined using a caliper (Fisher Scientific Co., Raleigh, NC). Measurements were discontinued after 120 days. Tumors were excised and either tumors were serially transplanted into other nude mice or tumor tissue was examined for morphological and/or functional characteristics. In some cases, tissues that had been carried as transplants were fixed in Bouin's solution, dehydrated, embedded in paraffin, and sectioned at 6 µm. Tissues were stained for the presence of the estrogen-inducible protein, lactoferrin, by methods described previously (15). Lactoferrin was also measured in the fluid that accumulated in some implanted tissues using Western blot techniques described previously (15). The lactoferrin antibody was graciously provided by Dr. Christina Teng, NIEHS.

Studies with DES Analogues and Steroidal Estrogens

Developmental Estrogenic Potency. Neonatal female CD-1 pups were given s.c. injections of hexestrol (Hex; Sigma), DES (Sigma), TF-DES (PCR, Inc., Gainesville, FL), and 17β-estradiol (Steraloids, Inc., Wilton, NH), at doses ranging from 0.002 to 2 µg/pup on days 1–4. The morning following the last injection (day 5), mice were sacrificed and the uterine weight/body weight ratio was determined. Estrogenic potency was expressed as the percentage of increase over control (untreated) mice.

Treatment for Tumor Induction. Outbred female CD-1 pups were given s.c. injections of corn oil or Hex, TF-DES, and 17β-estradiol dissolved in corn oil on days 1–5 of neonatal life using the same procedures that were described for DES-treated neonates. Mice were sacrificed from 8 to 12 months of age and reproductive tract tissues were examined by light microscopy.

RESULTS

Effect of Neonatal DES Treatment. A comparison of changes in the uterus of control and neonatally DES-exposed mice (days 1–5) are summarized in Table 1. There were no remarkable pathological alterations in DES mice until 8 months of age at which time atypical hyperplasia, microcystic endometrial hyperplasia with squamous metaplasia, and mixed cystic endometrial hyperplasia and squamous metaplasia were observed in the DES-exposed mice. By 12 months of age, 3 of 17 (18%) DES-exposed mice had cystic endometrial hyperplasia, 2 of 17 (12%) had atypical hyperplasia (Fig. 1), and 8 of 17 (47%) had uterine endometrial adenocarcinoma (Fig. 2). The incidence and severity of the lesions progressed with age until at 18 months 9 of 10 (90%) of the animals had uterine endometrial adenocarcinomas (Fig. 3). Seven of these tumors were primary to the corpus uteri while the remaining two probably originated in the uterine cervix. Some of these lesions had focal areas of squamous differentiation while the remaining tumors were composed of cuboidal or columnar cells. The tumors ranged from areas with neoplastic cells forming well defined glandular patterns to areas with a mixed population of neoplastic squamous, cuboidal, and columnar cells. The stroma of these lesions was fibrous and there were fewer stromal cells present than usual. The epithelium extended through the full thickness of the myometrium to the serosal surface in some lesions (Fig. 3). In other lesions there was piling up of cells in glands (Fig. 4). Some of the glands had an intact basal lamina whereas others were locally invasive within the adjacent stroma. At 12 and 18 months of age, uterine lesions were seen in mice receiving DES doses of 0.2 and 2 µg/pup, but they were not seen in doses below this level (Table 2).

Effects of Ovariectomy on Tumor Development. To determine whether endogenous ovarian steroid secretion contributed to neonatal DES-induced uterine tumors later in life, prepubertal (17 days old) control and neonatally DES-treated mice were ovariectomized and followed until 18 months of age. No tumors were observed in the ovariectomized mice at any age examined (1, 3, 10, 12, or 18 months). All uteri were hypoplastic except one DES-exposed uterus which had microcystic endometrial

| Table 1 Incidence of abnormalities in the uterus of mice treated with DES* during neonatal life |
|-----------------------------------------------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|
|                                              | Age (mos)* | 1     | 2     | 4     | 6     | 8     | 12    | 18    |
| Cystic endometrial hyperplasia                | Control   | DES    | Control | DES    | Control | DES    | Control | DES    | Control | DES    | Control | DES    |
| 0/15                                           | 0/18     | 0/10   | 0/10   | 0/10   | 0/10   | 0/10   | 0/12   | 0/12   | 0/5    | 2/5*   | 1/17   | 3/17*  | 2/10   | 1/10*  |
| Squamous metaplasia                            | Control   | DES    | Control | DES    | Control | DES    | Control | DES    | Control | DES    | Control | DES    |
| 0/15                                           | 0/18     | 0/10   | 0/10   | 0/10   | 0/10   | 0/10   | 0/10   | 0/10   | 0/10   | 0/10   | 0/10   | 0/10   |
| Adenomyosis                                    | Control   | DES    | Control | DES    | Control | DES    | Control | DES    | Control | DES    | Control | DES    |
| 0/15                                           | 0/18     | 0/10   | 0/10   | 0/10   | 0/10   | 0/10   | 0/12   | 0/12   | 0/5    | 0/5    | 0/7    | 0/17   | 2/17*  | 0/10   |
| Adenocarcinoma                                 | Control   | DES    | Control | DES    | Control | DES    | Control | DES    | Control | DES    | Control | DES    |
| 0/15                                           | 0/18     | 0/10   | 0/10   | 0/10   | 0/10   | 0/10   | 0/12   | 0/12   | 0/5    | 0/5    | 0/17   | 8/17*  | 0/10   |

* Mice were given injections of DES (2 µg/pup/day) on days 1–5 of neonatal life.
* Mice were sacrificed at indicated ages and reproductive tract tissues were processed and examined as described in “Materials and Methods.”
* 2 Statistical significance of DES-exposed animals to corresponding age-matched controls using Fisher exact test is: * P = 0.0026; ** P = 0.0002; * not significant.
Fig. 1. Atypical hyperplasia in the uterus of a 12-month-old mouse treated neonatally on days 1-5 with DES (2 μg/pup/day). Tissue was fixed in formalin, embedded in paraffin, sectioned at 6 μm, and stained with H & E. Note areas of atypical changes (arrows). × 100.

Fig. 2. Uterine adenocarcinoma in a 12-month-old mouse treated neonatally on days 1-5 with DES (2 μg/pup/day). Areas of neoplastic cells form relatively well-defined glandular patterns. Endometrial glands are crowded together with little intervening stroma. Some glands contain secretory material (S) and some are dilated but many glands appear to be nests of cells with no lumen (arrow). × 75.

Fig. 3. Uterine carcinoma in an 18-month-old mouse treated neonatally on days 1-5 with DES (2 μg/pup/day). (A) Low power view of the lesion. × 25. There is extension of uterine glands through the myometrium to the serosal surface (arrow). (B) Higher magnification (× 75) of the endometrial tumor. There is a mixed population of squamous, cuboidal, and columnar cells present in the lesion. This is a typical example of the uterine tumors observed at this age in DES-treated mice with intact ovaries and only neonatal exposure to the exogenous estrogen, DES. Note area of invasion of uterine glands through the myometrium (arrow). × 75.

Fig. 4. Uterine carcinoma in an 18-month-old mouse treated neonatally on days 1-5 with DES (2 μg/pup/day). Note that the cells are piling up in one gland with an intact basal lamina, whereas others are locally invasive within the adjacent stroma (arrow). × 100.

Atypical hyperplasia with focal inflammation. These data are summarized in Fig. 6.

Tumorigenesis Assay. To demonstrate the tumorigenic potential of the uterine lesions, minced uterine tissue from intact DES mice over 18 months of age (90% had uterine adenocarcinoma), were grown as s.c. implants in gonadectomized nude mice. Data are shown in Table 3. None (0 of 10) of the nude mice without 17β-estradiol supplementation had successful tumor growth, while 73% (11 of 15) of the nude mice receiving 17β-estradiol pellets showed successful tumor transplants. Nude mice with pellets of progesterone or dihydrotestosterone did not have successful transplanted tumors.1

In two cases, tumor transplants growing in nude mice with 17β-estradiol pellets were serially carried in other nude mice. The histological patterns of uterine tumors growing as transplants in nude mice resembled the original transplanted tumors (Fig. 5). The glandular pattern of the tumors was maintained. In fact, fluid accumulated in cystic pockets in some tumors. When the fluid was aspirated out of the tumor and analyzed by gel electrophoresis, the proteins of the fluid resembled proteins found in uterine luminal fluid. In addition, Western blot analysis of the fluid with an antibody to lactoferrin, an estrogen-inducible uterine protein isolated from immature female mice, indicated this protein was present. These data suggest that the uterine tumors grown as transplants in nude mice retained some differentiated structural and functional characteristics of normal uterine tissue.

Studies with DES Analogues and Steroidal Estrogens. The estrogenic potency of DES analogues and steroidal estrogens

1 Manuscript in preparation.
The histological pattern resembles that of the original transplanted tumors, but those transplanted in neonatal life and sacrificed at 12 months of age. Ovariectomy before puberty blocks the development of uterine carcinoma later in life. Carcinoma of mice treated with DES (2 µg/pup/day) on days 1-5 and sacrificed at various ages. Some mice were left intact while another group was ovariectomized prepubertally on day 17. Ovariectomy before puberty blocks their growth. Other transplants of tissue into nude mice require estrogen supplements for their continued neoplastic growth. It is proposed that DES acts as an inducer or initiator to transform uterine cells during early development and that ovarian estrogens act as promoters, thereafter, to stimulate proliferation of the DES-transformed cells in the adult mouse. Additional studies are under way to determine the mechanisms involved in the early transformation events. The establishment and characterization of several epithelial tumor cell lines from those DES-induced uterine tumors may help to answer these questions. Support for the idea that DES is acting as an initiator of transformation during neonatal development causing permanent changes in the uterine target cells is found in a study reported by Rustia and Shubik (16, 17) as well as in the earlier studies by Takasugi et al. (9) which described a persistently altered cell population in the vagina of mice exposed developmentally to estrogens. Furthermore, mice treated neonatally with DES provide evidence that there is an alteration in antigenic determinants in the DES-treated reproductive organs (12). Using this same mouse model, cervical adenocarcinoma has been reported in aged animals, although the number of mice observed was small (11). Taking all of the animal models into consideration (4, 11, 12, 14, 16, 18-22), the fact that DES induces the neoplastic transformation of Syrian hamster embryo fibroblasts in culture (23), it can be concluded that exposure of the developing reproductive tract to DES apparently affects the pattern of cell differentiation in the uterus and ultimately results in permanent neoplastic alterations in the target cells.

Studies comparing neonatal treatment of mice with various DES analogues (hexestrol and TF-DES) and steroidal estrogens show an apparent correlation of estrogenicity and uterine carcinogenicity in neonatal life. All compounds at the 2-µg/pup/day dose except 17β-estradiol were capable of inducing uterine carcinoma by 12 months of age. Higher doses (3x) of 17β-estradiol were also able to induce uterine tumors but the prevalence of lesions was lower than that induced by DES. In summary, this model is ideally suited to study the mechanisms of induction and expression of epithelial cancers by estrogens in their target tissues.

Table 2 Uterine tumors in mice exposed to various doses of diethylstilbestrol during neonatal life

<table>
<thead>
<tr>
<th>DES treatment (µg/pup/day)</th>
<th>% of animals with uterine neoplasia</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>0.002</td>
<td>0</td>
</tr>
<tr>
<td>0.02</td>
<td>0</td>
</tr>
<tr>
<td>0.2</td>
<td>14</td>
</tr>
<tr>
<td>2.0</td>
<td>50</td>
</tr>
</tbody>
</table>

Table 3 DES-induced uterine adenocarcinomas* grown in nude mice* as s.c. implants

<table>
<thead>
<tr>
<th>Condition of transplant</th>
<th>Tumor take incidence</th>
</tr>
</thead>
<tbody>
<tr>
<td>+Estradiol</td>
<td>11/15 (73)%</td>
</tr>
<tr>
<td>-Estradiol</td>
<td>0/10 (0)</td>
</tr>
</tbody>
</table>

*Left uterine horn was removed from neonatal DES-treated mice that were older than 18 months of age. Uterine tissue was minced and transplanted s.c. into the nude mice.

ACKNOWLEDGMENTS

The authors wish to thank Dr. Meta Bonner for her work with uterine tissues transplanted into nude mice, Julia V. Miller for her...
Table 4 Effects of various estrogenic compounds on the neonatal mouse uterus

<table>
<thead>
<tr>
<th>Compound</th>
<th>Structure</th>
<th>Estrogenic potency</th>
<th>Uterine lesions</th>
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</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>neonatal activity*</td>
<td>Squamous metaplasia</td>
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<td>DES</td>
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<td>2/22 (9)</td>
</tr>
<tr>
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<td>192</td>
<td>0/9 (0)</td>
</tr>
<tr>
<td>Hexestrol</td>
<td><img src="image4" alt="Structure" /></td>
<td>252</td>
<td>2/11 (18)</td>
</tr>
</tbody>
</table>

* Compound was injected s.c. into female mice on days 1–4 of neonatal life at various doses. Animals were sacrificed on day 5 and the uterine weight/body weight ratio was determined. Data are expressed as the percentage increase over controls at the 2 μg/pup/day dose. These determinations were made using a minimum of 10 animals/treatment.

† Numbers in parentheses, percentage of incidence.

‡ Prevalence of uterine adenocarcinoma at a higher dose (6 μg/pup/day) was 4 of 5 (80%) at 12 months of age.

References

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