New Approaches for Estimating Risk from Exposure to Diethylstilbestrol

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A subgroup from a National Institute of Environmental Health Sciences, workshop concerned with characterizing the effects of endocrine disruptors on human health at environmental exposure levels considered the question, If diethylstilbestrol (DES) were introduced into the market for human use today and likely to result in low-dose exposure of the human fetus, what would be required to assess risk? On the basis of an analysis of the quality of data on human DES exposure, the critical times and doses for inducing genital tract malformations and cancer must be determined. This would be facilitated through analysis of the ontogeny of estrogen receptor expression in the developing human genital tract. Models of low-dose estrogenic effects will have to be developed for human and rodent genital tract development. Mouse models offer many advantages over other potential animal models because of the wealth of the earlier literature, the availability of sensitive end points, the availability of mutant lines, and the possibility of generating genetically engineered model systems. Through multidisciplinary approaches, it should be possible to elucidate the cellular and molecular mechanisms of endocrine disruption elicited by estrogens during development and facilitate an assessment of risk to humans. *Key words:* carcinogenesis, clear cell carcinoma, diethylstilbestrol (DES), genital tract, human, teratogenesis. — *Environ Health Perspect* 107(suppl 4):625–630 (1999).
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This report is the product of a subgroup from a National Institute of Environmental Health Sciences, workshop concerned with characterizing the effects of endocrine disruptors on human health at environmental exposure levels. This workshop provided a forum to discuss methods and data needed to improve risk assessments of endocrine disruptors. This report addresses data on the health effects of diethylstilbestrol (DES) and how this information may be used to evaluate risks from exposure to weaker synthetic estrogens. The goal of this review is a re-evaluation of the risk assessment of the human DES experience, using the abundant experimental animal data to answer the following questions: How can we use the human and animal data to better anticipate adverse health effects from agents that are introduced in the future? How could we have anticipated the consequences of DES exposure from the information available when DES was approved for use in pregnant women? Can general lessons be drawn regarding animal-to-human extrapolation for endocrine disruptors? To answer these questions, a historical perspective is required.

Years before clinical use of DES in pregnant women, estrogens in general, and DES specifically, were known to induce breast cancer in postnatal mice and rats when pharmacologic doses were given chronically over long periods (*1–3*). The relationship of postnatal studies to possible transplacental carcinogenesis was certainly not appreciated in the 1930s and early 1940s. Indeed, transplacental carcinogenesis of DES or other estrogens was not considered or reported by investigators at that time. Although prior to 1945, estrogens were known to perturb urogenital development in fetal rodents (*4,5*) and were thought to cross the placenta in humans (*6,7*), direct evidence of teratogenicity of estrogens in humans was unknown until after the association between DES and vaginal adenocarcinoma was reported. In any case, despite the tragedy of the DES episode, the human DES clinical data offer an unprecedented opportunity to learn about the consequences of *in utero* exposure to a potent estrogen and thus to infer potential risks following exposure to less potent environmental estrogens.

If properly interpreted, lessons from the DES episode may prove invaluable for judging potential effects of compounds that have been or will be identified as potential endocrine disruptors. It will be important, however, to keep in mind the considerable differences in potency between such compounds when inferences are drawn concerning potential effects. For example, the carcinogenicity of DES was identified in a human study including only 8 cases and 32 controls (*8*). Normal sample size calculations would rule such a study as inadequate. However, because the cancer induced by DES (clear cell vaginal adenocarcinoma) was so rare in young women, the association between prenatal DES exposure and development of clear cell adenocarcinoma of the vagina was easily identified. Clearly, most chemicals with significantly less potent endocrine (e.g., estrogenic) effects will convey much-reduced risks, particularly at low doses. Thus, study designs for other endocrine disruptors will have to be more precise and more powerful, especially if the background incidence of a particular lesion is substantial. Given the history of the DES episode, we have considered this issue: If DES were introduced into the market for human use today and were likely to result in low-dose exposure of the human fetus, what would be required to assess risk of developing adverse health outcomes such as cancer or impaired reproductive potential? To answer this question, we have considered the following points:

- *Delineation of the critical times and doses for inducing genital tract malformations and cancer.* Critical time periods and doses must be determined for DES-induced malformations of the developing human Mullerian duct, Wolffian duct, urogenital sinus, and their organ derivatives. Given the plethora of teratogenic effects of DES on the developing male and female genital tracts (Tables 1,2), different lesions are expected to have different periods of temporal susceptibility as well as different dose levels required to induce such effects. The background information for such teratogenic studies can be estimated from the literature of classical embryologic studies. Retrospective studies on the incidence of

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Table 1. Effects of perinatal exposure to estrogenic substances in males.

Rat	Mouse	Human	Estrogenic effects	Notes
+	+	Not known	Decreased prostate and seminal vesicle secretion	High doses
+	+	Not known	Increased prostate weight	Low doses
Not known	+/–	+/–	Rete testes tumor	
Not known	+	+/–	Decreased sperm count	
Not known	+	Not known	Increased lactoferrin in seminal vesicle	High doses
+	+	+	Mullerian duct remnants in males, hypertrophic prostatic utricle	
Not known	+	+/–	Impaired reproduction	High doses
Not known	+	+	Anomalies of male external genitalia	
Not known	+	Not known	Thyroid effects	
Not known	+	Not known	Intersex	
Not known	+	Not known	Alterations in sex behavior	
+	+	–	Hypothalamic changes	

Table 2. Effects of perinatal exposure to estrogenic substances in females.

Rat	Mouse	Human	Estrogenic effects	Notes
+	+	Not known	Polyovulatory follicles	
+	+	Not relevant	Ovary-independent vaginal cornification	
–	+	+	Cervical/vaginal carcinoma	
Not known	+	+/–	Uterine carcinoma	
Not known	+	+/–	Immune dysfunction	More frequent autoimmune disease in women
+	+	Not known	Polycystic ovary	
+	+	+/–	Estrus/menstrual cycle disturbance	
+	+	Not known	Mammary tumors in daughters	Small increased risk in mothers
+	+	+	Vaginal adenosis	
+	+	+	Impaired myometrial development	T-shaped uterus in humans
Not known	+	+	Impaired reproduction	
+	+	Not known	Mammary gland alterations	
+	+	+	Mesonephric remnants	
Not known	+	Not known	Accelerated vaginal opening	Equivalent to puberty in humans
Not known	+	+	Oviductal malformations	
Not known	+	+	Uterine and cervical malformations	

certain lesions such as adenosis in women exposed *in utero* to DES in relationship to the initiation of DES treatment are helpful in establishing periods of susceptibility even though estimates of gestational ages of exposure may be inexact. Human cell lines are not particularly useful for determining the critical times and doses for inducing human genital tract malformations. However, analysis of DES effects on grafts of human fetal genital tracts in nude mice has proved to be a reliable method for assessing periods of susceptibility for the induction of different lesions (*9–13*). Indeed, this method is perhaps the only method for delineating the critical times and doses for inducing human genital tract malformations. Contrary to popular thought, a considerable amount of abortus material is available for such screening purposes. Clearly, it will be necessary to extend these studies to determine the specific times and doses of DES required to induce various lesions in the developing human genital tract. This will provide a relevant basis for judging the likelihood of similar effects from compounds identified as environmental endocrine disruptors. Recognizing that other developing organ systems such as the neuroendocrine and immune systems are also sensitive to estrogenic substances, critical periods for inducing adverse effects on these systems would also need to be established.

- *Ontogeny of estrogen receptor (ER) expression in the developing human genital tract.* The presumed mechanism of action of DES is through ER in the developing genital tract, even though action via other receptors and nonreceptor-mediated mechanisms should also be considered. Although the ontogeny of expression of ER has been studied in the mouse and rat, data are meager for the human genital tract (*14–16*). Future studies need to take into account both ER-alpha (ERα) and the recently discovered ER-beta (ERβ) (*17*). The literature is even more deficient for the ontogeny of androgen receptors and progesterone receptors, especially in the human fetal genital tract (*18,19*) . Such ontogenic studies will be critical for the interpretation of adverse effects of DES and other chemicals and drugs with hormonal activity.
- *Low-dose models of estrogenic effects on genital tract development.* Even though DES exposure levels of the developing human vary considerably, all clinical exposure of human fetuses falls into the high-dose range. High-dose animal studies mainly designed to duplicate the therapeutic doses prescribed for pregnant women have been extensively published for mouse and rat, although a few low-dose studies have been described (*20–22*). Low-dose animal models are now receiving attention (*23–25*) and indicate that outcomes from high- and low-dose exposure can be both qualitatively and quantatively different. Additional low dose–response animal work is required to assess the potential effects of less potent estrogenic compounds on the many end points previously described (Tables 1,2). Because of the wealth of DES data in both animals and humans, an animal model that is sensitive to DES effects at low doses may be useful in screening other compounds with potentially similar mechanisms of action. The ability to study low-dose effects on human urogenital tract development would be valuable. Such information in the human could be obtained by studying transplants of human fetal genital tracts in DES-treated nude mouse hosts (*10*). The transplant model of human fetal genital tracts could be used as either a screen or a confirmatory test for low-dose effects seen in animal studies. Such proposed use of human fetal genital tracts in DES-treated nude mouse hosts to delineate the lower end of the dose response in developing human genital tracts could provide the basis for assessing low-dose effects of environmental chemicals identified as having potential endocrine-disrupting effects.
- *Characterization of human fetal serum-binding proteins.* Calculation of relative binding affinity (RBA) and serum-modified access (SMA) is a powerful method for determining levels of free compound capable of eliciting estrogenic effects in a test system (*26*). This information is available for the fetal rat and mouse but is not available for the human fetus. Umbilical cord serum from full-term fetuses should be analyzed to assess RBA and SMA. Serum from first and second trimester human fetuses will be extremely difficult to obtain. Fetal primate serum may be a useful substitute.

What Is the Quality of Human DES Exposure Data?

Even though the dosing regimen recommended by Smith et al. (*27*) was in widespread use, this dosage pattern was far from universal, particularly since its efficacy had never been established. Thus, there are defined cohorts of women who received 1.4–17.9 g DES as a total dose during

pregnancy (*28*), even though a certain level of imprecision exists concerning timing of exposure and the numbers of individuals exposed *in utero* to specific maternal doses of DES. In any case, the human data available are primarily related to the high-dose regimens. With respect to clear cell vaginal adenocarcinoma, it is evident that for many patients even these high-dose exposures were insufficient to induce neoplasia in all but a small subset of patients. Thus, it would appear that there are dosages and/or periods of high-dose DES exposure that do not trigger neoplastic change in both humans and animals. For nonmalignant lesions in humans such as adenosis, cervical defects, and T-shaped uteri, the timing of exposure is important in generating genital tract abnormalities. Based upon the abundant human clinical data, the relationship between dosage and the development of nonmalignant lesions suggests that there are also DES doses below which adverse noncancer effects are not seen. However, for humans especially, there is a great need to accurately define the exact dose range and timing that elicit genital tract malformations and those doses that are below the threshold for eliciting adverse effects. Use of a nude mouse transplant system for human fetal genital tracts may be the only method to obtain this critical data. Acquisition of these types of data will permit relevant potency comparisons between DES and environmental compounds identified as having estrogenic activity.

The existence of cohorts exposed to DES at mean total maternal doses spanning more than an order of magnitude provide an opportunity to study the dose–response characteristics for relatively high-dose DES-induced effects. These data also provide the opportunity to compare human clinical data and dose–response data for DES-induced effects observed in animal studies. As part of the ongoing follow-up of DES-exposed cohorts, substantial numbers of exposed males and females have been studied. Depending on the timing of exposure and the total maternal DES dose administered, unequivocal (and more readily observed) effects seen in males and females include reproductive tract malformations, impaired reproduction, and vaginal carcinoma (*29–33*). More equivocal effects include decreased sperm count (*34–36*), immune dysfunction (*37*), alterations in sexual behavior (*38*), disturbed menstrual cycles (*39*), and testicular cancer (*40*). To date, DES-exposed males and females diagnosed with malignant or nonmalignant lesions include individuals in childhood and puberty, and adults less than 50 years of age. Substantial numbers of males and females in the DES adenosis cohort are just now reaching 50 years of age. This is the age when male and female reproductive tract neoplasias typically begin to occur. Additional follow-up of DES-exposed sons will be essential to establish whether they are at increased risk of testicular or prostate cancer. It is important to note that, in general, women have been more extensively studied than men because of the initial association between *in utero* DES exposure and vaginal cancer and also because of a greater interaction of women with health care services and providers. Furthermore, women have formed DES support groups and have successfully lobbied the government for studies of the adverse effects of DES.

It should also be noted that some of the adverse effects observed as a consequence of *in utero* exposure to DES occur against an extremely low background incidence of reproductive tract malformations and vaginal carcinoma. By following the DES-exposed cohorts, it will be challenging to determine if more prevalent conditions (i.e., thyroid effects, breast and prostate cancer, endometriosis, immune dysfunction) or conditions that increase in frequency with age (e.g., declining immune function, endometrial hyperplasia and cancer, ovarian cancer, benign prostatic hyperplasia, prostatic cancer) are increased as a result of *in utero* exposure to DES. Also, the possibility of third-generation effects has to be considered (*41*).

The lack of low-dose human DES exposure data might be addressed by the use of human fetal reproductive tract tissue transplanted to nude mouse hosts. This would permit a detailed study of the lower end of the dose–response curve. Previous use of such a model system of human fetal reproductive tract transplants has demonstrated that many of the high-dose DES effects observed in the epidemiologic studies can be induced experimentally in such transplants of human fetal reproductive tracts. The transplant model offers the possibility of extending dose-response studies in the human well below the DES doses used clinically. Additionally, such data would also serve as a bridge between the low-dose mouse data and potential low-dose human effects data. Because of the known potency of DES, acquiring these kinds of data would provide the most relevant basis for judging whether compounds identified as having estrogenic activity might be expected to be teratogenic in humans.

Possible Pharmaceutical Exposures to Endocrine-Disrupting Chemicals during Pregnancy

Environmental contamination by endocrine-disrupting agents has received considerable attention in the scientific and lay press, and the impacts of such agents on reproduction in wildlife has had a deleterious impact on many species (*42*). Humans can be exposed to endocrine disruptors through use of a variety of commonly used medications. Thus, potentials for exposing women of reproductive age to hormonally active drugs (estrogens, androgens, or progestins) include the following possibilities: *a*) inadvertent use of a drug in the luteal phase of a conceptive cycle, *b*) inadvertent administration of a drug during pregnancy in oligo/amenorrheic women, *c*) contraceptive failure coupled with continued use of birth control pills, *d*) inadvertent administration of a drug following nonhormonal contraceptive failure (intrauterine devices, condoms, diaphragms), and *e*) use of a drug in gynecologic or medical disease in women of child-bearing age. Inappropriate exposure to estrogens, androgens, and/or progestins can elicit severe malformations of the genital tract. Thus, low- or high-dose exposure to hormonally active compounds should be avoided at all costs, and if exposure occurs, any adverse outcomes should be monitored. Some currently used pharmaceuticals that may pose risks to the human fetus are given in Table 3.

Animal Models

Summary of the Models

The animal models in which the developmental effects of DES exposure have been studied mainly include the mouse, rat, and hamster. The mouse has been the most extensively studied species, and the size of the data set in mice is superior to those for all other species combined. This extensive body of evidence in the mouse extends back about 50 years. The perinatal DES-treated mouse model correlates remarkably well with the adverse effects observed in both male and female humans exposed *in utero* to DES. Tables 1 and 2 illustrate these effects and the correspondence in effects between rodent and human studies.

The Mouse As the Best Animal Model

Although the rat and hamster may be equally appropriate for modeling DES effects in humans, data in these species are not nearly as abundant as those for the mouse. Rat studies are only likely to further validate the existing mouse data. Another advantage of the mouse model is the wealth of genetic information that is available and the relative ease of using transgenic and gene knockout mice to study the mechanism by which DES produces adverse effects. In addition, genetically modified strains of mice might make it possible to study the interaction between direct DES effects, immune factors, and endogenous hormones in teratogenic or carcinogenic processes. Additional advantages of the mouse model include the

Table 3. Drugs potentially capable of eliciting adverse effects on development via disturbance of sex hormones.

- Combination estrogen/progestin (oral contraceptives and continuous combined hormone replacement therapy, continuous combined oral contraceptives)
  - C-19 norprogestins (modified testosterone)
    - High androgenicity - levonorgestrel
    - Intermediate androgenicity - norethindrone (acetate), ethynodiol diacetate, norgestrol
    - Low androgenicity - gestidine, norgestimate, desogestrel
  - C-21 progestins: medroxyprogesterone acetate
  - Estrogens
    - Ethinyl estradiol (20–35 μg)
    - Mestranol (35 μg)
    - Conjugated estrogens (0.625–2.5 mg)
- Progestin-only products
  - Medroxyprogesterone acetate (oral and/or depo)
  - Megestrol acetate (Megace) - for endometrial cancer
  - Norethindrone acetate (Micronor)
  - Levonorgestrel implants (Norplant)
  - Micronized progesterone (oral)
  - Progesterone transvaginal gel
  - Progesterone in oil for injection
- Antiandrogens
  - Spironolactone (receptor antagonist and synthesis inhibitor)
  - Flutamide (receptor antagonist)
  - Finasteride (5α-reductase inhibitor)
  - Cimetidine (tagamet, significant effects within the range of ulcer treatment)
  - Cyproterone acetate (formulated with ethanyl estradiol in Europe)
  - Letozole (aromatase inhibitor)
- Androgens
  - Danazol (androgen/anabolic for treatment of endometriosis, hereditary angioneurotic edema, fibrocystic breast disease, AIDS)
  - Estratest (1.25 mg + 2.5 mg methyltestosterone)
  - Depotestadiol (estradiol + testosterone for intramuscular use)
  - Testosterone (implants/gel/transdermal)
  - Oxandraolone (and other anabolic agents)
- Estrogens
  - Conjugated estrogens
  - Conjugated equine estrogens (equillin, equillinin, other equine estrogens)
  - Micronized estradiol
  - Transdermal estradiol
  - Transvaginal estradiol
- SERMs
  - DES
  - Clomiphene citrate
  - Tamoxifen
  - Nafoxidine
  - Raloxifene
  - Toremifene
  - Many others in development
- Antiprogestins
  - RU486 (receptor blocker)
  - Epostane (synthesis inhibitor)
- Other agents to which fetuses could be inadvertently exposed
  - Fluconazole (systemic antifungal)
  - Acyclovir (antiherpetic drug)
  - Famciclovir (antiherpetic drug)
  - Valcyclovir (antiherpetic drug)
  - Interferon α-n3

Abbreviations: DES, diethylstilbestrol; SERMs, selective estrogen-response modulators.

following: *a*) smaller amounts of the test compound are required for study; *b*) housing and animal care expenses are less than those for other rodents; and *c*) faster breeding to generate multigenerational studies is possible. Reproductive tract development is similar in mice and humans. Therefore, the DES mouse model can be used to study developmental exposure to a wide range of compounds to which pregnant women may be exposed including selective estrogen response modulators (SERMs).

Animal Models That Will Accommodate SERMs

The activity of SERMs can be studied in animal models to assess compounds such as clomiphene or tamoxifen (*43*). Potential SERM activity of a compound may induce a response in the human that is not induced in the animal model. This potential for false negative and false positive results can be reduced by using multiple animal models (mouse and rat) rather than relying on a single model in which SERM activity may be expressed. The danger is that all models may not be equally sensitive. Also, it is unclear whether a negative response is a result of decreased sensitivity. Although SERMs may be capable of inducing selective responses in specific animals, tissues, or conditions, as far as is known, all SERM activities have in common the binding to the ER. The use of receptor-binding assays is another approach to reliably screen for the effect of SERMs. These assays may include relative binding affinity analysis.

What Are the Most Sensitive End Points?

Assays for the most sensitive end points must be able to detect the low-dose ranges that are well defined for DES. In addition, the full dose–response range must be determined for each compound and end point. The low-dose range for DES effect is approximately 0.02 μg/kg/day for the fetal mouse prostate (*25*) and 0.01 μg/kg/day for the neonatal mouse uterus (*20,21,23*). Overall, this low-dose range is defined as estrogenic activity delivered to cells in approximately the same low range of natural estrogen (e.g., free estradiol) at physiologic levels. A procedure and an approach for a predicted dose at this level have been described (*26,44*). It is not known if human fetal reproductive tract tissues are as sensitive to DES as are fetal mouse tissues. The nude mouse/human fetal tissue transplant system may provide data on this critical issue.

Biomarkers

Molecular Markers

Patterning of the male and female genital tract has recently been shown to involve expression of *hox* and *wnt* genes (*45–48*). *hox* gene knockout (KO) studies have demonstrated profound disturbances in organogenesis and differentiation of the genital tract (*46*). DES has been shown to perturb the expression of several *hox* genes in the fetal Mullerian ducts when injected into pregnant mice (*49*). It will be useful to explore whether other exogenous estrogenic compounds share this activity. This new area of investigation requires further exploration to elucidate the role of these patterning genes in urogenital tract development in the mouse. Comparable studies in the human fetal reproductive tract are possible using the human fetal genital tract transplants to nude mice and would be desirable.

High Sensitivity Biomarkers of DES Action

Lactoferrin is a protein that is regulated by estrogen in the female mouse reproductive tract. In the uterus of the adult mouse, lactoferrin transcripts are stimulated approximately 300-fold by estradiol or DES (*50, 51*). The expression of lactoferrin is induced by DES in the uterus prenatally, neonatally, and in adult stages (*52*), thus making it a particularly attractive biomarker of DES action in the mouse. Lactoferrin has also been localized to a subset of epithelial cells of the human endometrium and is responsive to estrogen (*53*). Comparable markers for the human uterus following exposure to other endocrine-disrupting chemicals should be sought.

Engineered Assay Systems

Transgenic mice could be created using the lactoferrin promoter linked to green fluorescent protein. Estrogen action could be detected by external viewing through the body wall or via an intravaginal light detection system. In theory a single mouse could be used sequentially to test a series of separate compounds for their estrogenicity. This area of investigation is promising.

What Can Be Learned Mechanistically for the DES Data?

A central question is which DES effects are dependent upon ERα-mediated or ERβ-mediated mechanisms. Studies with ERα-KO, ERβ-KO, and ERα/ERβ double KO mice will be required to settle these issues. Teratogenicity and/or carcinogenicity could be ERα-mediated either in the initiation or promotional phases. If tumor or birth defect initiation is an ERα-dependent event, the ERα-KO mouse will be useful in verifying that the initiating event involves estrogenic compounds acting through ERα. If tumor or birth defect initiation is an ERβ-dependent event, the ERβ-KO mouse will be useful in verifying that the initiating event involves estrogenic compounds acting through ERα. More sophisticated models could be created in which perinatal DES exposure can be achieved in an ERα-KO context with ERβ fully active to initiate lesions in the absence of ERα. At a later point it would be possible to selectively reestablish ERα-mediated estrogenic sensitivity by splicing out a stop cassette to reconstitute ERα. Through use of the CRE-lox system in transgenic mice, it should be possible to create such a model. By similar methods the role of ERβ in estrogen-induced teratogenicity and/or carcinogenicity should be explored. Studies on genetic and epigenetic changes associated with DES exposure will also be useful in understanding the mechanism of action of DES and other endocrine-disrupting chemicals.

Collectively, the DES database in humans and rodents is likely to provide a highly relevant yardstick upon which to judge the potential estrogenic effects of compounds identified as environmental estrogens. The combination of known estrogenic potency with dose–response data for potential end points of concern in the mice and humans will be a useful tool for characterizing the effects of endocrine disrupters on human health at environmental exposure levels.

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