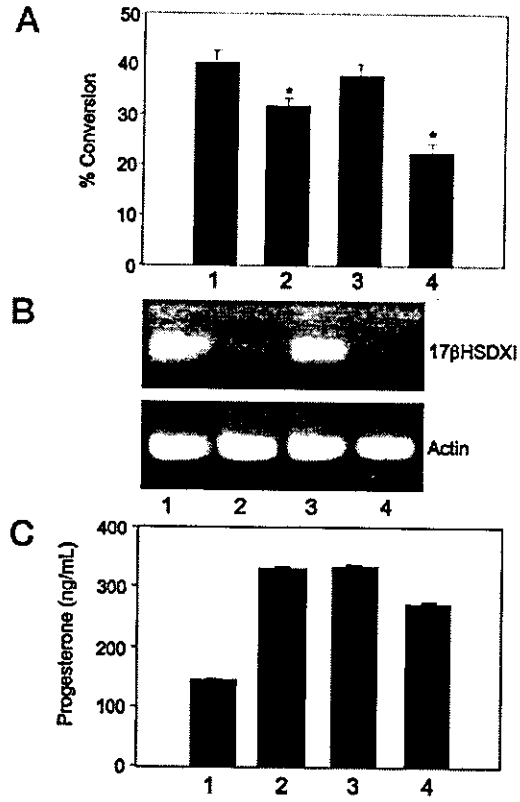

FIG. 6. Effect of cAMP and all-*trans*-retinoic acid treatment on 17βHSDXI activity (A) and mRNA levels (B) in mouse Y1 cells. Lane 1, No-treatment control; lane 2, cAMP; lane 3, all-*trans*-retinoic acid; and lane 4, cAMP plus all-*trans*-retinoic acid. As a control for treatment efficacy, progesterone production was measured in the media of these cells with corresponding treatment (C; n = 3, ± SEM). *, $P < 0.02$, compared with no treatment control.

the second half of gestation, leading to a peak in circulating 3α-Adiol-glucuronide levels during this time (27). In a subsequent study, it was shown that a failure to 5α-reduce androgens leads to excessive production of estrogens and thus to fetal mortality (28).

We also observed 17βHSDXI immunostaining coincident with staining for 17α-hydroxylase, a marker of fasciculata/reticularis cells of the adrenal (29). This pattern of expression is consistent with a regulatory role for 17βHSDXI in androgen synthesis/steroidogenesis. We suggest that 17βHSDXI may not participate directly in the steroid biosynthetic pathway because two-dimensional TLC did not reveal metabolism of a pool of over a dozen steroids generated from H295R cells using labeled cholesterol, but 11βHSD2 readily metabolized cortisol in this mixture (results not shown).

Although we observed evidence for 17βHSDXI message in the adrenal medulla, we were unable to confirm the presence of the protein by immunostaining. This could be due to the nontranslation of this message or contamination of the medulla with cortex cells during tissue collection. 17βHSDXI was also found in epithelia of the endometrium and small intestine. Androgens are thought to inhibit the growth of endometrial cells, suggesting that 17βHSDXI may be important in lowering active androgen levels in this setting (30). Further evidence for a role of 17βHSDXI in androgen action comes from our localization of this enzyme to the sebaceous gland in which previous studies have shown high 5α-reductase and 17βHSD activity (31, 32). In addition, 17βHSDXI is expressed at significant levels in lymphocytes in which recent evidence suggests metabolism of sex steroids (33). A large number of hydroxysteroid dehydrogenases have been described in the epithelium of the small intestine in which they may act on exogenous compounds to protect the organism from toxins of dietary origin. Airway epithelia also express significant amounts of the 17βHSDXI in adult and fetal tissues (13).

The liver is clearly the organ with the highest amount of 17βHSDXI and would most likely modulate plasma levels of 3α-Adiol, but paracrine effects of 17βHSDXI are also important in organs such as the heart in which fibroblasts may produce 3α-Adiol (34). Interestingly, 3α-Adiol has been shown to act as a neurosteroid (25) and prevent changes induced by adrenalectomy in the brain (26, 35). Clinical studies suggest that it modulates central γ-aminobutyric acid (GABA)ergic tone and that higher plasma concentrations could play a role in preventing anxiety (36). A decrease in cardiac 17βHSDXI could result in higher local 3α-Adiol and changes in GABAergic tone in the heart. This may have deleterious effects given that stimulation of cardiac GABA receptors has been shown to enhance the cardiotoxicity of some agents (37).

Collectively, these results point to a role for 17βHSDXI in androgen metabolism. Differences in cellular distribution and substrate specificity with other 17βHSD isoforms suggest that this enzyme may also have an important role to play in modulating the paracrine actions of 3α-Adiol. Furthermore, in the present work, regulation studies showed an inverse relationship between steroid production in Y1 cells and 17βHSDXI activity and gene expression. This observation, together with the finding of multiple potential SF-1-binding sites in the 17βHSDXI gene, is consistent with a role in steroidogenesis (38). 17βHSDXI could act by metabolizing compounds that stimulate steroid synthesis and/or by generating metabolites that inhibit it.

Acknowledgments

The authors thank Michelle Cinel for sequencing, Benjamin Lamont for help with making the 5'pA constructs, Professor Jock Findlay for comments during the study, and Professor John Funder for insight and encouragement throughout this work.

Received October 4, 2002. Accepted January 30, 2003.

Address all correspondence and requests for reprints to: Zygmunt Krozowski, Ph.D., Baker Heart Research Institute, P.O. Box 6492, St. Kilda Road, Central Melbourne, Victoria 8008, Australia. E-mail: zygmunt.krozowski@baker.edu.au.

References

1. **Stewart PM, Krozowski ZS** 1999 11βHydroxysteroid dehydrogenase. Vitam Horm 57:249–324
2. **Yau JL, Noble J, Kenyon CJ, Hibberd C, Kotelevtsev Y, Mullins JJ, Seckl JR** 2001 Lack of tissue glucocorticoid reactivation in 11β-hydroxysteroid dehydrogenase type 1 knockout mice ameliorates age-related learning impairments. Proc Natl Acad Sci USA 98:4716–4721
3. **Morton NM, Holmes MC, Fievet C, Staels B, Tailleux A, Mullins JJ, Seckl JR** 2001 Improved lipid and lipoprotein profile, hepatic insulin sensitivity and

glucose tolerance in 11β-hydroxysteroid dehydrogenase type 1 null mice. J Biol Chem 276:41293–41300
4. **Masuzaki H, Paterson J, Shinyama H, Morton NM, Mullins JJ, Seckl JR, Flier JS** 2001 A transgenic model of visceral obesity and the metabolic syndrome. Science 294:2166–2170
5. **Funder JW, Pearce PT, Smith R, Smith AI** 1988 Mineralocorticoid action: target tissue specificity is enzyme, not receptor, mediated. Science 242:583–585
6. **Stewart PM, Krozowski ZS, Gupta A, Milford DV, Howie AJ, Sheppard MC, Whorwood CB** 1996 Hypertension in the syndrome of apparent mineralocorticoid excess due to mutation of the 11β-hydroxysteroid dehydrogenase type 2 gene. Lancet 347:88–91
7. **Wilson RC, Krozowski ZS, Li K, Obeyesekere VR, Razzaghy-Azar M, Harbison MD, Wei JQ, Shackleton CH, Funder JW, New MI** 1995 A mutation in the HSD11B2 gene in a family with apparent mineralocorticoid excess. J Clin Endocrinol Metab 80:2263–2266
8. **Mune T, Rogerson FM, Nikkila H, Agarwal AK, White PC** 1995 Human hypertension caused by mutations in the kidney isozyme of 11β-hydroxysteroid dehydrogenase. Nat Genet 10:394–399
9. **Moghrabi N, Head JR, Andersson S** 1997 Cell type-specific expression of 17β-hydroxysteroid dehydrogenase type 2 in human placenta and fetal liver. J Clin Endocrinol Metab 82:3872–3878
10. **Geissler WM, Davis DL, Wu L, Bradshaw KD, Patel S, Mendonca BB, Elliston KO, Wilson JD, Russell DW, Andersson S** 1994 Male pseudohermaphroditism caused by mutations of testicular 17β-hydroxysteroid dehydrogenase 3. Nat Genet 7:34–39
11. **Li KX, Smith RE, Krozowski ZS** 1998 Cloning and expression of a novel tissue specific 17β-hydroxysteroid dehydrogenase. Endocr Res 24:663–667
12. **Haeseleer F, Palczewski K** 2000 Short-chain dehydrogenases/reductases in retina. Methods Enzymol 316:372–383
13. **Brereton P, Suzuki T, Sasano H, Li K, Duarte C, Obeyesekere V, Haeseleer F, Palczewski K, Smith I, Komesaroff P, Krozowski Z** 2001 Panlb (17βHSD11)-enzymatic activity and distribution in the lung. Mol Cell Endocrinol 171:111–117
14. **Albiston AL, Obeyesekere VR, Smith RE, Krozowski ZS** 1994 Cloning and tissue distribution of the human 11β-hydroxysteroid dehydrogenase type 2 enzyme. Mol Cell Endocrinol 105:R11–R17
15. **Biswas MG, Russell DW** 1997 Expression cloning and characterization of oxidative 17β- and 3α-hydroxysteroid dehydrogenases from rat and human prostate. J Biol Chem 272:15959–15966
16. **Chai Z, Sarcevic B, Mawson A, Toh BH** 2001 Set-related cell division autoantigen-1 (cda1) arrests cell growth. J Biol Chem 276:33665–33674
17. **Krozowski Z, Maguire JA, Stein-Oakley AN, Dowling J, Smith RE, Andrews RK** 1995 Immunohistochemical localization of the 11β-hydroxysteroid dehydrogenase type II enzyme in human kidney and placenta. J Clin Endocrinol Metab 80:2203–2209
18. **Krozowski Z** 1992 11βHydroxysteroid dehydrogenase and the short-chain alcohol dehydrogenase (SCAD) superfamily. Mol Cell Endocrinol 84:C25–C31
19. **Sachs AB, Bond MW, Kornberg RD** 1986 A single gene from yeast for both nuclear and cytoplasmic polyadenylate-binding proteins: domain structure and expression. Cell 45:827–835
20. **Wei CC, Balasta ML, Ren J, Goss DJ** 1998 Wheat germ poly(A) binding protein enhances the binding affinity of eukaryotic initiation factor 4F and (iso)4F for cap analogues. Biochemistry 37:1910–1916
21. **Cross SH, Charlton JA, Nan X, Bird AP** 1994 Purification of CpG islands using a methylated DNA binding column. Nat Genet 6:236–244
22. **Romert A, Tuvendal P, Simon A, Dencker L, Eriksson U** 1998 The identification of a 9-*cis* retinol dehydrogenase in the mouse embryo reveals a pathway for synthesis of 9-*cis* retinoic acid. Proc Natl Acad Sci USA 95:4404–4409
23. **Su J, Chai X, Kahn B, Napoli JL** 1998 cDNA cloning, tissue distribution, and substrate characteristics of a *cis*-retinol/3α-hydroxysterol short-chain dehydrogenase isozyme. J Biol Chem 273:17910–17916
24. **Nakhla AM, Ding VD, Khan MS, Romas NA, Rhodes L, Smith RG, Rosner W** 1995 5α-Androstan-3α, 17β-diol is a hormone: stimulation of cAMP accumulation in human and dog prostate. J Clin Endocrinol Metab 80:2259–2262
25. **Melcangi RC, Magnaghi V, Galbiati M, Ghelarducci B, Sebastiani L, Martini L** 2000 The action of steroid hormones on peripheral myelin proteins: a possible new tool for the rebuilding of myelin? J Neurocytol 29:327–339
26. **Rosellini RA, Svare BB, Rhodes ME, Frye CA** 2001 The testosterone metabolite and neurosteroid 3α-androstanediol may mediate the effects of testosterone on conditioned place preference. Brain Res Brain Res Rev 37:162–171
27. **Mahendroo MS, Cala KM, Russell DW** 1996 5α-Reduced androgens play a key role in murine parturition. Mol Endocrinol 10:380–392
28. **Mahendroo MS, Cala KM, Landrum DP, Russell DW** 1997 Fetal death in mice lacking 5α-reductase type 1 caused by estrogen excess. Mol Endocrinol 11: 917–927
29. **Sasano H, Mason JI, Sasano N** 1989 Immunohistochemical study of cytochrome P-45017α in human adrenocortical disorders. Hum Pathol 20:113–117
30. **Tuckerman EM, Okon MA, Li T, Laird SM** 2000 Do androgens have a direct effect on endometrial function? An *in vitro* study. Fertil Steril 74:771–779
31. **Takayasu S, Wakimoto H, Itami S, Sano S** 1980 Activity of testosterone 5α-reductase in various tissues of human skin. J Invest Dermatol 74:187–191
32. **Thiboutot D, Gilliland K, Light J, Lookingbill D** 1999 Androgen metabolism in sebaceous glands from subjects with and without acne. Arch Dermatol 135:1041–1045
33. **Zhou Z, Shackleton CH, Pahwa S, White PC, Speiser PW** 1998 Prominent sex steroid metabolism in human lymphocytes. Mol Cell Endocrinol 138:61–69
34. **Milewich L, Kaimal V, Shaw CB, Johnson AR** 1986 Androstenedione metabolism in human lung fibroblasts. J Steroid Biochem 24:893–897
35. **Frye CA, McCormick CM** 2000 The neurosteroid, 3α-androstanediol, prevents inhibitory avoidance deficits and pyknotic cells in the granule layer of the dentate gyrus induced by adrenalectomy in rats. Brain Res 855:166–170
36. **Barbaccia ML, Lello S, Sidiropoulou T, Cocco T, Sorge RP, Cocchiarale A, Piermarini V, Sabato AF, Trabucchi M, Romanini C** 2000 Plasma 5α-androstane-3α, 17βdiol, an endogenous steroid that positively modulates GABA(A) receptor function, and anxiety: a study in menopausal women. Psychoneuroendocrinology 25:659–675
37. **McLemore GL, Billingsley ML, Severs WB** 1994 Cardiac noradrenergic mechanisms mediate GABA-enhanced ouabain cardiotoxicity. Pharmacology 49: 343–350
38. **Hanley NA, Rainey WE, Wilson DI, Ball SG, Parker KL** 2001 Expression profiles of SF-1, DAX1, and CYP17 in the human fetal adrenal gland: potential interactions in gene regulation. Mol Endocrinol 15:57–68

Environmental Health Perspectives Volume 107, Supplement 4, August 1999

New Approaches for Estimating Risk from Exposure to Diethylstilbestrol

Gerald R. Cunha,[1] John-Gunnar Forsberg,[2] Robert Golden,[3] Arthur Haney,[4] Taisen Iguchi,[5] Retha Newbold,[6] Shanna Swan,[7] and Wade Welshons

[1]Anatomy Department and Reproductive Endocrinology Center, University of California, San Francisco, California USA; [2]Tornblad Institute Lund, Sweden; [3]ToxLogic, Potomac, Maryland USA; [4]Department of Obstetrics and Gynecology, Duke University Medical Center, Durham, North Carolina USA; [5]Department of Biology and Graduate School of Integrated Science, Yokohama City University, Yokohama, Japan; [6]Laboratory of Toxicology, Environmental Toxicology Program, National Institute of Environmental Health Sciences, Research Triangle Park, North Carolina USA; [7]Department of Family and Community Medicine, University of Missouri, Columbia, Missouri USA; [8]Department of Veterinary Biomedical Science, University of Missouri, Columbia, Missouri USA

- **What Is the Quality of Human DES Exposure Data?**
- **Possible Pharmaceutical Exposures to Endocrine- Disrupting Chemicals during Pregnancy**
- **Animal Models**
- **Biomarkers**
- **What Can Be Learned Mechanistically for the DES Data?**

Abstract

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). **http://ehpnet1.niehs.nih.gov/docs/1999/suppl-4/625-630cunha/abstract.html**

This report was developed at the Workshop on Characterizing the Effects of Endocrine Disruptors on Human Health at Environmental Exposure Levels held 11-13 May 1998 in Raleigh, North Carolina.

Address correspondence to G.R. Cunha, Anatomy Department and Reproductive Endocrinology Center, University of California San Francisco, CA 94143. Telephone: (415) 476-4140. Fax: (415) 502-2270. E-mail: **grcunha@itsa.ucsf.edu**

This work was funded in part by the following NIH grants: DK02397, DK51101, DK45861, DK52708, CA64872, CA59831, DK52721, DK51397, AG13784, AG15500.
Received 25 September 1998; accepted 24 March 1999.

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 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.

- http://ehp.niehs.nih.gov/members/1999/suppl-4/625-630cunha/cunhatab1.GIF

- http://ehp.niehs.nih.gov/members/1999/suppl-4/625-630cunha/cunhatab2.GIF

- *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 (ER) and the recently discovered ER-beta (ERß) (*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 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 ER-mediated or ERß-mediated mechanisms. Studies with ER-KO, ERß-KO, and ER/ERß double KO mice will be required to settle these issues. Teratogenicity and/or carcinogenicity could be ER-mediated either in the initiation or promotional phases. If tumor or birth defect initiation is an ER-dependent event, the ER-KO mouse will be useful in verifying that the initiating event involves estrogenic compounds acting through ER. If tumor or birth defect initiation is an ERß-dependent event, the ERß-KO mouse will be useful in verifying that the initiating event involves estrogenic compounds acting through ER. More sophisticated models could be created in which perinatal DES exposure can be achieved in an ER-KO context with ERß fully active to initiate lesions in the absence of ER. At a later point it would be possible to selectively reestablish ER-mediated estrogenic sensitivity by splicing out a stop cassette to reconstitute ER. 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 ERß 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.

REFERENCES AND NOTES

1. Gardner WU. Estrogens in carcinogenesis. Arch Pathol 27:138-170 (1939).

2. Geschickter CF. Diseases of the Breast. Philadelphia:J.B. Lippincott, 1943.

3. Shimkin MB. Hormones and mammary cancer in mice. In: Mammary Tumors in Mice (Moulton FR, ed). Washington, DC:American Association for the Advancement of Science, 1945;85-122.

4. Greene RR, Burrill MW, Ivy AC. The effects of estrogens on the antenatal sexual development of the rat. Am J Anat 67:305-345 (1939).

5. Greene RR. Hormonal factors in sex inversion: the effects of sex hormones on embryonic sexual structures of the rat. Biol Symp 9:105-123 (1940).

6. Karnaky KJ. Estrogenic tolerance of pregnant women. Am J Obstet Gynecol 53:312-316 (1947).

7. Fraenkel L, Papanicolaou GN. Growth, desquamation and involution of the vaginal epithelium of fetuses and children with a consideration of the related hormonal effects. Am J Anat 62:427-451 (1938).

8. Herbst AL, Ulfelder H, Poskanzer DC. Adenocarcinoma of the vagina: association of maternal stilbestrol therapy with tumor appearance in young women. N Engl J Med 284:878-881 (1971).

9. Yonemura CY, Cunha GR, Sugimura Y, Mee SL. Temporal and spatial factors in diethylstilbestrol-induced squamous metaplasia in the developing human prostate. II: Persistent changes after removal of diethylstilbestrol. Acta Anat (Basel) 153:1-11 (1995).

10. Taguchi O, Cunha GR, Robboy SJ. Experimental study of the effect of diethylstilbestrol on the development of the human female reproductive tract. Int J Biol Res Pregnancy 4:56-70 (1983).

11. Taguchi O, Cunha GR, Lawrence WD, Robboy SJ. Timing and irreversibility of Mullerian duct inhibition in the embryonic reproductive tract of the human male. Dev Biol 106:394-398 (1984).

12. Robboy SJ, Taguchi O, Cunha GR. Normal development of the human female reproductive tract and alterations resulting from experimental exposure to diethylstilbestrol. Hum Pathol 13:190-198 (1982).

13. Sugimura Y, Cunha GR, Yonemura CU, Kawamura J. Temporal and spatial factors in diethylstilbestrol-induced squamous metaplasia of the developing human prostate. Hum Pathol 19:133-139 (1988).

14. Brandenberger AW, Tee MK, Lee JY, Chao V, Jaffe RB. Tissue distribution of estrogen receptors * (ER-*) and ß (ER-ß) mRNA in the midgestational human fetus. J Clin Endocrinol Metab 82:3509-3512 (1997).

15. Glatstein IZ, Yeh J. Ontogeny of the estrogen receptor in the human fetal uterus. J Clin Endocrinol Metab 80:958-964 (1995).

16. Taguchi O, Cunha GR, Robboy SJ. Expression of nuclear estrogen-binding sites within developing human fetal vagina and urogenital sinus. Am J Anat 177:473-480 (1986).

17. Kuiper GG, Enmark E, Pelto-Huikko M, Nilsson S, Gustafsson JA. Cloning of a novel receptor expressed in rat prostate and ovary. Proc Natl Acad Sci USA 93:5925-5930 (1996).

18. Majumder PK, Kumar VL. Androgen receptor mRNA detection in the human foetal prostate. Int Urol Nephrol 29:633-635 (1997).

19. Levine AC, Wang JP, Ren M, Eliashvili E, Russell DW, Kirschenbaum A. Immunohistochemical localization of steroid 5 *-reductase 2 in the human male fetal reproductive tract and adult prostate. J Clin Endocrinol Metab 81:384-389 (1996).

20. McLachlan JA, Newbold RA, Shah HC, Hogan MD, Dixon RL. Reduced fertility in female mice exposed transplacentally to diethylstilbestrol (DES). Fertil Steril 38:364-371 (1982).

21. McLachlan JA, Newbold RR, Bullock BC. Long-term effects on the female mouse genital tract associated with prenatal exposure to diethylstilbestrol. Cancer Res 40:3988-3999 (1980).

22. Newbold R. Cellular and molecular effects of developmental exposure to diethylstilbestrol: implications for other environmental estrogens. Environ Health Perspect 103:83-87 (1995).

23. Halling A, Forsberg JG. Acute and permanent growth effects in the mouse uterus after neonatal treatment with estrogens. Reprod Toxicol 7:137-153 (1993).

24. vom Saal FS, Cooke PS, Buchanan DL, Palanza P, Thayer KA, Nagel SC, Parmigiani S, Welshons WV. A physiologically based approach to the study of bisphenol A and other estrogenic chemicals on the size of reproductive organs, daily sperm production, and behavior. Toxicol Ind Health 14:239-260 (1998).

25. vom Saal FS, Timms BG, Montano MM, Palanza P, Thayer KA, Nagel SC, Dhar MD, Ganjam VK, Parmigiani S, Welshons WV. Prostate enlargement in mice due to fetal exposure to low doses of estradiol or diethylstilbestrol and opposite effects at high doses. Proc Natl Acad Sci USA 94:2056-2061 (1997).

26. Nagel SC, vom Saal FS, Thayer KA, Dhar MG, Boechler M, Welshons WV. Relative binding affinity-serum modified access (RBA-SMA) assay predicts the relative *in vivo* bioactivity of the xenoestrogens bisphenol A and octylphenol. Environ Health Perspect 105:70-76 (1997).

27. Smith OW, Smith GVS, Hurwitz D. Increased excretion of pregnanediol in pregnancy from diethylstilbestrol with special reference to the prevention of late pregnancy accidents. Obstet Gynecol 51:411-415 (1946).

28. Golden RJ, Noller KL, Titus-Ernstoff L, Kaufman RH, Mittendorf R, Stillman R, Reese EA. Environmental endocrine modulators and human health: an assessment of the biological evidence. Crit Rev Toxicol 28:109-227 (1998).

29. Kaufman RH, Adam E, Binder GL, Gerthoffer EA. Upper genital tract changes and pregnancy outcome in offspring exposed *in utero* to diethylstilbestrol. Am J Obstet Gynecol 154:330-332 (1980).

30. Kaufman RH, Noller AE, Irwin JF, Gray M. Upper genital tract changes and infertility in diethylstilbestrol-exposed women. Am J Obstet Gynecol 154:1312-1318 (1986).

31. Herbst A, Bern H. Developmental Effects of DES in Pregnancy. New York:Thieme Stratton, 1981.

32. Herbst AL, Anderson D. Clear cell adenocarcinoma of the vagina and cervix secondary to intrauterine exposure to diethylstilbestrol. Semin Surg Oncol 6:343-346 (1990).

33. Hatch EE, Palmer JR, Titus-Ernstoff L, Noller KL, Kaufman RH, Mittendorf R, Robboy SJ, Hyer M, Cowan CM, Adam E, Colton T, Hartge P, Hoover RN. Cancer risk in women exposed to diethylstilbestrol *in utero*. JAMA 280:630-634 (1998).

34. Jensen TK, Toppari J, Keiding N, Skakkebaek NE. Do environmental estrogens contribute to the decline in male reproductive health? Clin Chem 41:1896-1901 (1995).

35. Bibbo M, Gill WB, Azizi F, Blough R, Fang VS, Rosenfield RL. Follow-up study of male and female offspring of DES-exposed mothers. Obstet Gynecol 49:1-8 (1977).

36. Gill W, Schumacher G, Hubby M, Blough R. Male genital tract changes in humans following intrauterine exposure to diethylstilbestrol. In: Developmental Effects of Diethylstilbestrol (DES) in Pregnancy (Herbst A, Bern HA, eds). New York:Thieme Stratton Inc, 1981;103-119.

37. Vingerhoets AJ, Assies J, Goodkin K, Van Heck GL, Bekker MH. Prenatal diethylstilbestrol exposure and self-reported immune-related diseases. Eur J Obstet Gynecol Reprod Biol 77:205-209 (1998).

38. Bekker MH, Heck GL, Vingerhoets AJ. Gender-identity, body-experience, sexuality, and the wish for having children in DES-daughters. Womens Health 24:65-82 (1996).

39. Hornsby PP, Wilcox AJ, Weinberg CR, Herbst AL. Effects on the menstrual cycle of *in utero* exposure to diethylstilbestrol. Am J Obstet Gynecol 170:709-15 (1994).

40. Marselos M, Tomatis L. Diethylstilboestrol. I: Pharmacology, toxicology and carcinogenicity in humans. Eur J Cancer 28A:1182-1189 (1992).

41. Newbold R, Hanson RB, Jefferson WN, Bullock BC, Haseman J, McLachlan JA. Increased tumors but uncompromised fertility in the female descendants of mice exposed developmentally to diethylstilbestrol. Carcinogenesis (in press).

42. Colborn T, vom Saal FS, Soto AM. Developmental effects of endocrine-disrupting chemicals in wildlife and humans. Environ Health Perspect 101:378-384 (1993).

43. Newbold RR, Jefferson WN, Padilla-Burgos E, Bullock BC. Uterine carcinoma in mice treated neonatally with tamoxifen. Carcinogenesis 18:2293-2298 (1997).

44. Nagel SC, vom Saal FS, Welshons WV. The effective free fraction of estradiol and xenoestrogens in human serum measured by whole cell uptake assays: physiology of delivery modifies estrogenic activity. Proc Soc Exp Biol Med 217:300-309 (1998).

45. Dolle P, Izpisua-Belmonte JC, Brown JM, Tickle C, Duboule D. HOX-4 genes and the morphogenesis of mammalian genitalia. Genes Dev 5:1767-1777 (1991).

46. Warot X, Fromental-Ramain C, Fraulob V, Chambon R, Dolle P. Gene dosage-dependent effects of the Hoxa-13 and Hoxd-13 mutations on morphogenesis of the terminal parts of the digestive and urogenital tracts. Development 124:4781-4797 (1997).