

Uterine Adenocarcinoma in Mice Treated Neonatally with Genistein

Retha R. Newbold,¹ Elizabeth Padilla Banks, Bill Bullock, and Wendy N. Jefferson

Developmental Endocrinology Section, Laboratory of Toxicology, Environmental Toxicology Program, Division of Intramural Research, National Institute of Environmental Health Sciences, Research Triangle Park, North Carolina 27709 [R. R. N., E. P. B., W. N. J.], and Department of Pathology, Wake Forest University School of Medicine, Wake Forest University, Winston-Salem, North Carolina 27157 [B. B.]

Abstract

The developing fetus is uniquely sensitive to perturbation with estrogenic chemicals. The carcinogenic effect of prenatal exposure to diethylstilbestrol (DES) is the classic example. Because phytoestrogen use in nutritional and pharmaceutical applications for infants and children is increasing, we investigated the carcinogenic potential of genistein, a naturally occurring plant estrogen in soy, in an experimental animal model previously reported to result in a high incidence of uterine adenocarcinoma after neonatal DES exposure. Outbred female CD-1 mice were treated on days 1–5 with equivalent estrogenic doses of DES (0.001 mg/kg/day) or genistein (50 mg/kg/day). At 18 months, the incidence of uterine adenocarcinoma was 35% for genistein and 31% for DES. These data suggest that genistein is carcinogenic if exposure occurs during critical periods of differentiation. Thus, the use of soy-based infant formulas in the absence of medical necessity and the marketing of soy products designed to appeal to children should be closely examined.

Introduction

Studies in our laboratory have shown long-term consequences of exposure to estrogens during critical periods of development (1). In particular, DES,² a potent synthetic estrogen, has been shown to cause adverse reproductive effects including structural abnormalities, infertility, and neoplasia after prenatal exposure (2). Furthermore, mice treated neonatally with 2 $\mu\text{g}/\text{pup}/\text{day}$ (1000 $\mu\text{g}/\text{kg}/\text{day}$) DES on days 1–5 have a 90–95% incidence of uterine carcinoma at 18 months of age (3). The background incidence of this lesion is rare in this strain of mouse (3). The naturally occurring phytoestrogen, genistein, is found in many soy products. Humans may be exposed to high levels of genistein during development through soy-based infant formulas and soy products marketed specifically for children (4). The concentrations of genistein and other isoflavones found in some of these soy formulas can far exceed the amount found in an adult diet (4). Infants consuming a diet of soy-based formula may be exposed to ~20–40 mg per day (4–6 mg/kg/day) of soy isoflavones, of which genistein makes up >65%, whereas adults consuming a moderate to large amount of soy in the diet are exposed to ~1 mg/kg/day soy isoflavones (4). Earlier reports suggested beneficial effects of genistein exposure early in life, e.g., breast cancer prevention (5, 6) and improved cholesterol synthesis rates (7), but an increasing number of reports are now describing long-term deleterious effects of genistein (8) and of another phytoestrogen, coumestrol (9, 10). One recent report showed an increase in carcinogen-induced mammary carcinogenesis after prenatal exposure of rats to genistein (11), and another associated a diet high in phytoestrogens with the development of

insulin-dependent diabetes (12). Still another report highlighted the increased incidence of hypospadias in male offspring of vegetarian mothers (13). To further study the effects of phytoestrogen exposure early in life, we treated mice neonatally with genistein or an equivalent estrogenic dose of DES and determined the long-term carcinogenic potential.

Materials and Methods

Animals. Adult female CD-1 [CrI:CD-1 (ICR) BR] mice were obtained from Charles River Breeding Laboratories (Raleigh, NC) and bred to male mice of the same strain in the breeding facility at the NIEHS (Research Triangle Park, NC). Vaginal plug detection was considered day 0 of pregnancy. Pregnant mice were individually housed in plastic cages with hardwood chip bedding under controlled lighting (12 h light and 12 h dark) and temperature (21–22°C) conditions. Mice were fed NIH 31 mouse chow, which contained a moderately low amount of genistein (46 $\mu\text{g}/\text{g}$; Ref. 14) and fresh water *ad libitum*. All animal procedures complied with NIEHS/NIH animal care guidelines. At delivery, pups from all litters were pooled, then separated by sex, and standardized to eight female pups per dam. Dams with male pups were used in another experiment.

Comparison of the Estrogenicity of Genistein and DES in Neonatal Pups. Female pups were given daily s.c. injections of genistein or DES dissolved in corn oil or of corn oil alone (as control) on days 1–5 of neonatal life. A minimum of eight pups per compound was used. On the afternoon of day 5, mice were killed and body weights and uterine weights recorded. Data are expressed as percentage uterine weight increase over values in corn oil-treated control mice. A dose response for genistein has been previously reported in the immature mouse uterotrophic bioassay (15).

Tumor Induction. Female pups were treated by daily s.c. injections on days 1–5 with genistein (50 mg/kg) or DES (0.001 mg/kg) dissolved in corn oil or with corn oil alone (as control). These doses are ~100 $\mu\text{g}/\text{pup}/\text{day}$ genistein and 0.002 $\mu\text{g}/\text{pup}/\text{day}$ DES and are equal in estrogenic potency. Mice were weaned on day 21 and housed four per cage. Mice were killed by cervical dislocation at 18 months of age. Reproductive tract tissues were removed, fixed in 10% neutral buffered formalin, embedded in paraffin, and sectioned at 6 μm . Tissue sections were stained with H&E and evaluated by light microscopy. If a microscopic lesion was observed, additional serial sections were made to include the entire area of pathological change.

Results

Comparison of Hormonal Activity of DES and Genistein in the Neonate. Genistein (50 mg/kg/day) and DES (0.001 mg/kg/day) induced a 202% and 190% gain in uterine weights, respectively, compared with control animals treated with corn oil (Table 1). Both compounds caused a significant increase in uterine wet weight compared with controls from this experiment by Fisher's exact test.

Reproductive Tract Abnormalities. A comparison of reproductive tract abnormalities observed in mice treated neonatally on days 1–5 with Genistein or DES is shown in Table 2. At 18 months of age, cystic ovaries were common in all of the treatment groups (46% in controls, 41% in the genistein group, and 58% in the DES group). Corpora lutea were absent in 100% (17 of 17) of the genistein-treated mice, whereas only 33% (4 of 12) of the DES-treated mice lacked corpora lutea. All 13 of the control mice had corpora lutea. In the

Received 2/6/01; accepted 4/3/01.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¹ To whom requests for reprints should be addressed, at National Institute of Environmental Health Sciences, MD E4-02, P. O. Box 12233, Research Triangle Park, NC 27709. Phone: (919) 541-0738; Fax: (919) 541-4634; E-mail: newbold1@niehs.nih.gov.

² The abbreviations used are: DES, diethylstilbestrol; NIEHS, National Institute of Environmental Health Sciences; PPL, progressive proliferative lesion; CEH, cystic endometrial hyperplasia.

Table 1 Uterotropic bioassay in day-5 mice treated neonatally with DES or with genistein

Mice were given s.c. injections of 50 mg/kg/day (100 µg/pup/day) genistein or 0.001 mg/kg/day (0.002 µg/pup/day) DES in corn oil on days 1–5 of neonatal life; controls were untreated. They were killed on day 5, and the uterine weight:body weight ratio determined. A minimum of eight pups/compound was included in each group.

Compound	Uterine wet weight:body weight ratio × 100 ^a	Estrogenic potency in neonates (% uterine wet weight gain) ^b
Control	0.109 ± 0.007	100
DES	0.207 ± 0.008 ^c	190
Genistein	0.220 ± 0.027 ^c	202

^a Data are expressed as the mean ± SE.

^b Data are expressed as the percentage increase over control values.

^c P < 0.05.

DES-treated mice, 17% (2 of 12) had ovarian stromal tumors; ovarian tumors were not seen in the control or genistein-treatment group.

Abnormalities in the oviduct were seen in both the DES and genistein treatment groups in this study (Table 2). As described previously with DES (16), the pattern of tubal plications of oviductal mucosa was distorted and characterized by lack of plications or by irregularities in the size and shape of the mucosal folds relative to the control animals. The mucosal folds had an adenomatous (gland-like) appearance but maintained connection with the oviductal lumen. This abnormality in proliferation was termed PPL in DES-treated animals (16) because it did not spread along the serosal surface nor metastasize. In this study, sections were not always available through the entire length of the oviduct for each animal, but PPL was observed in 50% (5 of 10) of the available sections of DES-treated mice, whereas all (14 of 14) of the genistein-treated mice had this abnormality.

The range of uterine abnormalities in the uteri of mice exposed neonatally to genistein or DES is also shown in Table 2. The incidence of CEH of the uterus was similar in both DES- and genistein-treatment groups [54% (7 of 13) and 47% (8 of 17), respectively]. A low incidence of CEH occurred in the control mice in this study [19% (3 of 16)]. Squamous metaplasia of the uterus occurred in 38% (5 of 13) of the DES and 64% (11 of 17) of the genistein groups. In addition to these uterine lesions, more severe pathologies occurred after neonatal exposure to both compounds. Atypical hyperplasia of the uterus occurred in 5% (1 of 17) of the genistein-treated mice. Furthermore, some animals had cellular alterations that progressed to uterine adenocarcinoma (Fig. 1, A and B). These tumors were usually well differentiated and characterized by irregularly shaped glands with little intervening stroma. Some lesions extended through the myometrium to the serosal surface of the uterus. Nuclear pleomorphism and mitotic figures were frequently observed. In genistein-treated mice, 35% (6 of 17) developed this tumor, and at the approximate equal estrogenic dose of DES, 31% (4 of 13) had uterine adenocarcinoma;

there were no cases of uterine adenocarcinoma in any controls in this study.

Discussion

This report describes the induction of benign and malignant lesions of the reproductive tract, including uterine adenocarcinoma in the reproductive tract of mice treated neonatally with the phytoestrogen genistein. Similar malignant lesions have never been observed in control mice of this strain in our laboratory. The dose of genistein used in this study is within the range to which humans may be exposed in soy-based infant formulas (4). In addition, mice exposed to a dose of DES that is approximately equal in estrogenic activity gave similar incidences of uterine adenocarcinoma under the same treatment conditions. The association of estrogenicity and carcinogenicity in the neonate is further supported by the results of another study from our lab that compared metabolites of estradiol (17); based on the day-5 uterotrophic bioassay, compounds with the highest estrogenic potency in the neonatal mouse uterus showed the highest percentage of uterine adenocarcinoma after neonatal exposure. The data shown in this report shows a close association between the estrogenic activity of the compound in the neonate and the incidence of uterine adenocarcinoma after neonatal exposure.

Of particular significance in this study is the incidence of uterine adenocarcinoma after neonatal exposure to genistein. This compound is readily available to many infants during the first year of life as a component of soy-based formulas. The amount of soy isoflavones found in some infant formulas, of which genistein and its conjugates account for >65%, approaches 40 mg/liter of formula (4). An infant consuming 1 liter of soy-based infant formula would ingest ~27 mg of genistein per day. The amount of genistein used in our study (50 mg) is slightly higher than the amount consumed by infants, but it is certainly within one order of magnitude of the level of human exposure. We are currently evaluating the carcinogenic potential of lower doses of genistein as well as investigating the effects of genistein if exposure occurs through the diet instead of s.c. injection as described in this study.

In another report from our laboratory, lower doses of genistein caused alterations in the ovary such as multiocyte follicles (18); a dose-related increase in multiocyte follicles starting at a dose of 5 mg/kg/day was observed in 2-month-old mice. Previous work by Iguchi *et al.* (19) has shown that multiocyte follicles resulting from neonatal DES exposure are less fertile than single oocyte follicles. This data suggest that fertility could also be affected in mice exposed neonatally to genistein. Future studies in our laboratory will investigate fertility in genistein-treated mice as well as mechanisms involved in the formation of multiocyte follicles.

Table 2 Incidence of benign and malignant abnormalities in 18-month-old mice treated neonatally with DES or with genistein

Outbred CD-1 mice were treated by s.c. injections on days 1–5 of neonatal life with 0.001 mg/kg/day (0.002 µg/pup/day) DES or 50 mg/kg/day (100 µg/pup/day) genistein; these are approximately equal estrogenic doses. Mice were allowed to age and were killed at 18 months. The numbers in parentheses represent percentage.

Treatment	Ovary/Oviduct	Uterus
Control	0/13 No CL ^a (0)	1/16 Uterine adenomyosis (6)
	6/13 Ovarian cysts (46)	3/16 CEH (19)
	0/13 PPL (0)	0/16 Uterine adenocarcinoma (0)
DES, 0.001 mg/kg/day	4/12 No CL (33)	7/13 CEH (54)
	7/12 Ovarian cysts (58)	5/13 Squamous metaplasia (38)
	5/10 PPL (50)	1/13 Uterine adenomyosis (8)
	2/12 Ovarian stromal tumors (17)	4/13 Uterine adenocarcinoma (31)
Genistein, 50 mg/kg/day	17/17 No CL (100)	8/17 CEH (47)
	7/17 Ovarian cysts (41)	11/17 Squamous metaplasia (64)
	14/14 PPL (100)	1/17 Atypical hyperplasia (5)
		6/17 Uterine adenocarcinoma (35)

^a CL, corpora lutea.

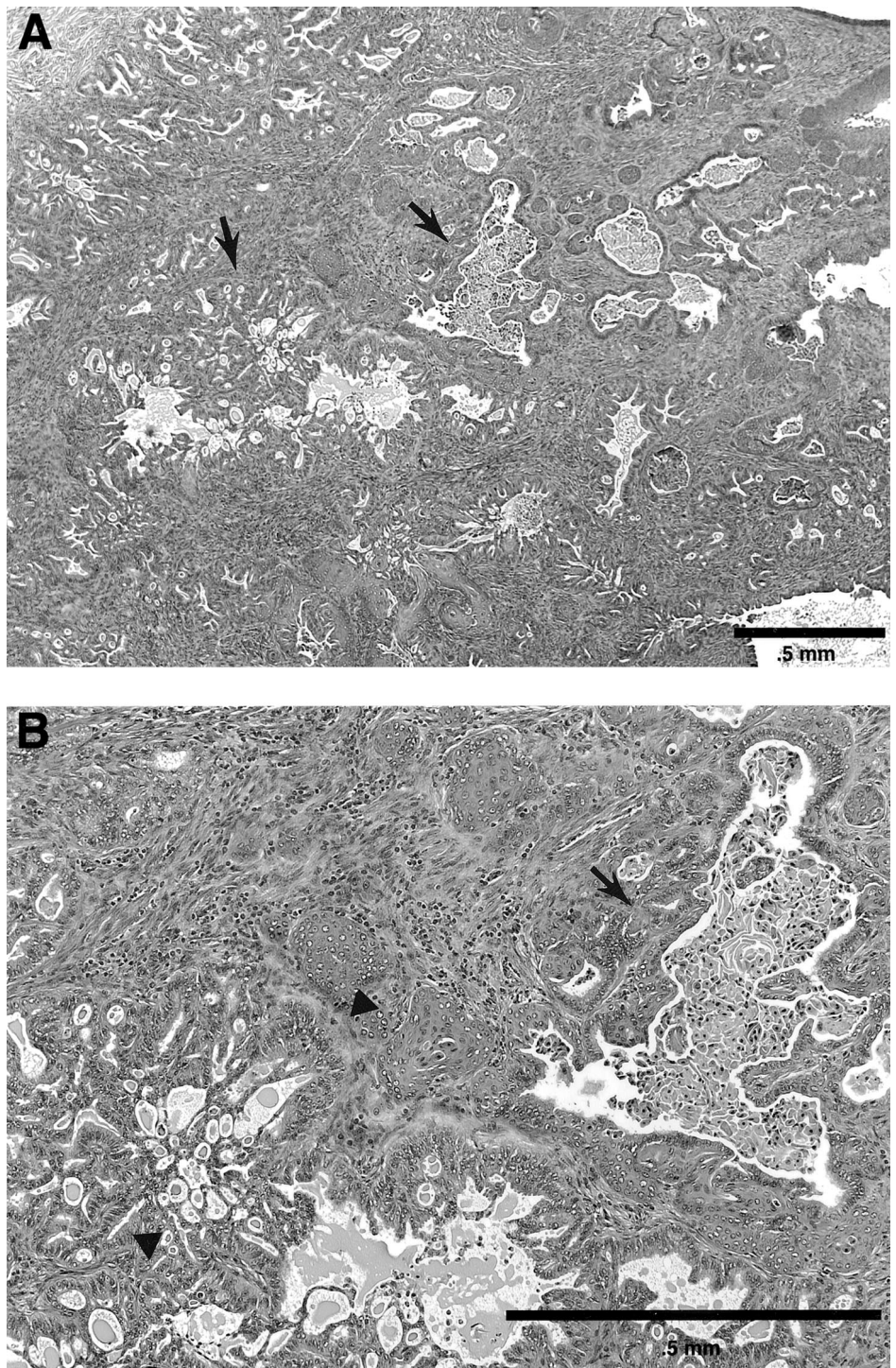


Fig. 1. *A*, well-differentiated invasive uterine adenocarcinoma in an 18-month-old mouse treated neonatally with genistein (100 mg/kg/day) on days 1–5 of life. Neoplastic endometrial tissue is present between strands of smooth muscle. There are irregularly shaped glands with little intervening stroma and eosinophilic material in the lumen (*arrow*). (H&E; bar, 0.5 mm.) *B*, higher magnification of uterine adenocarcinoma in an 18-month-old mouse treated neonatally with genistein (100 mg/kg/day). There are irregularly shaped glands, many with eosinophilic material in the lumen (*arrow*). Some irregularly shaped endometrial glands are packed together between bundles of smooth muscle (*arrowheads*). (H&E; bar, 0.5 mm.)

The findings of the present study raise concerns over the amount of phytoestrogens in soy-based infant formulas and other soy-based products that are fed to young children. Additional studies are needed to determine the potential effects in humans exposed to high quantities of phytoestrogens during critical stages of neonatal or early development. Ongoing National Toxicology Program (NTP) studies designed to address multigenerational effects of genistein,³ will help to determine the risks of developmental exposure to genistein and other endocrine-disrupting chemicals. The potential risks and benefits need

to be thoroughly assessed to determine the appropriate balance of exposures of these chemicals during development and the permanent effects that may follow.

References

1. Newbold, R. Cellular and molecular effects of developmental exposure to diethylstilbestrol: implications for other environmental estrogens. *Environ. Health Perspect.*, *103*: 83–87, 1995.
2. McLachlan, J. A., Newbold, R. R., and Bullock, B. C. Long-term effects on the female mouse genital tract associated with prenatal exposure to diethylstilbestrol. *Cancer Res.*, *40*: 3988–3999, 1980.
3. Newbold, R. R., Bullock, B. C., and McLachlan, J. A. Uterine adenocarcinoma in mice following developmental treatment with estrogens: a model for hormonal carcinogenesis. *Cancer Res.*, *50*: 7677–7681, 1990.

³ B. Delclos and R. R. Newbold. Dose range studies of genistein in the rat, submitted for publication.

4. Setchell, K. D., Zimmer-Nechemias, L., Cai, J., and Heubi, J. E. Isoflavone content of infant formulas and the metabolic fate of these phytoestrogens in early life. *Am. J. Clin. Nutr.*, *68*: 1453S–1461S, 1998.
5. Barnes, S. The chemopreventive properties of soy isoflavonoids in animal models of breast cancer. *Breast Cancer Res Treat.*, *46*: 169–179, 1997.
6. Lamartiniere, C. A., Zhang, J. X., and Cotroneo, M. S. Genistein studies in rats: potential for breast cancer prevention and reproductive and developmental toxicity. *Am. J. Clin. Nutr.*, *68*: 1400S–1405S, 1998.
7. Cruz, M. L., Wong, W. W., Mimouni, F., Hachey, D. L., Setchell, K. D., Klein, P. D., and Tsang, R. C. Effects of infant nutrition on cholesterol synthesis rates. *Pediatr. Res.*, *35*: 135–140, 1994.
8. Faber, K. A., and Hughes, C. L., Jr. Dose-response characteristics of neonatal exposure to genistein on pituitary responsiveness to gonadotropin releasing hormone and volume of the sexually dimorphic nucleus of the preoptic area (SDN-POA) in postpubertal castrated female rats. *Reprod. Toxicol.*, *7*: 35–39, 1993.
9. Burroughs, C. D., Mills, K. T., and Bern, H. A. Long-term genital tract changes in female mice treated neonatally with coumestrol. *Reprod. Toxicol.*, *4*: 127–135, 1990.
10. Whitten, P. L., Lewis, C., Russell, E., and Naftolin, F. Phytoestrogen influences on the development of behavior and gonadotropin function. *Proc. Soc. Exp. Biol. Med.*, *208*: 82–86, 1995.
11. Hilakivi-Clarke, L., Cho, E., Onojafe, I., Raygada, M., and Clarke, R. Maternal exposure to genistein during pregnancy increases carcinogen-induced mammary tumorigenesis in female rat offspring. *Oncol. Rep.*, *6*: 1089–1095, 1999.
12. Drash, A. L., Kramer, M. S., Swanson, J., and Udall, J. N. Infant feeding practices and their possible relationship to the etiology of diabetes mellitus. American Academy of Pediatrics Work Group on Cow's Milk Protein and Diabetes Mellitus. *Pediatrics*, *94*: 752–754, 1994.
13. North, K., and Golding, J. A maternal vegetarian diet in pregnancy is associated with hypospadias. The ALSPAC Study Team. Avon Longitudinal Study of Pregnancy and Childhood. *BJU Int.*, *85*: 107–113, 2000.
14. Thigpen, J. E., Setchell, K. D. R., Ahlmark, K. B., Locklear, J., Spahr, T., Caviness, G. F., Goelz, M. F., Haseman, J. K., Newbold, R. R., and Forsythe, D. B. Phytoestrogen content of purified, open- and closed- formula laboratory animal diets. *Lab. Anim. Sci.*, *49*: 530–536, 1999.
15. Jefferson, W. N., and Newbold, R. R. Potential endocrine-modulating effects of various phytoestrogens in the diet. *Nutrition*, *16*: 658–662, 2000.
16. Newbold, R. R., Bullock, B. C., and McLachlan, J. A. Progressive proliferative changes in the oviduct of mice following developmental exposure to diethylstilbestrol. *Teratog. Carcinog. Mutagen.*, *5*: 473–480, 1985.
17. Newbold, R. R., and Liehr, J. G. Induction of uterine adenocarcinoma in CD-1 mice by catechol estrogens. *Cancer Res.*, *60*: 235–237, 2000.
18. Jefferson, W. N., Padilla-Burgos, E., Miller, C., and Newbold, R. R. Endocrine modulating effects of various phytoestrogens. In: G. E. Dunaif, S. S. Olin, J. A. Scimeca, and J. A. Thomas (eds.), *Human Diet and Endocrine Modulation: Estrogenic and Carcinogenic Effects*, pp. 286–289. Washington, DC: ILSI Press, 1998.
19. Iguchi, T., Takasugi, N., Bern, H. A., and Mills, K. T. Frequent occurrence of polyovular follicles in ovaries of mice exposed neonatally to diethylstilbestrol. *Teratology*, *34*: 29–35, 1986.