Effects of Estrogenic Chemicals on Development

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It is an established concept in modern cancer research that structural and functional alterations of cellular genes are instrumental in the transformation of a normal cell into a malignant phenotype. Although to date no causal relationship has been proved to link steroid-hormone effects to the induction of chromosomal alterations in an animal system, steroid hormones have been shown to be capable of producing chromosomal abnormalities in cultured cells (*1,2*). In addition, Banduhn and Obe (*3*) showed that both diethylstilbestrol (DES) and estradiol are capable of inducing genomic mutations in cultured cells, and Wheeler et al. (*4*) showed that estradiol and DES are potent inhibitors of mitosis in *in vitro* cell cultures. A similar study by Tsutsui et al. (*5*) suggested that estrogen can induce two types of genetic changes, one involving numerical chromosome change (aneuploidy) with no evident DNA damage and another associated with structural chromosomal aberrations induced by estrogen catechol metabolites. Hillbertz-Nilsson and Forsberg (*6*) reported estrogen-induced aneuploidy in the epithelial cells from the uterine cervix of a neonatal mouse. Their report, along with those of Endo et al. (*7*) and Hajek et al. (*8,9*), suggests the possibility that perinatal exposure to estrogen results in chromosomal aberrations in the same target tissues in which neoplasia occurs after the *in vivo* administration of estrogen.

Evolution of Malignancy

Experimental studies of carcinogenesis and clinical evidence suggest that the development of a malignant tumor is a gradual evolutionary process during which tumor cells progressively acquire permanent qualitatively different characteristics (*10*). Genetic instability of tumor cells has been found to be greatly enhanced over that of normal cells, and amplification of DNA may be one of the mechanisms that leads to the emergence of clonal populations that have increasingly malignant properties (*11,12*). Amplification of genes could enable the host cells to escape growth control, to become mobile and invasive, or to escape immune surveillance. For instance, trisomy for chromosome 7 appears to be one of the nonrandom primary changes in gynecologic adenocarcinomas that is apparently associated with the early clinical stages of the disease. From the molecular viewpoint, chromosome 7 has several growth factor genes (e.g., epidermal growth factor [EGF] receptor), many genes for cell surface proteins, and at least two human protooncogenes (i.e., *HER2/neu* and *met*). If the extra chromosome 7 results in increased secretion of cellular growth factors, this could be an initial step in malignant transformation. Information supporting the exposure to steroid hormones and genetic alterations that ultimately lead to the development of malignant tumor has been gradually forthcoming.

Role of Estrogenic Compounds as Potential Initiators and Promoters of Hormonal Carcinogenesis

Using a mouse skin tumorigenesis model, Conti et al. (*13*) and Aldaz et al. (*14*) reported a positive correlation between histological and cytogenetic analysis with more aggressive and atypical tumors. They showed that 10 to 20 weeks of exposure to a promoter stimulated the progression of a papilloma (benign diploid lesion) to a hyperdiploid (neoplastic) lesion. Their results suggest that aneuploidy may play a mechanistic role in the sequence of events that lead to neoplasia in epithelial tissue. It has also been suggested that the chemical induction of skin tumors in mice can be subdivided into at least three stages: initiation, promotion, and tumor development (*14*). The progression from initiation to carcinoma is thought to involve the clonal expansion of initiated epidermal cells, which results in the formation of benign tumors. The conversion of benign tumors to carcinomas is thought to require an additional cellular (i.e., genomic) change. The hormonal induction of tumors may involve a similar process. Unlike chemical induction in the mouse skin tumorigenesis model, perinatal exposure to estrogen during a critical period during the development of the neonatal mouse resulted in a state of continuous proliferation of cervicovaginal epithelium, even in the absence of estrogen. However, similar to the chemical induction of skin tumors, perinatal exposure to estrogen may result in the selection of a clonal population. It is the development of cervicovaginal tumors in intact mice that indicates that a particular clonal population may be under the influence of estrogen (Figure 1).

The development of this particular clonal population and the subsequent development of tumors is time dependent (*15*) (Table 1). In addition, perinatal exposure to estrogen before 3 days after birth, followed by continuous secondary estrogen administration beginning at 10 days of age, reduces the latency period required for the nuclear DNA content of the cervicovaginal epithelium to increase (*9*). These data indicate that, like the human fetus, a critical

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Abbreviations used: ER, estrogen receptor; PR, progesterone receptor; PCB, polychlorinated biphenyl; DES, diethylstilbestrol; EGF, epidermal growth factor.

period exists in mice for the induction of cervicovaginal abnormalities. The critical period for the induction of nuclear DNA changes appears to be similar to the critical period for the induction of cervicovaginal tumors. In addition to the numerical chromosomal changes, a change in the expression of certain proteins occurs (Figure 1). In our neonatal mouse model after exposure to estrogen, the expression of *HER2/neu* (Figure 2) and c-*fos* was altered (*16*). Interestingly, the c-fos protein is considered to be a master switch because it is part of the primary genomic response to stimulation by extracellular signals. It transduces these signals by regulating a number of secondary genes, which remain to be identified. Perinatal exposure to estrogen during a critical period results in an alteration in the expression of c-*fos* and thus links hormonal carcinogenesis to the manipulation of a key function in the regulation of cell behavior (*16*). Recent studies by Liehr et al. (*17*) demonstrated a similar effect in hamster kidneys exposed to estrogens.

J. Gorski's presentation asked whether the variety of effects induced by estrogen exposure was due to a nontraditional effect of hormones or was it related to the classic estrogen receptor (ER) system. Gorski reported that the ER system exists in a 10-day rat fetus whether it is a future male or female rat. Western blots done on protein extracts from the reproductive tracts of both male and female rat fetuses on days 15, 17, and 19 postconception showed a marked increase of ER in the female reproductive tract with age. In contrast, the ER immunostaining decreased with age in the male. Using the more sensitive procedure of reverse transcriptase–polymerase chain reaction, they probed the rat fetus on days 0 to 5 and found that 1- and 2-cell-stage oocytes contained a signal that indicated the presence of an ER message. They speculated that this ER message was both maternal in origin and carried by the oocyte as well as made available to the embryo. This message gradually disappeared and reached its lowest level between the 5- to 8-cell stage; the ER mRNA was not detected at the morula stage but reappeared at the blastocyst stage. A repeat experiment showed that the progesterone receptor (PR) was not detected in any of the early stages, but was detected in the blastocyst stage. Therefore, these results indicate a possible role for estrogen receptors in both endogenous and exogenous exposure to estrogens during critical periods of development.

Table 1. Defining critical period for neonatal estrogen-induced tumor development.

Treatment[a] schedule, days treated[b]	Number of animals (%) with cervicovaginal lesions
1 – 5	8/17 (47%)
2 – 6	10/22 (45%)
3 – 7	2/24 (8%)
5 – 9	0/20 (0%)
10 – 14	0/23 (0%)

[a]Female BALB/c mice were injected (sc) daily with 25 µg of 17β-estradiol in 0.02 ml of sesame oil. [b]After birth.

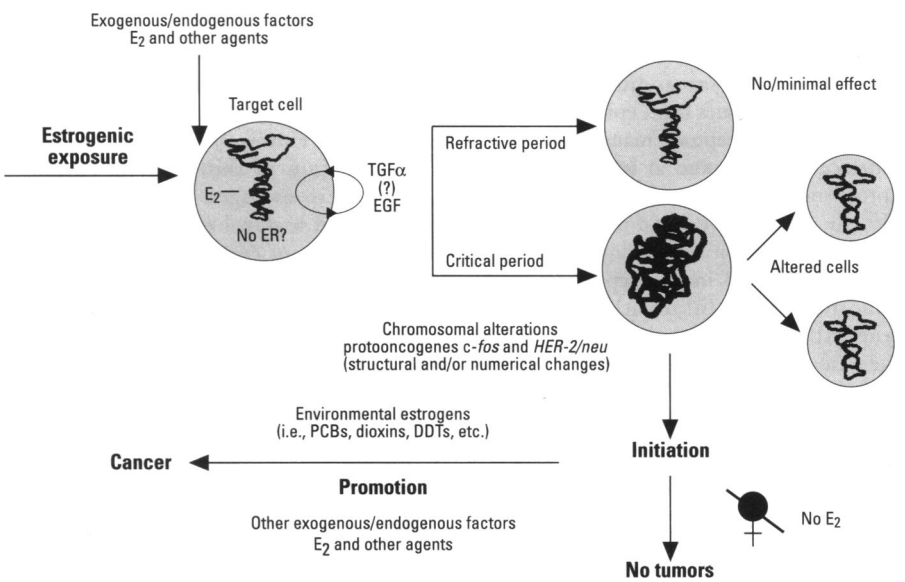

Figure 1. Multistep hormonal carcinogenesis.

Environmental Influences on Estrogen

D. Crews and associates stated in their presentation that exposure to specific environmental stimuli or physical conditions during developmental stages (i.e., embryonic and fetal) can influence the expression of genes such as the aromatase and reductase enzymes. They set out to determine how the environmental stimulus of temperature affected the cellular events controlling sex determination. Using turtles, they found that a precise relationship existed between the temperature at which turtle eggs were incubated and the male:female ratio of turtles born. With a variance of only about 1°C, Crews found that only male turtles hatched from eggs incubated at low temperatures and only females hatched from eggs incubated at higher temperatures; there were no hermaphrodites. His results suggested the existence of a mechanism that controlled steroid hormone feedback, but was itself controlled by temperature. How does the physical stimulus of temperature get transferred into a cellular event? It is known that specific temperature conditions can create specific enzyme patterns during this sensitive stage of development, particularly involving aromatase and reductase (e.g., 5α-reductase) activity. Crews' studies indicate the possible presence of a temperature-sensitive promoter that acts directly on the aromatase gene and complementary to the reductase gene to influence the production of estrogen. That is, the environmental temperature triggers the action of the mechanism controlling steroid hormone feedback and causes these genes to produce or not produce estrogen.

In extending his studies to the impact of direct exposure to environmental estrogen on development, Crews found that only two polychlorinated biphenyl (PCB) compounds had any effect. Cocktail mixtures of PCBs without these two effectors had no influence on enzyme or estrogen production. In addition, the two effectors were synergistic.

Effects of Perinatal Exposure to Estrogen on Nonreproductive Structures

T. Iguchi discussed the effect of perinatal exposure to estrogen on both reproductive and nonreproductive structures. Perinatal treatment of female mice with estrogenic compounds such as DES induced a continuous proliferation of the vaginal epithelium, even in the absence of estrogen.

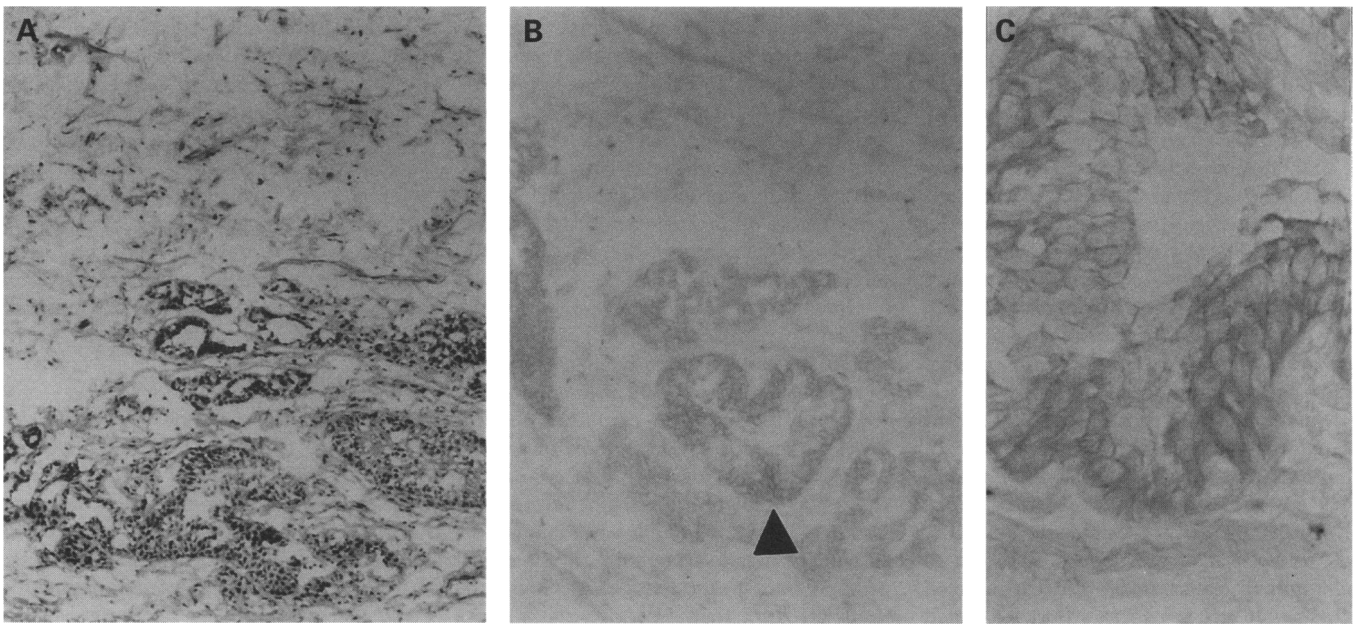

Figure 2. (*A*) H&E stained slide showing cervicovaginal lesions in a neonatally estrogenized 20-month-old mouse ×20. (*B*) The same area in *A* stained for the presence of *HER-2/neu* ×20. (*C*) Higher magnification of the highlighted area in *B*, ×40.

Iguchi and associates reported that this irreversibly changed vaginal epithelium persistently expressed higher levels of c-*jun* and c-*fos* mRNAs. Although not considered to be a reproductive structure, sexual dimorphism of the pelvis has been described in several mammals. Iguchi described sexual dimorphism in the mouse pelvis and the anococcygeous muscle. They reported that the pelvic bone is susceptible to castration, ovariectomization, and the addition of exogenous hormones. Neonatal tamoxifen treatment caused inhibition of pubic bone calcification, causing this bone to remain cartilaginous into adulthood.

Iguchi reported that the effect of sex steroids on the growth of the anococcygeous muscle varied according to gender. In males, castration reduced the size of the muscle, whereas the administration of testosterone resulted in partial restoration. In females, ovariectomy slightly increased the size of the anococcygeous muscle, whereas the administration of estrogen decreased the size of the muscle. However, when DES was administered neonatally, the muscle was widened, which again supports the hypothesis that estrogens have different effects on developing and adult tissue.

The permanent effects of exposure of neonatal rodents to steroid hormones have been the subject of scientific investigation since the 1930s (*18*). At the end of the 1960s, evidence began to emerge from clinical studies that treating pregnant women with DES for threatened abortion in the first trimester correlated with the appearance of vaginal adenocarcinoma in their young daughters. During the decade following the description of the initial six cases of vaginal adenocarcinoma in young women after exposure to DES or chemically related hormones *in utero*, a wealth of information became available concerning the pathology and pathogenesis of disorders related to prenatal exposure to these agents (*19*). Presently, it is estimated that *in utero* DES exposure of the female human fetus has resulted in approximately 519 documented cases of vaginal and cervical clear-cell adenocarcinoma (*20*). This represents only a small percentage of the as yet unknown total number of young women exposed to DES *in utero*. The population at risk has been estimated to be as great as 2 million females (*21,22*).

As reported by R. Newbold, studies of DES using the neonatal mouse have been and continue to be productive for a variety of reasons. She and her associates used the mouse model to study the effects of DES and demonstrated that the majority of DES-related developmental abnormalities observed in humans could be reproduced in mice. Conversely, she provided data useful as clinical guides for as yet unseen or unrecognized human conditions. These effects may also illustrate potential adverse health effects from lower potency estrogens.

It is important to consider the role of estrogens in the environment, especially their effects on development. For instance, many organochlorine pollutants such as PCBs present in the environment have the ability to weakly bind to the estrogen receptor (*23*). However, at present, it is unknown whether they also have the ability to induce tumorigenesis as observed for potent estrogens. Both environmental and genetic interactions play equally important roles in human disease.

Developmental Abnormalities Resulting from Exposure to Estrogenic Chemicals

Ando et al. (*24*) demonstrated that estrogenic chemical compounds can cross the placenta to the fetus and can be transferred via breast milk to newborns. In the female fetus, previous studies have indicated that the organs most susceptible to developmental effects from endocrine-disrupting chemicals include the mammary gland, fallopian tubes, uterus, cervix, and vagina (*18*). Recent findings demonstrated that 17α-estradiol, a weak estrogen, can cause reproductive abnormalities similar to those reported in BALB/c mice exposed perinatally to natural and synthetic estrogens. These findings raise the possibility of long-term consequences resulting from early exposure to weak environmental estrogens (*25*). Because of this possibility, exposure during a critical period of development to environmental estrogens such as organochlorine pollutants (e.g., PCBs), which have the ability to bind weakly to the ER, may induce abnormalities similar to those

resulting from potent estrogens such as DES. Are dioxins potent estrogens too?

Dioxins, once thought solely to be antiestrogenic, represent another family of ubiquitous environmental pollutants (*26,27*) all of which demonstrate a common mechanism of action. L. Birnbaum's presentation summarized the potential developmental toxic effects caused by dioxin. For instance, dioxin-exposure syndrome may include any or all of the following symptoms: wasting, thymic atrophy, fatty liver, enzyme induction, edema, chloracne, embryo/fetal toxicity, and teratogenicity. The biochemical effects of dioxin exposure include the induction of enzymes that are associated with intermediary metabolism and biotransformation. Dioxins modulate hormone systems, including that of the thyroid, and lead to changes in homeostasis. Dioxin-induced changes in growth factors lead to altered growth and development patterns. Dioxins also produce changes in such protooncogenes as c-*fos*, c-*jun*, and *ras*. Interestingly, these are the same protooncogenes influenced by perinatal exposure to estrogen. In summary, Birnbaum suggests that dioxins should be viewed as modulators of growth and development because they contain both estrogenic and antiestrogenic properties. What is interesting is the fact that dioxins do not interact with the ER to cause any of the above effects. However, further study may shed some light on the complexities of these compounds as growth dysregulators operating through their own receptor mechanism, AhRs.

In what other tissues could environmental exposure to estrogenic compounds potentially result in developmental abnormalities? S. Migliaccio and colleagues studied the effect of DES exposure on the developmental programming of bone cell metabolism. Osteoporosis is a condition that over decades robs the skeleton of resources until the bone is weak enough to sustain a spontaneous fracture. In the study of ER in bone tissue, estrogen was found to modulate the activity of bone cells *in vitro*. To address the question of whether changes in estrogen levels during development could affect skeletal characteristics, Migliaccio and co-workers used the neonatal mouse model to evaluate the potential effects of environmental estrogens on developing bone tissue (*33*). What they observed was the effects of DES on the length of the femur. In addition, perinatal exposure to DES affected the density of the bone tissue. Basically, the length of the femur decreased and the bone mass increased. She also observed that bone is more sensitive to the disruptive effects of DES at lower levels of exposure. Although no definitive study has been done with women exposed to DES *in utero*, there have been a few self-reported cases of spondylolisthesis.

Summary

The sum of the evidence supports the necessity to continue to investigate the developmental effects of estrogenic and antiestrogenic compounds when exposure occurs early in life. Additional studies will answer questions relevant to the molecular definition of the developmental or carcinogenic effects of estrogens such as hormone-induced gene alterations. These studies also support the need to use the neonatal mouse model to demonstrate the consequences of reproductive and nonreproductive stem-cell exposure to estrogenic compounds.

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