BIOCHEMICAL AND MOLECULAR CHANGES AT THE CELLULAR LEVEL IN RESPONSE TO EXPOSURE TO ENVIRONMENTAL ESTROGEN-LIKE CHEMICALS

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Estrogen-like chemicals are unique compared to nonestrogenic xenobiotics, because in addition to their chemical properties, the estrogenic property of these compounds allows them to act like sex hormones. Whether weak or strong, the estrogenic response of a chemical, if not overcome, will add extra estrogenic burden to the system. At elevated doses, natural estrogens and environmental estrogen-like chemicals are known to produce adverse effects. The source of extra or elevated concentration of estrogen could be either endogenous or exogenous. The potential of exposure for humans and animals to environmental estrogen-like chemicals is high. Only a limited number of estrogen-like compounds, such as diethylstilbestrol (DES), bisphenol A, nonylphenol, polychlorinated biphenyls (PCBs), and dichlorodiphenyltrichloroethane (DDT), have been used to assess the biochemical and molecular changes at the cellular level. Among them, DES is the most extensively studied estrogen-like chemical, and therefore this article is focused mainly on DES-related observations. In addition to estrogenic effects, environmental estrogen-like chemicals produce multiple and multitype genetic and/or nongenetic hits. Exposure of Syrian hamsters to stilbene estrogen (DES) produces several changes in the nuclei of target organ for carcinogenesis (kidney): (1) Products of nuclear redox reactions of DES modify transcription regulating proteins and DNA; (2) transcription is inhibited; (3) tyrosine phosphorylation of nuclear proteins, including RNA polymerase II, p53, and nuclear insulin-like growth factor-I receptor, is altered; and (4) DNA repair gene DNA polymerase β transcripts are decreased and mutated. Exposure of Noble rats to DES also produces several changes in the mammary gland: proliferative activity is drastically altered; the cell cycle of mammary epithelial cells is perturbed; telomeric length is attenuated; etc. It appears that some other estrogenic compounds, such as bisphenol A and nonylphenol, may also follow a similar pattern of effects to DES, because we have recently shown that these compounds alter cell cycle kinetics, produce telomeric associations, and produce chromosomal aberrations. Like DES, bisphenol A after metabolic activation is capable of binding to DNA. However, it should be noted that a particular or multitype hit(s) will depend upon the nature of the environmental estrogen-like chemical. The role of individual attack leading to a particular change is not clear at this stage. Consequences of these multitypes of attack on the nuclei of cells could be (1) nuclear toxicity/cell death; (2) repair of all the hits and then acting as normal cells; or (3) sustaining most of the hits and acting as unstable cells. Proliferation of the last type of cell is expected to result in transformed cells.

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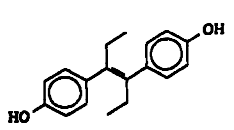
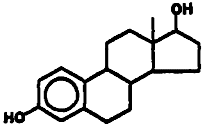
Ovarian estrogens (e.g., estradiol, estrone) occur naturally and are steroidal in nature. Nonovarian estrogens are either synthetic (diethylstilbestrol) or produced by plants (equol, coumestrol) or microbes (enterolactone). Synthetic estrogens are either steroidal or nonsteroidal in nature and can be categorized into two groups: chemicals synthesized (1) as estrogen for pharmacological and other purposes (e.g., diethylstilbestrol, 17α-ethinylestradiol, mestranol) and (2) not as estrogens, but with estrogenic properties that have been identified with time (bisphenol A, nonylphenol, chlorinated hydrocarbons) (structures are shown in Figure 1). The number of estrogen-like chemicals of the latter group has been growing rapidly. At present we know of more than a hundred chemicals that have estrogenic activity. A large number of pesticides and industrial chemicals possessing estrogen-like activity are ubiquitous in the environment and make their way into the food chain (Birnbaum, 1994; Davis et al., 1993; Colborn et al., 1993; Hunter & Kelsey, 1993; Soto et al., 1992, 1994; Wolff et al., 1993; White et al., 1994; Safe, 1995). Some of the pesticides that are estrogenic include endosulfan, 1-hydroxychlordene, dicofol, methoxychlor, and parathion (Soto et al., 1992, 1994). Some of these pesticides, such as methoxychlor, need to be metabolized before they can bind to the estrogen receptor (Ousterhout et al., 1981). The pesticide dichlorodiphenyltrichloroethane (DDT), even though banned and not in use, is still present in the environment due to previous widespread use and relatively long half-life (Wolff et al., 1993; Sharpe, 1995). In addition to pesticides, industrial chemicals such as some polybrominated biphenyls (PBBs) and polychlorinated biphenyls (PCBs), phthalate, and styrene have estrogenic activity and are found as contaminants in the environment (Birnbaum, 1994; Colborn et al., 1993; Davis et al., 1993; White et al., 1994; Jobling et al., 1995). Principal sources of some of the environmental estrogen-like chemicals are listed in Table 1.

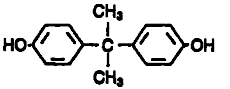
Natural estrogens play a major role in controlling reproduction in females and, to a lesser extent, in males. Additionally, physiological concentrations of estrogen are essential for the maintenance of cell growth and several other biological activities (Jensen, 1992). For example, normal physiological levels of estrogen are known to be involved in the control of cell proliferation, transcription, and DNA synthesis (Jensen, 1992). Besides estrogen-responsive organs (such as uterus, breast, and pituitary), estrogens exert their effect at multiple sites (kidney, liver, skeletal tissues, etc.) depending upon the species. Imbalance in the steady-state concentrations or pharmacological dose or concentration higher than the physiological level of estrogens is known to produce adverse effects (Hertz, 1985; IARC, 1979; Marselos & Tomatis, 1992a, 1992b). The source of extra or elevated concentration of estrogen could be both endogenous and exogenous. The potential for exposure of humans and animals to environmental estrogen-like chemi-



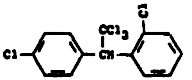
diethylstilbestrol



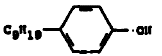
17 β-estradiol



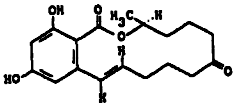
bisphenol A



o,p'-DDT



p-nonylphenol



Zearalenone

FIGURE 1. Structure of some of the natural and environmental estrogens.

TABLE 1. Partial list of the sources of environmental estrogen-like chemicals

Agricultural and industrial estrogen-like chemicals:
Pesticides: Kepone (chlordecone), methoxychlor, DDE, a metabolite of *o,p'*-DDT, endosulfan, toxaphene, dieldrin, dicofol, 1-hydroxychlordene
Polychlorinated industrial by-products: Some polychlorinated biphenyls (PCBs), polychlorinated dibenzodioxins, and chlorofluorocarbon
Alkylphenols: 6-bromo-naphthol-2, nonylphenol, 4-octylphenol, 4-nonylphenoxycarboxylic acid, bisphenol A
Polycyclic aromatic hydrocarbons: 3,9-dihydroxybenz[a]anthracene
Drugs: cimetidine, digitalis, diethylstilbestrol
Chemical constituents of oral contraceptives: 17α-ethinylestradiol, Equilenin, Mestranol
Natural and synthetic mycotoxins: zeranol, zearalenone
Microbial intestinal fauna: enterolactone
Plant estrogens: equol, diadzein, formononetin, biochanin A, genistein, coumesterol, giberellic acid

cals is high. There have been many reports indicating residues of PCBs, DDT, and other organochloride pesticides in human breast milk and adipose tissue (Kutz et al., 1991; Rogan et al., 1987). In addition, humans are exposed to estrogenic compounds present in vegetables, fruits, and meat on a regular basis (Li et al., 1985; Seychell, 1985; Markaverich et al., 1995). In addition to exogenous estrogen received as a constituent of oral contraceptives and postmenopausal therapy, the early onset of puberty, late age of menopause, each preovulatory surge, and an imbalance in aromatization of androstenedione may also provide an elevated level of natural estrogens and a longer exposure to them (Marshall, 1993). It has been known since the early 1950s that in animals elevated level of estrogens may act as xenobiotics, which produce both adverse reproductive effects (such as embryotoxicity and teratogenicity) and carcinogenicity (IARC, 1979; Marselos & Tomatis, 1992a, 1992b). However, recent epidemiological and laboratory findings have increased the growing concern that exposure to estrogenic chemicals in the environment might cause deleterious effects to both wildlife and humans (Bornstein et al., 1988; Pike & Spicer, 1992; Carlsen et al., 1992; Giwercman et al., 1993; Henderson et al., 1993; Kelce et al., 1995; Malone, 1993; Marshall, 1993; Poliner, 1993; Sharpe, 1995; Sharpe & Skakkeback, 1993; Stone, 1994; Wolff et al., 1993). For example, it has been reported that since 1938 sperm counts of men in the United States and 20 other countries have decreased by an average of 50% (Carlsen et al., 1992; Sharpe & Skakkeback, 1993). At the same time, testicular cancer has tripled. An increase in cryptorchidism (undescended testicles) and hypospadias (abnormal urethral opening) has also been reported (Giwercman et al., 1993). The incidence of endometriosis in the United States, Germany,

and other Western countries is increasing. For example, there were only 21 reported endometriosis cases in the world 70 years ago; today there are 5 million in the United States alone. Breast cancer incidence has been increasing approximately 1% per year over the past 50 yr (Feuer & Wun, 1992; Marshall, 1993; Poliner, 1993). American Caucasian women whose blood contained the most DDE (a metabolite of DDT) had four times the breast cancer risk of women who carried the least DDE (Wolff et al., 1993). A second epidemiological study also observed a positive association (although not statistically significant) between DDT and breast cancer risk in Caucasian and African-American women (Krieger et al., 1994). There is increasing evidence that exposure to PCB compounds may disrupt reproductive and endocrine function in fish, birds, and mammals, particularly during development (Birnbaum, 1994; Colborn et al., 1993; Bergeron et al., 1994). It is suspected that these conditions could be associated with elevated exposure to estrogen-like chemicals, either prenatally or during early postnatal life (Birnbaum, 1994; Davis et al., 1993; McLachlan, 1993). Adverse effects of estrogen-like chemicals have been extensively reviewed (Birnbaum, 1994; Colborn et al., 1993; Davis et al., 1993; Safe, 1995) and are not discussed in detail here. However, some examples of adverse effects of environmental estrogen-like chemicals are discussed later.

GENERAL TOXICITY/REPRODUCTIVE HAZARDS AND TERATOGENICITY/CARCINOGENICITY

DES is one of the environmental estrogens whose adverse effects to both animals and humans are widely reported (McLachlan et al., 1977; Bern, 1992; IARC, 1979; Marselos & Tomatis, 1992a, 1992b). Although general use of DES is banned, it is still used for advanced prostate cancer treatment, as a morning-after pill in emergency situations such as postcoital conception, and in cattle feeds as a growth promoter. DES is the only environmental estrogen for which transplacental carcinogenic action in both humans and animals has been proven (IARC, 1979). Adverse effects with DES exposure in both animals and humans have recently been reviewed (Marselos & Tomatis, 1992a, 1992b). In animals, estrogens (both natural and synthetic) act as both initiators and promoters of various types of cancers, depending upon their nature, strain, and species of animal (IARC, 1979). For example, DES causes liver cancer in Sprague-Dawley rats (Williams et al., 1993), zearalenone causes liver toxicity in Armenian hamsters (Coe et al., 1990), and estrone causes breast tumor in Noble rats (Cutts & Noble, 1964; Cutts, 1964). Synthetic (diethylstilbestrol) and natural (estradiol and estrone) estrogens have been implicated to induce bladder, ovarian, testicular, lymphatic, uterine, and prostatic tumors in mice

and rats; ovarian and mammary tumors in dogs; endometrial carcinoma in rabbits; and kidney and uterine tumors in hamsters (IARC, 1979; Ofner et al., 1992). Ongoing follow-up epidemiological studies appear to suggest a slightly increased risk of breast cancer in the population exposed to DES (Cody, 1991; Malone, 1993). Recently, exposure to environmental estrogen (DDE, a metabolite of DDT) has been implicated to be involved in the development of breast cancer in humans (Wolff et al., 1993; Krieger et al., 1994). There is also a growing concern that estrogenic compounds may be involved in the development of some disorders in male reproductive system function, including increased occurrence of prostatic and testicular cancers (Davis et al., 1993; Giwercman et al., 1993; Hunter & Kelsey, 1993; Santii et al., 1994; Sharpe, 1995).

Bisphenol A (BPA) is another example of an environmental estrogen-like chemical (NIOSH, 1985; Colborn et al., 1993; Krishnan et al., 1993). It is used in the manufacture of epoxy, polycarbonate, and corrosion-resistant unsaturated polyester-styrene resins required for food packaging materials in industrial processing (Knaak & Sullivan, 1966; National Toxicology Program, 1982; NIOSH, 1985, 1987). Leaching of bisphenol A from plastic products has been documented (Krishnan et al., 1993; Brotons et al., 1995). In humans absorption of BPA through the skin has been shown to produce extensive damage to kidneys, liver, spleen, pancreas, and lungs (Sax, 1975). High incidence of multinucleated hepatocellular giant cells was observed in male mice treated with both low and high doses of BPA (National Toxicology Program, 1982). The exposure of rats and mice to BPA also has been reported to produce adverse reproductive effects (Bond et al., 1980; Hardin et al., 1981; Morrissey et al., 1987; Atkinson, 1995). There is also an association between BPA exposure and cancer of the hemopoietic system in F344 rats and $B_6C_3F_1$ mice (National Toxicology Program, 1982; Ashby & Tennant, 1988).

A number of estrogenic alkylphenolic compounds (e.g., nonylphenol, 4-octylphenol, nonylphenoxycarboxylic acid), by-products of a variety of commercial products found in river and drinking water, are toxic to aquatic organisms (White et al., 1994). Reproductive impairment in marine mammals has been directly attributed to feeding on PCB-contaminated fishes (Reijnders, 1986). It has been recently observed that estrogenic effects of some PCBs can reverse the gonadal sex in reptile species that exhibit temperature-dependent sex determination (Bergeron et al., 1994). Neonatal exposure of mice to plant estrogens, coumestrol or zearalenone, results in reproductive abnormalities paralleling those seen after exposure to DES and steroidal estrogens (Burroughs et al., 1990a, 1990b; Williams et al., 1989). Reproductive effects such as oligospermia and sterility are observed in the workers exposed to the estrogenic pesticide chlordecone (Cohn et al., 1978). Exposure to envi-

ronmental estrogenic chemicals leading to precocious sexual development has also been previously reported (Perez-Comas, 1982).

The role of environmental estrogen-like chemicals in the etiology of some of the human cancers and reproductive health hazards has been implicated, although the linkage between these two processes is highly controversial (Safe, 1995). However, there is general agreement that human populations are continually exposed to a wide variety of environmental estrogen-like chemicals, and therefore it is important to understand the effects of environmental estrogen-like chemicals at the cellular level. Therefore, this review article critically evaluates the current status of knowledge of biochemical and molecular changes at the cellular level in response to the exposure of estrogen-like chemicals, which might help in understanding the mechanisms of some of the adverse effects. Estrogen-like chemicals are unique compared to non-estrogenic xenobiotics, because in addition to their chemical properties, the estrogenic property of these compounds allows them to act like sex hormones. Whether weak or strong, the estrogenic response of a chemical, if not overcome, will add additional estrogenic burden to the system. The adverse effect of this extra estrogenic pressure will depend upon the time, exposure, strain, and species. Additionally, some of the environmental estrogen-like chemicals have recently been shown to possess antiandrogenic effects (Kelce et al., 1995; Kelce, unpublished). However, this raises the question of how these chemicals can act simultaneously as agonists and antagonists for both estrogen and androgen actions. Only a limited number of estrogen-like compounds, such as DES, bisphenol A, nonylphenol, PCBs, and DDT, have been used to assess the biochemical and molecular changes at the cellular level. Among them, DES is the most extensively studied estrogen-like chemical, and therefore this review mainly focuses on DES-related observations.

CHANGES AT CELLULAR LEVELS ASSOCIATED WITH EXPOSURE TO DES

DES has attracted the interest of many researchers for the last 50 years, first as a promising new drug for medicinal and veterinary use, later for its carcinogenic/teratogenic effects, and now as a model compound for environmental estrogen-like chemicals. Most of the literature using stilbene estrogen is available for the Syrian hamster renal cancer and reproductive tract cancer in mice. We decided to utilize the data pertaining to these models to critically evaluate the mechanisms of adverse effects produced by environmental estrogen-like chemicals. Despite intensive efforts from several laboratories over the last half century, how stilbene estrogen (DES) and/or DES metabolites convert normal cells into transformed cells remains unknown. How DES con-

trols the proliferation of normal or transformed cells is not clear. Why tumors specifically develop in a particular organ of an animal species [such as kidney of Syrian hamsters (Kirkman & Bacon, 1952), mammary gland of Noble rat (Cutts & Noble, 1964; Cutts, 1964), reproductive tract of mice (Newbold et al., 1990; Bern, 1992), or liver of Sprague-Dawley rats and Armenian hamsters (Coe et al., 1990; Williams et al., 1993)] and not in other organs, in response to DES exposure, is also not clear.

Imbalance in Estrogenicity

It is logical to think that an increase in the hormonal activity by exogenous administration of estrogen may be involved in causing adverse effects. There are some evidence in support of this concept (Li et al., 1979, Bern, 1992; Cortes-Vizcaino & Llombart-Bosch, 1993). Contrary to this hypothesis, the data obtained from various laboratories show that hormonal potency alone can not be correlated with tumor incidence (Li & Li, 1984, 1987; Liehr, 1983; Liehr et al., 1992b) For instance, 17α-ethinylestradiol and 2-fluoroestradiol have an affinity for the estrogen receptor equal to that of DES and 17β-estradiol and produce a full estrogenic response as demonstrated by the induction of both the progesterone receptor and serum prolactin levels. However, both 17α-ethinylestradiol and 2-fluoroestradiol are weak carcinogens. While DES produces 100% kidney tumor incidence in hamsters, ethinylestradiol produces only 21% kidney tumor incidence, and 2-fluoroestradiol produces no kidney tumors (Li & Li, 1987; Liehr, 1983). Lack of effectiveness of the antiestrogens RU 39 411 or keoxifen in the prevention of estrogen-induced tumors in Syrian hamster has been observed (Liehr et al., 1992b). Recently, an estrogenic metabolite of DDT, *p,p'*-DDE, has been shown to inhibit androgen binding to the androgen receptor, androgen-induced transcriptional activity, and influence of androgen in developing, pubertal, and adult rats (Kelce et al., 1995). It is possible that DDE and related estrogen-like environmental chemicals may also produce abnormalities in male sex development through modulating the regulation of androgen receptor. These findings suggested that in addition to the estrogenic activity of environmental estrogen-like chemicals, other factor(s) may also be involved in the induction of environmental estrogen-induced carcinogenesis in hamster.

Metabolism as a Mechanism of Adverse Reactions

In the early 1980s, it was postulated that metabolism of stilbene estrogen may play a role in the induction of tumors in kidney (Metzler & McLachlan, 1978), and there have been extensive studies to investigate the role of DES metabolism in carcinogenesis (reviewed by Metzler, 1984; Li & Li, 1984, 1987; Liehr & Roy, 1990). Recent reports also have strongly implicated the role of estrone metabolism in

the etiology of breast cancer (Yu & Fishman, 1985; Swaneck & Fishman, 1988; Davis et al., 1993). Changes in estrone metabolism have been shown to substantially reduce incidence, size, and multiplicity of spontaneous tumors in mice (Michnovicz & Bradlow, 1991, 1992). Increased rate of hydroxylation of estrone at the 16α position has been suggested to play a role in the development of breast cancer and systemic lupus erythematosus (Swaneck & Fishman, 1988). Before we discuss further the role of metabolism, it is imperative to discuss in brief DES metabolism.

We and others have shown that both catechol and stilbene estrogens undergo microsomal cytochromes P-450-mediated redox cycling reactions (Liehr et al., 1986, 1990; Roy & Liehr, 1990; Roy et al., 1991a, 1991b, 1992) resulting in the formation of reactive metabolites. Microsomal or peroxidative DES metabolism has been extensively reviewed (Liehr & Roy, 1990; Metzler, 1984; Li & Li, 1987). Several reports exist concerning the binding of stilbene and steroidal estrogens to microsomal proteins (Metzler, 1984; Li & Li, 1990; Adams & Notides, 1986; Brueggmeier et al., 1984). However, correlation between tumor incidence and covalent binding by DES reactive metabolites to proteins has yet to be demonstrated.

Considering the unstable nature of estrogen reactive intermediates and the presence of abundant amounts of nucleophilic molecules capable of scavenging reactive intermediates in the main organelle of metabolic activation (endoplasmic reticulum) and in the cytoplasm, it seems unlikely that estrogen genotoxic metabolites will traverse the distance from the endoplasmic reticulum to the nucleus or mitochondrion, the sites of genotoxicity. We have recently shown that nuclei are capable of catalyzing redox cycling of estrogens, and estrogen reactive metabolites generated during nuclear redox cycling covalently bind to nuclear DNA along with histone and nonhistone nuclear proteins (Roy & Thomas, 1994; Roy & Pathak, 1993, 1995; Roy et al., 1995). This provides support for the concept that in the cell, the metabolic activation of estrogens into genotoxic metabolites occurs in close proximity to the site of genotoxicity. In support of this concept, we also have recently demonstrated that mitoplasts (i.e., mitochondria without outer layer of membrane) are able to convert stilbene estrogen (diethylstilbestrol, DES) to reactive metabolites, which covalently bind to mtDNA (Thomas & Roy, 1995).

Genotoxicity and Cytogenetic Changes

Covalent binding of DES to hamster kidney nuclear DNA was first shown by Liehr and his associates (Gladeck & Liehr, 1989) and has been extensively studied in his laboratory (reviewed by Liehr, 1990). Recently, Williams and his associates have also shown the ability of DES to bind to rat hepatic nuclear DNA (Williams et al., 1993;

Montandon & Williams, 1994) and DES quinone can produce mutations (Korah & Humayun, 1993). We have recently shown that DES can also bind in vivo to mtDNA of liver (target organ of cancer) of Sprague-Dawley rats, and the adduct level generated in the mtDNA was 15 times higher than that of the nuclear DNA (Thomas & Roy, 1994a). In Syrian hamsters, the level of mtDNA-DES adducts in the target organ of cancer (kidney) was several fold higher than that of the nuclear DNA (Thomas & Roy, 1994b). Demonstration of the ability of the mitochondria to oxidize DES to reactive metabolites, which covalently attack mitochondrial DNA, suggests that DES reactive metabolites may produce genetic instability in mitochondria by producing mutational changes in mt genome.

In addition to the DNA binding property, DES exposure produces aneuploidy (Tsutsi et al., 1983; Eastmond & Pinkel, 1990; Ebert et al., 1990; De Sario et al., 1990; Banerjee et al., 1992; Aizu-Yokota et al., 1994). How DES or other estrogens produce aneuploidy in vivo is not clear. Metzler and his associates have shown using an in vitro system that DES and metabolites of DES alter polymerization/depolymerization of purified microtubular proteins (Metzler et al., 1992) and binding of DES and its metabolites to tubular proteins in an in vitro system (Metzler et al., 1992). Therefore, one may possibly think that DES and/or DES metabolites may interfere with spindle apparatus during mitosis, resulting in abnormal segregation of chromosomes. However, whether such events occur in vivo or are responsible for the development of aneuploid cells in vivo remains to be shown. Microtubule disruptive activities of some natural estrogens do not correlate with their hormonal carcinogenesis. Estrone is known to stimulate growth and produce tumorigenesis, but it has no effect on the microtubule network (Aizu-Yokota et al., 1994). 17α-Estradiol is noncarcinogenic and hormonally much less active (Li & Li, 1984, 1987); however, its microtubule disruption potential is equal to that of DES or 17β-estradiol (Aizu-Yokota et al., 1994). In addition to aneuploidy, other cytogenetic changes, including chromosomal aberrations, have been shown to be produced by DES and estradiol (Banerjee et al., 1994; Ho & Roy, 1994; Endo et al., 1994). Whether aneuploidy, binding of DES and its metabolites to DNA and spindle proteins, or chromosomal aberrations play a role in the development of cancer remains to be ascertained.

Free Radicals and DNA Damages

Free radical generation by redox cycling of estrogen has been reviewed by Liehr and Roy (1990) and therefore it is discussed briefly here. We were first to demonstrate that microsomal cytochromes P-450-catalyzed redox cycling of DES produces superoxide radicals (Roy & Liehr, 1988a), followed by reports showing changes in free radical detoxifying enzymes (Roy & Liehr, 1988a, 1988b, 1989a, 1989b). Recently, several new investigations supporting the formation of organic

free radicals and active oxygen species from diethylstilbestrol have been reported (Roy et al., 1991a, 1991b, 1991c; Kodama et al., 1993). Changes in free radical detoxifying enzymes (Segura-Aguilar et al., 1990; Oberley et al., 1994) and oxidative damages to DNA and lipids in response to DES treatment (Roy & Liehr, 1989a, 1989b; Roy et al., 1991a, 1991b, 1991c; Ho & Roy, 1994; Wang & Liehr, 1994; Li & Trush, 1994; Ni & Yager, 1994) have also been reported. Formation of free radicals from DES may explain some of the cytogenetic changes observed in response to DES treatment (Banerjee et al., 1992, 1994).

Growth Factors/Inhibitory Factors/Protooncogenes

The effects of 17β-estradiol on growth factors, growth factor receptors, or protooncogenes in vitro using cell culture of mammary or pituitary cancer cell or uterine tissue have been extensively reviewed (Imagawa et al., 1991). Acute exposure to natural estrogen, 17β-estradiol, augments cellular protooncogenes, growth factors, and growth factor receptors in a temporal pattern consistent with their involvement in cell proliferation (Lingham et al., 1988; DiAugustine et al., 1992; Hyder et al., 1992; McLachlan et al., 1992; Nelson et al., 1994). Induction of transforming growth factor-α (TGF-α) and basic fibroblast growth factor (bFGF)-like proteins in hamster kidney slices, of TGF-α, platelet-derived growth factor (PDGF) and its receptor in the reproductive tract of CD-1 mice, and of TGF-β in rat granulosa cells in response to DES exposure has been observed (Beleh et al., 1993; Gray et al., 1995; Mulheron & Schomberg, 1992). Alteration of epidermal growth factor receptor (EGF-R) expression in hamster kidney (Narayan & Roy, unpublished) and in mouse reproductive tract tissue in response to DES exposure has also been reported (Bern, 1992; Iguchi et al., 1993). Recently, we have shown the enhanced expression of both plasma membrane and nuclear insulin-like growth factor I (IGF-1) receptors and an increase in the IGF-1 mediated phosphorylation in DES-treated hamster kidney membranes as compared to that of age-matched controls (Narayan & Roy, 1992, 1993; Chen & Roy, 1995, 1996; Chen et al., 1996). The higher expression of c-fos, c-jun, and c-myc in hamster kidney tumor tissues and of c-myc and mdm-2 in murine uterine adenocarcinoma cells compared to that of control has been observed (Liehr et al., 1992a; Risinger et al., 1994; Nelson et al., 1994). Also, neonatal treatment of DES to mice has been shown to increase the expression of c-myc in the prostate (Pylkkanen et al., 1993). Whether any growth factor receptors or oncogenes are involved in the onset of tumorigenesis by DES remains to be shown.

Changes at the Nuclear Level Indicating Genomic Instability

Chemical Modifications in Nuclear Proteins While it is widely believed that unrepaired DNA damage by chemical carcinogens is likely the major cause of neoplastic transformation, covalent modification in

the nuclear proteins associated with the regulation of gene expression or transcription can also play an important role in this process (Roy, 1990). The fact that transcriptionally active chromatin proteins are susceptible to attack by DES reactive metabolites (Roy & Pathak, 1993, 1995) provides support to this concept. In an in vitro system, DES quinone, one of the metabolites of DES, binds to pure nonhistone proteins, RNA polymerase, and DNA polymerase. DES metabolites have also been shown to bind to nuclear proteins in vivo (Roy et al., 1995). The level of in vivo DES binding to nuclear proteins in the kidney (target organ of cancer) of Syrian hamster was twofold or more higher than that observed in the liver or testis (nontarget organs). We have recently observed that treatment of hamsters with a carcinogenic dose of DES capable of producing in vivo covalent modifications in nonhistone proteins inhibited transcriptional activity (Palangat & Roy, 1995a, 1995b). Based on these data we suggest that nuclear redox cycling of DES through covalent modification in macromolecules of nuclear components (a schematic diagram is shown in Figure 2) may be a factor in producing genetic instability in the cells.

Increased Tyrosine Phosphorylation of Nuclear Matrix Proteins by DES A carcinogenic dose of DES, shown to produce cell proliferation with acute treatment and to produce 90–100% cancer in kidney with chronic treatment (Kirkman & Bacon, 1952; Purewal & Roy, 1994), increased the activity of kidney nuclear matrix protein tyrosine kinases

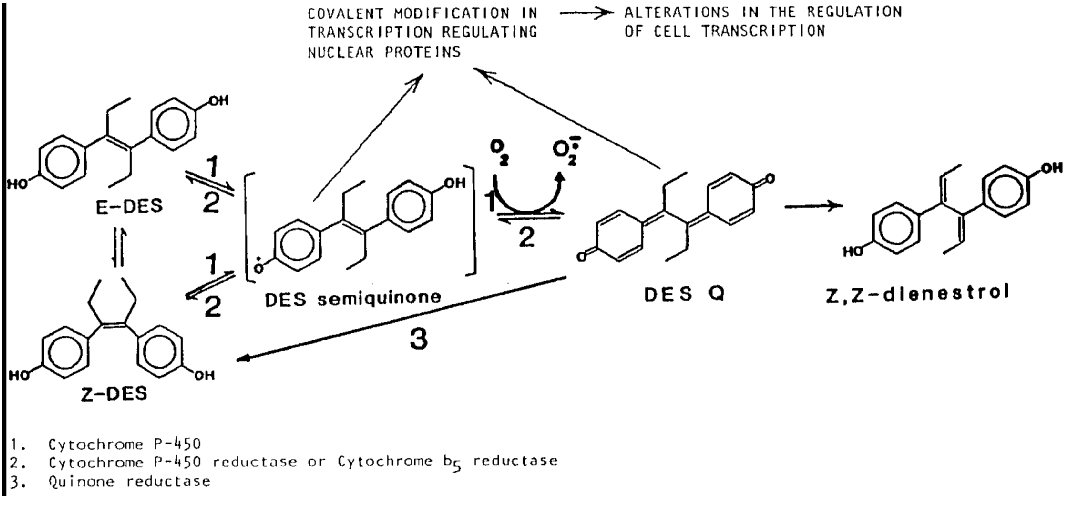


FIGURE 2. Nuclear redox cycling of diethylstilbestrol. Both *E*- and *Z*-DES are oxidized by cytochrome P-450 oxidase (1). The intermediacy of a DES semiquinone in this process is postulated but not demonstrated. The DES quinone is reduced via semiquinone by cytochrome P-450 reductase (2) or directly to *Z*-DES by quinone reductase (3). The DES quinone and semiquinone generated during nuclear redox cycling of DES are postulated to covalently bind to nuclear proteins and DNA.

by 2.5-fold over the control (Roy & Palangat, 1994; Palangat & Roy, 1994, 1995a, 1995b, 1996). Western blotting using the antibody of phosphotyrosine and of p53 revealed that tyrosine phosphorylation of p53 and several other phosphoproteins was increased in response to exposure to DES. Enhanced tyrosine phosphorylation of p53 by DES exposure coincides with that of DES-induced cell proliferation in the hamster kidney.

Transcriptional Defects We have recently observed the differential effect of exposure to a carcinogenic dose of DES on organelle transcriptional activity in nuclei isolated from hamster kidney (target organ of cancer) and liver (nontarget organ) (Palangat & Roy, 1995a). The inhibition of endogenous template transcription by in vivo treatment with DES seems to be due to alterations in chromatin template and transcription-regulating proteins. This conclusion is based on the findings that (1) both Western and Northern blotting experiments revealed that total RNA pol II amount or transcripts of RNA pol II was not decreased by DES treatment, (2) the phosphorylation of tyrosine residues of RNA pol II was decreased by 60% in response to DES exposure, and (3) an inhibitory effect of DES on the total RNA polymerase activity was observed in vitro, which is in agreement with the previous report (Oda et al., 1991).

DNA Repair Defects Despite rigorous attempts and screening of a large number of genes, defect(s) in any particular gene in DES-induced tumor samples from humans were not detected (Stone, 1993). Inhibition of DNA polymerase I activity by DES exposure has recently been reported (Oda et al., 1993). Our more recent studies revealed that DNA repair enzyme DNA pol β mRNA obtained from DES-induced kidney tumors has several mutations in the catalytic domain compared to that of age-matched control kidney (Yan & Roy, 1994, 1995; Roy et al., 1996). Types of mutational changes include base substitution, insertion, and frameshift. Aberrant splicing in a gene may cause insertions and deletions in mRNA. Alternatively, these mutations in DNA pol β can be caused by chromosomal rearrangements, since DES-treated hamster kidneys contain chromosomal aberrations, including deletions, inversions, and translocation (Banerjee et al., 1992, 1994). Furthermore, analyses of the expression of DNA pol β by Northern blotting and RT-PCR revealed that the transcripts of DNA pol β were seven times lower in DES-induced hamster kidney tumor tissues compared to that of control tissues (Yan & Roy, 1994, 1995; Roy et al., 1994). Based on these data, it is important to note that mutations in DNA pol β occurred in the catalytic domain and not in the DNA binding domain. Mutations in the mRNA might generate an impaired DNA pol β, which may be weak in DNA repair, incapable of repairing the lesions, or more prone to committing mistakes during DNA repair than normal DNA pol β. Defects in the DNA pol β-catalyzed

DNA repair system might lead to genetic instability through an increase in replication errors or may allow the accumulation of mutations due to impaired catalytic activity incapable of repairing the lesion. An impaired DNA repair system may specifically allow the accumulation of mutations in protooncogenes, tumor suppressor genes, or other cancer-associated genes, or may cause genetic instability. Mutations coupled with decrease in the expression of DNA pol β mRNA in DES-treated kidney compared to that of age-matched control suggest that the altered DNA pol β may play a key role in the development of DES-induced cancer.

Increased Rate of Cell Proliferation in the Target Organ of Carcinogenesis Syrian hamsters exposed to DES (22 mg, sc) for short periods, that is, 0, 5, 8, 15, and 30 d, revealed an increase (4.7-fold) in total proliferation in the epithelial cells of proximal tubules as compared with controls at d 8 of DES exposure (Purewal & Roy, 1994). The time window of increased cell proliferation coincides with that of DES-induced changes at the nuclear level (Roy et al., 1991c; Palangat & Roy, 1994, 1995a, 1995b). An increased level of cell proliferation of renal and hepatic cells in response to exposure to DES or other synthetic estrogens has also been reported (Oberley et al., 1989; Li et al., 1993, 1995; Ni & Yager, 1994). Recently, we have observed a second burst of focal increase in cell proliferation of epithelial cells of hamster renal cortex after 8 d of a second DES implant received on d 30 of treatment (Palangat & Roy, 1996). Focal increase in cell proliferation appears to be confined to the areas of renal cortex having undifferentiated cells, because we found tubule formation in the area of focal proliferation. DES-induced increase in cell proliferation may allow fixation of genetic instability.

Changes at Cellular Levels Associated with Exposure to Alkylphenols

Alkylphenols, such as bisphenol A, nonylphenol, and octylphenol, are widely used as plastic additives, surfactants, in industrial detergents, and in other formulated products such as paints, herbicides, and pesticides throughout the world (Soto et al., 1991, 1992; White et al., 1994). Nonylphenol and bisphenol A have been shown to leach from plastic used in food processing and wrapping (Gilbert et al., 1992; Krishnan et al., 1993). Recently, bisphenol A has also been reported to leach from lacquer coating in food cans (Brotons et al., 1995). The plastic monomer bisphenol A was found as a contaminant not only in the liquid of the preserved vegetables, but also in water autoclaved in these lacquer-coated cans. The amounts of bisphenol A ranged from 0 to 33 μg per can (Brotons et al., 1995). Presently, there is no direct evidence that the estrogenic activity possessed by this group of chemicals might be responsible for any deleterious effect in any species. However, the widespread use of alkylphenols and the persistence of their degradation products in the environment coupled with the con-

cern about inadvertent exposure of humans and wildlife to "estrogens" raises considerable disquiet. The relatively low estrogenic activity of alkylphenols does not rule out their potential toxicity after chronic exposure to animals or humans. Fish in the Detroit River's Trenton channel, near a chemical plant manufacturing alkylphenols, were reported to contain 40 μg *p-tert*-pentylphenol per gram of fat tissue, a concentration higher than that found in the river sediment (Shiraishi et al., 1989). The *p-tert*-pentylphenol concentration in carp adipose tissue was comparable to the alkylphenol concentration eliciting maximal cell proliferation in MCF-7 cells. This compound causes vaginal cornification in ovariectomized rats (Dodds & Lawson, 1938) and interacts with estrogen receptors (Mueller & Kim, 1978). The bioaccumulative properties of alkylphenols (Shiraishi et al., 1989) parallel those of chlordecone, which is sequestered in liver and adipose tissue, eliciting considerable estrogenic activity in spite of its low potency when compared to E_2 (Hammond et al., 1979; Egle et al., 1978). Reproductive effects such as oligospermia and sterility were reported in workers exposed to chlordecone (Cohn et al., 1978). Estrogenic effects of alkylphenol and chlordecone in MCF-7 cells occur at comparable doses (Soto et al., 1991). Based on bisphenol A or nonylphenol's weak estrogenic activity, a calculated theoretical dose of the order 10^5- to 10^6-fold higher of bisphenol A or nonylphenol will be required to produce the same biological effects as DES (0.1 mg/kg/d). According to the weak estrogenic concept, exposure to 0.1 mg/kg/d dose of nonylphenol or bisphenol A should not produce any biological effects in the mammary gland of animals. In contrast, in our recent studies a comparison of the proliferative activity of DES to those of an equivalent dose of nonylphenol or bisphenol A (0.1 mg/kg/d) revealed that even though bisphenol A and nonylphenol are weakly estrogenic and 10^5 to 10^6 less estrogenic than DES, their influence on proliferative activity in the epithelial cells of mammary gland is only one-third less than that of DES (Colerangle & Roy, 1995c, 1996a, 1996c). The 0.1 mg/kg/d dose of bisphenol is 30-fold lower than that of a specific migration limit of bisphenol A in food set by the European Union Commission (3 mg/kg) (Brotons et al., 1995), and only twofold higher than the reference dose (Rfd) set by the U.S. EPA (1995; 0.05 mg/kg/d). The weak estrogenic activity of bisphenol A does not explain its profound effects. A recent report also indicated a substantially higher activity for related alkylphenols in vivo than prior reports in vitro and was much more active relative to DES when measured in vivo (Nagel et al., 1995). These findings suggest that the exposure of alkylphenols may pose hazards to humans and other animals when target cells in situ become exposed to these levels of alkylphenols. In addition to their estrogenic activity, there is very limited information available on adverse effects at the cellular levels by these chemicals.

Metabolism and DNA Binding Property of Bisphenol A

Previous studies using sister chromatid exchange and *Salmonella* mutation assays suggested that bisphenol A (BPA) is a nongenotoxic chemical (Ivett et al., 1989; Ashby & Tennant, 1988). We have recently shown that BPA is chemically converted to bisphenol *o*-quinone. Bisphenol *o*-quinone or bisphenol A in the presence of peroxidase activation system is able to covalently bind to DNA (Atkinson & Roy, 1995a). Using the ^{32}P-postlabeling technique, we have also investigated covalent modifications in DNA caused by in vitro or in vivo exposure to BPA (Atkinson & Roy, 1995b; Atkinson, 1995). Administration of a single or multiple dose of 200 mg/kg of bisphenol A to CD1 male rats produced two major and several minor adducts in DNA of liver, kidney, and testis. DNA binding is inhibited by the inhibitors of cytochromes P-450. One of the DNA binding metabolite(s), both in vitro and in vivo, is bisphenol *o*-quinone (a scheme of bisphenol A metabolism is shown in Figure 3). These data suggest that estrogenicity coupled with genotoxicity of bisphenol A may be factors for the induction of adverse effects.

Perturbations of Cell Cycle by Bisphenol A and Nonylphenol A

Epidemiologists have been advocating the concept that estrogen-mediated increase in cell proliferation leading to disturbances in cell cycle may provide a necessary environment for the development of some cancers, particularly breast cancer in human (Preston & Martin et al., 1990; Pike & Spicer, 1992). If environmental estrogen-like

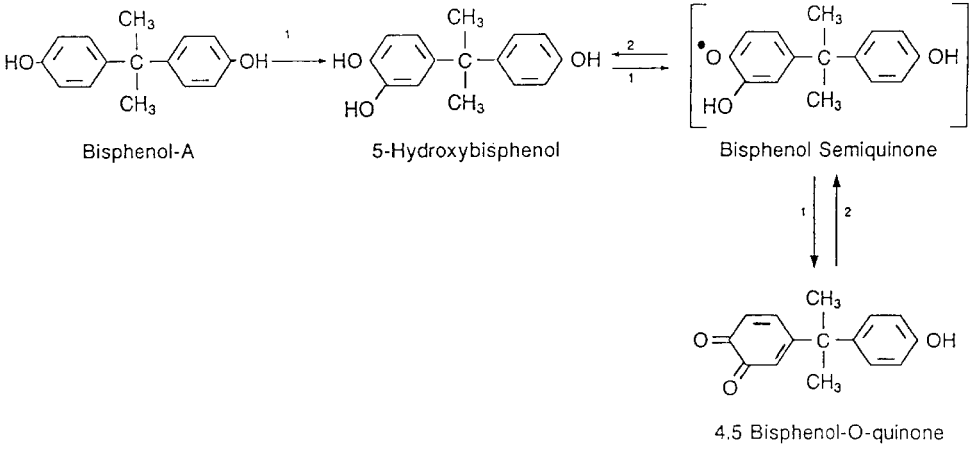


FIGURE 3. Redox cycling of bisphenol A. Bisphenol A is oxidized by cytochrome P-450 oxidase or peroxidase (1). The intermediacy of a bisphenol semiquinone in this process is postulated but not demonstrated. The bisphenol quinone is reduced via semiquinone by cytochrome P-450 reductase (2) to catechol bisphenol A. The bisphenol quinone and semiquinone generated during redox cycling are postulated to covalently bind to DNA.

chemical is a factor in the induction of cancer through alteration in cell cycle, then it is imperative to demonstrate that environmental estrogen exposure is able to alter cell cycle kinetics of the normal epithelial cells of the target organ. Environmental and natural estrogens have been shown to produce mitogenic effects in mammary cancer cells (Soto et al., 1992, 1994). However, estrogens fail to stimulate cell proliferation of normal mice mammary epithelial cells in vitro (Imagawa et al., 1991). There has been no detailed examination of cell cycle kinetics or quantitative changes in the morphology of individual mammary gland components in response to a carcinogenic dose of natural or environmental estrogen. Our recent study revealed that the exposure of female Noble rats to estrone, stilbene estrogen (DES), bisphenol A, or nonylphenol produced premature physiological aging of the mammary gland, which was evident from the presence of lobular structures (particularly lobule type 3) normally seen in the very aged rats and lactational-type lobules filled with secretory fluid, found in the mammary gland of pregnant rats (Holland & Roy, 1995, 1996; Colerangle & Roy, 1995a, 1995b). These findings suggest that physiological aging, not chronological aging, of the mammary gland of rats has been rapidly accelerated in response to exposure for a very short period to environmental estrogen. Additionally, a severalfold increase in cell numbers due to alterations in cell proliferation and in cell cycle kinetics in response to environmental estrogen exposure in the epithelial cells of the mammary gland compared to that of control reflected the exuberant growth in the mammary gland. The labeling index, growth fraction, and potential doubling time were altered in response to a single exposure of estrone, DES, bisphenol A, and nonylphenol to Noble rats. The maximum effect at the labeling index, growth fraction, and potential doubling time was produced by estrone, followed by DES, nonylphenol, and bisphenol A (Colerangle & Roy, 1995c, 1996c). Based on the proliferative activity and perturbation in cell cycle, we predict that the order of potential of developing genomic instability and mammary tumors will be estrone > DES > nonylphenol > bisphenol A; however, this remains to be tested. The exact mechanism by which nonylphenol might have induced cell proliferation and altered cell cycle is not clear. Alkylphenols over a range with an alkyl group in the *para* (or fourth) position on the phenol ring were able to stimulate the growth of MCF-7 cells (Soto et al., 1992). The alkylphenols are mitogenic, presumably as a result of their ability to bind to estrogen receptors (Soto et al., 1992; White et al., 1994). Alkylphenols have been shown to be estrogenic in fish, avian, and mammalian cells and mimic the effects of 17β-estradiol by binding to the estrogen receptor (White et al., 1994). The actions of alkylphenols were inhibited by estrogen antagonists (White et al., 1994). Moreover, the observation that the mutant receptor G-525R,

which is defective in estrogen binding (Danielian et al., 1993), is also insensitive to alkylphenols suggests that alkylphenols interact with a similar region of the hormone-binding domain as does 17β-estradiol. Inspite of the low binding activity of alkylphenols, it is striking that alkylphenols are able to stimulate a number of biological responses, such as a massive burst of proliferative activity as observed in our laboratory (Colerangle & Roy, 1995a, 1995b, 1995c, 1996a, 1996c) and specific gene transcription to the same extent as 17β-estradiol itself (White et al., 1994). These findings suggest that both transcriptional activation functions, TAF-1 and TAF-2, are functional when an alkylphenol is bound to the receptor. This is supported by the previously reported observation comparing the activity of the wild-type receptor with that of the deletion mutant MOR 121-599 (White et al., 1994). In chicken embryo fibroblast (CEF) cells, the TAF-2 activity exhibited by the deletion mutant receptor was induced as well by an alkylphenol as by 17β-estradiol, and maximum transcriptional activation by the wild-type receptor, which depends on TAF-1, was also induced similarly by either ligand (White et al., 1994). It seems remarkable that a molecule as structurally different from 17β-estradiol as alkylphenol is able to mimic the action of the natural hormone in inducing full transcriptional activity of the receptor (White et al., 1994). Estrogen-like chemicals have previously been shown to influence ovarian and pituitary hormone release (Safe, 1995); therefore, we can not exclude the possibility of the involvement of both peptide and steroid hormones in alkylphenol-induced proliferative activity in the mammary gland. Also, we do not rule out the possibility of involvement of other pathway(s) such as growth factors and growth-regulating protooncogenes for the effects observed in the mammary gland in response to alkylphenol exposure. Since we know that indeed a single exposure to environmental estrogen can perturb the cell cycle, now we will seek to understand whether perturbations of cell cycle would predispose the rat mammary gland to genetic instability.

Alkylphenols and Genomic Instability

Increased cell proliferation and alteration in cell cycle kinetics are considered important factors for the development of genetic instability (Preston-Martin et al., 1990). For example, cell proliferation may allow mitotic recombination to occur, which may result in more profound changes than those of a single mutation (Preston-Martin et al., 1990). Damage to DNA, such as incorporation of a wrong nucleotide, can occur during DNA synthesis. Damaged DNA is considered to be repaired during the G1 phase of the cell cycle. Some alkylphenols can directly produce DNA damage (Atkinson & Roy, 1995a, 1995b; Banerjee & Roy, 1996). As discussed earlier, the massive burst in the synthesis phase of the cell cycle in response to exposure to nonylphe-

nol or bisphenol A coupled with a decrease in DNA repair may allow DNA damage, acquired during synthesis, to accumulate and fix the genetic instability. These events may produce genetic instability, which could be a factor in the development of adverse effects in the mammary gland. It remains to be confirmed, however, that any one of these individual events occurs in direct response to environmental estrogen-like chemical exposure. Recently, we determined the effects of exposure of MCF-7 breast cancer cells to environmental estrogen-like chemicals (diethylstilbestrol, bisphenol A, nonylphenol) on telomeric associations. Telomere-deficient chromosomes show telomere–telomere associations. Most cancer cells exhibit telomeric associations. The telomeric associations have been implicated to contribute instability in the genome, presumably by facilitating homozygosity, translocation, amplification, and other rearrangements. Exposure of MCF-7 cells to DES, bisphenol A, or nonylphenol induced a dose-dependent increase in telomeric associations and chromatid breaks (Banerjee & Roy, 1996). Whether these effects are the result of a direct interaction of these chemicals at chromosome level or an indirect effect through interaction with nuclear proteins remains to be examined. We also have recently determined the length of the telomere in the mammary gland of Noble rats exposed to estrone or DES. A significant reduction in telomere length was observed in response to exposure of DES and estrone (Colerangle & Roy, 1996b). The major function of the telomere is to provide stability to chromosomes and protect them from illegitimate recombination. Thus, these findings suggest that exposure of cells to environmental estrogen-like chemicals may potentially be involved in the induction of instability in the genome through telomeric associations and/or reduction in telomeric length. Studies of perturbation in cell cycle coupled with genomic instability would provide insights as to how environmental or ovarian estrogen may produce adverse effects to human health and wild life.

SUMMARY

In addition to their estrogenic effect, environmental estrogen-like chemicals may produce multiple and multitype genetic and/or non-genetic hits. For example, we have shown several changes in the nuclei of target organ for carcinogenesis from animals exposed to stilbene estrogen (DES): Products of nuclear redox reactions of DES modify transcription-regulating proteins and DNA; transcription is inhibited; tyrosine phosphorylation of nuclear proteins, including RNA pol II, p53, and nuclear IGF-IR, is altered; DNA repair gene transcripts are decreased and mutated; telomeric length is attenuated; etc. Thus, stilbene estrogen produces multiple types of attack on the nuclei. The role of any individual attack leading to a particular change is not

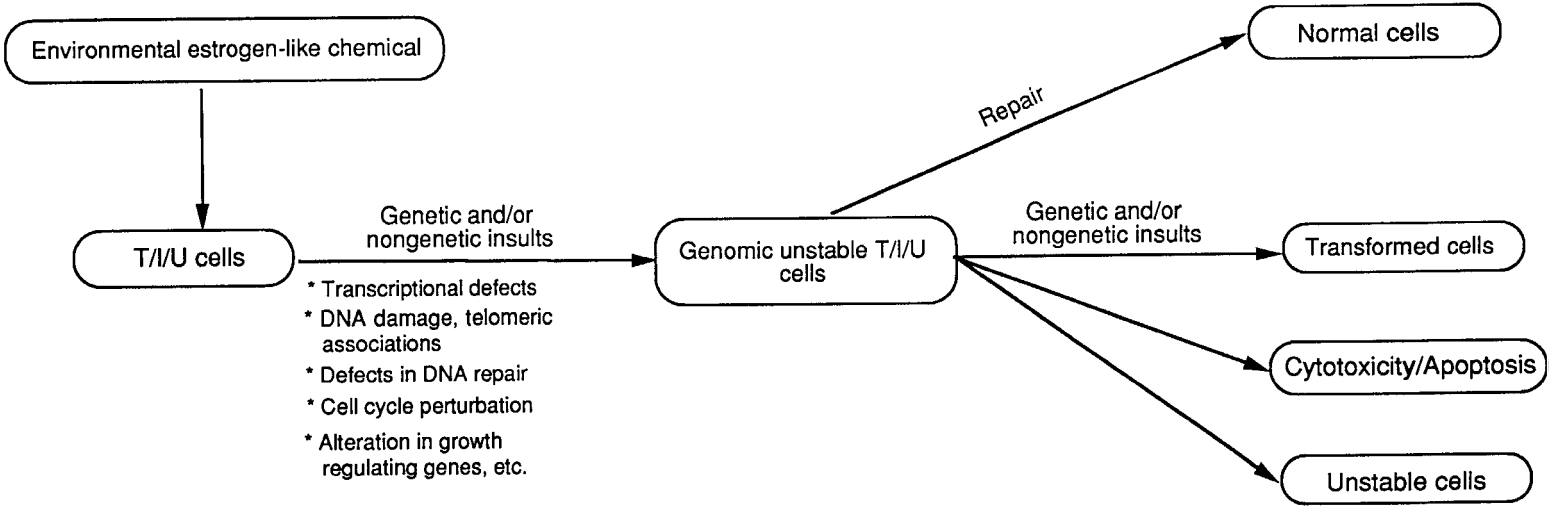


FIGURE 4. Scheme of possible mechanisms of adverse effects associated with exposure of environmental estrogen-like chemicals. T, differentiated cells; I, intermediate cells; U, undifferentiated cells.

clear at this stage. Consequences of these multitype attacks on the nuclei of cells could be nuclear toxicity/cell death, repair of all the hits and then acting as normal cells, or sustaining most of the hits and acting as unstable cells. Proliferation of the last type of cell is expected to result in transformed cells (a schematic representation is shown in Figure 4). It appears that some other estrogenic compounds such as bisphenol A and nonylphenol may also follow some of the pattern of effects similar to DES, because we have recently shown that these compounds alter cell cycle kinetics and produce telomeric associations and chromosomal aberrations. However, it should be noted that a particular type or multitype of hit(s) will depend upon the nature of the environmental estrogen-like chemical.

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