Developmental Effects of Dioxins

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The potent developmental toxicity of dioxin in multiple species has been known for a number of years. However, recent studies have indicated that dioxin also induces functional developmental defects, many of which are delayed. Subtle structural deficits, not detectable at birth, have also been described in multiple species and in both sexes. Certain defects have been reported not only in animals but also in children prenatally exposed to complex mixtures containing dioxinlike compounds. None of the effects can be attributed to modulation of any one endocrine system. For example, dioxin does not bind to the estrogen receptor, but it can cause effects that are both estrogenic and antiestrogenic. However, viewing dioxin and related compounds as endocrine disruptors that may alter multiple pathways sheds some light on the complexities of this potent class of growth dysregulators. — Environ Health Perspect 103(Suppl 7):89–94 (1995)

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Introduction

The dioxins represent a family of ubiquitous environmental pollutants (*1,2*). The prototype of this class of chemicals is 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD, "dioxin"), which is often called the most toxic man-made chemical. Other polyhalogenated congeners with bromine and/or chlorine in the four lateral positions also possess dioxinlike activity as do structurally related members of the polyhalogenated dibenzofurans, biphenyls, napthalenes, and azo-, and azoxy-benzenes (*3*). Of course, the majority of congeners within these classes do not have dioxinlike activity but may display their own spectrum of effects (*4*).

Dioxin and related chemicals cause a wide variety of responses ranging from frankly adverse effects, such as lethality, to biochemical changes, such as induction of drug-metabolizing enzymes. There are suggestions of sex and age differences in susceptibility to the lethal effects of dioxin, but these parameters have not been carefully examined. However, it is clear that the embryo/fetus of all species studied to date display similar sensitivity to dioxin-induced lethality. Death in the adult is usually preceded by severe body-weight loss known as the wasting syndrome.

Atrophy of the lymphoid tissue is a consistent result of exposure to relatively high doses of TCDD and related chemicals. In addition, the gonads of the adult display atrophy following high-dose exposure. Testicular atrophy has been demonstrated in rats (*5*), and recent studies have shown ovarian atrophy in the same species (K Rozman and A Cummings, personal communications). While the effects in these tissues may involve a hypoplastic response, hyperplasia occurs in the liver, gastric mucosa, and urinary tract. Changes in both proliferation and differentiation in the skin lead to chloracne, which is often used diagnostically for high-level dioxin poisoning. Alterations in cell proliferation and differentiation may underlie the immunotoxic, teratogenic, and carcinogenic response to this class of chemicals as well.

The biochemical effects of dioxin can be broken down into three types of responses involving enzymes, growth factors, and hormones. Induction of drug-metabolizing enzymes alters the biotransformation capacity and products of endogenous compounds, such as steroids, and xenobiotics. Changes in the levels of enzymes involved in proliferation and differentiation, as well as various pathways of intermediary metabolism, alter the biochemical state of the cell. Modulation of multiple growth factors [e.g., epidermal growth factor (EGF), transforming growth factor α (TGFα), transforming growth factor β (TGFβ)] and their receptors (e.g., EGF receptor), as well as cytokines (e.g., IL-1β) and protooncogene (e.g., c-*fos*, c-*jun*, c-*ras*) expression, has also been demonstrated following dioxin exposure, which leads to alterations in growth and differentiation.

Changes in both steroid and peptide hormones have also been shown to result from dioxin treatment. Levels of steroid hormones such as androgens have been shown to be lowered following dioxin exposure, probably due to changes in steroid-metabolizing enzymes (*6*). Estrogen receptor levels are decreased following dioxin exposure, although this is not a particularly sensitive response except in immature animals (*7*). Changes in the glucocorticoid receptor appear to be tissue specific. Thyroid hormones also appear to respond to dioxin treatment, although the response may be both dose and time dependent. Melatonin levels in the blood appear to decrease in response to TCDD (*8*). This is intriguing in light of the sequence similarity between the dioxin receptor and *per* (*9*), a Drosophila gene involved in the control of circadian rhythms. Peptide hormones (e.g., gastrin, insulin) and their receptors are also altered by dioxin treatment. Overall, dioxin modulation of hormones and their receptors leads to altered homeostasis. Viewing dioxin as an endocrine disruptor makes the tissue-specific,

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Abbreviations used: TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; dioxins, all approximate isostereomers of TCDD that have a common mechanism of action and bring about the same spectrum of responses; EGF, epidermal growth factor; TGF, transforming growth factor; IL-1β, interleukin-1β; GD, gestation day; DES, diethylstilbestrol; GFAP, glial fibrially acidic protein; PCB, polychlorinated biphenyls; T_3, triiodothyronine; T_4, thyroxine; PCDF, polychlorinated dibenzofuran; PCDD, polychlorinated dibenzo-*p*-dioxin.

stage-specific, and time-specific nature of its effects more comprehensible.

The similarity of dioxin to various hormones is reinforced by the receptor-mediated nature of dioxin's effects. For many years it was hypothesized that the dioxin or Ah (aryl hydrocarbon) receptor was a member of the steroid-hormone superfamily of receptors (*10*). However, it has recently been shown to be the first ligand-activated member of the helix–loop–helix family of regulatory proteins (*11,12*) functioning as a transcription factor (*9*). Most of the understanding of this mechanism comes from intensive studies on the regulation of CYP1A1 (*13,14*). While a similar mechanism appears to underlie dioxin-mediated expression of CYP1A2 (*15*) and GSTYa (*16*), there is little information on the molecular nature of transcriptional control of other dioxin-responsive genes.

Recent studies have led to the suggestion that the Ah receptor may function in the cytoplasm as well as in the nucleus (*17,18*). Increases in tyrosine phosphorylation appear to be a rapid and sensitive response to dioxin exposure, both *in vivo* (*19–22*) and *in vitro* (*19,23,24*). The possibility of two roles for the Ah receptor, both as a transcriptional enhancer and as a regulator of second messengers such as tyrosine phosphorylation, appears to be similar to what has been described for the steroid hormones (*25,26*). Dioxin treatment leads to biological amplification of Ah receptor-mediated responses via a cascade of growth factors and hormones. The use of the phrase "combinatorial complexity" (*27*) seems most appropriate.

Experimental Developmental Toxicity

There have been several recent reviews on the developmental toxicity of dioxin and related compounds (*28–30*). Until recently, it was believed that frank terata were not detected in any species other than mice except at doses that were overtly toxic to the dam. However, at such doses, decreased fetal growth and prenatal mortality were observed in rats, guinea pigs, hamsters, rabbits, and monkeys (*31*). Thymic and splenic atrophy were observed in the fetus in several species, and subcutaneous edema was noted at higher doses in mouse fetuses. Hemorrhage, both in the gastrointestinal tract and in the kidney, were observed in rats and hamsters (*32*). Recent studies from Walker et al. (*33*) have demonstrated developmental toxicity in fish as well, with dioxin inducing a syndrome called blue-sac disease that occurs in the swim-up stage of fish.

Structural defects following dioxin exposure have been reported in the mouse at doses that do not cause either maternal or fetal toxicity. The best described malformation is cleft palate. There is a critical window for the induction of this defect, with peak incidence following exposure on day 11 or 12 of gestation (*31*). In contrast, the induction of hydronephrosis does not appear to have a peak window of sensitivity during organogenesis and can even be induced lactationally (*34*). Hydronephrosis, however, is a more sensitive response than cleft palate, being induced at lower levels.

Both defects are due to altered differentiation and proliferation of epithelial cells in the respective tissues. The target for dioxin's effect in the developing palate is the medial edge epithelium. In normal development, the medial edge cells transform into mesenchyme (*35*) allowing fusion of the opposing palatal shelves following contact toward the end of organogenesis. The medial peridermal cells slough off the surface allowing the underlying mesenchyme to fuse. Dioxin treatment results in a failure of programmed cell death of the peridermal cells. In addition, the medial epithelium fails to transform into mesenchyme. Instead, these cells continue to proliferate and develop into a stratified, squamous, keratinizing epithelium with desmosomes and tonofilaments (*36,37*). The opposing shelves make contact, but fusion is impossible because of the intact epithelium. The altered proliferation and differentiation is accompanied by changes in the balance of various growth factors and receptors. For example, in the mouse, TCDD treatment both *in vivo* (*38–40*) and *in vitro* (*39*) results in elevated levels of the EGF receptor and TGFβ. Recent studies have demonstrated that exposure of the pregnant mouse to dioxin results in increased expression of the glucocorticoid receptor in the developing palate (*41*). Similar growth factor effects have been noted *in vitro* with exposure of embryonic human palates to TCDD (*42–44*). The Ah receptor has also been detected in the developing palate in both mouse and human tissue (*44,45*). In the palatal epithelium, this receptor is both nuclear and cytosolic. Exposure to TCDD, *in vivo* in the mouse and *in vitro* in the human palate, results in altered expression of the Ah receptor both in the epithelium and in the mesenchyme.

Dioxin-induced hydronephrosis is due to hyperplasia of the ureteric epithelium, leading to blockage of the ureteric lumen. Once the fetus begins to produce urine, the back pressure builds up due to blockage of urine outflow, and destruction of the renal parenchyma results (*46*). TCDD treatment results in elevated levels of EGF receptor in the ureteric epithelium (*47*), similar to what has been observed in the palatal epithelium (*39,44*). There is a possibility that dioxin also has direct effects on the developing kidney. Abbott and coworkers (*48*) noted effects on the extracellular matrix surrounding the Bowman's capsule; there was a decrease in both laminin and type IV collagen. Such a change could lead to a decrease in the functional integrity of the glomerulus and alteration of the normal filtration barrier.

The endocrine-disrupting nature of dioxin can be seen not only in the altered expression of growth factors and receptors in target tissues such as the palate and ureteric epithelium, but it also can be seen in the synergistic interactions demonstrated with other growth factors and hormones. Treatment with either triiodothyronine (T_3) or thyroxine (T_4) appears to potentiate the induction of cleft palate in the mouse (*49*). Retinoic acid, the physiologic active form of vitamin A, interacts synergistically with dioxin to induce cleft palate (*38,39,50,51*). These interactions are, however, tissue specific since retinoic acid does not have any effect on the induction of dioxin-induced hydronephrosis. A synergistic interaction of dioxin and hydrocortisone in the induction of cleft palate was noted several years ago (*52*). Recently, the basis for this interaction has been investigated (*41*) and appears to involve a feedback loop in which dioxin increases the levels of glucocorticoid receptor in the palate and hydrocortisone elevates the amount of Ah receptor. Blankenship et al. (*53*) have also shown that TCDD inappropriately accelerates differentiation in the early embryo.

Much of the recent interest in the developmental effects of dioxin and related compounds has stemmed from the studies conducted at the University of Wisconsin by Peterson and co-workers. In a series of studies (*54–56*), these investigators reported on the effects of prenatal exposure on male rat reproductive parameters. Basically, towards the end of organogenesis (gestation day 15), they exposed the pregnant Holtzman rat to low levels of dioxin and conducted functional assessments of male pups from birth through adulthood.

They reported decreases in the anogenital distance, accessory sex organ weights, and spermatogenesis at doses as low as 64 ng TCDD/kg body weight of the mother on GD15. In addition, they reported that the sexual behavior of the male offspring was both demasculinized and feminized and the regulatin of LH secretion was feminized. They have not been able to repeat some of their published observations on effects on circulating male hormone levels (*57*).

Studies from our laboratory have attempted to confirm and extend the findings of Peterson and coworkers. Using an experimental paradigm based on that of Mabley et al. (*54–56*), Gray et al. (*58,59*) treated pregnant Long-Evans rats on GD15 with 1 μg TCDD/kg body weight and examined both the male and female offspring at birth, puberty, and adulthood. A small number of Holtzman rats were also included in this study. In addition, pregnant Syrian golden hamsters were exposed on GD11, a comparable developmental stage to GD15 in the rat, with 2 μg TCDD/kg. As noted earlier by Mabley et al. (*54*), anogenital distance was reduced in the male rat pup at birth. However, the amount of reduction was less in the Long-Evans rat than in the Holtzman rat, and there was no effect on anogenital distance in the neonatal hamster (*59*). Serum testosterone levels were normal in the newborn rat in contrast to the original report of Mabley (*54*), but consistent with later publications from the same laboratory (*57*). *In vitro* testosterone production was also unaffected, not only just after birth but at puberty (*59*). In fact, puberty was delayed in both the male rat and hamster following prenatal and lactational exposure to dioxin as measured by a delay in preputial separation. Body weight was slightly (6%) but significantly decreased in the male pups at puberty.

Effects on sperm numbers were noted at puberty in rats and persisted throughout adulthood into at least middle age. Testicular sperm production was down 30% at puberty, but in adulthood was down only 5%. This was accompanied by a slight (5%) drop in testis weight at puberty. Serum testosterone levels were normal, but ventral prostate and seminal vesicle weights were reduced. Epididymal sperm counts were decreased 59% at puberty and were still down 30% at adulthood. Ejaculated sperm counts, measured by mating to satiety and collection of sperm from the female partners, were down 60% in both rats and hamsters prenatally and lactationally exposed to TCDD. No reduction in androgen receptor numbers were found in the seminal vesicle, ventral prostate, and epididymis. There were subtle alterations in male sexual behavior, similar to that previously observed by Mabley et al. (*55*). Gray et al. (*59*) observed no difference in latency to mount, but more mounts were necessary for ejaculation. Given the adverse effects of dioxin on the development of the urogenital tract, this altered male sexual behavior may well have a physical basis. No effect on female sex behavior (lordosis) in the castrated male Long-Evans rats was noted, in contrast to the results reported in the Holtzman rat (*55*). This could reflect a strain difference in lordotic tendencies. There was no difference in sex behavior in the male hamster offspring.

Reproductive alterations in the female offspring following prenatal and lactational exposure involved structural malformations in the urogenital tract (*58*). In both rats and hamsters, cleft phallus and hypospadias were present. Minimal to severe clefting of the phallus/clitoris was observed in the majority of female offspring. Hypospadias accompanied the severe clefting. Vaginal opening was incomplete and accompanied by a persistent thread of tissue across the vaginal orifice in many of the female rat pups. Vaginal opening was delayed in the hamster, who also evidenced an abnormal vaginal estrus. Litter sizes were reduced in the female hamsters following the prenatal and lactational dioxin exposure at 2 μg TCDD/kg. It is interesting to note that the combination of phallic clefting and hypospadias has also been observed in female offspring of DES-treated female rats (*60*).

The reported alterations in male sexual behavior, demasculinization and feminization, led to examination of sexually dimorphic behaviors in middle-aged male rat offspring following prenatal and lactational exposures to TCDD (D Miller, personal communication). Using the same paradigm (exposure on GD15 to 1 μg TCDD/kg in Long-Evans rats), there was no effect on motor activity in the male offspring. Females generally exhibit more rearing behavior than males, and TCDD caused no increase in this behavior in the male pups. Learning and memory also show sexual dimorphism. Spatial learning is usually greater in males than in females. No effects were seen using the Morris water maze, which examines this endpoint. Likewise, saccharin preference shows a male/female difference and was also unaffected by prenatal/lactational exposure. Recent studies also fail to report any change in the size of the sexually dimorphic nuclei in the hypothalamus (*61*). Thus, there is little evidence at this time for a direct impairment of the central nervous system following prenatal and lactational TCDD exposure.

Effects of this exposure paradigm were also examined for nonsexually dimorphic responses. Changes in glial fibrially acidic protein (GFAP) are often considered a marker of neurotoxicity (*62*). Miller and O'Callaghan (personal communication) failed to detect any changes in GFAP levels following prenatal and lactational TCDD exposure. However, the core body temperature appears to be permanently reduced in these rats, suggesting a downward shift in the set point for the control of body temperature (C Gordon, personal communication). This lowered body temperature, which persists until at least 18 months of age, occurs in the absence of any effect on metabolic rate. These adult male rats whose mothers were exposed to only 1 μg TCDD/kg on GD15 weigh 10% less than matched controls throughout their lives.

Few laboratory studies have been conducted on the functional developmental toxicity of compounds related to TCDD. However, Lundkvist (*63*) did examine the effects of prenatal and lactational exposure to a complex PCB mixture, Clophen A50, in guinea pigs. Similar to many of the results observed in rats and hamsters, there was a delay in sexual maturation in both male and female offspring. Vaginal opening was delayed in females, and there was a decrease in testis weight in the males. Some growth retardation was noted. Similar to the results of Gray et al. (*59*), there was no difference in plasma testosterone levels. Thus, studies in rats, hamsters, and guinea pigs all suggest a direct effect of dioxin and related compounds on the developing genitourinary tract. This would be compatible with the direct effect of dioxin on the ureteric epithelium, which is part of the genitourinary tract. Whether these effects are induced by late prenatal exposure or early postnatal lactational exposure is not fully resolved. It is clear that exposure late in organogenesis is more effective at inducing these effects than exposure earlier in gestation; exposure of rats on GD8 resulted in the same spectrum of responses but at a lower incidence and severity than following exposure on GD15 (*59*). However, Bjerke and Peterson (*64*) have conducted cross-fostering studies in the rat which indicate that the majority of these effects are induced by exposure before birth. It is

important to note that developmentally in many tissues the neonatal rodent is similar to a second- or third-trimester human fetus.

Functional developmental toxicity of TCDD has also been noted in the immune system. Atrophy of the fetal thymus has long been known following prenatal dioxin exposure in many species. Exposure of the pregnant mouse to TCDD towards the end of organogenesis (GD14) resulted in altered maturation of lymphocytes (*65,66*) and changes in the differentiation of T lymphocytes (*67*) at doses below that where thymic atrophy occurs. Analysis of thymic lymphocytes by flow cytometry demonstrated that prenatal exposure targeted immature $CD3^-$ cells in the both the Long-Evans and the Fisher rat (*68*). There were increases in the relative amount of $CD4^-CD8^-$ and $CD4^-CD8^+$ cells and a decrease in the percentage of $CD4^+CD8^+$ thymocytes, suggesting that TCDD acts on the $CD3^-CD4^+CD8^+$ population of immature cells in the fetal thymus. This suggests a decrease in thymic cellularity and a decrease in splenic T cells. The implications of an elevated percentage of maturing cytoxic/suppressor T cells is unclear. The persistence of these effects is currently under study.

Developmental Toxicity in People

The developmental effects of dioxins in humans are much less clear than in the rat and mouse. There have been no in-depth epidemiological studies of children born to women exposed to TCDD. (In fact, there have been few studies conducted of TCDD-exposed women at all; most of the studies have involved adult men). A recent report from the Netherlands examined the levels of circulating thyroid hormone in children and found an increase in T_4 levels associated with higher levels of PCDDs/PCDFs in their mother's breast milk (*69*). Children born to women exposed in the 1976 poisoning in Seveso, Italy, have no increase in obvious birth defects (*70*). However, there has not been a follow-up of that cohort to determine if functional developmental toxicity is present. In contrast, clear evidence of developmental toxicity has been found in two cohorts inadvertently exposed to complex mixtures of PCBs and PCDFs. In both the Yusho and Yu-Cheng poisonings in Japan and Taiwan, respectively, there was a increased incidence of stillbirths born to exposed women (*71*). The Yu-Cheng cohort continues to be intensively followed (*72*). The developmental delays present in the infants have persisted, with children now between the ages of 8 and 13 years of age evidencing behavioral problems (*73*), intellectual deficits (*74*), and growth retardation (*75*). While the ectodermal dysplasia syndrome (*76*) noted at birth has tended to resolve, other functional impairments have been noted; for example, the children appear to have hearing loss (*77*). A recent report has raised the concern about reproductive alterations; the boys appear to have significantly smaller penises when they reach puberty than matched controls (*78*). There is also a suggestion of immunosuppressive effects of the prenatal exposure to the PCB/PCDF mixtures, as the children have elevated incidences of respiratory infections and otitis (*76*). These findings are compatible with the recent report of immunological effects in children born to women with high PCB levels in their breast milk in Arctic Quebec (*79*). A preliminary report on this population suggested changes in T-cell subsets in the prenatally exposed infants.

Conclusions

Dioxin's developmental effects, both structural and functional, immediate and delayed, are complex and tend to resemble those seen with effects on multiple hormone systems. Recent studies have suggested that dioxin is an antiestrogen (*80*) because of its ability to block proliferation of the uterine lining in response to estrogen in the immature rodent and its ability to block estrogen response in breast cancer cell lines. However, dioxin is not an antiestrogen in the classical sense of blocking estrogen binding to the estrogen receptor. It may alter the metabolism of estrogen, potentially leading to a decrease in the active form of the hormone at target sites. It also leads to decreased numbers of estrogen receptors. Dioxin is also not an estrogen and does not bind to the estrogen receptor. It does not cause classical estrogenic responses, but some of the effects of dioxin do resemble those seen with potent synthetic estrogens such as DES. Estrogen appears to be necessary for dioxin to cause cancer in rat liver (*81*), and recent studies suggest that it may be necessary to allow the progression of TCDD-promoted endometriosis in mice (A Cummings, personal communication).

The appropriate way to view dioxin is as a modulator of growth and development. Depending on the tissue and stage of development, dioxin may be estrogenic or antiestrogenic. Its effects may resemble those of hypovitaminosis or hypervitaminosis A. Dioxin can cause either suppression or elevation of circulating thyroxin levels. It causes hypoplasia, hyperplasia, and metaplasia, as well as neoplasia. Dioxin increases the amounts of TGFα in some tissues and decreases it in others. The same can be said of both the glucocorticoid and the EGF receptors—down regulation versus enhanced expression—depending on the cell, tissue, and stage of development. Dioxin appears to perturb normal homeostasis and hormonal balance leading to alterations in proliferation and differentiation. In the adult, such changes may lead to cancer, immunosuppression, chloracne, and endometriosis. In the embryo/fetus, altered programming of developing tissues leads both to overt malformations, anomalies, fetal toxicity, and functional and structural deficits, which are often not detectable until later in life. Whether the multiple functional and subtle structural deficits detected at low levels of dioxin exposure in prenatally exposed experimental animals are occurring in the human population remains to be determined.

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