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Prenatal TCDD Exposure Predisposes for Mammary Cancer in Rats

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Abstract

Epidemiological data are conflicting in the link between 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) exposure and breast cancer causation. We have hypothesized that timing of exposure to endocrine disruptors, such as TCDD, will alter breast cancer susceptibility. Using a carcinogen induced rat mammary cancer model, we have shown that prenatal exposure to TCDD alters mammary gland differentiation and increases susceptibility for mammary cancer. Investigations into imprinting *via* DNA methylation mechanisms showed that there were no changes in protein expression in DNA methyltransferases, ER-alpha, ER-beta, GST-pi, or MDGI. Using 2-D gels and mass spectrometry, we have found seven proteins to be differentially regulated, including a decrease in superoxide dismutase 1 (SOD1). Down-regulation of SOD1 could provide an environment ill equipped to deal with subsequent free radical exposure. We conclude that prenatal TCDD can predispose for mammary cancer susceptibility in the adult offspring by altering the mammary proteome.

Keywords

Prenatal; TCDD; mammary cancer; superoxide dismutase; DNA methylation

Despite an explosion in new treatment options and increased attention to factors that have been shown to prevent cancer occurrence, breast cancer continues to be a major cause of cancer incidence and mortality in women. In 2006, an estimated 212,920 women will be diagnosed with invasive breast cancer in the United States, and a projected 41,430 cases will result in mortality [1]. With only 5-15% of all breast cancer etiology being attributed to hereditary causes, other causative factors are postulated to account for the high incidence of breast cancer [2]. One hypothesis is that environmental chemicals that are hormone agonists or antagonists are contributing to increased susceptibility for breast cancer. Hence, endocrine disruptors, especially those with the ability to alter estrogen signaling, are now being scrutinized for their possible role in the causation of breast cancer.

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2,3,7,8-Tetrachlorodibenzo-p-dioxin

Exposure to dioxins, such as 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), has been linked to a host of health maladies, including reproductive abnormalities, decreased immune function, cleft palate, embryonic toxicity, chloracne, thymic involution, a progressive and irreversible wasting syndrome, and carcinogenesis of multiple organs in animal models [reviewed in reference 3 & 4]. TCDD is a planar compound, consisting of two benzene rings joined by two oxygen atoms with four chlorine substituents located at the 2, 3, 7, and 8 positions. The hydrophobic structure of TCDD dictates a compound that is highly persistent, resistant to breakdown in both the environment and in the human body, with an estimated half-life of 7.6 years in humans [5]. After oral ingestion of TCDD, the compound is primarily sequestered in the liver and adipose tissue because of its lipophilic nature. Due to TCDD's anti-estrogenic effects and its classification as an endocrine disruptor, the fear is that persistent, low levels of exposure could have adverse effects on humans, specifically in the development of hormonally-dependent cancers such as breast cancer.

The basic molecular actions of TCDD are well documented. TCDD binds with high affinity to the cytosolic aromatic hydrocarbon receptor (AhR) [3]. AhR is a ligand-activated nuclear transcription factor that is held inactive in the cytosol by two chaperone heat shock proteins (HSP90), a small protein (p23), and an immunophilin-like protein (XAP2). The binding of TCDD to AhR causes the dissociation of this complex. The newly formed and active TCDD-AhR complex translocates to the nucleus where it partners with the aromatic hydrocarbon receptor nuclear translocator (ARNT). In the nucleus, this heterodimer acts as a nuclear transcription factor, binding to dioxin-responsive elements (DRE) and altering specific gene transcription. It is known to induce a number of drug metabolizing enzymes, including, but not limited to, cytochromes P450 1A1 and 1A2, glutathione *s*-transferase, and glucuronyl transferase. While the majority of the observed toxic effects of TCDD are thought to be mediated through the activated AhR, the exact mechanisms are presently unknown.

Proclaimed as the most potent man-made carcinogen based on LD₅₀ data in animals, the effects of TCDD on humans are much less clear. Collectively, epidemiological reports have failed to provide a consistent link between TCDD exposure and breast cancer causation. Drawing samples from the 1976 industrial explosion in Seveso, Italy, Bertazzi and coworkers found a lower than expected incidence of breast cancer in the most contaminated zones [6]. This finding has been attributed to the anti-estrogenic activity of TCDD and has led some to suggest the use of designer AhR-agonists, termed selective AhR modulators (sAhRMs), as chemotherapeutic agents against breast cancer [7]. From 1967 to 1987, the Middle Volga chemical plant in Chapaevsk, Russia produced hexachlorocyclohexane and other chlorine containing products. High levels of dioxins, including TCDD, were found in the air, soil, drinking water, and in cow's milk. In a study based on the population of this area, an increased incidence in the rates of breast cancer was observed [8]. This finding was coupled by increased incidences in reproductive difficulties, congenital morphogenetic conditions in children, and spontaneous abortions [8]. A meta-analysis of the literature on occupational exposure to TCDD failed to yield a statistically different rate ratio of breast cancer incidence between exposed and unexposed women [9]. These and other studies have led to opposing conclusions on the connection between TCDD exposure and breast cancer development. However, epidemiological data are complicated by a wide and varying range in the exposure window and dosage level of the subjects considered.

Timing of Exposure

Timing of exposure is a subtle point that may, in part, account for the inability to draw consistent conclusions from epidemiological data. Exposure during different windows of development

can have wholly different outcomes. For example, the lack of a correlation between TCDD exposure at a variety of ages and breast cancer development later in life does not necessarily mean that no correlation exists for any window of exposure. Perhaps the best illustration of the importance of timing of exposure comes from studies of radiation exposure in women. Exposure before adolescence (age 20) has been strongly associated with a dose-specific increase in excess relative risk (ERR_{ISV}) of developing breast cancer [10]. This dose-specific increase in ERR_{ISV} of developing breast cancer declines with increasing age at time of exposure after adolescence. These findings highlight a critical window of exposure in which exposure to radiation before adolescence creates a substantially higher risk of developing breast cancer later in life. This is contrasted by only a modest increase of breast cancer developing if exposure occurs after adolescence.

Further proof of timing of exposure has been observed in studies that examined exposure to the synthetic estrogen diethylstilbestrol (DES) and the soy isoflavone genistein. In animal models, prenatal DES exposure has been shown to cause increased multiplicity and incidence of mammary tumors in a dimethylbenz[a]anthracene (DMBA) model of rodent mammary cancer [11], but neonatal exposure to DES results in more mature and developed mammary glands and reduced incidence of spontaneously developing mammary tumors [12]. While genistein fed to pregnant rats does not alter susceptibility for chemically-induced mammary cancer in the offspring, dietary exposure to genistein during the neonatal/prepubertal period protects against DMBA-induced mammary cancer development in a dose-dependent manner [13,14]. Additionally, early first-term pregnancy has been shown to provide a protective effect in the development of breast cancer, while pregnancy later in life increases the risk of developing breast cancer later in life [15]. Clearly, chemical specificity and timing of exposure are critical determinants to consider and may be responsible, in part, for confounding results obtained from epidemiological studies.

Prenatal Exposure to TCDD

In order to lessen the incidence of confounding results due to multiple variables, we limited TCDD exposure to a very specific and critical window: the prenatal period. This allows for TCDD exposure during a critical period of mammary gland development. As shown by Sakakura *et al.*, the migration of the mammary epithelial bud into the fat pad occurs at approximately gestational days 16-17 [16]. Following the work of Gray and Ostby, pregnant dams were gavaged with 1 μg TCDD/kg body weight or an equivalent volume of sesame oil on day 15 post-conception [17,18]. This dose has been reported to be associated with a delay in the onset of puberty in female rats as well as reproductive abnormalities such as vaginal threads and complete to partial clefting of the phallus [17,18]. Then, to minimize the potential for lactational transfer of TCDD to offspring, we cross-fostered the newborns to surrogate dams on the day of birth. Coupling this mode of exposure to TCDD with a DMBA model of rodent mammary cancer, our lab has shown that prenatal exposure to TCDD results in an increased incidence of mammary adenocarcinomas in female offspring [18] (Fig. 1). This dose and window of exposure to TCDD were found to cause substantial alterations in mammary gland architecture and differentiation. Specifically, prenatal treatment with TCDD resulted in mammary glands that had a greater number of immature terminal end buds and fewer mature lobules at the time of DMBA exposure (day 50 postpartum) [18] (Fig. 2). In contrast, no morphological effects were observed in mammary glands from 21 day old female offspring exposed prenatally to TCDD [18]. Terminal end buds have been shown to play a key role in mammary cancer development, being identified as the structure of the breast that is the most immature, proliferative, and thus susceptible to carcinogenic insult [19]. As such, terminal end buds are often the site in which mammary cancer develops in rats. Similar structures have been observed in humans [20]. Lobules are more differentiated, and thus are less susceptible to carcinogenic insult [19]. Before menopause, pregnancy and increasing age transition the

structures of the breast from being dominated by the immature terminal end buds to the more mature lobules. Creating a gland where that progression is altered, with an excess of terminal end buds and a reduction in lobules, essentially results in a mammary gland that is more susceptible to carcinogenic insult. Similar results by Lewis *et al.* and Fenton *et al.* have been obtained in studies of combined prenatal and prepubertal (through lactation) exposure to TCDD [21,22].

In Holtzman rats exposed prenatally and prepubertally through lactation to TCDD and subsequently ovariectomized at nine weeks of age, Lewis and coworkers noted that the mammary glands of the TCDD-exposed females showed significant differences when compared to control females. In comparison to controls, glands from TCDD-exposed females showed a greater percentage of terminal end buds and a lower percentage of Lobules I and II at 11 weeks of age [21]. Similarly, Fenton *et al.* showed that female Long-Evans rats exposed perinatally to TCDD presented with a significantly stunted mammary gland when compared to control, age-matched females, an effect that began at four days postpartum [22]. Mammary glands from TCDD-treated females contained fewer primary branches from the collecting duct, delayed migration of the epithelium through the fat pad, and had fewer terminal branches and alveolar buds as compared to controls [22]. Even at 68 days of age, contrary to age-matched control females, TCDD-treated females retained terminal end buds [22].

In studying the effects of TCDD to the normal mammary differentiation process induced by pregnancy, Vorderstrasse *et al.* reported similar finding in C57B1/6J mice [23]. Pregnant females were dosed with 5 µg TCDD/kg body weight or an equivalent volume of peanut oil on days 0, 7, and 14 of pregnancy. Sacrifice during early pregnancy (day 9), mid-pregnancy (d12), late pregnancy (day 17), and parturition revealed statistically significant stunting of the mammary glands in animals treated with TCDD, a finding that was independent of the stage of pregnancy. TCDD exposed females showed less branching and fewer and smaller lobules as compared to control animals as early as day 9 of pregnancy. Further analysis at days 12 and 17 of pregnancy showed little to no lobule development. These studies provide substantial backing for the results that our lab obtained in a purely prenatal model of TCDD exposure. In summary, prenatal exposure to TCDD results in mammary glands that are less differentiated and more susceptible to carcinogenic exposure.

Defining the Molecular Mechanisms of TCDD

Since prenatal TCDD exposure resulted in long-lasting alterations to mammary gland morphology and increased susceptibility for mammary cancer, our lab hypothesized that this might be occurring *via* an imprinting mechanism. First, we investigated whether DNA (cytosine-5) methyltransferases (Dnmts) were altered. Dnmts function to enzymatically add methyl groups to DNA, usually to cytosine bases. DNA methylation is considered an epigenetic mechanism of controlling gene expression. Hypermethylation results in inactivating cellular genes, while hypomethylation results in allowing gene expression [24]. There are two patterns of methylation, 1) *de novo* methylation, which starts *in utero* and continues during critical periods of development, and 2) adult methylation that occurs in aging cells. The ontogeny of the Dnmt 3 family (a and b) implicates these proteins as being essential for *de novo* methylation, while Dnmt1 functions to maintain, rather than to establish, patterns of CpG methylation. Methylation of promoter CpG islands leads to the binding of methylated CpG binding proteins and transcription repressors to block transcription initiation [25]. Since down-stream events lead to expression of the Dnmt proteins and this is where the actions occur, we chose to use western blot analyses to measure the expression of these Dnmt proteins. Also, we measured protein levels of estrogen receptors alpha and beta (ER-alpha and beta), glutathione S-transferase-pi (GST-pi) and mammary derived growth inhibitor (MDGI), proteins whose DNA promoters are rich in CpG islands and are associated with early stage mammary cancer.

Western blot analyses for Dnmts 1, 3a and 3b, and ER-alpha, ER-beta, GST-pi and MDGI in mammary glands of 21 and 50 day old female offspring exposed at day 15 post-conception to sesame oil or TCDD (1 and 3 µg TCDD/kg BW to pregnant female rats) did not reveal any significant changes (data not shown). Since prenatal exposure to TCDD did not alter expression levels of these proteins, we conclude that imprinting *via* DNA methylation of the ERs, GST-pi and MDGI is not responsible for increased susceptibility of the mammary to TCDD. While we did not demonstrate prenatal TCDD altering Dnmts and DNA methylation, we do not discount the possibility of this treatment altering methylation of histones. The latter may be another opportunity for affecting altered *in utero* imprinting mechanisms.

Using traditional laboratory methods, such as immunoblotting, in attempts to elucidate the details of the molecular mechanisms of prenatal TCDD in the mammary gland have largely been unsuccessful guesswork. The use of relatively new, high-throughput, discovery technologies, such as DNA microarrays and proteomic technology, are attractive alternatives. Proteomic technologies have recently provided a variety of new methods that can be used for discovery work, including multidimensional-protein identification technologies (MudPIT), isotope-coded affinity tagging (ICAT), activity-based protein profiling (ABPP), and surface-enhanced laser desorption ionization time of flight (SELDI-TOF) profiling analysis. Despite the advent of these new technologies, two-dimensional gel electrophoresis (2-D gels) continues to be the workhorse of proteomics. 2-D gels are not without disadvantages, including limitations in the size, hydrophobicity, and isoelectric point of proteins resolved. Nevertheless, 2-D gels provide an established method of resolving hundreds of proteins on a single gel. Combining this with gel detection software for the warping and computer-based matching of protein spots between gels, and mass spectrometry protein identification, 2-D gels provide an excellent method for discovery work that can be used to decipher novel molecular mechanisms and pathways.

Recently, our lab has applied the use of discovery 2-D gel electrophoresis in attempts to elucidate the molecular mechanisms behind the action of prenatal exposure to TCDD in the mammary gland. Briefly, pregnant dams were administered 3 µg TCDD/kg body weight ($n=8$) or an equal volume of sesame oil ($n=8$) on day 15 post-conception. After birth, offspring were transferred to surrogate mothers in order to prevent additional exposure through lactation. Female offspring were killed and mammary glands were collected at 50 days post-partum. The mammary glands were homogenized and prepared according to Rowell *et al.* [26]. Protein (150 µg) from each sample was loaded onto 24-cm immobilized pH gradient (IPG) strips, pH 4-7. Samples were rehydrated overnight at room temperature before being subjected to isoelectric focusing. Strips were equilibrated and subjected to SDS-PAGE according to the manufacturer's protocol. Gels were fixed, stained, and destained before being imaged. Gel images were warped and matched using Progenesis software (Nonlinear Dynamics). The average values for normalized spot volume were used to identify differentially regulated proteins *via* ANOVA or the Generalized Method [26]. Protein spots determined to be differentially regulated were excised and subjected to trypsin digestion. Matrix-assisted laser desorption ionization time of flight (MALDI-TOF) identification of proteins was carried out using a Voyager Elite MS. The MASCOT program was used to produce probable protein identifications from the acquired MALDI-TOF spectra.

Via 2-D gel electrophoresis, seven proteins were found to be differentially regulated between TCDD-treated females and age-matched control females. Albumin and Rho GDP Dissociation Inhibitor (GDI) were found to be significantly up-regulated, and γ -fibrogenin, γ -actin 1 (ACTG1), RASP1, superoxide dismutase 1 (SOD1), and transthyretin (prealbumin) were significantly down-regulated (Fig. 3). Of particular interest is the down-regulation of SOD1. Since SOD1 is involved in the detoxication of superoxide anion radicals, a down-regulation in the mammary gland could provide an environment ill-equipped to deal with subsequent free

radical exposure. Without the first step of superoxide anion radical detoxication functioning at full capacity, mammary glands may be kept in a state of vulnerability, thereby increasing the possibility of cellular transformation occurring. Though the down-regulation of SOD1 was not extreme, exhibiting only a standardized 1.2-fold change, it has a p-value of less than 0.001 due to the tightness of variance. Additionally, immunoblot validation of SOD1 in different animals showed a very similar down-regulation, with a p-value of 0.018 (data not shown). In a protein with critical function and observed tight regulation, even a slight amount of differential regulation could be physiologically significant.

The down-regulation of SOD1 is of great interest due to the reports of TCDD causing oxidative stress and SOD1's role as the primary scavenger of superoxide anion radicals [27]. To have the gateway enzyme of superoxide anion radical detoxication diminished could result in deleterious effects, causing a potential build-up of damaging reactive oxygen species (ROS) within the mammary gland. Using transgenic *Sod1*^{-/-} mice, Elchuri *et al.* have shown that mice deficient in SOD1 experienced an increased incidence of hepatocellular carcinoma and a decreased lifespan in long-term studies [28].

However, most interesting is the idea that the differential regulation of SOD1 may be an indication to dysfunction deeper into the detoxication pathway. Several studies have shown such a correlation. In investigating the enzymatic activities of SOD2, glutathione peroxidase, and catalase in *Sod*^{-/-} mice, Elchuri and coworkers have shown age-dependent changes. SOD2 was observed to have an increase in activity at early ages (3 & 6 months) [28]. This was followed by a subsequent drop in activity, to an estimated 70% of control levels, in end-stage animals (11-23 months of age, when mice became ill and had to be euthanized). Glutathione peroxidase activity was persistently and progressively reduced compared to control animals while no changes were observed in the activity of catalase [28]. Hassoun *et al.* observed that subchronic exposure of TCDD to female Sprague-Dawley rats caused an increase in superoxide anion radical production and a dose-dependent suppression of SOD1 in the hippocampus and cerebral cortex [29]. At lower doses (10 ng TCDD/kg/d), increases in the activities of both catalase and glutathione peroxidase were observed. In contrast, higher dosages of TCDD (22 ng TCDD/kg/d and 46 ng TCDD/kg/d) showed a significant decrease in the activities of catalase and glutathione peroxidase in the same regions [29]. Additionally, in a study of the effects of TCDD on the rodent epididymis, TCDD was shown to cause an increase in the levels of ROS and a decrease in the enzymatic activities of SOD, catalase, glutathione peroxidase in the epididymal sperm and the epididymis [30].

Furthermore, in studying SOD1 expression through immunohistochemical staining of clinical breast carcinoma tissue, Iwase *et al.* reported a correlation between the intensity of SOD1 staining and the level of differentiation of the carcinoma [31]. Well differentiated tubular carcinomas stained more strongly for SOD1 than did poorly differentiated tubular carcinomas [31]. This indicates that the level of SOD1 present changes according to cell proliferation and differentiation in breast carcinoma [31]. If the observations in breast carcinoma apply to normal breast development and physiology, a global decrease in SOD1 in the breast could make those structures of the breast that are most proliferative and undifferentiated, such as the terminal end buds, at a greater susceptibility for cellular transformation. Such a situation would create a mammary gland that has reduced capability of dealing with the oxidative stress that is a part of normal cellular function. Future work includes the confirmation, *via* immunoblotting with different animals, of all proteins noted to be differentially regulated by 2-D gel analysis as well as defining the localization of each confirmed protein by immunohistochemical staining. Additionally, delving deeper into the pathway of superoxide anion radical detoxication, both by immunoblotting and surveying enzymatic activity, is planned.

Conclusions

In 1997, the International Agency for Research on Cancer (IARC) classified TCDD as being a human carcinogen (Group 1). Though an abundance of animal models have fully supported this classification, epidemiological data are often conflicting with regard to the causative association for a number of different cancers, including breast cancer. However, one of the key elements lacking in epidemiological studies is an isolated timing of exposure, a factor which may play a role in the conflicting results of these studies. By limiting TCDD exposure to the prenatal period, female offspring of gavaged rats were found to be predisposed for developing mammary cancer later in life in a DMBA-model of rodent mammary carcinogenesis [18]. Histological examination of the mammary glands showed that prenatal exposure to TCDD resulted in an increase in the number of terminal end buds and a reduction in the number of lobules, essentially creating an immature gland less able to handle carcinogenic insult [18]. This finding has been confirmed by similar studies in other labs, using models of prenatal treatment and lactational transfer of TCDD [21,22]. Investigations into imprinting *via* DNA methylation mechanisms showed that there were no changes in protein expression in DNA methyltransferases, ER-alpha, ER-beta, GST-pi, or MDGI. Employing discovery proteomics, we have identified a number of differentially regulated proteins that may shed light on TCDD's molecular action in the mammary gland and how this relates to a predisposition for the development of breast cancer later in life.

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Figure 1.

Ontogeny of palpable mammary tumors in female rats exposed prenatally to TCDD and treated with DMBA on day 50 post-partum. Pregnant female Sprague-Dawley CD rats were treated with 1 $\mu\text{g/kg}$ body weight or an equivalent volume of the solvent (sesame oil) on day 15 post-conception. DMBA was administered by gavage at 30 mg/kg body wt to 50-day-old female offspring. The TCDD/DMBA group and the sesame oil/DMBA group contained 29 and 30 female rats/group, respectively. (Figure from ref. 18.)

Figure 2. Number of mammary terminal end buds and lobules II in female rats exposed prenatally to TCDD. Terminal ductal structures were evaluated from mammary whole mounts of offspring from 50-day-old female rats treated prenatally with TCDD or sesame oil on Day 15 post-conception (eight animals/group). Values represent mean $\pm$ SEM. ^a $p < 0.05$ as compared to age-matched sesame oil-treated animals. (Figure from reference 18.)

Figure 3.

Two-dimensional gel analysis of proteins from mammary glands of 50 day old rats treated prenatally with 3 μ g TCDD/kg body weight. Seven proteins were determined to be differentially regulated: (A) Albumin, (B) Gamma-Fibrinogen, (C) Actin Gamma 1 (ACTG1), (D) RASP1, (E) Rho GDP Dissociation Inhibitor, (F) CuZn Superoxide Dismutase (SOD1), and (G) Transthyretin.