Ovarian Tumors in Rats Induced by Chronic 2,3,7,8-Tetrachlorodibenzo-p-Dioxin Treatment

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ABSTRACT

2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD) is a multispecies reproductive toxicant, and it has been recently classified by IARC as a known human carcinogen. Here, we report that TCDD promotes the development of ovarian tumors in an initiation-promotion model in female Sprague Dawley rats. Rats were initiated with diethylnitrosamine (DEN) or vehicle at 70 days of age. Starting 2 or 18 weeks after initiation, rats were exposed biweekly to TCDD at a daily average dose of 125 ng/kg/day for 14, 30, or 60 weeks continuously or for 30 weeks plus withdrawal periods of 16 or 30 weeks. Fifteen of 76 (20%) rats initiated with DEN and promoted with TCDD for various lengths of time developed ovarian sex cord-stromal tumors of Sertoli cell type, whereas no ovarian tumors developed in 86 rats used as vehicle controls or that received DEN alone or TCDD alone. The highest tumor incidence occurred in 6 of 14 rats (43%) after 60 weeks of continuous TCDD after DEN initiation. One of six rats developed a tumor by 30 weeks of exposure. Because most effects of TCDD can be attributed to its activation of the aryl hydrocarbon receptor (AhR), the presence and localization of AhR was determined in the rat ovary and in the ovarian tumors by reverse transcription-PCR, immunohistochemistry, and in situ hybridization. AhR was localized to oocytes, granulosa and thecal cells of growing follicles, surface epithelial cells, and epithelial cells lining single tubules in ovaries from adult control Sprague Dawley rats. Neoplastic cells in the ovarian tumors were also positive for both AhR message and protein. These results indicate that the ability of TCDD to cause ovarian tumors is dependent on initiation, length of promotion, and age of the animal when exposed and evaluated. The tumor type induced by TCDD in this experimental system is the same histological subtype as that reported from an early study of youngsters exposed during an industrial accident in Seveso, Italy.

INTRODUCTION

The polyhalogenated aromatic hydrocarbon TCDD2 is a ubiquitous environmental contaminant that produces a spectrum of biochemical and adverse biological effects in people and in a wide variety of experimental animal models, including reproductive, developmental, and carcinogenic effects (1, 2). TCDD has recently been classified as a known human carcinogen (3). Whereas TCDD is a multisite carcinogen in rodents, the most commonly studied model is liver tumor induction, particularly through initiation and promotion studies (4, 5). Using an initiation and promotion model, Lucier et al. (6) demonstrated that the ovary needs to be intact to observe the hepatocarcinogenic and proliferative action of TCDD in female rats. This finding suggested that the tumor-promoting effect of TCDD may be mediated either by direct modulation of ovarian function or through indirect endocrine modulation. Antiestrogenic activity has been ascribed to TCDD (7) through studies showing TCDD inhibits the uterotrophic action of estrogen (8), induces uterine atrophy, and causes reproductive failure in mice (9). TCDD also decreases expression of ER in the liver (10) and uterus (11).

TCDD may act directly on the ovary because the AhR message has been identified in rodent and primate ovaries by RT-PCR (12), and the general scientific consensus is that most, if not all, of the effects of TCDD are mediated by initial binding to this transcription factor (13, 14). Moreover, the binding affinity of polycyclic aromatic hydrocarbons to the AhR correlates with the ability of these chemicals to cause ovarian toxicity and ovarian tumors in a strain and species-dependent manner in mice (15). Thus, it is possible that TCDD elicits direct effects on the ovaries through the AhR that, in turn, modulates hormone levels, ER, or other ER-mediated pathways. Given the central role of the ovary and ovarian hormones in TCDD carcinogenicity in the liver, the purpose of this study was to determine the ovarian pathology within the framework of an initiation-promotion model and determine whether AhR is a factor in the ovarian pathology.

MATERIALS AND METHODS

Chemicals. TCDD dosing solutions were obtained from Radian Corp. (Morrisville, NC). Concentration and purity were controlled by gas chromatography. All other chemicals were obtained from Sigma Chemical Co. (St. Louis, MO), unless specified in the text. All PCR reagents were from Promega (Madison, WI).

Animals and Experimental Design. The study design has been described elsewhere (16). Briefly, at 70 days of age, female Sprague Dawley rats were initiated with a single dose of DEN i.p. at 175 mg/kg in saline vehicle (1 ml/kg body weight) or saline only. Two or 18 weeks after initiation, promotion was started with biweekly oral gavage of TCDD in corn oil at a dose of 1750 ng/kg, equivalent to 125 ng/kg/day. Controls received corn oil. The study included four treatment categories: (a) groups dosed continuously with TCDD or vehicle for 14 weeks, 30 weeks, or 60 weeks, beginning 2 weeks after initiation; (b) groups dosed continuously with TCDD for 30 weeks, followed by 16 or 30 weeks of treatment with corn oil alone; (c) groups dosed with TCDD for 14 or 30 weeks beginning 18 weeks after initiation; and (d) groups dosed with TCDD for 30 weeks, followed by 16 weeks of dosing with corn oil only beginning 18 weeks after initiation. Necropsies were performed 1 week after the last treatment. All time points included control animals receiving the vehicles saline and corn oil only and animals initiated only.

Serum was collected from cardiac puncture at necropsy. Serum levels of estradiol, progesterone, and testosterone were analyzed by RIA (Diagnostic Products Inc., Los Angeles, CA) from serum stored at −70 °C. A complete histomorphological examination was done on ovaries, uterus, cervix, vagina, and all grossly characterized masses of the reproductive tract at all time points of the study. Rats were categorized into stages of the estrous cycle by vaginal histology and correlated with uterine and ovarian histology.

Cell proliferation rates were determined by BrdUrd incorporation by mini osmotic pumps (Alzet model 2 ml, 10 ml/hr; Alza Corp., Palo Alto, CA) filled with 30 mg/ml BrdUrd implanted into each rat 7 days before necropsy. Nuclear BrdUrd incorporation was detected by immunohistochemistry using 1:50 dilution of mouse-anti-BrdUrd antibody (Becton Dickinson, San Jose, CA). Cell proliferation rates were generated by computer-assisted image analysis using a Microimage Video system and NIH image (v5.8). Positive (brown) nuclei and total nuclei were counted from at least five, ×40 fields per slide from four separate ovaries with a diagnosis of hyperplasia and three ovaries with a diagnosis of tumor.
AhR Studies. RT-PCR was performed on total RNA isolated from a single frozen rat ovary using TRI Reagent (Molecular Research Center, Inc., Cincinnati, OH). Primer pairs for RT-PCR were selected from the rat AhR (GenBank accession no. U09000), nucleotides 1399–1422 (5’-tcggggcgctgaagaagaca-gag-3’) for reverse primer and nucleotides 1073–1096 (5’-ggaagggtgctcagtc-aatgcac-3’) for forward primer and made by Bioserve Biotechnologies (Laurel, MD). RT-PCR was performed on 800 ng of total RNA. The PCR reaction was 35 cycles of 30 s at 94°C, 30 s annealing at 60°C, and 30 s at 72°C, followed by a 4-min extension at 72°C. Restriction enzyme digest using Fok I and BamHI (Promega) confirmed the AhR product.

In situ hybridization was performed using a digoxigenin-labeled probe generated from a 1.8-kb cDNA fragment of the mouse amino terminal DNA sequence subcloned into pBluescript II plasmid (Stratagene, La Jolla, CA), which was kindly provided by C. Bradfield (17) and used previously for in situ hybridization studies (18). The mouse sequence has nearly 100% homology to the rat AhR (19). The plasmid was linearized with XhoI or Xhol (Stratagene), then labeled with digoxigenin using an Ambion transcription kit (Austin, TX). Riboprobes were then reduced to 150–200 bp by alkaline hydrolysis and used at a final concentration of 1.0 ng/ml. The hybridization was performed on formalin-fixed, paraffin-embedded sections of mouse and rat liver (as controls), rat ovary, and ovarian tumor using methods described previously (20).

Immunolocalization of AhR was done on normal Sprague Dawley rat ovary and ovarian tumors using a standard avidin-biotin antiperoxidase detection system (Vector Laboratories, Burlingame, CA). TCDD-treated rat liver served as a positive control. AhR antibodies were generated from rabbits immunized with a synthetic peptide corresponding to amino acids 371–399 of the human AhR plus a COOH-terminal cysteine conjugated to maleimide-activated Injekt KLH (Pierce Chemical Co., Rockford, IL). The specificity of the immunopurified antibody (Immunopure Plus Protein A IgG purification kit; Pierce Chemical Co.) as well as the cross-reactivity with rat AhR was confirmed by Western blot analysis (data not shown) on normal rat liver. AhR was detected in 5-μm sections of fixed tissues following standard protocols. Positive staining was visualized with 3,3′-diaminobenzidine tetrachloride (Sigma Fast DAB tablet set; Sigma Chemical Co.) using 0.05% Toluidine Blue as counterstain (Fisher Scientific, Fair Lawn, NJ).

Statistical Analyses. Serum hormones were analyzed by ANOVA after log transformation of data (JMP; SAS Institute, Inc., Cary, NC). Tumor incidence data were analyzed by Fisher’s exact test.

RESULTS

Ovarian tumors developed in 15 of 76 (20%) rats initiated with DEN and promoted with TCDD, whereas no ovarian tumors developed in the 86 rats that served as vehicle controls or received DEN alone or TCDD alone (Table 1). The highest tumor incidence occurred in DEN-initiated rats treated with TCDD continuously for 60 weeks (6 of 14 rats). The tumor incidence in this group was statistically different (P < 0.05) from the DEN-initiated concurrent control group (0 of 11). The earliest time point of occurrence of this tumor was after 30 weeks of continuous TCDD treatment, with one of six animals developing this tumor (17% incidence). Tumors were also in rats in group II that were continuously dosed for 30 weeks with TCDD, but then kept for an additional 15 or 30 weeks on vehicle alone. In groups

Table 1 Incidence of ovarian tumors by TCDD

<table>
<thead>
<tr>
<th>Group</th>
<th>Time (weeks)</th>
<th>Vehicle initiated</th>
<th>DEN initiated</th>
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<tr>
<td></td>
<td>Control</td>
<td>TCDD</td>
<td>Control</td>
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<tr>
<td>I. 2 weeks after initiation</td>
<td>14</td>
<td>0/6</td>
<td>0/6</td>
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<td>Continuous dose</td>
<td>30</td>
<td>0/6</td>
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<tr>
<td>60</td>
<td>0/6</td>
<td>0/6</td>
<td>0/11</td>
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<tr>
<td>II. 2 weeks after initiation</td>
<td>30–16</td>
<td>0/6</td>
<td>0/6</td>
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<tr>
<td>Continuous dose</td>
<td>30–30</td>
<td>0/6</td>
<td>0/9</td>
</tr>
<tr>
<td>III. 18 weeks after initiation</td>
<td>14</td>
<td>1/6</td>
<td></td>
</tr>
<tr>
<td>Continuous dose</td>
<td>30</td>
<td>1/15c</td>
<td></td>
</tr>
<tr>
<td>IV. 18 weeks after initiation</td>
<td>30–16</td>
<td>4/12</td>
<td></td>
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<tr>
<td>Continuous dose</td>
<td>30–30</td>
<td>0/6</td>
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* Data represent the number of female Sprague Dawley rats with ovarian tumors per total number rats at study end. Rats were treated biweekly with TCDD at a daily average dose of 125 mg/kg/day for up to 61 weeks. Control rats received corn oil.
* Rats were treated at 10 weeks of age with a single dose of 175 mg DEN/kg (DEN-initiated) or saline (noninitiated).
* Significantly different from control (P < 0.05) by Fisher’s exact test.
* One rat had bilateral tumors.

Fig. 1. Sprague Dawley rat ovaries from the 60 week, DEN/TCDD treatment group. A, ovarian characteristic of Sertoli cell tumor (large arrow), with remnant ovarian follicle (small arrow); H&E. Bar, 200 μm. B, typical tubular pattern of ovarian Sertoli cell tumor at higher magnification; H&E. Bar, 50 μm. C, higher magnification of B demonstrating cell morphology. The large arrow points to a mitotic figure and the small arrow points to intracytoplasmic granules. Bar, 25 μm. D, nuclear BrdUrd incorporation in ovarian Sertoli cell tumor with typical heterogeneous brown staining of nuclei (arrow); hematoxylin counterstain. E, small tumor developing adjacent to follicles, both demonstrating marked staining for BrdUrd. Bar, 200 μm. F, higher magnification of follicle showing both typical granulosa cell morphology and aberrant Sertoli cell morphology with remnant oocyte still within the follicle. Bar, 50 μm.
III and IV in which rats were exposed to TCDD beginning 18 weeks after initiation, 1 of 6 rats was identified with an ovarian tumor after only 15 weeks of TCDD treatment. After 30 weeks, 1 of 15 rats developed an ovarian tumor. Four of 12 (33%) rats developed ovarian tumors when dosed for 30 weeks and then kept for an additional 17 weeks on vehicle only.

All tumors were diagnosed as ovarian sex cord-stromal tumors of Sertoli cell pattern. The microscopic pattern of the tumors was characterized by expansile nodules of densely packed, varying sized tubules (resembling seminiferous tubules) separated by a fine fibrovascular stroma (Fig. 1, A–C). Tubules were lined by one to three disorganized layers of elongated or polyhedral cells with abundant pale eosinophilic wispy cytoplasm and basally located nuclei. At least one-fourth of the cells in a tubule contained large clear intracytoplasmic vacuoles, and other cells contained numerous round, 1–2-μm diameter, bright eosinophilic intracytoplasmic granules (Fig. 1C). Nuclei were vesicular with a single small nucleolus. Benign tumors were diagnosed microscopically based on size (1-mm diameter or greater), distinct separation from ovarian stroma into nodular masses with partial or complete encapsulation, and compression of surrounding parenchyma.

Whereas most tumors were benign, ovarian Sertoli cell carcinomas were diagnosed in four rats in the 60-week continuous exposure group. Diagnosis of carcinoma was based on size, complete effacement of ovarian tissue, lack of a capsule, marked anaplasia, cellular and nuclear atypia, and pleomorphism of the tumor cells, although no metastasis occurred. Two of the carcinomas had been identified at necropsy. The largest tumor was located in the right ovary and measured 6 mm in diameter. One rat in the 60-week continuous TCDD exposure group had bilateral benign ovarian Sertoli cell tumors, whereas all other rats had unilateral ovarian tumors with contralateral ovarian atrophy or interstitial cell hyperplasia. Hyperplastic lesions were characterized by poorly demarcated, unencapsulated, 1-mm or less areas of Sertoli cell-like tubular proliferations. Hyperplastic Sertoli cell-like foci appeared concurrently with tumors, but did not precede tumor formation temporally (data not shown).

Ovaries from four rats with hyperplastic lesions and three rats with benign tumors were stained for BrdUrd incorporation as an indicator of cell proliferation (Fig. 1, D–F). Cells were stained for nuclear BrdUrd in hyperplastic foci in a random pattern with a mean of 4.75 ± 1 per 62 cells (7.6% proliferation rate). Neoplastic cells stained for nuclear BrdUrd in a much higher frequency, and in some tubules all cells incorporated BrdUrd. The mean number of positive nuclei was 30 ± 2.6 per 53 cells (78% proliferation rate).

The presence of ovarian AhR message was confirmed by RT-PCR (Fig. 2). AhR protein and message were localized by immunohistochemistry and in situ hybridization in a hyperplastic lesion, two benign tumors, and one malignant tumor. Punctate nuclear and cytoplasmic immunopositive staining and intense staining for message was present in the large hyperplastic and neoplastic pleomorphic cells lining tubules (Fig. 3). Additionally, AhR message and protein were localized to the nucleus and cytoplasm in oocytes, and granulosa and thecal cells of primordial and growing follicles in ovaries from a control rat, a noninitiated animal treated with TCDD for 60 weeks, and in an ovary from a DEN-initiated rat with 60 weeks of TCDD treatment that also contained a benign tumor (Fig. 4). Minimal to no staining for receptor was seen in granulosa cells or thecal cells of mature, antral follicles.

AhR protein and message were also localized the surface epithelial cells. An intense dark brown nuclear staining was found in the surface epithelial cells and in Sertoli-like stellate cells lining single tubules of about 250 μm diameter presumed to be cross-sections of aberrant
atretic follicles (Fig. 4C). These cells also had faint cytoplasmic staining. Such tubules may have represented precursor lesions given that similar structures, but surrounding an oocyte, were found within tumors (see Fig. 1, E and F).

No significant treatment-related changes in serum estradiol and progesterone levels or ratios of estradiol:progesterone were detected after data were log transformed and analyzed by ANOVA. No significant differences were detected when rats with tumors were compared with appropriate controls at 60 weeks. Testosterone levels were below the detection levels of the assay in all cases.

**DISCUSSION**

This is the first study to demonstrate that TCDD promotes the growth of ovarian sex cord-stromal tumors of the Sertoli cell variant in Sprague Dawley rats initiated with DEN. The experimental evidence supporting the tumor promotional effect of TCDD in this design include the observations that: (a) ovarian tumors were confined to the DEN/TCDD groups; (b) the highest incidence of tumors were found in rats with the longest promotion (60 weeks); and (c) more tumors had features characteristic of malignancy in this group. In contrast, no ovarian tumors developed in rats used as vehicle controls or that received DEN alone or TCDD alone. The time course analysis suggests that continuous exposure to TCDD (60 weeks) is most effective in promoting tumors. However, the occurrence of tumors in groups in which rats were older when promotion was started suggests that, in addition to the length of TCDD exposure, the development of ovarian tumors can be influenced by the length of time after DEN initiation, and/or by the age of the animal at the time of exposure. Such observations for the induction of ovarian cancer are consistent with those previously determined for liver tumors (21). Additionally, cell proliferation rates were dramatically increased in the ovarian tumors compared with hyperplastic foci and normal tissue, suggesting that TCDD promotes proliferation of preneoplastic cells, leading to tumors similar to that seen in promotion of liver foci (22).

The primary effect of TCDD on the adult female rat reproductive tract appears limited to the ovary, because no other significant lesions...
were found that could not be attributed to age or weight (data not shown). Given the specificity of effect in this study, TCDD appears to act directly on the ovary to promote tumor development. Previous studies have found that TCDD acts as an endocrine disrupter. For example, a single treatment of TCDD suppressed estradiol production by the ovary in immature rats stimulated with ovulatory doses of gonadotropins (23). Additionally, in primates, chronic administration of TCDD decreased serum estrogen and progesterone levels (24). Although changes in serum hormone levels may have occurred acutely but undetected in our study, there were no significant differences in serum hormone levels between the various treatment groups at any time point. Thus, it is likely that the tumors were promoted by TCDD directly acting on the ovary. The resulting ovarian changes may then secondarily impact hormonal balances. Indeed, Sertoli cell tumors in women may manifest clinically as hyperestrogenism (25).

Sertoli cell tumors belong to the class of sex cord-stromal tumors that includes the histological subtypes of granulosa cell tumors, thecomas, Sertoli cell tumors, and stromal tumors (26). Sertoli cell tumors or Sertoliform-like tumors occur as a rare spontaneous tumor in about 2% of Sprague Dawley rats (154 of 7748 rats) at 130 weeks of age (27). These tumors have also been induced by N-ethyl-N-nitrosourea but not by DEN at equivalent doses used in this study (28). Thus, the rarity of this tumor in rats and the initiation/promotion model used in this study are further support that TCDD promotes the development of Sertoli cell tumors in female rats.

The histomorphological observations of our study suggest that the ovarian Sertoli cell tumors are derived from granulosa cells, because a number of tumors contained both granulosa cells and Sertoli cells, and in some cases both cell phenotypes surrounded an oocyte. During development of the gonad, Sertoli cells and granulosa cells arise from the same progenitor sex cord cell while their function and fate become differentially determined in each sex. In the ovary, granulosa cells have a finite life span and die through apoptosis either during follicular development or after differentiating to a luteal cell after ovulation. In the testes, Sertoli cells normally have an indefinite life span as they support spermatogenesis. We propose that promotion by TCDD altered a fundamental apoptotic program of the DEN-initiated granulosa cell, allowing for survival and transdifferentiation to the male-patterned Sertoli cell. Indeed, TCDD has been shown to inhibit apoptosis in initiated hepatocytes in vivo (29) and in vitro (30). Moreover, this inhibition of apoptosis of selected subpopulations of hepatocytes has been proposed as critical mechanistic step in liver tumor promotion in these models. The observed tumor promotion in the rat ovary could follow a similar mechanism of action.

A dysmorphogenesis or transdifferentiation of follicular growth and atresia characterized by follicles containing remnants of oocytes surrounded by Sertoli cells has been described in the ovaries of mice deficient in both estrogen receptors α and β (31). As TCDD has been shown to down-regulate ER in the liver and uterus (10, 11), it is possible that TCDD interacts and down-regulates ER in the granulosa cells as it promotes tumor growth. Because most effects of TCDD can be attributed to its activation of the AhR, the presence and localization of AhR in undifferentiated granulosa and thecal cells, which are of sex cord-stromal lineage, and within the neoplastic cells in the ovarian tumors in this rodent study suggests that TCDD may directly promote the survival and growth of initiated cells through receptor-mediated events. The role of the AhR and its activation in mediating TCDD toxicity and potentially its carcinogenicity is complex because TCDD may mediate some toxic and carcinogenic effects independently of activation of the AhR (32). Nonetheless, the identification of the AhR message and protein within TCDD-promoted ovarian tumors coupled with the well-documented role of AhR in TCDD-mediated effects provide strong evidence that this receptor pathway may be involved in ovarian tumorigenesis. Moreover, the identification of the AhR message and protein in cells of immature follicles but not in differentiated cells suggests this receptor may have a physiological role in follicular cell function as well. However, additional studies of the role of AhR in ovarian function and in tumorigenesis is needed because the antibody used for the immunolocalization studies was a polyclonal peptide antibody that can cross-react with other unidentified proteins and because a systematic evaluation of expression in all tumors and in all ovarian cell types under various hormonal and cycle conditions has yet to be done.

An IARC working group concluded that TCDD seems to act similarly in rodents and people, and several epidemiological studies have shown an association between high dioxin exposure and adverse effects, including increased overall cancer risk in people (3). Results from two epidemiological studies further support a link between ovarian cancers in women and the ovarian cancers produced experimentally through TCDD promotion in the rat. The first study investigated cancer incidence in 20,000 young, 0–19-year-old, Seveso, Italy, residents within the first 10 years after exposure to TCDD in an industrial accident (33). One ovarian androblastoma and one ovarian tumor of germ cell origin were reported based on hospital discharge records. Sertoli cell tumors are also known as “androblastomas” or “arrhenoblastomas” and occur occasionally in women (25). However, no ovarian tumors were expected or found in the aged-match reference population, and the relative risk for ovarian tumor development was infinity in the TCDD-exposed group. Because no direct exposure measurements were available from the persons developing the tumors, the true exposure of the tumor-developing individuals is unknown. The second study investigated cancer incidence in a cohort of 334 women exposed to high levels of dioxins as well as hexachlorocyclohexane during the manufacturing of different herbicides and insecticides (34). An increased incidence of ovarian cancer (4 cases observed versus 2.6 cases expected; 95% CI, 0.41–3.96) was reported. However, no information is available on the exact types of tumors in this cohort. It is possible that the tumors occurred by random chance because there is a low number of cases and these tumor types can occur in young children as well as women. However, it may also be possible that the occurrence of ovarian tumors in these exposed human populations is truly significant. Given that our experimental studies produced ovarian tumors through TCDD promotion, that the AhR is present in follicular cells and tumors that are common between species, and that common ovarian tumor types are now reported in female rats, it is possible that exposure to TCDD and activation of the receptor may increase the risk for the development of ovarian cancer in women as is the case in the experimental model.

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