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2,3,7,8-Tetrachlorodibenzo-*p*-dioxin Has Both Pro-Carcinogenic and Anti-Carcinogenic Effects on Neuroendocrine Prostate Carcinoma Formation in TRAMP Mice

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Abstract

It is well established that the prototypical aryl hydrocarbon receptor (AHR) agonist 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) can both cause and protect against carcinogenesis in non-transgenic rodents. But because these animals almost never develop prostate cancer with old age or after carcinogen exposure, whether AHR activation can affect cancer of the *prostate* remained unknown. We used animals designed to develop this disease, Transgenic Adenocarcinoma of the Mouse Prostate (TRAMP) mice, to investigate the potential role of AHR signaling in prostate cancer development. We previously reported that AHR itself has prostate tumor suppressive functions in TRAMP mice; i.e., TRAMP mice in which *Ahr* was knocked out developed neuroendocrine prostate carcinomas (NEPC) with much greater frequency than did those with both *Ahr* alleles. In the present study we investigated effects of AHR activation by three different xenobiotics. *In utero* and lactational TCDD exposure significantly increased NEPC tumor incidence in TRAMP males, while chronic TCDD treatment in adulthood had the opposite effect, a significant reduction in NEPC incidence. Chronic treatment of adult TRAMP mice with the low-toxicity selective AHR modulators indole-3-carbinol or 3,3'-diindolylmethane did not significantly protect against these tumors. Thus, we demonstrate, for the first time, that ligand-

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dependent activation of the AHR can alter prostate cancer incidence. The nature of the responses depended on the timing of AHR activation and ligand structures.

Graphical abstract

Keywords

2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD); indole-3-carbinol; 3,3'-diindolylmethane; neuroendocrine prostate cancer; TRAMP mice

INTRODUCTION

2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) is a persistent environmental contaminant whose toxicity is aryl hydrocarbon receptor (AHR) mediated. The prostate of rats and mice is adversely affected by TCDD exposure in adulthood and especially by *in utero* and lactational (IUL) TCDD exposure. A single dose in adulthood reduces prostate weight (Moore et al., 1985), and IUL exposure inhibits prostatic bud formation, alters prostate ductal morphology, reduces prostate weight, and can cause ventral prostate agenesis (Ko et al., 2002; Lin et al., 2002, 2003; Mably et al., 1992; Roman and Peterson, 1998; Roman et al., 1998; Vezina et al., 2009). Mice with IUL TCDD exposure also fail to exhibit the normal age-related decline in prostate androgen dependence and, in senescence, their prostate contains cribriform structures (Fritz et al., 2005). But while more than 50 primary research publications describe *in vivo* effects of TCDD on the prostate of lab animals, none meaningfully address the question of whether TCDD causes prostate cancer. This information gap existed despite (1) multiple demonstrations that TCDD causes other cancers in lab animals (discussed below), (2) TCDD's classification as a human carcinogen (Huff et al., 1994; IARC, 1997), and (3) evidence that TCDD may cause prostate cancer in men (see Discussion).

Multiple TCDD carcinogenicity studies have been conducted using rodents and most report that TCDD causes cancer in one or more organs, while some show that TCDD can also protect against cancer (Knerr and Schrenk, 2006; Kociba et al., 1978). Whether TCDD is pro- or anti-carcinogenic depends on the organ and/or age at exposure (Knerr and Schrenk, 2006; Murray et al., 2014; Safe et al., 2013). None of these studies report any evidence of prostate cancer (Knerr and Schrenk, 2006). However, because rats and mice that have not been genetically engineered almost never develop prostate cancer spontaneously, or even in response to carcinogens (Grabowska et al., 2014; Sharma and Schreiber-Agus, 1999), the negative results (Knerr and Schrenk, 2006) are of dubious significance. To begin to address this information gap, we conducted experiments to determine effects of TCDD on prostate

cancer in animals genetically engineered to develop this disease. We chose Transgenic Adenocarcinoma of the Mouse Prostate (TRAMP) mice for these experiments.

TRAMP mice, in which the rat probasin gene promoter drives expression of simian virus 40 large T and small t antigens (Tag), are widely used to identify chemicals that stimulate or inhibit prostate cancer development (Grabowska et al., 2014). Two types of prostate tumors develop: papillary tumors, which develop from atypical hyperplasia of Tag (AHT) and which are benign, and neuroendocrine prostate carcinomas (NEPC), which grow rapidly and metastasize (Chiaverotti et al., 2008). The name “TRAMP” (A = adenocarcinoma) is a misnomer because the malignant tumors that develop are NEPCs rather than adenocarcinomas (Chiaverotti et al., 2008; Wang et al., 2011). In men, most prostate tumors are adenocarcinomas (Humphrey, 2012). Clinically, NEPC constitutes only 0.5–2% of prostate cancers initially, but NEPC tumor incidence rises to 10–20% after androgen-deprivation therapy, and most castration-resistant prostate adenocarcinomas display a mixed histology which includes features of neuroendocrine differentiation. NEPC is associated with aggressive disease, metastasis, and poor prognosis (Beltran et al., 2012; Komiya et al., 2009; Shtivelman et al., 2014). NEPC in TRAMP mice, therefore, models an aggressive form of cancer that contributes substantially to prostate cancer mortality in men.

In our initial investigation of how AHR signaling activity affects prostate cancer we discovered that knocking out *Ahr* greatly increased the incidence of NEPC in TRAMP mice (Fritz et al., 2007), presumably because AHR acts as a tumor suppressor. We then observed that a selective AHR modulator (SAHRM), 6-methyl-1,3,8-trichlorodibenzofuran, inhibited prostate tumor metastasis in TRAMP mice (Fritz et al., 2009). The fact that IUL TCDD exposure increased the incidence of prostatic cribriform structures in wild-type mice (Fritz et al., 2005) raised the possibility that such exposure might increase prostate cancer incidence in animals capable of developing this disease. In the present study we used TRAMP mice to test this hypothesis, i.e., that IUL TCDD exposure can increase prostate cancer incidence. We also tested the hypothesis that sustained AHR activation in adulthood by its prototypical agonist TCDD can protect against prostate cancer. Our rationale for hypothesizing a protective effect is that lifelong AHR signaling inactivation had the opposite effect, i.e., it increased NEPC incidence (Fritz et al., 2007). In addition, TCDD had been shown to inhibit proliferation of cultured prostate cancer cells (Jana et al., 1999; Morrow et al., 2004).

We also examined the effects of two low toxicity SAHRMs, indole-3-carbinol (I3C) and its metabolite 3,3'-diindolylmethane (DIM). These cruciferous vegetable constituents inhibit breast, colon, thyroid, and other cancers (Banerjee et al., 2011; Safe et al., 2013; Sarkar and Li, 2004; Weng et al., 2008), and both inhibit proliferation of PC-3 prostate cancer cells (Chinni et al., 2001; Li et al., 2003). We used TRAMP mice to test the hypothesis that DIM and I3C treatment in adulthood can inhibit prostate cancer.

This study is the first to determine whether TCDD affects prostate cancer in animals capable of developing this disease. We found IUL TCDD exposure to be procarcinogenic and TCDD treatment in adulthood to be anti-carcinogenic. In contrast, neither I3C nor DIM had statistically significant protective effects within the constraints of the experiment.

METHODS

Mouse stewardship

All experiments were conducted in accordance with University of Wisconsin Animal Care and Use Committee guidelines and the NIH Guide for the Care and Use of Laboratory Animals. Mice were housed in Udel® polysulphone cages with heat-treated chipped aspen bedding in rooms maintained at $20 \pm 1^\circ\text{C}$ and with 12 h light and dark cycles. Feed (5015 Mouse Diet, PMI Nutrition International, Brentwood, MO) and water were available *ad libitum*.

Mouse husbandry and genotyping

Ahr knockout mice were originally obtained from Dr. Christopher Bradfield (Department of Oncology, University of Wisconsin, Madison, WI), and maintained as heterozygotes on a C57BL/6J and/or albino C57BL/6J background in our lab (subsequently referred to as B6 *Ahr*^{+/-}). The mutant *Ahr* allele has a deleted exon 2 and does not produce any protein (Schmidt et al., 1996). *Ahr* genotype was determined by polymerase chain reaction (PCR) analysis of DNA extracted from ear punches taken prior to weaning; the procedure and primers have been described previously (Benedict et al., 2000).

TRAMP transgenic mice were obtained from Dr. George Wilding (Department of Medicine, University of Wisconsin, Madison, WI) who in turn obtained this strain from Dr. Norman Greenberg (then at the Department of Cell Biology, Baylor University, Houston, TX). These mice were from the same line used to establish the C57BL/6-Tg(TRAMP)8247Ng/J line available through The Jackson Laboratory (Bar Harbor, ME). In our lab, the colony was maintained on a C57BL/6J and/or albino C57BL/6J background (subsequently referred to as B6-TRAMP). TRAMP genotyping measured transgene copy number by quantitative PCR using a Roche LightCycler 1.5 and primers described previously (Greenberg et al., 1995). Keratin 8 primers were used as a loading control (Lin et al., 2002).

B6 *Ahr*^{+/-} mice were mated to B6-TRAMP^{+/+} mice to generate B6 *Ahr*^{+/-}; TRAMP^{+/-} offspring. These offspring were crossed to generate B6 *Ahr*^{+/-}; TRAMP^{+/+} males which were used for subsequent breeding (described below).

Chemicals

TCDD (98% purity) was purchased from Cambridge Isotope Laboratories (Andover, MA). I3C was purchased from Sigma-Aldrich (St. Louis, MO). DIM (99.8% purity) was synthesized in Dr. Stephen Safe's lab at Texas A&M University. *Caution: TCDD is exceptionally toxic and must be used with comprehensive safety precautions.*

IUL TCDD exposure experiment

Ahr mutant females (B6 *Ahr*^{+/-}; TRAMP^{-/-}) were mated to B6 *Ahr*^{+/-}; TRAMP^{+/+} males to generate B6-TRAMP hemizygous offspring with two, one, or zero copies of a functional *Ahr* gene. Dams were given a single oral dose of corn oil vehicle (5 ml/kg) or TCDD (1 µg/kg) on embryonic day (E) 13.5 to initiate IUL exposure. E0.5 is defined as the day after overnight mating. This dose of TCDD does not inhibit prostatic bud formation in C57BL/6J

mice as is observed at higher doses (Abbott et al., 2003). Dosing was on E13.5 to coincide with the onset of testosterone synthesis in fetal mouse testes (Pointis et al., 1979). Litter independence was achieved by keeping only one male of each genotype from any litter. Pups were weaned at 21 days of age and males were kept until 20 weeks of age, when *Ahr* genotype demonstrably alters prostate tumor incidence in B6-TRAMP mice (Fritz et al., 2007), then euthanized by CO₂ overdose. At necropsy, the presence of grossly evident prostate tumors and grossly evident metastases in the adjoining pelvic lymph nodes was determined.

Prostate tumors were identified as follows: NEPC tumors in B6-TRAMP mice start as small, dense, white spheres clearly distinct from the surrounding tissue and are clearly visible even when only 1 mm in diameter. As they grow and vascularize they become darker, and large ones are very dark due to both vascularization and necrosis. NEPC tumors have a clearly distinct boundary from the surrounding tissue, and no glandular structure is visible. The papillary tumors, in contrast, look distinctly different. They retain a glandular appearance, though with thickening of the stroma and/or enlargement of ducts. The boundary between papillary tumors and the surrounding tissue is not nearly as well defined, and these tumors are shaped irregularly. No records were made of the papillary tumors that may well have been present in the IUL TCDD experiment because its purpose was to determine the incidence of prostate cancer, not of benign prostate tumors.

Adult TCDD, I3C, or DIM treatment experiment

FVB/NJ (The Jackson Laboratory) females were mated to B6-TRAMP^{+/+} males to generate B6FVBF1-TRAMP hemizygous males, which develop prostate tumors at a younger age and higher incidence than B6-TRAMP hemizygous males (Chiaverotti et al., 2008). The adult treatment experiment was conducted using B6FVBF1-TRAMP mice, all of which were *Ahr*^{+/+}, because the higher tumor incidence on this background facilitates detection of possible protective effects. At 56 ± 1–2 days of age mice were randomly assigned to one of six treatment groups. Mice were dosed orally three times per week (every other weekday) with corn oil vehicle (5 ml/kg), I3C (10 or 100 mg/kg), or DIM (10 or 100 mg/kg). Mice treated with TCDD were given a single oral loading dose of 5 µg/kg, and a sustained maximum body burden of 5 µg TCDD/kg was achieved by orally administering a weekly maintenance dose of 1.8 µg/kg, based on a *t*_{1/2} of 11 days for whole body elimination of TCDD in C57BL/6J mice (Gasiewicz et al., 1983). Each treatment group consisted of 16–17 males. Body weights were recorded and mice were palpated the first dosing day of each week, under isoflurane anesthesia, until a palpable mass was detected in the prostate, at which time the animal was euthanized by CO₂ overdose. With experience, tumors were detected at roughly 250 mg. Prostate glands, prostate tumors, and grossly visible pelvic lymph node metastases were collected for histological analysis.

Prostate tumor type and histology

Prostate tissue, prostate tumors, and enlarged lymph nodes with suspected metastases were saved from each animal and processed for histological analysis. Tissues were placed in Bouin's fixative overnight at room temperature, incubated briefly in 50% ethanol, stored in 70% ethanol until paraffin-embedded, cut into 5 µm sections, and stained with hematoxylin

and eosin (H&E). Prostate pathology was scored as AHT, papillary tumor, or NEPC based on previously determined criteria (Chiaverotti et al., 2008). All metastases found in this study were located in pelvic lymph nodes, and the cellular morphology and growth pattern of each metastasis resembled that of the primary NEPC found in the same mouse. Additionally, several lymph node sections were stained by immunohistochemistry for Tag; each stained positively for Tag, demonstrating the prostatic origin of the lymph node metastases. Papillary tumors arising from AHT are not known to be metastatic (Chiaverotti et al., 2008), and in this study, no mouse exhibiting only AHT pathology/papillary tumors (no NEPC) was found to have a metastasis. Additional sections were stained by immunohistochemistry for Tag to assess whether transgene expression was altered by treatment with TCDD, I3C, or DIM. The anti-Tag antibody was purchased from Santa Cruz Biotechnology (cat # sc-147) and used as described previously (Schneider et al., 2014).

Statistical analysis

Results were analyzed for statistical significance using MStat 6.1 software (<http://mcardle.oncology.wisc.edu/mstat>). Time to palpable prostate tumor results are shown as Kaplan-Meier plots and were analyzed using log-rank tests; prostate tumor and metastatic incidences were analyzed using Pearson's chi-square test; and age at tumor detection was analyzed by the Wilcoxon Rank Sum test. Statistical significance was set at $p < 0.05$.

RESULTS

IUL TCDD exposure increased NEPC incidence in *Ahr*^{+/+} B6-TRAMP mice

To determine how activation of AHR by TCDD during the earliest stages of prostate development would affect prostate carcinogenesis and metastasis in B6-TRAMP *Ahr*^{+/+}, *Ahr*^{+/-}, and *Ahr*^{-/-} mice, dams were treated with a single oral dose of corn oil vehicle or TCDD (1 µg/kg) on E13.5. There was no apparent effect of *Ahr* genotype or TCDD exposure on litter size (results not shown). Males were aged to 20 weeks of age without additional treatment; consequently, the body burden of TCDD ($t_{1/2}$ =11 d; Gasiewicz et al., 1983) at the time of NEPC tumor development (Chiaverotti et al., 2008; Fritz et al., 2007) would have been negligible. No mice died or needed to be euthanized prior to termination of the study at 20 weeks of age.

Vehicle-exposed mice developed NEPC tumors with incidences similar to those observed previously (Fritz et al., 2007); deleting one or both *Ahr* alleles significantly increased the incidence of NEPC tumors (Fig. 1A). Activation of AHR by IUL TCDD exposure significantly increased NEPC tumor incidence in *Ahr*^{+/+} mice, from 13% in vehicle-exposed mice to 35% in TCDD-exposed mice (Fig. 1A). IUL TCDD exposure did not alter NEPC tumor incidence in the *Ahr*^{-/-} group, indicating that TCDD did not promote NEPC tumor development independently of AHR. Unexpectedly, IUL TCDD exposure did not increase NEPC tumor incidence in *Ahr*^{+/-} mice.

All tumors reported in this experiment were identified as NEPC, and all arose in the ventral prostate lobes. Furthermore, of the mice that developed NEPC tumors, the percentage that

also presented with metastatic disease in the pelvic lymph nodes was not different regardless of *Ahr* genotype or treatment (Fig. 1B).

Treatment in adulthood with TCDD (but not I3C or DIM) prolonged time to palpable prostate tumors in *Ahr*^{+/+} B6FVBF1-TRAMP mice

B6FVBF1 hemizygous TRAMP mice were dosed starting at eight weeks of age with corn oil, the AHR agonist TCDD (initial dose 5 µg/kg and weekly maintenance doses of 1.8 µg/kg), or the SAHRMs I3C and DIM (10 or 100 mg/kg three times per week). All mice were weighed weekly and no significant treatment-related effects on body weight were detected (results not shown).

Five of the 97 mice used in this experiment were euthanized prematurely, due to intestinal blockage, salivary gland tumor, sickness, or error. These losses were not treatment-related. By 31 weeks of age all other mice had developed a palpable mass in the prostate. Median tumor-free survival time for vehicle-treated mice was 20.3 weeks (Fig. 2A–C). The TCDD-treated group had a median prostate tumor-free survival time of 23.7 weeks and remained tumor-free significantly longer than vehicle-treated mice (Fig. 2A). The hazard ratio was 0.44. Mice treated with 10 or 100 mg I3C/kg had median tumor-free survival times of 22.4 and 20.4 weeks, respectively (Fig. 2B) while mice treated with 10 or 100 mg DIM/kg had median tumor-free survival times of 21.1 and 19.1 weeks, respectively (Fig. 2C). Although time to palpable prostate tumor in SAHRM-treated mice appeared to be increased after about 22 weeks of age, the only difference in tumor latency that approached statistical significance was the apparent increase at 10 mg I3C /kg ($p = 0.068$).

Treatment in adulthood with TCDD (but not I3C or DIM) decreased NEPC incidence in *Ahr*^{+/+} B6FVBF1-TRAMP mice

Upon detection of a palpable prostate mass the mice described above were euthanized. Prostate tissue, prostate tumors, and grossly visible metastases to pelvic lymph nodes were collected for histological analysis, which was used to categorize prostate pathology as NEPC, AHT, or papillary tumor. An example of an NEPC tumor is shown in Fig. 3A and 3B, and an example of a papillary tumor is shown in Fig. 3C and 3D; detailed descriptions of each tumor type are found in Chiaverotti et al. (2008). Some degree of AHT was detected in all mice, but the severity of AHT was not further graded. AHT lesions/papillary tumors have not been shown to metastasize (Chiaverotti et al., 2008) and therefore appear to have relatively little impact on the overall health of the mouse.

In vehicle-treated B6FVBF1-TRAMP mice, the palpable mass that triggered euthanasia was identified as an NEPC in 14 of 15 mice (93%). TCDD treatment significantly decreased NEPC incidence, to 50% (Fig. 4). To explore the possibility that this protective effect might result from possible inhibition by TCDD of TRAMP transgene expression, immunohistochemistry for Tag was conducted (see Supplementary Fig. 1 for examples). NEPC tumors, papillary tumors, and non-tumorous prostate tissue all stained variably, and no detectable differences in staining patterns were observed among any of the treatment groups (results not shown). These observations, though not definitive, suggest that the protective effect of TCDD against NEPC development was probably not caused by any

major reduction in the ability of TCDD-treated mice to express Tag in the cells in which these carcinomas originated.

In contrast to the clear anti-carcinogenic effect of TCDD, effects of I3C and DIM on NEPC incidence, if any, were minor. Each SAHRM slightly reduced NEPC incidence from the 93% seen in corn oil-treated mice (Fig. 4), yet the only difference that even began to approach statistical significance was the 73% incidence in mice given 10 mg I3C/kg ($p = 0.14$). NEPC incidence in mice treated with 100 mg I3C/kg, 10 mg DIM/kg, and 100 mg DIM/kg was 79, 80, and 88%, respectively.

Metastasis of NEPC to pelvic lymph nodes of *Ahr*^{+/+} B6FVBF1-TRAMP mice was not significantly affected by TCDD, I3C, or DIM treatment in adulthood

Of the 15 vehicle-treated mice, 14 of which developed NEPC, three presented with grossly visible metastasis in a pelvic lymph node at necropsy. No metastases were seen in any mouse treated in adulthood with either TCDD or 100 mg I3C/kg (Fig. 4). In contrast, no protection against metastasis was seen in DIM-treated mice, suggesting that I3C itself and/or I3C metabolites other than DIM were responsible for the anti-metastatic effect of I3C. The incidence of metastasis per treatment group is an index of overall disease status, one dependent on both the incidence of NEPC tumor formation and incidence of metastasis in mice with NEPC. The apparent protective effects of TCDD and of the high I3C dose approached statistical significance when considering all mice ($p = 0.06$ and $p = 0.08$, respectively) or only mice with NEPC ($p = 0.16$ and $p = 0.10$, respectively). Nevertheless, no treatment significantly ($p < 0.05$) altered the incidence of pelvic lymph node metastasis or the likelihood of NEPC metastasizing to pelvic lymph nodes (Fig. 4).

Age at which palpable NEPC tumors were detected in *Ahr*^{+/+} B6FVBF1-TRAMP mice was not affected by TCDD, I3C, or DIM treatment in adulthood

None of the treatment regimens altered the age at which palpable NEPCs were detected (Fig. 5). Within the TCDD, 100 mg I3C/kg, and 100 mg DIM/kg groups the age at which a palpable NEPC was detected was significantly less than the age at which a palpable papillary tumor was detected (Fig. 5). In the vehicle-treated control group the oldest mouse in which a palpable tumor was detected was the only one where the tumor was papillary and not NEPC. In the 10 mg/kg I3C and DIM groups age at detection was close to ($p = 0.08$ and 0.07 , respectively) but was not significantly different between palpable NEPCs and palpable papillary tumors. Across all treatment groups, the age at which a palpable NEPC was detected, 20.2 weeks (19.2–21.2, 95% CI), was significantly less than age at detection of palpable papillary tumors (26.3 weeks; 25.1–27.5, 95% CI) (Fig. 5). This suggests that TCDD treatment in adulthood increased prostate tumor-free survival time (Fig. 2) by reducing the incidence of NEPCs rather than by prolonging time to NEPC development (Figs. 4, 5).

DISCUSSION

IUL TCDD exposure causes an AHR-dependent increase in prostate cancer incidence in B6-TRAMP mice

The key new finding from the IUL TCDD exposure experiment is that TCDD significantly increased the incidence of NEPC tumors (though not metastasis) 2.6-fold in *Ahr*^{+/+} B6-TRAMP mice (Fig. 1). This is the first time any potent and persistent AHR agonist has been shown to increase the incidence of any form of prostate cancer in any laboratory animal model. As expected, TCDD had no effect on either NEPC tumor incidence or metastasis in *Ahr*^{-/-} B6-TRAMP mice, demonstrating that its carcinogenic effect is AHR-mediated. Failure of TCDD to increase NEPC tumor incidence in *Ahr*^{+/+} B6-TRAMP mice is surprising in light of its clear effect when both *Ahr* alleles were present; further research would be needed to learn why. While numerous publications describe a wide range of effects of IUL TCDD exposure on the prostate, the significant increase in prostate cancer incidence we observed is the first demonstration for TCDD of a fetal basis for adult prostate disease.

Multiple experiments demonstrate that TCDD treatment in adulthood can be both pro- and anti-carcinogenic (Knerr and Schrenk, 2006; Kociba et al., 1978), but we are aware of only two previous carcinogenicity studies in which TCDD exposure was via maternal treatment. Both publications (Brown et al., 1998; La Merrill et al., 2010) reported that TCDD affected the development of mammary tumors in mutagen-treated animals, yet neither demonstrated that cancer incidence per litter (or even per animal) was significantly affected. Our experiment with TRAMP mice is the first to demonstrate a statistically significant ($p < 0.05$), litter-independent effect of maternal TCDD treatment on cancer incidence in any organ. Additional research would be needed to elucidate the mechanisms responsible for this effect, including the extent to which the increased incidence of NEPC tumors is due to effects of TCDD itself versus consequences of displacement from AHR of endogenous ligands that may protect against tumorigenesis.

TCDD treatment in adulthood decreases prostate cancer incidence in B6FVBF1-TRAMP mice

TRAMP transgene expression occurs primarily from puberty onward (Greenberg et al., 1994). Because lifelong AHR signaling inactivation (via *Ahr* knockout) greatly increased NEPC incidence in TRAMP mice (Fritz et al., 2007), we hypothesized that activation of AHR signaling pathways in adulthood would protect against prostate cancer. B6FVBF1-TRAMP mice treated with TCDD starting at eight weeks of age (5 µg TCDD/kg maximum body burden) had significantly longer time to palpable tumor formation than corn oil-treated controls (Fig. 2A). More importantly, TCDD treatment in adulthood significantly reduced the incidence of NEPC found at necropsy (Fig. 4). The fact that TCDD had no effect on latency for NEPC tumors (Fig. 5), and the absence of histologically detectable NEPC from TCDD-treated mice euthanized due to a benign (papillary) prostate tumor, strongly suggests that the anti-carcinogenic effect of TCDD is due to prevention of NEPC formation rather than a delay in NEPC tumor appearance. This is the first report that any potent and persistent AHR agonist can protect against any form of prostate cancer in any animal model.

The absence of detectable pelvic lymph node metastasis from TCDD-treated mice with NEPC (0 of 8) was not significantly different ($p = 0.16$) from the incidence of metastasis in control mice with NEPC (3 of 14). Yet when all mice in these treatment groups are considered the complete protection against metastasis by TCDD was very close to being statistically significant ($p = 0.06$). These observations, particularly in light of our previous finding that 6-methyl-1,3,8-trichlorodibenzofuran significantly inhibited NEPC metastasis in TRAMP mice (Fritz et al., 2009), suggest that AHR signaling pathway activation may protect against prostate tumor metastasis. Additional research would be needed to determine how long the apparent protection against visibly obvious pelvic lymph node metastasis persists, and whether metastasis to other sites is also inhibited.

The mechanisms by which TCDD treatment in adulthood protected against prostate cancer remain unknown. Nevertheless, this finding indicates that research to determine if TCDD can protect against NEPC in other model systems, and against other forms of prostate cancer, is warranted. An experiment using mice reported to develop prostate adenocarcinomas is in progress (Moore et al., 2016). Finally, while TCDD itself is too toxic to be used to treat humans, the protective effect we found gives further impetus to continued exploration of whether low toxicity SAHRMs can reduce NEPC incidence. Results of testing two such chemicals are discussed below.

I3C and DIM treatment in adulthood had minimal effects on B6FVBF1-TRAMP mice

We gave I3C and DIM chronically, at two dose levels each, to B6FVBF1-TRAMP mice starting in young adulthood. Time to palpable tumor appeared to be increased in mice older than about 22 weeks of age (Fig. 2B,C); the percentage of mice with NEPC was slightly reduced in all four treatment groups; and no metastases were seen at the high dose of I3C (Fig. 4). However, none of these possible effects was statistically significant. If the trends we observed are evidence of real effects, it would take substantially more mice per treatment group than the small number we used ($n = 16-17$) to demonstrate statistical significance. In short, we found evidence but no “proof” (i.e., $p < 0.05$) that I3C and DIM may partially protect TRAMP mice against prostate cancer.

After our experiments were essentially completed, other labs reported finding significant protection by I3C and DIM against prostate cancer in B6-TRAMP mice (Cho et al., 2011; Wu et al., 2012, 2013). Our experiments differ in several ways. We used TRAMP mice on a B6FVBF1 background, where NEPC tumor development is far more aggressive (Chiaverotti et al., 2008; Gingrich et al., 1999). None of the other studies identified any of the prostate tumors as NEPC (the only prostate cancer present in TRAMP mice; Chiaverotti et al., 2008; Wang et al., 2011), or confirmed metastasis histologically. And ours is the only one to include histological examination of the ventral prostate, the site in TRAMP mice where NEPC development is most prevalent (Chiaverotti et al., 2008; Wang et al., 2011). While all four studies (including ours) present evidence for chemoprevention, none actually shows a statistically significant reduction in prostate cancer incidence¹. Whether any of the observed

¹Palpable tumor and metastasis incidences were said to be significantly reduced by I3C (Wu et al., 2012) and DIM (Wu et al., 2013), but we find no statistically significant effects using the same statistical procedure.

responses to I3C and DIM are due to their effects as SAHRMs or to other mechanisms would require additional experiments using *Ahr*^{-/-} TRAMP mice.

Implications for human health

Our observation that IUL TCDD exposure caused a statistically significant 2.6-fold increase in the incidence of NEPC in TRAMP mice raises the possibility that older men might be at increased risk of NEPC if their mothers had elevated body burdens of TCDD (or other potent and persistent AHR agonists) when they were pregnant and/or nursing. We are not aware of any studies that can yet address this possibility. The men born to women exposed to TCDD from an industrial accident in Seveso Italy are under 40 years of age (Wesselink et al., 2014), and the oldest men exposed to TCDD via maternal Agent Orange exposure are now only 50 years old (Institute of Medicine, 2016), well before most prostate cancers are detected. In light of our findings with TRAMP mice, we suggest that epidemiological investigations on any appropriate cohort examine not only (1) total prostate cancer incidence but also (2) prostate cancer mortality, (3) the incidence of NEPC at diagnosis, (4) the incidence of NEPC after androgen deprivation therapy (which greatly elevates NEPC incidence) and (5) neuroendocrine characteristics of late-stage adenocarcinomas.

Multiple epidemiology studies examine possible connections between TCDD exposure in adulthood and prostate cancer. Whether a convincing association exists is a contentious issue. Many publications present evidence of an increased incidence while many others find no association (Chang et al., 2014; Leng et al., 2014). Common study weaknesses include lack of individual exposure data, inadvertent or potentially deliberate errors in self-reporting of exposure status, possible differences in surveillance and therefore detection rates, study completion before most participants were old enough to develop clinical prostate cancer, etc. To compound matters, studies on the reliability of self-reporting of exposure as a useable index of TCDD exposure reached opposite conclusions (Centers for Disease Control Veterans Health Studies, 1988; Li et al., 2013). Most studies have been reviewed recently (Chang et al., 2014), though with a disproportionate emphasis on limitations of the studies that find associations. Amidst the conflicting claims, the Institute of Medicine finds “limited or suggestive evidence of an association between exposure to herbicides (including Agent Orange) and prostate cancer” (Institute of Medicine, 2016).

Obviously, the reduction we observed in NEPC incidence in TRAMP mice given TCDD in adulthood does not model an unchanged-or-increased prostate cancer incidence in men exposed as adults. Yet all epidemiology studies combined report what type of prostate cancer was found in only one man (Thiess et al., 1982), and collectively they report far less about mortality from this disease than its incidence. A key unanswered (and generally unasked) question is whether TCDD exposure in adulthood changes the 12–16% probability that a prostate cancer diagnosis will lead to a prostate cancer fatality. The incidence of NEPC at diagnosis and in untreated men is so low (0.5–2%; Shtivelman et al., 2014) that even if TCDD has the same effects in men as it does in TRAMP mice, a 50% reduction in tumor incidence would affect relatively few men and would be very difficult to detect. But if TCDD (or an appropriate SAHRM) were to halve the incidence of NEPC following androgen-deprivation therapy, and/or halve the incidence of adenocarcinomas with

neuroendocrine characteristics during the final stages of prostate cancer, such effects would reduce disease severity and prolong survival for large numbers of men. Reexamination of prostate histopathology from several of the epidemiology studies could well reveal such effects of TCDD if they are present. And if these pathologies are lessened in TCDD-exposed men, this would strongly suggest that relatively non-toxic SAHRMs that activate prostatic AHR signaling could be used chemotherapeutically to help protect men against NEPC.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

AHT	atypical hyperplasia of Tag
AHR	aryl hydrocarbon receptor
DIM	3,3'-diindolylmethane
I3C	indole-3-carbinol
IUL	<i>in utero</i> and lactational
NEPC	neuroendocrine prostate carcinoma
SAHRM	selective AHR modulator
Tag	simian virus 40 large T and small t antigens
TCDD	2,3,7,8-tetrachlorodibenzo- <i>p</i> -dioxin
TRAMP	Transgenic Adenocarcinoma of the Mouse Prostate

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HIGHLIGHTS

- TRAMP mice model aggressive neuroendocrine prostate carcinomas in men.
- *In utero*/lactational TCDD exposure raised prostate cancer incidence in TRAMP mice.
- TCDD treatment in adulthood lowered prostate cancer incidence in TRAMP mice.
- No significant protection was seen in TRAMP mice given I3C or DIM in adulthood.
- This is the first report that TCDD alters prostate cancer incidence in lab animals.

Fig. 1. IUL TCDD exposure increased neuroendocrine prostate carcinoma (NEPC) tumor incidence in B6-TRAMP mice containing both *Ahr* alleles. Male B6-TRAMP mice containing zero, one, or two functional copies of the *Ahr* gene were exposed *in utero* and via lactation to a single oral maternal dose of corn oil (vehicle, 5 ml/kg) or TCDD (1 µg/kg) on E13.5. Mice were euthanized at 20 weeks of age, and the incidence of NEPC tumors and incidence of metastasis to pelvic lymph nodes were determined. A) IUL TCDD exposure significantly increased the incidence of NEPC tumors in *Ahr*^{+/+} mice (asterisk). Loss of one or both

copies of the functional *Ahr* gene significantly increased NEPC tumor incidence in vehicle-exposed control mice (daggers), but IUL TCDD exposure did not alter NEPC tumor incidence in *Ahr*^{+/-} or *Ahr*^{-/-} mice. Numbers within each bar are the number of mice observed to have an NEPC tumor divided by the total number of mice. B) The incidence of pelvic lymph node metastasis in mice with NEPC tumors was not significantly affected by treatment or genotype. Numbers within each bar are the number of mice with a pelvic lymph node metastasis divided by the number of mice with a primary NEPC tumor.

Fig. 2.

Treatment with TCDD in adulthood prolonged prostate tumor-free survival. Male B6FVB/F1-TRAMP mice were treated beginning at eight weeks of age with either corn oil, an AHR agonist (TCDD), or a selective AHR modulator (I3C or DIM) for the duration of the study. Mice were palpated weekly and were considered tumor-free until a palpable mass was detected in the prostate. Control mice were dosed orally three times a week with corn oil vehicle (5 ml/kg). A) TCDD-treated mice were given a single oral loading dose (5 µg/kg) followed by a weekly maintenance dose (1.8 µg/kg). TCDD significantly prolonged tumor-

free survival (asterisk). Mice treated with B) I3C (10 or 100 mg/kg) or C) DIM (10 or 100 mg/kg) were orally dosed three times a week. Neither I3C nor DIM significantly altered tumor-free survival.

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Fig. 3.

Tumor types in prostate lobes of B6FVBF1-TRAMP mice as revealed by H&E staining. A representative example of an NEPC tumor is shown in panel A. These tumors are characterized by solid sheets of cells; prostate ducts with mild atypical hyperplasia of Tag (AHT; arrow) are present on the left. In a higher magnification image of the NEPC tumor (panel B), three prostate ducts with mild AHT (arrows) are surrounded by the tumor. A representative example of an isolated papillary tumor is shown in panel C, with a higher magnification image in panel D. These tumors develop from AHT and are benign.

Fig. 4.

Treatment with TCDD in adulthood protected against NEPC formation in B6FVBF1-TRAMP mice. Mice were treated as described in the legend to Fig. 2 until a palpable prostate mass was detected. Prostate tissue, prostate tumors, and grossly visible metastases to pelvic lymph nodes were then collected for histological analysis. Prostate pathology was categorized as AHT, papillary tumor, or NEPC based on examination of H&E-stained tissue sections. Numbers at the top of each gray bar are the number of mice with an NEPC tumor divided by the total number of mice assessed. TCDD treatment significantly decreased the incidence of NEPC tumors (asterisk). Numbers at the bottom of each black bar are the number of mice with pelvic lymph node metastasis divided by the number of mice with an NEPC tumor. No statistically significant changes were detected in the incidence of metastasis for any treatment group.

Fig. 5.

Latency to palpable NEPC tumors was shorter than latency to palpable papillary tumors and was unaffected by treating B6FVBF1-TRAMP mice in adulthood with TCDD, I3C, or DIM. Experimental conditions were as described in the legend to Fig. 4. Results are displayed as the ages (mean $\pm$ SE) at which palpable NEPC and papillary tumors (as later determined by histopathology) were detected; the N value for each category is shown at the base of each bar. Treatment with TCDD, I3C, or DIM did not change the age at which palpable NEPCs were detected. However, in the TCDD and high dose I3C and DIM treatment groups papillary tumors were palpable at a significantly later age (asterisks) than were NEPC tumors.