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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-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.

1. 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 >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

Abbreviations: AHT, atypical hyperplasia of Tag; AHR, aryl hydrocarbon receptor; DIM, 3,3′-diindolylmethane; I3C, indole-3-carbinol; IUL, *in utero* 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-*p*-dioxin; TRAMP, Transgenic Adenocarcinoma of the Mouse Prostate.

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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 pro-carcinogenic 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.

2. Methods

2.1. 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 ± 1 °C and with 12 h light and dark cycles. Feed (5015 Mouse Diet, PMI Nutrition International, Brentwood, MO) and water were available ad libitum.

2.2. 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).

2.3. 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.*

2.4. 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 CO_2 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,