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Fetal Mouse *Cyp1b1* and Transplacental Carcinogenesis from Maternal Exposure to Dibenzo[*a,l*]pyrene

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Abstract

Dibenzo[*a,l*]pyrene (DBP) is among the most potent carcinogenic polycyclic aromatic hydrocarbons (PAHs). Previously, we showed that DBP administration to pregnant mice resulted in high mortality of offspring from an aggressive T-cell lymphoma. All mice that survive to 10 months of age exhibit lung tumors with high multiplicity. Recombinant cytochrome P450 (cyp) 1b1 from mice and the homolog 1B1 in humans exhibit high activity toward the metabolic activation of DBP. Targeted disruption of the *cyp1b1* gene protects against most DBP-dependent cancers. Mice heterozygous (het) for the disrupted *cyp1b1* allele were used to examine the effect of *cyp1b1* gene dosage on DBP transplacental carcinogenesis. Dams were treated with 1 or 15 mg/kg of DBP or 50 mg/kg of benzo [a]pyrene (BP). *Cyp1b1*-null offspring did not develop lymphoma, whereas, wild-type and heterozygous siblings, born to dams given the high dose of DBP, exhibited significant mortalities between 10–30 weeks of age. At 10 months, all groups had lung adenomas or carcinomas (9.5, 40.3, 25.6 and 100% incidences for controls, BP, 1 and 15 mg/kg DBP, respectively). *Cyp1b1* status did not alter BP-dependent carcinogenesis. At 1 mg/kg DBP, *cyp1b1* status altered the incidence of lung tumors (19.0, 27.8 and 28.6% for nulls, heterozygous and wild-type, respectively). At 15 mg/kg, tumor multiplicities in *cyp1b1* wild-type (9.3) and heterozygous (9.5) offspring were nearly twice that of *cyp1b1*-null siblings (5.0). These data confirm that *cyp1b1* bioactivation of DBP occurs in fetal target tissues, following transplacental exposure, with the thymus and lung as primary and secondary targets, respectively.

Keywords

Cytochrome P4501B1; transplacental cancer; Polycyclic Aromatic Hydrocarbons; Lymphoma; Lung Cancer

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INTRODUCTION

The fetus and infant are at increased risk, relative to adults, upon exposure to many environmental chemicals. Yet, only exposures to ionizing radiation and diethylstilbesterol to pregnant women have been sufficiently well documented as causing cancer in their children [1,2]. However, an increasing number of carcinogens have been demonstrated to be effective transplacentally in animal models including arsenic [3], 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone [4], 3'-azido-3'-deoxythymidine [5] and cooked food mutagens such as 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine [6] and polycyclic aromatic hydrocarbons (PAH; ref. 7). These data, in addition to the fact that human epidemiological studies correlate maternal chemical exposure with cancer in children [1,2], highlight the importance of understanding the mechanism(s) of transplacental carcinogenesis.

We recently developed a mouse model of transplacental carcinogenesis in which maternal exposure to the potent PAH, DBP, resulted in high mortality in offspring at a relatively young age from a T-cell lymphoma. At 10 months, the offspring exhibited a 100% incidence of lung adenomas and carcinomas and most of the males had liver lesions as well [8–10].

Recombinant mouse *cyp1b1* and human CYP1B1 have the highest activity toward conversion of DBP to DBPDE, a “fjord” region diol-epoxide thought to be responsible for the high mutagenic and carcinogenic potency of DBP [11,12]. *In vivo* evidence also shows that disruption of the *cyp1b1* gene protected adult mice from DBP cancer at most sites [13,14].

We tested the hypothesis that DBP is transplacentally available to the fetus and is bioactivated by *cyp1b1* in thymus and lung. *Cyp1b1* is expressed at relatively high levels in both fetal thymus and lung [15,16]. The finding that fetal thymus in humans exhibits the highest CYP1B1 expression of any tissue [16] highlights the relevance of this model for human exposures. We bred *cyp1b1* heterozygotes (*cyp1b1*^{+/-}) so that all litters should have a 1:2:1 ratio of wild-type/heterozygote/null in order to assess fetal *cyp1b1* gene dosage on DBP transplacental carcinogenesis. *Cyp1b1* knockouts were completely resistant to DBP transplacental lymphoma mortality. In lung, *cyp1b1* genotype influenced tumor multiplicity only at the high dose of DBP, whereas tumor burden was unaffected by genotype in all other groups.

These results confirm that DBP transplacental lymphoma in mouse is due to *cyp1b1* bioactivation in fetal thymus. This finding gains further significance in human fetal risk assessment considering that, in humans, CYP1B1 expression is greater in thymus than in mouse during late stages of ontogeny.

METHODS

Mouse husbandry, carcinogen treatment, necropsy and pathology

Cyp1b1 null mice on both a B6129F1 and a 129 genetic background were obtained from the National Cancer Institute and bred to produce female heterozygote on a B6129F1 background and male heterozygote on a completely 129 genetic background. All colonies were housed in the Laboratory Animal Resource Center at Oregon State University at 20 ± 1° C and 50 ± 10% humidity and a light/dark cycle of 12 hr in micro-isolator cages with CareFRESH bedding (Absorption Corp., Bellingham, WA). During breeding, gestation and lactation, mice were fed powdered AIN93G diet (Research Diets, New Brunswick, NJ) *ad libitum*. When these mice were 8 weeks-old we bred the B6129F1 *cyp1b1*^{+/-} females to 129 *cyp1b1*^{+/-} males. Gestation day 0 was established by appearance of the vaginal plug. Offspring were fed pelleted AIN93G diet for the first three months and then AIN93M diet (Research Diets) *ad lib* until euthanized. On the 17th day of gestation, pregnant mice were treated with vehicle (corn oil, 5 ml/kg b.w.), 1 or 15 mg/kg DBP or 50 mg/kg benzo[*a*]pyrene (Midwest Research Inst., Kansas City, MO)

in corn oil by gavage. To monitor the health status of the mice, sentinels were housed in the colony and tested for viral or bacterial pathogens and parasites. Upon signs of morbidity, pain or distress the mice were euthanized with an overdose of CO₂, exsanguinated, and necropsied. At 10 months of age, any surviving mice were euthanized and necropsied. All procedures for treatment, housing and euthanasia of the mice used in this study have been approved by the Oregon State University Institutional Animal Care and Use Committee and successfully carried out by the investigators [8–10]. Tissues were fixed in 10% formalin, stained with H&E and analyzed by light microscopy. We previously identified the lymphoma to be a T-cell lymphoblastic lymphoma and the lung tumors as hyperplasia, adenoma, adenoma with progression, and carcinoma; the liver tumors as foci and adenoma [8].

Genotyping of mice for the *Cyp1b1* allele

At necropsy, an 8 mm ear-punch was collected and lysed overnight at 55°C in 100 µl of DirectPCR Lysis Reagent containing proteinase K (Viagen Biotech Inc., Los Angeles, CA), followed by 45 min at 85°C. The lysis reaction was centrifuged for 10 sec and used directly in a PCR reaction with allele-specific primers (Table 1) to permit one-tube genotyping for the wild-type *cyp1b1* allele. A 25 µl PCR reaction contained 1× GeneAmp buffer and 0.25 units AmpliTaq Gold polymerase (Promega Co., Madison, WI), 0.2 mM of each dNTP, 0.2 µM of each primer and 2 µl DNA. PCR cycling conditions were an initial 5 min 95°C enzyme activation step, followed by 35 cycles of: 30 sec at 95°C to denature the DNA; 30 sec at 55°C for primer annealing; 45 sec at 72°C for extension. A final cycle with a further 10 min extension at 72°C concluded the reaction. PCR products were separated on Novex® 8% TBE gels (Invitrogen Technologies, Carlsbad, CA). *Cyp1b1* heterozygotes yielded two PCR products of 365-bp and 460-bp (NEO), respectively. Wild-type *cyp1b1* mice had a single product of 365-bp. A molecular weight ladder of *MspI* cut pBR322 DNA (New England Biolabs, Ipswich, MA) and ethidium bromide (Sigma Chemical Co., St. Louis, MO) was used to stain the DNA, followed by UV visualization.

Statistical Analysis

Litter sizes per dam were compared between treatments using the exact Kruskal-Wallis test (SAS 9.1.3 Npar1way procedure). Because the experimental unit is the pregnant female, litters were accounted for in modeling of individual offspring data. Birth weights were compared between treatments using linear mixed models with litters as a random factor (SAS mixed procedure). Survival curves were compared between *cyp1b1* groups using a robust score test in cox proportional-hazard regression with litters as clusters in the model (refs. 8,17; using S-plus 7.0).

Lung tumor incidences in the three treatment groups (benzo[*a*]pyrene, DBP1 and DBP15) were each compared to the incidence in the control group using quasilielihood logistic regression where the observed variation between litters is used to account for overdispersion (SAS genmod procedure). For comparing tumor incidences between the *cyp1b1* gene dosages within the DBP1 treatment group, both logistic regression ignoring litters and quasilielihood logistic regression for grouped binomial data (accounting for litters) were used and both gave very similar results (SAS genmod procedure). Offspring tumor multiplicity was compared between *cyp1b1* gene dosages within the DBP15 treatment group with a linear mixed model fit to the log transformed tumor count for each offspring. Because there was large variability in the *cyp1b1* group differences from litter to litter ($p=0.0011$) the mixed model included a random litter-by-*cyp1b1* interaction (SAS Mixed procedure). Residuals (either simple or deviance residuals) were examined and found reasonable for each linear model and generalized linear model fit.

RESULTS

Treatments of dams with DBP did not result in any significant maternal or fetal toxicities. The PAHs had no significant effect on litter size ($p=0.43$, Kruskal-Wallis test) or birth weight ($p>0.5$) (data not shown). These results are consistent with previous studies in which no toxicity was observed at the 15 mg/Kg DBP maternal dose [8–10].

As previously observed, offspring born to mothers treated with 15 mg/kg DBP exhibited lymphoma-dependent mortality between 10–30 weeks of age (Figure 1A). In support of our hypothesis concerning the role of fetal *cyp1b1* in DBP transplacental carcinogenesis, we observed a clear pattern of decreasing survival times with increasing *cyb1b1* gene dosage (Figure 1B). None of the *cyp1b1*-null mice succumbed to the DBP transplacental lymphoma mortality. Siblings with both wild-type alleles exhibited sensitivity comparable to what was previously observed, whereas, mice heterozygous for wild-type *cyp1b1* allele exhibited a survival curve almost exactly intermediate between the nulls and the wild-type siblings. There is strong evidence of a trend consisting of decreasing probability of survival with increasing *cyp1b1* gene dosage ($p=0.0002$ score test ignoring litters and $p=0.018$ robust score test with litters as clusters). The number of litters and offspring, as well as their genotype ratio, is shown in Table 2.

We included a lower dose of DBP in case, at the high dose, *cyp1b1* is saturated and *cyp1a1* and *cyp1a2* could contribute. Again, this is doubtful as previous studies have shown *cyp1a* enzyme to be expressed at very low levels in fetal tissue during the final trimester [5,16,18], although it is still capable of responding to induction. The benzo[a]pyrene group was included as a negative control. Benzo[a]pyrene, like DBP, is a potent carcinogenic PAH, but is bioactivated primarily by *cyp1a1* and *cyp1a2*, rather than by *cyp1b1* [12,19,20]. For this reason, we expected to see few, if any, lymphomas and no significant difference between siblings of different *cyp1b1* genotypes with respect to benzo[a]pyrene-dependent transplacental carcinogenesis.

Neither benzo[a]pyrene nor the low dose of DBP produced any lymphoma-dependent mortality over the time-course examined (Figure 1A). All treatments produced higher incidence of lung adenomas and carcinomas than the spontaneous rate in controls and the rates in the BP ($p=0.01$, quasilielihood F-test) and DBP15 ($p<0.0001$) groups were significantly higher than the incidence in the controls at 10 months of age (Table 3). Due to overdispersion between litters, DBP1 is not significant ($p=0.11$) compared to controls. The observed incidences are consistent with there being an impact of genotype, but the differences are too small to be statistically significant. Interestingly, all mice born to mothers receiving 15 mg/kg DBP had 100% incidence of lung tumors at the end of the study, irrespective of genotype. However, only at the high dose of DBP was there a *cyp1b1* genotype-dependent impact lung tumor multiplicity (Figure 2; Table 3). Mice of the wild-type and heterozygous genotype had nearly double the numbers of tumors per mouse compared to the null offspring (9.3, 9.5, and 5.0; Table 3). After adjusting for litter variation (random litter-by-*cyp1b1* effect), there was some evidence of a *cyp1b1* effect ($p=0.08$).

DISCUSSION

Childhood cancer accounts for < 1% of all cancers but these 12,400 cases annually in the U.S. translate to the second leading cause of death (2,300) after accidents, in children in the U.S. [1,2]. Leukemias and lymphomas are the most common type of childhood cancer, followed by tumors of the nervous system. The etiology of 80–90% of childhood cancers is unknown [1, 21–23], but considerable evidence exists for maternal exposure to environmental chemicals as contributors to development of childhood leukemias and lymphomas [24–33]. The two

environmental *in utero* exposures that have definitively been linked with increased cancer in children and young adults are diethylstilbestrol and ionizing radiation [1]. In addition, it may well be that exposure *in utero* or during infancy may not result in cancer during childhood, but may predispose the individual to cancers developing later in life. Indeed, studies with DES in rodent models have shown that transplacental exposure significantly enhanced the risk of development of tumors in older animals upon exposure to chemical carcinogens [2]. In many of the rodent transplacental cancer models, *in utero* exposure produces cancers in middle aged adult offspring (reviewed in ref.7), that is, the significance of this research is not limited to childhood cancers.

With respect to the suitability as a model for human transplacental carcinogenesis, mice have turned out to be an excellent model in the case of DES [2]. Many environmental chemicals for which there are significant human exposures, are transplacental carcinogens in rodents including the food mutagen, 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine [6], benzo[*a*]pyrene [34], and the tobacco-specific carcinogen 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone [4]. The infant mouse has also been used as a cancer model with enhanced sensitivity compared to the adult for a number of chemical carcinogens such as diethylnitrosamine, PAHs and aflatoxin B₁ [35–37]. The sensitivity of the infant mouse to chemical carcinogens is relevant for the present discussion as we administered a very lipophilic carcinogen during late pregnancy in our model. We have recently performed a cross-fostering experiment to estimate how much of the DBP-dependent lymphoma mortality was due to *in utero* exposure and how much to exposure through nursing (21 days). The results indicate a slightly greater contribution from the much shorter *in utero* exposure (data not shown).

Currently, over 320,000 Americans have leukemia, lymphoma or myeloma with an estimated 135,500 new cases annually and 85,580 deaths [38]. Although the etiology of the majority of such cancers is unknown, chemical exposures have been identified as one definitive risk factor (reviewed in ref. 1). The mouse can serve as a useful model for human lymphoma [39] with the recognition that certain lymphoma pathologies are species-specific and molecular markers may be distinct. A classification scheme [40] devised under the direction of the Mouse Models of Human Cancers Consortium (MMHCC) has been published and can be used for comparison to the WHO classification of lymphomas [39]. We have, for the first time in any animal model, documented that exposure of pregnant mice to a single dose of a potent PAH, DBP, results in a very aggressive T-cell lymphoma in the offspring beginning at 10 weeks of age [8–10]. Our hypothesis is that maternal transfer to the fetus results in cyp1b1-dependent bioactivation to DBP-(–)-*anti*-(11*R*,12*S*,13*S*,14*R*)-dihydrodiol epoxide and other reactive metabolites of DBP in the thymus producing a number of DNA-adducts. This model should recapitulate human fetal exposure to PAHs from maternal diet and airborne particles including direct and second-hand tobacco smoke as a causative factor in leukemias/lymphomas in children and young adults.

We hypothesize that cyp1b1 bioactivation of DBP occurs in fetal target tissues, primarily thymus with the secondary target of lung and that this pathway plays a greater role than other potential means of bioactivation of PAHs, such as peroxidation [41] and the aldo-keto reductase pathways [42], both of which produce oxidative damage to DNA. In previous studies, utilizing DBP or 3-methylcholanthrene in models, in which either the dam or the fetus were Ahr “non-responsive” or “responsive”, it was shown that the responsive dam reduced the risk to the fetus apparently by decreasing the bioavailability through enhanced maternal metabolism and clearance [7,8].

However, the issue of fetal versus maternal metabolism in PAH-transplacental carcinogenesis is not settled. By utilizing crosses of *cyp1b1* heterozygote on the same genetic background as our DBP-transplacental model, we were able to demonstrate that DBP-dependent

transplacental lymphoma mortality was dependent upon the presence of at least one wild type *cyp1b1* allele and there was a very tight correlation between *cyp1b1* gene dosage and mortality. In the lung, the impact of the *cyp1b1* expression was apparent, but not as marked, possibly due to some contribution from CYPs in the 1a sub-family. It should be kept in mind, however, that lung tumor incidence and multiplicity determined at 10 months of age in the high dose DBP group is complicated from a statistical standpoint by the earlier lymphoma deaths. In offspring born to mothers treated with benzo[*a*]pyrene or low dose DBP, the statistical analysis is cleaner as we are not dealing with only a population of lymphoma survivors.

The results clearly show that *cyp1b1* genotype does not influence benzo[*a*]pyrene-dependent lung cancer in this model. Lung cancer is the major leading cause of cancer-related deaths for both sexes in the U.S. with 173,700 new cases in 2004 and 160,400 deaths. The five-year survival rate is poor for lung cancer (15%) and any prevention approach would be beneficial. CYP1B1 has been found to be, at least in part, under epigenetic control [43,44] and it has been suggested that CYP1B1 would make an excellent target for prevention strategies [45]. In our previous studies, we demonstrated that the addition of indole-3-carbinol, green tea or caffeine to the maternal diet during pregnancy and nursing significantly reduced lung cancer multiplicity in 10-month-old offspring from mothers treated with 15 mg/kg DBP [9,10]. The results presented in this study provide further evidence that fetal *cyp1b1* plays an important role in DBP transplacental carcinogenesis, primarily with lymphoma and to a lesser degree with lung tumors. We conclude that fetal *cyp1b1* could be a target for cancer prevention and additional work with specific inhibitors, such as 2,3,4,5-tetramethoxystilbene may prove fruitful in that respect. In addition, as with most other CYPs in humans, 1B1 exists in the population as a number of non-synonymous allelic variants [19,46–49]. The Swiss Protein entry for human CYP1B1⁷ () lists 21 non-synonymous gene variants, found in at least 25 allelic combinations.

The Cancer Genome Anatomy Project data for human CYP1B1⁷) identified 4 non-synonymous SNPs with >5% frequency in a least one population. The same non-synonymous SNPs were also most common in CYP1B1 data from the NIEHS Environmental Genome Project () [50].⁹ If humans recapitulate the responses observed here, transplacental exposure to carcinogenic PAHs, such as DBP or benzo[*a*]pyrene, will lead to lymphoma or lung cancer later in life. The CYP1B1 genetic polymorphism may play a role in the target and severity of the transplacental carcinogenesis.

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Figure 1. DBP-Dependent Lymphoma Mortality

A) Offspring, born to dams treated on the 17th day of gestation with corn oil (5 ml/Kg) (squares), benzo[a]pyrene (50 mg/Kg) (triangles), DBP (1 mg/Kg) (inverted triangles) or DBP (15 mg/Kg) (diamonds). B) Offspring of null (square), heterozygous (diamond), and wild-type (triangle) *cyp1b1* genotype, born to dams given 15 mg/kg DBP. All mice were euthanized upon signs of morbidity as described in Methods. The full genotype composition is given in Table 2.

Figure 2. Lung Tumor Multiplicity at 10 months

Offspring were euthanized at the conclusion of the study (10 months) and lung lesions (predominantly adenomas) quantified by histopathology. Offspring born to mother administered corn oil (control), 1 mg/kg DBP, 15 mg/kg DBP, and 50 mg/kg benzo[a]pyrene (BaP). Bars, group mean; dots, individual animals.

Table 1Primer Sequences for *cyp1b1* and Neomycin Selection Marker

<u>Marker</u>	<u>Primer</u>	<u>Product Size</u>
Cyp1b1 ^a	1b1-3 5'-ttt gcc tgt cac cat tcc ac-3'	
	1b1-3R 5'-acg act tgg gct taa tgg tc-3'	365 bp
Neomycin NEO-1	NEO-2 5'-tga atg aac tgc agg acg ag-3'	
	NEO-2 5'-cca cag tcg atg aat cca ga-3'	460 bp

^aWild-type mice (*Cyp1b1*^{+/+}) have a single band at 365 bp, knockout mice (*Cyp1b1*^{-/-}) a single band at 460 bp and the hets (*Cyp1b1*^{+/-}) both bands.

Table 2*Cyp1b1* Genotype Composition

Group	Dams	Offspring	Genotype Ratio
			($1b1^{-/-}$: $1b1^{+/-}$: $1b1^{+/+}$)
Control	7	42	1.24 : 2.20 : 0.56 ^a
Benzo[a]pyrene	11	77	0.88 : 1.92 : 1.20
DBP (1 mg/kg)	11	78	1.08 : 1.84 : 1.08
DBP (15 mg/kg)	11	79	0.88 : 2.04 : 1.16

^aThis is not significantly different from expectations (1:2:1) based on a X^2 test with a two-tailed *P* value.

Table 310 Month Lung Tumor Multiplicity Sorted by *cyp1b1* Genotype

	<u>Incidence</u>		<u>Multiplicity</u>
<u>Controls</u>			
Wild-type	0.0 %	(0/6)	N/A
Hets	13.0 %	(3/23)	1.00 ± 0.00
Nulls	7.7 %	(1/13)	1.00 ± 0.00
<u>BP (50 mg/Kg)</u>			
Wild-type	39.1 %	(9/23)	1.33 ± 0.15
Hets	40.5 %	(15/37)	1.33 ± 0.15
Nulls	41.2 %	(7/17)	1.43 ± 0.17
<u>DBP (1 mg/Kg)</u>			
Wild-type	28.6 %	(6/21)	1.00 ± 0.00
Hets	27.8 %	(10/36)	1.40 ± 0.20
Nulls	19.0 %	(4/21)	1.00 ± 0.00
<u>DBP (15 mg/Kg)</u>			
Wild-type	100.0 %	(10/10)	9.30 ± 1.46
Hets	100.0 %	(28/28)	9.54 ± 1.03
Nulls	100.0 %	(16/16)	5.00 ± 0.96