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. 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 homologue 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 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. 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 and 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, benzo(a)pyrene, 1 and 15 mg/kg DBP, respectively]. Cyp1b1 status did not alter benzo(a)pyrene-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.

The fetus and infant are at increased risk, relative to adults, upon exposure to many environmental chemicals. Yet, only exposures to ionizing radiation and diethylstilbestrol to pregnant women have been sufficiently well documented as causing cancer in their children (1, 2). However, an increasing number of carcinogens have been shown 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 epidemiologic 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 heterozygote (cyp1b1+/−) so that all litters should have a 1:2:1 ratio of wild-type/heterozygote/nulls to assess fetal cyp1b1 gene dosage on DBP transplacental carcinogenesis. Cyp1b1 knockouts were completely resistant to

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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.

Materials and 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 h in microisolator cages with CareFRESH bedding (Absorption Corp.). During breeding, gestation, and lactation, mice were fed powdered AIN93G diet (Research Diets) ad libitum. At necropsy, an 8-mm ear punch was collected and lysed overnight in 100 μL of DirectPCR Lysis Reagent containing proteinase K (Viagen Biotech, Inc.), followed by 45 min at 85°C. The lysis reaction at 55°C in 100 μL of each deoxynucleotide triphosphate, 0.2 μmol/L of each primer, and 2 μL DNA. PCR cycling conditions were an initial 5 min at 95°C enzyme activation step, followed by 35 cycles of 30 s at 95°C to denature the DNA, 30 s at 55°C for primer annealing, and 45 s 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% Tris-borate EDTA gels (Invitrogen Technologies). Cyp1b1 heterozygotes yielded two PCR products of 365 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) and ethidium bromide (Sigma Chemical Co.) 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 Nparlway 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 with the incidence in the control group using quasi-likelihood 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 quasi-likelihood 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

Table 1. Primer sequences for cyp1b1 and neomycin selection marker

<table>
<thead>
<tr>
<th>Marker</th>
<th>Primer</th>
<th>Product size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cyp1b1*</td>
<td>1b1-3, 5′-TTGCTCTGTCACATCTGAC-3′</td>
<td>365</td>
</tr>
<tr>
<td></td>
<td>1b1-3R, 5′-ACGACTTGGGCTTAATGGTC-3′</td>
<td></td>
</tr>
<tr>
<td>Neomycin</td>
<td>NEO-1, 5′-TGAAATGACTGAGAGGAGG-3′</td>
<td>460</td>
</tr>
<tr>
<td></td>
<td>NEO-2, 5′-CCACAGCTGATGAACTGACA-3′</td>
<td></td>
</tr>
</tbody>
</table>

*Wild-type mice (Cyp1b1+/−) have a single band at 365 bp, knockout mice (Cyp1b1−/−) a single band at 460 bp, and the heterozygote (Cyp1b1+/−) both bands.
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 and 30 weeks of age (Fig. 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 cyp1b1 gene dosage (Fig. 1B). None of the cyp1b1-null mice succumbed to the DBP transplacental lymphoma mortality. Siblings with both wild-type alleles exhibited sensitivity comparable with 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...
Table 2. Cyp1b1 genotype composition

| Group | Dams | Offspring | Genotype ratio (1b−/−:1b+/−:1b+/+)
<table>
<thead>
<tr>
<th></th>
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</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>7</td>
<td>42</td>
<td>1.24:2.20:0.56*</td>
</tr>
<tr>
<td>Benzo(a)pyrene</td>
<td>11</td>
<td>77</td>
<td>0.88:1.92:1.20</td>
</tr>
<tr>
<td>DBP (1 mg/kg)</td>
<td>11</td>
<td>78</td>
<td>1.08:1.84:1.08</td>
</tr>
<tr>
<td>DBP (15 mg/kg)</td>
<td>11</td>
<td>79</td>
<td>0.88:2.04:1.16</td>
</tr>
</tbody>
</table>

*This is not significantly different from expectations (1:2:1) based on a χ² test with a two-tailed P value.

Table 3. Ten-month lung tumor multiplicity sorted by cyp1b1 genotype

<table>
<thead>
<tr>
<th>Group</th>
<th>Incidence</th>
<th>Multiplicity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls</td>
<td>Wild-type</td>
<td>0.0% (0/6)</td>
</tr>
<tr>
<td></td>
<td>Heterozygous</td>
<td>13.0% (3/23)</td>
</tr>
<tr>
<td></td>
<td>Nulls</td>
<td>7.7% (1/13)</td>
</tr>
<tr>
<td>BP (50 mg/kg)</td>
<td>Wild-type</td>
<td>39.1% (9/23)</td>
</tr>
<tr>
<td></td>
<td>Heterozygous</td>
<td>40.5% (15/37)</td>
</tr>
<tr>
<td></td>
<td>Nulls</td>
<td>41.2% (7/17)</td>
</tr>
<tr>
<td>DBP (1 mg/kg)</td>
<td>Wild-type</td>
<td>28.6% (6/21)</td>
</tr>
<tr>
<td></td>
<td>Heterozygous</td>
<td>27.8% (10/36)</td>
</tr>
<tr>
<td></td>
<td>Nulls</td>
<td>19.0% (4/21)</td>
</tr>
<tr>
<td>DBP (15 mg/kg)</td>
<td>Wild-type</td>
<td>100.0% (10/10)</td>
</tr>
<tr>
<td></td>
<td>Heterozygous</td>
<td>100.0% (28/28)</td>
</tr>
<tr>
<td></td>
<td>Nulls</td>
<td>100.0% (16/16)</td>
</tr>
</tbody>
</table>

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 (Fig. 1A). All treatments produced higher incidence of lung adenomas and carcinomas than the spontaneous rate in controls, and the rates in the benzo(a)pyrene (P = 0.01, quasi-likelihood 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 significantly different than the controls, and the rates in the benzo(a)pyrene group was in- significance higher than the incidence in the controls at 10 months of age (Table 3). After adjusting for litter variation (random litter-by-sibling model), there was no 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 United States translate to the second leading cause of death (2,300) after accidents, in children in the United States (1, 2). Leukemias and lymphomas are the most common type of childhood cancer, followed by tumors of the nervous system. The etiology of 80% to 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 diethylstilbestrol 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-(methyleneamino)-1-(3-pyridyl)-1-butanone (4). The infant mouse has also been used as a cancer model with enhanced sensitivity compared with 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 done a cross-fostering experiment to estimate how much of the DBP-dependent lymphoma

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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 has been published and can be used for comparison with 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-(11R,12S,13S,14R)-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, using DBP or 3-methylcholanthrene in models, in which either the dam or the fetus were Ahr “nonresponsive” 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 using crosses of cyp1b1 heterozygote on the same genetic background as our DBP-transplacental model, we were able to show that DBP-dependent transplacental lymphoma mortality was dependent on 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 effect of the cyp1b1 expression was apparent, but not as marked, possibly due to some contribution from CYPs in the 1a subfamily. 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 the 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 United States with 173,700 new cases in 2004 and 160,400 deaths. The 5-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 showed 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 cytochrome P450 1B1 (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 nonsynonymous allelic variants (19, 46–49). The Swiss Protein entry for human CYP1B1 lists 21 nonsynonymous gene variants, found in at least 25 allelic combinations.

The Cancer Genome Anatomy Project data for human CYP1B1 identified four nonsynonymous SNPs with >5% frequency in a least one population. The same nonsynonymous SNPs were also most common in CYP1B1 data from the National Institute of Environmental Health Sciences Environmental Genome Project (50). 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.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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