Cytochrome P4501B1 Mediates Induction of Bone Marrow Cytotoxicity and Preleukemia Cells in Mice Treated with 7,12-Dimethylbenz[a]anthracene1

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ABSTRACT

Humans are exposed to polycyclic aromatic hydrocarbons (PAHs) through many environmental pollutants, especially cigarette smoke. These chemicals cause a variety of tumors and immunotoxic effects, as a consequence of bioactivation by P-450 cytochromes to dihydrodiol epoxides. The recently identified cytochrome P4501B1 (CYP1B1) bioactivates PAHs but is also a physiological regulator, as evidenced by linkage of CYP1B1 deficiency to congenital human glaucoma. This investigation demonstrates that CYP1B1 null mice are almost completely protected from the acute bone marrow cytotoxic and preleukemic effects of the prototypic PAH 7,12-dimethylbenz[a]anthracene (DMBA). CYP1B1 null mice did not produce the appreciable amounts of bone marrow DMBA dihydrodiol epoxide adducts present in wild-type mice, despite comparable hepatic inductions of the prominent PAH-metabolizing P-450 cytochrome, CYP1A1. Wild-type mice constitutively expressed low levels of bone marrow CYP1B1. These findings suggest that CYP1B1 is responsible for the formation of DMBA dihydrodiol epoxide in the bone marrow. Furthermore, this study substantiates the importance of DMBA dihydrodiol epoxide generation at the site of cancer initiation and suggests that tissue-specific constitutive CYP1B1 expression may contribute to cancer susceptibility in the human population.

INTRODUCTION

Numerous studies have suggested that high levels of PAH exposure increase the risk of developing certain types of human cancer (1). Exposure to cigarette smoke, which contains large amounts of PAHs, is positively correlated with an increased risk for developing lung cancer in adults (2). Epidemiological data have been ambiguous regarding a link between cigarette smoke and childhood lymphomas and leukemias. Some studies have indicated that the children born from mothers who smoke during pregnancy do not have an increased risk of developing cancer (3). Other studies indicate that maternal exposure to passive smoke increases the risk of childhood cancer, particularly leukemia and lymphoma (4). This is supported by the recent finding that in utero exposure to cigarette smoke increases the mutational spectrum in newborns’ lymphocytes at genomic regions associated with hematopoietic malignancies in early childhood (5). Furthermore, mutational hotspots in p53 that are attributable to develop-}

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The abbreviations used are: PAH, polycyclic aromatic hydrocarbon; CYP, cytochrome P-450; DMBA, 7,12-dimethylbenz[a]anthracene; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling; RT-PCR, reverse transcription-PCR.
mas in ~70% of wild-type mice but only 7% of CYP1B1-null mice (19). The levels of CYP1A1, which is more active in the metabolism of DMBM, were far greater than CYP1B1 in the major organs after DMBM treatment and were similar in wild-type and CYP1B1-null mice. This suggests that although CYP1A1-mediated metabolism may dictate the systemic pharmacokinetics of DMBM, activation by CYP1B1 in target tissues such as the bone marrow may be critical for the toxicity and carcinogenicity of DMBM.

In a recent study, we demonstrated that multipotent bone marrow stromal cells express high basal levels of CYP1B1 that metabolize DMBM to the mutagenic precursor DMBM-3,4-dihydrodiol (17). In this study, we have tested the hypothesis that reactive DMBM metabolites generated by CYP1B1 are responsible for a rapid depletion of bone marrow cells and the appearance of preleukemic cells in the bone marrow. We suggest that this mechanism initiates the high proportion of lymphoblastomas that result from repetitive administration of DMBM.

MATERIALS AND METHODS

Reagents. DMBM was purchased from Sigma Chemical (St. Louis, MO) and dissolved in olive oil at a concentration of 5 mg/ml for i.p. injection. For in vitro studies, a 10 mM stock solution of DMBM was prepared in DMSO. Culture medium consisted of RPMI 1640 supplemented with 5% fetal bovine serum (v/v; Intergen Co., Purchase, NY), 5 × 10⁻³ M 2-mercaptoethanol, 2 mM l-glutamine, 50 IU penicillin/ml, and 50 µg streptomycin/ml (w/v). Animals and Treatments. C57Bl/6 mice were purchased from The Jackson Laboratory (Bar Harbor, ME). CYP1B1-null mice were produced and characterized as described previously (19). Mating C57Bl/6 mice with CYP1B1-null mice generated CYP1B1 heterozygous mice. Animals were housed at the Association for Assessment and Accreditation of Laboratory Animal Care International-certified University of Wisconsin-Madison School of Veterinary Medicine Animal Care Facility and used in accordance with the NIH Guide for the Care and Use of Laboratory Animals. C57Bl/6 mice (5–8 weeks of age) were randomly selected and received injections i.p. of 10–200 mg/kg DMBM in olive oil. Control animals received injections of an equivalent volume of olive oil. In subsequent experiments, 50 mg/kg DMBM were used because this dose consistently resulted in peak bone marrow toxicity in wild-type mice but had no effect in CYP1B1-null mice.

Harvesting Bone Marrow Cells. Mice were sacrificed by inhalation of DMBM or oil vehicle. The femurs, tibias, and humeri were dissected free of muscle tissue, and the ends were removed with surgical scissors. For total bone marrow cell counts, cells from both femurs and a single tibia were flushed from the bones with culture medium using a syringe equipped with a 25-gauge needle. After centrifugation, RBCs were lysed in ACK buffer (150 mM NH₄Cl, 1.0 mM KHCO₃, and 100 mM Na₂EDTA, pH 7.3). Viable cells were identified and enumerated in a hemocytometer by their exclusion of 0.04% trypan blue (Fig. 1). An aliquot of 10⁵ cells was removed and stained with 1 µl of FITC-conjugated RB6–8C5, anti-B220 (PharMingen, Inc.), or sca-1 (PharMingen, Inc.). The standard opticals of the Coulter Profile II were used to separate and measure the fluorescence emissions from each cell. The data from 2 × 10⁵ cells were collected, transformed to standard FCS format using Pro2FCS software (Verity Software House, ME), and quantified using Win-midi 2.7 software (Joe Trotter; Scripps Institute). The remaining cells were used for DMBM-DNA adduct analysis. Bone marrow smears were prepared by cutting a tibia longitudinally, streaking the exposed bone marrow cells onto a glass slide with a fine sable hair brush, and allowing the cells to air dry at room temperature.

Histopathology. Sternums were removed, fixed in ice-cold 4% phosphate-buffered paraformaldehyde, decalcified for 30 min in Formacal-4 (Decal Co., Congers, NY), embedded in paraffin, sectioned at 2-µm thickness, and stained with H&E for evaluation of their bone marrow. Bone marrow smears were stained with Wright’s stain. Standard light microscopy was used to evaluate the sternum bone marrow sections and bone marrow smears.

Cell Cultures. The 70Z/3 preB cell line was generously provided by Dr. Paul Kincade (Oklahoma City, OK) and maintained in the same tissue culture medium described above. Primary bone marrow stromal cells were isolated and cultured in six-well plates as described elsewhere (21). Primary cultures from animals with different genetic backgrounds had similar cellular compositions, as determined by light microscopic examination. After 3–5 weeks of culture, the nonadherent cells were removed, and the remaining adherent stromal cells were used in experiments.

PreB Cells that remained adherent to the bone marrow stromal cells were dislodged by gently tapping the plate. The detached preB cells were collected in ice-cold PBS and combined with the original preB cell suspensions from the femurs, tibias, and humeri. PreB cell apoptosis assays. Exponentially multiplying 70Z/3 preB cells were centrifuged, counted with a hemocytometer, and diluted to 10⁴ cells in a total volume of 2.5 ml culture medium. Primary bone marrow stromal cell cultures were established in six-well tissue culture plates as described above and then incubated with 2.5 ml of the 70Z/3 preB cell suspension. After 24 h of incubation with either 10 µg DMBA or 0.1% vehicle control, the culture medium containing the suspended preB cells was removed and placed on ice. PreB cells that remained adherent to the bone marrow stromal cells were dislodged by gently tapping the plate. The detached preB cells were collected in ice-cold PBS and combined with the original preB cell suspensions from the same light microscopy revealed that the bone marrow stromal cells remained adherent to the well, whereas most of the preB cells where removed (>99%). The TUNEL assay was used to label apoptotic preB cells, according to the manufacturer’s directions (ApopTag-Fluorescence kit; Promega Corp., Madison, WI). The standard opticals of the Coulter Profile II (Hialeah, FL) flow cytometer was used to detect and enumerate fluorescent apoptotic cells. The data from 2 × 10⁵ cells were collected, transformed to standard FCS format using Pro2FCS software (Verity Software House), and quantified using Win-midi 2.7 software (Joe Trotter; Scripps Institute).

RT-PCR Amplification of CYP1B1 mRNA. Bone marrow cells were aspirated from the humeri using a syringe with a 25-gauge needle. Their total RNA was isolated with TRIzol reagent (Life Technologies, Inc., Grand Island, NY), according to the manufacturer’s directions. RNA isolated from three mice in the each group was pooled to increase the working amount of RNA. Total RNA was isolated from 70% confluent BMS2 cells as a reference for CYP1B1 mRNA expression. The Access RT-PCR System (Promega) was used to amplify CYP1B1 mRNA using an upstream (5′-GGCGTTTGGCTGTCAC- TACTCTG-3′) and a downstream (5′-AGGTGTGGCGTGTCACTAC7′) CYP1B1 primer. After 40 amplification cycles (1 min at 94°C, 1 min at 57°C, and 1 min at 72°C), 20 µl of each PCR reaction mixture were electrophoresed in a 2% agarose gel, the gel was stained with ethidium bromide, and the PCR products were visualized with UV light.

Western Immunoblots. A piece of liver from each mouse was flash-frozen in liquid nitrogen. Microsomes were prepared from thawed livers as described previously (22). Protein concentrations were determined by the BCA method (Pierce Chemical, Rockford, IL). Proteins were resolved by SDS-PAGE electrophoresis, transferred to nitrocellulose, and immunoblotted for CYP1A1 or CYP1B1. Bone marrow cell counts were statistically reduced in DMBM-treated wild-type mice and CYP1B1+/− mice (*, P < 0.05) compared with their oil vehicle controls. Data shown are the means of at least four mice from three separate experiments; bars, SD.
CYP1B1 as described previously (15). Immunoblot signals were quantified using a Molecular Dynamics (Sunnyvale, CA) Personal Densitometer SI and Image QuaNT software.

DMBA-DNA Adduct Analysis. Bone marrow cells isolated as described above were lysed, and their DNA was isolated, digested with nuclease P1, and postlabeled with $[^\gamma-33P]ATP$, and the mononucleotide adducts were analyzed by HPLC as described previously (23). The elution time for a standard of DMBA-3,4-dihydrodiol-1,2-epoxide-DNA adduct was used to identify the corresponding peak in the elution profiles of bone marrow cell DNA.

Statistical Analysis. ANOVA analysis was used for statistical comparison of control and DMBA-treated total bone marrow cell count data for each of the three mouse genotypes. A minimum of four animals was used for each data point. PreB cell apoptosis data were analyzed for significance within a mixed model ANOVA using the SAS statistical software program.

RESULTS

CYP1B1-Null Mice Are Protected against DMBA-induced Bone Marrow Cytotoxicity and Preleukemia. Mice were injected i.p. with a single 50-mg/kg dose of DMBA. This dose of DMBA is similar to those used to initiate tumors in rodent carcinogenesis models (24). Wild-type mice treated with DMBA had almost a 50% reduction in total bone marrow cells after 48 h as compared with controls (Fig. 1). Flow cytometry of bone marrow revealed a significant decrease ($P < 0.05$) in RB6–8C5-positive myeloid cells (mean ± SE of $14.6 \pm 1.4 \times 10^6$ versus $24.4 \pm 1.5 \times 10^6$) and B220-positive B lymphocytes (7.9 ± 0.5 × 10^6 versus 9.3 ± 0.6 × 10^6) in DMBA-treated versus control wild-type mice (mean ± SE of three and four mice, respectively). This level of reduction represents the maximum that can be attained with DMBA, as evidenced by no further suppression after treatment with 4-fold higher concentrations of DMBA or at examination at earlier time points. An increase ($P < 0.05$) in sca-1-positive stem cells was also noted in DMBA treated versus control wild-type mice (1.6 ± 0.1 × 10^6 versus 1.1 ± 0.1 × 10^6, respectively). In contrast, no reduction of total bone marrow cells was observed in CYP1B1-null mice treated with DMBA (Fig. 1). CYP1B1 heterozygous mice (CYP1B1+/−) demonstrated an intermediate response after DMBA treatment. The apparent differences in baseline cell numbers among the three groups of untreated mice do not necessarily reflect genotype differences, because the three groups of mice were not littermates and were not matched for age and weight. Other experiments with CYP1B1 (+/+) mice demonstrated that comparable variation can occur among mice from different litters or of different ages. As a result, the relevant comparisons in Fig. 1 are within, rather than across, the three genotypes of mice.

Histopathological examination of vehicle-treated CYP1B1-null mouse bone marrow revealed that they were similar to those of control wild-type mice (Fig. 2). The bone marrow of DMBA-treated CYP1B1-null mice could not be distinguished from vehicle controls and received a histological grading of normal (Fig. 2; Table 1). The decreased numbers of total bone marrow cells after DMBA administration in wild-type mice was reflected histopathologically as markedly hypocellular bone marrow, accompanied by dilated sinusoids containing mature RBCs. The most severe cell reductions occurred in those cells belonging to the granulocytic and erythroid series. This
loss of bone marrow cellularity was consistent in all DMBA-treated wild-type animals and resulted in an average histological grade of 3+ (4+ was the most severe). Along with the reduction in cell density, the bone marrow of DMBA-treated wild-type mice contained a greater proportion of large, dark blue-staining cells compared with vehicle-treated mice. These cellular characteristics are commonly associated with proliferating blast cells, and all DMBA-treated wild-type mice received a histological grading of 4+ for blast cells. The increase in blast cells is consistent with the increased number of sca-1-positive stem cells noted above. DMBA treatment failed to reduce the density of bone marrow cells in CYP1B1-null mice or to increase the number of blast cells. Treatment of CYP1B1+/− with DMBA resulted in an intermediate response, which is reflected by their histological grading (Table 1).

The blast-like cells in bone marrow smears from DMBA-treated wild-type animals contained a greater proportion of large cells with dark blue cytoplasm as compared with controls (Fig. 2, f and e, respectively). Cytochemical staining suggests that the majority of these blast cells were of granulocytic lineage.4 Although some of these cells were normal proliferating blasts, many exhibited evidence of dysplasia, as characterized by asynchronous maturation of the nucleus relative to the cytoplasm. More specifically, myeloblasts and promyelocytes, which are typically characterized by their azurophilic granules and round nuclei, were larger and darker stained than expected. The postmitotic granulocytes also appeared dysplastic, as distinguished by their enlarged size compared with the stage of nuclear maturation. In addition, these cells had very pleomorphic nuclei, as distinguished by their elongate, convoluted, or lobulated shapes. These cellular morphologies are characteristic of myelodysplastic bone marrow cells, which are one of the hallmarks of preleukemia (25). Because these myelodysplastic cells belong to the granulocytic series, the granulocytic:erythrocytic cell ratios in DMBA-treated wild-type mice were increased (92:8) compared with vehicle-treated mice (61:39; Table 1). The bone marrow smears from DMBA-treated CYP1B1-null mice could not be distinguished from control smears and, therefore, had a similar granulocytic:erythrocytic ratio as controls. Collectively, these findings demonstrate that CYP1B1 is required for the cytotoxic and preleukemic effects of DMBA in the bone marrow.

Expression of Hepatic CYP1A1 and Bone Marrow CYP1B1. CYP1A1 metabolizes DMBA more rapidly than CYP1B1 and produces much less of the 3,4-dihydrodiol metabolite (12). We, therefore, considered the possibility that differences in hepatic CYP1A1 might influence DMBA-mediated bone marrow toxicity. Liver microsomes were isolated from control or DMBA-treated mice and immunoblotted for CYP1A1. Fig. 3A demonstrates that low levels of constitutive hepatic CYP1A1 are substantially increased after DMBA treatment of wild-type, CYP1B1+/−, and CYP1B1-null mice. These levels of hepatic CYP1A1 were similar in all three groups of mice. Hepatic CYP1B1 was not detectable by immunoblotting in either wild-type or CYP1B1 heterozygous mice. Elsewhere, we have demonstrated that hepatic CYP1B1 is typically 20–100 times lower than hepatic CYP1A1, even after maximal inducing conditions (15).

We have demonstrated previously that cultured bone marrow stromal cells express appreciable amounts of DMBA-metabolizing CYP1B1 in vitro (17). We hypothesized that the in vivo bone marrow toxicity of DMBA might reflect the expression of CYP1B1 in the bone marrow. RT-PCR analysis demonstrates that CYP1B1 is constitutively expressed in wild-type mouse bone marrow (Fig. 3B). This level of CYP1B1 expression was much lower than in the BMS2 bone marrow stromal cell line. In a previous report, we show that cultured primary bone marrow stromal cells express 2–3-fold less CYP1B1 than BMS2 cells, and that none of these cells express detectable CYP1A1. These differences in CYP1B1 expression are likely attributable to the enrichment of stromal cells in primary bone marrow cultures relative to bone marrow in vivo (17).

CYP1B1-Null Mice Have Significantly Reduced Levels of Bone Marrow DMBA-DNA Adducts. CYP1B1-null mice are resistant to DMBA-induced bone marrow cytotoxicity and the generation of preleukemic cells. The constitutive bone marrow expression of CYP1B1 in wild-type mice and the similar hepatic CYP1A1 expression in all strains of mice examined suggest that CYP1B1-null mice are resistant because the responses require local activation of DMBA by CYP1B1. In contrast, hepatic metabolism presumably produces PAH dihydrodiol epoxide metabolites that are stabilized by lipoproteins in the blood (26). Hepatic metabolism should, however, be independent of the CYP1B1 genotype, because this form is absent in these livers. To investigate these possibilities, DNA was isolated from the bone marrow of DMBA-treated and control mice and analyzed for the presence of DNA adducts. These adducts can provide a measure of the amount and type of DMBA metabolites present in bone marrow. Dihydrodiol epoxide DNA adducts were detected in the bone marrow of wild-type mice after DMBA administration, with the 3,4-dihydrodiol-1,2-epoxide DNA adducts accounting for

<table>
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<tr>
<th>Genotype</th>
<th>Cell loss</th>
<th>Blast cells</th>
<th>M-E ratio</th>
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<tr>
<td>1B1 null</td>
<td>N</td>
<td>N</td>
<td>60:40</td>
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<tr>
<td>1B1 +/−</td>
<td>1+</td>
<td>1+</td>
<td>88:12</td>
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<tr>
<td>Wild type</td>
<td>3+</td>
<td>4+</td>
<td>92:9</td>
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* Results are the average from three mice in each group.
* 4+, most severe effect; N, normal.
* Blast cells from all cell lineages.
* M-E (granulocytic:erythrocytic) ratios in vehicle control treated mice were 60:40, 55:45, and 62:38 for 1B1 null, 1B1 +/−, and wild-type mice, respectively.

3 R. Raskin, personal communication.
most of the adducts present (Fig. 4). This pattern of dihydriodiol epoxide adducts is typical of DMBA activation by CYP1B1, rather than CYP1A1, which additionally produces more polar adducts (27). Bone marrow from DMBA-treated CYP1B1 null mice had <5% of the dihydriodiol epoxide DMBA-DNA adducts present in the bone marrow of DMBA-treated wild-type mice. These results indicate that CYP1B1-dependent DMBA metabolism is responsible for >95% of the total bone marrow DMBA-DNA adducts in wild-type mice. The correlations between these adduct levels, and both the cytotoxic and preleukemic actions of DMBA, suggest that these effects are dependent on DMBA-dihydriodiol epoxide production. The presence of CYP1B1 in bone marrow suggests that this activity, rather than hepatic metabolism by CYP1A1, is responsible for DMBA activation.

**Bone Marrow Stromal Cell CYP1B1 Is Required for preB Cell Apoptosis Induced by DMBA in Vitro.** Coculturing bone marrow stromal cells with preB cells constitutes an in vitro model for the bone marrow microenvironment in vivo (28). In addition, other investigators have demonstrated that DMBA causes preB cell apoptosis only when these cells were cocultured with bone marrow stromal cells (9). We hypothesized that CYP1B1 in bone marrow stromal cells produce reactive DMBA metabolites that caused preB cell apoptosis in vitro and possibly bone marrow hypocellularity in vivo. To investigate this possibility, primary bone marrow stromal cells established from CYP1B1 null, CYP1B1+/−, and wild-type mice were cocultured with a preB cell line. The cellular composition among these primary bone marrow stromal cell cultures was similar, as determined by light microscopic evaluation (data not shown). When cocultured with wild-type bone marrow stromal cells and 10 µM DMBA, 28% of preB cells were apoptotic. Consistent with our hypothesis, preB cells did not undergo apoptosis when cocultured with CYP1B1-null bone marrow stromal cells and DMBA. Similar to the intermediate bone marrow cytotoxicity seen in DMBA-treated CYP1B1+/− bone marrow stromal cells and DMBA. In the absence of DMBA, only ~2% of preB cells were apoptotic when cocultured with primary bone marrow stromal cells (Fig. 5), which was the same amount as preB cells cultured with DMBA in the absence of bone marrow stromal cells (data not shown). These findings demonstrate that bone marrow stromal cell expression of CYP1B1 is required for DMBA-induced preB cell apoptosis.

**DISCUSSION**

CYP1B1 is scarcely expressed in the liver but is expressed constitutively in many extrahepatic tissues. We demonstrate here that CYP1B1 deletion prevents profound cytotoxicity in the bone marrow produced by DMBA. This toxicity is reflected in wild-type mice by a 50% loss of myeloid cells, a smaller decrease in lymphocytes, but a resistance of blast cells. This protection conferred by CYP1B1 deletion was paralleled by a near-complete loss of DNA-DMBA dihydriodiol epoxide adducts in the bone marrow. These data strongly suggest that CYP1B1 is necessary to generate DMBA dihydriodiol epoxides in the bone marrow for toxicity at this site. This is supported by an earlier in vivo study in which benzo(a)pyrene caused less bone marrow toxicity in mice with a lower affinity Ah receptor and liver CYP1A1 activity than in mice with a high-affinity Ah receptor and high CYP1A1 activity (29). Mice that are CYP1B1+/+ are also resistant to the bone marrow toxicity of benzo(a)pyrene and exhibit a 5-fold higher induction of CYP1A1 in the liver as compared with equivalent DMBA treatments in the current study.6 Although compelling for bone marrow toxicity, these findings do not exclude the possibility that CYP1A1 plays an important role in liver DMBA activation by generating the 3,4-dihydriodiol metabolite, a proximate

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6 Galvin et al., unpublished results.
CYP1B1- AND DMBA-MEDIATED BONE MARROW CYTOTOXICITY
carcinogen. However, we hypothesize that CYP1A1-mediated metabolism of PAHs in the liver is largely protective through effective subsequent metabolism of the proximal dihydrodiols to mostly non-toxic products. Convincing evidence for this hypothesis is provided by the failure of DMBA to generate bone marrow adducts and cytotoxicity in CYP1B1-null mice despite comparable levels of liver CYP1A1 in all three mouse genotypes examined.

Previous work demonstrated that CYP1B1 in primary bone marrow stromal cells metabolizes DMBA, thus supporting the possibility that the bone marrow toxicity of DMBA may be attributable to local activation (17). In contrast, dihydrodiol epoxides of benzo(a)pyrene are stabilized by serum lipoproteins, suggesting that hepatic metabolism may be the prime source of toxicity in extrahepatic tissues (26). The present study indicates that this later mechanism is not effective in causing bone marrow toxicity.

Selective protection from the acute bone marrow toxicity of DMBA parallels our recent finding that CYP1B1-null mice are resistant to DMBA-induced lymphoblastic lymphomas (19). This resistance of CYP1B1-null mice implicates CYP1B1 as being essential for initiating the carcinogenic effect of DMBA in immune organs. A linkage to leukemia initiation is supported by the present finding of myelodysplastic bone marrow cells in DMBA-treated wild-type mice but not CYP1B1-null mice. Myelodysplastic cells are one of the distinguishing features of preleukemia, a syndrome that greatly increases the likelihood of leukemia development (30). Others have observed the preleukemic effect of DMBA (25), and chronic DMBA treatment results in leukemias and lymphomas (31, 32).

The carcinogenicity of DMBA has been linked to the covalent binding of DMBA dihydrodiol epoxides to DNA (10, 33). There is also evidence that DMBA initiates carcinogenesis without an intermediate dihydrodiol (34), possibly via DMBA radical cations (35). The data presented here suggest that the DMBA dihydrodiol epoxide DNA adducts in wild-type mouse bone marrow cause mutations that initiate leukemias and lymphomas. The near absence of bone marrow DMBA-DNA adducts in CYP1B1-null mice, combined with their resistance to DMBA-induced lymphoblastic lymphomas, strongly implicates DMBA metabolism by bone marrow CYP1B1 as being involved in immune cell carcinogenesis in wild-type mice. DMBA metabolites generated by CYP1B1 activity may have also removed immune cells that may be important for eliminating mutant cells generated by carcinogens.

We have demonstrated that bone marrow cells constitutively express CYP1B1 both in vitro and in vivo, and that wild-type and CYP1B1-null mice had comparable amounts of hepatic CYP1A1 after DMBA treatment. Despite these similar levels of hepatic CYP1A1, CYP1B1-null mice are resistant to DMBA-induced bone marrow toxicity, indicating that these sizable levels of CYP1A1 contribute little to this toxicity. Certainly, the conversion of DMBA to the 3,4-dihydrodiol precursor of the most mutagenic metabolite is favored by CYP1B1 relative to CYP1A1 (12). However, this selectivity does not apply to benzo(a)pyrene, which is readily activated to mutagenic precursors by CYP1A1 (13), but exhibits much less bone marrow toxicity than DMBA.6 That bone marrow CYP1B1 is sufficient for inducing toxicity was further established by in vitro studies that show DMBA-induced proB cell apoptosis is dependent on bone marrow stromal cell CYP1B1.

The constitutive and Ah receptor inducible levels of CYP1B1 and CYP1A1 may also be important for target organ toxicity. For instance, CYP1B1 is constitutively expressed in organs that are targets for the carcinogenicity of DMBA (uterus, ovary, skin, and bone marrow), whereas only CYP1A1 is expressed in liver, and this is dependent on Ah receptor activation (15–17). Because DMBA only modestly activates the Ah receptor, low levels of hepatic CYP1A1 are induced relative to potent Ah receptor ligands, such as benzo(a)pyrene. Thus, appreciable amounts of DMBA can reach the bone marrow for metabolism by CYP1B1, as evidenced by the large decrease in DMBA-DNA adducts in CYP1B1-null mice relative to wild-type mice. Moreover, DMBA-DNA adduct profiles in wild-type mouse bone marrow are nearly identical to those generated with DMBA in cultured bone marrow stromal cells, where we have demonstrated expression of CYP1B1 but not CYP1A1 (17, 36). Although we have demonstrated DMBA induction of CYP1B1 in bone stromal cells in vitro (17, 36), we have yet to determine the extent to which DMBA elevates bone marrow CYP1B1 in vivo above the observed constitutive expression. Efforts are under way to identify expression of CYP1B1 by specific cell types in vivo. Metabolism by the modest levels of hepatic CYP1A1 noted in this study may largely detoxify DMBA, presumably because of the selectivity of oxidation and effective phase 2 conjugation in the liver.

Progenitor leukocytes are potential targets of DMBA metabolites. In vivo and in vitro treatments with DMBA inhibit lymphocyte proliferation (37, 38). Cytochrome P-450 is implicated, because the inhibitor α-naphthoflavone alleviates this effect in vitro (38). Similarly, an active dihydrodiol epoxide metabolite of benzo(a)pyrene arrested the cell cycle of human lymphoblasts, eventually leading to their death (39). We were unable to detect a significant increase in TUNEL-positive apoptotic bone marrow cells in wild-type mice treated with DMBA, even when examined at time points as early as 12 h after DMBA administration. Nevertheless, bone marrow smears of DMBA-treated wild-type mice contained increased numbers of phagocytosed cells, suggesting that apoptotic cells may have been quickly removed in vivo by bone marrow macrophages. In addition, we have shown that cultured preB cells undergo apoptosis in vitro when these cells are incubated with wild-type bone marrow stromal cells, which metabolize DMBA via CYP1B1. Interestingly, CYP1B1+/− mice exhibit an intermediate bone marrow response to DMBA in vivo, whereas bone stromal cells from these mice exhibit intermediate activity in mediating DMBA-induced apoptosis in preB cells in vitro. The latter observation suggests that bioactivation of DMBA in CYP1B1+/− cells is less than in wild-type cells, probably because of diminished CYP1B1 expression.

DMBA damage to bone marrow stromal cells in vivo may also change their production of factors that regulate progenitor leukocyte survival. An absence of bone marrow stromal cell growth factors, including colony stimulating factors and interleukins 3 and 6, causes cell death of progenitor leukocytes (40). Alternatively, damage to bone marrow stromal cells may cause the release of factors, such as tumor necrosis factor-α and transforming growth factor-β, which can cause apoptosis of myeloid precursor cells (41). Future investigations will address whether damage to stromal cells in the bone marrow microenvironment is involved in the bone marrow toxicity of DMBA in vivo.

The results of this investigation indicate that CYP1B1 activity has important implications for human health. Many reports have suggested that PAHs containing environmental contaminants pose a risk for human cancer development (1), and a recent report demonstrated that human CYP1B1 activates many diverse procarcinogens, including the proximate carcinogen DMBA-3,4-dihydrodiol (42). CYP1A1 is more effective at metabolizing DMBA than CYP1B1 but produces less of the proximate carcinogen in mice. Although DNA adducts of PAHs have been demonstrated in circulating human leukocytes, it is controversial whether they influence the development of leukemias and lymphomas (5). A recent article substantiates the role of PAH metabolism and adduct formation in carcinogenesis by demonstrating that lung tumors arising among cigarette smokers contain mutations thought to be initiated by metabolites of benzo(a)pyrene (2, 6). Our
work suggests that human CYP1B1 polymorphisms could pose a risk factor for the development of cancers, including those of the immune system.

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References


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