

Bone Marrow Stromal Cell Cytochrome P4501B1 Is Required for Pre-B Cell Apoptosis Induced by 7,12-Dimethylbenz[a]anthracene¹

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Received June 28, 1999; accepted August 27, 1999

This paper is available online at <http://www.molpharm.org>

ABSTRACT

We previously demonstrated that murine bone marrow stromal cells express high levels of cytochrome P4501B1 (CYP1B1) that metabolizes 7,12-dimethylbenza[anthracene (DMBA), and that DMBA activates the Ah receptor (AhR) in these cells in vitro. More recently, we reported that CYP1B1 is required for DMBA-induced lymphoblastoma formation in vivo. In this study, we addressed the hypothesis that bone marrow stromal cell CYP1B1, and not AhR activation, is required for DMBA-induced pre-B-cell apoptosis. Although DMBA did not directly cause apoptosis in pre-B cells, dose-dependent apoptosis of pre-B cells was observed when they were cocultured with a bone marrow stromal cell line. The DMBA 3,4-dihydrodiol metabolite was more potent in effecting pre-B-cell apoptosis than DMBA, whereas the potent AhR agonist 2,3,7,8-tetrachlorod-

ibenzo-*p*-dioxin was inactive. Both pre-B cells and bone marrow stromal cells contained DMBA-diol-epoxide DNA adducts, indicating that reactive metabolites were transferred from stromal cells to pre-B cells. DMBA caused apoptosis when cocultured with primary bone marrow stromal cells isolated from AhR-null mice but not CYP1B1-null mice. When cocultured with AhR-null primary bone marrow stromal cells, DMBA induced approximately 50% of the pre-B-cell apoptosis seen with stromal cells from AhR-heterozygous mice. This reduced level of apoptosis parallels the decreased CYP1B1 expression in AhR-null mouse bone marrow stromal cells. These findings provide convincing evidence that bone marrow stromal cell CYP1B1 metabolism of DMBA, but not AhR activation, is required for DMBA-induced pre-B-cell apoptosis.

Polycyclic aromatic hydrocarbons (PAHs) are ubiquitous environmental contaminants produced as a byproduct of combustion. Major sources of human exposure are cigarette smoke, charbroiled foods, and automobile exhaust. These compounds are potent carcinogens in rodents, producing tumors of the mammary and hematopoietic systems (MacLeod et al., 1997). In addition, PAHs are immunosuppressive and cause a severe loss of bone marrow cellularity (Ward et al., 1984; White et al., 1994). Recent findings suggest that the bone marrow cytotoxicity of PAH may be the result of apoptosis of developing leukocytes (Hardin et al., 1992; Yamaguchi et al., 1997a,b). The latter effect is interesting in that

leukocytes typically contain at most low levels of cytochrome P450.

Apoptosis, or programmed cell death, is a well-defined physiological process that is particularly important in the bone marrow. Developing B lymphocytes that are potentially self-reactive, or have improperly rearranged their germ line genes, are eliminated by apoptosis (Rajewsky, 1996). In addition to its role in normal bone marrow lymphopoiesis, apoptosis of bone marrow cells also occurs after exposure to the prototypic polycyclic aromatic hydrocarbon 7,12-dimethylbenza[anthracene (DMBA) (Yamaguchi et al., 1997a). Although the particular bone marrow cell types undergoing apoptosis in vivo were not characterized, a previous investigation suggested that B-lymphocyte precursor cells are sensitive to DMBA (Ward et al., 1984). Using an in vitro model for B-cell maturation, other investigators recently demonstrated that DMBA induced apoptosis in precursor B cells when they were cocultured with bone marrow stromal cells

This work was supported by the University of Wisconsin School of Veterinary Medicine, by National Institute of Environmental Health Sciences Training Grants ES07015 and IF32E505827 (S.M.H.), and by U.S. Public Health Science Grants CA16265 (C.R.J.) and CA32484 (C.J.C.).

¹ This work was performed in the laboratories of Dr. Czuprynski and Dr. Jefcoate.

ABBREVIATIONS: PAH, polycyclic aromatic hydrocarbon; DMBA, 7,12-dimethylbenza[anthracene; CYP1B1, cytochrome P4501B1; CYP1A1, cytochrome P4501A1; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; TUNEL, TdT-mediated dUTP nick end labeling; PARP, poly-ADP-ribose polymerase; 1B1^{-/-}, CYP1B1 null; 1B1^{+/-}, CYP1B1 heterozygous; AhR^{-/-}, Ah receptor null; AhR^{+/-}, Ah receptor heterozygous.

(Yamaguchi et al., 1997a). This finding is paradoxical, because bone marrow stromal cells, which do not express detectable levels of cytochrome P4501A1 (CYP1A1) even after DMBA or 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) induction (Heidel et al., 1998), typically foster the maturation of leukocytes. These authors postulated that activation of the Ah receptor (AhR) was involved in this immunotoxic effect of DMBA (Yamaguchi et al., 1997b).

The AhR is a ligand-activated transcription factor that regulates multiple genes that contain the appropriate recognition elements (Schmidt and Bradfield, 1996; Rowlands and Gustafsson, 1997). Many of these genes code for enzymes, such as CYP1B1 and CYP1A1, which in turn can metabolize PAH to reactive metabolites. Bone marrow stromal cells express a functional AhR that is activated by DMBA and TCDD (Heidel et al., 1998; Lavin et al., 1998), suggesting that AhR activation might be involved in DMBA-induced pre-B-cell apoptosis. However, the potent AhR ligand TCDD did not cause pre-B-cell apoptosis when cultured with bone marrow stromal cells (Yamaguchi et al., 1997). Because DMBA is metabolized to mutagenic products by cytochrome P450s but TCDD is not, we hypothesized that DMBA metabolites, and not AhR activation, causes pre-B-cell apoptosis.

The possible role of bone marrow stromal cell metabolism of DMBA in the apoptosis of cocultured pre-B cells deserves careful consideration. Metabolism of DMBA to its most toxic metabolite (3,4-dihydrodiol-1,2-epoxide) requires two separate oxidations by CYP1B1. The first oxidation produces the 3,4-dihydrodiol, which is further metabolized by CYP1B1 to the highly mutagenic 3,4-dihydrodiol-1,2-epoxide metabolite. This metabolite covalently binds DNA, forming adducts that can result in cell-cycle arrest, apoptosis, or malignant transformation of the cell. We have previously demonstrated that bone marrow stromal cells express large amounts of CYP1B1 that metabolize DMBA to its 3,4-dihydrodiol metabolite (Heidel et al., 1998). Striking evidence of the importance for CYP1B1-dependent DMBA metabolites in immune cell tumors was provided by a recent investigation demonstrating that DMBA treatment resulted in lymphomas in only 7% of CYP1B1-null mice, versus 70% of wild-type mice (Buters et al., 1999). In a parallel study, we reported that CYP1B1-null mice were resistant to DMBA-induced bone marrow cytotoxicity in vivo (S.M. Heidel, P.S. MacWilliams, W.M. Baird, W.M. Dashwood, J.T.M. Buters, F.J. Gonzalez, M.C. Larsen, C.J. Czaprynski, and C.R. Jefcoate, unpublished observations). Because of the link between carcinogenicity and immunotoxicity of xenobiotics, we hypothesized that CYP1B1 metabolism of DMBA is similarly required for its ability to cause apoptosis of pre-B cells.

The overall goal of this study was to address the hypothesis that CYP1B1 metabolism by bone marrow stromal cells is required for DMBA-induced pre-B-cell apoptosis in vitro. This was done with a well-established in vitro model of B-cell development, in which pre-B cells were cocultured with bone marrow stromal cells and DMBA. We used a combination of tools (most notably bone marrow stromal cells from CYP1B1-null and AhR-null mice) to assess the potential contributions of both CYP1B1-mediated DMBA metabolism and AhR activation on pre-B-cell apoptosis. The results of these studies suggest that bone marrow stromal cell CYP1B1 but not the AhR is required for DMBA-induced pre-B-cell apoptosis in vitro.

Materials and Methods

Animals. Mice heterozygous for a disrupted AhR gene were generously provided by Dr. Chris Bradfield (Madison, WI). PCR amplification of tail DNA was used to genotype pups that resulted from the mating of heterozygous adult mice, as described previously (Schmidt et al., 1996). CYP1B1-null mice were produced and characterized as described previously (Buters et al., 1999). Heterozygous CYP1B1 mice were obtained by mating CYP1B1-deficient mice with AhR-positive mice. Animals were housed under microisolator caps at the American Association for Accreditation of Laboratory Animal Care-certified University of Wisconsin-Madison School of Veterinary Medicine Animal Care Facility and used in accordance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals*.

Cell Cultures. The 70Z/3 pre-B-cell line was purchased from the American Type Culture Collection, (Rockville, MD) and routinely grown in tissue culture medium that consisted of RPMI-1640 supplemented with 5% fetal bovine serum (v/v) (Intergen Co., Purchase, NY), 5×10^{-5} M 2-mercaptoethanol, 2 mM L-glutamine, 50 IU of penicillin/ml, and 50 mg of streptomycin/ml (w/v). Dr. Paul Kincade (Oklahoma City, OK) generously provided the BMS2 bone marrow stromal cell line (Pietrangeli et al., 1988), which was maintained as described elsewhere (Heidel et al., 1998). For apoptosis assays, BMS2 cells were seeded in a six-well tissue-culture plate at a density of 1.5×10^6 cells/well and cultured for 2 days in RPMI medium before being cocultured with 70Z/3 pre-B cells.

Primary bone marrow stromal cells were derived and cultured as previously described. Briefly, 3- to 5-week-old mice were euthanized, their femurs dissected free of muscle, and the ends of the femurs removed with a surgical scissors. The bone marrow cells were flushed from the femurs with a 25-gauge needle and ice-cold RPMI medium and diluted to 10^6 cells/ml of RPMI medium. Primary bone marrow stromal cultures were established by plating 2.5 ml of this cell suspension into each well of a six-well tissue-culture plate. Twice weekly, 75% of the conditioned medium was replaced with fresh RPMI medium. Individual colonies of adherent stromal cells grew to 60 to 90% confluence after 2 to 4 weeks of culture at 37°C with 5% CO₂. Light-microscopic examination revealed similar cellular compositions of primary bone marrow cell cultures from animals with different genetic backgrounds. Nonadherent cells were removed by gently tapping the plate, followed by a wash with warm PBS. This process was repeated a second time, and the remaining adherent stromal cells were used in experiments. Primary bone marrow stromal cells, established by methods similar to that described above, have been reported previously to support long-term lymphopoiesis in vitro (Whitlock et al., 1984).

Reagents. DMBA was purchased from Sigma Chemical Co. (St. Louis, MO), and DMBA 3,4-dihydrodiol was obtained from the NCI Chemical Carcinogen Reference Standard Repositories (Frederick, MD). TCDD was purchased from Cambridge Isotopes (Cambridge, MA). Stock solutions of 10 mM DMBA, 1 mM DMBA 3,4-dihydrodiol, and 100 μ M TCDD were prepared in embryo-fibroblast-quality dimethyl sulfoxide (Sigma). The anti-poly-ADP-ribose polymerase (PARP) monoclonal antibody used for immunoblotting was purchased from Chemicon International (clone C-2-10; Temecula, CA) and diluted 1:10,000 in buffer (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, and 0.05% Tween 20 (v/v)).

Pre-B-Cell Coculture Assays. Proliferating 70Z/3 pre-B cells were centrifuged, enumerated with a hemacytometer, and diluted to 10^6 cells in a total volume of 2.5 ml of RPMI medium. BMS2 cells or primary bone marrow stromal cells were cultured as described above, and 2.5 ml of the 70Z/3 pre-B-cell suspension was added to each well. Appropriate dilutions of DMBA, DMBA 3,4-dihydrodiol, or TCDD were then added to each well at the final concentrations indicated in the figure legends. After 24 or 48 h of incubation, the medium containing the suspended pre-B cells was removed and placed on ice. The plates were gently tapped to dislodge any pre-B

cells that remained adherent to the bone marrow stromal cells. The dislodged pre-B cells were collected in ice-cold PBS and combined with the original pre-B-cell suspensions from the same well. Visual examination of the culture wells with an overhead microscope revealed that most of the pre-B cells were removed (>99%) and that the bone marrow stromal cells remained adherent to the plastic surface. An aliquot of pre-B cells was removed at this point for PARP cleavage and DMBA-DNA adduct assays.

Apoptosis Assays. Apoptotic pre-B cells were labeled with a TdT-mediated dUTP nick end-labeling (TUNEL) assay according to the manufacturer's directions (apoptosis-fluorescein kit; Promega, Madison, WI). Apoptotic cells were then detected by flow cytometry with a Coulter (Hialeah, FL) Profile II flow cytometer. The standard optics of the Coulter Profile II were used to separate and measure the fluorescence emissions from each cell. The data from 2×10^4 cells were collected, transformed to standard forward cell scatter format with Pro2FCS software (Verity Software House, Topsham ME), and quantified with Winmidi 2.7 software (Joe Trotter, Scripps Institute, LaJolla, CA). As another measure of apoptosis, cells were analyzed for caspase activation by detecting the presence of cleaved PARP (Sallmann et al., 1997). Immunoblot analysis of cleaved PARP was performed as described previously.

DMBA-DNA Adduct Analysis. Cocultures of pre-B cells and BMS2 cells were treated with DMBA for 24 h. The pre-B cells were isolated as described above, and the BMS2 cells were removed by scraping in ice-cold PBS. The two types of cells were kept separate and lysed, and their DNA was isolated. The DNA was digested to single nucleotides postlabeled with [γ - 32 P]ATP and HPLC eluted as described previously (Melendez-Colon et al., 1997). Nucleotides that did not contain DMBA adducts eluted rapidly from the column within the first 5 min. Addition of a standard amount of DMBA 3,4-dihydrodiol-1,2-epoxide-DNA adduct was used to identify the peak that corresponded to this DNA adduct in pre-B cell and BMS2 cell DNA solutions.

Statistical Analysis. Apoptosis data were analyzed for statistical significance with a mixed-model ANOVA, with the SAS statistical software program. Each data point consists of a minimum of three experiments, with one to three replicates per experiment.

Results

DMBA-Induced Pre-B-Cell Apoptosis Requires Bone Marrow Stromal Cells. This study focused on DMBA-mediated pre-B-cell toxicity in vitro with the well-characterized 70Z/3 pre-B cell line (Pietrangel and Kincade, 1987). These cells are committed to the B-cell lineage but lack the surface immunoglobulin found on mature B cells. To model the complex interactions that occur in the bone marrow microenvironment, pre-B cells were cultured in vitro with the BMS2 bone marrow stromal cell line. When these cocultured cells were incubated with $1 \mu\text{M}$ DMBA for 24 h, the pre-B cells exhibited nuclear condensation and membrane blebbing (data not shown), which are classic morphological features of apoptosis (Wyllie, 1980). Further evidence of pre-B-cell apoptosis was provided by labeling the fragmented DNA cells with a TUNEL assay and enumerating the fluorescein-conjugated cells by flow cytometry. In the presence of BMS2 cells, about 40% of the pre-B cells were TUNEL positive after a 24-h incubation with $1 \mu\text{M}$ DMBA (Fig. 1). In the absence of BMS2 cells, even a 10-fold higher concentration of DMBA did not increase the number of apoptotic pre-B cells over that found in controls (data not shown). Because $10 \mu\text{M}$ DMBA did not cause BMS2 cells to undergo apoptosis (data not shown), we were confident that the TUNEL-positive cells were pre-B cells. Dose-response experiments established that

as little as $0.1 \mu\text{M}$ DMBA significantly ($p < .05$) increased pre-B-cell apoptosis (Fig. 2). These data are similar to a previous report that used propidium iodide staining to detect apoptosis in the BU-11 pre-B cell line (Yamaguchi et al., 1997a).

Further evidence that TUNEL-positive pre-B cells were apoptotic rather than necrotic was provided by PARP Western immunoblots of pre-B-cell lysates. Cleavage of 115-kDa native PARP to an 85-kDa fragment reflects caspase activation that occurs during apoptosis but not necrosis (Sallmann et al., 1997). Pre-B cells cocultured with BMS2 cells in the absence of DMBA contained mostly native PARP (>95% of total), indicating that these cells were not undergoing apoptosis (data not shown). Likewise, pre-B cells cultured for 24 h with $10 \mu\text{M}$ DMBA in the absence of BMS2 cells contained mostly native PARP (data not shown). In contrast, a dose-dependent effect of DMBA on PARP cleavage was observed when pre-B cells were cocultured with BMS2 cells in the presence of 0.01 to $10 \mu\text{M}$ DMBA (Fig. 3). As a positive control for apoptosis, pre-B cells were treated with the apoptosis-inducing agent staurosporine (Bertrand et al., 1994). Cell lysates from pre-B cells treated with staurosporine contained mostly the 85-kDa cleaved PARP fragment, indicating that most of the cells were apoptotic (data not shown). These observations confirm our TUNEL-assay results, which indicated that pre-B-cell apoptosis was DMBA-dose dependent and required BMS2 bone marrow stromal cells.

Bone Marrow Stromal Cell AhR Activation Does Not Induce Pre-B-Cell Apoptosis. We and others have demonstrated previously that bone marrow stromal cells possess a functional AhR that is activated by DMBA and TCDD (Heidel et al., 1998; Lavin et al., 1998) and that AhR activation may influence DMBA-induced pre-B-cell apoptosis (Yamaguchi et al., 1997b). In this portion of the study, we used the potent AhR ligand TCDD to address whether bone marrow stromal cell AhR activation would cause pre-B-cell apoptosis. TCDD has a much higher affinity for the AhR than DMBA

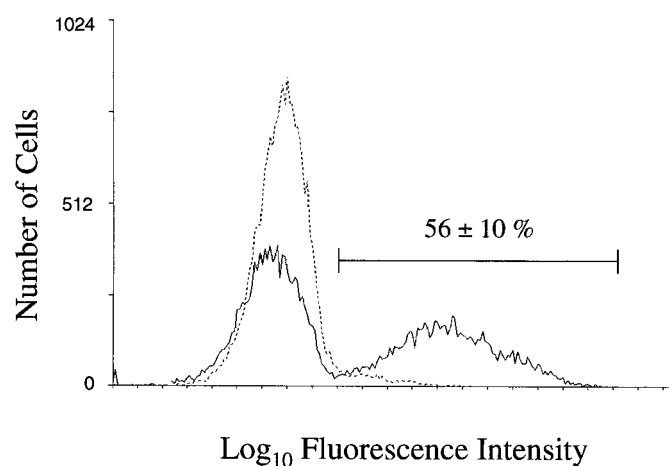


Fig. 1. DMBA-mediated pre-B-cell apoptosis is bone marrow stromal cell dependent. Pre-B cells were incubated with $1 \mu\text{M}$ DMBA for 24 h in the presence (solid line) or absence (dashed line) of bone marrow stromal cells (BMS2 cells). Pre-B cells were harvested, TUNEL labeled, and assessed by flow cytometry. The horizontal bar indicates the range of TUNEL-positive cells after incubation with DMBA in the presence ($56 \pm 10\%$ calculated from six separate experiments) or absence ($4 \pm 1\%$) of BMS2 cells. The latter is identical to the percentage of apoptotic pre-B cells observed when they were cultured in medium without BMS2 cells and DMBA.

but, unlike DMBA, is not metabolized by P450 cytochromes. Therefore, any toxicity exerted by TCDD can be completely attributed to AhR activation. Pre-B cells and BMS2 cells were cocultured with 10 nM TCDD, a concentration that has been demonstrated to maximally activate the AhR in BMS2 cells (Heidel et al., 1998). Pre-B-cell apoptosis was not observed by TUNEL assay after TCDD treatment (Fig. 4), thus indicating that bone marrow stromal cell AhR activation by itself does not cause pre-B-cell apoptosis.

3,4-Dihydrodiol Metabolite of DMBA Is More Potent than DMBA in Causing Pre-B-Cell Apoptosis. Metabolism of DMBA to the highly mutagenic and toxic 3,4-dihydrodiol-1,2-epoxide metabolite proceeds through the intermediate DMBA 3,4-dihydrodiol metabolite, which we have previously shown is produced by CYP1B1 activity in BMS2 cells (Heidel et al., 1998). If DMBA metabolism is involved in pre-B-cell apoptosis, then the 3,4-dihydrodiol DMBA metabolite should be more potent than DMBA. When we tested this possibility, approximately 70% of the pre-B cells were apoptotic when cocultured with BMS2 cells in the presence of 1 μ M 3,4-dihydrodiol, whereas only about 40% of the pre-B cells were apoptotic when cocultured with BMS2 cells and 1 μ M DMBA ($p < .05$; Fig. 4). Pre-B cells cultured with 1 μ M DMBA-3,4-dihydrodiol in the absence of BMS2 cells resulted in only control levels of apoptosis (data not shown). These results suggest that maximal pre-B-cell apoptosis requires

DMBA metabolism to the 3,4-dihydrodiol-1,2-epoxide metabolite by bone marrow stromal cells.

DMBA-DNA Adducts in Pre-B-Cells and Bone Marrow Stromal Cells. Our results suggest that DMBA metabolism by bone marrow stromal cell CYP1B1 is required for pre-B-cell apoptosis. It is known that the carcinogenic effect of DMBA requires DNA mutations that arise from the covalent binding of reactive metabolites to DNA forming DMBA-DNA adducts (Huberman and Sachs, 1977). We postulated that DMBA-DNA adduct formation may also be involved in pre-B-cell apoptosis. To investigate this possibility, pre-B cells were incubated with 1 μ M DMBA and BMS2 cells for 24 h and assessed for DNA adduct formation as described previously (Melendez-Colon et al., 1997). The total quantity of DMBA-DNA adducts per milligram of DNA was 34.6 pmol for pre-B cells and 15.1 pmol for BMS2 cells. The major peak indicated in the adduct profiles for both pre-B and BMS2 cells was identified as the syn-DMBA 3,4-dihydrodiol-1,2-epoxide deoxy-adenosine adduct, which represents approximately 50% of the total adducts for both cell types (Fig. 5). The remaining smaller peaks are DMBA dihydrodiol deoxy-adenosine or deoxy-guanosine adducts that were not further identified.

Bone Marrow Stromal Cell CYP1B1, but not the AhR, Is Required for Pre-B-Cell Apoptosis. The preceding experiments suggested that pre-B-cell apoptosis requires DMBA metabolism by CYP1B1 in BMS2 cells and not simply AhR activation. Previous results from our laboratory demonstrated that DMBA metabolism by bone marrow stromal cells occurs exclusively through CYP1B1 activity (Heidel et al., 1998). To further examine the relationship between CYP1B1-dependent DMBA metabolism and pre-B-cell apoptosis, we established primary bone marrow stromal cells from CYP1B1-null or CYP1B1-heterozygous mice. We reasoned that, if DMBA-induced pre-B-cell apoptosis was dependent on CYP1B1 activity, then these cells would not undergo apoptosis when cocultured with CYP1B1-null bone marrow stromal cells. The results confirmed this supposition, because 10 μ M DMBA caused significant numbers of apoptotic pre-B cells when they were cocultured with CYP1B1-heterozygous but not -null bone marrow stromal cells (Fig. 6A). Likewise, DMBA treatment increased the amount of the 85-kDa PARP cleavage product in pre-B cells incubated with CYP1B1-heterozygous but not CYP1B1-null bone marrow stromal cells (Fig. 6B). Taken together, the TUNEL and PARP cleavage data demonstrate that bone marrow stromal cell CYP1B1 is required for DMBA-induced pre-B-cell apoptosis.

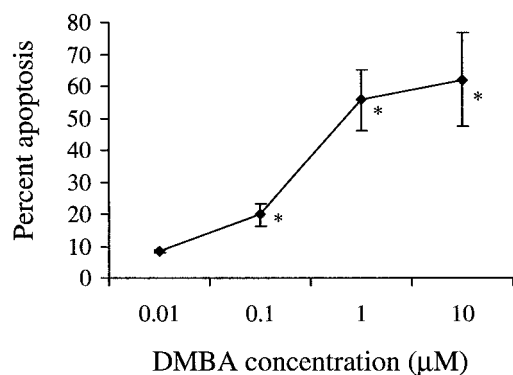


Fig. 2. Apoptosis of pre-B cells is DMBA-dose dependent. Pre-B cells were incubated with BMS2 cells for 24 h in presence of the indicated concentrations of DMBA. Each data point represents the mean \pm S.E. percentage of TUNEL-positive cells from six separate experiments. * $p < .05$, compared with control cultures ($4 \pm 1\%$, data not shown).

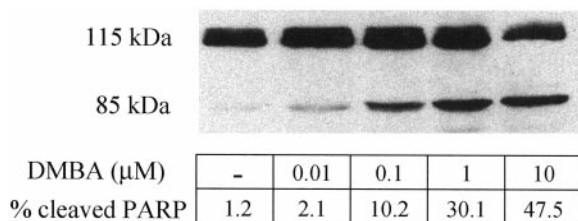


Fig. 3. PARP cleavage in pre-B cells cocultured with BMS2 cells and DMBA. Pre-B cells were cocultured with BMS2 cells and the indicated concentrations of DMBA for 24 h. Total protein was isolated, separated by SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose, and Western immunoblotted with an anti-PARP monoclonal antibody. Proteins were visualized with the enhanced chemiluminescence method and quantified with a Molecular Dynamics (Sunnyvale, CA) personal densitometer SI. Native (115-kDa) and cleaved (85-kDa) PARPs are shown. The values below the immunoblot indicate the percentage of cleaved PARP. This immunoblot is representative of results obtained in three other independent experiments.

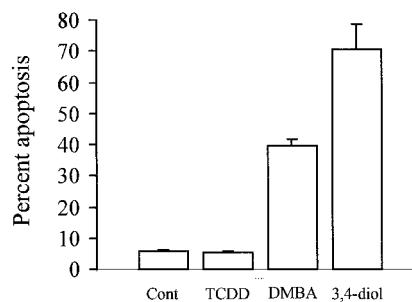


Fig. 4. Increased pre-B-cell apoptosis with DMBA 3,4-dihydrodiol. Pre-B cells were cocultured with BMS2 cells and 10 nM TCDD, 1 μ M DMBA, or 1 μ M DMBA 3,4-dihydrodiol for 24 h. Apoptotic pre-B cells were TUNEL labeled and quantified by flow cytometry as described in Fig. 1. The data shown are the means \pm S.E. of three separate experiments.

Discussion

This investigation demonstrates that bone marrow stromal cell CYP1B1 is required for DMBA-induced apoptosis of pre-B cells in vitro. Because the carcinogenic effect of DMBA requires metabolic activation by P450 cytochromes, it seems intuitive that metabolism would also be involved in immunotoxicity. Although many reports have stressed the importance of DMBA metabolism for immunotoxicity (White et al., 1985; Kawabata and White, 1989), others advocate a requirement for AhR activation (Yamaguchi et al., 1997a,b). Our study shows that primary bone marrow stromal cells and the BMS2 bone marrow stromal cell line have comparable abilities to facilitate DMBA-induced apoptosis of pre-B cells in vitro. The absence of pre-B-cell apoptosis when cocultured with CYP1B1-null primary bone marrow stromal cells and DMBA clearly demonstrates a requirement for CYP1B1-mediated DMBA metabolism. AhR activation is not required for this effect, because DMBA caused significant pre-B-cell apoptosis when cocultured with AhR-null primary bone marrow stromal cells. The percentage of apoptotic pre-B cells was reduced in the presence of AhR-null stromal cells compared with AhR-heterozygous stromal cells. This reduction parallels the decrease in CYP1B1 in the former because of the absence of AhR activation. These findings have important implications for the immunotoxicity of DMBA and perhaps other polycyclic aromatic hydrocarbons.

The importance of DMBA metabolism for pre-B-cell apoptosis was supported by the lack of apoptosis until these cells were incubated with bone marrow stromal cells. In a previ-

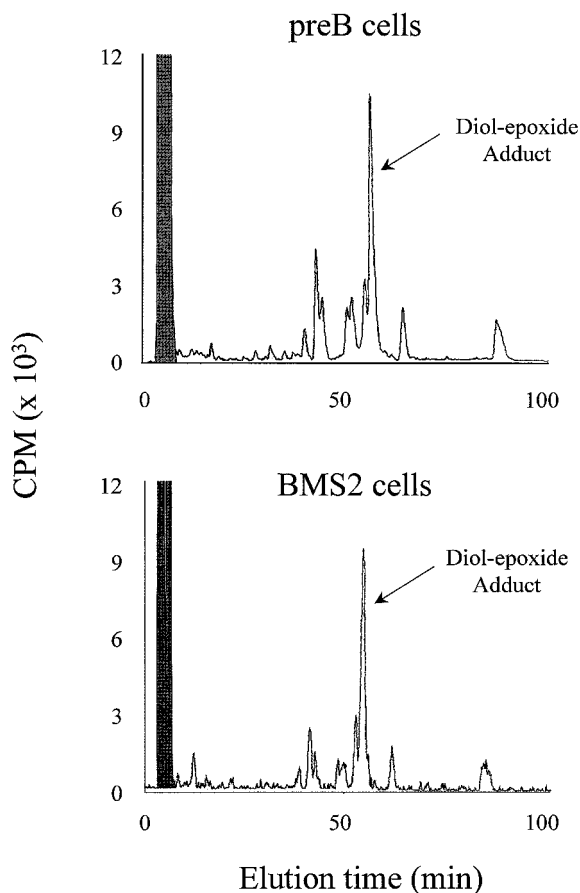


Fig. 5. DMBA-DNA adducts in pre-B cells and BMS2 cells. HPLC analysis of the ^{33}P -postlabeled DMBA-DNA adducts formed in pre-B cells (A) or BMS2 cells (B) cocultured for 24 h with $1\ \mu\text{M}$ DMBA. The total amounts of DMBA-DNA adducts per milligram DNA were $34.6\ \mu\text{mol}$ and $15.1\ \mu\text{mol}$ in pre-B cells and BMS2 cells, respectively. The arrow indicates the *syn*-DMBA 3,4-dihydrodiol-1,2-epoxide adduct with deoxyadenosine monophosphate, which accounted for about 50% of the total adducts present. Peaks that eluted within the first 5 min are breakthrough peaks of ^{33}P -labeled components of the postlabeling procedure. The DMBA adducts eluted after 20 min, and no adducts were present in vehicle-treated cells.

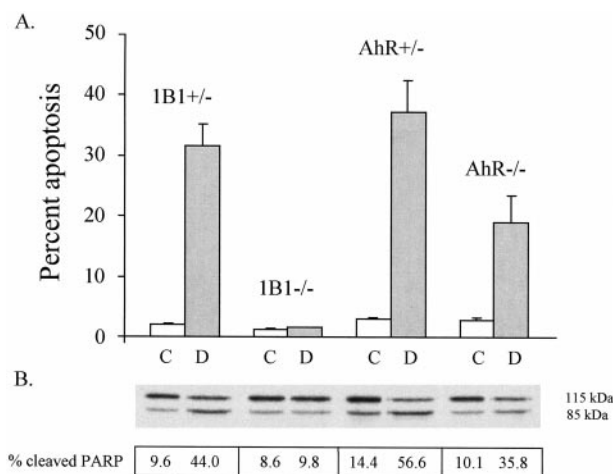


Fig. 6. Pre-B-cell apoptosis requires bone marrow stromal cell CYP1B1. A, primary bone marrow stromal cells were established from CYP1B1-heterozygous (1B1^{+/+}) or -null (1B1^{-/-}) mice and from AhR-heterozygous (AhR^{+/+}) or -null (AhR^{-/-}) mice, as described in *Materials and Methods*. Pre-B cells were incubated for 48 h with the indicated types of bone marrow stromal cells in the presence (shaded columns) or absence (open columns) of $10\ \mu\text{M}$ DMBA. The data shown are the results of a single experiment that is representative of two others. Apoptotic pre-B cells were TUNEL labeled and quantified by flow cytometry. DMBA treatment resulted in a significant ($p < .01$) increase in pre-B-cell apoptosis when incubated with all bone marrow stromal cells except those from CYP1B1-null mice ($p > .05$). B, pre-B-cell apoptosis as determined by PARP cleavage. Total protein was isolated from an aliquot of pre-B cells removed immediately before the TUNEL assay. Proteins were separated by SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose, and immunoblotted with anti-PARP monoclonal antibody. The enhanced chemiluminescence method was used to visualize proteins. The values below the immunoblot indicate the percentage cleaved PARP, as estimated with a Molecular Dynamics personal densitometer SI.

ous study, we demonstrated that CYP1B1 is the cytochrome P450 principally responsible for bone marrow stromal cell DMBA metabolism (Heidel et al., 1998). We have not been able to detect CYP1B1 expression or DMBA metabolism in pre-B cells (SMH, CJC, and CRJ, unpublished data), which is consistent with the previously reported absence of DMBA metabolism by murine lymphocytes (Thurmond et al., 1989). Further evidence for the importance of bone marrow stromal cell metabolism of DMBA was provided by our observation that DMBA 3,4-dihydrodiol, a CYP1B1-dependent DMBA metabolite that does not activate AhR, was significantly more potent than a 10-fold greater concentration of DMBA in causing pre-B-cell apoptosis. This observation is compatible with our earlier report that 15% of the DMBA is converted to DMBA 3,4-dihydrodiol by BMS2 cells (Heidel et al., 1998) and with the report of Ladics et al. (1991), who demonstrated that DMBA 3,4-dihydrodiol was more potent than DMBA in causing a reduction of antibody-forming B cells. The latter study did not examine the cell type that metabolized DMBA or whether apoptosis of B cells was responsible for their reduced numbers. The importance of stromal cell CYP1B1 was established by our results demonstrating that DMBA did not cause pre-B-cell apoptosis when cocultured with CYP1B1-null bone marrow stromal cells. Because similar cell types were present in CYP1B1-null and -heterozygous bone marrow stromal cell cultures (data not shown), it is likely that the lack of pre-B-cell apoptosis with CYP1B1-null bone marrow stromal cells was the result of a deficiency in DMBA metabolism and not variations in cell type among cultures. Our observations are consistent with a previous study, which demonstrated that the cytochrome P450 inhibitor α -naphthoflavone blocked the ability of bone marrow stromal cells to cause pre-B-cell apoptosis (Yamaguchi et al., 1997b). However, Yamaguchi et al. emphasized the role of α -naphthoflavone as an antagonist of the AhR.

In contrast to our findings of a stromal cell requirement, recent reports have demonstrated that DMBA and benzo[a]pyrene cause apoptosis in mature human B cell lines (Salas and Burchiel, 1998). These findings may be explained by differences between human and mouse B cells or because these studies used mature B cells as opposed to our precursor B cells. Separate findings have demonstrated constitutive CYP1B1 expression in peripheral blood mononuclear cells from normal human donors (Dassi et al., 1998; Spencer et al., 1999), suggesting that CYP1B1-dependent metabolites of DMBA and benzo[a]pyrene induce apoptosis of human B cell lines. This hypothesis is supported by metabolites of benzo[a]pyrene causing more human B cells to undergo apoptosis than benzo[a]pyrene itself and by α -naphthoflavone inhibiting apoptosis (Salas and Burchiel, 1998). The findings implicate CYP1B1 in mature human B cells as being important for xenobiotic metabolite toxicity within these cells. Whether precursor B cells in human bone marrow express CYP1B1 is not known.

Our studies demonstrate that the AhR does not have an obligatory function in DMBA-mediated pre-B-cell apoptosis. It has been reported previously that AhR activation by TCDD decreases bone marrow cellularity, possibly by altering bone marrow stem cells (Fine et al., 1989). We and others have previously demonstrated that both DMBA and the potent AhR ligand TCDD activate the AhR in bone marrow stromal cells (Heidel et al., 1998; Lavin et al., 1998). Other investi-

gators have proposed that bone marrow stromal cell AhR activation plays a more prominent role in pre-B-cell apoptosis induced by DMBA (Yamaguchi et al., 1997a,b). In contrast, our studies demonstrated that the AhR in stromal cells is not required for apoptosis of pre-B cells mediated by DMBA, although the AhR increases DMBA-induced pre-B-cell apoptosis by about 2-fold. This increase in apoptosis matches the 2-fold increase in CYP1B1 protein in bone marrow stromal cells after DMBA treatment (Heidel et al., 1998), suggesting that the AhR influences DMBA-induced apoptosis by increasing CYP1B1. In the light of the constitutive expression of CYP1B1 in bone marrow stromal cells (Heidel et al., 1998), it is unlikely that the AhR is critical for the bone marrow toxicity of DMBA. The possibility of ligand-free AhR regulation of CYP1B1 is not supported by a previous report that nuclear extracts from untreated BMS2 cells lack detectable binding to dioxin response elements (Lavin et al., 1998). Overall, our observations are consistent with older reports that show that immunotoxicity of DMBA in vivo is largely independent of the AhR (Thurmond et al., 1987). In addition, other polycyclic aromatic hydrocarbons, such as methylcholanthrene and fluoranthene, cause AhR-independent apoptosis of thymocytes and pre-B cells, respectively (Yamaguchi et al., 1996; Lutz et al., 1998).

Pre-B-cell apoptosis may be initiated by DMBA-DNA adducts formed through the covalent binding of reactive DMBA metabolites with DNA. We found that pre-B cells and BMS2 cells contained relatively similar levels of DMBA 3,4-dihydrodiol-1,2 epoxide DNA adducts and that the distribution of adducts arising from different epoxide isomers was similar in the two cell types. Because the pre-B cells lack CYP1B1, these data strongly suggest that the reactive DMBA epoxides were generated in BMS2 cells and then readily equilibrated between the two cell types. The increased level of adducts in pre-B cells might reflect their higher rate of proliferation, because it has been reported that benzo[a]pyrene metabolites preferentially attack replicating DNA (Melendez-Colon et al., 1997). Although it is well established that DMBA-DNA adduct formation is a critical step in the process of tumor initiation by DMBA (Harvey, 1991), the mechanisms by which DMBA-DNA adducts may cause pre-B-cell apoptosis are not understood. Other forms of DNA damage, including those resulting from γ -irradiation and chemotherapeutic agents, induce cell cycle arrest and apoptosis by increasing the amount of p53 (Lowe et al., 1993; Cui et al., 1995). Although we suspect that DMBA-DNA adducts might induce pre-B-cell apoptosis through the same pathway, we cannot exclude the possibility of an adduct-independent pathway. In addition, p53 levels were not elevated in pre-B cells undergoing DMBA-induced apoptosis (data not shown). This finding suggests that DMBA-induced apoptosis of pre-B cells may be p53 independent, which is similar to the p53-independent growth arrest of 3T3 fibroblasts by the polycyclic aromatic hydrocarbon benzo[a]pyrene (Vaziri and Faller, 1997).

Bone marrow stromal cell DMBA-DNA adducts may stimulate these cells to release a factor that causes apoptosis of pre-B cells. Candidate apoptosis-inducing factors produced by bone marrow stromal cells, including transforming growth factor- β and tumor necrosis factor- α , have been shown to cause apoptosis of bone marrow progenitor cells (Lotem and Sachs, 1992; Ashkenazi et al., 1998). It might also be possi-

bility that DMBA-DNA adducts impair bone marrow stromal cell release of growth factors required for pre-B-cell survival. It has been previously demonstrated that several growth factors [e.g., interleukins 3 or 6, and granulocyte-macrophage colony-stimulating factor) produced by bone marrow stromal cells can rescue bone marrow progenitor cells from apoptosis (Williams et al., 1990; Lotem and Sachs, 1992). Because the 70Z/3 pre-B cells used in this study are not stromal cell dependent, however, it is unlikely that the pre-B-cell apoptosis we observed resulted from a deficit in growth-factor production. Future studies should clarify whether DMBA-DNA adducts in pre-B cells, bone marrow stromal cells, or both are required for pre-B-cell apoptosis.

In summary, this study provides convincing evidence that bone marrow CYP1B1 is critical for the pre-B-cell toxicity of DMBA. In contrast, a functional AhR is not required for pre-B-cell apoptosis, although it does amplify the response to DMBA, probably by mediating the induction of CYP1B1. These observations may explain previous reports that the immunotoxicity of polycyclic aromatic hydrocarbons is frequently more severe in low- than in high-affinity AhR mice (Thurmond et al., 1987). Because hepatic CYP1A1 is less readily induced in low-affinity AhR mice, more PAH is diverted to other tissues, such as the bone marrow. Local metabolism by bone marrow stromal cell CYP1B1, and not hepatic CYP1A1 activity, is critical for the pre-B-cell toxicity of DMBA. Because human CYP1B1 activates many diverse procarcinogens to mutagenic metabolites (Shimada et al., 1996), it is possible that human bone marrow cytotoxicity has a similar dependence on CYP1B1-mediated metabolism of polycyclic aromatic hydrocarbons. Further investigations concerning CYP1B1 will help to resolve these important issues.

Acknowledgments

We thank Dr. Chris Bradfield (Madison, WI) and Frank Gonzalez (Frederick, MD) for providing the AhR-null and P4501B1-null mice, respectively. We greatly appreciate Dr. William Baird (Corvallis, OR) for performing the DMBA-DNA adduct analysis. We thank Michele Larson and David Alexander for technical assistance and advice and Steven Giles for assistance in preparing the figures and final manuscript.

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