

Bone Marrow Stromal Cells Constitutively Express High Levels of Cytochrome P4501B1 that Metabolize 7,12-Dimethylbenz[a]anthracene

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ABSTRACT

The polycyclic aromatic hydrocarbon 7,12-dimethylbenz[a]anthracene (DMBA) is a potent carcinogen that produces immunotoxic effects in bone marrow. Here, we show that bone marrow stromal cells metabolize DMBA to such products as 3,4-dihydrodiol, the precursor to the most mutagenic DMBA metabolite. The BMS2 bone marrow stromal cell line constitutively expressed higher levels of CYP1B1 protein and mRNA than C3H10T $\frac{1}{2}$ mouse embryo fibroblasts. BMS2 cells also produced a DMBA metabolite profile that was consistent with CYP1B1 activity. Treatment with the potent aryl hydrocarbon receptor (AhR) ligand 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) induced a ~2-fold increase in CYP1B1 mRNA, protein, and activity in BMS2 cells. Two forms of the AhR (97 and 104 kDa) and the AhR nuclear translocator were detected in BMS2

cells. The AhR translocated to the nucleus after treatment with TCDD or DMBA but was ~5 times slower with DMBA. Primary bone marrow stromal (BMS) cell cultures established from AhR^{-/-} mice showed similar basal CYP1B1 expression and activity as cell cultures established from heterozygous littermates or C57BL/6 mice. However, primary BMS cells from AhR^{-/-} mice did not exhibit increased CYP1B1 protein expression after incubation with TCDD. BMS cells therefore constitutively express functional CYP1B1 that is not dependent on the AhR. This contrasts with embryo fibroblasts from the same mouse strain, in which basal CYP1B1 expression is AhR dependent. We therefore conclude that bone marrow toxicity may be mediated by CYP1B1-dependent DMBA metabolism, which is regulated by factors other than the AhR.

PAHs are environmental contaminants formed during incomplete combustion. Human exposure to these compounds occurs primarily through cigarette smoking, the inhalation of polluted air, and ingestion of charbroiled foods (Davila *et al.*, 1995). These compounds are known to be carcinogens (Pelkonen and Nebert, 1982) and cause immunosuppression in laboratory animals (White *et al.*, 1994; Davila *et al.*, 1995). DMBA is one of the most potent carcinogenic and immunosuppressive PAHs. Mice treated with DMBA exhibit signs of immunotoxicity that include reduced spleen, bone marrow (Ward *et al.*, 1984), and thymus cellularity (Thurmond *et al.*, 1987) and decreased resistance to *Listeria monocytogenes* infection (Ward *et al.*, 1984) and tumor growth (Dean *et al.*, 1986). Although these *in vivo* studies demonstrate that DMBA cause a generalized reduction of immune cells that

results in decreased immune surveillance, they do not provide a mechanistic explanation of how DMBA produces these effects.

Recent reports suggest that the bone marrow toxicity of DMBA may be dependent on the AhR in BMS cells (Yamaguchi *et al.*, 1997a, 1997b). DMBA treatment of pre-B cells cultured with BMS cells caused apoptosis of pre-B cells, whereas DMBA treatment of pre-B cells in the absence of BMS cells did not. The cytochrome P450 and AhR antagonist α -naphthoflavone blocked BMS cell-dependent pre-B cell apoptosis. Because neither BMS cells nor pre-B cells express CYP1A1 and only BMS cells express the AhR, the authors postulated that BMS cell AhR activation was necessary for DMBA-induced pre-B cell apoptosis. However, DMBA is a relatively weak AhR ligand (Bigelow and Nebert, 1982), and the potent AhR ligand TCDD did not cause pre-B cell apoptosis. These findings suggest that an event other than AhR activation is involved in DMBA-induced pre-B cell apoptosis.

Metabolism of DMBA may play a role in BMS cell-depen-

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ABBREVIATIONS: PAH, polycyclic aromatic hydrocarbon; AhR^{-/-} BMS, primary bone marrow stromal cells prepared from AhR null mice; AhR^{+/-} BMS, primary bone marrow stromal cells prepared from AhR heterozygote mice; AhR, aryl hydrocarbon receptor; ARNT, aryl hydrocarbon receptor nuclear translocator; BMS, bone marrow stromal; C57-BMS, primary bone marrow stromal cells prepared from C57BL/6 mice; DMBA, 7,12-dimethylbenz[a]anthracene; DMSO, dimethylsulfoxide; EGTA, ethylene glycol bis(β -aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; FBS, fetal bovine serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; SDS, sodium dodecyl sulfate; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin.

dent pre-B cell apoptosis. It is well established that metabolism of PAHs is required for them to be carcinogenic (Pelkonen and Nebert, 1982) and that a correlation exists between the carcinogenic and immunotoxic potential of PAHs (White and Holsapple, 1984; White *et al.*, 1985). From this relationship, it seems likely that PAH metabolism is required for immunosuppression and that the most potent toxic effects would be produced by metabolism within the target tissue. In accordance with this paradigm, bone marrow cells (Heidel *et al.*, 1997; O'Dowd, 1987) and splenic microsomes (Kawabata and White, 1989) metabolized DMBA. In addition, the 3,4-dihydrodiol metabolite of DMBA was 65-fold more potent than DMBA in causing a reduction in the number of antibody-producing splenic B cells (Ladics *et al.*, 1991). Furthermore, the cytochrome p450 antagonist α -naphthoflavone prevented the reduction of antibody-producing B cells generated by DMBA (Ladics *et al.*, 1991). These data provide convincing evidence that cytochrome P450-dependent metabolism of DMBA was required for mature B cell toxicity. However, a requirement for metabolism of DMBA in bone marrow progenitor B cell toxicity remains to be demonstrated.

In the current study, we sought to better understand the potential contributions of cytochrome P450-dependent metabolism and AhR activation in DMBA-induced bone marrow toxicity. To accomplish this, we characterized BMS cell cytochrome P450 expression, DMBA metabolism, and AhR activation.

Materials and Methods

Animals. Dr. Albee Messing (University of Wisconsin, Madison, WI) generously donated C57BL/6 mice for this study. Mice heterozygous for a disrupted AhR gene were generated (Schmidt *et al.*, 1996) and provided by Dr. Chris Bradfield (University of Wisconsin, Madison, WI). Breeding of AhR heterozygous adult mice produced pups that were null, heterozygous, or homozygous for the wild-type AhR allele. Pups were genotyped by PCR amplification of tail DNA for the presence of a 669-bp AhR gene product (AhR^{+/+}, 453-1122), a 459-bp neomycin gene product (AhR^{-/-}, 782-1240), or both (AhR^{+/-}). The 3-4-week-old pups were used to establish primary BMS cell cultures.

Antibody and cDNA probes. Rabbit antibodies to mouse AhR and mouse ARNT were generously provided by Dr. Rick Pollenz (University of South Carolina, Charleston, SC). Monoclonal anti-GAPDH was purchased from Biodesign International (Kennebunk, ME). Rabbit antibodies to CYP1B1 and CYP1A1 were prepared in our laboratory as described previously (Pottenger and Jefcoate, 1990). All primary antibodies were used at a concentration of 1 μ g/ml in antibody dilution buffer [10 mM Tris-HCl, pH 8.0, 150 mM NaCl, and 0.05% Tween 20 (v/v)]. A 1028-bp mouse CYP1B1 probe was obtained by *Sma*I restriction endonuclease digestion of a cDNA-containing plasmid (Savas *et al.*, 1994). Mouse GAPDH cDNA probe was prepared by PCR amplification of control DNA using primers purchased from Stratagene (La Jolla, CA).

Cell culture and treatments. BMS2 cells were provided generously by Dr. Paul Kincade (Oklahoma Medical Research Foundation, Oklahoma City, OK) (Pietrangeli *et al.*, 1988). C3H/10T $\frac{1}{2}$ cells were purchased from the American Type Culture Collection (Rockville, MD). BMS2 cells were grown in RPMI1640 with 5% FBS (v/v) (Intergen, Purchase, NY) and C3H/10T $\frac{1}{2}$ cells were grown in Dulbecco's modified Eagle's medium with 7% FBS. All media were supplemented with 5×10^{-5} M 2-mercaptoethanol, 2 mM L-glutamine, 50 IU/ml penicillin, and 50 mg streptomycin/ml. When monolayers of BMS2 cells and C3H/10T $\frac{1}{2}$ cells were 70-80% confluent, the conditioned media were removed, and the cells were incubated with fresh

media containing 10 μ M DMBA [or 0.1% DMSO (v/v) or 10 nM TCDD as controls] for the times indicated in the figure legends.

Primary BMS cell cultures were established in six-well tissue culture plates (Whitlock and Witte, 1982) with RPMI-1640 and 5% FBS. Using this method, primary BMS cells retain their ability to support long term lymphopoiesis *in vitro* (Whitlock *et al.*, 1984). The cultures initially consisted of many different individual adherent cell colonies that grew to 60-90% confluence after 2-3 weeks of culture. At this time, the nonadherent cells were removed by gentle pipetting, and the remaining adherent BMS cells were used in experiments. In some experiments that used primary BMS cells, TRIzol reagent (GIBCO BRL, Bethesda, MD) was used to isolate total cellular protein, mRNA, and DNA.

Preparation of microsomes and cytoplasmic and nuclear extracts. CYP1B1 and CYP1A1 Western immunoblots were performed on microsomes isolated as described previously (Pottenger and Jefcoate, 1990). AhR and ARNT Western immunoblots were performed on cytosolic and nuclear fractions isolated from BMS2 cells. To prepare the fractions, BMS2 cells were treated for 1 hr with 10 μ M DMBA, 10 nM TCDD, or 0.1% DMSO vehicle control; washed with ice-cold phosphate-buffered saline; and removed from the flask with a cell scraper. After centrifugation at $500 \times g$, BMS2 cell pellets were resuspended in lysis buffer [25 mM 3-(*N*-morpholino)propane-sulfonic acid, pH 7.4, 0.02% Na₂N₃ (w/v), 10% glycerol (v/v), 1 mM Na₂EDTA, 5 mM EGTA, 0.5% Tween-20 (v/v), 2 mM NaVO₄, 1 mM NaF, 20 mM Na₂MoO₄, 11.7 μ M leupeptin, 100 units/ml aprotinin, 5 μ g/ml soybean trypsin inhibitor, and 27 μ M 1-chloro-3-tosylamido-7-amino-L-2-heptanone] and incubated on ice for 30 min. The supernatant from a $325 \times g$ centrifugation was collected and designated the cytosolic fraction. The remaining pellet (nuclear fraction) was washed three times with cold lysis buffer and sonicated on ice (four 15-sec bursts at 40% power, Sonicator Cell Disrupter; Heat Systems-Ultrasonics, Plainview, NY). Protein concentrations were determined according to the bicinchoninic acid method (Pierce Chemical, Rockford, IL).

Western immunoblots. Total cell, microsomal, cytoplasmic, or nuclear proteins were resolved in a 0.75-mm 7.5% SDS-polyacrylamide gel according to standard methods (Sambrook *et al.*, 1989), and transferred to nitrocellulose membrane (Amersham, Arlington Heights, IL) using a Hoeffler TE51 transfer apparatus at 500 mA for 1 hr. The membranes were incubated for 2 hr with primary antibody (1 μ g/ml), and the immunoreactive proteins were visualized with the enhanced chemiluminescence detection method (Amersham). Immunoblots that were probed with more than one primary antibody were incubated in stripping solution (100 mM 2-mercaptoethanol, 62.5 mM Tris-HCl, pH 6.7, 2% SDS) for 30 min at 50° and washed twice (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.1% Tween 20) before incubation with a different primary antibody. Immunoblot signals were

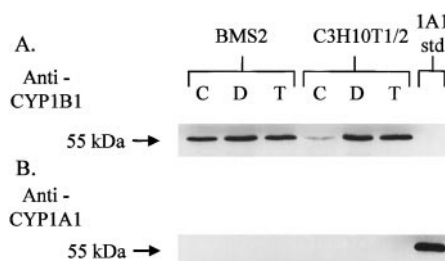


Fig. 1. Immunoblot analysis of CYP1B1 in BMS2 cells and C3H10T $\frac{1}{2}$ cells. Microsomal proteins were isolated after 24-hr treatments with 0.1% DMSO vehicle (C), 10 μ M DMBA (D), or 10 nM TCDD (T). All lanes were loaded with 10 μ g of protein, except for the far right lane, which received 2 ng of CYP1A1 (IA1 std) as a control. Proteins were separated on SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose, and incubated with anti-CYP1B1 or anti-CYP1A1. The enhanced chemiluminescence method was used to visualize proteins. The immunoblot was first probed with anti-CYP1B1 antibody (A) and then stripped and reprobed with anti-CYP1A1 antibody (B).

quantified using a Molecular Dynamics (Sunnyvale, CA) Personal Densitometer SI and ImageQuant software.

RNA isolation and Northern blot analysis. Total RNA was isolated, quantified, and electrophoresed through a 1% agarose gel containing formaldehyde according to standard methods (Sambrook *et al.*, 1989). RNA was transferred from the gel to Hybond-N⁺ membrane (Amersham) and fixed by baking for 2 hr at 80°. cDNA probes were labeled with α -³²P-dCTP (3000 Ci/mmol) using Prime-a-Gene (Promega, Madison, WI) and purified with G50 Nick Columns (Pharmacia, Piscataway, NJ). Prehybridization of the membranes was performed for 2 hr at 42° in a buffer of 750 mM NaCl, 75 mM Na₃ citrate, pH 7.0, 50% deionized formamide (v/v), 0.1% SDS (w/v), 100 μ g of denatured salmon sperm DNA/ml, 0.5% Ficoll 400 (w/v), 0.5% polyvinylpyrrolidone (w/v), and 0.5% bovine serum albumin (w/v). Prehybridization and hybridization were carried out in the same buffer, with the exception that a ³²P-labeled probe was added for hybridization. Blots were washed using standard methods (Sambrook *et al.*, 1989), and the amount of specific ³²P-labeled cDNA hybridization was quantified using a Molecular Dynamics PhosphorImager and ImageQuant software.

Metabolism assays. To assess the effect of prior cytochrome P450 induction on DMBA metabolism, cells were treated with 10 nM TCDD for 24 hr at 37°. Control cells were treated with 0.1% DMSO (vehicle control). Conditioned medium was removed and replaced with fresh medium containing 1 or 10 μ M DMBA. After a 1-hr incubation at 37°, the medium containing DMBA and metabolites was removed from the cells and incubated for 2 hr at 37° with β -glucuronidase solution (2000 IU of β -glucuronidase/ml, 0.5 M sodium acetate, pH 5.2, and 5.7 mM ascorbic acid). Cortisol was added to each sample as an internal standard, and the DMBA metabolites were extracted with ethyl acetate/acetone-containing dithiothreitol (at ratios of 2:1:0.003). The solvent phase was dried down under nitrogen gas and resuspended in 100 μ l of methanol. Separation and quantification of DMBA metabolites were performed by high performance liquid chromatography analysis as described previously (Savas *et al.*, 1993). Metabolic activities were normalized to the number of cells present in each well after completion of the DMBA metabolism assay. Cells were detached by treating them for 1–5 min with 0.05% trypsin (w/v) in calcium and magnesium-free Hanks' balanced salt solution, and the trypsin was neutralized by adding RPMI media containing 5% FBS. Cells were enumerated using a hemacytometer.

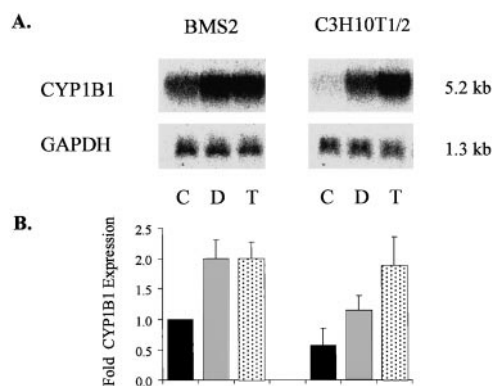


Fig. 2. Northern analysis of CYP1B1 in BMS2 cells and C3H10T1/2 cells. Cells were treated for 24 hr with 0.1% DMSO as a vehicle control (C), 10 μ M DMBA (D), or 10 nM TCDD (T). A, Total RNA (10 μ g/lane) that was electrophoresed in a denaturing agarose gel, transferred to nitrocellulose, and hybridized with ³²P-labeled cDNA probes for CYP1B1 and GAPDH. B, Level of CYP1B1 mRNA expression relative to control BMS2 cells (C). Bars, mean \pm standard deviation expression of three separate experiments, as quantified using a Molecular Dynamics PhosphorImager. Loading differences were corrected using GAPDH as an internal control.

Results

BMS cells express CYP1B1. Western immunoblot analysis demonstrated that the BMS2 mouse BMS cell line constitutively expresses CYP1B1 protein (Fig. 1). BMS2 cells had ~4-fold greater constitutive levels of 55-kDa CYP1B1 protein than the C3H10T1/2 mouse embryo fibroblast cell line, which are known to constitutively express CYP1B1 (Pottenger and Jefcoate, 1990). Treatment of BMS2 cells with DMBA or TCDD increased CYP1B1 protein by only 1.3-fold (1.3 ± 0.1 as an average of three separate experiments) and 1.4-fold (1.4 ± 0.3), respectively, relative to the vehicle control (0.1% DMSO). Incubation with DMSO had no effect compared with BMS2 cells incubated in medium alone (data not shown). In agreement with previous reports (Pottenger and Jefcoate, 1990; Savas *et al.*, 1993), CYP1B1 protein increased by 5- and 6-fold, respectively, in C3H10T1/2 cells incubated with DMBA or TCDD. These induced levels of CYP1B1 in C3H10T1/2 cells were comparable to those in BMS2 cells after induction with DMBA or TCDD. CYP1A1 protein was not detected in either cell line.

Northern blot analysis demonstrated that BMS2 cells expressed about twice the amount of constitutive CYP1B1 mRNA as did C3H10T1/2 cells and that expression increased ~2-fold in BMS2 cells and ~4-fold in C3H10T1/2 cells after a 24-hr treatment with TCDD (Fig. 2). These estimates of CYP1B1 mRNA induction correlate well with the increases in CYP1B1 protein expression in response to TCDD treatment (Fig. 1). DMBA treatment induced a 2-fold increase in CYP1B1 mRNA in both BMS2 cells and C3H10T1/2 cells (Fig. 2). These observations are consistent with the hypothesis that AhR activation of transcription is capable of regulating CYP1B1 induction in BMS2 cells.

To show that the BMS2 BMS cell line accurately reflects their *in vivo* counterparts, we investigated CYP1B1 expression in primary bone marrow cultures established from C57BL/6 mice (C57-BMS) (Whitlock and Witte, 1982). These primary bone marrow cultures support long term lymphopoiesis *in vitro* (Whitlock *et al.*, 1984). Light microscopy revealed that C57-BMS cultures consisted of a heterogeneous population of adherent cells and that many cells were morphologically similar to BMS2 cells (data not shown). As shown in Fig. 3, C57-BMS constitutively expressed 3-fold more constitutive microsomal CYP1B1 protein than C3H10T1/2 cells and demonstrated a 2-fold increase of CYP1B1 after a 24-hr treat-

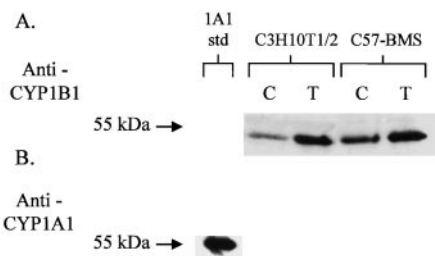


Fig. 3. Immunoblot analysis of CYP1B1 in C57-BMS cells and C3H10T1/2 cells. Primary BMS cells from C57BL/6 mice (C57-BMS) were established as described in Materials and Methods. After 24-hr treatments with 0.1% DMSO as a vehicle control (C) or 10 nM TCDD (T), microsomal proteins were isolated and Western immunoblotted. As a standard, 2 ng of purified CYP1A1 (IA1 std) was included in a separate lane. The immunoblot was first probed with anti-CYP1B1 (A) and then stripped and reprobed with anti-CYP1A1 (B). Proteins were visualized according to the enhanced chemiluminescence method.

ment with TCDD. CYP1A1 was not detected in C57-BMS cells. These results are comparable to those obtained with BMS2 cells.

BMS cells metabolize DMBA. Table 1 demonstrates that BMS2 cells metabolize DMBA with a regioselectivity that is very similar to that of C3H10T½ cells. DMBA metabolism in C3H10T½ cells has been previously characterized to be entirely due to CYP1B1 (Pottenger and Jefcoate, 1990; Savas *et al.*, 1997). The proximate carcinogen DMBA-3,4-dihydrodiol was a similar fraction of the total dihydrodiols (13–19%) produced by both BMS2 and C3H10T½ cells, whereas DMBA-5,6-dihydrodiol was absent (Table 1). Although the DMBA-dihydrodiol metabolite patterns were similar, the amounts of DMBA-phenols produced by BMS2 cells were consistently higher than those produced by C3H10T½ cells. This suggests that BMS2 cells may have less epoxide hydrolase than C3H10T½ cells, similar to what has been previously reported for mouse endometrial stromal cells (Savas *et al.*, 1993). Pretreatment with the potent AhR ligand TCDD increased the amount of each metabolite produced, although the percent of each metabolite was essentially unchanged. These findings imply that BMC cell CYP1B1 metabolizes DMBA under both basal conditions and after TCDD treatment.

To determine whether primary BMS cells metabolize DMBA, cultures were established from C57Bl/6 mice (C57-BMS) (Whitlock and Witte, 1982). For simplicity, we compared the major products DMBA-8,9-dihydrodiol and DMBA-10,11-dihydrodiol with those produced by BMS2 cells. Control C57-BMS cells (0.1% DMSO treated) produced ~30% of the DMBA-8,9-dihydrodiol and ~40% of the DMBA-10,11-dihydrodiol produced by BMS2 cells (Fig. 4). TCDD treatment increased the amounts of both DMBA-8,9-dihydrodiol and DMBA-10,11-dihydrodiol metabolites ~2-fold in C57-BMS cells. These results demonstrate that C57-BMS cells metabolize DMBA, producing a metabolite profile that is consistent with CYP1B1 activity.

AhR is functional in BMS cells. Induction of CYP1B1 after TCDD or DMBA treatment suggests that the AhR is active in BMS cells. In addition, the high constitutive expression of CYP1B1 in BMS2 cells might be due to constitutive activation of the AhR complex. To further investigate these possibilities, cytoplasmic and nuclear extracts from BMS2 cells were immunoblotted for the AhR and ARNT. Fig. 5A shows that BMS2 cells express 97- and 104-kDa AhR proteins, which correspond with the predicted Ah^{b-1} and Ah^d

alleles for this cell line, which was derived from a C57Bl/6 × DBA mouse (Pietrangeli *et al.*, 1988). The AhR proteins were clearly and reproducibly detectable (~5% of total) in the nucleus under control conditions (Fig. 5A, lane 7). Levels of DMBA and TCDD that maximally induce CYP1B1 were examined for their effect on AhR translocation. Approximately 20% (Fig. 5A, lane 8) and 70% (Fig. 5A, lane 9) of the total cellular AhR translocated to the nuclear fraction after a 1-hr treatment with DMBA or TCDD, respectively. Corresponding reductions in cytoplasmic AhR to 65% (Fig. 5A, lane 2) and 23% (Fig. 5A, lane 3) of control levels were observed after treatment with DMBA or TCDD, respectively. The substantially lower translocation of AhR in response to DMBA than TCDD, at DMBA and TCDD concentrations that fully induce CYP1B1, is noteworthy. After DMBA treatment, selective translocation of the two AhR allelotypes was observed, with more of the 97-kDa AhR than the 104-kDa AhR being depleted from the cytosol and appearing in the nucleus (Fig. 5A, lanes 8 and 9). This difference was consistently observed in four separate experiments. In the particular experiment illustrated in Fig. 5, a slight degree of selective translocation was also observed with TCDD that was not observed in the three other experiments. After 6 hr of treatment with DMBA or TCDD, most of the AhR was depleted, although this was most extensive after incubation with TCDD. These observations are in agreement with a previous report of AhR down-regulation after ≥ 2 hr of treatment with AhR ligands (Pollenz, 1996). Immunoblotting for the GAPDH cytosolic marker demonstrated that the nuclear fractions were completely free of cytosolic protein contamination.

In the nucleus, ARNT forms a heterodimer with ligand-bound AhR, resulting in a functional AhR complex. BMS2 cells express an 87-kDa ARNT protein (Fig. 5B) that was predominantly found in the nuclear fraction (75% of total ARNT) of untreated cells. More ARNT was found in the nucleus after a 1-hr exposure of cells to DMBA or TCDD, which parallels AhR translocation. ARNT has been shown by immunofluorescence to be a nuclear protein in most cells examined (Pollenz *et al.*, 1994; Abbott and Probst, 1995). It therefore is likely that the cytosolic ARNT signal we detected was due to the release of some ARNT protein from the nucleus into the cytosolic fraction during cell fractionation.

AhR-deficient BMS cells express CYP1B1. These findings demonstrate that BMS cells express CYP1B1 and a functional AhR but do not establish whether the AhR is required for CYP1B1 expression. To address this question,

TABLE 1
DMBA metabolism by BMS2 cells and C3H10T½ cells

Cells	TCDD ^a	DMBA metabolite ^b				Phenols ^{c,d}	Total
		Dihydrodiols					
		5,6–	8,9–	10,11–	3,4–		
<i>pmol / 10⁶ cells / hr</i>							
BMS2	–	0	5 (24)	2 (9)	3 (14)	11 (53)	21
	+	0	12 (28)	4 (8)	6 (13)	22 (51)	44
10T1/2	–	0	1 (30)	1 (19)	1 (19)	1 (32)	4
	+	0	5 (28)	3 (18)	3 (16)	7 (38)	18

Numbers in parentheses are percent total metabolism.

^a Cells were treated for 24 hours with 0.1% DMSO vehicle (control) or 10 nM TCDD.

^b Values represent the mean of duplicate determinations from separate experiments.

^c Total identifiable DMBA phenols, which include 2-, 3-, and 4-OH DMBA.

^d 7- and 12-hydroxymethyl metabolites were not detectable above background.

primary BMS cell cultures were established from AhR-deficient mice (AhR^{-/-} BMS) and their AhR heterozygous (AhR^{+/-} BMS) littermates. BMS2 cells were included in these experiments as a control. No obvious variation in the composition of cell types was apparent between AhR^{-/-} BMS cells and AhR^{+/-} BMS cells, and many of the cells in the primary BMS cultures were morphologically similar to BMS2 cells (data not shown). Fig. 6 illustrates that AhR^{-/-} BMS cells constitutively expressed CYP1B1 protein at levels ~60% and ~75% of those observed in BMS2 and AhR^{+/-} BMS cells, respectively. CYP1A1 protein was not detected in either AhR^{-/-} BMS or AhR^{+/-} BMS cells. As a control, we detected no immunoblot signal for CYP1B1 in primary BMS cells obtained from CYP1B1-deficient mice (data not shown). A 24-hr treatment with TCDD had no effect on CYP1B1 in AhR^{-/-} BMS cells but resulted in a 2-fold induction of CYP1B1 in AhR^{+/-} BMS cells. These findings indicate that the constitutive expression of CYP1B1 in BMS cells is largely AhR independent, whereas TCDD induction of CYP1B1 is AhR dependent. Moreover, AhR-independent constitutive CYP1B1 expression is consistent with the low levels of the AhR in the nucleus of control BMS2 cells (Fig. 5A, lane 7).

Metabolism of DMBA was investigated in AhR^{+/-} BMS and AhR^{-/-} BMS cells. The amounts of 8,9-dihydrodiol and 10,11-dihydrodiol metabolites produced were compared with those produced by BMS2 cells. Other metabolites were not significantly detectable over background conditions (no cells). Both AhR^{+/-} BMS and AhR^{-/-} BMS cells metabolized DMBA, producing 8,9-dihydrodiol and 10,11-dihydrodiol metabolites although at much lower levels than the BMS2 cell line (Fig. 7). A 24-hr pretreatment with TCDD resulted in a 2-fold increase in DMBA metabolism by AhR^{+/-} BMS cells but had no effect on DMBA metabolism by AhR^{-/-} BMS cells. In this experiment, a product eluted at the approximate time expected for 5,6-dihydrodiol. However, this product was present in comparable amounts in the absence of cells, did not increase in any of the cells after TCDD treatment, and was not consistently present in other experiments. From these and previous results, we conclude that primary BMS cells metabolize DMBA to a metabolite profile that is consistent with CYP1B1 activity.

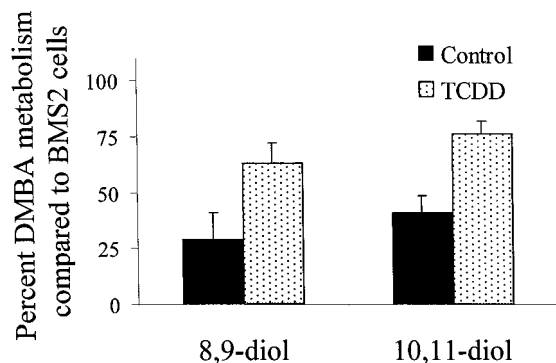


Fig. 4. DMBA metabolism by C57-BMS. Primary BMS cells from C57Bl/6 mice (C57-BMS) were established as described in Materials and Methods. Nonadherent cells were removed by washing, and adherent cells were treated for 24 hr with 0.1% DMSO as a vehicle control (Control) or 10 nM TCDD. The monolayers were then washed and incubated for 1 hr with 1 μ M DMBA, and the DMBA metabolites analyzed as described in Materials and Methods. The relative amounts of DMBA-8,9-dihydrodiol and DMBA-10,11-dihydrodiol produced by C57-BMS were compared with those produced by BMS2 cells treated for 24 hr with 0.1% DMSO (100%). Bars, mean \pm standard deviation of a single experiment.

Discussion

The results presented here demonstrate that BMS cells constitutively express high levels of CYP1B1, which is capable of metabolizing DMBA *in vitro*. Although constitutive CYP1B1 expression was largely AhR independent, activation of the AhR increased CYP1B1 expression and DMBA metabolism. The former observation differs from previous investigations that indicated that constitutive CYP1B1 expression in embryo fibroblasts is dependent on AhR activation (Zhang *et al.*, 1998). These findings have important implications for bone marrow toxicology and physiology and suggest that BMS cells may regulate CYP1B1 expression in a manner different from that previously described in embryo fibroblasts (Alexander *et al.*, 1997; Zhang *et al.*, 1998).

We clearly demonstrated that mouse BMS cells metabolize DMBA *in vitro*. Metabolites produced include a substantial proportion of 3,4 dihydrodiol (15% of total metabolites produced by BMS2 cells), the precursor to the most toxic product. The profile of DMBA metabolites formed (Table 1, Figs. 3 and 7) strongly suggests that CYP1B1 was the predominant P450 cytochrome catalyzing DMBA metabolism. This conclusion is based on similarities in the proportions of DMBA metabolites produced by BMS2 cells and C3H10T $\frac{1}{2}$ cells. In particular, production of the 3,4-dihydrodiol and 10,11-dihydrodiol DMBA metabolites has been reported for recombinant mouse CYP1B1 but not for recombinant mouse CYP1A1 (Savas *et al.*, 1997). Moreover, neither BMS2 nor primary BMS cells produced appreciable amounts of DMBA-5,6-dihydrodiol, a major predicted product of CYP1A1 (Wilson *et al.*, 1984). To our knowledge, this is the first report that mouse BMS cells metabolize DMBA, although human mononuclear bone marrow cells have been reported previously to metabolize DMBA to the 3,4-dihydrodiol product (O'Dowd, 1987).

Western and Northern blot analyses confirmed that mouse BMS cells constitutively express high levels of CYP1B1, which is only slightly induced by TCDD (1.4–2-fold), but no detectable CYP1A1. The basal levels of CYP1B1 in BMS2 and C57-BMS cells were consistently higher than in C3H10T $\frac{1}{2}$ cells, whereas TCDD-induced levels were comparable in all three cell types.

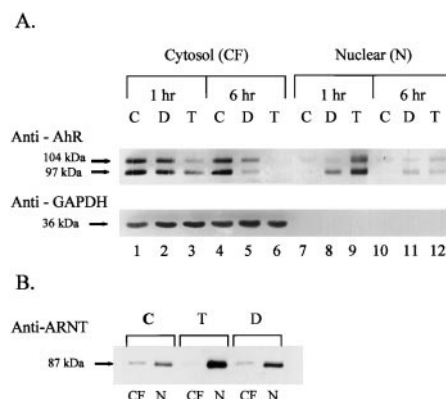


Fig. 5. Immunoblot analysis of cytosolic and nuclear proteins from BMS2 cells treated with DMBA or TCDD. Cytosolic (CF) and nuclear (N) protein fractions were isolated after treatments with 0.1% DMSO vehicle (C), 10 μ M DMBA (D), or 10 nM TCDD (T) for 1 or 6 hr (A) and 1 hr (B). Proteins (20 μ g/lane) were separated by SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose, and incubated with anti-AhR or anti-GAPDH (A) and with anti-ARNT (B). Visualization of immunoreactive proteins was achieved by the enhanced chemiluminescence method.

The levels of constitutive CYP1B1 in BMS2 cells are higher than those previously reported for other mouse tissues (Savas *et al.*, 1994, 1993) and human cells (Kress and Greenlee, 1997).

Based on our results, we hypothesize that AhR activation increases CYP1B1 expression in BMS cells. In support of this statement, we observed that BMS2 cells (1) express immunodetectable ARNT and AhR proteins (97 and 104 kDa) that are primarily cytosolic (only ~5% nuclear) under basal conditions, (2) exhibit nuclear translocation of AhR proteins after exposure to DMBA or TCDD, and (3) increase CYP1B1 expression after treatment with DMBA or TCDD and (4) TCDD did not induce CYP1B1 expression in AhR^{-/-} BMS cells. Our observations are supported by previous findings that suggest the AhR is active in BMS2 cells (Yamaguchi *et al.*, 1997b; Lavin *et al.*, 1998).

Although AhR activation increased CYP1B1 expression, the constitutive regulation of CYP1B1 in BMS cells appeared to be largely AhR independent. AhR^{-/-} BMS cells expressed levels of CYP1B1 protein that were similar (60–75%) to those in AhR^{+/-} BMS cells and in the BMS2 cell line. From these observations, it seems that AhR activation is responsible for at most 25–40% of constitutive CYP1B1 expression in BMS cells. The constitutive expression of CYP1B1 in AhR^{-/-} BMS is in contrast to a recent report by our laboratory that demonstrated that CYP1B1 expression in mouse embryo fibroblasts was dependent on the presence of the AhR (Zhang *et al.*, 1998). These separate findings suggest that the constitutive expression of CYP1B1 is regulated by a factor or factors other than the AhR in BMS cells. Because TCDD-induced levels of CYP1B1 are similar in mouse BMS cells and C3H10T $\frac{1}{2}$ embryo fibroblasts, it is possible that AhR activation overrides other cell-type-specific mechanisms to provide a maximal level of transcription.

The primary BMS cells used in this study were adherent

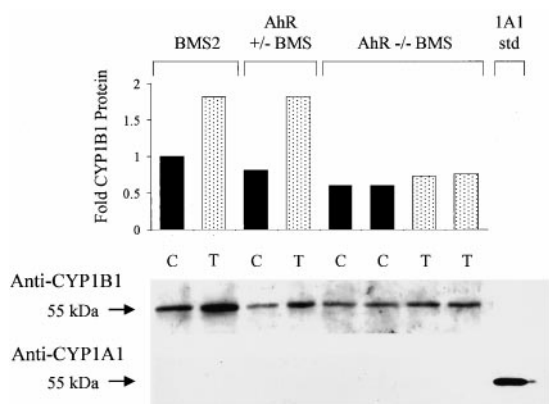


Fig. 6. Immunoblot analysis of CYP1B1 in BMS2 cells AhR^{+/-} BMS, and AhR^{-/-} BMS cells. Primary BMS cells from AhR heterozygote mice (AhR^{+/-} BMS) and AhR-deficient mice (AhR^{-/-} BMS) were established as described in Materials and Methods. These cells were treated for 24 hr with 0.1% DMSO as a vehicle control (C) or 10 nM TCDD (T). Cells then were incubated for 1 hr with 1 μ M DMBA, and the medium was removed for analysis of DMBA metabolites. Immediately thereafter, total protein was isolated using TRIzol reagent and Western immunoblotted for CYP1B1. The immunoblot was stripped and probed with anti-CYP1A1. Proteins were visualized with the enhanced chemiluminescence method and quantified using a Molecular Dynamics Personal Densitometer SI. Bars, level of CYP1B1 protein relative to control BMS2 cells (C). The lanes for AhR^{-/-} BMS cells represent two separate cultures within the same experiment. Amounts of protein loaded were 10 μ g/lane for AhR^{+/-} BMS cells, 20 μ g/lane for BMS2 cells and AhR^{+/-} BMS cells, and 2 ng for purified CYP1A1 (1A1 std).

cells prepared from Whitlock-Witte cultures (Whitlock and Witte, 1982). These types of cell cultures are reported to consist primarily of very large nonphagocytic fibroblastoid cells and macrophages (Witte *et al.*, 1987). Stromal fibroblasts from mouse endometrium (Savas *et al.*, 1993), mouse embryos (Pottenger and Jefcoate, 1990; Alexander *et al.*, 1997), and rat mammary glands (Christou *et al.*, 1995) also constitutively express CYP1B1 although at lower levels than BMS cells. Because both primary BMS cultures and BMS2 cells expressed comparable levels of constitutive and inducible CYP1B1, we conclude that most of the primary cells are CYP1B1-expressing fibroblast-like cells. However, primary BMS cell cultures, but not BMS2 cells, exhibit nonspecific DMBA oxidation in DMBA metabolism experiments (data not shown). We attribute this difference to the heterogeneity of cells in primary BMS cultures, particularly macrophages that could release peroxidative products (Johansson *et al.*,

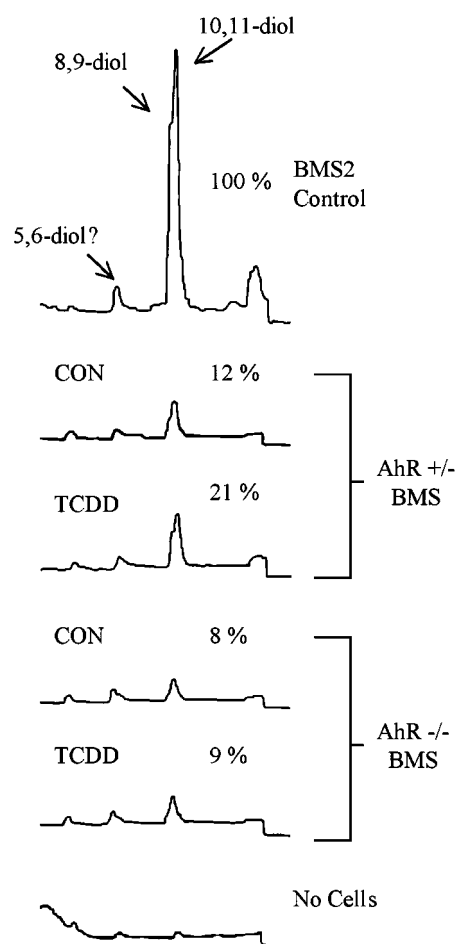


Fig. 7. Metabolism of DMBA by AhR^{+/-} BMS and AhR^{-/-} BMS cells. Chromatograms of DMBA metabolites demonstrate that primary BMS cells established from AhR-deficient mice (AhR^{-/-} BMS) and their AhR heterozygous littermates (AhR^{+/-} BMS) metabolized DMBA producing 8,9-dihydrodiol and 10,11-dihydrodiol metabolites. Primary BMS cells were established and analyzed by high performance liquid chromatography for DMBA metabolism as described in Materials and Methods. Cells were pretreated for 24 hr with either 0.1% DMSO vehicle control (CON) or 10 nM TCDD before the DMBA metabolism assay. Peaks corresponding to the 8,9- and 10,11-dihydrodiol DMBA metabolites are indicated along with a potential 5,6-dihydrodiol metabolite. The percentages indicate the amounts of combined DMBA-8,9-dihydrodiol and DMBA-10,11-dihydrodiol produced by AhR^{-/-} BMS and AhR^{+/-} BMS relative to those produced by BMS2 cells treated for 24 hr with 0.1% DMSO (100%).

1995) that attack DMBA. Moreover, these nonspecific oxidation products likely reduced the amounts of specific DMBA metabolites formed by CYP1B1 in primary BMS cultures.

Bone marrow stromal cell expression of CYP1B1 has important implications for bone marrow toxicology and physiology. Metabolic activation of environmental contaminants by bone marrow CYP1B1 could increase the effective concentrations of activated toxicants in bone marrow. This is important because the human ortholog of CYP1B1 recently has been shown to activate many diverse procarcinogens to mutagenic metabolites (Shimada *et al.*, 1996). The high constitutive expression of CYP1B1 means that activation of toxic compounds in the bone marrow is not dependent on AhR activation and therefore might precede AhR-dependent up-regulation of detoxifying enzymes in distant organs such as the liver and kidney. Furthermore, the high constitutive levels of CYP1B1 suggest that it may play a role in maintaining bone marrow physiology. This supposition is supported by the expression of catalytically active CYP1B1 in the absence of an AhR (AhR^{-/-} BMS cells), a finding that has not been reported for any other tissue. Future experiments using CYP1B1-deficient mice will help determine whether CYP1B1 has a physiological role in bone marrow and whether the bone marrow toxicity of DMBA is dependent on the presence of CYP1B1.

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