Bone Marrow Stromal-B Cell Interactions in Polycyclic Aromatic Hydrocarbon-Induced Pro/Pre-B Cell Apoptosis

Lenka L. Allan,† Koren K. Mann,‡ Raymond A. Matulka,§ Heui-Young Ryu,† Jennifer J. Schlezinger,*, and David H. Sherr*,†

*Department of Environmental Health, †Department of Microbiology, Boston University Schools of Medicine and Public Health, Boston, Massachusetts; ‡Lady Davis Institute for Medical Research, Montreal, Canada; and §The Burdock Group, Vero Beach, Florida

Received July 24, 2003; accepted September 5, 2003

Environmental polycyclic aromatic hydrocarbons (PAH) and related halogenated hydrocarbons are immunotoxic in a variety of systems. In a model system of B lymphopoiesis, PAH exposure rapidly induces apoptosis in CD43+ pre-B and CD43+ pro/pre-B cells. Apoptosis induction by 7,12-dimethylbenz[a]anthracene (DMBA) is dependent upon AhR bone marrow stromal cells and likely involves DMBA metabolism within the stromal cell. However, it is not known if PAH-treated stromal cells release free metabolites or soluble factors that may directly induce B cell death or if the effector death signal is delivered by stromal cell-B cell contact. Here, we demonstrate that supernatants from DMBA-treated bone marrow stromal cells contain an activity capable of inducing apoptosis in pro/pre-B cells cocultured with stromal cells. This activity (1) is not produced when stromal cells are cotreated with DMBA and e-naphthaloflavone (α-NF), an aryl hydrocarbon receptor (AhR) and cytochrome P-450 inhibitor, (2) is ≥ 50 kDa, (3) is trypsin and heat sensitive, and (4) is dependent on AhR stromal cells, which in turn deliver the effector death signal to pro/pre-B cells. The results (1) argue against a role for a soluble, stromal cell-derived cytokine as the effector of PAH-induced pro/pre-B cell death, (2) exclude the possibility of a free metabolite acting directly on AhR pro/pre-B cell targets, and (3) suggest the elaboration by stromal cells of a relatively stable, DMBA metabolite-protein complex capable of acting on other stromal cells at some distance. Collectively, these studies suggest that, while stromal cell products, e.g., metabolite-protein complexes, may affect the function of distant stromal cells, the effector death signal delivered by stromal cells to bone marrow B cells is mediated by cell–cell contact.

Key Words: polycyclic aromatic hydrocarbon; B cell apoptosis; stromal cells.

Polycyclic aromatic hydrocarbons (PAH) and related halogenated hydrocarbons (HAH) are both carcinogenic and immunosuppressive. For example, the prototypic PAHs benzo[a]pyrene (B[a]P) and 7,12 dimethylbenz[a]anthracene (DMBA) induce a variety of tumors in animals (Rigdon and Neal, 1969) and compromise the immune system by suppressing cytokine production, B and T cell mitogen responses, tumor-specific CD8+ T cell induction, and B cell antibody production (Burchiel et al., 1992, 1993; Davilla et al., 1996; Thurmond et al., 1988; White et al., 1985; Wojdani et al., 1984).

The apparent correlation between PAH carcinogenicity and immunotoxicity suggests that overlapping signaling pathways may mediate these divergent biologic outcomes. Two elements in the pathway to malignant transformation are the aryl hydrocarbon receptor (AhR), which transforms into a transcription factor when bound by ligand, and PAH metabolites, production of which is facilitated by AhR-regulated cytochrome P-450 monooxygenases (Christou et al., 1987; Uno et al., 2001). There is a considerable body of evidence supporting the hypothesis that the AhR influences PAH-mediated immunosuppression (Dertinger et al., 2001; Kerkvliet, 1995; Laisoa et al., 2002; Lawrence et al., 1996; Mann et al., 1999; Near et al., 1999; Staples et al., 1998; Thurmond et al., 2000; Vorderstrasse and Kerkvliet, 2001; Yamaguchi et al., 1997a,b), as well as PAH-induced malignant transformation (Poland et al., 1974; Safe and Krishan, 1995). Similarly, PAH metabolites are both highly carcinogenic and immunosuppressive. Indeed, some PAH metabolites suppress antigen-specific T and B cell responses or compromise lymphocyte development at doses lower than those required to induce immunosuppression with the respective parent compounds (Davilla et al., 1996; Kawai bata and White, 1987; Ladics et al., 1991; Mann et al., 1999; Thurmond et al., 1988).

Of particular concern for environmental exposures is the extreme sensitivity of the developing immune system to PAHs and HAHs (Holladay and Smith, 1995; Lai et al., 2000; Urso and Johnson, 1988). In a series of studies using an in vitro model of B lymphopoiesis, our laboratory demonstrated that PAHs (B[a]P and DMBA) rapidly induce apoptosis in primary pre-B cells or in a nontransformed CD43+ pro/pre-B cell line.
AhR (Hardin et al., 1992; Mann et al., 1999, 2001; Near et al., 1999; Quadri et al., 2000; Yamaguchi et al., 1997a,b). However, PAH toxicity in this system is not due to direct effects of PAH on the B cells, which do not express the AhR, but rather is mediated indirectly through PAH-treated AhR− bone marrow stromal cells. The ability of common environmental pollutants such as B[a]P to effect changes in bone marrow stromal cell activity has implications for multiple hematopoietic cell types, since the growth and/or development of all eight hematopoietic lineages is dependent on these stromal elements.

Induction of the stromal cell-derived, pro/pre-B cell-directed death signal with relatively low PAH doses (i.e., 10−6–10−5 M) is blocked by AhR inhibitors (Quadri et al., 2000; Yamaguchi et al., 1997b) and requires that the stromal cells express a functional AhR (Mann et al., 1999; Near et al., 1999). Furthermore, there appears to be some role for PAH metabolism in the induction of the death signal, in that poorly metabolized AhR ligands such as 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) and related PCBs do not induce apoptosis while a DMBA metabolite, DMBA-3,4-dihydrodiol, is a potent inducer of apoptosis. Furthermore, a role for a stromal cell monoxygenase, CYP1B1, in induction of apoptosis in the transformed pre-B cell line, 70Z3, has been demonstrated (Heidel et al., 1999, 2000). Again, these results suggest a similarity between PAH carcinogenicity and immunotoxicity.

The nature of the death signal that PAH-treated stromal cells deliver to pre- or pro/pre-B cells is not known. Work in our laboratory suggests that it is not mediated by Fas ligand, TNF-α, TNF-β, lymphotoxin β, TGF-β1, β2, or β3, glucocorticoids, or other obvious inducers of lymphocyte apoptosis (data not shown). It has been suggested by studies performed in a related system that at least some of the toxicity is mediated by direct effects of PAH metabolites on B cells (Heidel et al., 1999). However, no data are available that directly support this hypothesis.

In the present study, the potential for DMBA-treated stromal cells to deliver a death signal to pro/pre-B cells via a soluble apoptosis-inducing activity was evaluated. Of particular interest was the possibility that stromal cells could generate metabolites that directly activate an early B cell death pathway.

MATERIAL AND METHODS

Cell lines. Cell lines where maintained at 37°C in 10% CO2 in Dulbecco’s modified Eagle’s medium (DMEM) (BioWhittaker, Walkersville, MD) supplemented with 5% fetal bovine serum (FBS), 2 mM L-glutamine, and 5 × 10−3 M 2-mercaptoethanol (Life Technologies, Grand Island, NY). Pro/pre-B cell lines were obtained by harvesting stromal cell-adherent B cells from primary Whitlock-Witte cultures (Whitlock et al., 1984; Yamaguchi et al., 1997a) and transferring them to monolayers of a cloned bone marrow stromal cell line, BMS2 (Pietrangeli et al., 1988), kindly provided by Dr. P. Kincade. Cells of one such line, termed BU-11, uniformly express the late pro/B/early pre-B cell marker CD43 and contain a rearranged immunoglobulin heavy chain gene (Yamaguchi et al., 1997b). AhR−/− mice with a C57BL/6 background were obtained from Jackson Laboratories (Bar Harbor, ME). To generate primary bone marrow stromal cells from wild-type and AhR−/− littermates, AhR−/− heterozygote breeding pairs were established and offspring analyzed for AhR-specific PCR analysis of tail DNA. Primary bone marrow stromal cell cultures were obtained from femurs of AhR+/− and AhR− male mice as previously described (Whitlock et al., 1984). Briefly, bone marrow plugs were flushed from the femurs of 4- to 6-week-old mice and RBC-depleted bone marrow cells were cultured in RPMI-1640 supplemented with 10% FBS, L-glutamine, 2-mercaptoethanol, and penicillin/streptomycin (Gibco/BRL) at an approximate cell density of 2–4 × 105/ml. Nonadherent cells were removed 7 days later by vigorous washing. Adherent cells were treated with 0.05% trypsin–0.53 mM EDTA (Gibco/BRL), harvested, and transferred to fresh tissue culture flasks containing a 1:1 ratio of fresh media and conditioned media.

Hepa-1c1c7 (Hepa-1) cells, kindly provided by Dr. James Whitlock (Stanford University), were maintained in DMEM supplemented with 5% FBS, 2 mM L-glutamine, and 5 × 10−3 M β-mercaptoethanol. Hepa-1 cells were plated at a cell density of 105 cells/ml and treated the next day with vehicle (dimethylsulfoxide, DMSO), 10−5–10−3 M DMBA, or conditioned media (supernatants) from vehicle- or 10−5 M DMBA-treated BMS2 cells. Eighteen hours later, the monolayers were washed with phosphate-buffered saline (PBS) (Gibco/BRL). Cells were detached with 0.05% trypsin–0.53 mM EDTA, harvested, and stored at −80°C until use.

Production of stromal cell conditioned supernatant. BMS2 cells (0.6 × 105/ml) were plated in 10 ml DMEM as described above and treated the next day with vehicle (0.01% DMSO final concentration) or 10−5–10−3 M DMBA (Sigma/Aldrich Chemical Co., Milwaukee WI) dissolved in DMSO. Eighteen hours later all media were removed. Plate-adherent BMS2 monolayers were washed extensively with four changes of PBS, and fresh media were added (a 1:1 ratio of RPMI-1640 and DMEM supplemented with 5% FBS, L-glutamine, and 2-mercaptoethanol). After 24 h, conditioned supernatants were removed and sterile filtered through a 0.22 μm syringe filter (Fisherbrand, Fisher Scientific, Pittsburgh, PA).

Dialysis/ultrafiltration. Supernatants from vehicle- or BMS2-treated cells were dialyzed using either Spectra/Por 6 (MWCO 1,000) or Spectra/Por CE membranes against a 10-fold excess of 1:1 RPMI/DMEM media for 18 h with one change of media (Spectrum Laboratories, Inc., Dominguez, CA). Alternatively, supernatants were concentrated using a Centricron-10 microconcentrator (10 kDa membrane exclusion) (Amicon Corp., Danvers, MA), a Biomax PB 50,000 NMWL ultrafiltration membrane (50 kDa membrane exclusion) (Millipore Co., Bedford, MA) or an Amicon Ultrafiltration cell (50 kDa membrane exclusion) to one-tenth the starting volume. For some experiments, concentrated supernatants were heat inactivated by incubating in a 60°C water bath for one h, incubated for one h at 37°C as a control, or digested with an equal volume of 0.25% trypsin for one h at 37°C. Following the treatment, concentrates were reconstituted with media to the original volumes, and retentates and filtrates were added to BU-11/BMS2 cultures.

Induction and quantitation of apoptosis. Apoptosis induction and quantitation was performed as described previously (Ryu et al., 2003). Briefly, BMC2 cells were plated at a density of 0.4 × 105/ml in 24-well culture dishes (Costar, Corning Inc., NY) and allowed to adhere overnight. In experiments where primary stromal cells were used in place of BMC2 cells, AhR− or AhR+ stromal cells were seeded at a density of 104 cells/ml and allowed to adhere for 48 h. Subsequently, BU-11 cells were added to stromal cells at a density of 0.5 × 105/ml and allowed to settle for 6–8 h before treating individual wells with vehicle (0.01% DMSO), 10−5–10−4 M DMBA dissolved in DMSO, or conditioned supernatants from vehicle- or BMS2-treated BMC2 cells. In experiments where BU-11 were cultured in the absence of the supporting BMC2 cells for less than 48 h, the media was supplemented with recombinant interleukin-7 (IL-7) to maintain BU-11 viability. When adding BMC2 supernatants, > 95% the media was removed from BU-11/BMS2 cultures and...
Semiquantitative RT-PCR. Total RNA was prepared from Hepa-1 cell pellets using the Qiagen RNeasy kit (Qiagen Inc. Valencia, CA). Five μg of total RNA was combined with 50 ng of random hexamer primer and water in a volume of 11 μl and annealed at 70°C for 10 min. The primed RNA was chilled on ice and reverse transcribed using 160 units of SuperScript II reverse transcriptase in PCR buffer (5 mM MgCl₂, 20 mM DTT, and 2 mM dNTPs) at 42°C for 50 min followed by 70°C for 10 min. After chilling on ice, 1.6 units of RNase H were added, and the mixture was incubated for 20 min at 37°C. All enzymes were obtained from Life Technologies/Invitrogen Corp. (Carlsbad, CA). The PCR was conducted using 2 μl of cDNA in 10 × PCR buffer, 1.5 mM MgCl₂, 0.2 μM dNTP mix, 5 units Taq polymerase and 0.2 μM CYP1A1-specific primers (sense 5′-TTCCTGAGACCTCCTGGCATTTGCT-GGCC-3′/ antisense 5′-CCGATGACCTTTCGCT-GATCC-3′) or β-actin-specific primers (sense 5′-GTGTCGACAAAGCCGCTCC-GCATGTG-3′/ antisense 5′-CATTTAGAAGGGTGTCGTGGCCGAGATC-3′). Twenty-two cycles of amplification were performed in a programmed thermocycler (Barnstead/Thermolyne, Dubuque, IA), and 5 μl of each product was separated on a 1% agarose gel and visualized with ethidium bromide. Images were captured by digital photography (Kodak Transilluminator and Kodak DC290 Digital Camera). Relative band intensities were determined with the Kodak Digital Sciences ID program. CYP1A1-band intensity was normalized to the corresponding sample β-actin band intensity.

Statistics. Statistical analyses were performed with Statview (SAS Institute, Cary, NC). The Student’s t-test and one-factor ANOVAs were used to analyze the data. For the ANOVAs, the Dunnett’s or Tukey/Kramer multiple comparisons tests were used to determine significant differences.

RESULTS

Conditioned Supernatants from DMBA-Treated Stromal Cells Induce Pro/Pre-B Cell Apoptosis in BU-11/BMS2 Cocultures

Previous studies demonstrated that AhR negative pro/pre-B cells (BU-11) maintained on AhR⁺ primary or cloned bone marrow-derived stromal cells undergo apoptosis when cultures are exposed to PAH such as DMBA or B[a]P (Mann et al., 1999, 2001; Near et al., 1999; Quadri et al., 2000; Yamaguchi et al., 1997a,b). BU-11 cells grown in the presence of rIL-7 but in the absence of stromal cells are resistant to DMBA (Yamaguchi et al., 1997a,b). Furthermore, addition of rIL-7 to cocultures of BU-11 and BMS2 cells does not protect BU-11 cells from DMBA-induced apoptosis (Yamaguchi et al., 1997b). These data demonstrate that pro/pre-B cell apoptosis is not due to a loss of stromal cell function (e.g., loss of IL-7 secretion) but rather is mediated by the active production of a stromal cell-dependent death signal.

To determine if BU-11 cell apoptosis is mediated by an activity released into culture media, BMS2 cells were treated with vehicle (0.01% DMSO) or 10⁻⁵–10⁻⁷ M DMBA for 24 h. Media were removed and cells washed extensively prior to addition of fresh media. Conditioned supernatants were harvested with an equal volume of conditioned supernatant from vehicle- or DMBA-treated BMS2 cells. Forty-eight h later, BU-11 cells were harvested and washed once with cold PBS containing 5% FBS and 1% sodium azide. Cells then were resuspended in 0.3 ml of hypotonic buffer consisting of 50 μg/ml propidium iodide (P.I.) (Sigma Chemical Co., St. Louis, MO), 1% sodium citrate, and 0.1% Triton X-100 and analyzed in a Becton Dickinson FACScan flow cytometer. Cells undergoing DNA fragmentation and apoptosis are weaker in P.I. fluorescence than those in the G₀/G₁ phase of cell cycle (Mann et al., 1999, 2001).

FIG. 1. Conditioned supernatants from DMBA-treated BMS2 cells induce apoptosis in BU-11 pro/pre-B cells maintained on stromal cell monolayers. BU-11 cells grown on BMS2 monolayers were treated with vehicle, DMBA, or supernatants from vehicle- or DMBA-treated BMS2 cells. Forty-eight h later, BU-11 cells were harvested, and the percentage of apoptotic cells was assessed by P.I. staining and flow cytometry. (A) BU-11/BMS2 cocultures were treated with vehicle, 10⁻⁵–10⁻⁷ M DMBA (“Control”), supernatants from vehicle-treated BMS2 cells, or supernatants from BMS2 cells treated with 10⁻⁵–10⁻⁷ M DMBA (“Supe”). Data from three experiments are expressed as mean ± SE. An asterisk denotes statistical significance relative to corresponding vehicle controls (p < 0.05; Dunnett’s test). (B) BU-11/BMS2 cocultures were treated with supernatants from stromal cells from vehicle- or 10⁻⁵ M DMBA-treated BMS2 cells. The supernatants were added neat or diluted 3:1, 1:1, 1:3, or 1:10 in fresh media prior to addition to BU-11/BMS2 apoptosis assay cocultures. Data from three experiments are expressed as the mean ± SE. An asterisk denotes statistical significance relative to corresponding vehicle controls (p < 0.02).
BU-11/BMS2 cultures, conditioned supernatants from BMS2 cells treated with $10^{-5}$ or $10^{-6}$ M DMBA induced a level of BU-11 cell apoptosis approximately the same as that induced by adding DMBA directly to cocultures (Fig. 1A, right histograms). Similar results were obtained when BU-11 cells were cultured on primary bone marrow stromal cells (below).

More than 30% of the BU-11 cells underwent apoptosis when conditioned supernatants from DMBA-treated BMS2 stromal cells were diluted as much as 1:3 (Fig. 1B, $p < 0.01$). A lower, but statistically significant level of apoptosis was seen when these supernatants were diluted as much as 1:10 ($p < 0.02$). These results indicate that DMBA-treated stromal cells elaborate a soluble factor(s) capable of inducing pro/pre-B cell death in BU-11/BMS2 cocultures.

**α-Naphthoflavone (α-NF) Blocks Elaboration of Stromal Cell-Derived, Apoptosis-Inducing Activity**

BU-11 cell apoptosis induced by addition of DMBA directly to BU-11/BMS2 cocultures is blocked by α-naphthoflavone (α-NF), an AhR and cytochrome P-4501A1 inhibitor (Mann et al., 1999; Quadri et al., 2000; Yamaguchi et al., 1997b). To determine if α-NF similarly blocks release of the death-inducing activity, BMS2 cells were pretreated with vehicle or $10^{-6}$ M α-NF. Vehicle or $10^{-5}$–$10^{-6}$ M DMBA was added 1 h later, and cultures were incubated for 18 h. Cells were then washed extensively and incubated for 24 h in fresh media, and conditioned supernatants were collected and tested for apoptosis-inducing activity as in Figure 1. As seen when added directly to DMBA-treated cocultures (Mann et al., 1999; Quadri et al., 2000; Yamaguchi et al., 1997b), α-NF completely blocked the induction of the soluble death-inducing activity from DMBA-treated BMS2 cells (Fig. 2). These results suggest a role for either the AhR or PAH-metabolizing enzymes in production of an apoptosis-inducing activity.

**Characterization of Apoptosis-Inducing Activity**

To begin to characterize the stromal cell-derived apoptosis-inducing activity, conditioned supernatants were prepared from vehicle- or DMBA-treated BMS2 cells and then added directly to BU-11/BMS2 cocultures or dialyzed for 18 h in 1-kDa exclusion dialysis membranes prior to addition to cocultures. As in previous experiments, supernatants from DMBA-treated stromal cells contained significant levels of apoptosis-inducing activity (Fig. 3A, left histograms). These supernatants retained this activity when dialyzed overnight (Fig. 3A, right histograms), indicating that the apoptosis-inducing activity is relatively stable and is larger than 1 kDa. This result is not consistent with a role for the carryover of free DMBA or a free DMBA metabolite to the BU-11/BMS2 cocultures. Indeed, maintenance of DMBA in media for 18 h ablated its ability to induce pro/pre-B cell apoptosis (data not shown).

To estimate the size of the factor(s) responsible for this activity, supernatants from vehicle- or DMBA-treated BMS2 cells were concentrated with one of two commercial preparations of 50-kDa exclusion filters. The supernatant retentate was diluted back to the original volume of the conditioned supernatant, and both the filtrate and retentate were tested for apoptosis-inducing activity in BU-11/BMS2 cocultures. Significant apoptosis-inducing activity was present in the retentate, while the filtrate had no such activity (Fig. 3B). Similar results were obtained with 5-kDa and 10-kDa exclusion filters (not shown). Therefore, the death-inducing activity is $\geq 50$ kDa.

To determine if the apoptosis-inducing activity is associated with protein, conditioned media from vehicle- or DMBA-treated BMS2 cells were concentrated using a 50-kDa exclusion filter and treated with or without trypsin for 1 h at 37°C. All supernatants were then diluted back to the original volume of conditioned media and added to BU-11/BMS2 cocultures. Incubation of supernatant from DMBA-treated BMS2 cells at 37°C for 1 h had no effect on its ability to induce apoptosis in BU-11 cultures (Fig. 4, left histograms). In contrast, treatment of supernatant from DMBA-treated BMS2 cells with trypsin for 1 h at 37°C completely eliminated its ability to induce BU-11 cell apoptosis (Fig. 4, middle histograms), indicating a role for a protein component in the apoptosis-inducing activity. Maintenance of the supernatant for 1 h at 60°C similarly ablated apoptosis-inducing activity (Fig. 4, right histograms), a result consistent with a role for a heat-labile protein.

**Stromal Cell-Derived, Apoptosis-Inducing Activity Requires AhR Stromal Cells**

To determine if the apoptosis-inducing activity could act directly on pro/pre-B cells, supernatants from vehicle- or
DMBA-treated BMS2 cells were added to BU-11 cells grown in rIL-7 in the absence of stromal cells. While supernatant from vehicle- or 10^{-5} M DMBA-treated BMS2 cells were left untreated (“Untreated Supe”), dialyzed against fresh media overnight using a 1-kDa molecular cutoff dialysis membrane (“Dialyzed Supe”), or concentrated to one-tenth the original volume using a 50-kDa cutoff ultrafiltration membrane. Dialyzed supernatants, filtrates, or retentates restored to the original volume of the supernatant were added to BU-11/BMS2 cocultures, and BU-11 cell apoptosis was assayed forty-eight h later as in Figure 1. (A) Data from three experiments are expressed as the mean ± SE. An asterisk indicates a statistically significant level of apoptosis compared with corresponding vehicle controls (p < 0.01; Student’s t test). (B) Data from a separate series of three experiments are expressed as the mean ± SE. An asterisk indicates a significant level of apoptosis (p < 0.01).

To determine if production of the supernatant activity bypasses the previously described requirement for AhR in stromal cells, supernatants from vehicle- or DMBA-treated BMS2 cells were added to cocultures of BU-11 cells and primary bone marrow stromal cells derived from wild-type AhR^{+/+} or littermate AhR^{-/-} mice. BU-11 cell apoptosis was assayed 48 h later. Supernatants from DMBA-treated BMS2 cells readily induced apoptosis in BU-11 cells cultured on primary AhR^{+/+} bone

FIG. 3. DMBA-induced, stromal cell-derived apoptosis activity is retained by dialysis and is ≥ 50 kDa. Supernatants from vehicle- or 10^{-5} M DMBA-treated BMS2 cells were left untreated (“Untreated Supe”), dialyzed against fresh media overnight using a 1-kDa molecular cutoff dialysis membrane (“Dialyzed Supe”), or concentrated to one-tenth the original volume using a 50-kDa cutoff ultrafiltration membrane. Dialyzed supernatants, filtrates, or retentates restored to the original volume of the supernatant were added to BU-11/BMS2 cocultures, and BU-11 cell apoptosis was assayed forty-eight h later as in Figure 1. (A) Data from three experiments are expressed as the mean ± SE. An asterisk indicates a statistically significant level of apoptosis compared with corresponding vehicle controls (p < 0.01; Student’s t test). (B) Data from a separate series of three experiments are expressed as the mean ± SE. An asterisk indicates a significant level of apoptosis (p < 0.01).
marrow stromal cells (Fig. 6). The percentage of BU-11 cells undergoing apoptosis was significantly reduced ($p < 0.001$), but not completely abrogated ($p < 0.01$), when these pro/pre-B cells were cultured with supernatants from vehicle- or 10–5 M DMBA-treated BMS2 cells. These data are reminiscent of those obtained when adding DMBA-3,4-dihydrodiol, an early DMBA metabolite, directly to BU-11/stromal cell cocultures in that both an AhR-dependent and an AhR-independent component are evident (Mann et al., 1999).

Stromal Cell-Derived Apoptosis-Inducing Activity Is Associated with a CYP1A1-Inducing Activity

Although a role for protein was demonstrated by trypsin and heat inactivation of the apoptosis-inducing activity and is supported by its apparent size ($\geq 50$ kDa), the requirement for AhR$^{-/-}$ stromal cells in the apoptosis assay coculture simultaneously suggests a role for a relatively small AhR ligand which requires an AhR$^{+/+}$ target stromal cell to mediate its activity. These apparently contradictory observations would be resolved if the apoptosis-inducing activity reflects a DMBA metabolite(s) associated with and stabilized by an $\geq 50$ kDa protein(s). Supporting this possibility, some metabolites of DMBA, such as DMBA-3,4-dihydrodiol, both activate the AhR and induce apoptosis in BU-11/BMS2 cocultures (Mann et al., 1999). If such a metabolite contributes to the apoptosis-inducing activity in the supernatant, it would be expected that treatment of cells with supernatants or $\geq 50$ kDa fractions of supernatants from DMBA-treated stromal cells would induce AhR-regulated, CYP1A1 gene transcription. Since little or no CYP1A1 mRNA is induced by AhR ligands in BMS2 cells (Mann et al., 1999), a well-characterized CYP1A1-inducible murine hepatic line, Hepa-1, was used for these studies.

As expected, treatment of Hepa-1 cells with $10^{-5}$–$10^{-7}$ M DMBA for 18 h, as a positive control, significantly induced CYP1A1 mRNA (Fig. 7A, lanes 1–5, and Fig. 7B). Similarly, supernatants from DMBA- but not vehicle-treated BMS2 cells induced significant levels of CYP1A1 mRNA (Fig. 7A, lanes 6 and 7, and Fig. 7B). Furthermore, the apoptosis-inducing CYP1A1-inducing activity in supernatants from DMBA-treated stromal cells was retained following overnight dialysis or concentration with a $> 50$ kDa Amicon filtration unit (Fig. 7C). Collectively, these data support the working hypothesis that a DMBA-metabolite(s) produced by treatment of stromal cells with $10^{-6}$ M DMBA and associated with a $\geq 50$ kDa protein(s) mediates pro/pre-B cell apoptosis in BU-11/BMS2 cocultures. However, the target of this putative complex is not the pro/pre-B cells themselves but rather the AhR$^+$ stromal cells capable of delivering a death signal to the associated lymphocytes, presumably through cell–cell contact.

**DISCUSSION**

In a series of studies, we have evaluated the effects of common and prototypic PAHs on the developing immune system (Hardin et al., 1992; Mann et al., 1999, 2001; Near et al., 1999; Quadri et al., 2000; Ryu et al., 2003; Yamaguchi et al., 1997a,b). Although these studies focused on developing B cells as the ultimate targets of PAH toxicity, the results indicate that the immediate targets of PAHs are AhR$^+$ bone marrow stromal cells. Since all eight lineages of bone marrow hematopoietic cells interact with stromal cells in the bone marrow microenvironment, the results suggest that the in vivo effects of PAH exposure may not be limited to the developing B cell compartment. Indeed, injection of either B(2)22P or DMBA into mice results in the rapid destruction of the entire bone marrow hematopoietic compartment (Yamaguchi et al., 1997a).

The in vitro model of this bone marrow toxicity indicates that apoptosis is responsible for depletion of at least the B cell compartment. Several components of the intracellular apoptosis pathway activated in either nontransformed, stromal cell-dependent pro/pre-B cells (Mann et al., 1999, 2001; Ryu et al., 2003) or in transformed 7023 pre-B cells (Heidel et al., 1999; Page et al., 2002, 2003) following culture with stromal cells and PAH have been elucidated. For example, we have shown that down-regulation of NF-κB and NF-κB-regulated c-myc is required for maximal pro/pre-B cell apoptosis (Mann et al., 2001; Ryu et al., 2003). Unlike B cell receptor-mediated clonal deletion signals (Wu et al., 1996a,b, 1999), pro/pre-B cell apoptosis induced by PAHs does not involve upregulation of p21$^{WAF1}$ or p27$^{Kip1}$, indicating a potentially unique death signaling pathway activated by a stromal cell-derived death stimulus (Ryu et al., 2003). Other investigators have shown the involvement of caspase-3, -8, and -9 in DMBA-induced apoptosis in a transformed pre-B cell line (Page et al., 2002). Similarly, some
of the effects of PAH on stromal cells required for induction of the death signal are known. For example, bone marrow stromal cells must activate their AhR in order to generate the death signal (Mann et al., 1999; Near et al., 1999; Quadri et al., 2000; Yamaguchi et al., 1997b). In addition, PAH metabolism likely plays a role in generating the stromal cell-derived death signal (Heidel et al., 1999; Mann et al., 1999).

Despite this information, little is known of the signal delivered by the PAH-treated stromal cell to the target B cell. Three general possibilities were considered in the design of the experiments presented here: (1) elaboration of a soluble protein (e.g., a cytokine), (2) production of a PAH metabolite that is directly toxic to early B cells, and (3) delivery of a death signal via cell–cell contact.

If DMBA-treated stromal cells produce a soluble death-inducing protein, it would be predicted that supernatants from treated cells would induce pro/pre-B cell death. The ability to induce death in BU-11/BMS2 cultures with supernatant from DMBA-treated BMS2 cells initially seemed consistent with this hypothesis. Experiments with size exclusion filters indicated that the death-inducing activity was ≥50 kDa, ruling out the possibility of DMBA contamination or free DMBA metabolites as the mediators of the apoptosis signal. Furthermore, the activity contained in the ≥50 kDa fraction was ablated completely with trypsin or 60°C heat treatment, clearly indicating the involvement of one or more proteins.

Despite these results, several experiments indicated that elaboration of a conventional soluble protein such as a cytokine either was not involved or not sufficient for pro/pre-B cell apoptosis. Experiments with several recombinant cytokines associated with cell death (e.g., TNF-α, TNF-β, lymphotxin-β, TGF-β1, β2, β3) or their respective receptor knockout mice failed to identify a soluble cytokine capable of inducing pre-B or pro/pre-B cell death (data not shown). Furthermore, induction of pro/pre-B cell apoptosis was diminished significantly when BU-11 cells were cultured with supernatants from DMBA-treated stromal cells and AhR⁺ bone marrow stromal cells. This partial AhR-dependency would not be expected of an apoptosis-inducing effector cytokine. Rather, it is reminiscent of results obtained with DMBA-3,4-dihydrodiol, in that BU-11 cell apoptosis induced by that AhR-binding metabolite was only partially AhR-dependent (Mann et al., 1999). Furthermore, the production of a CYP1A1-inducing activity by DMBA-treated stromal cells (Fig. 7) suggests the presence of either DMBA or an AhR-activating DMBA metabolite, e.g., DMBA-3,4-dihydrodiol or DMBA-9,10-diolepodixode (Mann et al., 1999).

The most likely explanation for these results is the association of a DMBA metabolite with a ≥50 kDa protein(s) such that the putative metabolite is stabilized and capable of acting at some distance in cell cultures. The ability of α-NF, an AhR and CYP1A1 inhibitor, to block production of the death-inducing activity strongly suggests that the stromal cell is required to produce the metabolite, which putatively associates with protein. It is not known if the metabolite is bound to proteins present in the culture supernatant or proteins produced by stromal cells themselves. However, the retention of death-

![FIG. 7. Stromal cell-derived, apoptosis-inducing activity is associated with a CYP1A1-inducing activity. Hepa-1 cell monolayers were treated with vehicle, 10⁻³–10⁻⁷ M DMBA, or conditioned supernatants from vehicle or 10⁻⁵ M DMBA-treated BMS2 cells. Eighteen h later, total RNA was isolated, 5 µg was reverse transcribed, and the cDNA was subjected to PCR amplification with CYP1A1- and β-actin-specific primers. (A) Data from a representative experiment (from a total of three experiments) are shown. (B) CYP1A1 band densities were normalized to β-actin band densities. Data from three experiments are presented as the mean ± SE. An asterisk (*) indicates a significant increase in band densities relative to the corresponding vehicle controls (p < 0.05; Dunnett’s test). (C) Hep-1 cells were treated with untreated conditioned supernatant from vehicle or DMBA-treated BMS2 cells, conditioned supernatants dialyzed overnight against fresh media using 1-kDa cutoff dialysis tubing, or concentrated with a >50-kDa Amicon filter and reconstituted to their original volumes. Representative data from a total of three experiments are presented.](https://academic.oup.com/toxsci/article-abstract/76/2/357/1686031/fig7)
inducing activity only in fractions \( \geq 50 \text{kDa} \) suggests some limitation in what protein(s) is targeted. The identities of the protein(s) involved in stabilizing the putative DMBA metabolite and the metabolite mediating the death-inducing activity are currently under investigation.

Finally, the results suggest that products of PAH-exposed stromal cells, i.e., metabolite-protein complexes, may affect the function of distant cells, including stromal elements, within the bone marrow microenvironment. Such an interaction between stromal cells may represent a positive feedback loop in effect at higher PAH doses (e.g., \( \geq 10^{-6} \text{ M} \) DMBA). Moreover, the failure of concentrated supernatant from DMBA-treated stromal cells to kill pro/pre-B cells grown in the absence of stromal cells and the failure of pro/pre-B cells to die when separated from DMBA-treated stromal cells by a culture trans-well (not shown) indicate that the production of a soluble death-inducing effector factor is not likely and that stromal cell-pro/pre-B cell contact is required, particularly at lower DMBA doses (< \( 10^{-6} \text{ M} \)), for initiation of the B cell suicide program. The nature of this cell–cell signaling is under study.

REFERENCES


