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## 7,12-Dimethylbenz[a]anthracene Induces Apoptosis in Murine Pre-B Cells through a Caspase-8–Dependent Pathway

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### ABSTRACT

Polycyclic aromatic hydrocarbons (PAHs) have been demonstrated to cause a variety of tumors and immunosuppressive effects. Our laboratory, and others, have demonstrated that coculture of progenitor B lymphocytes (pre-B cells) with bone marrow stromal cells and the model PAH 7,12-dimethylbenz[a]anthracene (DMBA) results in pre-B cell apoptosis. In this study we investigated the molecular events that precede apoptosis in DMBA-treated 70Z/3 cells, a pre-B cell line. Using caspase activity assays and immunoblotting techniques, we determined the temporal pattern of caspase expression in the

pre-B cells. Using caspase inhibitors, we demonstrated that DMBA-mediated pre-B cell apoptosis is dependent on activation of caspase-8, whereas caspase-9 activation is essential for maximal apoptosis. We also demonstrated that DMBA activated PKR, an interferon-inducible protein kinase, in pre-B cells. PKR in turn can activate caspase-8 independently of death receptor ligation. As a result of these studies, we propose a novel PKR-dependent pathway for activation of apoptosis in DMBA-treated pre-B cells.

Polycyclic aromatic hydrocarbons (PAHs) are environmental contaminants that are produced during incomplete combustion of carbonaceous materials (Collins et al., 1998). They are introduced into the environment from a variety of sources, including forest fires, volcanic eruptions, the decay of organic matter, the combustion of fossil fuels, and tobacco smoke (White et al., 1994; Phillips, 1999). It has long been known that PAHs are carcinogenic. However, the PAH itself is an inert, hydrophobic molecule. To become carcinogenic PAHs must be metabolized by the cytochrome P450 family of monooxygenases (Phillips, 1983) to reactive diol-epoxide metabolites, which bind covalently to DNA. These adducts then lead to mutations and ultimately tumor formation (Phillips and Grover, 1994).

Many members of the PAH family are also immunotoxic, with 7,12-dimethylbenz[a]anthracene (DMBA) and benzo[a]pyrene (B[a]P) being the most extensively studied compounds (Ward et al., 1984; Burchiel et al., 1990; White et al., 1994). Several potential mechanisms have been proposed to

explain the immunotoxicity of PAHs. Because immunosuppressive PAHs bind to the aryl hydrocarbon receptor (Okey et al., 1984a,b), aryl hydrocarbon receptor binding, and subsequent activation of aryl hydrocarbon-responsive genes, can potentially play a role in immunotoxicity (Yamaguchi et al., 1997a,b; Near et al., 1999). However, compounds that have the same affinity for the aryl hydrocarbon receptor may or may not have the same effects on the immune system (Okey et al., 1984a,b).

In our laboratory, and others, it has been observed that PAHs must be metabolically activated to be immunotoxic (White et al., 1985; Heidel et al., 1999, 2000; Mann et al., 1999). Apoptosis does not occur in pre-B (70Z/3) cells incubated with DMBA in the absence of stromal (BMS2) cells. We have demonstrated that CYP1B1 present in bone marrow stromal cells activates DMBA to an immunotoxic metabolite that induces apoptosis in pre-B cells (Heidel et al., 1998). This parallels the DMBA-induced loss of myeloid and lymphoid cells in bone marrow in vivo, which is totally dependent on CYP1B1. Apoptosis does not occur in pre-B (70Z/3) cells incubated with DMBA in the absence of stromal (BMS2) cells. The focus of the present study was to determine the molecular events that lead to apoptosis in pre-B cells exposed to DMBA while cocultured with bone marrow stromal cells.

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This work was done primarily in the laboratory of C.J.C.

**ABBREVIATIONS:** PAH, polycyclic aromatic hydrocarbon; DMBA, 7,12-dimethylbenz[a]anthracene; B[a]P, benzo[a]pyrene; FasL, Fas Ligand; TNF, tumor necrosis factor; PKR, interferon-inducible protein kinase; Z-VAD-fmk, *N*-benzyloxycarbonyl-Val-Ala-Asp-fluoromethyl ketone; Z-IETD-fmk, *N*-benzyloxycarbonyl-Ile-Glu-Thr-Asp-fluoromethyl ketone; Z-LEHD-fmk, *N*-benzyloxycarbonyl-Leu-Glu-His-Asp-fluoromethyl ketone; PBS, phosphate-buffered saline.

Apoptosis, or programmed cell death, occurs as a normal part of the hematopoietic program. Cells undergoing apoptosis ultimately exhibit nuclear condensation, followed by internucleosomal DNA cleavage and encapsulation of the DNA fragments (i.e., apoptotic bodies), which can be phagocytosed by nearby cells (Kerr et al., 1972). Biochemical characteristics of apoptotic cells include a reduction in the mitochondrial transmembrane potential, cleavage of DNA into internucleosomal fragments, cleavage of poly(ADP-ribose) polymerase, and activation of the caspase cascade (Kerr et al., 1972; Wyllie et al., 1984; Lazebnik et al., 1994; Zamzami et al., 1995). The latter is the key step in apoptotic cell death. Caspases are cysteine-activated proteases that are synthesized in an inactive pro-form and are cleaved to activate their proteolytic activity (Nicholson and Thornberry, 1997). The two main initiator caspases are caspase-8 and caspase-9, which both serve to activate the chief effector caspase, caspase-3.

Caspase-8 is typically activated in response to receptor-mediated signals such as the binding of Fas to Fas Ligand (FasL), or TNFR1 by TNF- $\alpha$ . The caspase-8 pathway is used by the immune system to maintain the T-cell repertoire, and to delete autoreactive lymphocytes (Green and Scott, 1994; Osborne, 1996). For example, the binding of FasL to Fas results in the cleavage of pro-caspase-8 to its active form, which then initiates a protease cascade that results in the cleavage and activation of caspase-3 (Zhuang et al., 1999; Tang et al., 2000).

In contrast, caspase-9 is activated in response to cytochrome *c* release from mitochondria, which can be stimulated by DNA damage, ionizing radiation, and hypoxia (Slee et al., 1999; Perkins et al., 2000). This initiation process is poorly understood, although the proteins BAX and *BclII*, which are located in the mitochondrial membrane, are involved and probably affect the membrane potential. Upon release from mitochondria, cytochrome *c* binds apoptotic protease-activating factor-1 in an ATP-dependent manner. Apoptotic protease-activating factor-1 then binds pro-caspase-9 via a caspase recruiting domain, resulting in cleavage of pro-caspase-9 to its active form. Caspase-9 in turn activates a protease cascade that results in the activation of effector caspase-3. In addition, there is cross talk between the caspase-8 and -9 pathways. For example, caspase-8 can cleave the protein bid, which then stimulates the release of cytochrome *c* from mitochondria, leading to activation of caspase-9 (Luo et al., 1998).

The overall goal of the present study was to determine the respective contributions of the caspase-8 and -9 pathways to apoptosis in pre-B cells, when they are cocultured with DMBA and bone marrow stromal cells. We present this as a model for DMBA-induced loss of bone marrow lymphoid cells in vivo. We present evidence that apoptosis in pre-B cells, under these circumstances, is largely dependent on the activation of caspase-8. Caspase-9 is also activated by DMBA through caspase-8-independent mechanisms and by caspase-8-mediated cleavage of bid. We further demonstrate that caspase-8 activation is independent of Fas signaling, and that one potential activator of caspase-8 is the interferon-inducible protein kinase (PKR).

## Materials and Methods

**Cell Culture and Treatments.** The 70Z/3 cell line was purchased from American Type Tissue Collection (Manassas, VA) and

routinely grown in culture medium that consisted of RPMI 1640 supplemented with 5% fetal bovine serum (v/v) (Intergen, Purchase, NY),  $5 \times 10^{-5}$  M  $\beta$ -mercaptoethanol, 2 mM L-glutamine, 50 IU of penicillin/ml, and 50 mg of streptomycin/ml (w/v). The BMS2 bone marrow stromal cell line was generously provided by Dr. Paul Kincaide (Oklahoma Medical Research Foundation, Oklahoma City, OK), and maintained as described above. For caspase activity assays, the BMS2 cells were grown to confluence in T-25 flasks and 70Z/3 cells ( $4 \times 10^6$ ) were added in 5 ml of media. For flow cytometry, the BMS2 cells were grown to confluence in T-25 flasks and 70Z/3 cells ( $2 \times 10^6$ ) were added in 5 ml of media.

DMBA was purchased from Sigma-Aldrich (St. Louis, MO) and dissolved in dimethyl sulfoxide. The caspase inhibitors Z-VAD-fmk, Z-IETD-fmk, and Z-LEHD-fmk were purchased from Alexis Biochemicals (San Diego, CA) and dissolved in dimethyl sulfoxide.

**Propidium Iodide Staining.** 70Z/3 pre-B cells were removed from confluent BMS2 cells in T-25 culture flasks by gentle agitation as described previously (Heidel et al., 1999). Cells were then centrifuged for 5 min at 1200 rpm and washed once in ice-cold PBS + 2% bovine serum albumin. Cells were fixed in 1 ml of 80% ice-cold ethanol for 30 min at  $-20^\circ\text{C}$  then centrifuged for 5 min at 1200 rpm and resuspended in phosphate-citric acid buffer (0.192 M  $\text{Na}_2\text{HPO}_4$  and 4 mM citric acid, pH 7.8) for 5 min at room temperature. After centrifugation, the cells were resuspended in 0.5 ml of propidium iodide staining solution (33  $\mu\text{g}/\text{ml}$  propidium iodide; Sigma-Aldrich), 1 mg/ml RNase A, and 0.2% Triton X-100, in PBS) and analyzed in a FACScan flow cytometer (BD Biosciences, San Jose, CA). Because cells undergoing DNA fragmentation and apoptosis exhibit weaker propidium iodide fluorescence than cells in the  $G_0/G_1$  cell cycle, a decrease in propidium iodide fluorescence is indicative of the morphological changes consistent of apoptosis (Yamaguchi et al., 1997a).

**Caspase Assays.** Caspase activity was quantified using the caspase colorimetric assay (R & D Systems, Minneapolis, MN). Briefly, cells were centrifuged, washed in ice-cold PBS, and resuspended in 50  $\mu\text{l}$  of cell lysis buffer. An aliquot (5  $\mu\text{l}$ ) was removed and the total protein concentration estimated using the bicinchoninic acid protein assay kit (Pierce Chemical, Rockford, IL). Total protein (125  $\mu\text{g}$ ) was then placed in a flat bottom 96-well plate and dilution buffer was added to a volume of 50  $\mu\text{l}$ . Caspase reaction buffer ( $2 \times$ , 50  $\mu\text{l}$ ) was then added to each well, and caspase substrates were added to a final concentration of 200  $\mu\text{M}$ . The plates were covered, incubated at  $37^\circ\text{C}$  for 3 h, and read on an EL-312 microplate reader (Bio-Tek, Winooski, VT) at  $A_{405}$ . The data were analyzed using Prism software (GraphPad Software, San Diego, CA).

**Western Immunoblots.** Total cell lysates were prepared using MPER cell lysis buffer (Pierce Chemical), and total protein was estimated using the BCA protein assay kit (Pierce Chemical). Equal amounts of total protein were loaded onto Tris-HCl gels (Bio-Rad, Hercules, CA), electrophoresed, and transferred to nitrocellulose membranes (Amersham Biosciences, Piscataway, NJ). Equal loading and transfer of proteins were verified by Ponceau-S (Sigma-Aldrich) staining. The membranes were then blocked, washed, and probed with the appropriate concentration of antibody. The immunoreactive proteins were visualized with the Super signal picoWest system (Pierce Chemical). Relative band intensities were determined using 1D Image Analysis software (Eastman Kodak, Rochester, NY).

## Results

**Caspase Activation.** Previous studies have demonstrated that addition of DMBA induces apoptosis in 70Z/3 cells cocultured with BMS2 stromal cells (Heidel et al., 1999). In the present study, we sought to determine the relationship between DMBA treatment and caspase activation in 70Z/3 cells cocultured with BMS2 cells. We observed that caspase-8 was activated to a greater extent than caspase-9 at all DMBA concentrations tested (Fig. 1). Caspase-3 was also activated

very strongly. The addition of a pan-caspase inhibitor (Z-VAD-fmk) prevented the activation of all three caspases tested.

To further compare the roles of caspase-8 and -9 in DMBA-mediated apoptosis, inhibitors of specific caspases were added, and apoptosis of 70Z/3 cells was assessed by propidium iodide staining and flow cytometry (Fig. 2). A caspase-8 inhibitor (Z-IETD-fmk) completely blocked DMBA-mediated apoptosis, as did the pan-caspase inhibitor (Z-VAD-fmk). Addition of a caspase-9 inhibitor (Z-LEHD-fmk) resulted in a lesser, but statistically significant ( $p < 0.05$ ) reduction in apoptosis.

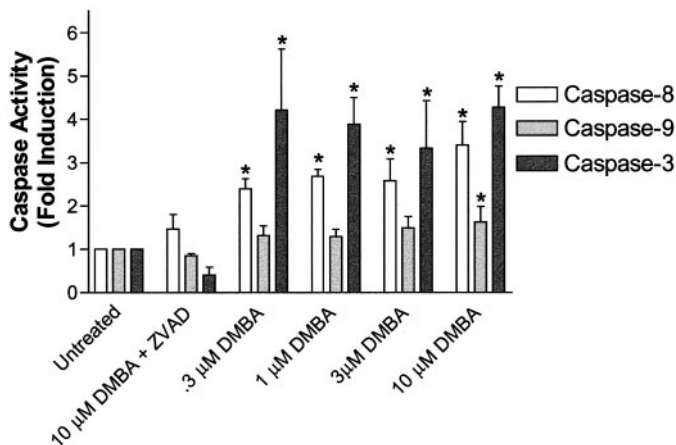
#### Time Course of Caspase Activation and Apoptosis.

We next examined the time course of caspase activation in response to DMBA treatment (Fig. 3). Caspase-8 was activated within 1 h and reached a 4-fold peak of activation at 2.5 h. Caspase-9 was activated within 1.5 h and exhibited steady activation (~2-fold) throughout the 18-h time course. The effector caspase caspase-3 was activated more slowly, reaching a 2-fold peak of activation at 18 h.

To determine the temporal relationship between caspase activation and apoptosis, the percentage of apoptotic 70Z/3 cells after DMBA treatment was determined at various time points after addition of DMBA (Fig. 4). The percentage of apoptotic cells began increasing at about 15 h, and continued to increase through 24 h, the last time point measured. Induction of apoptosis most closely paralleled the induction of caspase-3. This is consistent with the role of caspase-3 as the main effector caspase.

#### Detection of Caspase Activation by Immunoblotting.

In addition to assaying caspase activity, we also detected caspase cleavage by Western blotting. Lysates from DMBA-treated 70Z/3 cells were probed with an antibody against caspase-8 (Fig. 5), which binds both pro-caspase-8 and the active p18 fragment of the enzyme. The active form of caspase-8 was detected at all DMBA concentrations tested. The lowest concentration of DMBA (0.3  $\mu\text{M}$ ) actually had the highest amount of caspase-8 cleavage.

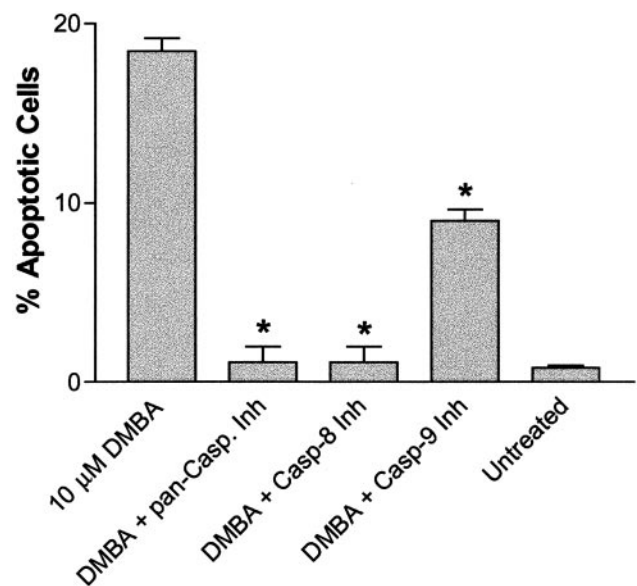


**Fig. 1.** Activation of caspase activity after treatment of 70Z/3 cells with DMBA. 70Z/3 cells were cocultured with BMS2 cells and the indicated concentrations of DMBA for 24 h. In some cases the pan-caspase inhibitor Z-VAD-FMK (50  $\mu\text{M}$ ) was added. The cells were collected, lysates were prepared, and caspase-8, -9, and -3 activity was determined using the appropriate substrates. The data are expressed as the fold increase in caspase activity compared with untreated cells. Each point represents the mean  $\pm$  S.E.M. of three independent experiments. Data were analyzed using an unpaired  $t$  test. The asterisks indicate data that are statistically different from untreated cells ( $p < 0.05$ ).

The explanation for this paradoxical result may be that because DMBA must be metabolized to be activated, the parent compound (DMBA) competes with an intermediate compound for metabolism, at higher concentrations of DMBA there may in fact be a lower concentration of the active metabolite. An alternate explanation may be that cleavage of pan-caspase-8 does not correlate with the activity of caspase-8. In the activity assays, a dose-response relationship was observed with regard to caspase-8 activation (Fig. 1). Perhaps cofactors of caspase-8 are also regulated by DMBA that affect caspase-8 activity. If DMBA treatment up-regulates a cofactor that increases the activity of caspase-8, relative to the amount of the p18 active subunit then the amount of p18 subunit would not correlate quantitatively with caspase-8 activation.

The pan-caspase inhibitor Z-VAD-fmk, and the caspase-8 inhibitor Z-IETD-fmk completely abrogated the apoptotic pre-B cell response to DMBA (Fig. 2). These inhibitors also blocked the activation of caspase-8. The caspase-9 inhibitor Z-LEHD-fmk did not inhibit the DMBA-induced activation of caspase-8. The active p35 form of caspase-9 was also detected after DMBA treatment; however, it did not seem to be induced as strongly as caspase-8 (Fig. 6). Addition of any of the three caspase inhibitors, including the caspase-8 inhibitor, blocked DMBA-induced activation of caspase-9.

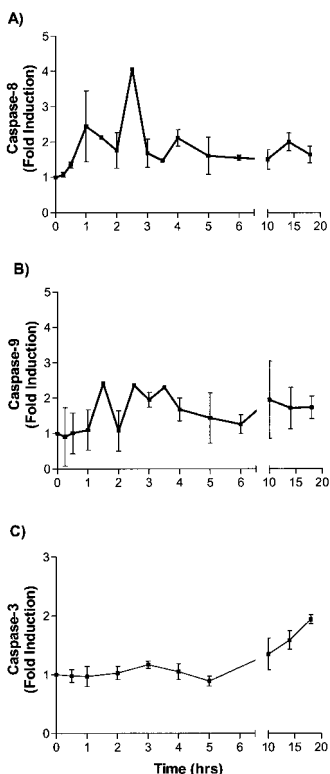
**Bcl-2 Levels after Treatment with DMBA.** We also investigated levels of the antiapoptotic protein bcl2 by Western blotting (Fig. 7). Bcl-2 levels were increased in a dose-dependent manner after DMBA treatment, compared with untreated 70Z/3 cells. This observation is important because induction of bcl2 results in decreased cytochrome *c* release



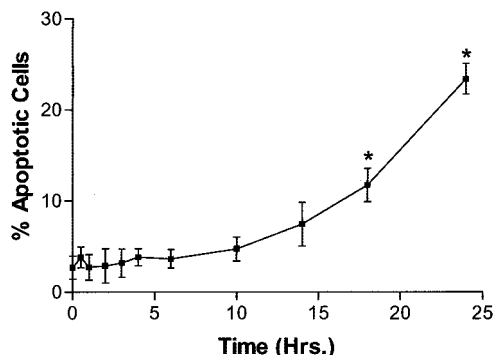
**Fig. 2.** Inhibition of apoptosis in DMBA-treated 70Z/3 cells incubated with specific caspase inhibitors. 70Z/3 cells were cocultured with BMS2 cells and DMBA for 24 h. The pan-caspase inhibitor (Z-VAD-fmk) (50  $\mu\text{M}$ ), the caspase-8 inhibitor (Z-IETD-fmk) (30  $\mu\text{M}$ ), and the caspase-9 inhibitor (Z-LEHD-fmk) (30  $\mu\text{M}$ ) were added to the indicated samples. After 24 h the 70Z/3 cells were collected, stained with propidium iodide, and analyzed by flow cytometry as detailed under *Materials and Methods*. The data are expressed as the percentage of apoptotic cells in each cell population. Each point represents the mean  $\pm$  S.E.M. of four independent experiments. Data were analyzed using an unpaired  $t$  test. The asterisks indicate data that are statistically different from 10  $\mu\text{M}$  DMBA-treated cells ( $p < 0.05$ ).

from mitochondria, which in turn could be expected to result in decreased activation of caspase-9. This observation is consistent with our earlier results, which suggest that caspase-8 is the main caspase pathway used in DMBA-induced apoptosis.

**Bid Cleavage after Treatment with DMBA.** Bid is a protein that, once cleaved by active caspase-8, can stimulate the release of cytochrome *c* from mitochondria, which leads to activation of caspase-9 (Luo et al., 1998; Gross et al., 1999).

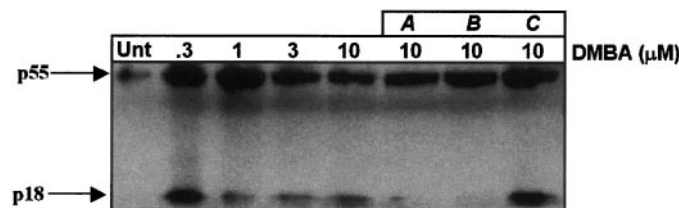


**Fig. 3.** Temporal patterns of caspase activation in DMBA-treated 70Z/3 cells. 70Z/3 cells were cocultured with BMS2 cells and DMBA (3  $\mu$ M) for the indicated times. The cells were collected and lysates prepared and assayed for caspase-8 (A), -9 (B), and -3 (C) activity using the appropriate substrates. The data are expressed as the fold increase in caspase activity compared with untreated cells. Each data point represents the mean  $\pm$  S.E.M. of three independent experiments.

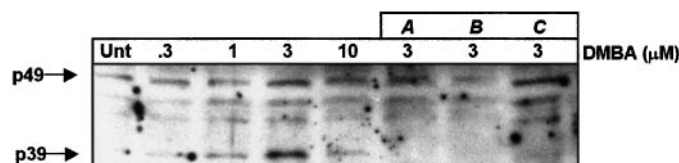


**Fig. 4.** Time course of apoptosis in DMBA-treated 70Z/3 cells. 70Z/3 cells were cocultured with BMS2 cells and DMBA (3  $\mu$ M) for the indicated times. The 70Z/3 cells were collected, stained with propidium iodide, and analyzed by flow cytometry as detailed under *Materials and Methods*. Each point represents the mean  $\pm$  S.E.M. Percentage of apoptotic cells for three replicates from one representative experiment is shown. Similar results were obtained in a second experiment. The asterisks indicate data that are statistically different from untreated cells ( $p < 0.05$ ).

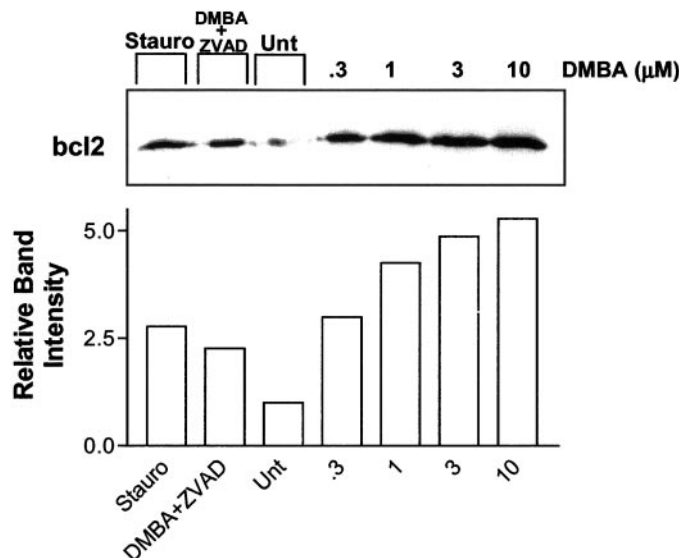
Because we could block activation of caspase-9 using a caspase-8 inhibitor, we sought to determine whether bid was cleaved in response to DMBA treatment. As illustrated in Fig. 8A, bid cleavage by 70Z/3 cells cocultured with BMS2 cells increased with the concentration of DMBA and was blocked by addition of a pan-caspase inhibitor (Z-VAD-fmk).



**Fig. 5.** Western blot of caspase-8 activation in DMBA-treated 70Z/3 cells. The cells were cocultured with BMS2 cells and treated with the indicated concentration of DMBA for 1.5 h. Whole cell lysates were then prepared, and activation of caspase-8 was analyzed by immunoblotting as described under *Materials and Methods*. The pro-form (p55) and active fragment of caspase-8 (p18) are indicated by arrows. A, 10  $\mu$ M DMBA plus 40  $\mu$ M Z-VAD-fmk (pan-caspase inhibitor). B, 10  $\mu$ M DMBA plus 40  $\mu$ M Z-IETD-fmk (caspase-8 inhibitor). C, 10  $\mu$ M DMBA plus 40  $\mu$ M Z-LEHD-fmk (caspase-9 inhibitor). The data are from one representative experiment of three that were performed.



**Fig. 6.** Western blot of caspase-9 activation in DMBA-treated 70Z/3 cells. The cells were cocultured with BMS2 cells and the indicated concentrations of DMBA for 15 h. Lysates were then prepared and activation of caspase-9 was analyzed by immunoblotting as described under *Materials and Methods*. A, 3  $\mu$ M DMBA plus 40  $\mu$ M Z-VAD-fmk (pan-caspase inhibitor). B, 3  $\mu$ M DMBA plus 40  $\mu$ M Z-IETD-fmk (caspase-8 inhibitor). C, 3  $\mu$ M DMBA plus 40  $\mu$ M Z-LEHD-fmk (caspase-9 inhibitor). The data are from one representative experiment of two that were performed.



**Fig. 7.** Incubation of 70Z/3 cells with DMBA causes up-regulation of Bcl-2. 70Z/3 cells were cocultured with BMS2 cells and the indicated concentration of DMBA for 24 h. The pan-caspase inhibitor Z-VAD-fmk (50  $\mu$ M) and staurosporine (200 nM, 2 h) were added as controls. Lysates were then prepared from the 70Z/3 cells, and BclII levels were analyzed by immunoblotting as described under *Materials and Methods*. Relative band intensity is expressed as arbitrary intensity values. The data are from one representative experiment of three that were performed.

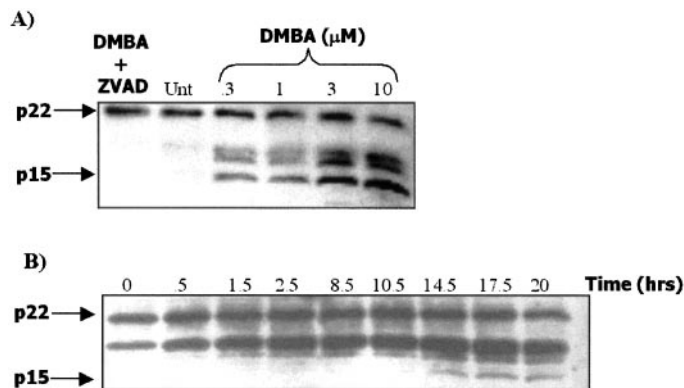
A time course evaluation indicated that bid cleavage was first observed at 14.5 h (Fig. 8B). These temporal data support the hypothesis that one component of caspase-9 activation is the result of caspase-8-mediated cleavage of bid.

**Role of FasL in DMBA-Mediated Cell Death.** Because the data suggested a predominant role for caspase-8 in 70Z/3 cell apoptosis, we next considered the possibility that this reflected FasL expression or secretion by the BMS2 cells. To test this hypothesis, we attempted to block DMBA-mediated apoptosis in 70Z/3 cells, cocultured with BMS2 cells, by adding a neutralizing monoclonal antibody to FasL. Although this anti-FasL monoclonal antibody blocked 70Z/3 cell apoptosis induced by recombinant FasL, it did not block apoptosis mediated by DMBA (Fig. 9). We also tested the WEHI 231 cell line, a pre-B cell line reported to be resistant to Fas-mediated apoptosis, for its susceptibility to DMBA-mediated apoptosis in our coculture system (Mueller and Scott, 2000). We found that DMBA-induced apoptosis in WEHI 231 cells cocultured with BMS2 cells (data not shown), providing further evidence that FasL release and Fas-mediated signaling are not required for DMBA-mediated pre-B cell apoptosis.

**PKR Activation in Response to DMBA Treatment.** It has been reported elsewhere that PKR can trigger apoptosis through activation of caspase-8, that is independent of both Fas and TNF- $\alpha$  receptors (Gil and Esteban, 2000). Based on these prior observations, we sought to determine the effect of DMBA treatment on PKR expression in 70Z/3 cells cocultured with BMS2 cells. PKR was induced in 70Z/3 cells cocultured with BMS2 and 0.3 to 10  $\mu$ M DMBA for 1 h (Fig. 10A). We observed increased PKR expression before the earliest changes in caspase activation. After 24 h of DMBA treatment the expression of PKR was quite different. We observed a dose-dependent decrease in the amount of PKR expression (Fig. 10B). Suppression of PKR seemed to be caspase-dependent, because it was blocked by the addition of a pan-caspase inhibitor (Z-VAD-FMK).

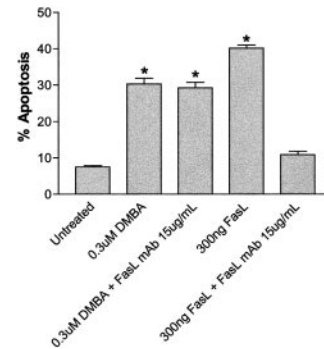
## Discussion

We and others have previously reported on the coculture of pre-B cells with bone marrow stromal cells as an *in vitro*

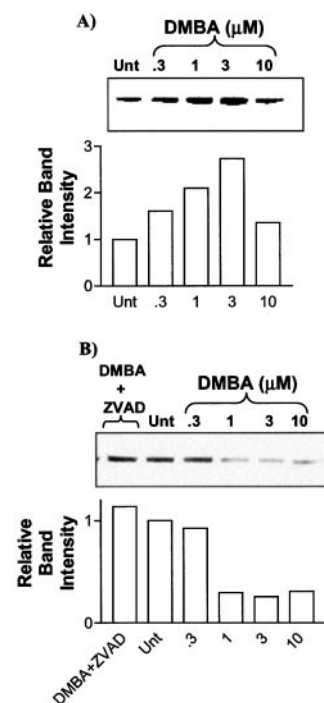


**Fig. 8.** Bid protein cleavage in 70Z/3 cells. A, 70Z/3 cells were cocultured with BMS2 cells and the indicated concentration of DMBA for 24 h. The pan-caspase inhibitor Z-VAD-fmk (50  $\mu$ M) was added to the indicated cells. In B, 70Z/3 cells were incubated with BMS2 cells and 3  $\mu$ M DMBA for the indicated times. Lysates were prepared from the 70Z/3 cells and cleavage of bid analyzed by immunoblotting according to the protocols under *Materials and Methods*. Cleaved bid (p15) and uncleaved bid (p22) are indicated by the arrows. The data are from one representative experiment of three that were performed.

model for investigating mechanisms of DMBA-induced immunotoxicity. Using this model, we have demonstrated that apoptosis of pre-B cells is dependent on metabolism of DMBA by CYP1B1 in bone marrow stromal cells. In the present study, we used this model system to determine the molecular events that precede apoptosis in 70Z/3 cells. Our results demonstrated an essential role for caspase-8 in DMBA-induced apoptosis in 70Z/3 cells. These findings are interesting in light of the fact that DMBA is metabolized into diol-epoxide compounds that have the ability to form adducts



**Fig. 9.** Neutralizing antibody to FasL fails to block apoptosis in DMBA-treated 70Z/3 cells. 70Z/3 cells were cocultured with BMS2 and the indicated compounds for 24 h. The 70Z/3 cells were collected, stained with propidium iodide, and analyzed by flow cytometry. The data are expressed as the percentage of apoptotic cells in each cell population. Each data point represents the mean  $\pm$  S.E.M. of three independent experiments. Data were analyzed using an unpaired *t* test. The asterisks indicate data that are statistically different from untreated cells ( $p < 0.05$ ).



**Fig. 10.** Modulation of PKR in 70Z/3 cells. A, 70Z/3 cells were cultured with BMS2 cells and treated with DMBA for 1 h. B, 70Z/3 cells were cocultured with BMS2 cells and treated with DMBA for 24 h. The indicated cells also received the pan-caspase inhibitor Z-VAD-fmk (50  $\mu$ M) for 24 h. Lysates were prepared from the 70Z/3 cells, and levels of PKR were determined by immunoblotting. Relative band intensity is expressed as arbitrary intensity values. The data presented are representative of three independent experiments.

with DNA (Melendez-Colon et al., 2000). DNA damage and chemotherapeutic drugs are generally thought to cause apoptosis through mitochondrial release of cytochrome *c* in a caspase-9–dependent pathway (Kawahara et al., 2000; Ochs and Kaina, 2000; Perkins et al., 2000). Although it is clear that DMBA does form DNA adducts in 70Z/3 cells (Heidel et al., 2000) and caspase-9 does contribute to maximal levels of apoptosis, it does not seem to be essential for apoptosis in 70Z/3 cells.

Typically, caspase-8 activation follows ligation of death receptors on the cell surface (Fas, TNFR1, DR3, DR4, and DR5) by their cognate ligands (Boldin et al., 1996; Medema et al., 1997). Two types of death receptor-mediated apoptosis have been identified, type I and type II (Scaffidi et al., 1998; Schmitz et al., 1999). Type I apoptosis is characterized by rapid activation of caspase-8 at the death inducing signaling complex followed by caspase-3 activation that is not sensitive to *bcl2* overexpression. In contrast, there is little death inducing signaling complex formation in type II apoptosis. Instead, the signal from the death receptor is amplified through the mitochondrial pathway and can be blocked by overexpression of antiapoptotic *bcl2* family members. In type II apoptosis, it is believed that mitochondrial activation is mediated by rapid, caspase-8 cleavage of *bid*. Cleaved *bid* then stimulates cytochrome *c* release from mitochondria, resulting in the activation of caspase-9, which subsequently cleaves caspase-3. In our system, we observed activation of caspase-8 at 1 h that peaked at 2.5 h. Caspase-9 activation was observed at 1.5 h but did not reach the magnitude of caspase-8 activation. In addition, cleavage of *bid* was observed beginning at 14.5 h after DMBA treatment, and induction of *bcl2* was also observed. These observations do not conform with either the type I or type II definitions of apoptosis.

Inhibition of caspase-8 resulted in complete inhibition of apoptosis after DMBA treatment. In contrast, inhibition of caspase-9 decreased apoptosis by 49% compared with control DMBA-treated cells. In addition, treatment with DMBA and the caspase-8 inhibitor blocked the cleavage of caspase-9, as measured by Western blot at 24 h. These data support the conclusion that activation of caspase-8 is the salient event in DMBA-mediated apoptosis of pre-B cells.

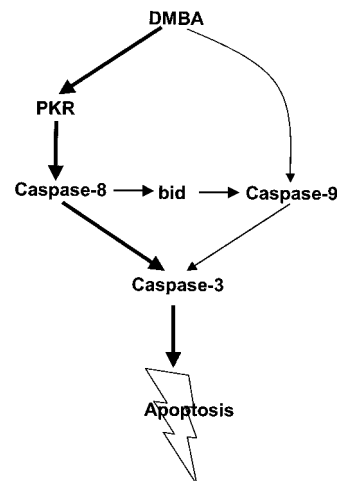
We considered the possibility that DMBA treatment could stimulate 70Z/3 cell apoptosis by the Fas/FasL pathway, as has been demonstrated for other types of toxic events (Kasibhatla et al., 1998; Mo and Beck, 1999). Because a neutralizing antibody against FasL did not decrease apoptosis after DMBA treatment, we can exclude a role for FasL. However, this experiment does not exclude activation of caspase-8 by some other member of the TNF receptor family. This observation, and the lack of correlation between the temporal pattern of caspase activation and the two best-characterized pathways of death receptor-mediated apoptosis, led us to hypothesize that DMBA-induced activation of caspase-8 was independent of death receptors.

We next considered the role of PKR, an interferon-inducible protein kinase, capable of activating caspase-8 independent of death receptor ligation (Gil and Esteban, 2000). We have found that PKR is transiently up-regulated by DMBA treatment in 70Z/3 cells. We hypothesize that up-regulation of PKR may be responsible, in part, for the caspase-8 activation that we observed. PKR expression is up-regulated after 1 h of DMBA treatment. This effect is transient; at 24 h after

treatment, PKR expression is decreased compared with untreated levels. The increase in PKR expression correlates with the increases in caspase-8 activity observed within 3 h of DMBA administration. The rapid activation of PKR may be the reason that we observe apoptosis primarily through a caspase-8–dependent pathway, as opposed to a caspase-9–dependent pathway. The timing of PKR activation may be indicative of a mechanism that is independent of appreciable DMBA metabolism and DNA adduct formation. The decrease in PKR levels after 3 h correlates with a decrease in caspase-8 activity at 3.5 h.

Future studies will focus on determining the mechanism used by DMBA to up-regulate PKR levels. Disruption of intracellular calcium stores has been observed in lymphocytes in response to treatment with PAHs (Davis and Burchiel, 1992; Salas and Burchiel, 1998). PKR activation has been demonstrated after depletion of  $Ca^{2+}$  stores in the endoplasmic reticulum (Srivastava et al., 1995). Perhaps, activation of PKR and subsequent caspase cleavage are driven by the release of calcium from intracellular stores in response to DMBA treatment.

We suggest that treatment of pre-B cells with DMBA results in the rapid activation of caspase-8 by PKR and the subsequent activation of caspase-9 by caspase-8–mediated cleavage of *bid* (Fig. 11). However, it is likely that there is a small amount of caspase-9 that is activated independently of caspase-8 in response to DMBA treatment. Supporting this hypothesis is the fact that a caspase-8 inhibitor completely blocks DMBA-mediated apoptosis, whereas a caspase-9 inhibitor reduces DMBA-mediated apoptosis by only 49% (Fig. 2). When caspase-9 is blocked by a specific inhibitor, DMBA is still able to induce apoptosis through activation of caspase-8, albeit at a diminished level. The results of our immunoblots further support this hypothesis. A caspase-9 inhibitor did not block DMBA-induced activation of caspase-8; however, treatment with a caspase-8 inhibitor blocked the activation of caspase-9. Although these results are similar to type II apoptosis induced by death receptors,



**Fig. 11.** Diagram of proposed caspase activation scheme in 70Z/3 cells treated with DMBA. Caspase-8 is activated by DMBA-induced activation of PKR. Caspase-8 then directly activates caspase-3 and also cleaves *bid*, which leads to cleavage of caspase-9. Caspase-9 then cleaves caspase-3 resulting in maximal apoptosis. It is likely that caspase-9 activation independent of caspase-8 activation also makes a small contribution to a maximal apoptotic effect.

there are several important differences. In our system, we observed a relatively rapid activation of caspase-8 that, by itself, is able to mediate significant levels of apoptotic cell death. We believe that DMBA-mediated apoptosis is induced through a complex process that includes caspase-8 activation, and caspase-8-dependent caspase-9 pathways. In addition, there is probably activation of caspase-9 through a caspase-8-independent mechanism. The additive effect of these caspase pathways is essential for maximal pre-B cell apoptosis after DMBA treatment.

In summary, we have proposed a novel mechanism of apoptosis in pre-B cells after treatment with DMBA, a member of the PAH family. This mechanism is independent of death receptor activation and proceeds primarily through caspase-8. This is significant because PAHs, in addition to being known carcinogens, are also potent immunotoxicants. DMBA and B[a]P are two of the most widely studied PAHs. B[a]P is a naturally occurring compound that can be found in the environment, whereas DMBA is a synthetic PAH that is not naturally occurring (Hardin et al., 1992). Mechanistically, the two prototypic PAHs seem to act in a similar manner. Determining the underlying mechanisms of immunotoxicity of PAHs will aid in the evaluation of their effects on human health.

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