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Environmental Chemical-Induced Bone Marrow B Cell Apoptosis: Death Receptor-Independent Activation of a Caspase-3 to Caspase-8 Pathway

Heui-Young Ryu, Jessica K. Emberley, Jennifer J. Schlezinger, Lenka L. Allan, Songqing Na, and David H. Sherr

Department of Environmental Health, Boston University School of Public Health (H.-Y.R., J.J.S., D.H.S.) and Department of Microbiology, Boston University School of Medicine (J.K.E., L.L.A.), Boston, Massachusetts; and Eli Lilly Co., Indianapolis, Indiana (S.N.)

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ABSTRACT

Programmed cell death is a critical process in B lymphocyte development. Premature apoptosis in developing B cells could affect the repertoire and number of mature B cells produced. Of particular concern is the ability of environmentally ubiquitous polycyclic aromatic hydrocarbons (PAH) to induce B cell apoptosis within the bone marrow microenvironment in a clonally nonspecific way. Here, models of bone marrow B cell development were used to assess the role of the "extrinsic" apoptosis pathway in PAH-induced apoptosis and to compare PAH-induced apoptosis with that induced during clonal deletion. As demonstrated previously with a nontransformed pro-/pre-B cell line, primary pro-B cells cultured on bone marrow stromal cells underwent apoptosis after exposure to a prototypic PAH, 7,12-dimethylbenz[*a*]anthracene (DMBA). Apoptosis was preceded by cleavage of caspase-3 (4–6 h) and caspase-8 (6–8 h) and their respective substrates, α-fodrin and Bid. Inhibition of caspase-3 blocked caspase-8 activation and apoptosis. Furthermore, a pan-caspase inhibitor blocked apoptosis and activation of both caspases-3 and -8. Cells from mice defective in tumor necrosis factor (TNF)-α, TNF-β, lymphotoxin-β, or TNFR1, TNFR2, Fas, or death receptor 6 were as susceptible to apoptosis signaling as wild-type cells. These results suggest a complex death receptor-independent B cell apoptosis pathway in which caspase-8 is activated downstream of caspase-3.

Apoptosis is a critical event in the deletion of autoimmune B lymphocytes as they enter the periphery from the bone marrow (Defrance et al., 2002). Some of the signaling pathway leading to immature B cell death and clonal deletion has been mapped in model systems in which transformed cells (e.g., WEHI-231) (Wu et al., 1996a, 1998; Andjelic and Liou, 1998; Doi et al., 1999; Ruiz-Vela et al., 1999) or immature splenic B cells (Andjelic and Liou, 1998; Tian et al., 2001) were induced to undergo apoptosis after immunoglobulin cross-linking. In these systems, contributions of nuclear factor-κB and c-Myc down-regulation (Wu et al., 1996a,b); p53, $p27^{Kip1}$, and $p21^{WAF1}$ up-regulation (Wu et al., 1998); mitochondrial activation (Doi et al., 1999); and protease (calpain, cathepsin, and caspase) activation (Ruiz-Vela et al., 1999) have begun to be defined. Our laboratory has investigated previously whether B lymphocytes earlier in development are similarly susceptible to apoptosis (Yamaguchi et al., 1997a; Mann et al., 1999, 2001; Ryu et al., 2003). Because pro- and pre-B cells do not express surface Ig, prototypic polycyclic aromatic hydrocarbons (PAH) such as benzo[*a*]pyrene or dimethylbenz[*a*]anthracene (DMBA) was used to induce apoptosis in these early B cells. Studies with PAH are particularly relevant because these ubiquitous environmental pollutants are profoundly immunosuppressive (Dean et al., 1986; Thurmond et al., 1987), and much of their immunotoxicity is directed toward B cells (Hardin et al., 1992; Page et al., 2003).

Using B cell/bone marrow stromal cell coculture systems

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ABBREVIATIONS: PAH, polycyclic aromatic hydrocarbon; DMBA, 7,12-dimethylbenz[*a*]anthracene; FBS, fetal bovine serum; TNF, tumor necrosis factor; rIL, recombinant interleukin; TNFR, tumor necrosis factor receptor; tBid, truncated Bid; FITC, fluorescein isothiocyanate; PE, phycoerythrin; DMSO, dimethyl sulfoxide; FMK, fluoromethyl ketone; PIPES, 1,4-piperazinediethanesulfonic acid; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; LPS, lipopolysaccharide; RT-PCR, reverse-transcriptase polymerase chain reaction; bp, base pair(s); ELISA, enzyme-linked immunosorbent assay; ANOVA, analysis of variance; DR, death receptor; Vh, vehicle.

containing either primary pre-B cells in Whitlock/Witte cultures or a nontransformed, stromal cell-dependent primary CD43$^+$ pro-/pre-B cell line (BU-11), it was shown that relatively low DMBA doses (≥10 nM) rapidly induce pre- or pro-/pre-B cell apoptosis (Yamaguchi et al., 1997a; Mann et al., 1999, 2001; Ryu et al., 2003). Like the clonal deletion pathway, down-regulation of nuclear factor-κB and c-Myc and up-regulation of p53 contribute to PAH-induced pro-/pre-B cell death (Mann et al., 2001; Ryu et al., 2003). However, unlike clonal deletion, up-regulation of p27^{kip1} and p21^{waf1} plays no role in PAH-induced apoptosis (Ryu et al., 2003).

Caspase activation is a hallmark of apoptosis in many cell types, including immature B lymphocytes undergoing clonal deletion (Ruiz-Vela et al., 1999). Caspases are grouped by phylogenetic analysis into three major classes: inflammatory (caspases-1, -4, -5, -11, and -12), initiator (caspases-2, -8/10, and -9), and effector (caspases-3, -6, and -7). In many cases, caspase cascades can be assigned to one of two non-mutually exclusive pathways on the basis of the initiator caspase activated and the contribution of death receptors in caspase activation (Nicholson, 1999). The "extrinsic pathway" is frequently induced by ligation of TNFR family death receptors and requires early activation of caspase-8, the most proximal caspase in this pathway (Medema et al., 1997). Downstream targets of caspase-8 include procaspase-3 and Bid, the truncated form of which (tBid) translocates to and induces cytochrome *c* release from mitochondria (Gross et al., 1999). TNFR family members also may be activated independently of ligands resulting in caspase-8 activation (Aragane et al., 1998; Micheau et al., 1999; Chen and Lai, 2001).

The "intrinsic pathway" is believed to be induced by stress (e.g., cytotoxic agents, irradiation) rather than by specific extrinsic cytokines. This pathway involves caspase-8–independent mitochondrial membrane potential depolarization ($\Delta\Psi_m$) and/or permeabilization (Jiang and Wang, 2004) and the formation of an "apoptosome", a death complex composed of cytochrome *c*, Apaf-1, and caspase-9. The apoptosome targets effector caspases-3 and -7. It is noteworthy that caspase-6 may be activated by caspase-3, which in turn activates caspase-8 in an apoptosis amplification loop (Slee et al., 1999; Belka et al., 2000; Wieder et al., 2001; Cowling and Downward, 2002; Murphy et al., 2004).

Given these models of caspase signaling, it was postulated that the determination of a role for caspase-8 in apoptosis and the signal through which it may be activated (i.e., death receptors and/or caspase-3) would provide insight into whether and at what developmental stage developing B cells are mature enough to have functional "extrinsic" or "intrinsic" apoptosis pathways. In addition, these studies could determine whether the apoptotic pathway initiated during clonal deletion is activated inappropriately by environmental chemicals. Therefore, studies were designed to determine a putative role for TNFR family death receptors, caspase-8, and caspase-3 in PAH-induced bone marrow stromal cell-dependent B cell apoptosis using a pro-/pre-B cell line and primary pro-B cells.

Materials and Methods

Cell Culture. Stromal cell-dependent, CD43$^+$ (pro-/pre-B) BU-11 cells expressing rearranged cytoplasmic Ig heavy chains (Yamaguchi et al., 1997a; Mann et al., 1999) were cocultured on cloned BMS2 bone marrow-derived stromal cells (kindly provided by Dr. P. Kincade, Oklahoma Medical Research Foundation, Oklahoma City, OK) in 50% RPMI 1640 medium and 50% Dulbecco's modified Eagle's medium (Mediatech, Herndon, VA) containing 5% fetal bovine serum (FBS) (Hyclone, Logan, UT), 2 mM L-glutamine (Mediatech), 0.01 mM 2-mercaptoethanol (Sigma Chemical, St. Louis, MO), and 0.5 μg/ml plasmocin, an antimycoplasma reagent (Invitrogen, Carlsbad, CA) at 37°C in a humidified 5% CO_2 incubator.

Primary bone marrow pro-B cell cultures were prepared from wild-type B6.129SF2/J and age-matched B6.129S6-Tnf^{tm1Gk1}/J (TNF-$\alpha^{-/-}$) or B6.129S-Tnfrsf1a^{tm1Imx}/Tnfrsf1b^{tm1Imx}/J (TNFR1$^{-/-}$/TNFR2$^{-/-}$) mice (The Jackson Laboratory, Bar Harbor, ME), wild-type BALB/c and age-matched BALB/c-*lpr* mice (the generous gifts of Dr. A. Marshak-Rothstein, Boston University School of Medicine, Boston, MA), B6.129-DR6$^{-/-}$ mice and their wild-type littermates (Schmidt et al., 2003), or C57BL/6 mice essentially as described previously (Tze et al., 2000). Bone marrow was flushed from the femurs of 4- to 6-week-old male mice. Red blood cells were lysed by incubation in 0.17 M NH_4Cl, 10 mM $KHCO_3$, and 1 mM EDTA at 37°C for 5 min. The remaining cells were cultured for 5 to 7 days in RPMI containing 10% FBS, penicillin/streptomycin (Mediatech), L-glutamine, 2-mercaptoethanol, and 16 ng/ml murine rIL-7 (Research Diagnostics, Flanders, NJ). For isolation of stromal cells, murine rIL-7 was not included in the media. B cells were stained with FITC-conjugated B220-specific (clone, RA3-6B2; BD PharMingen, San Diego, CA) and PE-conjugated CD43-specific (clone, S7; BD PharMingen) antibodies or with FITC-conjugated rat IgG_{2a} and PE-conjugated rat IgG_{2a} (clone, R35-95; BD PharMingen) as controls, fixed in 1.5% paraformaldehyde, and analyzed on a FACScan flow cytometer (BD Biosciences, San Jose, CA). At least 95% of the cells expressed CD43 and B220.

Experimental Treatment. BMS2 cells or primary bone marrow stromal cells were cultured for 24 h in 24-well plates or T75 flasks in Dulbecco's modified Eagle's medium containing 5% FBS to form a monolayer that was approximately 75% confluent. BU-11 cells or primary pro-B cells were added in RPMI containing 5% FBS and allowed to associate with the stromal cells for 24 h. Stromal cell monolayers or B cell/stromal cell cocultures were treated in duplicate wells or flasks with vehicle (0.1% acetone) or DMBA (1 μM; Sigma) for 2 to 24 h. DMSO (0.1%), the pan caspase inhibitor VAD-FMK, the caspase-3 inhibitor DEVD-FMK, or a control peptide FA-FMK (15–30 μM; Calbiochem, San Diego, CA) was added to cocultures 30 min before acetone (vehicle) or DMBA treatment.

Apoptosis Assays. BU-11 cells and primary pro-B cells were harvested and washed once with ice-cold PBS containing 5% FBS and 0.01 M sodium azide (Sigma). For propidium iodide staining, cells were resuspended in 0.15 ml of hypotonic buffer containing 50 μg/ml propidium iodide (Sigma), 0.1% sodium citrate, and 0.1% Triton X-100 and analyzed by flow cytometry. Cells undergoing DNA fragmentation (i.e., apoptosis) were shown to have a lower propidium iodide fluorescence than those in the typical G_0/G_1 stages of cell cycle (Yamaguchi et al., 1997a; Mann et al., 1999). For Annexin V staining, cells were resuspended in 0.2 ml of Annexin V binding buffer containing 10 mM HEPES, pH 7.4, 140 mM NaCl, and 2.5 mM $CaCl_2$. Next, 2.5 μl of Annexin V-PE (BD PharMingen) was added. Cells were incubated for 15 min in the dark at room temperature and were analyzed by flow cytometry within an hour. Annexin V and propidium iodide staining yielded equivalent results. Data from duplicates were averaged and used as a single representation of the percentage of apoptotic cells for any given treatment. Experiments were performed with a minimum of three mice.

Immunoblotting. BU-11 cells or primary pro-B cells were harvested and washed once in cold PBS. Cells were resuspended in lysis buffer containing 50 mM PIPES/NaOH, pH 6.5, 2 mM EDTA, 0.1% CHAPS, 5 mM dithiothreitol, and protease inhibitor cocktail for mammalian cells (1:200 dilution; Sigma) and incubated on ice for 15 min. The extracts were cleared by centrifugation at 14,000 rpm for

10 min at 4°C. Supernatants were collected, placed in aliquots, and frozen at −80°C until use. Protein concentrations were determined using the Bradford assay.

Total proteins (50–80 μg) were resolved on 6% (α-fodrin) or 15% gels, transferred to a 0.2-μm nitrocellulose membrane, and incubated with primary antibody. Primary antibodies included monoclonal mouse anti-α-fodrin (Chemicon International, Temecula, CA), polyclonal rat anti-Bid (R&D Systems, Minneapolis, MN), polyclonal rabbit anti-cleaved caspase-3 (Cell Signaling Technology Inc., Beverly, MA), or polyclonal rat anti-caspase-8 (Axxora, San Diego, CA). Immunoreactive bands were detected using horseradish peroxidase-conjugated secondary antibodies (Bio-Rad, Hercules, CA) followed by ECL. To control for equal protein loading, blots were stripped and reprobed with a β-actin–specific antibody (Sigma) or α-tubulin–specific antibody (EMD Biosciences, San Diego, CA) and analyzed as above.

Caspase Activity Assays. Bone marrow B cells were harvested and washed once in ice-cold PBS. Cytosolic proteins were prepared according to the manufacturer's instructions (Apoalert; BD Biosciences Clontech, Palo Alto, CA). In brief, BU-11 cells were resuspended in 50 μl of chilled cell lysis buffer, incubated on ice for 10 min, and centrifuged at 14,000 rpm for 10 min at 4°C. Supernatants were collected, and caspase activity was determined immediately. Protein concentrations were determined using the Bradford assay. Cytosolic proteins (50 μg) were incubated with reaction buffer containing 10 mM dithiothreitol and chromophore *p*-nitroaniline-conjugated DEVD or IETD substrate (final concentration, 200 μM) at 37°C for 2 h. *p*-Nitroaniline standard solution was diluted to a final concentration of 0 to 200 μM with cell lysis buffer to generate a standard curve. The concentration (micromolar) of free *p*-nitroaniline released from caspase substrate was measured at 405 nm in a microplate reader (Bio-Tek Instruments, Winooski, VT).

Analysis of TNFR Ligand Expression. For RNA analysis, stromal cells were trypsinized and washed once in complete medium and once in ice-cold PBS. Immature dendritic cells were produced by culture of bone marrow cells with rGM-CSF and rIL-4 for 7 days. These cells were treated with LPS (1 μg/ml) for 6 h as a positive control for TNF-α, TNF-β, and lymphotoxin-β (LT-β) mRNA expression. Total RNA was isolated (RNAzol; Tel-Test Inc., Friendswood, TX), and 5 μg was reverse-transcribed (Superscript First Strand Synthesis System for RT-PCR; Invitrogen, Carlsbad, CA). The cDNA was subjected to PCR amplification with TNF-α, TNF-β, LT-β (36 cycles) and β-actin–specific (26 cycles) primers. The primer sequences were as follows (Reddy et al., 2001): TNF-α: sense, ATGAGCACAGAAAGCATGATCCGCGAC (700 bp); antisense, TCACAGAGCAATGACTCCAAAGTAGACCTG; TNF-β: sense, CCCATGGCATCCTGAAAC (485 bp); antisense, GGAGGCCTGGAATCCAAT; LT-β: sense, TCGGGTTGAGAAGATCATTGG (640 bp); antisense, GCTCGTGTACCATAACGACC; and β-actin: sense, GTCGTCGACAACGGCTCCGGCATGTG (256 bp); antisense, CATTGTAGAAGGTGTGGTGCCAGATC.

For analysis of membrane-bound TNF-α, stromal cells were trypsinized for 5 min and washed once in complete medium and once in cold PBS. RAW 264.7 cells that were treated with LPS (1 μg/ml) for 4 h were included as a positive control. Cells were stained with anti-TNF-α-PE (clone, MP6-XT22; BD PharMingen) or PE-conjugated rat IgG_1 (clone, R3-34; BD PharMingen), fixed in 1.5% paraformaldehyde, and analyzed by flow cytometry. For analysis of secreted TNF-α, cell-free supernatants were collected, and TNF-α production was determined by ELISA (BD PharMingen).

Statistics. Statistical analyses were performed with Statview (SAS Institute, Cary, NC). At least three experiments were performed in each BU-11 cell protocol. Experiments with pro-B cells were performed with a minimum of three mice, and cells from each mouse were maintained separately. Each treatment within an experiment using either BU-11 cells or primary pro-B cells was performed in duplicate wells, and each well was assayed independently. Results from duplicate wells within each experiment were averaged before statistical analysis. Data from a minimum of three experiments were averaged and are presented as means ± S.E.. The Student's *t* test and one-factor ANOVAs were used to analyze the data. For ANOVAs, the Dunnett's or Scheffé's multiple comparisons tests were used to determine significant differences.

Results

DMBA Rapidly Induces Apoptosis in Primary Pro-B Lymphocytes. Previous studies demonstrated that a nontransformed pro-/pre-B cell line (BU-11), or primary pre-B cells, cocultured with bone marrow stromal cells, undergo apoptosis when the cultures are exposed to DMBA (Yamaguchi et al., 1997a; Mann et al., 1999, 2001; Ryu et al., 2003). To determine whether earlier primary B cells (i.e., those at the pro-B cell stage) similarly express an intact apoptosis signaling pathway, bone marrow-derived $B220^+/CD43^+$ B cell populations were expanded in rIL-7. Culture of bone marrow cells with rIL-7 for at least 5 days resulted in a highly enriched pro-B cell population, >95% of which expressed B220 and CD43 (Fig. 1A). These B cells loosely adhered to and, in some cultures, grew under the stromal cell monolayer. Cultures of either primary pro-B or BU-11 cells on bone marrow stromal cell (BMS2) monolayers were treated with vehicle (0.1% acetone) or DMBA (1 μM) for 2 to 18 h. This dose of DMBA was chosen because it induces significant apoptosis that is completely aryl hydrocarbon receptor-dependent (Mann et al., 1999). Apoptosis was quantified by propidium iodide staining and flow cytometry.

Primary pro-B cells (Fig. 1B) and BU-11 cells (Fig. 1C) generally exhibited a relatively low level of background apoptosis (<5%). Treatment with DMBA for 12 h consistently induced apoptosis in a significant fraction of both bone marrow B cell types (Fig. 1, B and C). Time course experiments indicated a trend toward increased apoptosis 6 to 8 h after DMBA treatment of BU-11 cultures that reached statistical significance 10 h after treatment (Fig. 1D). Likewise, significant apoptosis was induced in primary pro-B cells within 10 h of treatment (Vh, 4.4 ± 1.3%; DMBA, 16.5 ± 2.5%, $p < 0.01$, Student's *t* test). These results demonstrate that bone marrow B cells become responsive to DMBA-dependent death signals at an early stage of development (i.e., at the pro-B cell stage). Furthermore, they support the use of primary pro-B cells from mice deficient in apoptosis-signaling components to map out the PAH-induced apoptosis signaling pathway.

DMBA Activates Caspase-3 in Developing B Lymphocytes. Caspase-3 is considered to be the primary apoptosis executioner with the broadest substrate repertoire of the effector caspases (Slee et al., 2001). Among the substrates for caspase-3 are caspases-2 and -6, which may participate in an amplification loop leading to the activation of what is otherwise considered to be an initiator caspase, caspase-8 (Slee et al., 1999; Cowling and Downward, 2002). To determine the role of caspase-3 in DMBA-induced pro-/pre-B cell apoptosis, BU-11/BMS2 cocultures were treated with vehicle (0.1% acetone) or DMBA (1 μM) for 2 to 18 h. B cells were analyzed for caspase-3 activation by immunoblotting for cleaved caspase-3, by a colorimetric assay for cleavage of the caspase-3 peptide substrate DEVD, and by immunoblotting for endogenous cleaved α-fodrin, a specific caspase-3 substrate.

The appearance of the active 17-kDa caspase-3 fragment was detected in BU-11 cells 4 to 6 h after DMBA treatment (Fig. 2A). In the colorimetric assay, caspase-3-like activity increased 4 to 6 h after DMBA treatment and reached statistical significance 8 h after treatment (Fig. 2B). As expected from these results, endogenous cleavage of α-fodrin, a caspase-3 substrate, was observed after DMBA treatment (Fig. 2C, left). Similar data were obtained with primary pro-B cells (Fig. 2C, right). α-Fodrin cleavage was chosen as a marker for caspase-3 activity because its cleavage is mediated solely by caspase-3. Whereas poly(ADP-ribose) polymer-

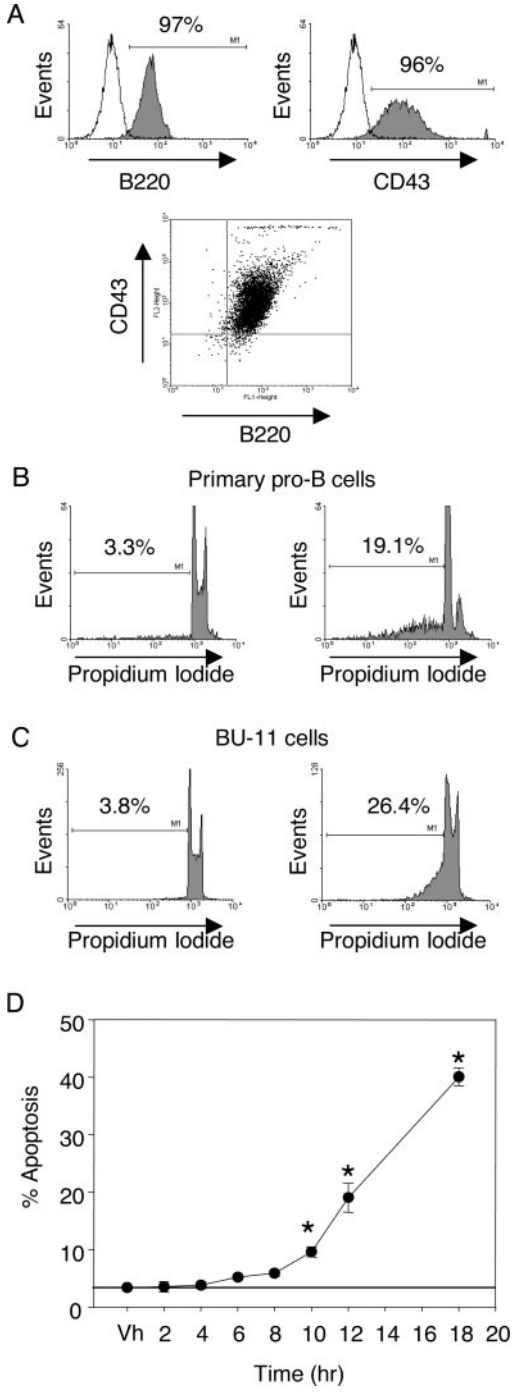

Fig. 1. DMBA induces apoptosis in primary bone marrow pro-B cells and in a nontransformed pro-/pre-B cell line (BU-11). A, bone marrow cells were cultured for 5 days with murine rIL-7 and then stained with FITC-conjugated rat anti-mouse B220 antibody and PE-conjugated rat anti-mouse CD43 antibody. Data are representative of 15 experiments. Cocultures of BMS2 with primary pro-B cells (B) or BU-11 cells (C) were treated with vehicle (0.1% acetone) or DMBA (1 μM). B cells were harvested after 12 h, propidium iodide-stained, and analyzed for apoptosis by flow cytometry. D, BU-11/BMS2 cocultures were treated with vehicle (0.1% acetone) or DMBA (1 μM). BU-11 cells were harvested after 2 to 18 h and analyzed for apoptosis by propidium iodide staining. Data are presented as means ± S.E. from at least four experiments. ⋆, statistically different from Vh ($p < 0.05$, ANOVA, Dunnett's).

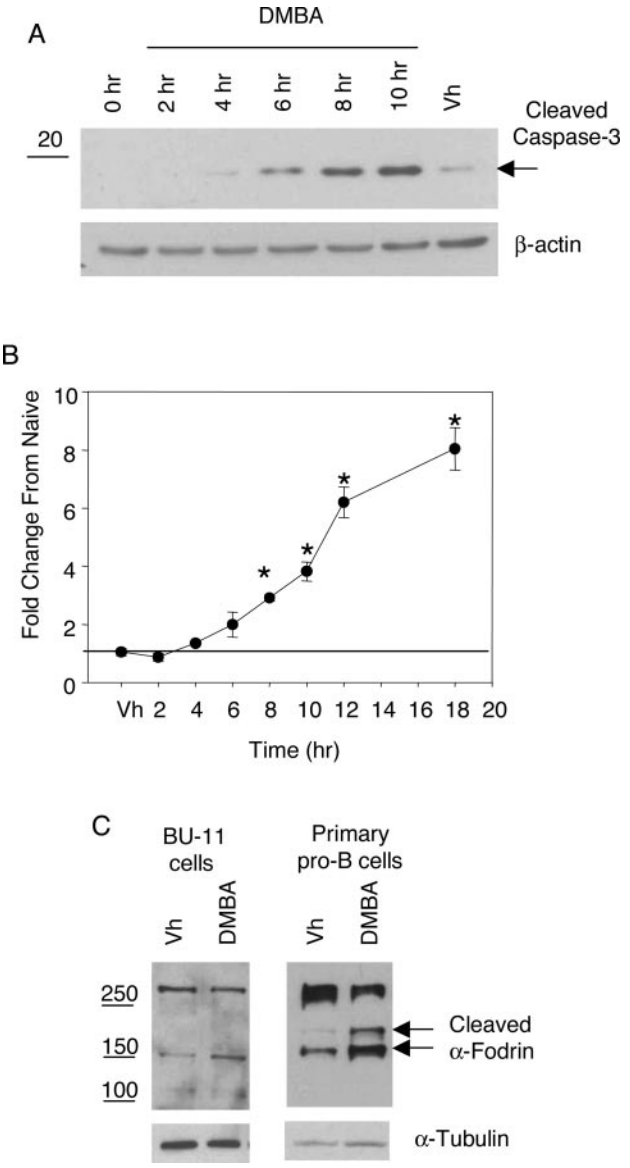

Fig. 2. Caspase-3 is activated in bone marrow B cells after DMBA treatment. BU-11/BMS2 cocultures were treated with vehicle (0.1% acetone) or DMBA (1 μM), and BU-11 cells were harvested after 2 to 18 h. A, total proteins were extracted and analyzed for active caspase-3 fragments (arrow) and for β-actin by immunoblotting. Data are representative of four experiments. B, cytosolic proteins were extracted, and caspase-3-like activity was measured using *p*-nitroaniline-conjugated DEVD substrate. Data are presented as the average fold increase in caspase-3-like activity relative to the activity in untreated cells ± S.E. from three to four experiments. The activity in untreated cells was 22.9 ± 2.8 μM *p*-nitroaniline. ⋆, statistically different from Vh ($p < 0.05$, ANOVA, Dunnett's). C, primary bone marrow pro-B cells were isolated from bone marrow from C57BL/6 mice by culturing with murine rIL-7 for 5 days. Primary pro-B cells or BU-11 cells were cocultured with BMS2 cells, treated with vehicle (0.1% acetone) or DMBA (1 μM), and harvested after 10 h. Cytoplasmic proteins were extracted and analyzed for cleaved α-fodrin and for α-tubulin by immunoblotting. Data are representative of three experiments.

ase cleavage occurs in both BU-11 cells and primary pro-B cells after DMBA treatment (data not shown), this cleavage may occur as a results of either caspase-3 or caspase-7 activation (Slee et al., 2001).

If caspase-3 activity plays a causal role in DMBA-induced B cell death, it would be predicted that a caspase-3 inhibitor, DEVD-FMK, would block apoptosis. To test this prediction, BU-11/BMS2 or primary pro-B/BMS2 cell cocultures were treated with vehicle (0.1% DMSO) or DEVD-FMK (30 μM) for 30 min before treatment with acetone (0.1%) or DMBA (1 μM). BU-11 cells were harvested 24 h later and analyzed for apoptosis by flow cytometry.

DEVD-FMK reduced the level of DMBA-induced BU-11 cell death by 80% (Fig. 3A). Likewise, DEVD-FMK suppressed DMBA-induced apoptosis 67% in primary pro-B cells (Fig. 3B). It is interesting that DEVD-FMK also seemed to suppress the spontaneous apoptosis seen in the bone marrow B cell cultures (Fig. 3, A and B). FA-FMK, a peptide frequently used as a negative control but that can suppress cathepsin B and caspases-2 and -9 at higher doses (Lopez-Hernandez et al., 2003), had no effect on DMBA-induced apoptosis in BU-11 cells (data not shown). FA-FMK was significantly toxic to the primary pro-B cells and therefore could not be used as a control with these cells. These data are consistent with a role for caspase-3 in early bone marrow B cell apoptosis induced with DMBA.

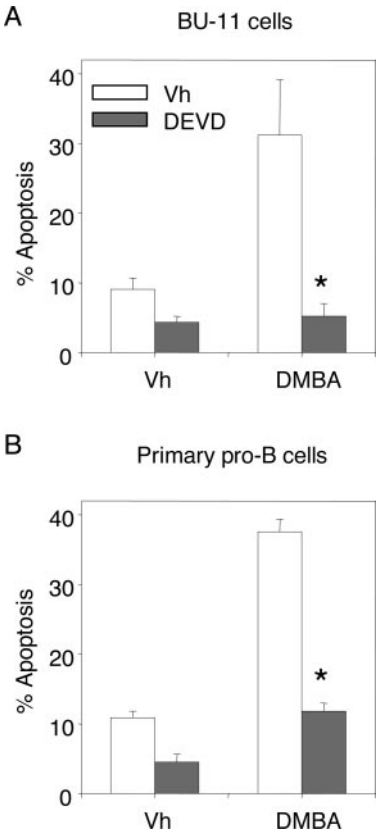

Fig. 3. DEVD-FMK blocks DMBA-induced apoptosis in BU-11 cells (A) and in primary pro-B cells (B). Primary bone marrow pro-B cells were isolated from bone marrow from C57BL/6 mice by culturing with murine rIL-7 for 5 days. Primary pro-B cells or BU-11 cells were cocultured with BMS2 cells, pretreated for 30 min with either vehicle (0.1% DMSO) or DEVD-FMK (30 μM), and treated with vehicle (0.1% acetone) or DMBA (1 μM). B cells were harvested after 24 h and analyzed for apoptosis by propidium iodide staining. Data are presented as the average percentage of cells undergoing apoptosis $\pm$ S.E. from three to five experiments. $\star$, significantly different from cultures pretreated with DMSO and then with DMBA (Student's t test, $p < 0.05$).

Caspase-8 Is Activated during DMBA-Induced Apoptosis. In general, the apoptotic process is activated by initiator caspases such as caspase-8. However, caspase-8 also can be activated by a caspase-3-dependent mechanism (Slee et al., 1999; Cowling and Downward, 2002). To determine whether caspase-8 is involved in PAH-induced apoptosis in bone marrow B cells, BU-11/BMS2 cocultures were treated with vehicle (0.1% acetone) or DMBA (1 μM) for 2 to 18 h. Caspase-8 activation in the B cells then was determined by immunoblotting for cleaved caspase-8, by a colorimetric assay for cleavage of the caspase-8 peptide substrate IETD, and by immunoblotting for truncated Bid, an endogenous caspase-8 substrate.

An increase in the formation of 40-kDa cleaved caspase-8 fragments was evident 6 to 8 h after DMBA treatment (Fig. 4A). In the colorimetric assay, an increase in caspase-8–like activity began 6 to 8 h after DMBA treatment, reached statistical significance after 10 h, and continued to increase through 20 h after treatment with DMBA (Fig. 4B). Furthermore, cleavage of Bid was evident in both BU-11 cells and primary pro-B cells 10 h after DMBA treatment (Fig. 4C). These data indicate that caspase-8 is activated after DMBA exposure.

Caspase-8 Activation in Bone Marrow B Cells Is Not Mediated by TNF-α, TNF-β, LT-β, TNFR1, TNFR2, Fas, or Death Receptor 6. The caspase-8-dependent extrinsic apoptosis pathway most commonly is activated by TNF family members through TNFR-like death receptors (DRs) (Medema et al., 1997). BU-11 cells have a functional extrinsic apoptotic response as they undergo apoptosis when exposed to FasL (data not shown). To address the possible role of death receptors and their ligands in caspase-8 activation in specific and in DMBA-induced apoptosis in bone marrow B cells in general, the contributions of TNF-α, TNF-β, and LT-β from stromal cells and of TNFR1, TNFR2, Fas, and DR6 on bone marrow B cells to PAH-induced apoptosis were investigated.

BMS2 cells were treated with vehicle (0.1% acetone) or DMBA (1 μM) for 1 to 16 h, and steady-state levels of TNF-α, TNF-β, and LT-β mRNA were determined by RT-PCR. Whereas significant levels of TNF-α, TNF-β, and LT-β mRNA were readily detected in LPS-activated primary murine dendritic cells, no signal was observed in vehicle or DMBA-treated BMS2 stromal cells (Fig. 5A). As would be expected from these results, TNF-α was detected on the surface of LPS-activated RAW 264.7 cells but not on BMS2 or primary bone marrow stromal cells (Fig. 5B). Likewise, DMBA did not induce TNF-α secretion, as measured by ELISA, in either BMS2 (Fig. 5C) or primary bone marrow stromal cells (data not shown). Furthermore, when BU-11 cells were cocultured with primary bone marrow stromal cells isolated from wild-type or TNF-$\alpha^{-/-}$ mice and treated with DMBA (1 μM) for 24 h, there were no significant differences in the ability of primary bone marrow stromal cells from wild-type or TNF-$\alpha^{-/-}$ mice to contribute to BU-11 cell apoptosis (Fig. 5D). Finally, a potential TNFR ligand autocrine feedback loop described in other systems (Kasibhatla et al., 1998; Herr et al., 2000) seemed not to be involved in DMBA-induced primary pro-B cell apoptosis because pro-B cells from TNF-$\alpha^{-/-}$ mice were as sensitive to DMBA-depen-

dent death signals as pro-B cells from wild-type controls (data not shown).

These results support the conclusion that these TNF family ligands do not play a role in DMBA-induced pro- or pro-/pre-B cell death. However, caspase-8 activation also may occur in the absence of an exogenous death receptor ligand through fas-associated death domain-dependent aggregation of TNFR family members followed by autocatalysis of caspase-8 (Aragane et al., 1998; Micheau et al., 1999; Chen and Lai, 2001). To determine the likelihood that such a mechanism contributes to apoptosis in the current system, primary pro-B cells from wild-type, TNFR1$^{-/-}$/TNFR2$^{-/-}$, or BALB/c-*lpr* mice were cocultured with BMS2 stromal cells and treated with vehicle (0.1% acetone) or DMBA (1 μM) for 24 h. B cells were stained with propidium iodide, and apoptosis was quantified by flow cytometry.

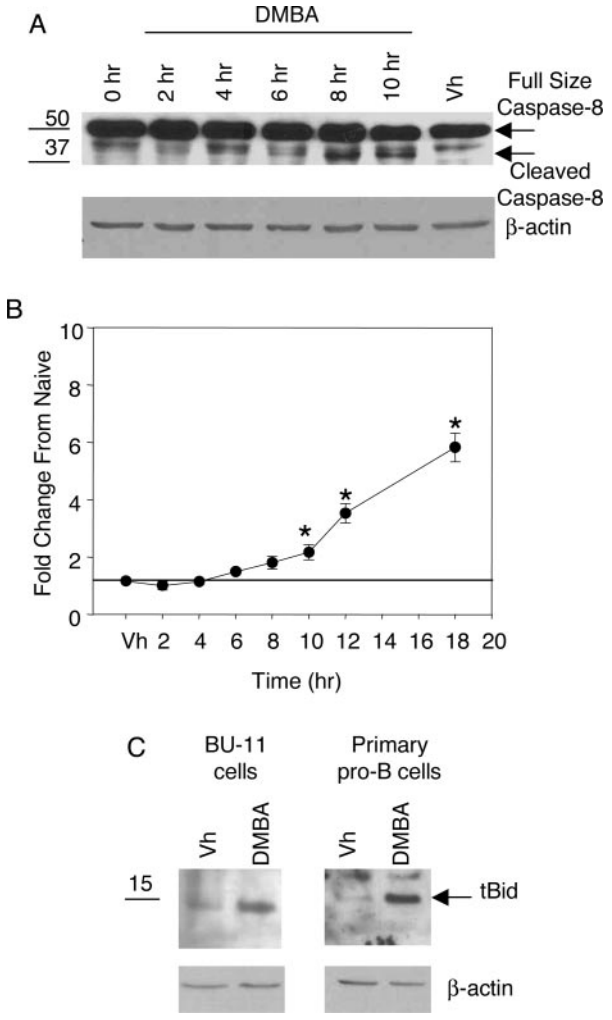

Fig. 4. Caspase-8 is activated in bone marrow B cells after DMBA treatment. BU-11/BMS2 cocultures were treated with vehicle (0.1% acetone) or DMBA (1 μM) and BU-11 cells were harvested after 2 to 18 h. A, total proteins were extracted and analyzed for caspase-8 fragments and β-actin by immunoblotting. Data are representative of four experiments. B, cytosolic proteins were extracted, and caspase-8–like activity was measured using *p*-nitroaniline-conjugated IETD substrate. Data are presented as the average fold increase in caspase-8-like activity relative to the activity in untreated cells ± S.E. from four to six experiments. The activity in untreated cells was 8.4 ± 1.3 μM *p*-nitroaniline. ⋆, statistically different from Vh ($p < 0.05$, ANOVA, Dunnett's). C, primary pro-B cells were isolated from bone marrow from C57BL/6 mice by culturing with murine rIL-7 for 5 days. Primary pro-B cells or BU-11 cells were cocultured with BMS2 cells, treated with vehicle (0.1% acetone) or DMBA (1 μM), and harvested after 10 h. Cytoplasmic proteins were extracted and analyzed for tBid and β-actin by immunoblotting. Data are representative of three experiments.

DMBA induced a significant amount of apoptosis in age-matched wild-type B6.129SF2/J and TNFR1$^{-/-}$/R2$^{-/-}$ primary pro-B cells with no significant differences between the wild-type and TNFR$^{-/-}$/R2$^{-/-}$ primary pro-B cells (Fig. 6A). Likewise, DMBA induced significant levels of apoptosis in BALB/c wild-type and BALB/c-*lpr* primary pro-B cells with no significant differences observed between the wild-type and BALB/c-*lpr* primary pro-B cells (Fig. 6B).

Analysis of a potential role for DR6 was of particular interest because this recently described TNFR-like death receptor is expressed on resting, mature B cells (Sheikh and Fornace, 2000) and because its genomic deletion results in increased mature B cell proliferation and reduced apoptosis (Schmidt et al., 2003). Furthermore, our preliminary studies indicated an up-regulation of DR6 on BU-11 cells after coculture on BMS2 cells and treatment with DMBA (data not shown). To test a possible role for DR6 in PAH-induced apoptosis, primary pro-B cells from wild-type and DR6$^{-/-}$ littermates were cocultured with BMS2 stromal cells, exposed to DMBA, and assayed for apoptosis as above.

Whereas the percentage of pro-B cells undergoing apoptosis was somewhat lower in this series of experiments than was seen previously, a significant percentage of pro-B cells from both wild-type and DR6$^{-/-}$ littermates underwent apoptosis after exposure to DMBA (Fig. 6C). However, no differences were observed between the DMBA-treated wild-type and DR6$^{-/-}$ littermate groups. In addition to the fact that caspase-3 seems to be activated before caspase-8 (Figs. 2 and 4), results here are consistent with the hypothesis that DMBA-induced apoptosis and caspase-8 activation are not initiated by death signaling through TNFR1, TNFR2, Fas, or DR6.

Caspase-8 Is Not the Initiator Caspase in DMBA-Induced Pro-/Pre-B Cell Apoptosis. Caspase-8 also may be activated by other caspases, notably caspase-6 via caspase-3 (Slee et al., 1999; Belka et al., 2000; Wieder et al., 2001; Cowling and Downward, 2002; Murphy et al., 2004). Because death receptors did not seem to be involved in caspase-8 activation, the contribution of an alternative, caspase-3–dependent pathway was investigated. BU-11/BMS2 cell cocultures were treated with vehicle (0.1% DMSO), FA-FMK (15 μM) as a putative negative control, VAD-FMK (15 μM), a pan-caspase inhibitor, or DEVD-FMK (15 μM), a caspase-3 inhibitor, 30 min before treatment with vehicle (0.1% acetone) or DMBA (1 μM). Limiting inhibitor doses (15 μM; e.g., the lowest dose of DEVD-FMK that completely suppressed apoptosis at 10 h), which are significantly lower than those used in other publications (Andjelic and Liou, 1998; Doi et al., 1999), were used to maximize inhibitor specificity. BU-11 cells were harvested after a 10-h treatment with DMBA and analyzed for apoptosis by propidium iodide staining and flow cytometry, for caspase activation by immunblotting for cleaved caspases-3 and -8, and for caspase-8 activity by immunoblotting for truncated Bid.

A significant percentage of BU-11 cells underwent apoptosis after DMBA exposure at this early time point (Fig. 7A). Apoptosis was blocked by treatment with either VAD-FMK or DEVD-FMK but not with FA-FMK (Fig. 7A).

Formation of active caspase-3 fragments was reduced sig-

nificantly in the presence of VAD-FMK (Fig. 7B), suggesting that an upstream caspase is required for caspase-3 activation. Because these peptide inhibitors block the activity and not the cleavage of caspases, DMBA-induced cleavage of caspase-3 was not expected to be and was not inhibited by the caspase-3 inhibitor DEVD-FMK (Fig. 7B). We were surprised to find that the "control" FA-FMK peptide slightly, although insignificantly, reduced caspase-3 formation without significantly reducing apoptosis (Fig. 7, A and B). This effect on caspase signaling may be caused by its ability to suppress caspases-2 and -9 or cathepsin B (Lopez-Hernandez et al., 2003).

If caspase-8 cleavage is dependent on caspase-3 activity, then it would be predicted that inhibition of caspase-3 would decrease caspase-8 cleavage and activity. Indeed, 15 μM DEVD-FMK completely blocked cleavage of caspase-8 (Fig. 7C) and formation of truncated Bid (Fig. 7D). The pan-caspase inhibitor VAD-FMK also inhibited both caspase-8 and Bid cleavage, again supporting the hypothesis that upstream caspases seem to control both caspase-3 and caspase-8 activation. Consistent with its minimal inhibition of caspase-3 cleavage (Fig. 7B) and the hypothesis that caspase-3 lies upstream of caspase-8, FA-FMK slightly although insignificantly reduced cleavage of caspase-8 (Fig. 7C) and formation of truncated Bid (Fig. 7D). These results support the hypothesis that DMBA-induced caspase-8 activation occurs as part of an amplification loop rather than as an initiating event in the apoptotic process.

Discussion

Studies performed with transformed and primary immature sIg$^+$ B cells have begun mapping apoptosis pathways invoked during clonal deletion (Wu et al., 1996a, 1998; Andjelic and Liou, 1998; Doi et al., 1999; Ruiz-Vela et al., 1999; Tian et al., 2001). Our previous studies with nontransformed, bone marrow stromal cell-dependent, primary pre-B cells and pro-/pre-B cell lines have shown that a similar but clearly distinct set of events leads to apoptosis at an earlier stage of B cell development when cultures are exposed to immunosuppressive environmental chemicals (Yamaguchi et al., 1997a; Mann et al., 1999, 2001; Ryu et al., 2003). The work presented herein was designed to extend these studies by analyzing the role of caspases in clonally nonrestricted, PAH-induced apoptosis in bone marrow B cells. These studies contribute to our understanding of when the capacity to undergo apoptosis is acquired during B cell development and how environmental chemicals, represented by DMBA, inappropriately activate apoptotic pathways, leading to immunosuppression (Dean et al., 1986; Thurmond et al., 1987).

To take advantage of mutant mouse strains defective in genes important to apoptosis and to study earlier stages in B cell development, studies were extended to primary pro-B cells expanded from bone marrow. To model events taking place in the bone marrow microenvironment, these primary pro-B cells were maintained on bone marrow stromal cells during DMBA treatment. As with primary pre-B cells

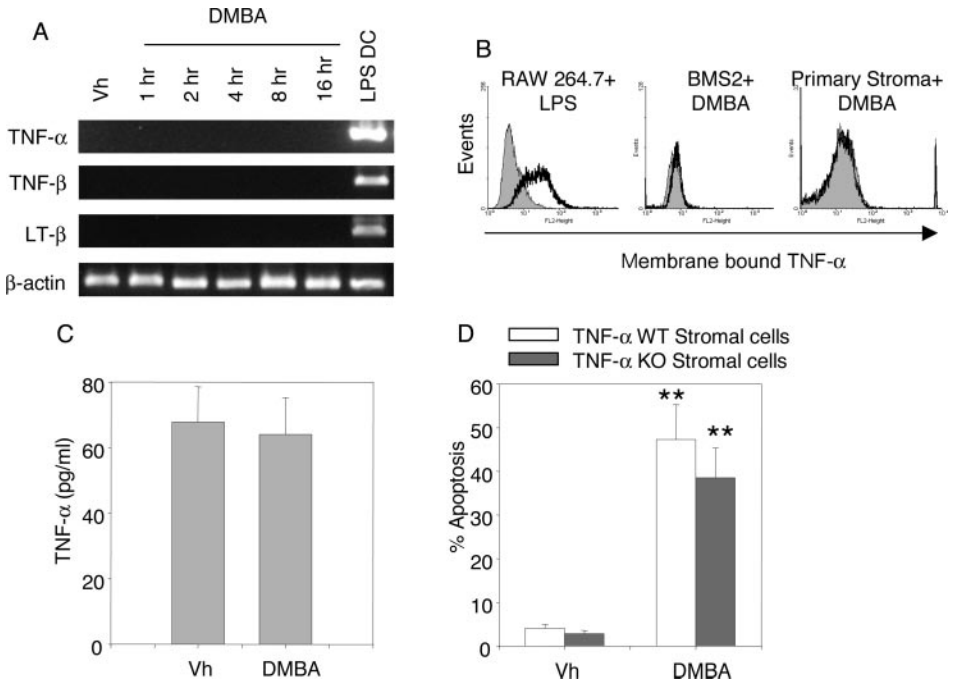

Fig. 5. TNF-α expression is not required for DMBA-induced bone marrow B cell apoptosis. Primary stromal cell cultures were prepared from mice by culturing cells expunged from bone marrow for 7 days. BMS2 cells or primary bone marrow stromal cells from C57BL/6 mice were treated with vehicle (0.1% acetone) or DMBA (1 μM) for 1 to 16 h. Stromal cells were harvested at the times indicated and were analyzed for TNF-α, TNF-β, and LT-β mRNA by RT-PCR (A) or were harvested at 16 h to assay for membrane-bound TNF-α by flow cytometry (B). Primary mouse dendritic cells treated with LPS (1 μg/ml) for 6 h or RAW 264.7 cells treated with LPS for 4 h were included as positive controls. The data are representative of three experiments. C, primary bone marrow stromal cells were treated for 16 h. Supernatants were collected and analyzed for secreted TNF-α by ELISA as described under *Materials and Methods*. D, BU-11 cells were cocultured with primary bone marrow stromal cells from either TNF-$\alpha^{-/-}$ or age-matched B6129F2/J mice and treated with vehicle (0.1% acetone) or DMBA (1 μM). BU-11 cells were harvested after 24 h and analyzed for apoptosis by Annexin V staining. The data are presented as the mean $\pm$ S.E. from three to four individual mice. $\star\star$, significantly different from Vh-treated controls ($p < 0.01$, ANOVA, Scheffé's). DMBA-induced apoptosis was not significantly different in BU-11 cells cultured with TNF-$\alpha^{+/+}$ or TNF-$\alpha^{-/-}$ primary stromal cells ($p > 0.5$, ANOVA, Scheffé's).

(Yamaguchi et al., 1997a) and the BU-11 pro-/pre-B cell line (Mann et al., 1999), primary pro-B cells grown in rIL-7 in the absence of stromal cells were completely resistant to PAH-induced apoptosis (data not shown). This result is consistent with the hypothesis that stromal cells deliver a death signal to bone marrow B cells. However, the exact nature of this signal is unknown. Attempts to identify a soluble stromal cell "death factor" revealed what is likely to be DMBA metabolite-protein complexes in the supernatant of DMBA-treated stromal cells that can act at a distance but still require stromal cells to deliver a death signal to stromal cell-adherent B cells (Allan et al., 2003). This result, together with the inability of DMBA-treated stromal cells to kill B cells separated by permeable membranes (Yamaguchi et al., 1997b), suggests that either an as-yet-unidentified cytokine-like factor or a toxic DMBA metabolite is delivered through cell-cell contact to bone marrow B cells. Although both of these possibilities are being considered, any putative death-inducing,

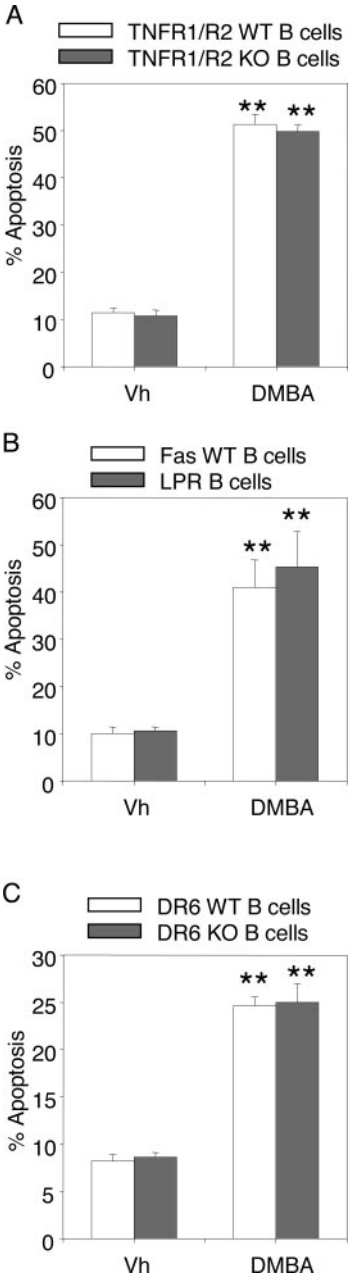

Fig. 6. TNFR1/R2, Fas, and DR6 do not contribute to DMBA-induced B cell death. Primary bone marrow pro-B cells were isolated from bone marrow aspirates from TNFR1/R2$^{-/-}$ and wild-type, age-matched B6129F2/J mice (A), BALB/c-*lpr* and wild-type, age-matched BALB/c mice (B), or from DR6$^{-/-}$ and wild-type littermates (C) by culturing with murine rIL-7 for 5 days. Primary pro-B cell/BMS2 cocultures were treated with vehicle (0.1% acetone) or DMBA (1 μM) for 24 h and analyzed for apoptosis by Annexin V staining. The data are presented as mean ± S.E. from 4 to 10 individual mice. $\star\star$, significantly different from Vh-treated controls ($p < 0.01$, ANOVA, Scheffé's). DMBA-induced apoptosis was not significantly different in TNFR1/R2$^{+/+}$ compared with TNFR1/R2$^{-/-}$ primary pro-B cells ($p > 0.4$, ANOVA, Scheffé's), in BALB/c-*lpr* compared with wild-type BALB/c primary pro-B cells ($p > 0.9$, ANOVA, Scheffé's), or in wild-type littermate compared with DR6$^{-/-}$ pro-B cells ($p > 0.9$, ANOVA, Scheffé's).

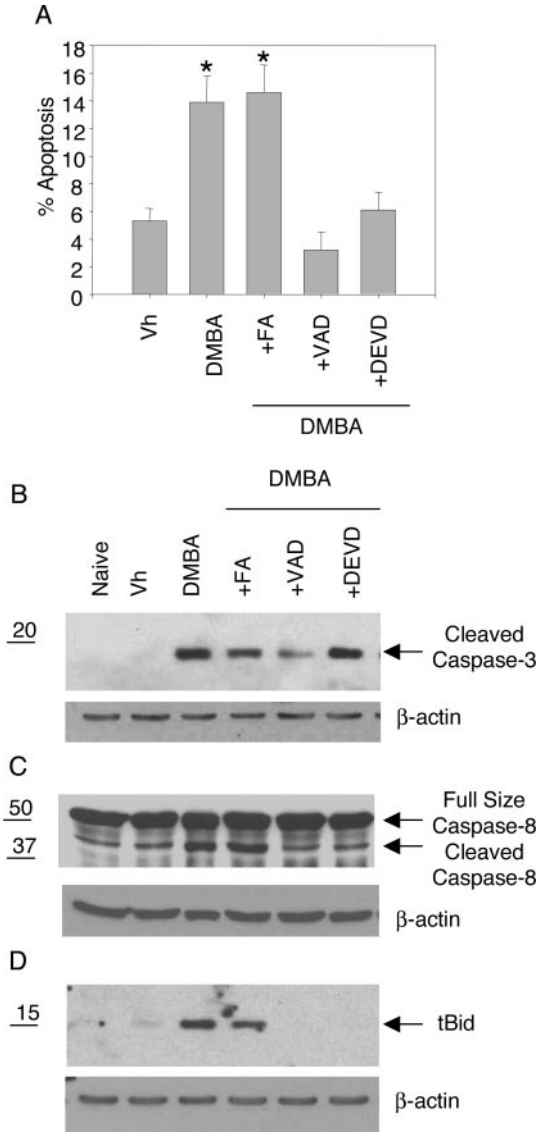

Fig. 7. Inhibition of caspase-3 activation blocks caspase-8 cleavage. BU-11/BMS2 cocultures were pretreated for 30 min with vehicle (0.1% DMSO), or FA-FMK (15 μM, negative control peptide), VAD-FMK (15 μM), or DEVD-FMK (15 μM) before treatment with vehicle (0.1%, acetone) or DMBA (1 μM) for 10 h. An aliquot was analyzed for apoptosis by propidium iodide staining (A). Data are presented as the mean ± S.E. of three to four experiments. $\star$, significantly different from Vh-treated controls ($p < 0.05$, ANOVA, Dunnett's). Total proteins were extracted from an aliquot for analysis of caspase-3 cleavage (B), caspase-8 cleavage (C), or Bid cleavage (D), and β-actin by immunoblotting. Data are representative of three to four experiments.

membrane-bound cytokine is not likely to be among the TNF family members studied herein (see below).

In addition to the demonstration of stromal cell dependence, the validity of the primary pro-B cell system was supported further by similarities in the magnitude and kinetics of DMBA-induced apoptosis compared with what had been observed in the BU-11 cell system. Similar caspase activation, shown by cleavage of endogenous caspase substrates and reduction in apoptosis by caspase inhibitors, also occurred in primary pro-B cells.

Caspases have been assigned to either the extrinsic or intrinsic pathway. However, significant crossover can occur that leads to amplification of the apoptotic process. For example, caspase-8 can participate in either pathway. Caspase-8, activated by the extrinsic pathway through a death receptor, may directly activate caspase-3 or may activate Bid, leading to activation of the intrinsic mitochondrial pathway (Gross et al., 1999). Furthermore, once the intrinsic pathway has been activated, caspase-8 may be activated by a caspase-3–dependent mechanism through caspase-6 (Slee et al., 1999; Cowling and Downward, 2002; Murphy et al., 2004). The current studies, therefore, were centered on the possible activation of caspase-8 and the role of caspase-3 and/or death receptors in that activation.

Caspase-8 was activated within 6 to 8 h of DMBA exposure as assessed by 1) the appearance of a 40-kDa cleaved caspase-8 fragment; 2) cleavage of the caspase-8 peptide substrate IETD; and 3) cleavage of Bid, an endogenous caspase-8 substrate. It is interesting that the activation of caspase-3, as assessed by similar criteria (i.e., appearance of the active caspase-3 fragment, cleavage of a peptide substrate, and endogenous α-fodrin cleavage), preceded that of caspase-8. These results suggest that caspase-8 activation occurs downstream of caspase-3 activation, presumably via the intrinsic pathway. Our observations are reminiscent of those obtained with a diverse group of toxicants, including ionizing radiation (Belka et al., 2000), chemotherapeutic agents (Wieder et al., 2001), and celecoxib (Jendrossek et al., 2003), all of which induce apoptosis through a mitochondria-dependent process. As would be predicted if mitochondrial activation preceded caspase-8 activation, inhibition of caspase-3 with DEVD-FMK blocked caspase-8 activation and apoptosis. Inhibition of Bid cleavage with the caspase-3 inhibitor further suggests that caspase-8 may amplify a mitochondria-initiated intrinsic pathway through the formation of tBid (Gross et al., 1999).

These results may be contrasted with those obtained with a transformed, stromal cell-independent pre-B cell line, 70Z3 (Page et al., 2002). In studies with these transformed pre-B cells, DMBA induced a minimal and transient level of caspase-8 activity that preceded the relatively late (approximately 15–20 h) induction of caspase-3 activity. Other characteristics, including a longer period of time until apoptosis is evident (e.g., 15–20 h), a smaller percentage of cells that undergo apoptosis (approximately 25% at 24 h), and the use of higher DMBA doses (e.g., 3 μM) to induce apoptosis, suggest that the transformed cells are more resistant to apoptosis signals in general and that they may activate alternative pathways when exposed to PAH in specific.

Studies in several systems suggest how the extrinsic mitochondrial pathway activates caspase-8 through caspase-3. Most studies implicate caspase-6 as an intermediary. In cell-free studies, caspases-6 and -8 were activated after cytochrome *c* treatment (Slee et al., 1999). Expression of a catalytically inactive caspase-6 mutant prevented caspase-8 activation in COS-7 cells in response to serum starvation (Cowling and Downward, 2002). Caspase-8 activated by caspase-6 is catalytically competent, despite the lack of a dimerization stimulus (Murphy et al., 2004). Preliminary data obtained in the present system indicate that DMBA induces the release of cytochrome *c* from mitochondria followed by caspase-6 activation and cleavage of its endogenous substrate lamin (data not shown). Studies are underway to determine whether this putative caspase-6 activation is causally linked to caspase-8 activity.

The likely contribution of caspase-3 to caspase-8 activation in and of itself does not rule out a role for TNFR family members in DMBA-induced apoptosis. Indeed, in vivo studies with DMBA suggest a role for TNFRs in the elimination of at least some hematopoietic cell types in the bone marrow (Page et al., 2002). In the absence of specific analysis of the fate of bone marrow B cells in particular (Page et al., 2002), it is difficult to tell whether TNFRs were in fact involved in DMBA-induced B cell death in vivo. However, several approaches described herein failed to implicate TNFR family members in early B cell apoptosis: 1) TNF-α, TNF-β, and LT-β mRNA were not detected by RT-PCR after DMBA treatment; 2) TNF-α, as assessed by ELISA or surface expression, was not induced in DMBA-treated primary bone marrow stromal cells or in BMS2 cells; 3) inhibitory TNF-Ig and Fas-Ig failed to block apoptosis (data not shown); 4) bone marrow stromal cells from TNF-$\alpha^{-/-}$ mice were as effective at inducing apoptosis as cells from wild-type mice; and 5) pro-B cells from TNFR1$^{-/-}$/TNFR2$^{-/-}$ double knockout mice, Fas-defective BALB/c-*lpr* mice, or DR6$^{-/-}$ mice were as susceptible to DMBA-induced apoptosis as wild-type cells. Although these studies do not rule out the contribution of other as-yet-uncharacterized TNFR family members, they argue that at least the well-described death receptors do not contribute to DMBA-induced apoptosis under these conditions. The apparent disparity between the current studies and some in vivo studies (Page et al., 2002) could reflect the lack of information on the effects of DMBA treatment on B cell subsets in vivo or on a systemic stress response in vivo secondary to DMBA toxicity as measured relatively late (48 h) after DMBA exposure.

In summary, the studies presented herein strongly suggest that PAH-induced apoptosis is mediated primarily by the activation of elements of the intrinsic pathway and not by death receptors. Likewise, the apoptotic pathway activated during B cell clonal deletion involves mitochondrial activation, followed by activation of caspases-3 and -9, poly(ADP-ribose) polymerase cleavage, and DNA fragmentation (Doi et al., 1999; Ruiz-Vela et al., 1999). Caspase-8 activation after DMBA treatment seems to result from the activation of caspase-3, leading to the cleavage of Bid and the activation of a positive feedback loop. Because a pan-caspase inhibitor blocked activation of both caspase-3 and caspase-8, it is postulated that an initiator caspase(s) upstream of caspase-3 is required for DMBA-induced bone marrow B cell apoptosis. Finally, these data and preliminary data indicating changes in cytochrome *c* release suggest that the apoptotic pathway induced by DMBA shares key elements with the mitochondria-dependent pathway activated during clonal deletion.

Therefore, it seems as though immunosuppressive environmental chemicals activate some but not all of the elements of the apoptosis pathway that signal clonal deletion.

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Address correspondence to: Dr. David H Sherr, Boston University School of Public Health, Dept. of Environmental Health, 715 Albany Street, R-408, Boston, MA 02118. E-mail: dsherr@bu.edu