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# The Role of NF-κB as a Survival Factor in Environmental Chemical-Induced Pre-B Cell Apoptosis

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## ABSTRACT

Polycyclic aromatic hydrocarbons (PAH) are ubiquitous environmental chemicals that suppress the immune system at multiple levels, including at the level of B cell development in the bone marrow microenvironment. Specifically, PAH induce preB cell apoptosis in primary bone marrow cultures and in cocultures of an early preB cell line (BU-11) and a bone marrow stromal cell line (BMS2). Previous studies focused on the molecular mechanisms through which PAH induce stromal cells to deliver an apoptosis signal to adjacent preB cells. Apoptosis signaling within the preB cell itself was not investigated. Here, the role of NF-κB, a lymphocyte survival factor, in PAH-induced preB cell apoptosis was assessed. Analysis of DNA-binding proteins extracted from the nuclei of untreated BU-11 cells indicated DNA-binding complexes comprising NF-κB subunits p50, c-Rel, and/or Rel A. NF-κB down-regulation with previously described inhibitors induced BU-11 cell apoptosis, demonstrating that the default apoptosis pathway blocked by NF-κB is functional at this early stage in B cell development. Similarly, exposure of BU-11/BMS2 cocultures to 7,12-dimethylbenz[*a*]anthracene (DMBA), a prototypic PAH, down-regulated nuclear Rel A and c-Rel before overt apoptosis. Finally, ectopic expression of Rel A or c-Rel rescued BU-11 cells from DMBA-induced apoptosis. These results extend previous observations by demonstrating that 1) NF-κB is a survival factor at an earlier stage of B cell development than previously appreciated and 2) NF-κB down-regulation is likely to be part of the molecular mechanism resulting in PAH-induced preB cell apoptosis. These results suggest nonclonally restricted, PAH-mediated suppression of B lymphopoiesis.

Polycyclic aromatic hydrocarbons (PAH), relatively common environmental contaminants, are immunotoxic (Day et al., 1990; Davis et al., 1991; Ladics et al., 1992; Temple et al., 1993; Szczeklik et al., 1994; Davilla et al., 1996). In animal models and/or human lymphocyte cultures, PAH decrease resistance to infectious agents and transplantable tumors, impair B and T lymphocyte proliferation, inhibit B cell antibody responses, suppress cytokine production, and decrease bone marrow cellularity. However, the molecular mechanisms through which these outcomes are effected have not been adequately described. To dissect the intracellular signals activated by PAH and resulting in adverse effects on the immune system, we have evaluated the effects of low PAH doses on early preB cell growth with in vitro models of B cell development in the bone marrow microenvironment (Hinoshita et al., 1992; Yamaguchi et al., 1997a,b; Mann et al., 1999; Near et al., 1999). In primary bone marrow cultures and in a coculture system consisting of an early ($CD43^+$) preB cell line (BU-11) and a cloned bone marrow stromal cell line on which preB cells depend for growth (BMS2), it was shown that low doses ($\geq 10^{-8}$ M) of prototypic PAH (benzo[*a*]pyrene and 7,12-dimethylbenz[*a*]anthracene) rapidly induce preB cell apoptosis. In evaluating the role of stromal cells in PAH-induced preB cell apoptosis, it was shown that 1) stromal cells are required for preB cell apoptosis (Yamaguchi et al., 1997a,b), 2) PAH-treated stromal cells or liver parenchymal cells deliver a death signal to adjacent preB cells (Mann et al., 1999; Near et al., 1999), 3) induction of this death signal is, at low PAH doses, dependent on activation of the aryl hydrocarbon receptor/transcription factor within stromal cells (Mann et al., 1999; Near et al., 1999), and 4) PAH metabolism within stromal cells likely plays a role in the generation of the apoptosis signal (Mann et al., 1999). Recent results in a related system in which apoptosis of a stromal cell-independent preB cell line (70Z/3) was induced

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**ABBREVIATIONS:** PAH, polycyclic aromatic hydrocarbon(s); DMBA, 7,12-dimethylbenz[*a*]anthracene; EMSA, electromobility gel shift assay(s); NF-κB, nuclear factor κB; IκB, inhibitor κB; MG-132, Z-Leu-Leu-Leu-CHO; PDTC, pyrrolidinedithiocarbamate; PI, propidium iodide; sIg, surface Ig; TPCK, *N*-tosyl-L-phenylalanine chloromethyl ketone; PBS, phosphate-buffered saline; BrdU, bromodeoxyuridine; PAGE, polyacrylamide gel electrophoresis; IL, interleukin.

with DMBA are consistent with the latter conclusion (Heidel et al., 1998, 1999).

These studies were extended herein to evaluate the molecular signals activated within early preB cells that result in their demise following delivery of the stromal cell-derived, PAH-induced apoptosis signal. In particular, the role of NF-κB, a survival factor in several mature cell types (Antwerp et al., 1996; Beg and Baltimore, 1996; Wang et al., 1996; Wu et al., 1996a,b; Karin, 1998), was investigated. Our previous studies indicated that cross-linking surface Ig receptors on cells of an immature B cell line, WEHI-231, resulted in NF-κB down-regulation and apoptosis induction (Wu et al., 1996a,b). These results implicate NF-κB modulation in B cell clonal deletion induced by high-affinity Ig receptor interactions with autoantigens. Although clonal deletion begins when immature B cells acquire Ig heavy-chain and surrogate light-chain receptors, it is formally possible that the NF-κB-dependent apoptosis pathway is intact at an earlier stage of B cell development. Consequently, PAH-induced preB cell apoptosis could be mediated by a clonally nonrestricted down-regulation of NF-κB. This hypothesis was tested by 1) determining whether an early (i.e., $CD43^+$) stromal cell-dependent preB cell line constitutively expresses nuclear, DNA-binding NF-κB, 2) testing if NF-κB down-regulation with specific inhibitors induces BU-11 cell apoptosis, 3) analyzing NF-κB-DNA binding following exposure of BU-11/BMS2 cell cultures to DMBA, and 4) attempting to rescue BU-11 cells from DMBA-induced apoptosis by transfection with the NF-κB subunits c-Rel or Rel A (p65). The data are consistent with NF-κB expression at an earlier point in B cell development than previously demonstrated and with a critical role for NF-κB regulation in PAH-induced early preB cell apoptosis.

## Materials and Methods

**Cell Culture and Treatments.** The stromal cell-independent late preB line 70Z/3 and the $sIg^+$ WEHI-231 line were maintained in RPMI 1640 media with 10% fetal bovine serum, penicillin/streptomycin, L-glutamine, and 2-mercaptoethanol at 37°C in a humidified 10% $CO_2$ chamber. The stromal cell-dependent C57BL/6-derived BU-11 cell line has been previously characterized (Near et al., 1999; Mann et al., 1999; Yamaguchi et al., 1997a,b). BU-11 cells express both CD43 and B220 and do not contain rearranged Ig heavy chain genes. Therefore they represent B cells at the transition point between the pro- and early preB cell stages (Hardy et al., 1991). For convenience, it is referred to as an early preB cell line. BMS2 is a culture dish-adherent, cloned bone marrow stromal cell line which supports preB cell growth (Pietrangeli et al., 1988). Cultures of BU-11 cells maintained on BMS2 cell monolayers were grown in RPMI 1640 media with 5% fetal bovine serum, penicillin/streptomycin, L-glutamine, and 2-mercaptoethanol. Cocultures were treated with vehicle (acetone, final concentration of 0.1%), DMBA (Sigma/BRL Chemical Co., St. Louis, MO), 125 to 12.5 μM *N*-tosyl-L-phenylalanine chloromethyl ketone (TPCK; Sigma/BRL), 50 to 0.5 μM pyrrolidinedithiocarbamate (PDTC; Sigma), 10 to 2.5 μM lactacystin (Alexis Biochemicals, San Diego, CA), or 0.1 to 0.4 μM Z-Leu-Leu-Leu-CHO (MG-132; Biomol Inc., Plymouth Meeting, PA). BU-11 cells were harvested 12 to 24 h later, and the percentage of cells undergoing apoptosis was quantitated by propidium iodide staining and flow cytometry.

**Apoptosis Quantitation.** BU-11 cells were harvested by gentle pipetting and washed once with cold PBS containing 5% fetal bovine serum and 1% sodium azide. Cells were resuspended in 0.5 ml of hypotonic buffer containing 50 μg/ml propidium iodide (PI; Sigma), 1% sodium citrate, and 0.1% Triton X-100 and analyzed in a Becton Dickinson (San Jose, CA) FACScan flow cytometer. Cells undergoing apoptosis were weaker in PI fluorescence than those in the typical $G_0/G_1$ cell cycle (Yamaguchi et al., 1997a,b; Mann et al., 1999; Near et al., 1999). In all experiments, a decrease in PI fluorescence correlated with morphologic changes characteristic of apoptosis, i.e., a decrease in cell size as detected by a decrease in forward light scatter and by DNA fragmentation as visualized in agarose gels (data not shown).

**Nuclear Protein Extraction.** BU-11 cells were gently washed off bone marrow stromal cell monolayers and washed in PBS. $CD45^+$ BU-11 cells constituted more than 95% of these populations (data not shown). BU-11, WEHI-231, or 70Z/3 cells were resuspended in $P_{10}$EG lysis buffer containing 10 mM sodium phosphate, 0.75 mM EDTA, 10% glycerol, and 0.03% Triton X-100. Plasma membranes were removed by gentle pipetting, and the quality of nuclear isolation was determined by visual inspection. Nuclei were centrifuged for 5 min at 1100*g* at 4°C. Supernatants were removed and pellets were resuspended in cold nuclear lysis buffer containing 0.1% Triton X-100, 150 mM NaCl, 25 mM Tris-HCl, 1 μg/ml aprotinin, 10 μg/ml leupeptin, 50 mM NaF, 1 mM EDTA, 1 mM sodium orthovanadate, and 1 mM phenylmethylsulfonyl fluoride. Samples were incubated for 60 min on ice and subsequently centrifuged for 15 min at 13,000*g* at 4°C. The supernatant containing nuclear protein was removed and stored at −80°C. The absence of contaminating cytosolic proteins was confirmed by western immunoblotting using antibody specific for a cytosolic IκB protein.

**In Vitro UV Cross-Linking, Immunoprecipitation, and Analysis of DNA-NF-κB Subunit Complexes.** Analysis of DNA-NF-κB complexes was performed essentially as we previously described (Ballard et al., 1990; Molitor et al., 1990; Doerre et al., 1993). Oligonucleotide probes and primers (Life Technologies, Gaithesburg, MD) were annealed by mixing probe and primer in 2× annealing buffer (100 mM NaCl, 20 mM Tris, pH 7.5, and 100 mM EDTA), heating to 85°C for 3 min, cooling in a 500-ml beaker of 85°C water for 2 h, and incubating at 4°C overnight. The annealed probe was then stored at −20°C. Probe and primer sequences are as follows: κBPD 5′-CAA CGG CAG GGG AAT TCC CCT CTC CTT-3′; 3′-G GGA GAG GAA-5′.

For labeling with [α-$^{32}$P]dCTP and bromodeoxyuridine (BrdU), 5 pmol of annealed probe was added to a mixture of 5× oligonucleotide-labeling buffer-UV (100 mM Hepes, pH 6.6; 10 mM $MgCl_2$; 1 mM dithiothreitol; 400 μg/μl bovine serum albumin; 500 μM each dATP, dGTP, and dTTP; and 500 μM BrdU), 2 U of Klenow enzyme (Roche Molecular Biochemicals, Indianapolis, IN), and 50 μCi [α-$^{32}$P]dCTP, and the mixture was incubated at 14°C for 4 h. An additional 1 μl of unlabeled dCTP was then added as a "cold chase", and the mixture was incubated at room temperature for 30 min. Labeled probe was separated from free label using a Centri·Spin$^{-20}$ column (Princeton Separations, Adelphia, NJ). Counts per minute were determined and the probe was diluted to 100,000 cpm/μl and stored at 4°C.

To cross-link probe and NF-κB subunits, nuclear extracts (10–40 μg) were incubated in a reaction mixture of 10× buffer (200 mM Hepes, pH 7.9, 10 mM EDTA, and 50% glycerol), 1 μl of random hexamers, 1 μl of poly-dIdC, 5 μg of bovine serum albumin, and 0.1 mM dithiothreitol for 5 min at room temperature. Labeled probe (200,000–500,000 cpm) was added to the reaction mixture, which then was incubated for 15 min at room temperature. The mixture was UV irradiated for 15 min. SDS-PAGE sample buffer was added and the samples were incubated at 95°C for 5 min, loaded into a 10% SDS polyacrylamide gel, and electrophoresed overnight at 46 V. The gel was fixed for 20 min in 5% methanol/5% acetic acid and then for 40 min in 50% methanol/10% acetic acid. The gel was dried for 1 to 2 h and exposed to photographic film at −80°C. For experiments testing the specificity of protein-DNA complexing, 20-fold excess "cold" wild-type oligonucleotide or mutant oligonucleotide (5′-CAA CGG CAG **ATC TA**T CTC CCT CTC CTT-3′) was added along with the [$^{32}$P]dCTP/BrdU-labeled wild-type oligonucleotide.

For immunoprecipitation of cross-linked probe-NF-κB complexes,

probe-nuclear protein mixtures were processed as described (Ballard et al., 1990; Molitor et al., 1990; Doerre et al., 1993). After cross-linking but before addition of SDS-PAGE sample buffer, radioimmune precipitation buffer containing phenylmethylsulfonyl fluoride and protease inhibitors (aprotinin, leupeptin, and sodium orthovanadate) was added to a volume of 200 $\mu$l. Rel A-, c-Rel-, p52-, or p50-specific antibody (1–2 $\mu$l; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) was added, and the solution was incubated on ice for 30 to 60 min. Complexes then were absorbed onto protein A-Sepharose (Sigma) by the addition of 25 $\mu$l of protein A-Sepharose (50% slurry in water) to the mixture. The mixture was rocked at 4°C for 1 h, centrifuged at 7500*g*, and the supernatant removed. After washing in radioimmune precipitation buffer, the pellet was resuspended in 25 $\mu$l of SDS-PAGE sample buffer and incubated at 95°C for 5 min. Those samples not immunoprecipitated were combined with SDS-PAGE sample buffer, heated to 95°C for 5 min, and stored at −20°C until immunoprecipitates were ready for loading. All samples were then analyzed by SDS-PAGE as above.

**Stable Transfections.** BU-11 cells ($5 \times 10^7$) were washed once in cold PBS, centrifuged at 180*g* for 5 min, resuspended in 0.8 ml of cold PBS, and incubated on ice for 10 min. Plasmid DNA was added to the cells at the following concentrations: 10 $\mu$g of *pRC-neo* alone, 20 $\mu$g of *RSV-p65* (*Rel A*) and 10 $\mu$g of *pRC-neo,* or 20 $\mu$g of *pEVRF2-cRel* and 10 $\mu$g of *pRC-neo*. Plasmid DNA was kindly provided by Dr. Gail Sonenshein (Boston University, Boston, MA). After another 10-min incubation on ice, cells were electroporated at 180 V/960 $\mu$F. Then cells were placed in prewarmed Dulbecco's modified Eagle's media supplemented with 5% fetal bovine serum, penicillin/streptomycin, L-glutamine, 2-mercaptoethanol, and 2 ng/ml recombinant murine rIL-7 (Peprotech, Rocky Hill, NJ) and cultured for 48 h at 37°C. BU-11 cells were then transferred to *pRC-neo*-transfected (i.e., G418/geneticin-resistant) BMS2 monolayers in 48-well culture plates, and 0.5 mg/ml geneticin (Life Technologies) was added. Every 2 days, half of the culture medium was removed and replaced with fresh medium supplemented with 1 mg/ml geneticin. Cells were cultured in this selection media for 2 weeks. Positively selected lines were expanded and Rel A or c-Rel expression assessed by immunoblotting.

**Statistics.** Results were compared using the paired Student's *t* test (Fig. 3B) or by analysis of variance in combination with Dunnett's multiple comparisons test when several experimental samples were compared with a single control group (Table 1 and Fig. 5).

## Results

**Early PreB Cells Express Nuclear NF-κB.** Expression of activated NF-κB subunits Rel A and c-Rel has been demonstrated in mature T and B lymphocytes (Molitor et al., 1990; Bryan et al., 1994; Miyamoto et al., 1994). Most studies attempting to place NF-κB activation at an earlier stage of B cell development have used Abelson virus-transformed preB cell lines in which the *abl* oncogene may have influenced NF-κB activity (Klug et al., 1994). Indeed, the immortalization of cells is frequently accompanied by, and dependent upon, increased NF-κB activity (Sovak et al., 1997). In one study, late preB cells maintained with stromal cells and IL-7 were shown to express modest levels of active, i.e., DNA-binding, NF-κB subunits Rel A, c-Rel, and p50 (Kistler et al., 1998). To determine whether NF-κB is constitutively active at an earlier stage in B cell development, i.e., at the $CD43^+$ "early" preB cell stage (Hardy et al., 1991), the identities of DNA-binding, nuclear NF-κB subunits were evaluated in BU-11 cells.

BU-11 cells were isolated from BU-11/BMS2 cocultures, and nuclear proteins were extracted. Nuclear proteins were also extracted from WEHI-231 cells as a positive control. Proteins were incubated with $^{32}$P- and BrdU-labeled NF-κB probe, and those capable of binding the palindromic NF-κB site were identified by SDS-PAGE under reducing conditions following UV cross-linking. Nuclear extracts from both WEHI-231 and BU-11 cells consistently formed three bands of protein-DNA complexes (Fig. 1, bands A–C), although the intensity of the largest of these complexes (band A) varied from experiment to experiment. Formation of all three bands was inhibited by addition of 20X cold wild-type but not mutated NF-κB oligonucleotide (Fig. 1, A and B), demonstrating specific protein binding to the NF-κB element. This inhibition of band A formation in BU-11 cell extracts can more easily be seen in Fig. 1B, for which films were overexposed to emphasize band A. Incomplete inhibition of band formation with cold wild-type probe in some experiments (e.g., Fig. 1B, BU-11 cell extract) most likely reflects the thermodynamics of using unlabeled oligonucleotide to block the irreversible interaction between the NF-κB subunit(s) and $^{32}$P- and BrdU-labeled oligonucleotide after UV cross-linking (Molitor et al., 1990; Doerre et al., 1993). These results are consistent with those previously reported indicating NF-κB-DNA complexes of approximately 50 to 55, 75 to 80, and 125 to 135 kDa following cross-linking of nuclear T cell extracts with $^{32}$P- and BrdU-labeled NF-κB probes (Molitor et al., 1990).

A comparison of DNA-protein complexes formed by extracts from BU-11 cells, from the stromal cell-independent, preB cell line 70Z/3 and from the $sIg^+$ immature B cell line WEHI-231 indicated three bands (Fig. 2, bands A–C) of similar molecular masses (50–55, 75–80, and 125–135 kDa) in all 3 lines. 70Z/3 cell extracts expressed the lowest and WEHI-231 cell extracts expressed the highest levels of protein-DNA complexes represented by these bands (Fig. 2, lanes 1–3).

Given the documented expression of nuclear, DNA-binding complexes containing p50, Rel A, and c-Rel in WEHI-231 cells, late preB cells, and mature T cells (Molitor et al., 1990; Wu et al., 1996; Kistler et al., 1998), and the apparent molecular masses of complexes visualized as bands A, B, and C (NF-κB-DNA complexes tend to run more slowly than the molecular masses of individual NF-κB subunits would predict) (Ballard et al., 1990; Molitor et al., 1990; Doerre et al., 1993), we postulated that band A represents complexes of probe and two NF-κB subunits, band B represents complexes of probe plus Rel A (65 kDa) and/or c-Rel (67 kDa), and band

TABLE 1

NF-κB inhibitors induce apoptosis in BU-11 cells

BU-11 cells were cultured on BMS2 cell monolayers and treated with titered doses of NF-κB inhibitors as indicated. Twenty four hours later, BU-11 cells were harvested and stained with PI, and the percentage of cells undergoing apoptosis was quantitated by flow cytometry. Data from three to five experiments are presented as the average percentage of apoptosis ± S.E. BMS2 cells were not affected by these treatments (data not shown).

| Treatment | Percentage of Apoptotic Pre-B Cells |
|---|---|
| Vehicle | 12 ± 1 |
| 125 $\mu$M TPCK | 64 ± 8* |
| 62.5 $\mu$M TPCK | 68 ± 6* |
| 12.5 $\mu$M TPCK | 20 ± 2* |
| 50 $\mu$M PDTC | 85 ± 2* |
| 5.0 $\mu$M PDTC | 65 ± 6* |
| 0.5 $\mu$M PDTC | 25 ± 4* |
| 10 $\mu$M Lactacystin | 86 ± 3* |
| 5.0 $\mu$M Lactacystin | 52 ± 7* |
| 2.5 $\mu$M Lactacystin | 7 ± 1 |
| 0.4 $\mu$M MG-132 | 58 ± 1* |
| 0.2 $\mu$M MG-132 | 19 ± 1* |
| 0.1 $\mu$M MG-132 | 9 ± 1 |

* Significant level of apoptosis relative to vehicle-treated groups, $p < 0.01$ (analysis of variance with Dunnett's multiple comparisons test).

C represents complexes of probe and p50 and/or p52. To test this hypothesis, protein-DNA complexes were immunoprecipitated with p50-, p52-, Rel A- or c-Rel-specific antibodies and resolved by SDS-PAGE.

Immunoprecipitation with p50-specific antibody resulted in the formation of a single band comigrating with band C (Fig. 2, lane 4). Anti-p52 antibody did not immunoprecipitate oligonucleotide probe-containing complexes (lane 5). Immunoprecipitation with Rel A- or c-Rel-specific antibodies resulted in the formation of bands migrating at approximately 75 (lane 6) and 80 kDa (lane 7), respectively, and comigrating with band B. Co-immunoprecipitation of the 75- and 80-kDa bands with a band of approximately 50 to 55 kDa (band C) strongly suggests constitutive association of Rel A and c-Rel subunits with p50. Consistent with previous studies (Ballard et al., 1990; Molitor et al., 1990; Doerre et al., 1993), these antibodies were unable to precipitate complexes comigrating with band A. However, since band A formation was readily inhibited with cold wild-type but not mutant NF-κB probe (Fig. 1, A and B), the failure to precipitate a 125- to 135-kDa complex likely reflects stearic hindrance in a complex of two NF-κB subunits and NF-κB probe. From these results it was concluded that band C represents p50-containing complexes, band B represents both Rel A- and c-Rel-containing complexes, and band A likely represents complexes of two NF-κB subunits. Collectively, these results demonstrate constitutive NF-κB activity at the early preB cell stage of development.

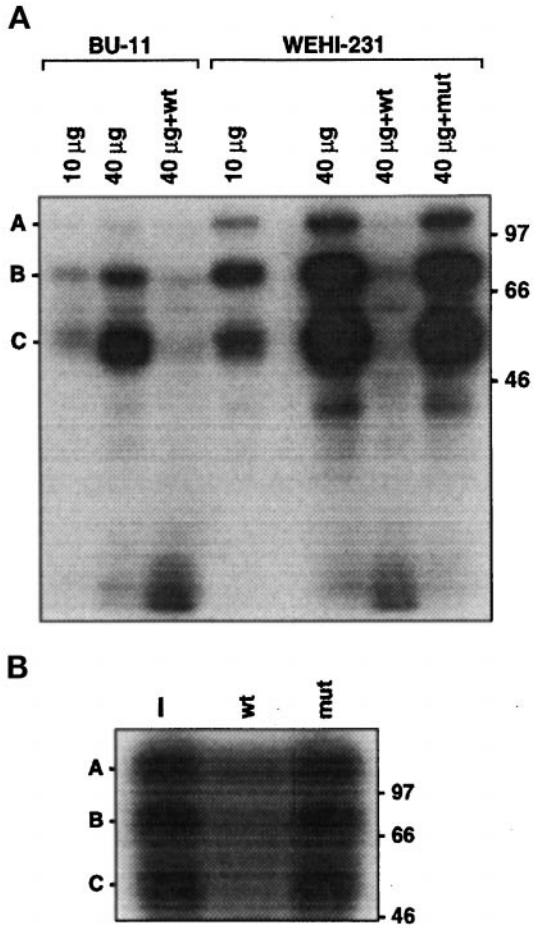


**Fig. 1.** BU-11 preB lymphocytes express nuclear, DNA-binding NF-κB. Nuclear protein extracts from BU-11 (A and B) and WEHI-231 (A) cells were incubated with $^{32}$P- and BrdU-labeled NF-κB oligonucleotide probe. Proteins and probe were cross-linked with UV light, and the resulting covalently linked complexes were resolved by loading 10 to 40 μg into and electrophoresing the complexes through SDS-polyacrylamide gels followed by autoradiography. Where indicated, 20-fold excess unlabeled wild-type (wt) or mutant (mut) NF-κB oligonucleotide probes were added along with labeled probe. A and B, data from two representative experiments are presented. Bands A, B, and C represent complexes with apparent molecular masses of 125 to 135, 75 to 80, and 50 to 55 kDa, respectively. The data in Fig. 1B were generated with BU-11 cell extracts. The autoradiograph in Fig. 1B was overexposed to highlight band A.

**NF-κB Down-regulation Induces BU-11 Cell Death.** Because BU-11 cells constitutively express DNA-binding, nuclear NF-κB subunits, it was hypothesized that down-regulation of NF-κB activity would activate a default apoptosis pathway in BU-11 cells. Therefore, NF-κB inhibitors were added to BU-11/BMS2 cultures, and the percentage of apoptotic BU-11 cells was quantitated 24 h later. Four NF-κB inhibitors were used. TPCK is a serine-threonine protease inhibitor that blocks the normally rapid turnover of IκB-α and retains NF-κB in the cytoplasm (Miyamoto et al., 1994). PDTC is a metal chelator and antioxidant that inhibits NF-κB activity by blocking NF-κB activation or inhibiting NF-κB/DNA binding (Schreck et al., 1992). Lactacystin and MG-132 are specific inhibitors of the 20S proteasome responsible for degradation of the NF-κB inhibitor IκB (Dick et al., 1996). Although these compounds inhibit NF-κB activation via different mechanisms, all four NF-κB inhibitors induced BU-11 cell apoptosis in a dose-dependent manner (Table 1). Stromal cell viability was unchanged by treatment with these concentrations of TPCK, PDTC, lactacystin, or MG-132 (data not shown). As with most pharmacologic inhibitors, the possibility that at least some of these agents affect other signaling pathways cannot formally be excluded. It was concluded, therefore, that the default apoptosis pathway blocked by NF-κB is intact and active at this early stage in B cell development.

**Down-regulation of Constitutive NF-κB Activity after DMBA Treatment Precedes BU-11 Cell Apoptosis.** Since these studies indicated that NF-κB acts as a survival factor in early preB cells, it was postulated that NF-κB DNA

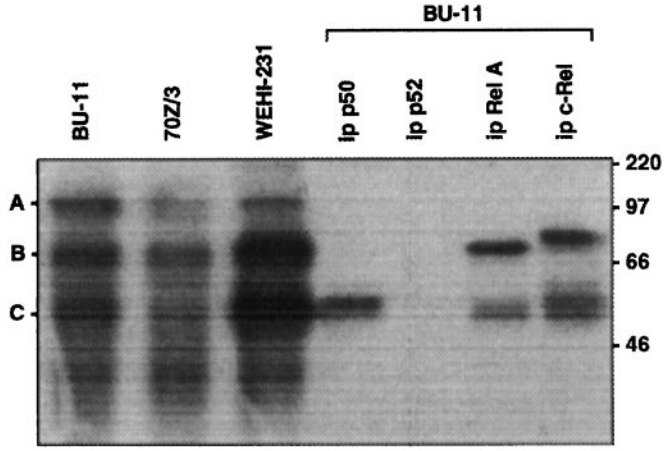


**Fig. 2.** DNA-binding complexes contain p50, c-rel, and/or Rel A. Nuclear protein extracts from BU-11, 70Z/3, and WEHI-231 cells were incubated with $^{32}$P- and BrdU-labeled NF-κB oligonucleotide probe. Proteins and probe were cross-linked with UV light; the resulting complexes were resolved by SDS-PAGE and were visualized by autoradiography (lanes 1–3). For immunoprecipitation experiments (lanes 4–7), complexes were treated with antibodies specific for the p50, p52, Rel A, and c-Rel subunits of NF-κB followed by absorption onto and elution from protein A-Sepharose beads before SDS-PAGE.

binding activity would decrease in BU-11 cells following exposure of BU-11/BMS2 cultures to DMBA, a treatment that induces BU-11 cell apoptosis within 18 to 24 h (Yamaguchi et al., 1997a,b; Mann et al., 1999; Near et al., 1999). BU-11/BMS2 cultures were treated with vehicle or $10^{-6}$ M DMBA for 12, 14, or 16 h. BU-11 cells were harvested, nuclear protein extracted, and DNA-binding NF-κB subunits assessed as in Figs. 1 and 2. Consistent with previous experiments, nuclear extracts from vehicle-treated cells formed three DNA-binding complexes (Fig. 3A, bands A–C). The intensity of bands A and B was significantly decreased 12 to 16 h after DMBA treatment, with the most profound decrease seen in band A. Although band C tended to decrease following DMBA treatment, this decrease did not reach statistical significance in four experiments. Relative band intensities are summarized in Fig. 3B. A similar decrease in κB oligonucleotide binding by nuclear proteins 12 to 14 h after DMBA ($10^{-6}$ M) exposure was seen in conventional EMSAs (data not shown). Western blotting for Rel A protein indicated constant levels of the NF-κB subunit at these time points (data not shown), indicating that down-regulation of NF-κB activity is not mediated by a decrease in subunit protein levels.

At these time points, BU-11 cells did not show overt signs of apoptosis (i.e., morphologic changes or a decrease in PI staining). Furthermore, nuclear protein binding to Oct-1 and SP-1 consensus binding sites, as evaluated in Oct-1- and SP-1-specific EMSAs, was not affected by DMBA treatment (data not shown). This observation indicates that a decrease in NF-κB/DNA binding was not a result of nonspecific degradation of transcription factors early in the apoptosis process. These results, together with those previously presented, are consistent with the hypothesis that NF-κB is a survival factor in BU-11 cells, that DMBA-induced apoptosis is preceded by a subunit-specific decrease in nuclear NF-κB expression, and that down-regulation of Rel A and c-Rel in particular are likely involved in triggering BU-11 cell apoptosis following DMBA exposure.

**Ectopic Expression of Rel A or c-Rel Rescues BU-11 Cells from DMBA-Induced Apoptosis.** If a decrease in nuclear Rel A and c-Rel expression leads to early preB cell death, then high level Rel A or c-Rel expression enforced through stable transfection of Rel A- or c-Rel-encoding plasmids should block DMBA-induced BU-11 cell apoptosis. Therefore, BU-11 cells were transfected with control (*neo*), *Rel A* (*pRSV-p65*), or *c-Rel* (*pEVRF2-c-Rel*) expression plasmids. Stable lines were selected in G418. Two control lines and two lines expressing high Rel A (Fig. 4A) or c-Rel (Fig. 4B) levels were selected for further study. These lines were cultured on BMS2 cell monolayers and exposed to vehicle or titered doses ($10^{-6}$–$10^{-8}$ M) of DMBA. BU-11 cells were harvested 24 h later, and the percentage of cells undergoing apoptosis was quantitated by PI staining and flow cytometry. Interestingly, Rel A- and c-Rel-transfected lines exhibited significantly lower levels of spontaneous apoptosis than neo-transfected lines (Fig. 5; $p < 0.001$), suggesting that NF-κB

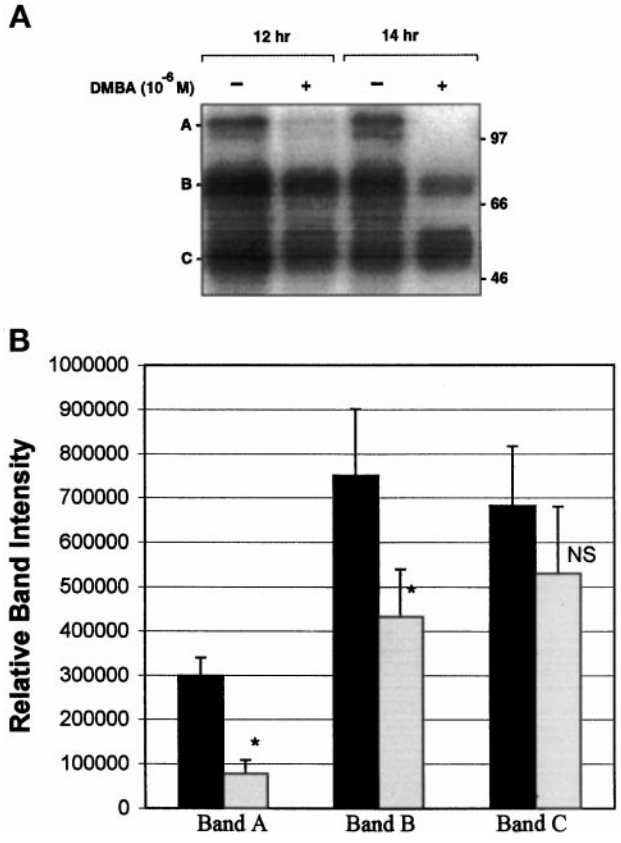


**Fig. 3.** DMBA treatment of BU-11/BMS2 cultures down-regulates NF-κB in BU-11 cells. Cultures of BU-11 cells growing on BMS2 stromal cell monolayers were treated with vehicle (0.1% acetone) or $10^{-6}$ M DMBA for 12, 14, or 16 h. BU-11 cells were harvested. An aliquot of cells was stained with PI, and apoptosis was quantitated by flow cytometry. The percentages of cells from vehicle- and DMBA-treated cultures exhibiting changes in morphology or decreased PI staining at these times were similar (5–12%). Nuclear extracts were prepared from the remaining cells and complexes of NF-κB subunits and probe assessed by in vitro cross-linking and SDS-PAGE as in Figs. 1 and 2. A, a representative autoradiograph from a total of four independent experiments. B, relative intensities of bands A, B, and C 12 to 16 h after DMBA treatment. Data are pooled from four experiments. *Significant reduction in band intensity, $p < 0.04$ (two-tailed, paired Student's *t* test). NS, not significant.

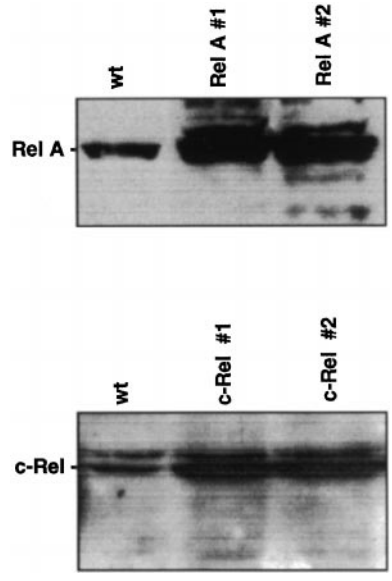


**Fig. 4.** Ectopic expression of Rel A or c-Rel in transfected BU-11 clones. BU-11 cells were transfected with *pRC-neo* alone (neo), *RSV-p65* and 10 μg of *pRC-neo* (*Rel A*), or 20 μg of *pEVRF2-cRel* and 10 μg *pRC-neo* (*c-Rel*) and stable clones selected with geneticin. Clones were grown on BMS2 monolayers and screened by Western immunoblotting for Rel A or c-Rel expression. Rel A and c-Rel expression were comparable in nontransfected and *pRC-neo*-transfected clones. Data from a representative experiment are presented.

nominally regulates cell survival at this early stage in B cell development. DMBA exposure significantly increased the percentage of neo-transfected preB cells undergoing apoptosis from a background of 8 to 10% to as high as 52 to 54% in a dose-dependent fashion (Fig. 5; $p < 0.001$ at all DMBA doses). The percentages of cells undergoing apoptosis following exposure to $10^{-6}$ to $10^{-8}$ M DMBA were comparable with those previously reported for wild-type BU-11 cells (Yamaguchi et al., 1997a,b; Mann et al., 1999; Near et al., 1999). Furthermore, as compared with apoptosis induced in neo-transfected lines, apoptosis induced with the highest dose of DMBA used, $10^{-6}$ M, was significantly but not completely attenuated in both Rel A- and c-Rel-transfected lines ($p <$ 0.001 for all transfectants). Notably, Rel A- and c-Rel-transfected clones were completely resistant to death signals induced at lower DMBA doses ($10^{-7}$ or $10^{-8}$ M; $p < 0.001$ for all transfectants at all DMBA doses). These results support the hypothesis that the NF-κB-regulated apoptosis pathway is intact in early preB cells and demonstrate that ectopic expression of either Rel A or c-Rel blocks DMBA-dependent activation of this default apoptosis pathway.

## Discussion

Previous studies in our laboratory focused on the mechanisms through which PAH induce preB cell apoptosis in cultures that model hematopoietic cell growth in the bone marrow microenvironment (Yamaguchi et al., 1997a,b; Mann et al., 1999; Near et al., 1999). These studies demonstrated that activation of the aryl hydrocarbon receptor/transcription factor in bone marrow stromal cells by PAH or PAH metabolites induces a signal that activates a death program in adjacent preB cells. Studies presented here extend previous work by investigating the nature of the intracellular signals that commit preB cells to death by apoptosis. More specifically, the role of NF-κB in BU-11 cell apoptosis induction was assessed.

NF-κB is of particular interest since it has been shown to block apoptosis induced by TNF-α, IL-1α and chemotherapeutic agents in many cell types (Beg and Baltimore, 1996; Van Antwerp et al., 1996; Wang et al., 1996, 1998). Our previous experience with NF-κB as a survival factor has involved analysis of apoptosis induced in immature, $sIg^+$ WEHI-231 B cells by cross-linking surface Ig receptors, i.e., by mimicking clonal deletion following autoantigen recognition (Wu et al., 1996a,b). In the WEHI-231 system, exposure of cells to the NF-κB inhibitors TPCK and PDTC or signaling through the surface Ig receptor down-regulated constitutive activity of Rel A- and/or c-Rel-containing complexes and rapidly induced WEHI-231 cell apoptosis. While a similar pathway is plausible for PAH-dependent preB cell apoptosis, the evidence for constitutive NF-κB activity in nonvirally transformed late preB cells was limited to one study (Kistler et al., 1998), and the role of NF-κB in earlier preB cell survival was unknown. Therefore, three goals were set for the present studies: 1) to determine whether BU-11 cells express nuclear, DNA-binding NF-κB complexes, 2) to determine whether an NF-κB-inhibited apoptosis pathway is intact at this early stage in B cell development, and 3) to determine whether NF-κB down-regulation precedes and is likely to trigger PAH-induced preB cell apoptosis.

The DNA binding activity of NF-κB from BU-11 early preB cells was assessed by UV cross-linking of nuclear proteins to a $^{32}$P- and BrdU-labeled NF-κB probe. Initial studies revealed three bands similar in migration rates to those obtained with stromal cell-independent 70Z/3 cells and WEHI-231 cells (Figs. 1 and 2) and described in previous studies with mature human T cells (Molitor et al., 1990). Specific inhibition of band formation with unlabeled NF-κB wild-type but not mutated probe confirmed that each band represents NF-κB subunit-containing complexes (Fig. 1). NF-κB-specific complexes were also identified by EMSA (data not shown). These results are consistent with those obtained with virally transformed preB cells (Sen and Baltimore, 1986; Guerrini et al., 1998) or IL-7-stimulated late preB cells (Kistler et al., 1998). Therefore, the first criterion supporting a role for

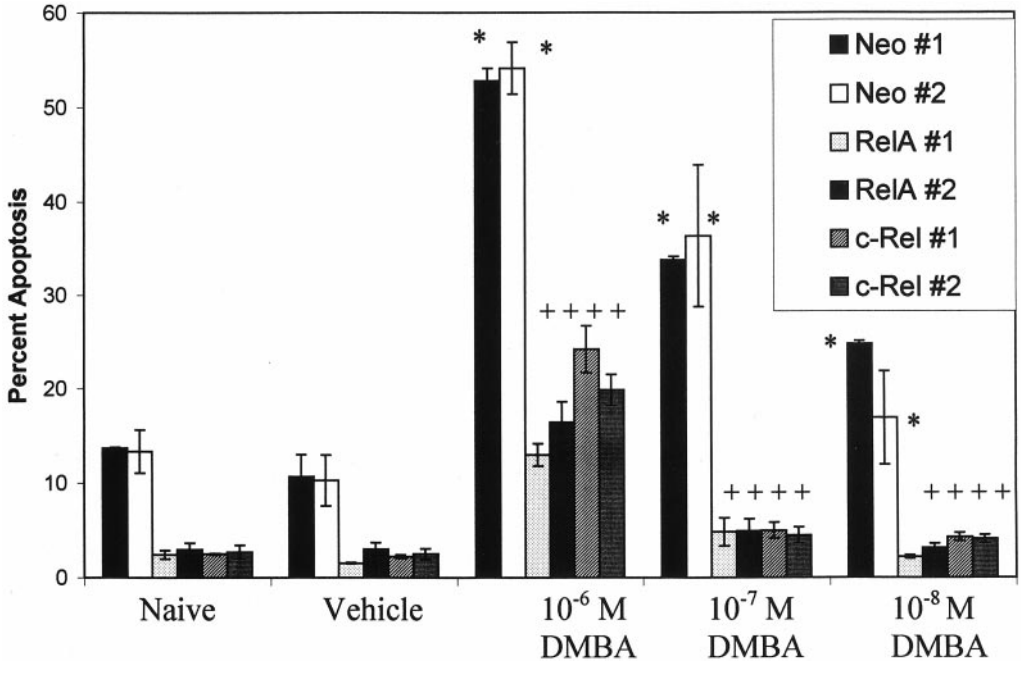


**Fig. 5.** Ectopic expression of Rel A or c-Rel inhibits DMBA-induced BU-11 cell apoptosis. Two randomly selected *pRC-neo*-transfected clones, two clones expressing elevated Rel A levels, and two clones expressing elevated c-Rel levels were maintained on BMS2 monolayers and treated with vehicle or $10^{-6}$ to $10^{-8}$ M DMBA for 24 h. Transfected BU-11 cells were then harvested and the percentage of apoptotic cells assessed by PI staining and flow cytometry. Data are averaged from three experiments and are presented as arithmetic means ± S.E. Rel A- and c-Rel-transfected lines exhibited significantly lower levels of spontaneous apoptosis than neo-transfected lines ($p <$ 0.001). All statistics were determined by analysis of variance in combination with Dunnett's multiple comparisons test. *Significant level of DMBA-induced apoptosis as compared with vehicle controls ($p <$ 0.001). $^+$Significantly lower levels of apoptosis as compared with neo-transfected controls treated with the same DMBA dose ($p < 0.001$).

NF-κB in PAH-induced apoptosis, i.e., constitutive NF-κB activity in the BU-11 early preB cell line, was met. Since early preB cells do not express Igκ light chains, the data also suggest that NF-κB, named for its role in Igκ chain induction (Sen and Baltimore, 1986), plays an important role before Igκ synthesis.

Immunoprecipitation experiments demonstrated that band C is formed by p50-containing complexes while band B is formed by Rel A- and c-Rel-containing complexes. Nuclear expression of either Rel A- or c-Rel-containing complexes generally indicates NF-κB-mediated transcriptional activity (Miyamoto and Verma, 1995). As has been our collective experience with other cell lines (Ballard et al., 1990; Molitor et al., 1990; Doerre et al., 1993), putative NF-κB dimers forming band A specifically bind NF-κB oligonucleotide probes but are not immunoprecipitable with antibodies against NF-κB subunits (Fig. 2 and data not shown). This result likely reflects stearic constraints, which prevent binding of subunit-specific antibodies to complexes of two NF-κB subunits covalently bound to oligonucleotide probe. While this technical limitation precludes definitive identification of NF-κB subunits constituting this high molecular mass complex, its apparent size (125–135 kDa) is consistent with p50/Rel A, p50/c-Rel, Rel A/Rel A, and/or c-Rel/c-Rel dimers. Importantly, all of these dimers are transcriptionally active (Miyamoto and Verma, 1995). Nevertheless, the presence of p50/p50 homodimers cannot be formally excluded.

A minor band resolving just above band B in some experiments suggested a p52-expressing complex (e.g., Fig. 3B). However, p52-specific antibody did not precipitate an NF-κB probe-binding protein (Fig. 2). While still under investigation, proteins that form this band appear not to contribute to PAH-induced apoptosis since the band density did not change significantly following DMBA exposure.

Given the constitutive expression of nuclear NF-κB complexes, it was predicted that NF-κB down-regulation with specific inhibitors would induce cell death. Indeed, this was shown to be the case (Table 1). BU-11 cell apoptosis was detectable as early as 8 h after exposure to NF-κB-inhibitors (data not shown). Hence, a default apoptosis pathway blocked by NF-κB is intact in this early preB cell line. A role for this apoptosis pathway in DMBA-induced preB cell apoptosis was suggested by the specific down-regulation of NF-κB before overt apoptosis, as detected by UV cross-linking studies (Fig. 3) or by EMSA (data not shown). Furthermore, the ability to decrease spontaneous apoptosis significantly and to rescue BU-11 cells completely from low-dose DMBA-induced apoptosis by ectopic expression of either Rel A or c-Rel supports the conclusions that NF-κB in early preB cells tightly regulates cell survival and that DMBA-induced NF-κB down-regulation is causally related to apoptosis induction.

Interestingly, Rel A activation may induce transcription of the NF-κB inhibitor IκB-α, thereby initiating a negative feedback loop (LeBail et al., 1993; Read et al., 1994; Sun et al., 1994). Similarly, constitutive ectopic Rel A expression may induce IκB in BU-11 cells. However, the induction of IκB may not be sufficient to overcome the high levels of ectopic Rel A in transfectants. Alternatively or in addition, chronic Rel A expression can stimulate synthesis of underphosphorylated (i.e., inactive) IκB thereby potentiating Rel A activity (Bitko and Barik, 1998). Perhaps ectopic expression also leads to synthesis of hypophosphorylated IκB. The regulation of IκB and other upstream NF-κB regulators in wild-type and Rel A-transfected BU-11 cells before and following DMBA exposure is under investigation.

The results presented here bear an obvious resemblance to results obtained in the WEHI-231 system. Both BU-11 and WEHI-231 cells rapidly undergo apoptosis when exposed to NF-κB inhibitors, both down-regulate Rel A and c-Rel before overt apoptosis, and both are rescued by ectopic c-Rel expression (Wu et al., 1996a,b). In WEHI-231 cells, at least one down-stream target of NF-κB modulation is c-*myc*, down-regulation of which leads to apoptosis (Wu et al., 1996a). Preliminary experiments in the BU-11 system similarly point to c-Myc down-regulation as a trigger for apoptosis (K. K. Mann, unpublished). Since c-Myc represses $p27^{kip}$ expression, confirmation of DMBA-induced c-Myc down-regulation following DMBA exposure in BU-11 cells would support the hypothesis that de-repression of $p27^{kip}$ alters cell cycle and initiates apoptosis. Furthermore, it would be concluded that the apoptosis pathway activated during the $sIg^{+}$ immature B cell stage for the purpose of autoantigen-specific clonal deletion is competent at the early preB cell stage. The premature and nonclonally restricted activation of this pathway following PAH exposure in the bone marrow microenvironment would be expected to skew the B cell repertoire and to compromise the development of a competent humoral immune system.

Finally, it should be pointed out that the studies presented here were performed with an in vitro model of B lymphopoiesis. The dependence of BU-11 cells on stromal cell monolayers suggests that this model more accurately mimics the bone marrow microenvironment than previously described models using stromal cell-independent cell lines (e.g., 70Z/3) or exogenous growth factor-dependent primary preB cells. The previous demonstration of bone marrow hematopoietic cell apoptosis following in vivo exposure to PAH (DMBA or benzo[*a*]pyrene; Yamaguchi et al., 1997a) supports the validity of the BU-11/BMS2 system. However, further experimentation is required to determine whether, as predicted by the current model, PAH-induced preB cell apoptosis in vivo is mediated by NF-κB down-regulation.





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