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Fas Resistance of Leukemic Eosinophils Is Due to Activation of NF-κB by Fas Ligation

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TNF family receptors can lead to the activation of NF-κB and this can be a prosurvival signal in some cells. Although activation of NF-κB by ligation of Fas (CD95/Apo-1), a member of the TNFR family, has been observed in a few studies, Fas-mediated NF-κB activation has not previously been shown to protect cells from apoptosis. We examined the Fas-induced NF-κB activation and its antiapoptotic effects in a leukemic eosinophil cell line, AML14.3D10, an AML14 subline resistant to Fas-mediated apoptosis. EMSA and supershift assays showed that agonist anti-Fas (CH11) induced nuclear translocation of NF-κB heterodimer p65(ReA)/p50 in these cells in both a time- and dose-dependent fashion. The influence of NF-κB on the induction of apoptosis was studied using pharmacological proteasome inhibitors and an inhibitor of IκBα phosphorylation to block IκBα dissociation and degradation. These inhibitors at least partially inhibited NF-κB activation and augmented CH11-induced cell death. Stable transfection and overexpression of IκBα in 3D10 cells inhibited CH11-induced NF-κB activation and completely abrogated Fas resistance. Increases in caspase-8 and caspase-3 cleavage induced by CH11 and in consequent apoptotic killing were observed in these cells. Furthermore, while Fas-stimulation of resistant control 3D10 cells led to increases in the antiapoptotic proteins cellular inhibitor of apoptosis protein-1 and X-linked inhibitor of apoptosis protein, Fas-induced apoptosis in IκBα-overexpressing cells led to the downmodulation of both of these proteins, as well as that of the Bcl-2 family protein, Bcl-xL. These data suggest that the resistance of these leukemic eosinophils to Fas-mediated killing is due to induced NF-κB activation. The Journal of Immunology, 2002, 169: 3536–3544.

Ligation of death receptors of the TNFR family can initiate signaling pathways leading to cell death or cell survival. Although TNF itself was named for its ability to induce cell death, it has been known for several years that TNF-α stimulation also can induce activation of the transcription factor NF-κB (reviewed in Ref. 1). Although activation of NF-κB has been thought to be part of the apoptotic induction, recent evidence suggests that in most circumstances, NF-κB activation is a prosurvival response. Many normal cells are not killed by TNF and this may be related to NF-κB activation; blockade of NF-κB sensitizes cells to TNF and augments induced apoptotic cell death (2–4). Fas (CD95/Apo-1) is a member of the TNFR family of proteins, but the ability of Fas to activate NF-κB has been variably reported (5, 6) and dissociated from any protective effect (6–10). Indeed, death occurs in these cell types despite the activation of NF-κB (9, 10), and inhibition of NF-κB has little effect on apoptosis (7). In leukemic or neoplastic cells, resistance to Fas has been attributed to deficiencies in constituents of the Fas pathway, including decreased surface expression of Fas (11), or to the presence of increased levels of antiapoptotic proteins such as Fas-associated phosphatase-1 (FAP-1) (12) or certain members of the Bcl-2 family (13). However, to our knowledge Fas resistance in such cells has not been directly linked to activation of NF-κB induced by Fas itself.

The prototypical NF-κB is composed of a p65 (ReA) and a p50 subunit and is sequestered in the cytoplasm as an inactive form bound to IκB inhibitory protein, particularly IκBα (1). Upon stimulation by a variety of extracellular agents, IκBα is phosphorylated at serines 32 and 36, leading to its polyubiquitination; this in turn leads to recognition and degradation of IκBα by the 26S proteasome (14–19). The disassociation of IκB exposes the nuclear localization sequence of NF-κB, and it is transported into the nucleus where it can activate expression of a wide variety of genes (15–19). Notably, two protein families contain NF-κB-inducible, anti-apoptotic family members—the inhibitor of apoptosis proteins (IAPs) and the Bcl-2 family (20–23). NF-κB-mediated regulation of the prosurvival Bcl-2 protein, Bcl-xL, for example, has been shown to be important in survival signaling in both B (21) and T lymphocytes (23). Recent studies have shown that NF-κB target gene products of the IAP family can inhibit the proteolytic activities of caspases (reviewed in Ref. 10) and can prevent apoptosis induced by Fas ligation (24). Recently, overexpression of IκBα in endothelial cells suppressed expression of iap genes and sensitized these cells to TNF-α-induced apoptosis (25). In this study, we show that ligation of the Fas receptors on the eosinophilic cell line AML14.3D10 (hereafter referred to as “3D10” cells) induced a distinctive pattern of activation of NF-κB, and that these cells were resistant to Fas-mediated killing. Pharmacologic blockade of NF-κB activation or overexpression of the physiologic NF-κB inhibitor protein IκBα abrogated the Fas resistance of the 3D10 cells.

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3 Abbreviations used in this paper: FAP-1, Fas-associated phosphatase-1; IAP, inhibitor of apoptosis protein; PSI, proteasome inhibitor 1; LC, lactacystin; PI, propidium iodide; pAML, parental AML14 cell line; PBE, peripheral blood eosinophil; CDE, cord blood-derived eosinophil; GFP, green fluorescence protein; MG132, carboxyoxo-L-leucyl-L-leucyl-L-leucinal; BAY 11-7085, (E)-(4-t-butylphenylsulfonyl)-2-propenenitrile; c-IAP, cellular IAP; XIAP, X-linked IAP; c-FLIP, cellular FLIP.
cells. In IκBα-overexpressing cells, both caspase-8 and caspase-3 were activated following anti-Fas treatment and nearly all the cells were killed, while in vector control there was little or no caspase activation and the cells remained resistant to Fas ligation. This suggests that NF-κB activation is critical for protection of the 3D10 eosinophils from Fas-mediated apoptosis and that in these cells Fas itself induces NF-κB activation.

**Materials and Methods**

**Cell culture**

Eosinophilic AML14 cell lines, the parental AML14 cell line and the AML14.3D10 subline, were generated and kindly provided by Drs. C. Paul and M. Baumann (Wright State University, Dayton, OH) (26, 27). The parental AML14 cell line (pAML) was established from a patient with FAB M2 acute myeloid leukemia (26). The 3D10 subline was isolated originally as a line with an advanced eosinophil phenotype and a doubling time of 48 h without cytokine supplementation (27). Cells were maintained in RPMI (Medicago, Hamburg, MN) and from Medicago International (Watertown, MA), respectively. The IκBα phosphorylation inhibitor (E)-3-[4-(4-tetrahydro-3-aminopyridyl)-2-propeninylurate (BAI 11-7085), proteasome inhibitors carboxenzoxy-t-leucyl-t-leucyl-t-leucyl-1-leucinal (MG132), proteasome inhibitor I (PSI), and lactacystin (LC) were purchased from Calbiochem. Plasmid DNA constructs of pCMV-IκBα and pCMV vector only were generally only gifts of Dr. D. Ballard (Vanderbilt University, Nashville, TN) and provided by Drs. C. Paul and S. Baumann. FabCD10 eosinophils from Fas-mediated apoptosis and that in these constructs of pCMV-IκBα and -2 and to X-linked IAP (XIAP) were obtained from R&D System (Minneapolis, MN) and from MBL International (Watertown, MA), respectively. The IκBα phosphorylation inhibitor (E)-3-[4-(4-tetrahydro-3-aminopyridyl)-2-propeninylurate (BAI 11-7085), proteasome inhibitors carboxenzoxy-t-leucyl-t-leucyl-t-leucyl-1-leucinal (MG132), proteasome inhibitor I (PSI), and lactacystin (LC) were purchased from Calbiochem. Plasmid DNA constructs of pCMV-IκBα and pCMV vector only were generally only gifts of Dr. D. Ballard (Vanderbilt University, Nashville, TN) and provided by Drs. C. Paul and S. Baumann.

**Induction of apoptosis**

Fas-induced cell death was determined by both trypsin blue exclusion assay and flow cytometric analyses. Briefly, AML14.3D10 cells were seeded at a density of 3 x 10⁶ cells/ml/well in 48-well cell culture plates. The mouse IgG monoclonal anti-Fas Ab, CH11, was used primarily at a range of 100 μg/ml (1-5 μg/ml) to 1 μg/ml (100 ng/ml) final concentrations. Each cell sample was divided for trypsin blue exclusion assays and for standard propidium iodide (PI) DNA analyses after 24, 48, and 72 h. Total cell death was determined by trypsin blue (0.2%) exclusion using a conventional light microscope. The remaining cells were centrifuged at 200 x g for 10 min and resuspended in hypotonic PI solution (50 μg/ml PI in 0.1% Na citrate, 0.1% Triton X-100). To ensure cell lysis, cells were stored overnight in the dark at 4°C before flow cytometric analysis. At least 5000 nuclei were examined for each sample to determine percentage of subG1 DNA content. In preliminary experiments, hypotonic PI analyses of cell samples closely correlated with other DNA fragmentation and morphologic criteria of apoptosis. Percentages (±SE) of cell death or survival (as percentage of viability) reported in the results are derived from the flow cytometric analyses.

**Western blot analysis**

Equal amounts of protein were separated by SDS-PAGE mini-gel electrophoresis and transferred onto nitrocellulose membrane (0.2-μm pore size; Sigma-Aldrich) using a semidry electrophoretic transfer system (Bio-Rad, Hercules, CA). Blots were stained with Ponceau S to check the quality of the protein and the transfer efficiency. Immunoblotting was performed according to the ECL Western blotting protocol (Amersham Pharmacia Biotech, Arlington Heights, IL). Briefly, blots were blocked in 5% nonfat dry milk in 1× TBS-Tween solution for 1 h followed by a 1-h incubation with the appropriate primary Ab. Blots were then washed for 30 min with four changes of 1× TBS-Tween solution followed by a 1-h incubation with the appropriate HRP-conjugated secondary Ab. Blots were washed again and incubated for 1 min with ECL detection reagents. The results were visualized by exposing blots to autoradiographic film (Kodak, Rochester, NY).

**Pharmacologic inhibition of the NF-κB activation**

AML14.3D10 cells were cultured at a density of 1 x 10⁶/ml and were preincubated for 1 h with IκBα phosphorylation inhibitor BAY 11-7085 (29), or proteasome inhibitors LC (30), MG132 (31), or PSI (32) at a range of concentrations (0.1–20 μM) before addition of TNF family ligands or Abs. Optimal doses, at which augmentation of Fas-mediated killing was greatest with the least background toxicity of inhibitors alone, were calculated and used in certain experiments as described.

**Extraction of nuclear protein**

Cells were passed and grown overnight at −7 x 10⁶ cells/ml in cell culture flasks. After the treatments, the cell nuclear extracts were prepared according to a published method (33) with some modifications. Unless indicated otherwise, all procedures were performed at 4°C. Briefly, 10 x 10⁶ cells were harvested by centrifugation and washed twice with ice-cold Dulbecco’s PBS buffer. The pellet was resuspended in 1× packed cell volume of buffer A (10 mM HEPES pH 7.9, 1.5 mM MgCl₂, 10 mM KCl, 0.5 mM PMSF, 0.5 mM DTT) and incubated on ice for 10 min. The supernatant was discarded after centrifugation at 1300 rpm for 7 min and 1× original packed cell volume of buffer A was added. The cell suspension was transferred to a 50-ml “woodrage” centrifuge tube and centrifuged at 8500 rpm for 20 min in a Beckman JX 13.1 rotor (Beckman Instruments, Palo Alto, CA). The supernatant was removed and set aside as the cytoplasmic extract. The pellet was gently washed with buffer A an additional time and resuspended in 1× original packed cell volume of buffer C (20 mM HEPES (pH 7.9), 25% glycerol, 1.5 mM MgCl₂, 420 mM KCl, 0.2 mM EDTA, 0.5 mM PMSF, 0.5 mM DTT). The suspension was stirred on a rocking platform for 30 min and then centrifuged in a Beckman rotor JA-17 (Beckman Instruments) at 12,500 rpm for 30 min. The supernatant was collected without disturbing the pellet and placed in dialysis tubing (Life Technologies, Grand Island, NY). Dialysis was performed for 1 h against three changes of 200 ml of buffer D (20 mM HEPES (pH 7.9), 20% glycerol, 100 mM KCl, 0.2 mM EDTA, 0.5 mM PMSF, 0.5 mM DTT). Following dialysis, the nuclear extract was clarified by centrifugation at 14,000 rpm for 20 min in an Eppendorf microcentrifuge tube (Brinkman Instruments, Westbury, NY). Protease inhibitors including leupeptin, anti-pain, chymostatin, and pepstatin A (Sigma-Aldrich) were added immediately (5 μg/ml each) to extracts before saving them at −80°C.

**EMSA**

The details of the EMSA have been described elsewhere (34). The procedure was performed with some modification. Double-stranded NF-κB synthetic oligonucleotides 5'–AGT TGA GGG GAC TTT CCC AGG C-3' were purchased from Promega (Madison, WI) and end-labeled with [γ-32P]ATP (Amersham Pharmacia Biotech) and T4 polynucleotide kinase (NEB, Beverly, MA). A 200-fold excess of unlabeled NF-κB probe and unrelated oligonucleotide probes for CArG was used to assess the specificity of the DNA-binding reaction. Binding reactions were performed on ice in a total volume of 15 μl DNA binding buffer (6 μl probe, 5% Ficoll, 300 μg/ml poly(dI-dC) (Promega). DNA-protein binding was initiated by adding 4 μg of nuclear extract. A total of 200-fold excess of “cold” (unlabeled) NF-κB probe was used as a specific competitor. Electrophoresis was performed for 3 h at 100 V in 0.5 × Tris-borate-EDTA running buffer in a 4°C cold room. The dried gel was visualized via exposure to high performance autoradiography film. The supershift analyses were performed by incubating the DNA-binding reactions with optimal concentrations (determined previously) of Abs to p65, p50, RelB, and c-rel for an additional 20 min on ice before electrophoresis.

**Luciferase reporter assay**

Cells were transfected with the NF-κB transcription reporter plasmid DNA pNF-κB-Luc (Clontech Laboratories) using GenePORTER transfection kit (Gene Therapy Systems, San Diego, CA) essentially according the method...
described in detail below. Transfection efficiency was assessed by cotransfection with pEGFP-C1. After 24 h following transfection, cells were serum starved for 8 h and treated with either TNF-α (5 ng/ml) or anti-Fas Ab CH11 (1 μg/ml). Cells were harvested 16 h after treatment and analyzed for luciferase activity using a luminometor and luciferase reporter assay kit obtained from BD Biosciences as previously described (35).

**Stable transfection of pCMV-IκBα**

Wild-type human IκBα cDNA was cloned into a mammalian expression vector, pCMV, and was used to transfect AML14.3D10 cells by using the GenePORTER kit. Briefly, 1 × 10^6 cells/well were plated on a 12-well tissue culture plate. The pEGFP-C1 plasmid DNA was used as the reporter gene and cotransfected at a ratio of 3:1. After 6 wk of selection for neomycin resistance in medium containing 1200 μg/ml G418 (Sigma-Aldrich), the positively transfected 3D10 cells were examined by FACS. The dead cells were discarded by Percoll gradient centrifugation. The positive cells were subsequently maintained in media supplemented with 400 μg/ml G418. Cell viability and proliferation were carefully monitored for at least 2 mo before the first experiments and during the experimental period. Over this period, the stable transfectants also exhibited almost identical viability and proliferative capacity compared with untransfected 3D10 cells.

**Results**

The TNF superfamily includes TNF-α, Fas ligand, TRAIL, and IL-1β (36), and interactions between TNF family ligands and their receptors are major regulatory mechanisms for hemopoietic cells (37). Specifically, TNF-α, Fas ligand, and TRAIL interactions with their receptors are considered to be death signals to many cells. In this study, we have examined the signaling effects of the agonist anti-Fas Ab CH11, as well as TRAIL, on 3D10 eosinophils. We analyzed caspase (-8 and -3) cleavage, cell death or survival, IkBα degradation, and NF-κB nuclear translocation following stimulation of these receptors under control conditions or in the presence of various inhibitors of NF-κB activation. We also determined the effects of Fas stimulation on the expression of antiapoptotic, NF-κB target proteins, Bcl-xL, c-FLIP, c-IAP1, c-IAP2, and XIAP. Finally, we examined these events in cells overexpressing the physiologic inhibitor of NF-κB, IκBα.

**AML14.3D10 cells are resistant to apoptosis induced by anti-Fas Ab CH11**

AML14.3D10 eosinophils express surface levels of Fas which are equivalent to those expressed by the pAML, and to peripheral blood eosinophils (PBEs) and mature, differentiated cord blood-derived eosinophils (CDEs). Minor (nonstatistical) differences in surface expression (Fig. 1A) did not correlate to resistance or susceptibility. Of the death ligands tested, only TRAIL induced death of large numbers of 3D10 cells (Fig. 1B). Percentage of cell death was 42.4 ± 6.6%, 67.7 ± 4.2%, and 80.9 ± 3.9% after 24-, 48-, and 72-h treatments, respectively. In contrast, there was significantly less cell death induced by anti-Fas (CH11) after 24-, 48-, and 72-h treatments (8.2 ± 0.67%, 12.9 ± 0.76%, and 17.3 ± 1.2%, respectively). Only the 3D10 cells demonstrated resistance to apoptosis induced by CH11 in comparisons with PBEs, the pAML, and mature, differentiated CDEs (Fig. 1B). All eosinophil cell types were resistant to TNF-α-induced cell death (data not shown).

**Caspase-8 and caspase-3 activation by CH-11 is suppressed in 3D10 eosinophils**

Caspase activation has been described as a critical event(s) in apoptotic cell death. TNF superfamily-induced cell apoptosis leads to the proteolytic activation of both upstream caspases (e.g., caspase-8) and downstream caspases (e.g., caspase-3) (38). Western blot assays were performed to examine caspase-8 and caspase-3 activation/cleavage induced by CH11 or TRAIL in 3D10 cells. Cells were treated with either 1 μg/ml CH11 or 100 ng/ml TRAIL for 1–24 h. No substantial CH11-induced procaspase-8 or procaspase-3 degradation was observed during this period (Fig. 2A) or during periods extending to 72 h (data not shown). Because these cells are sensitive to TRAIL, and TRAIL ligation has been shown to activate caspases-8 and -3 in sensitive cells (39, 40), we used TRAIL-induced apoptosis of 3D10 cells as a “positive control” to examine cleavage of these caspases. Cleavage of procaspase-8 (both isoforms; Ref. 28) was detected as early as 1 h and cleavage of procaspase-3 was detectable by 4–6 h after treatment with TRAIL (Fig. 2B). Anti-actin was used to monitor the equivalent protein loading in gels as shown (Fig. 2).

**NF-κB activation is induced by Fas ligation in 3D10 eosinophils**

NF-κB can be an important factor in the suppression of apoptosis in several cell types (2–4, 41–44). We examined NF-κB activation induced by anti-Fas, as well as TNF-α and TRAIL in the 3D10 eosinophils used in the apoptosis assays described above. EMSA analyses (Fig. 3) showed that NF-κB is activated in 3D10 cells after a 1-h treatment with 1 μg/ml CH11, but not after treatment with 100 ng/ml of TRAIL (even after 2 h; data not shown); no translocation of NF-κB was observed in control IgM-treated group (Fig. 3). TNF-α-induced nuclear translocation of NF-κB in these cells occurred as early as 5 min and reached a peak around 20–30 min (data not shown). Treatment of Fas-susceptible parental AML14 cells did not induce NF-κB translocation above baseline.
NF-κB revealed that the p65/p50 heterodimer is the activated form of the activation of NF-κB. This treatment induces the optimal responses in these experiments. The mouse IgM protein (1 μg/ml) again showed no induction of NF-κB activation (Fig. 4).

Identification of CH11-induced NF-κB subunits in 3D10 eosinophils

There are five members of the NF-κB/Rel family of proteins that have been found expressed in mammalian cells. These NF-κB/Rel subunits are p65/RelA, c-Rel, RelB, p105/NF-κB1 (which can be processed to p50), and p100/NF-κB2 (which can be processed to p52; reviewed in Ref. 1). These subunits usually exist as protein dimers such as the heterodimer, p65/p50, or the homodimer, p50/p50. We examined the NF-κB subunits in 3D10 cells in supershift assays using Abs specific for p65, p50, RelB, and c-Rel. Nuclear extract was prepared from 3D10 cells treated with 1 μg/ml monoclonal anti-Fas CH11 for 1 h. This treatment induces the optimal activation of NF-κB as described above. These experiments revealed that the p65/p50 heterodimer is the activated form of the NF-κB induced by CH11 in 3D10 eosinophils (Fig. 4). Faint bands in some lanes could be shifted by anti-p50 Ab only and could represent endogenous p50/p50 homodimers (Fig. 4, open arrow). However, these bands were inconsistent in their presence and responses in these experiments. The mouse IgM protein (1 μg/ml) again showed no induction of NF-κB activation (Fig. 4).

Activation of NF-κB in 3D10 cells stimulated by CH11 is time- and dose-dependent

To determine details of the regulation of anti-Fas-induced NF-κB activation, we examined the CH11 dose response and the time course of NF-κB nuclear translocation. EMSAs were performed using the nuclear extracts prepared from 3D10 cells treated either for 1 h with different doses of CH11 ranging from 0.01–2.0 μg/ml, or with 1 μg/ml CH11 for periods from 10 min to 3 h. The results show that the minimum dose of CH11 required for NF-κB activation is 0.1 μg/ml, and no significant increase occurs after treatment with >1 μg/ml (Fig. 5A). The peak of NF-κB translocation was seen at 60 min after treatment with 1 μg/ml CH11 and decreases greatly after 3 h (Fig. 5B).

Blockade of NF-κB activation leads to an increase in CH11-induced apoptosis in 3D10 eosinophils

Comparison of the apoptotic effects of CH11 and TRAIL on 3D10 cells suggests that there is a correlation between resistance to apoptosis and NF-κB activation. Treatment of 3D10 eosinophils with TRAIL leads to apoptosis but not activation of NF-κB (see Figs. 1 and 3). In contrast, 3D10 cells have shown strong resistance to TRAIL or 1 μg/ml CH11. BAY 11-7085 inhibits IκB phosphorylation, preventing the degradation of IκBα and release of the activated form of NF-κB. LC, MG132, and PSI were used to treat 3D10 cells. A range of doses of inhibitors was determined by toxicity assays and inhibitor alone controls were also performed. Fig. 6 shows the results of analyses of apoptosis of 3D10 cells pretreated with 1 μg/ml CH11 for 1 h before adding 1 μg/ml CH11. BAY 11-7085 inhibits IκBα phosphorylation, preventing the degradation of IκBα and release of the activated form of NF-κB. LC, MG132, and PSI are proteasome inhibitors which are specific for the 20S and/or 26S proteasome complex and inhibit NF-κB activation by blocking IκBα degradation. The dosages of inhibitors were determined by toxicity assays and inhibitor alone controls were also performed. Fig. 6A shows the results of analyses of apoptosis of 3D10 cells pretreated with 1 μg/ml CH11 for 1 h before adding 1 μg/ml CH11. BAY 11-7085 inhibits IκBα phosphorylation, preventing the degradation of IκBα and release of the activated form of NF-κB. LC, MG132, and PSI are proteasome inhibitors which are specific for the 20S and/or 26S proteasome complex and inhibit NF-κB activation by blocking IκBα degradation. The dosages of inhibitors were determined by toxicity assays and inhibitor alone controls were also performed. Fig. 6A shows the results of analyses of apoptosis of 3D10 cells pretreated

FIGURE 2. Caspase-8 and -3 activation induced by anti-Fas and TRAIL in 3D10 cells. Cells were treated as described in Fig. 1, and Western blot analyses of caspase-8 and caspase-3 cleavage/activation were performed using the ECL protocol described. A, Western blot analyses of caspase-8 and caspase-3 activation in CH11-treated 3D10 cells are shown in top and middle panels, respectively. The bottom panel is actin control performed on same blot. B, Western blot analyses of TRAIL-induced caspase-8 and caspase-3 activation is shown in top and middle panels; bottom panel is actin control.

FIGURE 3. NF-κB activation induced by anti-Fas in 3D10 cells. 3D10 cells were cultured at 5 × 10⁶ cells/ml and stimulated with 100 ng/ml TRAIL or 1 μg/ml CH11 for 1 h. A total of 1 μg/ml mouse IgM was used as the control. A total of 4 μg of each nuclear extract protein was used for EMSA analysis. For each treatment group, lane 1 represents nuclear extract proteins binding to the ³²P-labeled NF-κB probe, lane 2 represents self-competition, and lane 3 represents irrelevant ³²P-labeled anti-G probe binding reaction. Specific complexes (NF-κB), nonspecific (ns) bands, and free probe are indicated.
with BAY 11-7085 (2.5 μM) before treatment with 1 μg/ml of CH11 for 24, 48, or 72 h. Dramatically increased induction of apoptosis was observed at all time points. Similar results were seen for LC, MG132, and PSI, although background killing of cells (with inhibitor only) were somewhat higher with MG132 and PSI (data not shown).

EMSAs were performed following the treatment with the inhibitors which exhibited the least background toxicity, namely BAY 11-7085 and LC. These results demonstrated that inhibition of the NF-κB activation occurs when cells were treated with the optimal doses of either 2.5 μM BAY 11-7085 (Fig. 6A) or 1 μM LC (data not shown). Both BAY 11-7085 and LC blocked NF-κB translocation in a dose-dependent manner; the most effective blockade of NF-κB translocation induced by CH11 was observed with either 10–20 μM of BAY 11-7085 or with 10 μM of LC. However, background killing by inhibitors alone were greater at these latter doses. These observations correlated well with Western blot analyses of IκB degradation (Fig. 6C), and suggested that some maintenance of IκB expression (at 2.5 μM and above) was sufficient to inhibit NF-κB-mediated protection. Furthermore, Fas-induced NF-κB activation, as measured by luciferase reporter assay, was almost completely abrogated by BAY 11-7085 treatment (data not shown).

Overexpression of IκBα blocks CH11-induced NF-κB activation and inhibits 3D10 cell resistance to CH11

To more rigorously test the role of NF-κB activation in 3D10 eosinophils, we created the stably transfected cell line,
AML14.3D10-IκBα, by cotransfecting 3D10 cells with pCMV-IκBα and pEGFP-C1. After transfection, cells were selected with G418 as described above, and green fluorescence protein (GFP) expression in the cells was analyzed via flow cytometry. These data showed that ~86% of the cells were positive for GFP as compared with the control cells (Fig. 7). The selected cells were maintained in media with 400 μg/ml of G418 as described above. The stably transfected pCMV vector control cell line was generated using the same method. Transfection of cells had no significant effect on surface Fas expression (Fig. 8D). IκBα-transfected expressed almost identical amounts of Fas as control cells, and vector-only cells remained Fas-resistant. IκBα is the physiological inhibitor of NF-κB and only upon the phosphorylation and degradation of IκBα can NF-κB p65/p50 translocation occur. The overexpression of IκBα in 3D10 eosinophils inhibits depletion of cytosolic IκBα as compared with control group that showed significant reduction of IκBα after treatment with 1 μg/ml of CH11 for 30 min (Fig. 8A). The CH11-induced NF-κB activation was inhibited in IκBα-transfected 3D10 cells (Fig. 8B), while transfection with vector alone had no effect on NF-κB activation by CH11 treatment. Many recent studies have shown that activation of NF-κB can lead to the antiapoptotic proteins such as XIAP, c-IAP1, c-IAP2, survivin, and others (20). These antiapoptotic proteins can inhibit the proteolytic activity of caspases, blocking caspase activation (cleavage) and suppressing apoptosis (20). To examine the apoptotic effect of NF-κB inhibition in IκBα-overexpressing cells, we compared CH11-induced caspase activation and cell death in 3D10 cells transfected with the pCMV control vector with pCMV-IκBα-transfected cells after treatment with 1 μg/ml CH11. Western blot analyses revealed that while neither caspase-8 nor caspase-3 activation were observed in the vector control group (Fig. 9A), inhibition of NF-κB activation via IκBα overexpression led to a dramatic increase in cleavage of the proforms of both upstream caspase-8 and downstream caspase-3 (Fig. 9B). Enumeration of viable cells after treatment of these two groups with CH11 (1 μg/ml) for 24, 48, and 72 h correlated closely with caspase activation, and <10% of IκBα-overexpressing cells were viable, while >80% of control (vector-only) eosinophils survived CH11 treatment (Fig. 9, C and D).

**Fas-induced NF-κB activation leads to selective increases in antiapoptotic proteins**

To specifically identify potential targets of NF-κB-mediated protection in Fas-stimulated 3D10 cells, we examined expression of several proteins for 12 h following CH11 treatment of cells stably transfected with the pCMV control vector or with pCMV-IκBα. The results of these analyses are shown in Fig. 10. Western blot analyses consistently showed increased expression in both c-IAP1 and XIAP, but not in the other potential NF-κB targets, Bcl-xL, c-FLIP, or c-IAP2. Both c-IAP1 and XIAP showed increased levels of expression by 2 or 3 h, and levels above baseline were maintained for most or all of the 12-h period. Interestingly, both of these proteins, along with Bcl-xL, appeared down-regulated or degraded following anti-Fas treatment of IκBα-overexpressing cells (Fig. 10). Both Bcl-xL and XIAP were rapidly down-regulated with decreased levels evident by 2–3 h, while c-IAP1 decreases appeared somewhat later, ~6–12 h (Fig. 10).

**FIGURE 7.** Flow cytometric analysis of 3D10 cells stably transfected with pCMV-IκBα. A, Cells transfected with pCMV vector only. B, Cells cotransfected with pCMV-IκBα and pCMV-EGFP at a 3:1 ratio. After 6 wk of selection, all nuclei were stained using Hoechst 33342 dye (Sigma-Aldrich). C, Cytometric analyses showed that >86% of cells were positive for GFP. GFP positivity gradually increased to >90% and remained so for the duration of these experiments. D, Cytometric analyses of surface Fas/CD95 expression on nontransfected cells (red, nontransf.), on cells transfected with pCMV + EGFP only (blue, vector only), and on cells transfected with pCMV-IκBα + EGFP (green, IκBα-transf.). Cells were stained with PE-conjugated anti-Fas Ab; results are representative of four or more experiments, which showed no significant differences in Fas expression.

**FIGURE 8.** Stable transfection of pCMV-IκBα blocks the measurable degradation of IκBα and inhibits NF-κB activation. A, Western blot analyses of CH11-induced IκBα degradation: IκBα degradation in 3D10 cells transfected with pCMV vector (top) and IκBα degradation in 3D10 cells transfected with pCMV-IκBα (bottom). B, EMSA performed as above using pCMV-IκBα-transfected 3D10 cells. C, EMSA directly comparing Fas-induced control, nontransfected cells (C) with Fas-induced cells transfected with pCMV vector only (V) or with IκBα (I). As suggested by IκBα degradation in A, transfection with vector only did not affect CH11-induced NF-κB activation.
Discussion

Activation of NF-κB is now an accepted mechanism of protection from apoptosis for some cell types. Inhibition of NF-κB in such cells may lead to increased cell death through a variety of mechanisms. In this study, we present novel data that directly attribute Fas resistance of 3D10 eosinophils to NF-κB activation resulting from Fas ligation itself, and show that inhibition of the nuclear translocation of p65/p50 negates the Fas resistance of these cells.

Previous studies have demonstrated Fas-mediated NF-κB activation, but have dissociated one from the other. Using SV80 fibroblasts transfected with the cDNA encoding human Fas, Rensing-Ehl et al. (6) first demonstrated that anti-Fas induced NF-κB translocation to the nucleus. However, these cells were Fas-sensitive and inhibition of NF-κB had no effect on Fas-mediated cell death. Although NF-κB was activated by Fas ligation in resistant human bladder carcinoma T24 cells (7), again contrary to our observations, inhibition of NF-κB did not alter cell resistance or sensitivity. Furthermore, using sensitive Jurkat cells transfected with CD40-Fas fusion protein (CD40 extracellular domain and Fas intracellular and transmembrane domains), Ponton et al. (7) showed that stimulation of NF-κB binding activity by extracellular Fas ligation was unrelated to Fas sensitivity. Nevertheless, in agreement with our results, both of these studies (6, 7), as well as a recent report of CD40-induced NF-κB regulation of Bcl-2 family proteins (21), implicate the heterodimer p65/p50 as a prominent NF-κB complex in these interactions.

In another recent study, Fas ligation of dissociated cortical neuronoblasts was accompanied by nuclear translocation of the RelA/p65 subunit of NF-κB as detected by immunofluorescence (10). Nevertheless, ligation of Fas killed these cells, and condensed and fragmented apoptotic nuclei also were immunoreactive for p65, directly dissociating the Fas-mediated NF-κB activation from protection. Similarly, stimulation of TNFR or Fas on the surface of CEM-C7 T cells led not only to the activation of NF-κB, but to apoptotic death of the cells (9). Most recently, EMSA, as well as microarray analyses of the transcriptional effects of anti-Fas (and TNF-α) induction of HT29 colon carcinoma cells, confirmed activation of NF-κB (p65/p50) by Fas ligation (45). However, again NF-κB induction failed to protect these cells, and both TNF-α and anti-Fas induced cell death. In further contrast to our findings, the

FIGURE 9. Inhibition of NF-κB activation by IκBα overexpression abolishes Fas-resistance of 3D10 eosinophils. A, Western blot results of CH11-induced caspase-8 and caspase-3 cleavage in pCMV vector-transfected 3D10 cells. B, Western blot analyses of CH11-induced caspase-8 and caspase-3 cleavage in pCMV-IκBα-transfected 3D10 cells. C, CH11 killing assay on pCMV vector-transfected 3D10 cells. D, Anti-Fas survival assay using pCMV-IκBα-transfected 3D10 cells. Both cell lines were cultured at 1 × 10⁶ cells/ml and treated with 1 μg/ml CH11. A total of 1 μg/ml mouse IgM was used as the control for both assays. Cell survival was analyzed at 24, 48, or 72 h using cyotmetric measurement of hypotonic PI staining and expressed as percentage of viability ± SE.

FIGURE 10. Expression of antiapoptotic proteins in 3D10 cells transfected with pCMV vector only or with pCMV-IκBα. CH11 induction of NF-κB in cells transfected with pCMV vector only (left panel) had no discernable effects on expression on Bcl-xL, c-FLIP, or c-IAP2 (or on the control protein actin), but up-regulated both c-IAP1 and XIAP. In 3D10 cells transfected with pCMV-IκBα (right panel), no up-regulation of any protein occurred, but down-regulation of Bcl-xL, c-FLIP, and c-IAP did occur over the 12-h observation period. Results are representative of at least three separate experiments.
latter authors did not observe IκB degradation (for up to 4 h) after stimulation and suggested that anti-Fas treatment led to NF-κB activation through a different mechanism (45). In 3D10 eosinophils stimulated with anti-Fas, IκB-α degradation progressed through the 2-h time point (Fig. 9). Thus, it is possible that IκB degradation in response to Fas-ligation, and perhaps the protective capacity of Fas-mediated NF-κB activation, varies according to cell type.

Upstream mechanisms of Fas-induced activation of NF-κB are unknown, but receptor-associated proteins generally thought to be involved in NF-κB activation by TNFR family proteins include receptor-interacting proteins and TNFR-associating proteins (46). NF-κB inducing kinase may link death receptor signaling to the IκB kinases (47). As we have shown in this study, the kinetics of Fas-induced NF-κB (Fig. 5) differ substantially from those of TNF-α where maximum nuclear translocation could be seen 20–30 min or earlier (data not shown). Our data regarding the kinetics of Fas- and TNF-induced NF-κB activation confirm, in part, those of another direct comparison of Fas and TNF (9), and suggest that pathway intermediates in Fas-induced NF-κB activation may differ from those of TNF or other death receptors. Another molecule which associates with the cytoplasmic region of Fas is FAP-1. Indeed, FAP-1 is the only known molecule which associates with the negative regulatory domain of Fas (48, 49), and it is strongly expressed by 3D10 eosinophils (data not shown). Although the mechanism(s) by which FAP-1 inhibits apoptosis are still unclear, FAP-1 can interact with IκBα and enhance NF-κB activation (50). Current evidence suggests that Tyr42 phosphorylation of IκBα protects against its inducible degradation (51, 52). Nakai et al. (53) showed that FAP-1 enhanced NF-κB activation via the common neurotrophic receptor in transfected 293T cells. Furthermore, they have hypothesized that dephosphorylation of Tyr42 of IκBα by FAP-1 leads to an increase in the “receptivity” of IκBα for serine phosphorylation and subsequent NF-κB activation (53). We are currently investigating the potential role of FAP-1 in Fas-induced NF-κB in our eosinophil systems.

Finally, we are also continuing investigations of the transcriptional targets of Fas-induced NF-κB. Transcriptional profiling recently has suggested that among such genes, at least two (apoptosis inhibitor 2 (c-IAP2) and the cytoprotective manganese superoxide dismutase) are up-regulated by both TNF-α and anti-Fas signaling (44). The former belongs to the IAP family, which has been implicated in suppression of apoptosis induced by a variety of signals (20). These proteins can directly inhibit caspases in vitro, but their in vivo roles are largely undefined. In a study of TNF-induced apoptosis of transfected HT1080 fibrosarcoma cells, Wang et al. (54) found that activation of NF-κB blocked the activation of caspase-8. Furthermore, they demonstrated that c-IAP1 (and c-IAP2) may play roles in blocking the cleavage and activation of both caspase-8 and caspase-3. Both c-IAP1 and c-IAP2 have been shown to directly bind to caspase-3 and -7, and inhibited their proteolytic activation in a cell-free system (55). Recently, the susceptibility of human enterocytes to Fas-induced apoptosis was attributed to c-IAP1 and -2, and blockade of their synthesis with cycloheximide augmented Fas-mediated killing (56). XIAP also has been shown to be up-regulated by TNFR stimulation and to directly inhibit caspase-3 (and -7) in some cells (57). Our results support these observations, but the in vivo specificity of interactions (i.e., which IAP inhibits which caspase) within this group of caspases and inhibitors is still unclear.

Other antiapoptotic proteins, which may be transcriptional targets of NF-κB, but of controversial relevance in Fas-mediated cell death (58–61), include members of the growing mitochondria-associated Bcl-2 family. NF-κB can directly regulate the expression of prosurvival members such as Bcl-xL and are required for rescuing certain cell types from apoptosis (21–23). Although Fas-mediated apoptosis of some cells can bypass significant mitochondrial involvement and, thus, the antiapoptotic effects of Bcl-xL, in other cells Bcl-xL can contribute to inactivation of caspase-8 at the mitochondrial surface (62) or inhibit Fas-mediated apoptosis by preventing mitochondrial release of the IAP inhibitor, Smac/DIABLO (63). Although we have not observed consistent Fas-mediated up-regulation of Bcl-xL in wild-type 3D10 cells, a clear pattern of degradation or down-regulation was observed in the IκBα-transfected cells, and this could contribute to augmented death of these cells, as has been previously suggested (64). Finally, it is possible that the combined effect of several NF-κB-regulated proteins may be required for rescue from Fas-mediated apoptosis.

Whether blockade of Fas-induced apoptosis occurs in 3D10 eosinophils through antiapoptotic effects of Bcl-2 proteins, via caspase inhibition, or by some other mechanism(s), it is clear that in these cells, NF-κB activation is critical to cell survival following Fas ligation. This may have important implications in the therapeutic approaches using apoptotic machinery in both inflammatory diseases and hematological malignancies. Furthermore, these data suggest that the AML14.3D10 cell line may provide a useful model for studying antiapoptotic pathways involving NF-κB activation via TNF family receptor ligation.

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