CD95 (Fas)-induced Caspase-mediated Proteolysis of NF-κB

Rajani Ravi, Asheesh Bedi, Ephraim J. Fuchs, and Atul Bedi
Johns Hopkins Oncology Center, Division of Experimental Therapeutics and Pharmacology, Baltimore, Maryland 21287

Abstract

Activation of the nuclear factor (NF)-κB transcription factor is instrumental for the immune response and the survival of peripheral activated T cells. We demonstrate that ligation of CD95 (Fas/APO1), a potent apoptotic stimulus in lymphocytes, results in repression of NF-κB activity in Jurkat T cells by inducing the proteolytic cleavage of NF-κB p65 (Rel A) and p50. Inhibition of caspase-3-related proteases by a specific acetylated aldehyde (Ac-DEVD-CHO) prevented CD95-induced cleavage of p65 (RelA) or p50 and restored the inducibility of NF-κB in cells treated with an antibody against CD95. The addition of recombinant caspase-3 also resulted in proteolytic cleavage of RelA p65 and p50 in vitro. TNF-α treatment, unlike CD95 ligation, did not result in the death of Jurkat cells but did so in the presence of IκBαM, a transdominant inhibitor of NF-κB. These results suggest that intact, functional NF-κB maintains the survival of activated T cells, and that CD95-induced cleavage of NF-κB subunits sensitizes T cells to apoptosis and, hence, facilitates the decay of an immune response.

Introduction

The immune response entails a cascade of cellular and molecular events commencing with T-cell proliferation and the generation of effector T cells, and culminating with the death of the majority of activated T cells and persistence of a small cadre of T cells responsible for immunological memory (1). The activation and decay phases of the immune response involve certain key receptor-ligand interactions. Activation of T cells requires at least two signals (2). Signal one is generated by ligation of the clonotypic T-cell receptor by a complex of the antigenic peptide in the groove of a major histocompatibility complex molecule on the surface of the antigen-presenting cell. A second, or costimulatory, signal involves ligation of the CD28 receptor on the T cell by members of the B7 family of molecules expressed by the antigen-presenting cell. A prominent function of CD28 ligation in a primary immune response is the maintenance of T-cell survival until decay of the immune response (3). Apoptosis of ATCs occurs upon ligation of the CD95 (Fas/APO-1) cell surface receptor, a member of the TNF receptor superfamily, whose ligand, CD95L, is expressed on activated but not resting T cells (4). CD95L interactions are required for T-cell homeostasis, because mice deficient in either molecule accumulate excessive numbers of CD4+CD8−, B220+ T cells that are otherwise marked for apoptosis (5).

As the signals responsible for T-cell activation and apoptosis become better defined, the intracellular mediators of these signals are increasingly sought after as targets for therapeutic intervention. One important mediator of the immune response is NF-κB, a family of heterodimeric transcription factors that has a well-established role in the development and activation of T cells (6–9). The survival of peripheral ATCs is also dependent upon NF-κB activity because excessive ATC apoptosis is observed in transgenic mice expressing constitutively high levels of IκBα, an inhibitor of NF-κB (9, 10). NF-κB comprises two subunits (p65 or RelA and p50), which are held together by two molecules of IκBα (11, 12). Although the events leading to NF-κB activation upon T-cell activation have been well defined, the signals leading to its decay at the tail-end of an immune response have yet to be elucidated. One possibility is that signals through CD95 are somehow involved with its repression. The CD95L promoter contains an NF-κB binding site, and expression of CD95L is induced upon T-cell activation (4). CD95 ligation initiates the sequential activation of a cascade of cysteine proteases, or caspases, which are evolutionarily conserved mediators of cell death in response to diverse stimuli (16–21). Putative recognition sequences for caspase-3-related proteases are present in the amino-acid sequences of NF-κB p65 (RelA) and NF-κB p50 (22–24). These observations prompted us to investigate the possibility that CD95-transduced signals may be responsible for the autoregulatory repression of NF-κB in ATCs by caspase-mediated cleavage of RelA p65 or p50.

Materials and Methods

Cell Lines. A transdominant negative NH2- and COOH-terminal phosphorylation mutant of IκBα (IκBαM) has been shown to repress transcriptional activation mediated by all five NF-κB/Rel subunits (25, 26). The generation of Jurkat T cells transduced with the IκBαM retrovirus (pLixBαMSN) has been described previously (26). IκBαM was constructed by combining the NH2- and COOH-terminal phosphorylation mutants of IκBα into a single cDNA (26). Jurkat cells expressing IκBαM (IκBαM Jurkat) and control cells transduced with empty vector (Jurkat-LXSN) were a generous gift from Drs. Douglas Green (La Jolla Institute for Allergy and Immunology, La Jolla, CA) and Inder M. Verma (Salk Institute, La Jolla, CA; Ref. 26). IκBαM expression in pools of infected cells was confirmed by immunoblot analysis of IκBα; as described previously, murine IκBαM migrated faster than endogenous IκBα on SDS-PAGE (26). Control Jurkat cells were grown in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum and 100 units/ml penicillin/streptomycin. IκBαM Jurkat cells were grown in the same medium supplemented with G418 (500 μg/ml).

Assessment of Apoptosis in Response to TNF-α or Anti-CD95 Antibody. Jurkat cells transduced with IκBαM (IκBαM Jurkat) or empty LXSN vector (control Jurkat; 5 × 10⁴/ml) were treated with permutations of recombinant human TNF-α (100 ng/ml; Genzyme Diagnostics, Cambridge, MA), anti-human CD95 antibody (1 μg/ml; United Biomedical, Inc., Lake Placid, NY), and PHA (10 μM), as described. Cells were assessed for apoptosis 6 and 24 h later by Hoechst staining of nuclei and survival by trypan blue dye exclusion. The cell viability was measured by scoring at least 200 cells in each group, and the average percentage of viability was calculated from three different experiments.
EMSA of NF-κB DNA Binding Activity. Nuclear extracts were prepared from Jurkat T cells, as described (27). Cells were washed with PBS and buffer A (10 mM HEPES (pH 7.9), 1.5 mM MgCl₂, 10 mM KCl, and 0.5 mM DTT) at 4°C. Cells were resuspended in buffer A (supplemented with 0.1% NP40, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 10 μg/ml pepstatin, and 1 mM PMSF), incubated on ice for 15 min, and subjected to centrifugation at 2000 rpm for 10 min. Following removal of the supernatant fraction (cytosolic proteins), pellets (crude nuclei) were lysed with buffer B (20 mM HEPES (pH 7.9), 20% (v/v) glycerol, 0.42 M NaCl, 1.0 mM EDTA, 0.5 mM PMSF, and 0.5 mM DTT), incubated on ice for 30 min, and centrifuged at 12,400 rpm. The supernatant (nuclear extract) was collected, and protein concentrations were measured by standard bicinchoninic acid assay (Pierce Chemical Company, Rockford, IL).

Oligonucleotides (Santa Cruz Biotechnology, Inc.) containing the NF-κB/Rel consensus binding site (5'-GGG GAC TTT CCC-3') and mutants containing a G→C substitution in the consensus sequence (5'-GGC GAC TTT CCC-3') were 5' end-labeled using poly nucleotide kinase and [γ-32P]ATP. Nuclear extracts (10 μg protein) were incubated at 25°C for 20 min with 1 μl of labeled probe (20,000 cpm) in incubation buffer (10 mM NaCl, 1 mM EDTA, 1 mM β-mercaptoethanol, 2% glycerol, and 2 μg of poly(dl-dC)], and subjected to 5% PAGE. Labeled probe without protein was used as a negative control for each EMSA reaction. The specificity of NF-κB binding to the labeled probe (NF-κB consensus-binding site) was further confirmed by competition with excess unlabeled oligonucleotide.

Effect of Anti-CD95 Antibody on TNF-α- or PHA-stimulated NF-κB Activity. Jurkat cells transfected with either IκBαM (IκBαM Jurkat) or empty LXS vector (control Jurkat) were preincubated with or without 1 μg/ml anti-CD95 antibody for 15 min, followed by the addition of either 100 ng/ml human TNF-α or 10 ng/ml PHA. Cells in each group were harvested after 15, 30, and 60 min, and nuclear extracts were analyzed for NF-κB activation by EMSA.

Effect of Caspase-3-related Proteases on NF-κB Activity. p65 (Rel A), and p50. Jurkat T cells (10⁶/ml) were preincubated for 1 h with or without an inhibitor of caspase-3-related proteases [Ac-DEVD-CHO (Bachem, Torrance, CA; 300 μM)], followed by the addition of 1 μg/ml anti-CD95 antibody 30 min before exposure to 10 ng/ml PHA. NF-κB DNA binding activity was measured in each group using EMSA as described above.

Lysates from control (nonapoptotic) Jurkat cells (100 μg) were treated with recombinant caspase-3 for 1 h at 37°C in 20 μl of incubation buffer (20 mM HEPES (pH 7.5), 10 mM KCl, 1.5 mM MgCl₂, 1 mM sodium EGTA, 1 mM sodium EDTA, 20% (v/v) glycerol, 0.1% BSA, 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 10 μg/ml pepstatin A); Reactions were terminated by the addition of 20 μg of Laemmli buffer and subjected to immunoblot analyses for p65 (Rel A) and NF-κB p50. Recombinant caspase-3 was a generous gift from Drs. Donald W. Nicholson and Nancy A. Thornberry, Merck Frosst Center for Therapeutic Research (Pointe Claire-Dorval, Quebec, Canada; apopain, 40.6 units/μl; 0.15 unit/μg; 1 unit represents 1 pmol aminomethylcoumarin liberated from Ac-DEVD-aminomethylcoumarin per minute at 25°C at saturating substrate concentration; Ref. 22).

Immunoblot Analysis of NF-κB (p65 or p50) and IκBα. Cytosolic protein was prepared by lysing cells in lysis buffer (10 mM HEPES (pH 7.9), 1.5 mM MgCl₂, 10 mM KCl, 0.5 mM DTT, 0.1% NP40, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 10 μg/ml aprotinin, and 10 μg/ml pepstatin) for 15 min on ice. Cytosolic protein (supernatant fraction) was isolated by centrifugation at 15,000 × g for 10 min at 4°C. Whole-cell lysates were prepared by the addition of lysis buffer [50 mM Tris (pH 7.5), 137 mM NaCl, 5 mM MgCl₂, 1% Triton X-100, 50 mM β-glycerophosphate, 2 mM EDTA, 10 mM EGTA, 1 mM DTT, 1 mM NaVO₄, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 10 μg/ml pepstatin, and 1 mM PMSF], incubation on ice for 20 min, and centrifugation at 15,000 × g for 10 min at 4°C. Protein samples were quantified by biocinchoninic acid assay (Pierce). Fifty μg of cytosolic or whole-cell protein samples were subjected to 12.5% SDS-PAGE, transferred to nitrocellulose, and probed with antibodies against the COOH terminus of IκBα, the NH2 terminus of NF-κB p65 (RelA), or the nuclear localization signal of NF-κB p50, respectively (Santa Cruz Biotechnology). Immunoblots were developed with horseradish peroxidase-conjugated anti-rabbit IgG antibody (Santa Cruz Biotechnology) using enhanced chemiluminescence (Amer sham, Arlington Heights, IL).

Results

In cells that are resistant to TNF-α-mediated death, protection from apoptosis is mediated by TNF-α-mediated activation of NF-κB (25, 26, 28, 29). Thus, although Jurkat cells resist TNF-α, they are sensitized to TNF-α-induced apoptosis by transduction of IκBαM (Fig. 1). In contrast, control Jurkat and IκBαM Jurkat cells were equally susceptible to CD95-induced apoptosis (Fig. 1). Treatment with activators of NF-κB, including TNF-α or PHA, did not inhibit CD95-induced death (Fig. 1).

The effect of CD95 ligation on TNF-α- or PHA-induced activation of NF-κB was examined in Jurkat T cells transfected with IκBαM (IκBαM Jurkat) or pLXS (control Jurkat) using EMSAs. Treatment of control Jurkat cells with 100 ng/ml TNF-α or 10 ng/ml PHA resulted in rapid activation of NF-κB (Figs. 2 and 3). The specificity of NF-κB binding to the DNA consensus sequence was confirmed by competition with excess wild-type unlabeled oligonucleotide and the absence of binding to a mutant (G→C substitution) κB oligonucleotide (Fig. 3). IκBαM Jurkat cells failed to induce NF-κB in response to either agent (Fig. 2). Pretreatment with anti-CD95 antibody resulted in repression of TNF-α- or PHA-induced NF-κB activation, such that binding to the κB consensus oligonucleotide in control Jurkat cells was similar to the binding activity in IκBαM-transduced cells treated identically (Figs. 2 and 3).

CD95 ligand-dependent recruitment of FADD/MORT1 (Fas-associated protein with death domain) results in the formation of Fas/FADD complexes that transduce the sequential activation of a cascade of cysteine proteases (caspases) including FLICE (caspase-8), interleukin 1β converting enzyme (ICE/caspase-1), and CPP32β (caspase-3; Refs. 19–21). Because caspase-3 is required for CD95-
induced apoptosis, we investigated whether CD95-mediated repression of NF-κB involves caspase-3-related proteolysis (22). Pretreatment with Ac-DEVD-CHO, a specific inhibitor of caspase-3-related proteases, restored the TNF-α- or PHA-stimulated activation of NF-κB in Jurkat cells in the presence of anti-CD95 antibody (Fig. 3). These results indicate that CD95-induced repression of NF-κB requires caspase-3-related proteolysis. Activation of NF-κB by TNF-α or PHA involves phosphorylation of IκBα at serine residues 32 and 36 and subsequent degradation by ubiquitin-mediated proteolysis (11–13). NH2-terminal deletions of IκBα, resulting in loss of the critical phosphorylation residues, result in truncated forms of IκBα that resist degradation and function as trans-dominant repressors of NF-κB (25, 26). Previous reports have hypothesized that such an NH2-terminal truncated form of IκBα may be generated from cleavage of IκBα by interleukin 1β converting enzyme-like proteases (30). To determine whether caspase-3-related proteolytic cleavage of IκBα was involved in CD95-induced repression of NF-κB, immunoblot analyses of IκBα were conducted on cytosolic fractions of Jurkat cells stimulated with TNF-α in the presence or absence anti-CD95 antibody. TNF-α treatment resulted in the rapid degradation of endogenous IκBα within 15 min of exposure (Fig. 4A). Concomitant exposure to anti-CD95 antibody had no detectable effect on TNF-α-stimulated degradation of IκBα (Fig. 4A). Treatment of Jurkat cells with anti-CD95 antibody alone did not induce either cleavage or degradation of IκBα (Fig. 4A). These results indicated that CD95-induced caspase-mediated repression of NF-κB was not due to prevention of IκBα degradation or cleavage of IκBα into a transdominant repressor.

Caspase-3-related proteases have been shown to recognize and cleave critical substrates that contain a DXDD consensus tetrapeptide motif (22). Putative recognition sequences for caspase-3-related proteases are present in the amino-acid sequence of NF-κB p65/RelA (ASVD469-N470; DTDD294-R295, DCDR379-G380) and NF-κB p50 (DVSD370-S371;Refs. 23 and 24). This raised the possibility that CD95-induced activation of caspase-3-related proteases results in cleavage of RelA p65 and/or p50. To determine whether p65 (RelA) or p50 is cleaved by caspase-3-related proteolysis, immunoblot analyses were conducted on whole-cell lysates of Jurkat cells treated with anti-CD95 antibody in the presence or absence of Ac-DEVD-CHO, using antibodies directed against either the NH2 terminus of human p65 (RelA) or the nuclear localization signal of NF-κB p50. Treatment with anti-CD95 antibody resulted in COOH-terminal cleavage of an Mr ~10,000 fragment to generate a truncated RelA product of Mr ~55,000 (Fig. 4B). p65 cleavage was induced within 1 h of exposure to anti-CD95 antibody and was inhibited by Ac-DEVD-CHO (Fig. 4B). In addition, anti-CD95 antibody also induced caspase-mediated proteolysis of the NF-κB p50, resulting in the cleavage of an Mr ~15,000 COOH-terminal fragment and generation of a truncated product of Mr ~35,000 (Fig. 4B). In contrast, IκBα lacks the consensus DXDD motifs and was not cleaved in response to CD95 ligation (Fig. 4A). These observations indicate that CD95-induced repression of NF-κB involves caspase-mediated cleavage of both p65/RelA and p50.

To directly address whether RelA p65 or p50 are substrates for caspase-3 proteolysis, lysates from control (nonapoptotic) Jurkat cells were incubated with recombinant caspase-3 in vitro (22). Efficient cleavage of Rel A p65 and p50 was observed within 2 h of incubation with caspase-3, with generation of RelA or p50 fragments that comigrated with the product obtained from CD95-induced apoptotic cells (Fig. 4B). Caspase-3-mediated cleavage of p65 and p50 in control cell lysates was inhibited by co-incubation with Ac-DEVD-CHO.

Discussion

The decay of an immune response is characterized by apoptosis of the majority of T cells that were activated by the immunogen. In this
Fig. 4. A. CD95/Fas/APO-1 ligation does not alter IxBa degradation or induce IxBa proteolysis. Immunoblot analysis of Jurkat cells treated with anti-CD95/Fas antibody (1.0 μg/ml) with or without TNF-α (50 ng) is shown. Cells were treated for the indicated times, and cytoplasmic protein extracts were resolved by 12.5% SDS-PAGE. After transfer to nitrocellulose, the blots were probed with an antibody against the COOH terminus of IxBa. B. CD95/Fas-induced caspase-mediated cleavage of NF-κB p65 (RelA) and NF-κB p50. Immunoblot analysis of Jurkat cells treated with anti-CD95/Fas antibody (1.0 μg/ml) in the presence or absence of Ac-DEVD-CHO (300 μM) is shown. Control cell lysates were also treated with recombinant caspase-3 (rec-caspase-3; apopain) in vitro. Protein extracts were subjected to 10% SDS-PAGE, and blots were probed with an antibody against the NH2 terminus of NF-κB p65 (RelA) or the nuclear localization sequence of NF-κB p50. Arrows, positions of the full-length NF-κB p65 (RelA) or p50 and the truncated products of RelA (Δ RelA; Mr, 55,000) and p50 (Δ NF-κB; Mr, 35,000).

In this study, we have characterized an important molecular mechanism by which CD95/Fas meditates apoptosis of activated T cells. CD95 belongs to the TNF receptor superfamily which includes TNF-α, a potent activator of NF-κB (4). Most members of the TNF receptor family contain a TRAF domain that mediates activation of NF-κB (29, 31). TRAF2 signaling results in phosphorylation of IκBα and subsequent proteosomal degradation (29, 31). The released NF-κB enters the nucleus and activates genes possessing the NF-κB-binding consensus sequence, including proteins that protect cells from TNF-induced cytotoxicity (25, 26, 28, 29). In contrast, CD95/Fas does not contain a TRAF domain and consequently lacks the ability to activate NF-κB (4). In addition to its inability to stimulate NF-κB, our results indicate that CD95 actually represses NF-κB via caspase-mediated cleavage of NF-κB p65 (RelA) and p50. This novel mechanism of terminating NF-κB-dependent transcription of genes that promote cell survival may underlie the increased potency of CD95-mediated apoptosis in comparison to other members of its TNF receptor superfamily. CD95-mediated repression of NF-κB activity may not only be a key component for induction of activation induced T-cell death but may also be an important autoregulatory mechanism by which CD95 terminates the expression of NF-κB-driven lymphokines and receptors responsible for the immune response. A proposed schematic of such an autoregulatory loop between NF-κB and CD95 is shown in Fig. 5. Therefore, the activity of caspase-3-related proteases may determine the duration of NF-κB activity in activated T or B cells and hence play a critical role in the duration and potency of an immune response.

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References

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