Tumor Necrosis Factor-α-induced Cell Killing and Activation of Transcription Factor NF-κB Are Uncoupled in L929 Cells*

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The cytokine TNF-α plays a pivotal role in a variety of inflammatory, immunological, and pathological processes. Two well-studied receptors for TNF-α are TNF-R55 and TNF-R75. These receptors belong to the rapidly growing family of TNF receptors, including the recently discovered LT-β receptor, DR3/WSL-1/TRAMP, RANK, and TRAIL (for reviews, see Refs. 1 and 2). Binding of the extracellular ligand leads to trimerization of the receptors and association of numerous cytoplasmic proteins to the intracellular receptor domain. This binding is induced by ligation of the TNF-R55 or clustering of the intracellular C-terminal domains, which are sufficient for the induction of cell death, which coincides with the activation of transcription factor NF-κB (3).

NF-κB is normally retained in its inactive state in the cytoplasm of cells by interaction with an inhibitory IκB molecule (for review, see Ref. 4). Triggering of cells with a variety of inflammatory cytokines, including TNF-α, induces phosphorylation, ubiquitinylation, and degradation of IκB (for reviews, see Refs. 5 and 6). This allows the DNA-binding dimer to enter the nucleus, to bind to its cognate DNA, and to induce the transcription of its target genes (for a review, see Ref. 7). The target genes encode a variety of proteins involved in immune responses, cell growth, and apoptosis. Among those are the anti-apoptotic proteins Mn-superoxide dismutase, the zinc finger protein A20, and c-IAP2 (8). Previous studies showed that the activation of NF-κB can counteract TNF-α-induced cell death (for a review, see Ref. 9). Fibroblasts from mice lacking the transactivating p65 subunit are more sensitive against the cytopathic effect of TNF-α than fibroblasts from p65<sup>−/−</sup> mice (10). The inhibition of NF-κB in various cell types by stable overexpression of a transdominant negative form of IκBα renders those cells more susceptible to cell killing by TNF-α (11–13). However, NF-κB has also apoptosis-promoting activities. Expression of high levels of the NF-κB subunit c-Rel in bone marrow cells leads to apoptosis and autophagocytic cell death (14). Accordingly, the overexpression of the viral and cellular anti-apoptotic proteins E1B 19K and Bcl-2 impairs NF-κB-dependent transactivation (15, 16).

In this study, we investigated the impact of NF-κB activation on TNF-α-mediated cell killing in murine L929 fibrosarcoma cells. This widely used cell line is of particular interest because TNF-α-induced cell death occurs without co-apoptotic stimuli, such as cycloheximide or actinomycin D. Using several different strategies, we demonstrated that TNF-α-triggered cell killing and activation of transcription factor NF-κB are uncoupled in L929 cells.

EXPERIMENTAL PROCEDURES

Cell Culture and Stable Transfections—Murine L929 fibrosarcoma cells were maintained in Dulbecco’s modified Eagle’s medium containing 10% FCS and 1% (v/v) penicillin/streptomycin. All cells were grown in an incubator at 37 °C and 5% CO₂. The plasmid RSV-IκB<sub>α</sub>-S32/36A (17) into the HindIII fragment from the plasmid CMV-IκB<sub>α</sub>-S32/36A (17) into the HindIII site of a eukaryotic RSV expression vector. The NF-κB-dependent reporter gene construct p(IL6<sub>350hu</sub>)-350hu.IL6 was described previously (18). The plasmids were purified on CsCl gradients, and the L929 cells were transfected with CaCl₂ as described (16). Stably transfected cells were selected in 200 µg/ml G418 for several weeks.

Electrophoretic Mobility Shift Assays (EMSA)—Murine L929A fibrosarcoma cells were stimulated with recombinant TNF-α (Boehringer Mannheim) for 20 min at the indicated concentrations, washed twice with cold TBS buffer (25 mM Tris-HCl, pH 7.4, 137 mM NaCl, 5 mM KCl, 0.7 mM CaCl₂, 0.1 mM MgCl₂), and scraped off with a rubber policeman. After centrifugation for 3 min at 3000 rpm, the total cellular proteins were extracted from the pellet by resuspension in TOTEX buffer (20 mM Heps/KOH, pH 7.9, 0.5% NaCl, 20% (v/v) glycerol, 1% (v/v) Nonidet P-40, 1 mM MgCl₂, 0.5 mM EDTA, 0.1 mM EGTA, 1 mM phenylmethylsulfonyl fluoride). The tubes were incubated on ice for 30 min, and equal amounts of the protein contained in the supernatant were tested for NF-κB DNA binding activity as described (19).

Western Blot Analysis and Luciferase Assays—For Western blotting, the proteins were separated by SDS-polyacrylamide gel electrophoresis and transferred onto a polyvinylidene difluoride membrane (Millipore, Bedford, MA) using a semidry blot apparatus (Bio-Rad). The detection of IκB-α proteins was done as described (19). For the determination of

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Role of NF-κB in TNF-α-induced Killing of L929 Cells

RESULTS

BA Reduces TNF-α-induced Cytotoxicity but Not the Activation of NF-κB—To study the linkage between TNF-α-induced NF-κB activation and cell death in L929 cells, we investigated the effect of BA, which prevents the generation of mitochondrial permeability transition pores during the apoptotic process (21). The addition of 50 μM BA clearly decreased the cytotoxic effects of various concentrations of TNF-α on L929 cells (Fig. 1A). In contrast, the TNF-α-induced DNA binding activity of NF-κB was not influenced by BA at various concentrations (Fig. 1B). The influence of BA on TNF-α-stimulated NF-κB-dependent transcription was tested by adding BA, TNF-α, or a combination of both to a pool of L929 cells stably transfected with a NF-κB-dependent luciferase reporter gene. Subsequently, the luciferase activity was determined. Bars indicate S. D.

A

B

C

FIG. 1. Influence of BA on TNF-α-induced cell killing and NF-κB activation. A, the indicated concentrations of TNF-α were added alone or together with 50 μM BA to L929 cells. After 20 h, cell viability was determined using the MTT assay. Bars indicate the S. D. from three experiments. B, L929 cells were incubated for 20 min with the indicated combinations of TNF-α and/or BA. Subsequently, total cell extracts were prepared and tested for DNA binding of activated NF-κB by EMSA. The filled arrowhead indicates the location of the DNA-NF-κB complex, the circle indicates the position of a constitutively DNA-binding protein, and the open arrowhead points to the unbound oligonucleotide. C, TNF-α and/or BA were incubated at the indicated combinations for 8 h with a pool of L929 cells stably transfected with a NF-κB-dependent luciferase reporter gene. Subsequently, the luciferase activity was determined. Bars indicate S. D.
time point, suggesting that DEX interferes in L929 cells with NF-κB-mediated transactivation rather than detectably influencing the IκB-a levels. The effect of DEX on TNF-α-induced cell death was tested by adding DEX, TNF-α, or a combination of both to the cells. When cell viability was measured after various incubation times (Fig. 2C), it was found that DEX significantly protected the L929 cells from cell death, indicating that NF-κB activation does not protect this cell line from TNF-α-induced cell death.

Stable Overexpression of IκB-a Abrogates TNF-α-induced Transcription of IL-6 without Affecting Cell Killing—In order to inhibit NF-κB in a highly specific way, L929 cells were stably transfected with the plasmid RSV-IκB-a S32A/S36A, an expression vector encoding a transdominant negative human IκB-a mutant. Because serines 32 and 36 in this mutant were changed to alanines, the inducible phosphorylation and subsequent degradation was prevented, thus conferring an increased half-life to this IκB-a form (17). Several L929 cell clones were tested for the expression of IκB-α protein in Western blot experiments (Fig. 3A). Three of the cell clones (2D1, 3A6, and 3B1) were found to constitutively express the transdominant negative form of human IκB-α, which migrates slightly slower than the endogenous murine IκB-α protein from L929 cells. L929 control cells that were stably transfected with the empty expression vector did not contain this slower migrating IκB-α band (Fig. 3A). These IκB-α-overexpressing cell clones were
initiated by the trimerization of the TNF receptor. This leads to the induced association of the intracellular TRADD protein and subsequent recruitment of further proteins, including TRAF-2 and MORT1/FADD. The molecular events leading to NF-κB activation and cell killing diverge relatively early in this signal cascade. The expression of a transdominant negative form of FADD inhibits TNF-R55-mediated cell death but not κB-dependent transcription (26). TRAF-2-deficient mice display a functional NF-κB activation and an increased sensitivity to TNF-α, suggesting an additional NF-κB-independent pathway of cell protection that is mediated by TRAF-2 (27).

In this study, we show that the TNF-α-triggered activation of NF-κB and the induction of cell death are uncoupled events in L929 cells. A protective role of NF-κB would imply that the inhibition of this transcription factor would lead to enhanced cell death. However, even the partial inhibition of NF-κB transactivation by DEX was accompanied by a decrease in cell killing. In the case of L929 cells, the DEX-mediated repression of NF-κB seems to be independent from up-regulation of IκB and can probably be attributed to the recently proposed repression of transactivation (28). Conversely, the activation of NF-κB does not promote killing of L929 cells, because BA allowed the full activation of NF-κB while reducing the cytotoxic effects of TNF-α. Evidence for the uncoupling of NF-κB activation and cytotoxicity in L929 cells was obtained by the unchanged susceptibilities to cell death in cell lines stably overexpressing a transdominant negative variant of IκB-α. The role of NF-κB as a promoter or attenuator of cell killing may therefore depend on the nature of the apoptosis-inducing stimulus as well as on the cell type. Accordingly, the inhibition of NF-κB activation by IκB-α overexpression did not alter the sensitivity of the human breast cancer cell line MCF7 toward TNF-α (29), whereas the HT1080 fibrosarcoma cells and Jurkat T-cells were more susceptible to the detrimental effects of TNF-α (12, 13).

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Fig. 4. TNF-α-induced IL-6 production and cell killing of IκB-α-overexpressing cell clones. A, the indicated cell clones were stimulated for 10 h with 2000 units/ml TNF-α, and subsequently, IL-6 production was measured by enzyme-linked immunosorbent assay. Bars indicate the S. D. obtained from four independent experiments. B, the indicated cell clones were treated with TNF-α for the indicated periods. Cell viability was determined with the MTT assay. The viability of untreated cells was set as 100%. A representative experiment is shown.

The role of NF-κB in the regulation of apoptosis does not yield a homogeneous picture yet. There are numerous examples showing apoptosis-promoting as well as apoptosis-antagonizing effects of NF-κB activation (for a review, see Ref. 9). Some inducers of cell death, such as TNF-α, directly activate NF-κB; others may influence κB-dependent gene expression by alternative pathways. In this respect, it was shown that an activated form of Caspase-3 can cleave the unphosphorylated IκB-α protein, which leads to the generation of a constitutive inhibitor of κB-dependent gene expression (25). The events leading to TNF-α-triggered cell death and NF-κB activation are further characterized by testing the TNF-α-induced DNA binding activity of NF-κB by EMSAs. The induced DNA binding capacity of all IκB-α-overexpressing clones was strongly impaired when compared with L929 control cells (Fig. 3B and data not shown). The effect of IκB-α overexpression on the transcription of the endogenous NF-κB target gene IL-6 was tested by measuring the TNF-α-triggered production of this cytokine. In contrast to the L929 control cells, treatment of the three IκB-α-overexpressing cell clones with TNF-α did not result in strongly elevated IL-6 levels (Fig. 4A). In summary, these results demonstrate that the L929 cell clones 2D1, 3A6, and 3B1 are unable to significantly activate the transcriptional activity of NF-κB. However, TNF-α killed these cell clones with the same efficiency and kinetics as the L929 control cells (Fig. 4B), indicating that NF-κB activation neither activates nor represses TNF-α-mediated cell killing, thus establishing that both events are unrelated and uncoupled in L929 cells.

DISCUSSION

The role of NF-κB in the regulation of apoptosis does not yield a homogeneous picture yet. There are numerous examples showing apoptosis-promoting as well as apoptosis-antagonizing effects of NF-κB activation (for a review, see Ref. 9). Some inducers of cell death, such as TNF-α, directly activate NF-κB; others may influence κB-dependent gene expression by alternative pathways. In this respect, it was shown that an activated form of Caspase-3 can cleave the unphosphorylated IκB-α protein, which leads to the generation of a constitutive inhibitor of κB-dependent gene expression (25). The events leading to TNF-α-triggered cell death and NF-κB activation are
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