IxBα Overexpression in Human Breast Carcinoma MCF7 Cells Inhibits Nuclear Factor-κB Activation but Not Tumor Necrosis Factor-α-induced Apoptosis*

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Nuclear factor-κB (NF-κB) is one of major component induced by tumor necrosis factor-α (TNF), and its role in the signaling of TNF-induced cell death remains controversial. In order to delineate whether the involvement of NF-κB activation is required for triggering of the apoptotic signal of TNF, we inhibited the nuclear translocation of this transcription factor in TNF-sensitive MCF7 cells by introducing a human MAD-3 mutant cDNA coding for a mutated IxBα that is resistant to both phosphorylation and proteolytic degradation and that behaves as a potent dominant negative IxBα protein. Our results demonstrated that the mutated IxBα was stably expressed in the transfected MCF7 cells and blocked the TNF-induced NF-κB nuclear translocation. Indeed, TNF treatment of these cells induced the proteolysis of only the endogenous IxBα but not the mutated IxBα. The nuclear NF-κB released from the endogenous IxBα within 30 min of TNF treatment was rapidly inhibited by the mutated IxBα. There was no significant difference either in cell viability or in the kinetics of cell death between control cells and the mutated IxBα transfected cells. Furthermore, electron microscopic analysis showed that the cell death induced by TNF in both control and mutated IxBα transfected cells was apoptotic. The inhibition of NF-κB translocation in mutated IxBα-transfected cells persisted throughout the same time course that apoptosis was occurring. Our data provide direct evidence that the inhibition of NF-κB did not alter TNF-induced apoptosis in MCF7 cells and support the view that TNF-mediated apoptosis is NF-κB independent.

Cytokine-dependent activation of transcription factors such as NF-κB is one of the mechanisms by which signals are transmitted from the extracellular surface to the nucleus to enhance the transcription of specific genes (1, 2). The activation of cytoplasmic NF-κB heterodimer consisting of p50 and p65 polypeptides has been shown to require the degradation of a putative dominant negative IxB protein. Our results demonstrated that the mutated IxBα was stably expressed in the transfected MCF7 cells and blocked the TNF-induced NF-κB nuclear translocation. Indeed, TNF treatment of these cells induced the proteolysis of only the endogenous IxBα but not the mutated IxBα. The nuclear NF-κB released from the endogenous IxBα within 30 min of TNF treatment was rapidly inhibited by the mutated IxBα. There was no significant difference either in cell viability or in the kinetics of cell death between control cells and the mutated IxBα transfected cells. Furthermore, electron microscopic analysis showed that the cell death induced by TNF in both control and mutated IxBα transfected cells was apoptotic. The inhibition of NF-κB translocation in mutated IxBα-transfected cells persisted throughout the same time course that apoptosis was occurring. Our data provide direct evidence that the inhibition of NF-κB did not alter TNF-induced apoptosis in MCF7 cells and support the view that TNF-mediated apoptosis is NF-κB independent.

Genes (3–5). Several proteins, collectively termed IxB, share the property of retaining NF-κB dimers and preventing their translocation to the nucleus (6). To date, the most extensively studied IxB protein is IxBα (37 kDa) encoded by the human MAD-3 gene or its homologues in different species (7). The mechanisms that lead to the degradation of IxB proteins are poorly understood, but involve changes in the phosphorylation state of IxB (8, 9). Two serines in the N-terminal domain of IxBα, Ser-32 and Ser-36, were shown to be critical for IxBα stability. Substitution of Ser-32 and Ser-36 by alanine residue rendered IxBα undegradable by cellular activators (10–12). Among the many proteins exhibiting IxB function, IxBα is the only inhibitor that in response to cell stimulation dissociates from the NF-κB heterodimer complex, with kinetics matching NF-κB translocation to the nucleus (13, 14). It was therefore suggested that the inducible activation of NF-κB is mainly regulated by NF-κB/IxBα dissociation (6, 9, 15).

Tumor necrosis factor-α (TNF),1 originally described for its antitumor activity, is one of the cytokines known to activate NF-κB within minutes, leading to the transcriptional activation of various important cellular and viral genes (16, 17). The activation of NF-κB is considered integral to the transfer of the TNF signal to the nucleus (18). Both TNF receptors (p55 TNF-R1 and p75 TNF-R2) independently mediate NF-κB activation by TNF (19–22). The nature of signaling mechanisms mediating the effects of TNF on NF-κB activation remains poorly defined. It has been shown that TNF first activates phosphatidylcholine-specific phospholipase C and leads to the sequential activation of an acidic sphingomyelinase and the production of ceramide, which in turn causes the activation of NF-κB (23, 24). Mutagenesis studies have identified an 80-amino acid region within the cytoplasmic domain of p55 TNF-R1 that is required for initiation of both apoptosis and NF-κB activation (25). However, several recent studies debated the involvement of NF-κB activation in TNF-induced apoptosis. The report of Dhaibo et al. (26) suggested that ceramide mediated the effects of TNF on growth inhibition of Jurkat lymphoblastic leukemia cells, but was unable to activate NF-κB. In addition, TNF was reported to be capable of activating NF-κB in different cell models resistant to its cytotoxic action (27, 28). In order to directly examine whether the NF-κB activation is an essential requirement for triggering the apoptotic signal of TNF, we chose an approach based on the inhibition of the translocation of this transcription factor by introducing a dominant-negative human MAD-3 mutant construct into the TNF-sensitive MCF7 cells. In the present report, we describe the consequences of the stable expression of the mutated IxBα on NF-κB activation and TNF-mediated cell killing.

1 The abbreviations used are: TNF, tumor necrosis factor-α; EMSA, electrophoretic mobility shift assay.

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Experimental Procedures

Transfection of MCF7 Cells with Mutated MAD-3 cDNA and Cell Culture—The MAD-3 double point mutant (positions 32 and 36) construct was described by Traeckner et al. (11) and was a kind gift by Patrick A. Baeuerle, Tularik, Inc., San Francisco. The empty vector used for the generation of control cells was the pcDNA3 purchased from Invitrogen. The transfection of human breast carcinoma cell line MCF7 with the expression constructs was performed by the calcium phosphate precipitation method (29). 1000 cells were plated per 10-cm tissue culture plates. After 10–14 days selection in growth medium containing 200 µg/ml G418 (Sigma), four to five resistant colonies were isolated from each plate and examined for IκBα expression by Southern blot analysis, and the positive clones were maintained in culture medium with 100 µg/ml G418 for more than 2 months. All cell lines were routinely cultured in RPMI 1640 medium containing 5% fetal calf serum, 1% penicillin-streptomycin, 1% l-glutamine at 37 °C in a humidified atmosphere with 5% CO₂.

Determination of Cell Viability—Cells viability was determined using crystal-violet staining method as described previously (28). Absorbance (A), which was proportional to cell viability, was measured at 540 nm. TNF-mediated cell lysis was assessed by comparing the viability of untreated cells with that of treated cells using the following calculation: cell viability (%) = 100 × (A1/A0), cell lysis (%) = 1 – cell viability (%), where A1 and A0 were the absorbance obtained from TNF-treated and untreated cells, respectively. The mean value of quadruplicate was used for analysis. Highly purified (>99%) recombinant TNF (specific activity 6.63 × 10⁶ units/mg of protein) was kindly provided by A. G. Knoll (Ludwigshafen, Germany).

Nuclear Extracts and Electrophoretic Mobility Shift Assays (EMSA)—Transfected MCF7 cells (15 × 10⁶) were incubated in the presence or absence of 50 ng/ml TNF. The cells were then trypsinized and washed with phosphate-buffered saline. Nuclear extracts were prepared according to the procedure of Dignam et al. (30). Gel mobility shift assays were performed with a synthetic double-stranded 31-mer oligonucleotide containing the kB sequences of the human immunodeficiency virus long terminal repeat, 5'-end-labeled with [γ-^32P]ATP using the T4 kinase (31).

Southern Blot and Northern Blot Analysis—Genomic DNA was extracted from transfected MCF7 cells and digested by HindIII/XbaI enzymes before electrophoresed (10 µg) in a 0.8% agarose gel and transferred to nylon membrane hybrid-N (Amersham Corp.). Total RNA (15 µg) was electrophoresed in a 1.2% agarose gel and transferred to nitrocellulose membrane hybond-C (Amersham). The membranes were hybridized overnight at 42 °C with the probe labeled with [α-^32P]dCTP using a megaprime DNA labeling system (Amersham). The hybridized membranes were washed and exposed to Hyperfilm-MP (Amersham). The blot of RNA was stripped by boiling in 0.1% SDS and probed again with β-actin probe to confirm equal loading of RNA samples.

Western Blotting—Determination of IκBα content in MCF7 cell clones was performed by Western blotting of cytosolic protein extracts using a specific monoclonal antibody for IκBα, MAD10B antibody (32). The MAD10B antibody recognizes both wild-type and mutated IκBα. The cytosolic fractions of MCF7 cells used for EMSA analysis (as described previously) were denatured by boiling in SDS and mercaptoethanol. Equal amounts of protein extracts (50 µg) were subjected to 10% polyacrylamide gel electrophoresis in denaturing conditions (33). Fractionated proteins were transferred onto polyvinylidene difluoride membranes using the Hoeffer semi-phor system. The efficiency of the electrottransfer was assessed by Ponceau Red staining of the polyvinylidene difluoride membranes. IκBα protein was revealed with MAD10B hybridoma supernatant diluted 400-fold. The antigen-antibody complex was visualized by enhanced chemiluminescence method (ECL, Amersham) using the horseradish peroxidase-coupled anti-mouse antibody (Biodesign).

Morphological Examination by Electron Microscopy—Control and TNF-treated cells (1 × 10⁵) fixed with 2% glutaraldehyde in phosphate-buffered saline were pelleted at low speed. The pellet was washed in Sorensen buffer (67 mM phosphate buffer, pH 7.4), post-fixed in 2% osmium tetroxide, dehydrated with graded ethanol and propylene oxide, and included in “Epon” resin by usual techniques. Sections of cells were stained with uranyl acetate and lead citrate and observed with a Zeiss EM 902 electron microscope. Enhanced contrast was obtained by selecting elastic electrons using the slit of a spectrophotometer.

RESULTS AND DISCUSSION

The TNF-sensitive human breast carcinoma MCF7 cell line was used in this study to examine the effect of the inhibition of NF-κB activation on its susceptibility to the cytotoxic action of TNF. As shown in Fig. 1A, MCF7 cells were highly sensitive to the cytotoxicity of TNF. Following 72 h of exposure to TNF, optimal lysis (>75%) of MCF7 cells was obtained at 50–100 ng/ml of TNF. The results of EMSA indicate that in the absence of TNF, MCF7 cells showed no constitutive activation of NF-κB (Fig. 1B, lane 1). After 90-min incubation, TNF induced in these cells a significant activation of NF-κB (Fig. 1B, lane 2). The specific binding of NF-κB to DNA could be abrogated with an excess of unlabeled probe (Fig. 1B, lane 3). The fast migrating κB-binding protein detected in both TNF-treated and untreated cells was not selective for κB sequence (Fig. 1B, lanes 1 and 2), since its binding was not abrogated by an excess of unlabeled probe (Fig. 1B, lane 3).

In order to inhibit TNF-induced NF-κB translocation to the nucleus, we transfected MCF7 cells with the mutated MAD-3 cDNA which was unsusceptible to phosphorylation at positions 32 and 36 and which was found to resist degradation in transient transfections (11). The stable transfected clones of the control vector pcDNA3 (pcN-) and of the mutated MAD-3 gene (MAD-) were first screened by Southern blot analysis. As shown in Fig. 2A, the control clones (pcN-112 and pcN-183) contained only the endogenous wild-type IκBα gene, while the mutated IκBα transfected clones (MAD-1001, -1706, -1904, -1906) contained an additional band representative of the mutated exogenous IκBα gene. EMSA analysis (Fig. 2B) demonstrated that the introduction of exogenous IκBα mutant led to a significant suppression of TNF-induced NF-κB activation in the four representative MAD-3 transfected clones as compared with the level of NF-κB translocation after 90-min treatment with TNF in the control pcN-112 and pcN-183 cells.

To examine the stability and the efficiency of the NF-κB inhibition in MAD-3 mutant transfecants during a long term incubation with TNF, kinetic analysis of NF-κB translocation was performed. The treatment of control pcN-183 cells with TNF for 30 min (Fig. 3A, lane 2) resulted in a significant NF-κB...
translocation that further persisted and accumulated until after at least 24-h treatment (Fig. 3A, lanes 3 and 4). In contrast, in MAD-1906 cells, after 30-min incubation with TNF (Fig. 3A, lane 6), only marginally activated NF-κB was observed in the nuclear extract, that probably corresponded to the NF-κB released from rapidly degraded endogenous IκBα. No further activation of NF-κB could be detected after 4 h (Fig. 3A, lane 7) or 24 h (Fig. 3A, lane 8) treatment with TNF, thus suggesting that the NF-κB was inhibited by a stabilized association with the mutated IκBα.

It has been shown in various cell lines that the endogenous IκBα is rapidly degraded as a consequence of cell stimulation by TNF or phorbol esters (3, 5, 13, 34). As a result, NF-κB translocates to the nucleus, where it participates in the initiation of the transcription of numerous genes. One of the target genes of NF-κB is IκBα itself (35, 36). IκBα degradation is followed by its de novo synthesis as a consequence of the early NF-κB activation (4). In an attempt to compare the stability of

FIG. 2. A, Southern blot analysis of IκBα-transfected MCF7 cells. MCF7 cells were transfected by control pcDNA3 vector (pcN-) or mutated IκBα (MAD-) and the stable transfectants were obtained. Genomic DNA was extracted from transfected clones, digested by HindIII/XbaI, and subjected to a 0.8% agarose gel as described under "Experimental Procedures." Digested genomic DNA (10 μg/lane) was then transferred to nylon membrane and hybridized with 32P-labeled specific IκBα cDNA probe. B, EMSA study of transfected cells. Transfected MCF7 cells (10 × 10^6) were incubated for 90 min in the presence or absence of TNF (50 ng/ml). Nuclear proteins (15 μg) extracted from untreated cells (-) or TNF-treated cells (+) were subjected to electrophoretic mobility shift assay as described under "Experimental Procedures."
erated a reduced electrophoretic mobility product. Thus the slower migrating band probably corresponded to the product of the mutated MAD-3 cDNA. As already mentioned, treatment of MAD-1906 cells with TNF resulted in a small increase in the amount of the mutated IκBα and a total and persistent disappearance of the endogenous IκB-α. Thus, the mutated IκB-α was not degraded in response to TNF. Treatment of MAD-1906 cells with TNF for 30 min induced a faint NF-κB translocation (Fig. 3A, lane 6). This was probably due to the degradation of the endogenous IκBα in these cells. However, the lack of re-synthesis of the endogenous IκBα at the 4-h or later time points suggests that this faint NF-κB nuclear translocation was not sufficient to enhance IκBα transcription. At the 4- and 24-h time points, NF-κB was no longer detectable in nuclei of MAD-1906 cells (Fig. 3A, lanes 7 and 8). Concomitantly, the amount of mutated IκBα was increased and persisted in the cytosolic fraction of these cells (Fig. 3B, lanes 7 and 8). These observations suggest that the nuclear NF-κB released from the endogenous IκBα within 30 min of TNF treatment was rapidly inhibited by the mutated IκBα. The lack of further endogenous IκBα synthesis may be the consequence of the inhibition of NF-κB, since the IκBα itself is one of the target genes of NF-κB.

Together, these results demonstrated that the mutated IκBα was stably expressed in the transfected MCF7 cells and that TNF treatment of the MAD-1906 cells induced the proteolysis of only the endogenous IκBα but not the mutated IκBα. Additionally, the endogenous IκBα served as a marker of NF-κB activity. The results shown in Fig. 3B demonstrated that in the MAD-1906 cells, the endogenous IκBα was not re-synthesized in response to TNF, in contrast to what occurred in control cells. We conclude from these results that the mutated IκBα inhibited efficiently NF-κB nuclear translocation and activation in the MAD-1906 cells.

To further test the functional effect of the inhibition of NF-κB translocation in the MAD-3-transfected clones, we studied the expression of one of the TNF-inducible genes, mitochondrial manganese superoxide dismutase (37), in these cells. The mitochondrial manganese superoxide dismutase gene presents potential κB site(s) in its promoter region and the induction of its expression is closely associated with NF-κB activation by TNF (38, 39). The results of Northern blot analysis (Fig. 4) showed that TNF significantly induced the expression of mitochondrial manganese superoxide dismutase mRNA in control clones (pcN-112 and pcN-183). In contrast, no induction of this gene was observed in the four mutant MAD-3-transfected clones (MAD-1001, MAD-1706, MAD-1904, and MAD-1906). This correlated with the inhibition of the NF-κB activation in these MAD-3-transfected cells. Therefore, at least two known NF-κB target genes, IκBα and mitochondrial manganese superoxide dismutase, were negatively regulated in mutated MAD-3-transfected clones, indicating a functional inhibition of NF-κB in these cells.

A kinetic study was then performed to determine the sensitivity of the mutated MAD-3-transfected cells to the cytotoxic effect of TNF. When the transfected cells were incubated with 50 ng/ml of TNF during 6–72 h (Fig. 5), there was no significant difference between control (pcN-183) and the MAD-3-transfected (MAD-1904 and MAD-1906) cells, neither in the cell viability nor in the kinetics of cell death. After 48-h incubation with TNF, we even observed a slightly more elevated cell lysis in the two MAD-3 transfected clones as compared with the control pcN-183 cells. Furthermore, in order to examine the sensitivity of these transfected cells to short term treatment of TNF and the nature of cell death, the cells were treated with TNF for 24 h, and the electron micrograph analysis was performed. The results (Fig. 6) showed that the cell death induced by TNF in both control and MAD-3-transfected clones was apoptotic with the dense and vacuolated cytoplasm and the condensation of the chromatins along the nuclear membrane. To verify if inhibition of NF-κB persisted throughout the whole time course of the apoptotic process, we treated the nuclear extract of control (pcN-183) and MAD-3-transfected (MAD-1906) cells for NF-κB binding activity by EMSA between 24 and 72 h of TNF treatment. The treatment of control pcN-183 cells with TNF for 48 h (Fig. 7, lane 3) resulted in an accumulated NF-κB translocation as compared with 2-h short time treatment with TNF (Fig. 7, lane 2). The activation of NF-κB persisted until 72 h (Fig. 7, lane 4) but at a lower level due to the important cell lysis at this time point. In contrast, no activation of NF-κB could be detected in MAD-1906 cells after 2-h treatment with TNF (Fig. 7, lane 6), neither at 48 h nor at 72 h (Fig. 7, lanes 7 and 8). The possibility of a re-activation of NF-κB in MAD-3-transfected cells during a prolonged TNF incubation (24 h -72 h) can therefore be ruled out. The nuclear translocation of NF-κB is blocked in these cells throughout the same time course that apoptosis is occurring. These data

\[ C. Zhenzi, M. Körner, N. Tarantino, and S. Chouaib, unpublished data. \]
clearly indicated that the inhibition of NF-κB activation had no effect on TNF-mediated apoptotic cell death.

It is admitted that TNF signaling involves multiple second messenger pathways that function independently or coordinately to transduce distinct biological responses of TNF. Our results directly demonstrated that NF-κB activation is not required for induction of apoptosis by TNF. This is in agreement with the reports of Hsu et al. (40) indicating that TNF-R1-associated death domain protein (TRADD) directly interacts with one of the TNF-R2-associated factors (TRAF2) and the Fas-associated factor (FADD) to induce NF-κB activation and apoptosis, respectively. A TRAF2 mutant acts as a dominant-negative inhibitor of TNF-mediated NF-κB activation, but does not affect TNF-induced apoptosis. Conversely, a FADD mutant is a dominant-negative inhibitor of TNF-induced apoptosis, but does not inhibit NF-κB activation. Thus, it is suggested that TNF-R1 may utilize distinct TRADD-dependent mechanisms to activate signaling pathways for NF-κB activation and apoptosis. TNF has been shown to mediate its action through activation of several other transcriptional factors, including c-Jun/AP-1, c-Fos, c-Myc, IRF-1, and early growth response gene (Egr-1) (for review, see Ref. 17). However, Egr-1, c-Fos, c-Jun, and c-Myc have been implicated in cell proliferation. Therefore, the nuclear factors distinct from NF-κB that are capable of mediating TNF-induced apoptotic signal still remain to be identified.

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