Biochemical and Biophysical Research Communications **263,** 107–112 (1999)
Article ID bbrc.1999.1288, available online at http://www.idealibrary.com on IDEAL®

IκBα Is Essential for Maintaining Basal c-Jun N-Terminal Kinase (JNK) Activation and Regulating JNK-Mediated Resistance to Tumor Necrosis Factor Cytotoxicity in L929 Cells

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Received August 4, 1999

Early activation of c-Jun N-terminal kinase (JNK) is believed to block apoptosis in response to death signals such as tumor necrosis factor (TNF). Brief exposure of murine L929 fibroblasts to anisomycin for 1 hr to activate JNK resulted in resistance to TNF killing. TNF rapidly induced cytoplasmic shrinkage in control cells, but not in the anisomycin-pretreated L929 cells. However, the induced TNF resistance was suppressed in the L929 cells which were engineered to stably inhibit IκBα protein expression by antisense mRNA (~80% reduction in protein expression). No constitutive NF-κB nuclear translocation and increased TNF resistance were found in these IκBα antisense cells. Notably, these cells had a significantly reduced basal level of JNK activation (50–70%), compared to vector control cells. Furthermore, brief exposure of L929 cells to wortmannin, an inhibitor of phosphatidylinositol 3-kinase (PI3-kinase), resulted in resistance to TNF killing, probably due to preconsumption of caspases by wortmannin. Nonetheless, wortmannin-induced TNF resistance was suppressed in the IκBα antisense cells. Thus, these observations indicate that IκBα is essential for maintaining the basal level of JNK activation and regulating the JNK-induced TNF resistance.

c-Jun N-terminal kinase (JNK) (or stress-activated protein kinase) has been shown to play dual roles in regulating cell survival and apoptosis (1–5). JNK1 and JNK2 regulate region-specific apoptosis during early brain development (1). Roulston *et al.* demonstrated that early activation of JNK increases cell survival in response to tumor necrosis factor (TNF or TNF-α) (2). In contrast, a persistent activation of JNK induces cell death (3). A defect in JNK activation is associated with resistance to TNF cytotoxicity in breast MCF-7 cells (4). JNK activation also contributes to TRAIL-induced apoptosis (5). However, in contrast to these observations, Fas induces JNK activation, and this activation has no effect on Fas-mediated apoptosis in human colonic epithelial cells (6). Also, activation of JNK and p38 kinase in calphostin C-induced apoptosis does not contribute to cell death (7).

In this study, the role of JNK activation in regulation of TNF cytotoxicity was investigated. When murine L929 fibroblasts were briefly exposed to anisomycin (8), a potent agonist of JNK, for 1 hr, the cells became resistant to killing by TNF in the presence or absence of actinomycin D (ActD). In contrast, a prolonged exposure of L929 cells to anisomycin induced cell death, and anisomycin synergistically enhanced the TNF killing.

Interestingly, the anisomycin-mediated TNF resistance was significantly suppressed in L929 cells which were engineered to stably express antisense mRNA for blocking IκBα protein expression. IκBα is a specific inhibitor of nuclear factor κB (NF-κB). IκBα binds NF-κB in the cytoplasm and prevents NF-κB nuclear translocation and activation (9). IκBα may translocate independently to the nucleus and prevents the binding of NF-κB to nuclear DNA (10). IκBα promotes the export of NF-κB from nucleus to cytoplasm (10). Here, a novel function of IκBα in controlling the basal level of JNK activation and regulating JNK-mediated TNF resistance is described.

MATERIALS AND METHODS

L929 fibroblasts and stable transfectants with IκBα *cDNA.* The mouse full-length IκBα cDNA (Genbank accession AA517353) was obtained from Genome Sciences (St. Louis, MO), and the DNA sequence was determined. The coding region of this cDNA was amplified by polymerase chain reaction (PCR) and ligated, either in a sense or an antisense direction, with a mammalian expression vector pcDNA3.1.TOPO (Invitrogen, San Diego, CA) to the TA cloning site.

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0006-291X/99 $30.00

Synthetic PCR primers were: forward 5′-ATGTTTCAGCCAGCTGGG; reverse 5′-TAATGTCAGACGCTGGCC. The sense construct was tagged with a v5 epitope at the *C*-terminus. TNF-sensitive L929 cells were cultured in RPMI-1640 medium (Mediatech, Washington, DC), supplemented with 10% fetal bovine serum (FBS) (Sigma, St. Louis, MO), in a 5% CO_2, humidified 37°C incubator as described previously (11). L929 cells were electroporated with the sense or antisense IκBα cDNA construct (or the empty pcDNA3.1 vector), and neo-resistant cells were established by G418 selection (12, 13). The antisense mRNA-mediated suppression of IκBα protein expression was determined by Western blotting using specific antibodies (Pharmingen, San Diego, CA; Santa Cruz Biotechnology, Santa Cruz, CA). Similarly, expression of the v5-tagged IκBα protein in L929 cells was determined by Western blotting using anti-v5 antibodies (Invitrogen).

Where indicated, the levels of apoptosis regulatory and inhibitory proteins in the established stable transfectants were examined by Western blotting. Specific antibodies used were against Bad (14), Bcl-2 (14), Bcl-xL (14), CAS (cellular apoptosis susceptibility protein) (15), caspase 8 (16), Fas ligand (FasL), p53, TIAR (an RNA binding protein) (17), p65 (RelA) NF-κB, and RIP (14) (Transduction Laboratories, Lexington, KY and Santa Cruz Biotechnology). As control for protein loading into gels, antibodies against the house-keeping α-tubulin (Accurate Antibodies, Westbury, NY) were used in the Western blotting.

Anisomycin activation of JNK and regulation cellular sensitivity to TNF. To activate JNK, L929 cells (2.5×10^5/ml) were cultured in 96-well microtiter plates (Corning Glass Works, Corning, NY) overnight, and exposed to anisomycin (0–40 μM; Sigma, St. Louis, MO) for 1 hr. The cells were washed with phosphate-buffered saline (PBS) once, and then treated with recombinant human TNF (1000–4000 units/ml; Genzyme, Boston, MA), or TNF (2.5–10 units/ml) in the presence of actinomycin D (ActD; 1 μg/ml) (11–13) for 16–24 hr. The cells were stained with crystal violet and lysed with 33% acetic acid, and the absorbance was determined at OD 580 nm using a microtiter plate reader (TECAN, Research Triangle Park, NC) (11–13). The extent of cell death was calculated as: % Cell Death = [(OD from control cells − OD from TNF-treated cells)/OD from control cells] × 100 (11–13). Where indicated, anisomycin-mediated resistance to TNF killing was calculated as: % Resistance to TNF Killing = [1 − (% TNF killing of anisomycin-treated cells/% TNF killing of control cells)] × 100.

Similar experiments were performed by exposure of the established L929 transfectants to anisomycin for 1 hr, followed by washing with PBS once and exposure to TNF/ActD for 16–24 hr and determining the extent of cell death (11–13).

In some experiments, the established L929 transfectants were pretreated with wortmannin (Sigma), an inhibitor of phosphatidylinositol 3-kinase (PI3-kinase) (18–20), for 1 hr, followed by washing with PBS once, and exposure to TNF/ActD for 16–24 hr.

Western blotting. To examine JNK activation, untransfected L929 cells or the stable transfectants were cultured in 60 mm petri dishes overnight, and treated with anisomycin (40 μM) for 0, 20, 40, 60 and 120 min. The cells were lysed with a lysis buffer (11) and subjected to SDS-PAGE and Western blotting using specific antibodies against JNK or a phosphorylated JNK peptide (Santa Cruz Biotechnology). Additionally, the L929 stable transfectants were pretreated with anisomycin for 1 hr, followed by exposure to TNF (4000 units/ml) for various indicated times. The extent of TNF-mediated IκBα degradation and JNK phosphorylation was determined by Western blotting using antibodies against IκBα and the phosphorylated JNK peptide, respectively (Santa Cruz Biotechnology).

Fluorescent microscopy. Where indicated, the stable L929 transfectants were cultured on cover glasses overnight, and treated with or without anisomycin (40 μM) for 1 hr, followed by exposure to TNF (4000 units/ml) for 0, 15, 30, 60 and 120 min. The cells were fixed with 3.3% formaldehyde (in PBS) for 15 min at room temperature, permeabilized with 0.1% Triton-X100 (Sigma), washed 3 times with PBS, and incubated with antibodies against p65 (RelA) NF-κB (Santa Cruz Biotechnology) for 1 hr at room temperature. The cover glasses were washed 3 times with PBS, incubated with rodamine-conjugated anti-goat antibodies for 1 hr, again washed 3 times with PBS, and subjected to fluorescent microscopy.

RESULTS

Anisomycin-mediated TNF-resistance. To activate JNK, untransfected or control L929 cells were exposed to anisomycin for 1 hr. These cells were then washed with PBS once and exposed to TNF or TNF/ActD for 24 hr. The anisomycin-stimulated cells became resistant to TNF killing in the presence or absence of ActD (Fig. 1A). Anisomycin alone failed to induce L929 cell death during this short-term treatment. However, a sustained activation of JNK in L929 cells by continuous stimulation with anisomycin for longer than 2 hr caused cell death. Also, TNF killing of L929 cells was significantly enhanced in the continuous presence of anisomycin (data not shown).

As determined by immunostaining and fluorescent microscopy, TNF rapidly induced NF-κB nuclear translocation and reduced the volume of L929 cells after treatment for 15 min (Fig. 1B). In contrast, TNF mediated NF-κB nuclear translocation in the anisomycin-pretreated cells, without reducing the cell volume (Fig. 1B). Anisomycin alone failed to mediate NF-κB nuclear translocation in L929 cells.

Anisomycin-mediated TNF resistance is suppressed in the IκBα *antisense-expressing L929 cells.* To examine the role of IκBα in regulating anisomycin-mediated TNF-resistance, L929 cells were electroporated with an IκBα sense or antisense cDNA/pcDNA3.1 construct or the empty pcDNA3.1 vector, and stable transfectants were established by G418 selection. IκBα protein expression was suppressed approximately by 80% in the antisense transfectants, compared to empty vector transfectants (Fig. 2A). Also, the expression of several apoptosis regulatory and effector proteins was not altered in the IκBα antisense cells, compared to the vector control cells (Fig. 2A). These proteins were Bad, Bcl-2, Bcl-xL, CAS, caspase 8, FasL, p53, TIAR, NF-κB, and RIP. Despite the suppression of IκBα expression, no significant NF-κB nuclear translocation was observed in the IκBα antisense cells, compared to the vector control cells (Fig. 2A).

Compared to the empty vector-transfected cells, the sense or antisense expression of IκBα failed to alter cellular sensitivity to TNF-mediated death. For example, TNF at 5 units/ml (in the presence of ActD) was sufficient to achieve 50% killing (EC50) of these cell transfectants.

Exposure of the IκBα sense or the vector control cells to anisomycin for 1 hr, followed by washing with PBS once and exposure to TNF/ActD for 16–24 hr, resulted in resistance to TNF/ActD killing (Fig. 2B). In contrast,

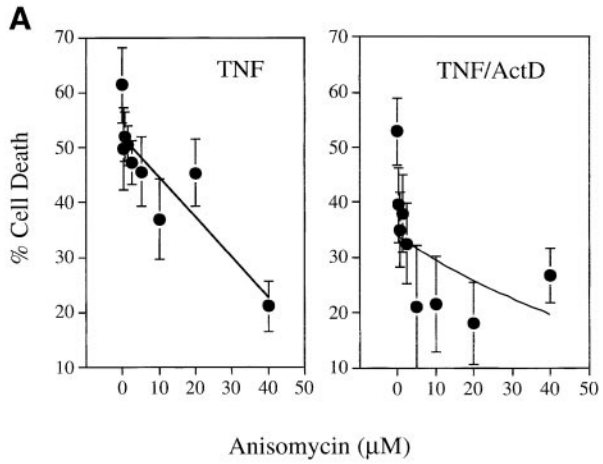

FIG. 1. Anisomycin-mediated TNF-resistance in L929 fibroblasts. (A) L929 cells were pretreated with or without anisomycin for 1 hr, followed by washing with PBS once, and exposure to TNF (1000 units/ml) or TNF (5 units/ml)/ActD (1 μg/ml) for 24 hr. Anisomycin mediated a dose-dependent resistance to TNF and TNF/ActD killing of L929 cells. (B) Compared to control cells, TNF mediated p65 (RelA) NF-κB nuclear translocation and reduced the cell volume of L929 after treatment for 15 min, as determined by immunostaining and fluorescent microscopy. TNF also induced NF-κB nuclear translocation but failed to reduce the volume of anisomycin-pretreated cells (40 μM for 1 hr). TNF concentration was increased to 4000 units/ml in this short-term assay.

the anisomycin-mediated TNF-resistance was significantly suppressed (by approximately 50%) in the IκBα antisense cells (Fig. 2B). Expression of the v5-tagged IκBα protein in the IκBα sense L929 cells was observed, as determined by Western blotting (data not shown).

The basal level of JNK activation was reduced in the IκBα *antisense cells, and both anisomycin and TNF increased* IκBα *expression in these cells.* Compared to the vector control cells, the IκBα antisense L929 cells had a reduced basal level of JNK activation by 50–70% (Fig. 3). No significant differences in the expression of JNK were found between the vector control and the IκBα antisense cells, and anisomycin failed to modulate JNK expression in these cells (Fig. 3). Upon activation of JNK with anisomycin, a greater extent of JNK activation was observed in the IκBα antisense cells (~120% increase) than in the vector control cells (~45% increase) (Fig. 3).

TNF-mediated JNK activation occurred in both the vector control and the IκBα antisense cells, pretreated with or without anisomycin (Fig. 4A). Again, the basal level of JNK activation was suppressed in the IκBα antisense cells (Fig. 4A).

TNF increased the expression of IκBα protein in the IκBα antisense cells after treatment for 20 min (Fig. 4B). However, TNF-mediated the subsequent IκBα degradation, as mediated by TNF, was less efficient in these cells (~50% in 40 min) than in the vector control cells (~80% in 20 min) (Fig. 4B). Similarly, anisomycin increased IκBα protein expression in the IκBα antisense cells, and TNF also induced IκBα degradation (~50% in 20 min) in these anisomycin-pretreated cells (Fig. 4B).

In parallel with IκBα degradation, TNF induced NF-κB nuclear translocation in both the vector control and the IκBα antisense cells, pretreated with or without anisomycin, as determined by immunostaining and fluorescent microscopy (data not shown).

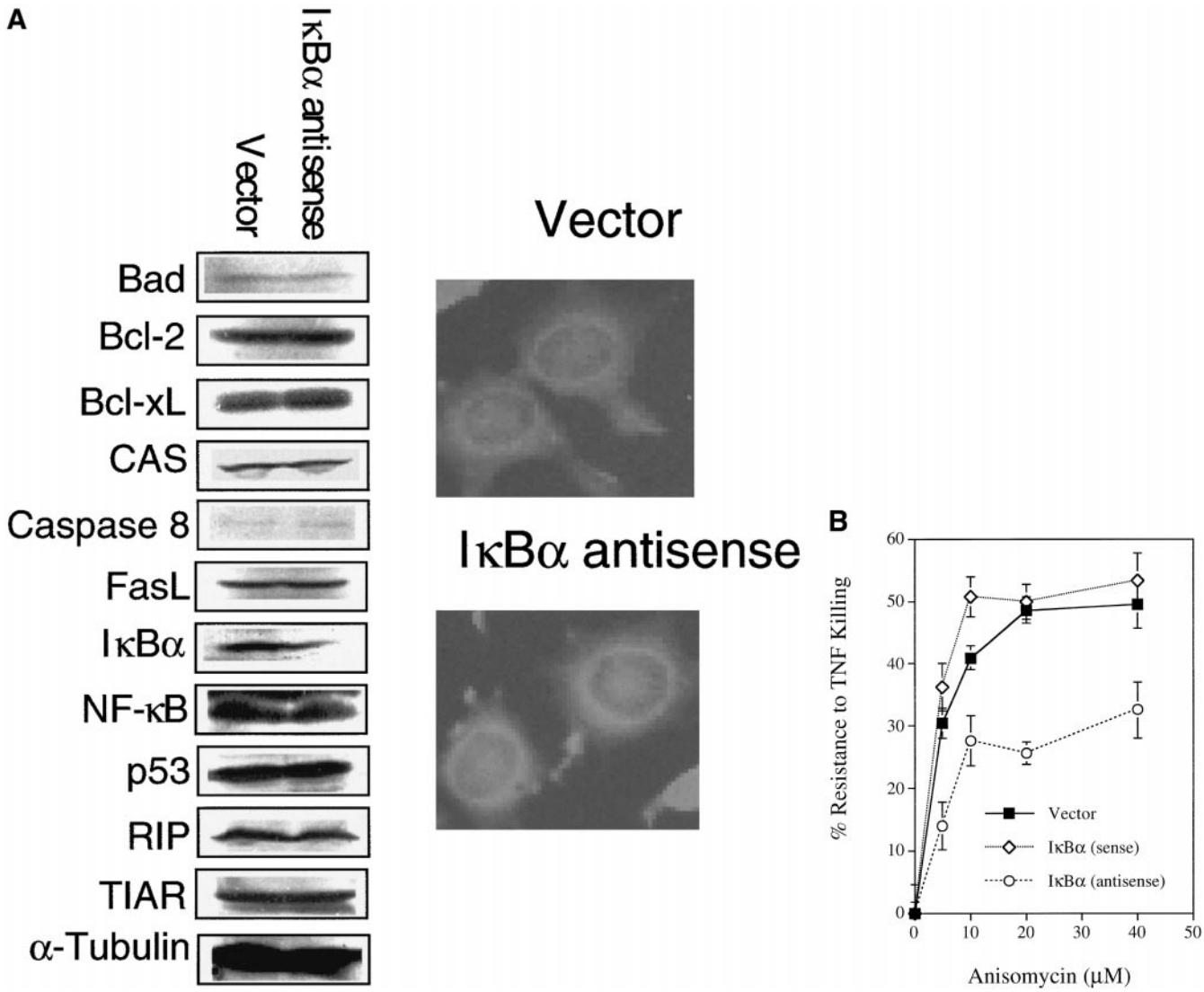

FIG. 2. Anisomycin-mediated TNF-resistance was suppressed in the IκBα antisense-expressing L929 cells. L929 transfectants were engineered to stably express v5-tagged IκBα protein, or to suppress IκBα protein expression by antisense mRNA. Control cells were transfected with an empty vector only. (A) Compared to vector control cells, expression of Bad, Bcl-2, Bcl-xL, CAS, caspase 8, FasL, p53, TIAR, NF-κB, and RIP was not altered in the IκBα antisense cells, as determined by Western blotting using specific antibodies (left panel). However, IκBα protein expression was inhibited by ~80% in the IκBα antisense cells. For protein loading control, expression of the house-keeping α-tubulin was examined. Despite the suppression of IκBα, no significant NF-κB (p65) activation or nuclear translocation was observed in the antisense-expressing cells, compared to vector control cells (right panel). The results were determined by immunostaining of cells with anti-p65 and anti-goat rodamine antibodies. (B) Exposure of the above established cells to anisomycin for 1 hr, followed by washing with PBS once, and exposure to TNF (5 units/ml)/ActD (1 μg/ml) for 24 hr resulted in resistance to TNF killing of the IκBα sense and vector control cells. However, anisomycin-mediated TNF-resistance was significantly suppressed (by approximately 50%) in the IκBα antisense cells. (Data not shown for the expressed v5-tagged IκBα protein in the IκBα sense cells.)

Wortmannin-mediated TNF resistance was suppressed in the IκBα *antisense cells.* Wortmannin has been shown to activate caspase 3 and increase TNF-mediated caspase 3 activation (19, 20). Pre-exposure of the vector control L929 cells to wortmannin for 1 hr resulted in resistance to TNF/ActD killing, whereas the resistance was significantly suppressed in the IκBα antisense cells (Fig. 5). Anisomycin increased the TNF resistance with wortmannin in the vector control cells in an additive manner; however, this effect was not observed in the IκBα antisense cells (Fig. 5).

DISCUSSION

In this report a novel function of IκBα is described. Pre-activation of JNK by anisomycin rendered L929 cells resistant to killing by TNF and TNF/ActD. The induced TNF resistance was significantly suppressed

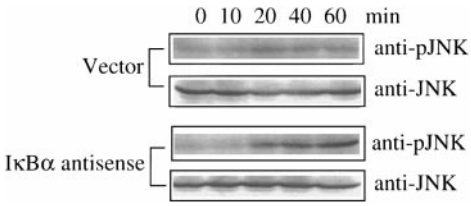

FIG. 3. The basal level of JNK activation was suppressed in the IκBα antisense L929 cells. Exposure of IκBα antisense or vector control L929 cells to anisomycin (40 μM) resulted in JNK activation, as determined using anti-JNK phosphopeptide antibodies. Anti-JNK antibodies were also used as controls. Anisomycin failed to modulate the expression of JNK in both IκBα antisense or vector control cells. Compared to control cells, the basal level of JNK activation was reduced in the IκBα antisense-expressing cells (50–70% reduction). Anisomycin induced a greater JNK activation in the IκBα antisense cells (~120% increase) than in the vector control cells (~45% increase).

in the IκBα antisense-expressing cells, indicating that IκBα regulates the JNK-induced TNF resistance. Furthermore, IκBα is essential for maintaining the basal level of JNK activation in L929 cells, since the basal JNK activation was significantly suppressed in the IκBα antisense cells.

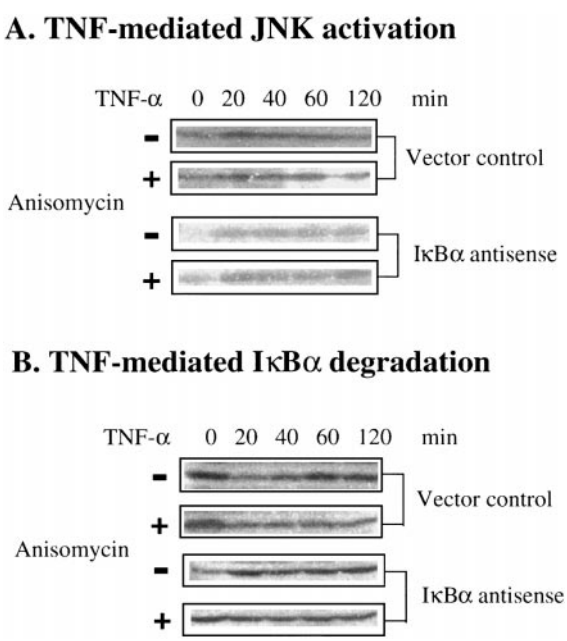

FIG. 4. Anisomycin and TNF increased IκBα expression in the IκBα antisense cells. (A) TNF (4000 units/ml) mediated JNK activation in both IκBα antisense and vector control L929 cells. Pretreatment of these cells with anisomycin (40 μM) for 1 hr, followed by exposure to TNF, also resulted in JNK activation. The basal level of JNK activation was significantly reduced in the IκBα antisense cells. (B) TNF induced IκBα degradation (~80% degradation in 20 min) in the vector control cells, pretreated with or without anisomycin (40 μM; 1 hr). In contrast, TNF induced IκBα expression in the IκBα antisense cells in 20 min, followed by subsequent IκBα degradation (~50%). Pretreatment of the IκBα antisense cells with anisomycin for 1 hr resulted in increased IκBα expression, and the subsequent TNF-mediated IκBα degradation also occurred (~50%).

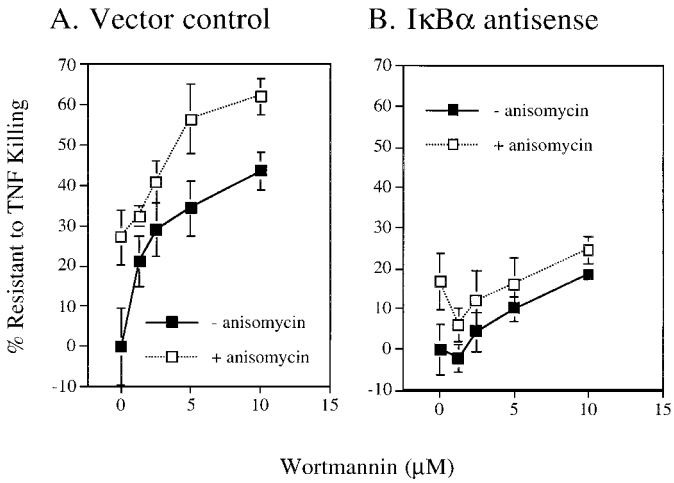

FIG. 5. Wortmannin increased TNF resistance in the vector control cells but not in the IκBα antisense cells. Both the vector control and the IκBα antisense L929 cells were pretreated with wortmannin for 1 hr, and then coincubated with anisomycin (40 μM) for 1 hr. These cells were washed once with PBS and exposed to TNF (5 units/ml)/ActD (1 μg/ml) for 16–24 hr.

NF-κB plays a critical role in blocking various cell death by TNF, ionizing radiation and anti-cancer drugs (21–26). However, NF-κB is not involved in the JNK-mediated TNF resistance. Suppression of IκBα expression by antisense mRNA is expected to induce constitutive NF-κB activation and increase TNF resistance in L929 cells. However, these events did not occur in the IκBα antisense-expressing cells. It is likely that blocking of NF-κB activation is achieved by other proteins such as the RelA-associated inhibitor and the NF-κB-inhibiting protein ABIN (27, 28), as well as other IκB proteins (IκBβ, IκBγ and IκBε). Anisomycin alone could not induce NF-κB activation in L929 cells. Anisomycin mediates early JNK activation, which leads to TNF resistance even in the presence of ActD. That is, prevention of NF-κB-mediated protective gene transcription by ActD fails to abolish the JNK-mediated TNF resistance.

Both anisomycin and TNF were shown to increase the expression of IκBα protein in the IκBα antisense-expressing cells. Tzen *et al.* demonstrated that TNF and anisomycin upregulate IκBα gene expression (29). TNF-mediated NF-κB activation was not impaired in the IκBα antisense-expressing or empty vector control cells, pretreated with or without anisomycin. Again, these observations suggest that JNK-mediated TNF resistance is not involved in NF-κB activation.

Despite its protective role against cell death, this function of NF-κB remains controversial. Induction of NF-κB activation by a dominant negative IκBα fails to increase the sensitivity of various cancer cells to TNF and chemotherapeutic agents (30). Hyperoxia-mediated NF-κB activation does not protect epithelial cells from death (31). Activation of NF-κB contributes

to glutamate-elicited neuronal cell death, and aspirin inhibits the cell death by blocking NF-κB activation (32). The zinc finger protein A20 blocks IκBα degradation and prevents NF-κB activation in endothelial cells; however, these events fail to sensitize these cells to TNF killing (33). Overexpression of IκBα in MCF7 breast cancer cells inhibits NF-kB activation but does not block TNF-mediated death (34).

Wortmannin activates caspase 3 and increases TNF-mediated caspase 3 activation (19, 20). Pretreatment of L929 cells with wortmannin for 1 hr resulted in TNF resistance. The resistance could be due to consumption of caspases in wortmannin-pretreated cells. However, wortmannin-increased TNF resistance was suppressed in the IκBα antisense-expressing cells, suggesting that IκBα is involved in controlling caspase activation. This possibility remains to be established.

Overall, this study demonstrates a novel role of IκBα in regulating JNK-mediated cellular resistance to TNF killing, as well as maintaining the basal level of JNK activation. This function is independent of NF-κB.

ACKNOWLEDGMENTS

I appreciate the technical assistance of John Heath and Nicole Zevotek, Dr. Robert S. Aronstam's kind assistance in processing graphs, and the research support from the Guthrie Foundation for Education and Research.

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