Tumor Necrosis Factor Induces Phosphorylation and Translocation of BAD through a Phosphatidylinositol-3-OH Kinase-dependent Pathway

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EXPERIMENTAL PROCEDURES

Tumor necrosis factor (TNF) induced the phosphorylation of BAD at serine 136 in HeLa cells under conditions that are not cytotoxic. BAD phosphorylation by TNF was dependent on phosphatidylinositol-3-OH kinase (PI3K) and was accompanied by the translocation of BAD from the mitochondria to the cytosol. Blocking the phosphorylation of BAD and its translocation to the cytosol with the PI3K inhibitor wortmannin activated caspase-3 and markedly potentiated the cytotoxicity of TNF. Transient transfection with a PI3K dominant negative mutant or a dominant negative mutant of the serine-threonine kinase Akt, the downstream target of PI3K and the enzyme that phosphorylates BAD, similarly potentiated the cytotoxicity of TNF. By contrast, transfection with a constitutively active Akt mutant protected against the cytotoxicity of TNF in the presence of wortmannin. Phosphorylation of BAD prevents its interaction with the antiapoptotic protein Bcl-XL. Transfection with a Bcl-XL expression vector protected against the cytotoxicity of TNF in the presence of wortmannin. The mechanism by which the inhibition of the phosphorylation of BAD is likely linked to the induction of lethal mitochondrial damage in TNF-intoxicated cells is discussed.

Phosphatidylinositol-3-OH kinase (PI3K)\(^1\) is a member of a signaling cascade that eventuates in the phosphorylation of the proapoptotic protein BAD (1, 2). Such growth factors as NGF and interleukin-3 are presumed to promote cell survival as a consequence of the PI3K-mediated activation of the serine-threonine kinase Akt, which in turn phosphorylates BAD (3, 4). Whereas BAD can be phosphorylated at either the Ser-112 or threonine kinase Akt, which in turn phosphorylates BAD (3, 4).

Notably, phospho-BAD-136 cannot bind either Bcl-X\(\text{L}\) or Bcl-2 (5). It is postulated that by binding to Bcl-X\(\text{L}\) and Bcl-2 BAD antagonizes their antiapoptotic activity. Upon inducing its phosphorylation, growth factors lead to the dissociation of BAD from Bcl-X\(\text{L}\) and Bcl-2 and thereby promote cell survival by allowing the unhindered action of these proteins.

The PI3K inhibitor wortmannin enhanced the activation of caspase-3 that was induced by TNF or anti-Fas (8). Here we demonstrate that TNF acts like other growth factors to promote the phosphorylation of BAD at Ser-136. As a result, there is the translocation of BAD from the mitochondria to the cytosol. Moreover, the phosphorylation of BAD by TNF occurs by a PI3K-dependent pathway and is necessary to prevent the cytotoxicity of this cytokine. Inhibition of PI3K prevents both the phosphorylation of BAD and its translocation from mitochondria to the cytosol, effects that are accompanied by substantially enhanced cell killing by TNF.
transfection efficiencies (10–15%) and number of β-galactosidase-positive cells/well (2500–3000 cells/well).

**Detection of Caspase-3 Activity**—The assay is based on the ability of the active enzyme to cleave the chromophore pNA from the enzyme substrate DEVD-p-nitroanilide. Cells were extracted with 2% Nonidet P-40 buffer (0.5 M sucrose, 1 mM dithiothreitol, 2 mM EDTA, 0.1% CHAPS, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml pepstatin, 10 μg/ml leupeptin). DEVD-p-nitroanilide was added to a final concentration of 50 μM, and the reaction was incubated for 1 h at 37 °C. The samples were then transferred to a 96-well plate, and absorbance measurements were made in a 96-well plate reader at 405 nm.

**Isolation of Cytosol and Mitochondrial Fractions**—Cells were plated in 25-cm² flasks at 5.0 × 10⁶ cells/flask. After treatment, the cells were harvested by trypsinization followed by centrifugation at 600 × g for 10 min at 4 °C. The cell pellets were washed once in PBS and then resuspended in 3 volumes of isolation buffer (20 mM Hepes, pH 7.4, 10 mM KCl, 1.5 mM MgCl₂, 1 mM sodium EDTA, 1 mM dithiothreitol, and 10 mM phenylmethylsulfonyl fluoride, 10 μM leupeptin, 10 μM aprotinin in 250 mM sucrose). After chilling on ice for 3 min, the cells were disrupted by 40 strokes of a glass homogenizer. The homogenate was centrifuged twice at 2,500 × g at 4 °C to remove unbroken cells and nuclei. The mitochondria were then pelleted by centrifugation at 12,000 × g at 4 °C for 30 min. The supernatant was removed through 0.2-μm Ultrafree MC filters (Millipore) followed by centrifugation at 100,000 × g at 4 °C to give cytosolic protein. Mitochondrial and cytosolic fractions (25 μg of protein) were separated on 12% SDS-polyacrylamide electrophoresis gels and electroblotted onto nitrocellulose membranes. Phospho-BAD-136, phospho-BAD-112, and BAD were detected by rabbit polyclonal antibody to a dilution of 1:500 (New England Biolabs). Secondary goat antirabbit horseradish peroxidase-labeled antibody (1:2000) was detected by enhanced chemiluminescence.

**Nuclear Extract Preparation and Electrophoretic Mobility Shift Assay**—At the end of the specified incubation time, cells (15–20 × 10⁶/sample) were collected by centrifugation at 359 × g for 5 min at 4 °C. After washing once with PBS, the cell pellet was suspended in 0.5 ml of buffer A (10 mM HEPES-NaOH, pH 7.8, 150 mM KCl, 2 mM MgCl₂, 0.1 mM EDTA, 1 mM dithiothreitol, and 1 mM phenylmethylsulfonyl fluoride). The suspension was transferred to a microcentrifuge tube and centrifuged at 750 × g for 5 min at room temperature. The supernatant was removed by aspiration, and the pellet was resuspended in 0.2 ml of buffer A. After 10 min on ice, Nonidet P-40 was added to 0.5%, and the suspension was centrifuged at 1330 × g for 15 min. The resultant nuclear pellet was suspended in 15 μl of buffer B (20 mM HEPES-NaOH, pH 7.9, 1.5 mM MgCl₂, 0.5 mM dithiothreitol, 0.42 mM NaCl, 0.2 mM EDTA, 25% glycerol, and 0.5 mM phenylmethylsulfonyl fluoride). After 15 min on ice with vigorous stirring, the suspension was centrifuged at 16,300 × g for 10 min. Fifteen μl of the resultant supernatant was diluted with 75 μl of buffer C (20 mM HEPES-NaOH, pH 7.9, 50 mM KCl, 0.2 mM EDTA, 0.5 mM dithiothreitol, and 0.5 mM phenylmethylsulfonyl fluoride) to obtain the final nuclear extract preparation. Electrophoretic mobility shift assays for NF-κB in nuclei was performed by using the gel shift assay system (Promega) according to the manufacturer’s instructions. The reaction mixture contained 2 μl of 5× gel shift binding buffer, 1 μl of [32P]-labeled NF-κB consensus oligonucleotide probe, 2 μl of water, and 5 μl of nuclear extract (1 μg of protein). Relative band densities were obtained by densitometric analysis (Amersham Pharmacia Biotech).

**Treatments**—Human recombinant TNF (Sigma) was dissolved in PBS and added for the times indicated to a final concentration of 10 ng/ml. Wortmannin (Sigma) was dissolved in Me₂SO and added at a final concentration of 10 μM. Rapamycin was dissolved in MeSO and added at a 0.2% volume to a final concentration of 50 nM. Wortmannin (Sigma) was dissolved in Me₂SO and added at a 0.2% volume to a final concentration of 50 nM. In all experiments the cells were pre-treated for 30 min with wortmannin, LY294002, or rapamycin before the addition of TNF. Control experiments demonstrated that MeSO at the concentration used was without measurable effect on the parameters examined. Protein synthesis was measured as the incorporation of [3H]leucine into an acid-insoluble precipitate as described previously (9).

**RESULTS**

**Effect of TNF on BAD Phosphorylation**—HeLa cells maintained a basal level of phosphorylation of BAD at Ser-136 (Fig. 1a, left panel). Within 30 min of treatment with TNF, however, there was a marked increase in BAD phosphorylation (phospho-BAD-136), an effect that increased further to reach a maximum within 2 h (Fig. 1a, left panel). The PI3K inhibitor wortmannin totally inhibited the induction by TNF of BAD phosphorylation at Ser-136 (Fig. 1a, right panel). In fact, there was a progressive decrease in the content of phospho-BAD-136 in the presence of both TNF and wortmannin over the 2 h time course of the experiment. Importantly, treatment of HeLa cells with either TNF alone or TNF together with wortmannin produced no change in the total content of BAD (Fig. 1b, left and right panel, respectively). In addition, no staining was detected when an antibody was used against the Ser-112-phosphorylated form of BAD, either in control, TNF, or TNF and wortmannin-treated cells (data not shown).

**Inhibition of PI3-kinase Potentiates the Cytotoxicity of TNF**—TNF alone killed few HeLa cells over a 6-h time course (Fig. 4a, closed squares). As measured by trypan blue uptake, less than 9% of the cells were dead after 4 h, and after 6 h, only 11% of the cells had died. By contrast, cells pretreated for 30 min with 50 nM wortmannin and then exposed to TNF exhibited substantial cell killing (Fig. 4a, closed circles). After 4 h, more than 40% of the cells were dead. Cell killing reached almost 70% after 6 h. Importantly, wortmannin alone (in the absence of TNF) had no effect on the viability of the HeLa cells after 6 h (Fig. 4a, closed triangles).

The resistance of cells to the cytotoxicity of TNF has been attributed to the activation of the transcription factor NFκB (10). The dependence of the killing produced by TNF on an inhibition of either transcription or translation is interpreted in turn as a consequence of the inhibition of the expression of...
HeLa cells to the cytosol is inhibited by wortmannin. HeLa cells (5.0 × 10^6) were either treated with TNF (10 ng/ml) alone or pretreated for 30 min with 50 nM wortmannin followed by the addition of TNF. At the times indicated, cytosolic fractions were prepared, and the levels of BAD-P136 and BAD were determined by Western blotting.

![Fig. 2](image)

**Fig. 2.** The translocation of phosphorylated BAD from the mitochondria to the cytosol is inhibited by wortmannin. HeLa cells (5.0 × 10^6) were either treated with TNF (10 ng/ml) alone or pretreated for 30 min with 50 nM wortmannin followed by the addition of TNF. At the times indicated, mitochondria were prepared, and the levels of BAD-P136 and BAD were determined by Western blotting.

lymphoma cells (17) to induce apoptosis by activation of caspase-3. The cytotoxicity of TNF was enhanced by wortmannin, with 31% of the cells staining positive for β-galactosidase as compared with untreated wells. By contrast, transfection with a constitutively active Akt mutant protected against the cytotoxicity induced by TNF in the presence of wortmannin. Cotransfection with pCDNA-LacZ and pCDNA-Akt(+) resulted in 86% of cells staining positive for β-galactosidase in the presence of TNF and wortmannin as compared with untreated wells. In cultures cotransfected with pCDNA-LacZ and pCDNA, only 27% of cells stained positive for β-galactosidase upon treatment with TNF and wortmannin as compared with the untreated wells. Thus expression of the constitutively active Akt is able to bypass the block at PI3K signaling brought about by wortmannin.

**Overexpression of Bel-X\textsubscript{L} Protects against TNF-induced Cytotoxicity in the Presence of Wortmannin**—It is hypothesized that the increased cytotoxicity of TNF upon inhibition of PI3K relates to the inhibition of BAD phosphorylation by Akt and its subsequent dissociation from antiapoptotic proteins such as Bel-X\textsubscript{L}. It was of interest, therefore, to explore the effect of the overexpression of Bel-X\textsubscript{L} in HeLa cells on the cytotoxicity of TNF in the presence of the PI3K inhibitors used above. Table IV shows the results of the transient transfection of HeLa cells with a Bel-X\textsubscript{L} expression vector (pCDNA-BclX\textsubscript{L}). Treatment of cells cotransfected with pCDNA-LacZ and pCDNA with TNF in the presence of either wortmannin or LY294002 resulted in only 33 and 29%, respectively, of the number of β-galactosidase-positive cells as compared with untreated wells. By contrast, overexpression of Bel-X\textsubscript{L} afforded significant protection against the cytotoxicity induced by TNF in the presence of either wortmannin or LY294002. In cultures cotransfected with pCDNA-LacZ and pCDNA-BclX\textsubscript{L}, and treated with TNF and either wortmannin or LY294002, 85 and 81%, respectively, of the cells stained positive for β-galactosidase as compared with untreated wells.

**Inhibition of PI3-kinase Potentiates the Activation of Caspase-3**—The cytotoxicity data were mirrored by the activity of the apoptotic protease caspase-3. Treatment of HeLa cells with TNF alone resulted in a 1.5-fold increase over the control value in the activity of caspase-3 (Fig. 4b, closed squares). By contrast, the combination of TNF and wortmannin produced a 12-fold increase in the activity of caspase-3 after 6 h (Fig. 4b, closed circles). Again, wortmannin alone (in the absence of TNF) had no effect on caspase-3 activity (Fig. 4b, closed triangles).

**DISCUSSION**

We have shown that BAD is phosphorylated on Ser-136 in response to the treatment of HeLa cells with TNF. This phosphorylation is accompanied by the translocation of BAD from the mitochondria to the cytosol. Importantly, the phosphorylation and translocation of BAD occurs in the absence of any cell killing by TNF. In turn, inhibition of PI3K by wortmannin prevented both the phosphorylation and translocation of BAD, a result that was now reflected in substantial cell killing in response to TNF. Transient transfection with a PI3K dominant negative mutant or a dominant negative mutant of the serine-threonine kinase Akt, the downstream target of PI3K and the
enzyme that phosphorylates BAD, similarly potentiated the cytotoxicity of TNF. By contrast, transfection with a constitutively active Akt mutant protected against the cytotoxicity of TNF in the presence of wortmannin. Phosphorylation of BAD prevents its interaction with the antiapoptotic protein Bcl-XL. Transfection with a Bcl-XL expression vector protected against the cytotoxicity of TNF in the presence of wortmannin.

Two concerns, at least, are raised by these results. First, the mechanism of PI3K activation by TNF needs to be considered. Second, we need to address the mechanism by which BAD phosphorylation promotes cell survival or, alternatively, the mechanism by which inhibition of BAD phosphorylation promotes cell killing.

The TNF superfamily of receptors includes, in addition to the two TNF receptors (TNF-R1 and TNF-R2), the Fas receptor (CD95), CD40, the lymphotoxin β-receptor, and nerve growth factor (13). NGF and anti-CD40 have also been shown to activate PI3K (14–16). The exposure of PC-12 cells to NGF causes their differentiation (14). Inhibition of PI3K with wortmannin or with a dominant negative mutant of PI3K inhibited the neurite outgrowth in PC-12 cells that was induced by NGF, an effect that was accompanied by the death by apoptosis of the cells. In this regard, it is noteworthy that interleukin-2 and -3 and insulin-like growth factor-I promoted cell survival by a mechanism that depended on activation of PI3K (1, 6, 17).

Expression of a dominant negative inhibitor of PI3K was able to potentiate TNF-induced cytotoxicity as did wortmannin or LY294002. In addition, the ability of a dominant negative inhibitor of Akt and constitutively active Akt to potentiate and inhibit TNF-induced cell killing, respectively, most likely means that it is Akt activation by PI3-kinase that is critical for
\[ \text{TABLE III} \\
\text{Phosphorylation of BAD by TNF} \]

HeLa cells were cotransfected with 0.5 \( \mu \text{g} \) of pCDNA-LacZ and 5 \( \mu \text{g} \) of one of the indicated constructs. 48 h post-transfection, cells were either left untreated or treated with TNF alone or TNF and wortmannin for 6 h. Cells were then stained for \( \beta \)-galactosidase, and the number of blue cells was counted. Results are the mean \( \pm \) S.D. from two experiments.

| \( \beta \)-Galactosidase-positive cells | TNF | TNF + wortmannin |
|----------------------------------------|-----|------------------|
| pCDNA-LacZ+pcDNA                      | 98  \pm 8 | 27  \pm 5 |
| pCDNA-LacZ+PI3K(−)                    | 37  \pm 9 | 26  \pm 9 |
| pCDNA-LacZ+Akt(−)                     | 31  \pm 8 | 27  \pm 10 |
| pCDNA-LacZ+Akt(+)                     | 99  \pm 7 | 86  \pm 6 |

\[ \text{TABLE IV} \\
\text{TNF-induced cytotoxicity by PI3-kinase inhibition is prevented by Bcl-X\textsubscript{L}} \]

HeLa cells were cotransfected with 0.5 \( \mu \text{g} \) of pCDNA-LacZ and 5 \( \mu \text{g} \) of pCDNA-Bcl-X\textsubscript{L}. 48 h post-transfection, cells were either left untreated or treated with TNF and LY294002 or TNF and wortmannin for 6 h. Cells were then stained for \( \beta \)-galactosidase, and the number of blue cells was counted. Results are the mean \( \pm \) S.D. from two experiments.

| \( \beta \)-Galactosidase (+) cells | TNF + wortmannin | TNF + LY294002 |
|-----------------------------------|------------------|----------------|
| pCDNA-LacZ+pcDNA                 | 33  \pm 12 | 29  \pm 9 |
| pCDNA-LacZ+Bcl-X\textsubscript{L} | 85  \pm 13 | 81  \pm 10 |

inhibition of TNF-induced cytotoxicity and not PI3-kinase activity per se.

The precise mechanism by which the cell surface receptors for any of these agents (TNF, NGF, interleukin-2) trigger the activation of PI3K is not fully understood. The increase in PI3K upon the activation of the CD40 receptor was independent of the activation of NF-\( \kappa \)-B and, thus, would not seem to depend on the action of Tra2 (15). Recent work suggests that PI3K may be activated by Ras (18-20). However, there is at present little evidence of a connection between TNF and Ras. Thus, the mechanism of PI3K activation by TNF remains enigmatic and a focus of our current efforts.

By contrast, a mechanism can be readily proposed by which the phosphorylation of BAD promotes the survival of cells exposed to TNF. We have shown previously that the cytotoxicity of TNF depends on induction of the mitochondrial permeability transition (MPT) (21). Thus, we can rephrase our concern as the mechanism by which BAD phosphorylation prevents induction of the MPT in cells exposed to TNF. The overexpression of the proapoptotic protein Bax killed Jurkat cells as a consequence of induction of the MPT (22), and purified Bax induced the MPT in isolated mitochondria \textit{in vitro}.\textsuperscript{2} Bax, Bcl-X\textsubscript{L}, and Bcl-2 are present constitutively in HeLa cells.\textsuperscript{3} Bax is located in the cytosol and upon the initiation of the apoptotic process, has been reported to translocate to the mitochondria (23, 24). We hypothesize that upon treatment with TNF the phosphorylation of BAD prevents its interaction with Bcl-X\textsubscript{L} or Bcl-2, thereby allowing these proteins to complex with Bax (Fig. 6). When Bax is bound to either Bcl-X\textsubscript{L} or Bcl-2, it would be incapable of inducing the MPT and, thus, of killing the cells. Conversely, when the phosphorylation of BAD is inhibited, the continued interaction of BAD with either Bcl-X\textsubscript{L} or Bcl-2 prevents the interaction of these proteins with

\textsuperscript{2} J. G. Pastorino and J. L. Farber, submitted for publication.

\textsuperscript{3} M. Tafani and J. G. Pastorino, unpublished observations.

FIG. 6. Possible mechanism by which inhibition of PI3-kinase potentiates the cytotoxicity of TNF. TNFR, TNF receptor.

Bax. Bax then moves to the mitochondria and induces the MPT, a result that causes the death of the cells. In this way, there is a kind of apoptotic thermostat that determines the fate of the cell. Cell survival or cell death is determined by the relative level of BAD phosphorylation and, hence, by the amount of Bcl-X\textsubscript{L} or Bcl-2 that is available at the mitochondria to bind and neutralize the proapoptotic protein Bax, thereby inhibiting mitochondrial dysfunction and the initiation of an apoptotic cascade.

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