

Published in final edited form as:

Biochem Biophys Res Commun. 2006 November 17; 350(2): 463–471. doi:10.1016/j.bbrc.2006.09.077.

PTEN enhances TNF-induced apoptosis through modulation of nuclear factor-kappa B signaling pathway in human glioma Cells

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Abstract

The PTEN tumor suppressor gene modulates cell growth and survival known to be regulated by the activation of the transcription factor NF- κ B, suggesting PTEN might affect the NF- κ B activation pathway. We found that PTEN inhibited NF- κ B activation induced by TNF. The suppression of NF- κ B activation correlated with sequential inhibition of the tumor necrosis factor-induced expression of NF- κ B-regulated anti-apoptotic (IAP1, IAP2, Bcl-2, Bcl-xL, cFLIP, Bfl-1/A1, and survivin) gene products. Downregulation of the antiapoptotic genes by PTEN increased TNF-induced apoptosis, as indicated by caspase activation, TUNEL, annexin staining, and estrase assay. We conclude that the ectopic expression of PTEN enhances TNF-induced apoptosis and downregulates the proliferation of glioma cells through the suppression of various molecules including NF κ B, and various mediators of cellular survival and proliferation and that this targets might be essential for its central role in the growth and survival of glioma cancer cells.

Keywords

PTEN; TNF- α ; NF κ B; apoptosis

Introduction

Gliomas remain one of the human tumors most refractory to treatment, despite continuing advances in radiotherapy, chemotherapy, and surgical techniques. The most malignant subtype, glioblastoma multiforme, is particularly aggressive, with a median survival of less than 1 year seen in most series of patients, even for patients treated with aggressive surgery, radiotherapy, and chemotherapy regimens (1–3). Consequently, various alternate approaches to the treatment of gliomas, including molecular therapies are attracting much interest. Resistance of tumor cells to the induction of apoptosis is an important reason for the failure of anticancer treatments in patients with gliomas, and several factors working in concert have been implicated as sources of this treatment resistance. One factor that has frequently been implicated in the development

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of tumor cell resistance to apoptosis is nuclear factor kappa B (NF κ B). NF κ B regulates the expression of many genes that are essential for cell growth and differentiation, such as cytokines, growth factors and their receptors, and adhesion molecules (4–8). It also up regulates the expression of genes that actively participate in controlling cell survival (Bcl-2, Bcl-xL, survivin, and the inhibitor-of-apoptosis [IAP] family) (9–11), angiogenesis (interleukin (IL)-8, basic fibroblast growth factor, and vascular endothelial growth factor) (12,13); and metastasis (matrix metalloproteases [MMPs] 2, 7, and 9) (14). Consistent with its regulatory role, the constitutive activation of NF κ B is frequently observed in different types of cancers (15) and has been correlated with cancer cell resistance to radiation- and chemotherapeutic agent-induced apoptosis (5). Conversely, the antagonism of NF κ B is an important step in initiating the transcription of specific genes that facilitate apoptosis.

Natural resistance to tumor necrosis factor (TNF)- α is another cause of treatment failure in patients with gliomas. TNF- α was first observed in the serum of endotoxin-treated mice, in which it was found to induce tumor necrosis *in vivo* and to selectively kill transformed and neoplastic cell lines *in vitro* (16). TNF- α signaling is transduced through its receptors to simultaneously elicit two opposing effects: the induction of apoptosis and the transcription of antiapoptotic genes, such as the genes that encode NF κ B and activator protein 1 (AP-1) (17, 18). Although in certain cell types and under certain conditions, TNF- α can induce apoptosis, its clinical use has been limited because of the natural resistance of numerous tumor cells to TNF-induced apoptosis noted above (19–21).

PTEN is a tumor suppressor gene inactivated in many common malignancies, including glioblastoma, melanoma, and endometrial, lung, and prostate cancer (22–26). PTEN is believed to regulate cell survival signaling through the phosphatidylinositol 3-kinase (PI3K)/Akt pathway. In particular, PTEN dephosphorylates the D3 position of the key lipid second messenger phosphatidylinositol 3,4,5-triphosphate (PIP3) (27–28). PIP3, produced by PI3K once activated by receptor tyrosine kinases, activates Ras, or G proteins, and stimulates several downstream targets, including the serine/threonine protein kinase Akt (also known as protein kinase B) (22–26). Activated Akt protects cells from apoptotic death by phosphorylating substrates such as BAD, procaspase-9, and forkhead transcription family members (29–31). Finally, multiple laboratories have shown that the PI3K/Akt pathway provides cell survival signals, in part, through the activation of the NF κ B transcription factor (32–35).

To better understand the role of PTEN in the resistance of glioma cells to apoptotic agents and to formulate potential therapies that alter PTEN expression, it is important to obtain greater insight into the effect of PTEN expression on cellular processes. The role of TNF- α in activating NF κ B has been well established. In this study, we tested our hypothesis that PTEN mediates its effects by modulating NF κ B and enhanced TNF-mediated apoptosis in glioma cells, which confirmed our hypothesis.

Materials and Methods

Materials

Tissue culture reagents and Lipofectamine were purchased from Invitrogen Life Technologies Inc. (Carlsbad, CA). Anti-PTEN, anti-p50, anti-p65, anti-poly (ADP-ribose) polymerase (PARP), anti-inhibitor of apoptosis protein 1 (IAP1), anti-IAP2, anti-Bcl-2, anti-Bcl-xL, and anti-Bfl-1/A1 antibodies were obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Anti- β -actin antibody was purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO).

Cell Culture and Retroviral Gene Construction and Stable Transfections

U251 and U87 human glioblastoma cells (American Type Culture Collection, Manassas, VA), which have been shown previously to have a mutated *PTEN* gene (36), were maintained in culture medium (Dulbecco's modified Eagle medium/F12, 5% fetal bovine serum) in a humidified atmosphere containing 5% CO₂ at 37° C. The *PTEN* gene was stably expressed in U251 and U87 glioma cells as previously described (37).

Electrophoretic Mobility Shift Assay

U251 and U87 cells either expressing PTEN or vector alone were treated with 1 nM TNF for the indicated times (37) and incubated for 15 min at room temperature with radiolabeled NFκB-binding probe. For the supershift assays anti-p-50 and anti-p-65 antibodies were added to the incubation mixtures for 5 min before the radiolabeled probe was added. The protein-DNA complexes were then resolved on 5% nondenaturing polyacrylamide gels and visualized by autoradiography.

Immunoblotting

Cells were washed with ice-cold phosphate-buffered saline and lysed in ice-cold lysis buffer containing 1% Triton X-100, 50mM HEPES, pH 7.4, 150mM MgCl₂, 1mM EGTA, 100mM NaF, 10mM Na-pyrophosphate, 1mM Na₃VO₄, 10% glycerol, 1mM phenylmethyl sulfonyl fluoride, and 10 ug/ml aprotinin. Proteins were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), electroblotted to poly vinylidene difluoride membranes (Millipore, Billerica, MA), and then probed with various primary antibodies. After incubation with horseradish peroxidase-conjugated secondary antibodies, specific proteins were detected by chemiluminescence (Amersham Pharmacia Biotech, Piscataway, NJ).

Live/Dead assay

To measure the effect of PTEN on TNF-induced apoptosis, we performed the Live/Dead assay (Molecular Probes), which determines intracellular esterase activity and plasma membrane integrity. This assay employs calcein, a polyanionic dye, which is retained within live cells and produces green fluorescence. It also employs the ethidium bromide homodimer dye (red fluorescence), which can enter the cells through damaged membranes and bind to nucleic acids but is excluded by the intact plasma membrane of live cells. Briefly, 1 x 10⁵ cells were treated with 1 nM TNF for 16 h at 37° C. Cells were stained with the Live/Dead reagent (5 μM ethidium bromide homodimer, 5 μM calcein-AM) and then incubated at 37° C for 30 min. Cells were analyzed under a fluorescence microscope (Labophot-2).

Annexin V Assay

An early indicator of apoptosis is the rapid translocation and accumulation of the membrane phospholipid phosphatidylserine from the cytoplasmic interface to the extracellular surface. This loss of membrane asymmetry can be detected by exploiting the binding properties of annexin V. In our quantification of cell apoptosis, we employed the annexin V antibody conjugated with FITC fluorescence dye. Briefly, 1 x 10⁵ cells were treated with 1 nM TNFα for 16 h at 37° C and then subjected to annexin V staining. Cells were washed in phosphate-buffered saline, resuspended in 100 μl of binding buffer containing FITC-conjugated anti-annexin V antibody (Santa Cruz Biotechnology), and then analyzed by flow cytometry (FACSCaliber; BD Bio-sciences, San Diego, CA).

TUNEL Assay

We also assayed cytotoxicity using the terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end-labeling (TUNEL) method, which examines DNA strand

breaks during apoptosis, in which we used the Roche in situ cell death detection reagent (Roche Applied Science, Indianapolis, IN). Briefly, 1×10^5 cells were treated with 1nM TNF for 16 h at 37 °C. Thereafter, cells were washed with phosphate-buffered saline, air-dried, fixed with 4% paraformaldehyde, and then permeabilized with 0.1% Triton-X 100 in 0.1% sodium citrate. After being washed, cells were incubated with reaction mixture for 60 min at 37°C. Stained cells were mounted with mounting medium purchased from Sigma Chemical and analyzed under a fluorescence microscope (Labophot-2; Nikon, Tokyo, Japan). Pictures were captured using a Photometrics Coolsnap CF color camera (Nikon, Lewisville, TX) and MetaMorph version 4.6.5 software (Universal Imaging, Downingtown, PA).

Results

PTEN Inhibits the Induction of NF κ B Activation by TNF

To determine the effect of PTEN on TNF-induced NF- κ B activation, we examined if TNF- α would activate NF κ B in an electrophoretic mobility shift assay. Treatment of cells with 0.1nM TNF- α led to NF κ B activation in a time-dependent manner in both cell lines (Fig. 1A and 1B). Further, the protein-DNA complex was supershifted by p50 and p65 antibody during NF κ B activation (Fig. 1C). PTEN expression in U251 and U87 glioma cell lines significantly inhibited TNF's ability to activate NF κ B, revealing PTEN as a putative primary player in the regulation of the DNA-binding activity of NF κ B.

PTEN Potentiates TNF-induced Apoptosis

Activation of NF κ B by TNF and other chemotherapeutic agents leads to resistance to apoptosis (39–41) so we first determined the potential of PTEN to enhance apoptosis induced by TNF using the live and dead assay, 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide. The Live and Dead assay (which measures intracellular esterase activity and plasma membrane integrity) showed that PTEN up-regulated TNF- α induced cytotoxicity, in that there was an increase in the proportion of cells showing cytotoxicity from 1% to 9% for U251 cells and from 1% to 11% for U87 cells. When PTEN and TNF- α were used together, the proportion of U251 cells showing cytotoxicity increased from 2% to 26% and the proportion of U87 cells showing cytotoxicity increased from 1% to 34% (Fig. 2A). Whether this increased cytotoxicity was due to apoptosis was investigated by Annexin V staining technique. Annexin V staining indicated that PTEN up-regulated TNF-induced early apoptosis ((Fig. 2B). TNF- α induced apoptosis is affected when PTEN suppresses NF κ B. That is, stimulation with TNF- α alone did not induce a greater proportion of annexin V–positive U251 and U87 cells, but PTEN expression dramatically enhanced the proportion of apoptotic U251 cells, from 3.1% to 28.6%, and of U87 cells, from 4.8% to 30.4% (Fig. 2B). PTEN by itself also had little effect on the induction of annexin V–positive cells.

TUNEL staining confirmed that TNF-induced apoptosis was enhanced by PTEN expression (Fig. 2C). This assay showed that PTEN upregulated TNF- α induced apoptosis such that the proportion of apoptotic U251 cells increased from 2% to 20% and the proportion of apoptotic U87 cells increased from 1% to 34%. When PTEN and TNF- α were used together, the proportions of apoptotic U251 and U87 cells increased from 3% to 42% and from 4% to 49%, respectively (Fig. 2C).

Effect on apoptotic proteins

Immunoblot analysis of the extracts from cells treated with TNF clearly showed activation of the downstream caspases leading to cleavage of the 118 kDa PARP protein into an 87 kDa fragment in PTEN expressing cells, another hallmark of cells undergoing apoptosis (Figure 2D). Where as control cells did not show any PARP cleavage in response to TNF treatment.

The combined results from this study therefore showed that PTEN enhances TNF- α induced apoptotic effects.

PTEN Down-modulated TNF-induced NF- κ B-dependent Antiapoptotic Gene Expression

Because NF- κ B regulates the expression of the anti-apoptotic proteins such as IAP1/2 (42, 43), Bcl-2 (44), Bcl-xL (45), cFLIP (46), Bfl-1/A1 (47), and survivin (48), we examined whether PTEN can modulate the expression of these anti-apoptotic gene products induced by TNF in U251 and U87 cells. The results of western blot analysis showed that TNF induced the expression of most of the anti-apoptotic genes studied in glioma cells and the expression of PTEN in these cells suppressed the expression of these anti-apoptotic proteins (Fig. 3A and 3B).

Discussion

The present study showed that PTEN targets the antiapoptotic gene NF κ B induced by TNF- α by suppressing several TNF-induced antiapoptotic gene products, thereby confirming our hypothesis that PTEN mediates its effects by modulating NF κ B and identifying a function of PTEN in its role as a tumor suppressor. Because NF κ B is a transcriptional activator and its antiapoptotic function is mediated by the induction of several genes (49), PTEN's suppression of NF κ B in order to induce apoptosis by TNF- α is a unique finding that suggests a broader regulatory role for PTEN than has previously been anticipated.

In human glioma cell lines, NF κ B is constitutively activated and confers resistance to TNF-induced apoptosis. (50–51). Conversely, inhibition of NF κ B activation prevents cell cycle progression and inhibits the growth of U251 glioma cells treated with TNF in a PI3K/Akt pathway-dependent manner (52–54). PTEN is a crucial phosphatase involved in the regulation of Akt phosphorylation, in that the presence of an active PTEN protein blocks Akt phosphorylation by the dephosphorylation of the PI3K product PIP3 (25). In an effort to make glioma cells sensitive to TNF-induced apoptosis and block NF κ B activation as well as to inhibit proliferation, we therefore used PTEN as a negative regulator of the PI3K/Akt/NF κ B pathway. In this way, we showed that PTEN could regulate the DNA-binding activity of NF κ B, as evidenced by TNF's failure to activate NF κ B in PTEN-expressing U251 and U87 glioma cell lines.

Numerous studies have shown that the activation of NF κ B causes cell death pathways to be blocked (55). Further, NF κ B must be activated to protect cells from the apoptotic cascade induced by TNF α and other stimuli (56–60). In particular, TNF α binding to the TNF receptor (TNFR) can initiate apoptosis and activate the NF κ B transcription factor, which in turn suppresses apoptosis by an unknown mechanism. One clue to this was the finding that NF κ B activation blocks the activation of caspase-8, a cell death executing protein. In addition, the stimulation of cells with TNF α , a potent inflammatory cytokine, generates two types of signals: one that initiates programmed cell death (61) and one that leads to activation of the NF κ B transcription factor (62), which subsequently activates the inflammatory response. How specific cell types fare in the face of this differential activity depends upon the balance between the two signals. For example, the direct inhibition of NF κ B or one of the upstream signaling moieties during TNF α activation results in apoptosis in various cell types that are naturally resistant to TNF-induced apoptosis. Similarly, fibroblasts and macrophages from NF κ B subunit p65-deficient mice are more sensitive to TNF-induced apoptosis. Logically it follows from this that activating NF κ B induces the expression of genes that counteract apoptotic signals and prevent cell death. Other antiapoptotic genes have been shown to be activated by NF κ B, including the Bcl-2 homologues A1/Bfl-1, Bcl-xL, IEX-1, and XIAP.

In this study we used PTEN to counteract the NF κ B activation by TNF- α via the PI3K/Akt pathway, as reported earlier by our group (63), to subsequently inhibit the expression of several antiapoptotic gene products. The expression of PTEN inhibited the expression of these antiapoptotic genes and sensitized the glioma cells to TNF-induced apoptosis. Therefore, the antagonism of NF κ B by PTEN in TNF-resistant cells sensitized cells to TNF-induced apoptosis, which is important to increasing the efficacy of the chemotherapy against malignant gliomas. In summary, we have provided evidence that PTEN expression targets the transcription factors NF κ -B and that this target might be essential for its central role in the growth and survival of glioma cancer cells. In view of the relative inability of standard therapies to successfully eradicate malignant gliomas, the hope for cure ultimately rests totally or partially on identifying such targets for therapy.

Acknowledgments

This work was supported by grants from the University Cancer Foundation at The University of Texas M. D. Anderson Cancer Center to D.K. grant R01 CA-056041 from the National Institute of Health to WKA Yung and a grant from the Gilland Foundation to W. K. A. Yung. We thank Betty P. Notzon (Department of Scientific publications, M. D. Anderson) for editorial assistance, Dr. John F. de Groot (Department of Neuro-Oncology, M. D. Anderson) for critical reading of the manuscript.

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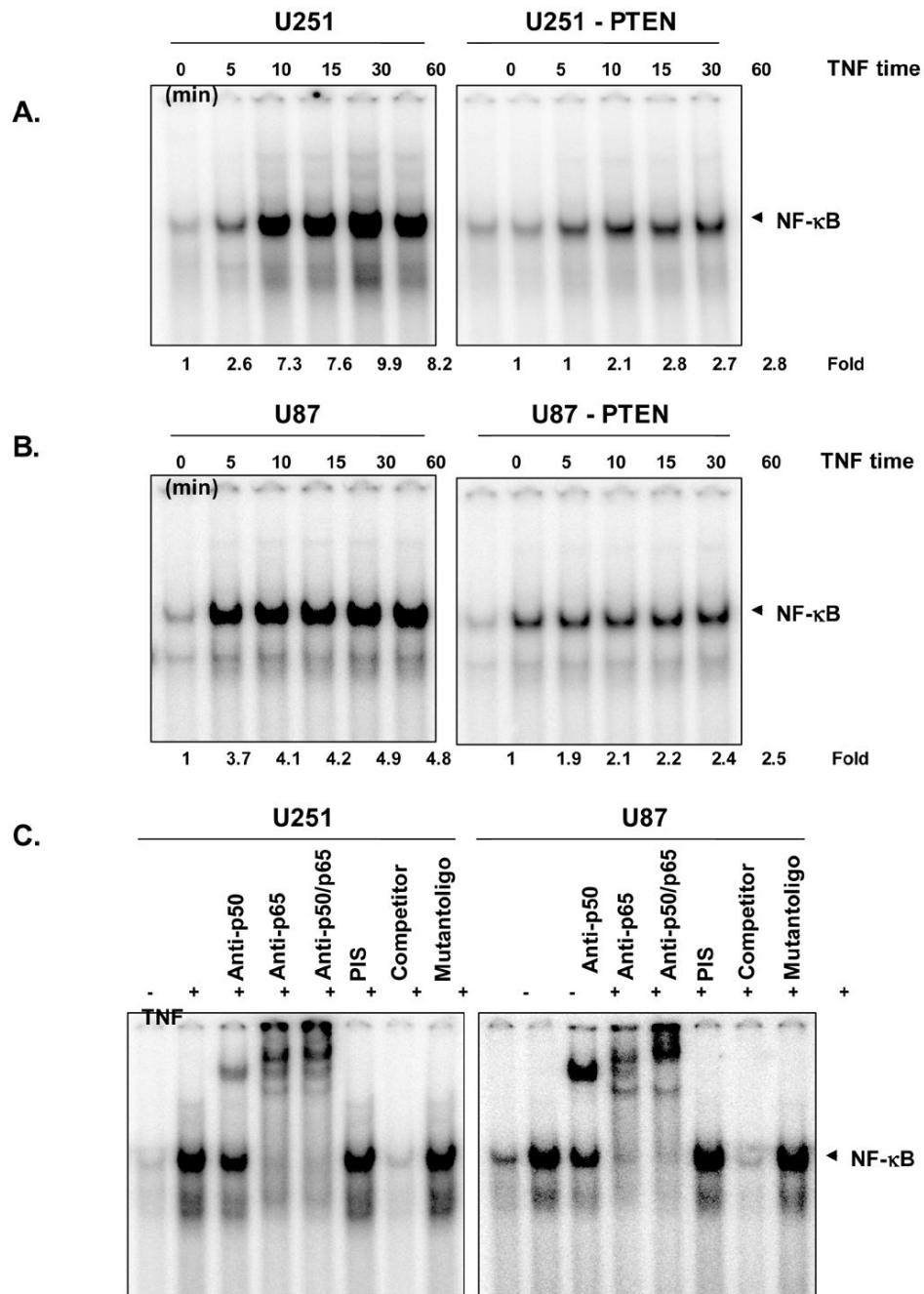
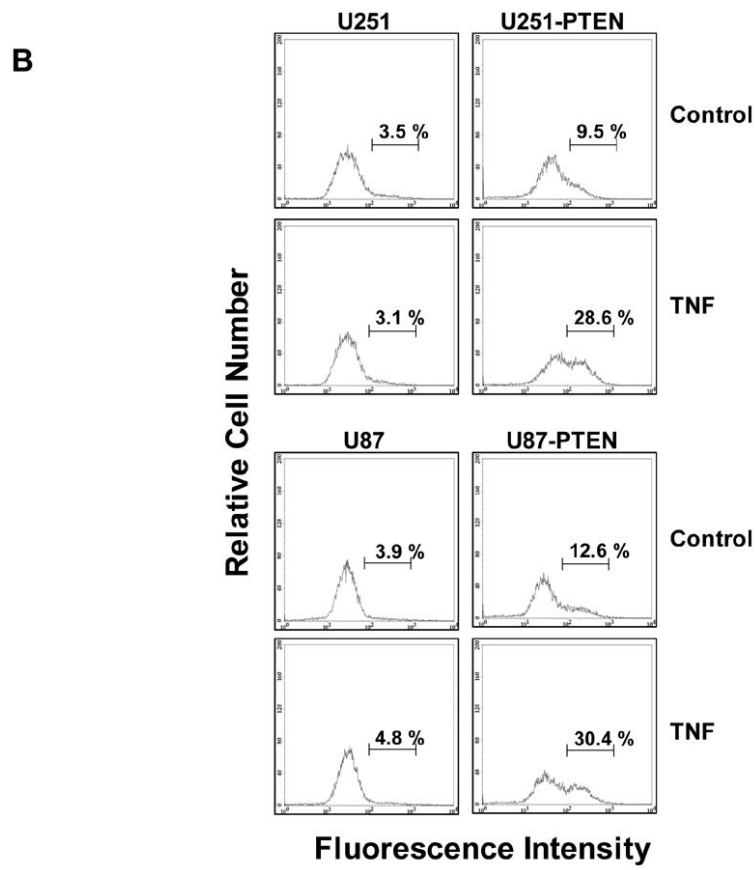
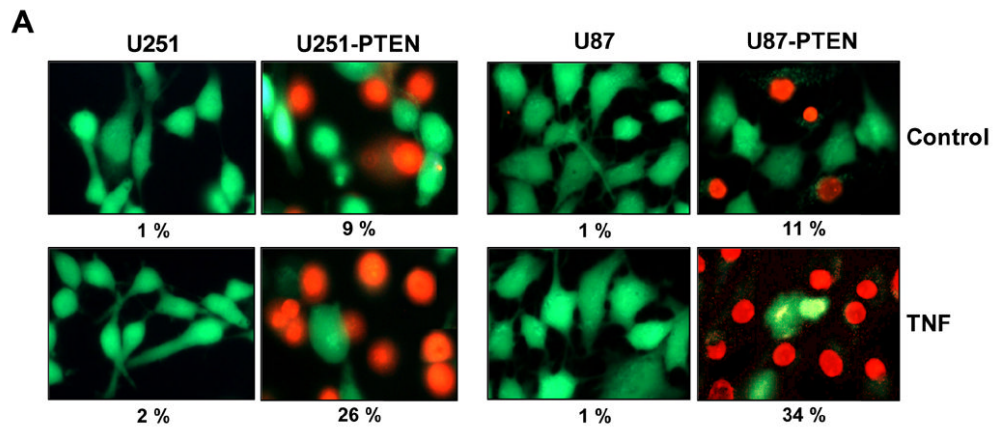


Fig. 1. Effect of PTEN on TNF-induced NF κ B activity

A, U251/U251-PTEN and B, U87/U87-PTEN cells were treated with TNF for various times before harvesting. Nuclear extracts were prepared and assayed for NF κ B activation by an electrophoretic mobility shift assay, as described in Materials and Methods. C, for the supershift assays, antibodies was added to the incubation mixtures before the probe. These data are representative of three independent experiments.



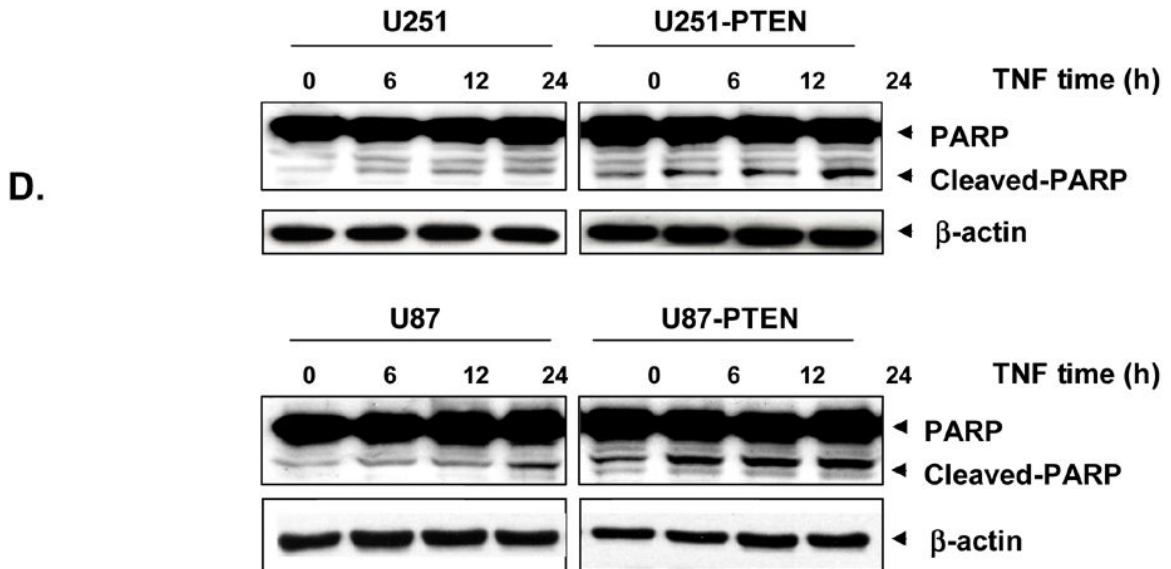
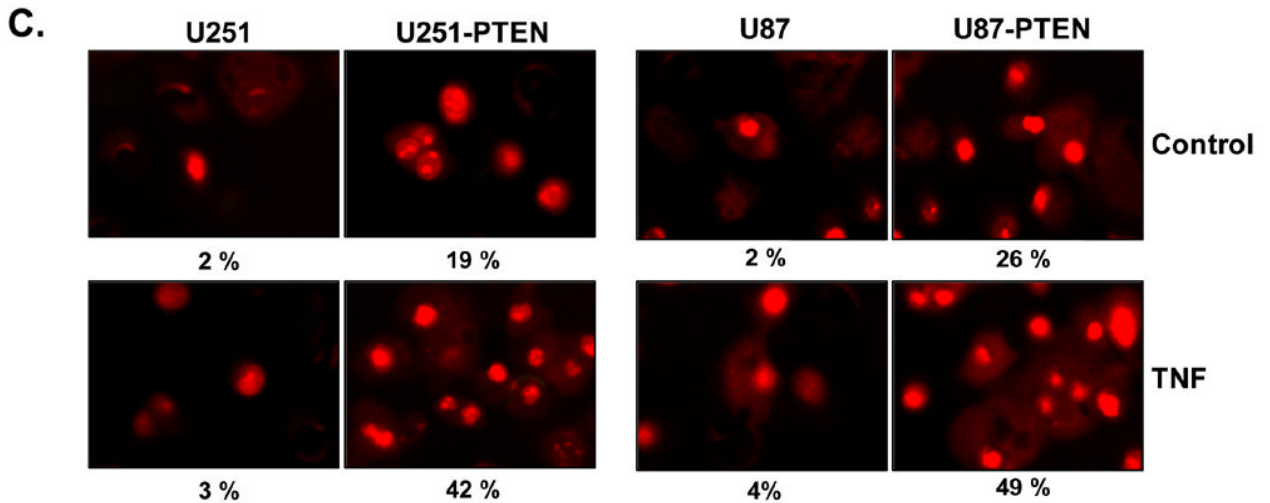


Fig. 2. PTEN sensitizes cells to TNF-induced apoptosis

A, U251/U251-PTEN and U87/U87-PTEN cells (1×10^5 cells/ml) were treated with 1 nM TNF for 16 h. Cells were stained with the Live/Dead reagent for 30 min. Cells were analyzed under a fluorescence microscope. *B*, U251/U251-PTEN and U87/U87-PTEN cells were treated with 1 nM TNF for 16 h and then underwent annexin V staining. Cells were washed, incubated with FITC-conjugated anti-annexin V antibody, and then analyzed by flow cytometry. *C*, U251/U251-PTEN and, U87/U87-PTEN cells (1×10^5 cells/well) were treated with 1 nM TNF for 16 h. Cells were washed with phosphate-buffered saline, air-dried, fixed, permeabilized, and then stained with TUNEL assay reagent, after which they were analyzed under a fluorescence microscope. *D*, U251/U251-PTEN and, U87/U87-PTEN cells (5×10^5 cells/well) were treated

with 1 nM TNF for the times indicated, and whole-cell lysates were subjected to SDS-PAGE. Western blot analysis was performed using anti-PARP antibody. β -actin was blotted as a loading control.

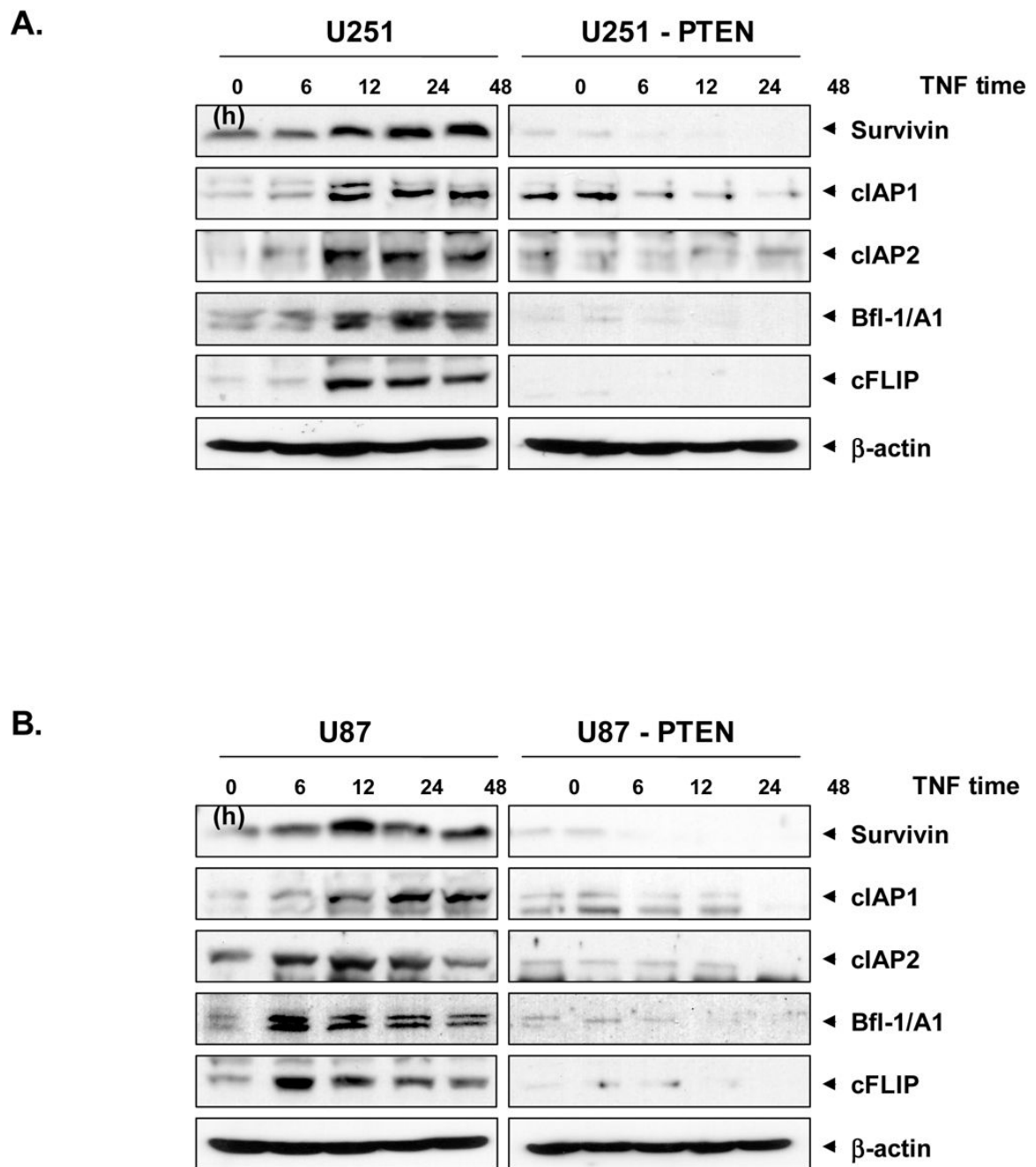


Fig. 3. PTEN inhibits the TNF-induced expression of NF- κ B -dependent genes
 U251/U251 -PTEN (A) and U87/U87-PTEN (B) cells were treated with 1 nM TNF for the times indicated. Whole-cell extracts were prepared and analyzed in Western blots using antibodies against IAP1/2, cFLIP, Bfl-1/A1 and survivin. As shown, PTEN inhibited the NF κ B-dependent gene expression induced by TNF in U251 and U87 cells.