Diverse TNFα-induced death pathways are enhanced by inhibition of NF-κB

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Abstract. TNFα was initially described as inducing necrotic death in tumors in vivo, and more recently as a cytokine that mediates cyt protection and inflammation. The anti-tumor effects of TNFα are poorly characterized because TNFα-induced death of human tumor cells has largely been studied in the presence of agents that block transcription or protein synthesis. Also, most reports in model cell systems describe apoptosis within relatively early time points as the principal mode of cell death induced by TNFα. We investigated the cytotoxic effects of 10 ng/ml TNFα on human tumor cells of different histological types without concomitant exposure to these inhibitors. Eleven of 21 human tumor cell lines underwent TNFα-induced cell death which ranged from 41% to complete loss of viability. Only one cell line demonstrated caspase-dependent apoptosis within 24 h. Nine cell lines underwent death between 48 h and 21 days. Seven of these lines underwent caspase-3 independent death consistent with necrosis. One tumor line exhibited characteristics of senescence following TNFα exposure. Nine of 9 cell lines activated NF-κB following TNFα exposure by 24 h. In all cell lines studied, with the exception of the epidermoid carcinoma cell line that underwent early apoptosis, expression of one or more NF-κB target genes was demonstrated at 24-96 h.

BMS-345541, a specific IKK inhibitor, increased TNFα killing in TNFα resistant tumor cell lines by increasing apoptosis, suggesting that inhibition of NF-κB may be an effective strategy to enhance the tumoricidal effects of TNFα.

Introduction

In 1975, Carswell et al., identified tumor necrosis factor-alpha (TNFα) as a cytokine that induces anti-tumor effects in mice (1,2). TNFα is now recognized as a multifunctional cytokine that mediates a spectrum of biological effects alone or in combination with other cytokines. These effects include anti-microbial and anti-tumor immunity, inflammation, cell proliferation, differentiation, and death. TNFα is the prototypical member of a superfamily of 19 related proapoptotic and proinflammatory cytokines that includes lymphotoxin-α (LTα)/TNFα, Fas ligand (FasL), TNF-related apoptosis-inducing ligand (TRAIL), and CD40 ligand (CD40L) (3,4). Most members of the TNF superfamily are potent activators of nuclear factor κB (NF-κB), a transcription factor first reported in B cells and subsequently described in many host responses, including host immune defenses and tissue- and cell-specific responses to stress through the induction of pro-survival and antioxidant responses (5). TNF superfamily ligands can also bind to corresponding death receptors [TNFR1, Fas (CD95), TRAMP (DR3), TRAIL-R1 (DR4), TRAIL-R2 (DR5), DR6, and EDAR] and induce cell death through apoptotic and necrotic pathways (4,6,7).

TNFα mediates its effects through two receptors: TNFR1 (TNF receptor 1) and TNFR2 (TNF receptor 2) (8). TNFα induces cell proliferation, survival, or death by binding TNFR1 (8). Binding of TNFα to TNFR1 results in the sequential formation of two distinct signaling complexes (complexes I and II) which mediate cell survival and apoptosis, respectively (4,9). The rapidly formed plasma membrane bound complex I comprised of TNFR1, TRADD, RIP1 (receptor interactive protein), and TRAF2 (TNF receptor associated factor 2) triggers cell survival and inhibits TNFα-

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mediated apoptosis through IκB kinase (IKK) dependent activation of the transcription factor NF-κB (4,10). NF-κB dimers containing RelA or c-Rel are retained in an inactive cytoplasmic complex by a family of inhibitory proteins, the IκBs, which include IκBa, IκBβ, and IκBγ (11-13,15). Following TNFα activation of TNFR1, the IκBs are phosphorylated by the IKK complex, which is comprised of two catalytic subunits, IKKα and IKKβ, and a regulatory subunit IKKγ or NEMO. The phosphorylation of IκBa by IKK targets the IκBs for rapid ubiquitin-mediated proteasomal degradation allowing NF-κB dimers to translocate to the nucleus where they stimulate the expression of numerous pro-survival target genes (10,13-23). Expression of these NF-κB target genes has been variously implicated as a principal and/or specific mediator of the TNFα pro-survival signal based on studies using genetic models or cell lines. These genes include c-IAP1 (BIRC2), XIAP, GADD45B, COX2 (PTSG2), A20 (TNFAIP3), and SOD2. However, the relationship of the specific temporal expression of these genes to clonogenic human tumor cell resistance/sensitivity to TNFα is unknown.

Although apoptosis is the most widely studied type of cell death induced by TNFα, it can also activate a pathway mediated through TNFR1 resulting in a necrotic cell death (7). TNFα binding to TNFR-1 leads to the enhanced production of reactive oxygen species (ROS) in mitochondria. The accumulation of ROS damages the mitochondria and causes the cells to undergo necrosis in which the cells swell and collapse resulting in extensive leakage of their contents (6,7,24-29). The precise intracellular events that dictate cell survival or cell death following TNFα binding are as yet unknown.

Regional perfusion of TNFα and/or local gene therapy with TNFα producing vectors has demonstrated therapeutic promise in clinical trials. These strategies employ high local concentrations of TNFα and damage the tumor vasculature. The clinical use of TNFα is currently limited to perfusion or under investigation as a locally delivered gene therapy (30-32). This is because high concentrations of TNFα are not practical for systemic administration due to toxicity (33-35). Therefore, understanding the spectrum of tumor cell killing and mechanisms underlying TNFα cytotoxicity in human tumors might expand the use of TNFα, other death ligands of the TNF superfamily, or small molecule mimetic drugs for systemic treatment of cancer. We report that TNFα is cytotoxic to 11 of 21 human tumor cell lines and most cells undergo the majority of death due to a caspase independent process resembling necrosis. Chemical inhibition of NF-κB activation by the IKK inhibitor BMS345541 enhanced TNFα cell killing by increasing apoptosis. These results provide strategies to enhance the clinical effectiveness of TNFα and other death ligands.

Materials and methods

Cell culture. Cells were cultured in appropriate media (Invitrogen, CA) supplemented with 10-20% fetal bovine serum, 2 mM L-glutamine, 100 U/ml penicillin/streptomycin. All cell lines were maintained at 37°C in a humidified chamber with 7% CO2.

Cell viability assays

MTS. Cells were seeded into 96-well plates at 2 x 10⁴ cells/well, incubated overnight and subsequently treated with 10 ng/ml TNFα. MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] reagent (Promega, Madison, WI) was added 48 h after initiation of treatment and plates were read at an absorbance of 490 nm 1-2 h later using a microplate reader. All treatments were performed in triplicate.

Clonogenic assay. To assess the ability of cells to form colonies, 200 cells were plated into P-60 plates in triplicate. After 24 h, cells were either left untreated or treated with 10 ng/ml TNFα. When colonies reached at least 50 cells (2-3 weeks), cells were fixed in ice-cold methanol and stained with crystal violet. The number of colonies in treated plates was normalized to untreated controls.

Propidium iodide staining. Cells were plated in 6-well plates at 1 x 10⁵ cells/plate in growth medium. After 24 h, cells were either left untreated or treated with 10 ng/ml TNFα. At 24, 48 or 96 h, cells were harvested, washed in cold PBS and stained for 5 min in 1 μg/ml propidium iodide (Sigma, St. Louis, MO) followed by FACS analysis.

Staining broad range caspases with FITC-VAD. To assess the general activation of caspases, cells were stained with FITC-tagged caspase inhibitor Val-Ala-Asp-fluoromethyl ketone (Promega) for 30 min at 37°C, washed and analysed by FACS for percentage of FITC-positive cells.

Active caspase-3 assay. To determine the percentage of cells with activated caspase-3, cells were harvested, washed once with PBS, fixed in CytofixCytoperm (PharMingen, San Diego, CA) solution and washed with PermWash buffer (PharMingen). Cells were then stained with PE-conjugated rabbit anti-active caspase-3 antibody according to the manufacturer’s protocol (PharMingen) and subjected to FACS analysis. Caspase activation was blocked by adding 20 μM ZVAD-fmk 15 min before treatment with TNFα.

Flow cytometry. Data were collected on a FACScan instrument using CellQuest Software (Becton-Dickinson, Franklin Lakes, NJ). At least 10,000 events were collected for each sample. FlowJo Software (FlowJo, LLC, Ashland, OR) was used for data analysis. Experiments were repeated 2-4 times per cell line with consistent results.

Transmission electron microscopy (TEM). Cells were trypsinized, washed in serum-free media, fixed in 2.5% gluteraldehyde/4% paraformaldehyde/0.1M sodium cacodylate, mounted and sectioned, and stained with 1% uranyl acetate. TEM was performed using an FEI Tecnai F30 at 300 KV. All micrographs were taken at magnification x4060.

Senescence assays

Carboxyfluorescein succinimidyl ester (CFSE) stain dilution assay. SCCS8 cells were plated at 1 x 10⁴ cells/P-100 plate. Twenty-four hours later cells were stained with 1 μg/ml 5(6)-carboxy-fluorescein diacetate, succinimidyl ester
cells were left untreated or treated for 5 or 24 h with TNF
vector pRL-SV40. Cells were allowed to recover for 24 h
construct and 0.001 μg/well of Renilla luciferase control
System, Promega). The NF-κB activation was per the manufacturer's protocol (Promega Gel Shift Assay
κB luciferase assay for NF-κB activation. Measurement of
κB was performed using the Dual-Luciferase
System (Promega) according to the manufacturer's
protocol. Briefly, cells were plated in 12-well dishes
(5x10^5 cells/plate) and 18-24 h later were co-transfected
with 0.01 μg/well of NF-κB firefly luciferase reporter
κB and 0.001 μg/well of Renilla luciferase control
vector pRL-SV40. Cells were allowed to recover for 24 h
and then replenisned with serum-free medium. Next day,
cells were left untreated or treated for 5 or 24 h with TNFα.
Subsequently, they were washed and lysed in situ for 30 min
with gentle agitation using the manufacturer's lysis buffer. A
volume of 20 μl of each lysate was used to test for levels
of firefly luciferase activity and normalized with the levels
of Renilla luciferase activity.

Inhibition of IKK with BMS-345541. To determine the effect
of the IKK inhibitor, BMS-345541 (Calbiochem, San Diego,
CA), on cell viability and caspase activation, cells were
treated with 2.5 mM BMS-345541 alone, 10 ng/ml TNFα
alone, or pretreated with inhibitor for 30 min before treat-
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mRNA isolation. Poly-A+ mRNAs were directly isolated
from cells using the MACS mRNA Isolation Kit (Miltenyi
Biotec Inc., Auburn, CA) according to the manufacturer's
protocol. Briefly, cells were lysed, mixed with the Micro-
Beads conjugated to Oligo(dT) and then loaded onto the
MACS magnetic columns. The columns were washed and
bound mRNAs eluted with hot (65°C) RNase-free water.
mRNA was used in the SuperScript-One-Step RT-PCR
System (Life Technologies, Rockville, MD).

QRT-PCR measurements of NF-κB-dependent genes. Real-
time PCR experiments were performed with 7 genes in the
12 cell lines presented in Table I. cDNA was synthesized
using Superscript II(r) reverse transcriptase (Invitrogen Life
Technologies, Carlsbad, CA) following the manufacturer's
instructions. Quantitative PCR was performed on an ABI
7700 system (Applied Biosystems, Foster City, CA) using
SYBR Green PCR reagents in a 25 μl reaction mixture
containing 2.5 μl 10xSYBR Green PCR buffer, 0.25 μl
10 mM primers, 2 μl dNTP mix, 3 μl 25 mM MgCl2, 0.25 μl
AmpErase, 0.125 μl AmpliTaq Gold and 2.5 μl of the 1:10
diluted cDNA. Primers for selected genes were designed
based on UniGene reference sequences using PrimerExpress
software (Applied Biosystems). PCR was performed for 40
cycles at 95°C for 15 sec and 60°C for 1 min after initial incu-
bations at 50°C for 2 min and 95°C for 10 min. All samples
were amplified in triplicate reactions. The expression of
each individual gene was calculated based on the difference
between amplification of the individual mRNA template and
the internal control (GAPDH) mRNA template (dct) as
described in the manufacturer's instructions (Applied Bio-
systems). Fold induction was calculated as 2^ΔΔct where ddct
values were differences between dct values of control samples
and samples treated by TNFα. Standard deviations were
calculated according to manufacturer's instructions. Intervals
of fold-induction values correspond to ddct+sd and ddct-sd.

Results

TNFα decreases survival in 2/21 human tumor cell lines
measured by MTS. We investigated the cytotoxic effects of
TNFα (10 ng/ml), in human tumor cell lines without the
concomitant use of protein synthesis inhibitors employed
in many reports of TNFα cytotoxicity assays to enhance
TNFα killing, presumably by inhibiting NF-κB target gene
expression (1,2,10,36-38). Twenty-one cell lines representing
9 tumor histological types were employed in our study: i)
breast: MCF7, MDA-MB435 and MDA-MB231; ii) head and
neck: SQ20B, SCC58 and SCC35; iii) prostatic: Panc1,
BxPC3 and MiaPaCa2; iv) sarcoma: STSAR90, STSAR11;
v) melanoma: U1Mel; vi) prostate: LNCaP, DU145 and PC3;
vi) glioma: T98g and D54; vii) esophageal: Seg1 and Bic;
and ix) colon: WiDr and HCT116. We employed several
assays to assess loss of viability. We measured cell death that
occurs within 48 h by MTS and demonstrated that only 2
of 21 (9.5%) tumor cell lines showed a loss of viability >30%.
However, 18 of 21 (85.7%) exhibited >80% survival at 48 h
of exposure to TNFα (Fig. 1). (cell counts at 48 h were
not different in TNFα-treated and control cells where MTS
indicates >100% survival - data not shown). The clonogenic
assay reflects apoptosis, necrosis and senescence. Results
from the clonogenic assay measured at 21 days showed <70%
cell survival in 11 of 21 cell lines [including cell(s) death
measured by MTS]. Eight of these 11 cell lines demonstrated
<31% survival. MTS measures survival at 48 h and clono-
genic survival measures cell death at 3 weeks. These results
are consistent with TNFα induction of cell death beyond
48 h. We investigated TNFα mediated effects in cell lines
SQ20B, SCC58, MiaPaCa2, STSAR90, STSAR11, Panc1,
LNCaP and BxPC3 based on time of cell death and appearance of cell morphology in vitro at time points from
48 h to 3 weeks.
Table I. TNFα induction of anti-apoptotic genes is cell type specific.

<table>
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<th>A20</th>
<th>CIAP1</th>
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mRNA was extracted at 24 h after 10 ng/ml TNFα and real-time PCR was performed on 12 cell lines for 7 anti-apoptotic genes. Results are reported as fold changes with upper and lower limits derived from independent experiments (see Materials and methods). Gene list: A20, TNFα induced protein 3; CIAP1, baculoviral IAP repeat-containing protein 2; COX2, prostaglandin-endoperoxide synthase 2; GADD45B growth-arrest-and DNA damage inducible gene; IKBA, nuclear factor kappa B inhibitor; SOD2, superoxide dismutase 2; XIAP, baculoviral IAP repeat containing protein 4.

Figure 1. Cell viability assays. Tumor cells were cultured with and without 10 ng/ml TNFα and assessed for cell viability by MTS assay at 48 h after treatment (light bars) and clonogenic assay (dark bars) 21 days after treatment for temporal evaluation of cell death in response to TNFα. Cell viability of TNF-treated samples was normalized to untreated samples.
Human tumors undergo early (<48 h) apoptosis and late (>48 h) necrosis induced by TNFα. Only one tumor cell line we studied, SQ20B, underwent >50% death within 48 h as determined by MTS. We performed survival studies in this cell line with propidium iodide (PI) staining as a measure of TNFα-induced cell death and assayed for caspase-3 activation as a measure of apoptosis. In SQ20B cells treated with TNFα for 48 h, 63 and 57% of cells stained positive for active caspase-3 and PI, respectively (Fig. 2a), and TEM confirmed the presence of apoptotic cells (Fig. 2b). Pretreatment with Z-VAD-fmk, a broad caspase inhibitor reduced TNFα-induced cell death to <5%. These results demonstrate that cell death in SQ20B cells is mediated through caspase-dependent apoptosis. Next we measured cell death with PI staining at 24 and 96 h after TNFα treatment (Fig. 2c) of Panc1, STSAR11, STSAR90, LNCaP, BxPC3 and MiaPaCa2, cell lines that each had >60% viability at 48 h by MTS but demonstrated <30% clonogenic survival, as well as MiaPaCa2 which demonstrated an intermediate level of TNFα sensitivity. Anti-active caspase-3 antibody staining was performed at 24, 48 and 96 h in these cell lines (Fig. 2d). SQ20B cells were studied as a positive control. Seventy percent of SQ20B cells stained...
human tumors undergo senescence induced by TNFα. SCC58 cells demonstrated <5% cell death, as measured by PI staining, despite undergoing >80% clonogenic death. Visual examination of SCC58 cells 4 days or more after TNFα treatment demonstrated the presence of many scattered individual non-colony forming tumor cells (data not shown).

To study the proliferative potential of these tumor cell lines in response to TNFα, we employed CFDA SE (carboxy-fluorescein diacetate succinimidyl ester) staining. CFDA SE forms a dye-protein adduct that is retained by the cells and is inherited by the daughter cells after cell division. The signal intensity of a cell after one cell division is half of the initial intensity. The proliferation control shows the decreased intensity of normally proliferating cells 4 days after staining with CFDA SE. After 4 days of treatment with 10 ng/ml TNFα, 36.9% of the cells have retained the dye at full intensity, indicating that this population of cells is not proliferating. Increased SA-β-gal staining of SCC58 at 96 h after 10 ng/ml TNFα, shows that the reduction in proliferation is likely due to senescence. Real-time PCR of SCC58 for p21 gene expression (c) at 24 and 96 h shows a 4- and 8-fold increase in the percentage of positively stained cells 96 h after TNFα treatment (Fig. 3b).

A 4- and 8-fold increase in induction of p21 at 24 and 96 h, respectively, was also observed (Fig. 3c). Taken together, these results are consistent with the induction of senescence in a population of SCC58 tumor cells.

NF-κB induction increased following TNFα treatment. To detect whether TNFα affects the binding of NF-κB sequences in nuclear extracts, we used an electrophoretic mobility shift assay (EMSA) in the TNFα-sensitive and -resistant tumor cell lines. Fig. 4a shows images of EMSAs representative of two TNFα-sensitive cell types (SQ20B and MiaPaCa2), and three TNFα-resistant cell types (T98g, D54 and HCT116) showing that binding is detectable in all the cell lines. Moreover, increased NF-κB binding activity was observed in all tumor cell lines treated with TNFα for 1, 5, 24 and 96 h. To evaluate the effect of TNFα on NF-κB-dependent transcription, an NFκB-responsive luciferase reporter was transfected into 5 cell lines resistant to the cytotoxic effects of TNFα; HCT116, Seg1, WiDr, T98g and D54 (<30% loss of viability in a clonogenic assay). NFκB-dependent luciferase activity was increased in all 5 cell lines at both 5 and 24 h after TNFα treatment (Fig. 4b) with a range of 3 to 9-fold induction. In 4 cell lines susceptible to TNFα; SQ20B, Panc1, STSAR90 and MiaPaCa2 (loss of viability >30% in clonogenic assay), activity was increased in all 4 cell lines at both 5 and 24 h (Fig. 4b) after TNFα treatment, with a range of 6 to 63-fold induction. These results are consistent with those of the gel shift data, suggesting that NF-κB is activated in response to TNFα in both resistant and sensitive cell lines.

NF-κB related gene expression is induced independently of cell type or late (>48 h) survival response to TNFα. Expression of seven NF-κB target genes (c-IAP, x-IAP, GADD45A, BCL2, A20, C0X2, and IκBa) previously reported to block TNFα induction of death was measured at 24 h using real-time PCR in 12 cell lines (Table I). The pattern of gene expression was independent of cell type or survival response, suggesting that induction of NF-κB target genes, alone or in combination, is insufficient to prevent late cell death.
Treatment of TNFα-resistant tumor cell lines with the IKKβ inhibitor BMS-345541 increases TNFα mediated cell death by apoptosis. Genetic data with Rel A cells or expression of a non-degradable IκBα indicates that blockade of NF-κB signaling enhances TNFα mediated cell death. To investigate the effects of the inhibition of NF-κB activation on TNFα-induced cell death, we used BMS-34551, a highly selective and specific IKKβ inhibitor (IC₅₀ 0.3 μM). We chose 10 μM as the concentration for the inhibition of NF-κB activation based on results from the luciferase reporter assay, and verified the suppression of induction of NF-κB target genes by BMS-34551 in several cell lines (see Fig. 5a and b). To test whether BMS-34551 increases TNFα-induced tumor cell death, we studied 6 TNFα-resistant 6 cell lines (T98g, DU145, WiDr, PC3, HCT116 and Seg1) after pretreatment with 10 μM BMS-34551. Staining with PI and FITC-VAD was assessed at 24 h after treatment with TNFα alone, BMS-34551 alone, or BMS-34551 followed by TNFα. As demonstrated in Fig. 5c, pretreatment with BMS-34551 followed by TNFα resulted in a 25-75% increase in cell death compared to control samples and samples treated with inhibitor alone or TNFα alone. In addition, the cells that underwent cell death stained positive for PI and FITC-VAD, indicating that the increase in cell death observed after 24 h occurs through a caspase-dependent apoptotic pathway. These results demonstrate that combining TNFα with pretreatment with BMS-34551 increases TNFα-induced cell killing of human tumor cells.

Discussion

Recent studies of the effects of TNFα on cell survival in genetically defined cell lines report that TNFα activates NF-κB and promotes survival through activation of NF-κB target genes (14-16,18,19,22,43-47). These results and those obtained from studies of TNFα-induced cell death that employ actinomycin D or cyclohexamide to induce TNFα-mediated death have led some investigators to conclude that TNFα is not directly cytotoxic to tumor cells. These conclusions and the clinical toxicity reported at high TNFα concentrations have shifted the focus away from TNFα as a potential systemic anti-cancer treatment.

We report that 11 of 21 human tumor cell lines exhibit cell death ranging from 41% to complete loss of viability at 21 days after treatment with TNFα. Only one of the 21 tumor cell lines in our study exhibited cell death >50% in a short-term MTS assay. This cell line, SQ20B, underwent caspase-3 dependent TNFα-induced apoptosis, which is reported in
most investigations of TNFα killing. By contrast, in the 8 tumor cell lines studied up to 6 days after TNFα treatment, relatively little cell death occurred as measured by MTS and PI staining; however, these tumor cells eventually underwent clonogenic cell death either through necrosis or induction of senescence. These results suggest that TNFα induces a large proportion of cell death in human tumor cells at 4-21 days. Our results are concordant with those of Fiers et al. (7), who reported cell death mediated by necrosis following TNFα in L929 cells. In the SCC58 cell line, treatment with TNFα resulted in induction of a senescent phenotype in a subpopulation of tumor cells, which included growth arrest, activation of cyclin inhibitor p21, and positive SA-β-Gal staining. TNFα treatment has been reported to result in prolonged cell growth inhibition in some tumor epithelial cells (48), in premature senescence in human diploid fibroblasts, and a senescence-like phenotype has been described in human leukemic cells (49). This is the first report of a TNFα-induced senescence phenotype in a human solid tumor cell line.

Many reports have linked NF-κB activation to resistance to TNFα mediated cytotoxicity. Interestingly, Tschopp and colleagues (26) reported that a TNFα-sensitive line did not activate NF-κB compared with a resistant derivative cell line. By contrast, we observed NF-κB activation following TNFα exposure in all cell lines investigated. Reports concerning NF-κB target genes that block apoptosis have identified inhibitors of activated effector caspases x-IAP, c-IAP, anti-

Figure 5. IKKβ inhibition suppresses TNFα-induced activation of NF-κB and increases TNFα-induced cell death. (a) Shows the results of the luciferase reporter assay in MiaPaCa2 cells after pretreatment with the IKK inhibitor, BMS-345541, at 5 and 10 μM demonstrating the suppression of TNFα-induced activation of NF-κB in a dose-dependent manner. (b) BMS-345541 prevents TNFα-induced upregulation of the NF-κB target gene A20 in MiaPaCa2 cells. (c) Inhibition of NF-κB activation by BMS-345541 increases TNFα-induced cell death. Cells were treated with 10 μM BMS-345541 alone, TNFα 10 ng/ml, or pretreated with the inhibitor for 30 min before treatment with 10 ng/ml TNFα, and cells were stained with FITC-VAD for caspases (light bars) or propidium iodide for cell death (dark bars) and evaluated by flow cytometry. Results are representative of 2-4 experiments per cell line. Treatment with BMS-345541 plus TNFα increases caspase-dependent cell death in all cell lines studied.
oxidant genes, including manganese superoxide dismutase (SOD2), and GADD45B, and COX2 as pivotal mediators in suppressing TNFα-mediated apoptosis (15,18-20). Consistent with these reports, our data demonstrate that, with the exception of SQ20B, all of the cell lines studied herein are resistant to TNFα-induced apoptosis, and all express anti-apoptotic genes. It is of interest that many cell lines undergo non-apoptotic death at later time points, suggesting that when apoptosis is blocked, death from TNFα still occurs and early measurement of TNFα induced death may underestimate the potential clinical efficacy of death ligands. Although we investigated a range of human tumor cell types, we recognize that one limitation of our study is that these lines might not be representative of the range of human tumors in vivo. Most studies on the mechanisms of TNFα killing have been carried out in cells derived from genetic model systems or single genetically modified cell lines. Our results raise questions as to the relevance of these systems to tumor therapy with TNFα and other death ligands.

We also noted that TNFα killing is enhanced 25-75% by pretreatment with the IKKβ inhibitor BMS-345541. The enhanced killing was mediated by an increase in apoptosis. These results, considered with the work of Karin and Lin (16), suggest that the efficacy of TNFα may be increased by chemically blocking NF-κB activation. Phase I trials of systemic TNFα reported significant toxicities, including hypotension, fever, nausea and in some cases, death. TNFα is currently employed in infusion therapy for limb sarcoma and melanoma. TNFα is in Phase III gene therapy trials as a locally administered radio-iodinable adeno-vector for locally unresectable pancreatic cancer (personal communication, Mark Thornton, GenVec). Improving the therapeutic index of TNFα by inhibiting NF-κB could allow the use of TNFα as a systemic anti-cancer agent or radio- or chemo-sensitizer.

In summary, TNFα kills human tumor cells by apoptosis, necrosis and senescence. In all cell lines examined, both sensitive and resistant to TNFα treatment, the NF-κB pathway is activated in response to TNFα. Chemical inhibition of IKKβ and NF-κB, which increases TNFα-induced apoptosis, is a therapeutic approach in cancer therapy that should be investigated.

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References


