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

MUKTA KATDARE[1*], ELENA V. EFIMOVA[2*], EDWARDINE LABAY[2], NIKOLAI N. KHODAREV[2], THOMAS E. DARGA[2], MICHAEL GAROFALO[3], SATOAKI NAKAMURA[4], DONALD W. KUFE[5], MITCHELL C. POSNER[1] and RALPH R. WEICHSELBAUM[2]

Departments of [1]Surgery, Section of General Surgery, [2]Radiation and Cellular Oncology, 5841 S. Maryland Ave., University of Chicago Hospitals, Chicago, IL 60637; [3]Department of Radiation Oncology, University of Maryland, 22 S. Greene St., Baltimore, MD, USA; [4]Department of Radiation Oncology, Osaka University Graduate School of Medicine, Osaka, Japan; [5]Department of Medical Oncology, Dana Farber Cancer Institute, 44 Binney St., Boston, MA, USA

Received May 8, 2007; Accepted July 26, 2007

Abstract. TNFα was initially described as inducing necrotic death in tumors *in vivo*, and more recently as a cytokine that mediates cytoprotection 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.

Correspondence to: Dr Ralph R. Weichselbaum, MC 9006, Department of Radiation and Cellular Oncology, 5841 S. Maryland Ave., Chicago, IL 60637, USA
E-mail: rrw@radonc.bsd.uchicago.edu

*Contributed equally

Key words: TNFα, NF-κB, necrosis, senescence, apoptosis

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-

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 IκBα, IκBß, 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 IKKß, and a regulatory subunit IKKγ or NEMO. The phosphorylation of IκBα 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% CO_2.

Cell viability assays

MTS. Cells were seeded into 96-well plates at $2x10^3$ 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 $1x10^5$ 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

Carboxyfluoroscein succinimidyl ester (CFSE stain) dilution assay. SCC58 cells were plated at $1x10^6$cells/P-100 plate. Twenty-four hours later cells were stained with 1 μg/ml 5(6)-carboxy-fluorescein diacetate, succinimidyl ester

(CFDA SE) (Molecular Probes, Invitrogen, CA) for 15 min at 37˚C. Cells were washed and either collected immediately (proliferating cell control) or grown for an additional 4 days with or without TNFα before they were collected, washed and subjected to FACS analysis for CFDA SE dilution as an indicator of cell division.

Senescence-associated-ß-gal staining. Attached cells were fixed in 2% formaldehyde/0.2% glutaraldehyde for 5 min and stained for senescence-associated-ß-gal (SA-ß-gal) activity using X-gal (5-bromo-4-chloro-3-indolyl ß-D-galactoside) at pH 6.0, as described (48). The percentage of SA-ß-gal positive cells was determined by bright-field microscopy after scoring 100-1000 cells for each sample.

NF-κB activation

Electrophoretic mobility shift assay (EMSA). Equal amounts of nuclear extracts were prepared at 5, 24 and 96 h after 10 ng/ml TNFα treatment, and gel shift assays were performed as per the manufacturer's protocol (Promega Gel Shift Assay System, Promega). The NF-κB consensus oligonucleotide sequence provided in the kit was used in the binding reaction.

Luciferase assay for NF-κB activation. Measurement of NF-κB activation was performed using the Dual-Luciferase Assay 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 construct and 0.001 μg/well of Renilla luciferase control vector pRL-SV40. Cells were allowed to recover for 24 h and then replenished 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 treatment with 10 ng/ml TNFα. Twenty-four hours later cells were harvested and subjected to analysis for PI and FITC-VAD staining.

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 $MgCl_2$, 0.25 μl AmpErase, 0.125 μl Amplitag 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 incubations 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 Biosystems). Fold induction was calculated as 2^{-ddct} 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) pancreatic: Panc1, BxPC3 and MiaPaCa2; iv) sarcoma: STSAR90, STSAR11; v) melanoma: U1Mel; vi) prostate: LNCaP, DU145 and PC3; vii) glioma: T98g and D54; viii) 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%. Conversely, 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 clonogenic 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.

Cell line	A20	CIAP1	COX2	GADD45B	IKBA	SOD2	XIAP
SQ20B	5.4 (6.7-4.4)	1.1 (1.4-0.9)	4.4 (5.5-3.5)	1.1 (1.4-0.9)	3.6 (4.9-2.6)	1.4 (1.8-1.1)	1.4 (1.8-1.1)
SCC58	3.1 (3.6-2.7)	1.4 (1.6-1.2)	1.6 (1.9-1.4)	0.6 (2.0-1.4)	1.6 (2.0-1.4)	2.6 (2.8-2.3)	2.0 (2.1-1.8)
STSAR90	11.0 (17.1-7.1)	2.3 (3.7-1.5)	1.8 (3.4-1.0)	0.9 (1.4-0.6)	4.0 (6.4-2.5)	2.5 (4.0-1.5)	1.3 (2.0-0.9)
PANC1	6.5 (8.7-4.6)	2.3 (8.7-4.8)	1.5 (2.9-0.8)	2.3 (3.5-1.5)	3.4 (6.1-1.9)	4.4 (6.2-3.1)	0.8 (1.4-0.4)
BxPC3	11.0 (14.3-8.4)	2.6 (3.6-4.2)	5.1 (6.2-4.2)	2.2 (3.0-1.6)	8.4 (12.2-5.7)	4.3 (6.0-3.1)	1.2 (1.8-0.8)
MiaPaCa2	23.5 (43.6-12.6)	1.8 (4.9-0.7)	3.4 (6.3-1.8)	3.1 (5.3-1.9)	2.6 (5.9-1.1)	7.1 (12.9-3.9)	1.1 (6.9-0.2)
T98g	76.6 (181.6-32.3)	9.8 (21.0-4.5)	3.6 (6.3-2.3)	1.6 (3.2-0.8)	3.6 (7.1-1.9)	19.9 (37.3-10.6)	4.2 (9.5-1.8)
DU145	9.8 (21.3-4.5)	3.3 (7.1-1.5)	18.0 (48.0-6.8)	5.1 (10.3-2.6)	2.8 (6.6-1.2)	3.5 (7.4-1.6)	1.7 (2.3-1.3)
WiDr	15.9 (23.2-10.9)	0.6 (0.7-0.5)	5.2 (9.6-2.8)	0.9 (1.6-0.5)	3.2 (6.3-1.6)	2.5 (4.3-1.5)	3.5 (7.3-1.7)
SEG1	1.5 (1.7-1.3)	0.6 (0.7-0.5)	0.6 (0.7-0.5)	0.4 (06-0.4)	0.9 (1.1-0.7)	1.5 (1.6-1.3)	2.2 (2.7-1.7)
D54	7.9 13.2-4.8)	1.7 (3.2-0.9)	1.9 (9.0-0.4)	1.0 (1.5-0.7)	3.4 (5.1-2.3)	1.2 (2.1-0.7)	0.8 (1.4-0.5)
PC3	2.9 (3.5-2.4)	1.3 (1.5-1.1)	1.2 (2.2-0.7)	1.1 (1.3-0.9)	2.9 (6.3-1.3)	1.7 (2.2-1.3)	1.0 (1.1-0.9)

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.

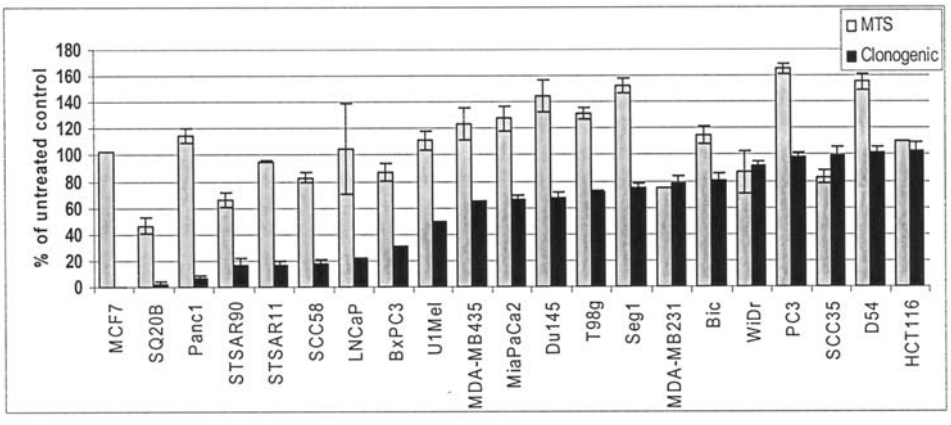

Figure 1. Cell viability assays. Tumor cells were cultured with and without 10 ng/ml TNFα and assessed for cell viability by MTS assay at 48 h after treatment (light bars) and clonogenic assay (dark bars) 21 days after treatment for temporal evaluation of cell death in response to TNFα. Cell viability of TNF-treated samples was normalized to untreated samples.

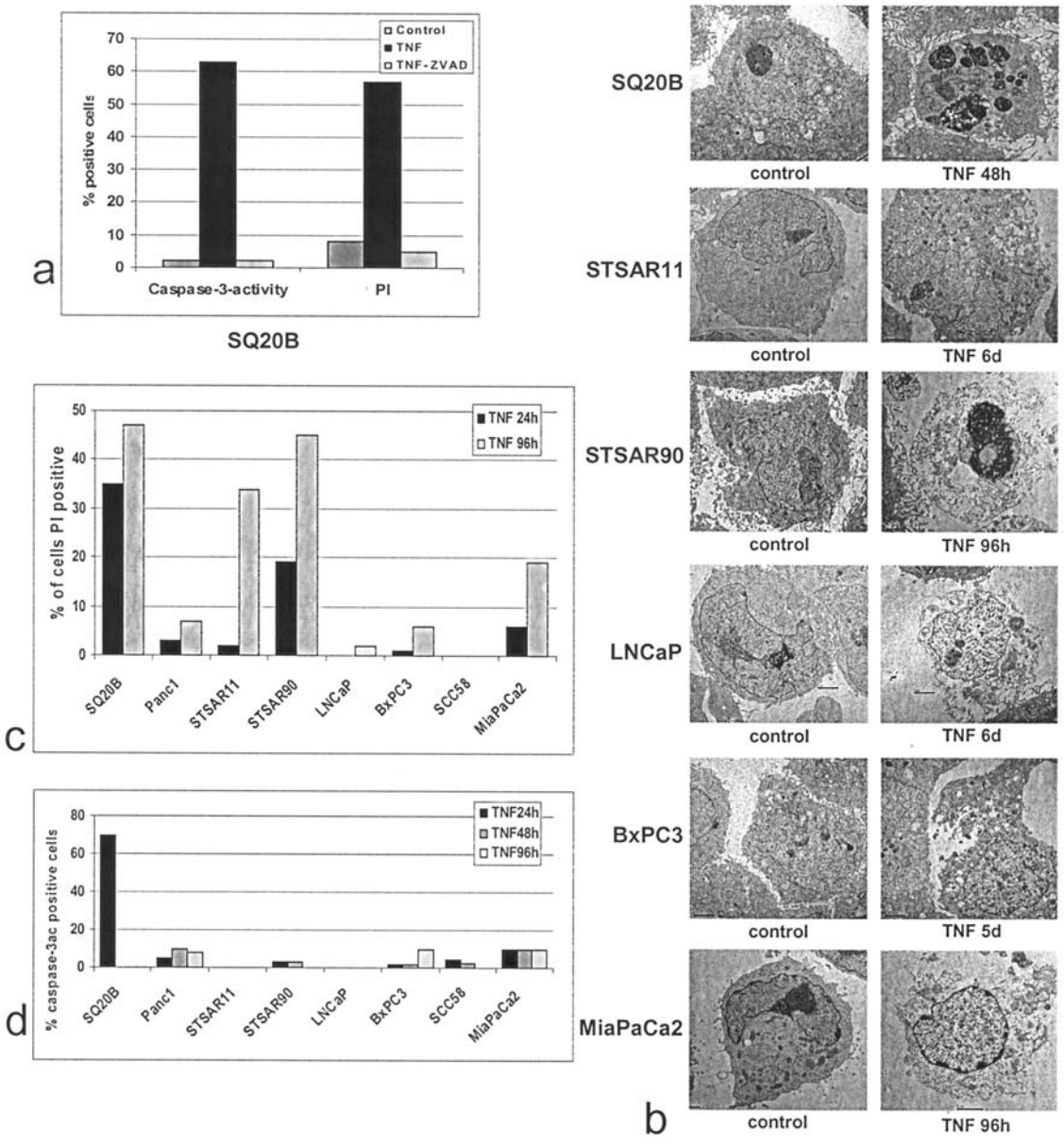

Figure 2. Caspase dependence in cell death. (a) SQ20B cells were cultured without any additions (control-dark gray bars), with 10 ng/ml TNFα alone (black bars) and with TNFα and ZVAD-fmk to block caspase activation (light gray bars) for 48 h and assessed for caspase-3 activity by staining with PE-conjugated anti-active caspase-3 antibody, and for overall cell death with PI (propidium iodide) using flow cytometry. Cell death with concomitant caspase-3 activation is abrogated by addition of Z-VAD-fmk, demonstrating an apoptotic death in SQ20B. (b) Late cell death in TNFα treated cells is characteristic of necrosis. TEM images of STSAR90, STSAR11, LNCaP, BxPC3 and MiaPaCa2 after 10 ng/ml TNFα demonstrate characteristics of necrosis (cytoplasmic swelling, rupture of the plasma membrane and dilation of cytoplasmic organelles, with the nucleus relatively intact). SQ20B cells demonstrate apoptotic death, as revealed by the formation of apoptotic bodies. (c) Seven cell lines chosen for low (<70%) clonogenic survival (along with SQ20B as a positive control for caspase-dependent death) were stained for 24 h (black bars) and 96 h (light gray bars) after 10 ng/ml TNFα with PI for overall cell death, and with PE-conjugated anti-active caspase-3 antibody for caspase-3 activity (d) at 24 h (black bars), 48 h (dark gray bars) and 96 h (light gray bars) using flow cytometry. None of the TNFα-sensitive cell lines demonstrates caspase-3 activation >10%, indicating caspase-independent cell death.

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 SCC58, 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

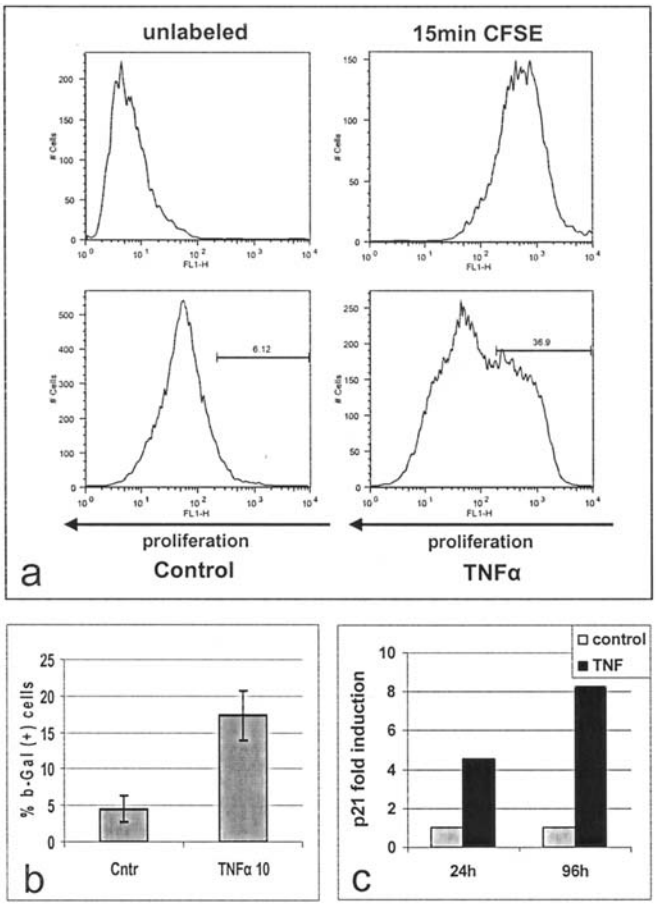

Figure 3. Senescence in SCC58. SCC58 cells were stained with CFDA SE to evaluate proliferation (a). Unlabeled cells have baseline signal intensity. Staining with CFDA SE of untreated cells at 15 min shows the initial intensity of staining. The proliferation control shows the decreased intensity of normally 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-ß-gal staining of SCC58 at 96 h after 10 ng/ml TNFα (b), 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 after 10 ng/ml TNFα also indicates senescence.

positive for caspase-3 activation, but <10% positive staining was observed in Panc1, STSAR11, STSAR90, LNCaP, BxPC3, SCC58 and MiaPaCa2 cells. Taken together, these data suggest that, in these cells, TNFα-induced death occurs mostly after 48 h and is predominantly caspase-3-independent. To study cell death in this group of cell lines, we performed TEM on STSAR11, STSAR90, LNCaP, BxPC3 and MiaPaCa2 cells at 96 h to 6 days after TNFα treatment at time points determined by observation of cells in culture. The results revealed a loss of membrane integrity, vacuolization, and an intact nucleus consistent with a necrotic cell death (Fig. 2b) (7,39). No apoptotic cells were noted in any of these cell lines on TEM. Considered together, these results suggest that in these cell lines that undergo TNFα-induced cell death late, death is caspase-independent and consistent with necrosis.

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 (carboxyfluorescein 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. Fig. 3a shows that at 96 h, 36.9% of the cells treated with TNFα have the same level of intensity as control cells treated with CFDA SE for 15 min, indicating that a subpopulation of TNFα treated cells have undergone growth arrest. To test whether these cells undergo senescence with TNFα, we evaluated growth arrest/senescence by measuring two markers of senescence; SA-ß-Gal (senescence associated ßGal) staining and induction of cyclin inhibitor p21 (40-42). SA-ß-Gal staining revealed a 4-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). NF-κB-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, GADD45B, SOD2, A20, C0X2, and IκBα) 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.

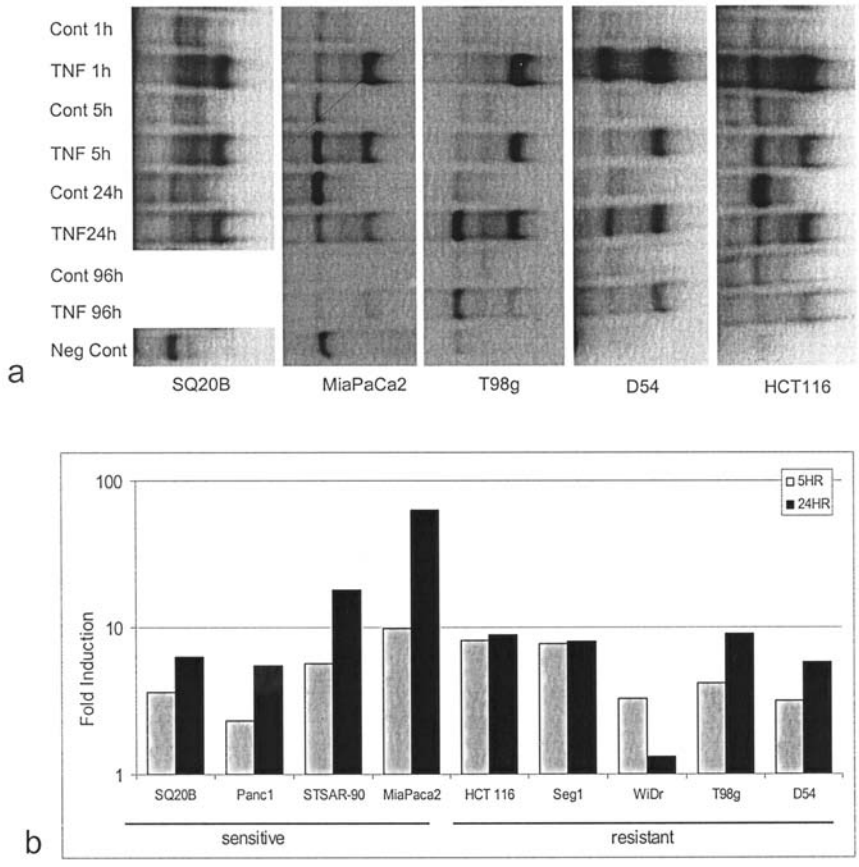

Figure 4. NF-κB is activated in all cell lines studied. (a) The binding of NF-κB to DNA promoter sequences was evaluated using EMSA in nuclear extracts taken at 5, 24 and 96 h after treatment with 10 ng/ml TNFα. No extracts were taken from SQ20B at 96 h due to early cell death in this cell line. (a) Shows that NF-κB binding increases in response to TNFα in both TNFα sensitive (SQ20B and MiaPaCa2) and TNFα resistant (HCT116, T98g and D54) cell lines. These images are representative of all cell lines studied. (b) TNFα induces NF-κB transcriptional activation. Measurement of NF-κB transcriptional activation was performed using the Dual-Luciferase Assay System (Promega) (in the same cell lines used above) at 5 h (light bars) and 24 h (dark bars) following treatment with 10 ng/ml TNFα. Results are reported as fold-induction of luciferase production by the luciferase reporter plasmid. All cell lines studied demonstrated activated NF-κB transcription after TNFα treatment at both time points with varying temporal profiles.

Treatment of TNFα-resistant tumor cell lines with the IKKß inhibitor BMS-345541 increases TNFα mediated cell death by apoptosis. Genetic data with Rel A cells or expression of a non-degradable IKBα 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 IKKß inhibitor (IC_{50} 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-345541 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-345541. 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-345541 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-345541 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

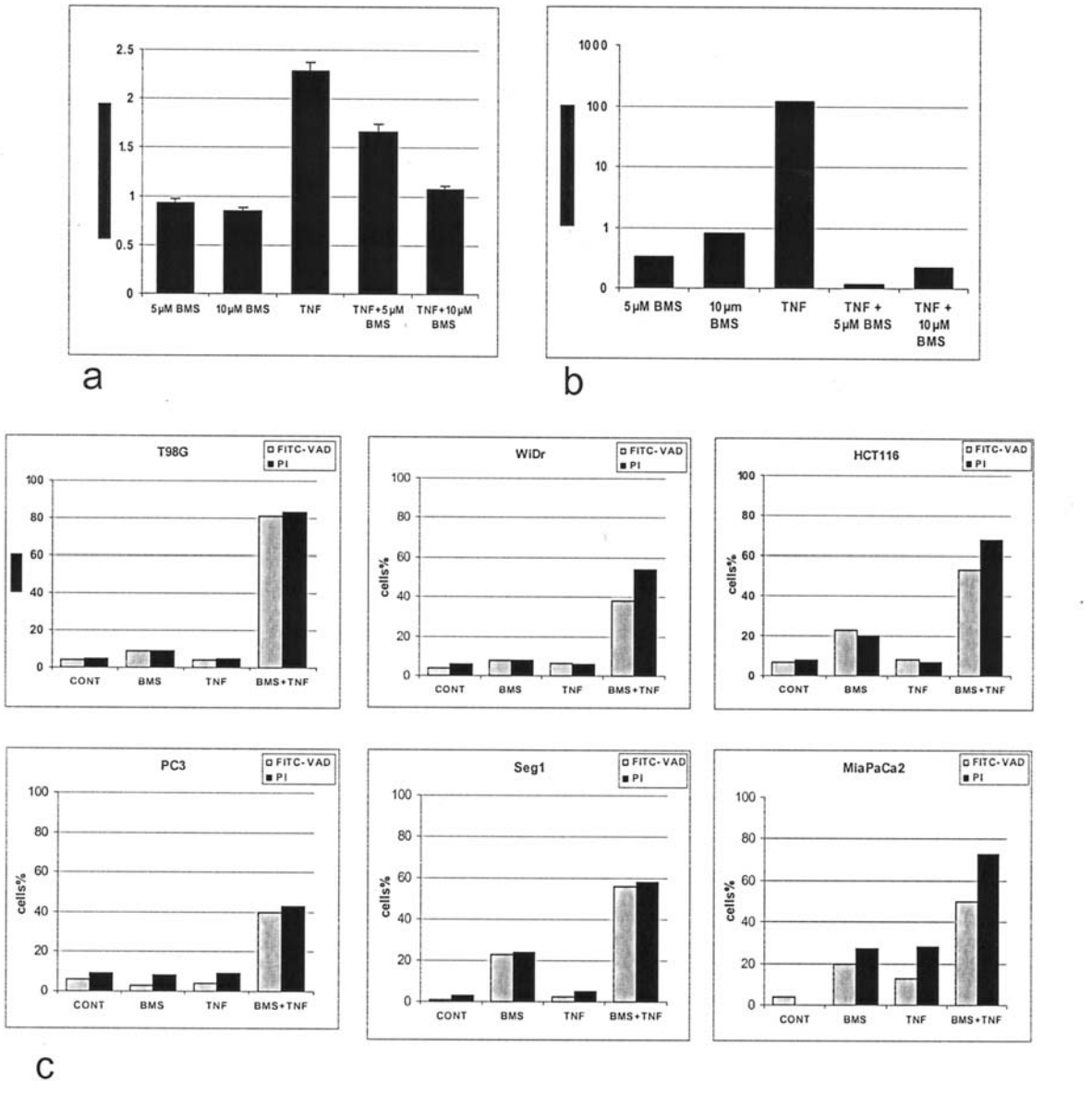

Figure 5. IKKß inhibition suppresses TNFα-induced activation of NF-κB and increases TNFα-induced cell death. (a) Shows the results of the luciferase reporter assay in MiaPaCa2 cells after pretreatment with the IKK inhibitor, BMS-345541, at 5 and 10 μM demonstrating the suppression of TNFα-induced activation 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.

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 sub-population of tumor cells, which included growth arrest, activation of cyclin inhibitor p21, and positive SA-ß-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 collegues (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-

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 underwent 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 IKKß 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 infusional therapy for limb sarcoma and melanoma. TNFα is in Phase III gene therapy trials as a locally administered radio-inducible 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 IKKß and NF-κB, which increases TNFα-induced apoptosis, is a therapeutic approach in cancer therapy that should be investigated.

Acknowledgements

This work was supported by NCI grant CA111423, by endowments from The Chicago Tumor Institute and The Center for Radiation Therapy, and by a generous gift from Mr. and Mrs. Vincent W. Foglia.

References

1. Carswell EA, Old LJ, Kassel RL, Green S, Fiore N and Williamson B: An endotoxin-induced serum factor that causes necrosis of tumors. Proc Natl Acad Sci USA 72: 3666-3670, 1975.
2. Old LJ: Tumor necrosis factor (TNF). Science 230: 630-632, 1985.
3. Aggarwal BB: Signalling pathways of the TNF superfamily: a double-edged sword. Nat Rev Immunol 3: 745-756, 2003.
4. Micheau O and Tschopp J: Induction of TNF receptor I-mediated apoptosis via two sequential signaling complexes. Cell 114: 181-190, 2003.
5. Karin M and Ben-Neriah Y: Phosphorylation meets ubiquitination: the control of NF-[kappa]B activity. Annu Rev Immunol 18: 621-663, 2000.
6. Denecker G, Vercammen D, Steemans M, Vanden Berghe T, Brouckaert G, van Loo G, Zhivotovsky B, Fiers W, Grooten J, Declercq W and Vandenabeele P: Death receptor-induced apoptotic and necrotic cell death: differential role of caspases and mitochondria. Cell Death Differ 8: 829-840, 2001.
7. Fiers W, Beyaert R, Declercq W and Vandenabeele P: More than one way to die: apoptosis, necrosis and reactive oxygen damage. Oncogene 18: 7719-7730, 1999.
8. Wajant H, Pfizenmaier K and Scheurich P: Tumor necrosis factor signaling. Cell Death Differ 10: 45-65, 2003.
9. Barnhart BC and Peter ME: The TNF receptor 1: a split personality complex. Cell 114: 148-150, 2003.
10. Kreuz S, Siegmund D, Scheurich P and Wajant H: NF-kappaB inducers upregulate cFLIP, a cycloheximide-sensitive inhibitor of death receptor signaling. Mol Cell Biol 21: 3964-3973, 2001.
11. Chen G and Goeddel DV: TNF-R1 signaling: a beautiful pathway. Science 296: 1634-1635, 2002.
12. Liu ZG, Hsu H, Goeddel DV and Karin M: Dissection of TNF receptor 1 effector functions: JNK activation is not linked to apoptosis while NF-kappaB activation prevents cell death. Cell 87: 565-576, 1996.
13. Ravi R and Bedi A: NF-kappaB in cancer - a friend turned foe. Drug Resist Updat 7: 53-67, 2004.
14. Barkett M and Gilmore TD: Control of apoptosis by Rel/NF-kappaB transcription factors. Oncogene 18: 6910-6924, 1999.
15. Beg AA and Baltimore D: An essential role for NF-kappaB in preventing TNF-alpha-induced cell death. Science 274: 782-784, 1996.
16. Karin M and Lin A: NF-kappaB at the crossroads of life and death. Nat Immunol 3: 221-227, 2002.
17. Lin A and Karin M: NF-kappaB in cancer: a marked target. Semin Cancer Biol 13: 107-114, 2003.
18. Luo JL, Kamata H and Karin M: IKK/NF-kappaB signaling: balancing life and death - a new approach to cancer therapy. J Clin Invest 115: 2625-2632, 2005.
19. Luo JL, Kamata H and Karin M: The anti-death machinery in IKK/NF-kappaB signaling. J Clin Immunol 25: 541-550, 2005.
20. Shishodia S and Aggarwal BB: Nuclear factor-kappaB activation: a question of life or death. J Biochem Mol Biol 35: 28-40, 2002.
21. Tanaka H, Matsumura I, Ezoe S, Satoh Y, Sakamaki T, Albanese C, Machii T, Pestell RG and Kanakura Y: E2F1 and c-Myc potentiate apoptosis through inhibition of NF-kappaB activity that facilitates MnSOD-mediated ROS elimination. Mol Cell 9: 1017-1029, 2002.
22. Tang G, Minemoto Y, Dibling B, Purcell NH, Li Z, Karin M and Lin A: Inhibition of JNK activation through NF-kappaB target genes. Nature 414: 313-317, 2001.
23. Zhang SQ, Kovalenko A, Cantarella G and Wallach D: Recruitment of the IKK signalosome to the p55 TNF receptor: RIP and A20 bind to NEMO (IKKgamma) upon receptor stimulation. Immunity 12: 301-311, 2000.
24. Goossens V, Grooten J, De Vos K and Fiers W: Direct evidence for tumor necrosis factor-induced mitochondrial reactive oxygen intermediates and their involvement in cytotoxicity. Proc Natl Acad Sci USA 92: 8115-8119, 1995.
25. Schulze-Osthoff K, Bakker AC, Vanhaesebroeck B, Beyaert R, Jacob WA and Fiers W: Cytotoxic activity of tumor necrosis factor is mediated by early damage of mitochondrial functions. Evidence for the involvement of mitochondrial radical generation. J Biol Chem 267: 5317-5323, 1992.
26. Schulze-Osthoff K, Beyaert R, van devoorde V, Haegeman G and Fiers W: Depletion of the mitochondrial electron transport abrogates the cytotoxic and gene-inductive effects of TNF. EMBO J 12: 3095-3104, 1993.
27. Shen HM and Pervaiz S: TNF receptor superfamily-induced cell death: redox-dependent execution. FASEB J 20: 1589-1598, 2006.
28. Vercammen D, Beyaert R, Denecker G, Goossens V, Van Loo G, Declercq W, Grooten J, Fiers W and Vandenabeele P: Inhibition of caspases increases the sensitivity of L929 cells to necrosis mediated by tumor necrosis factor. J Exp Med 187: 1477-1485, 1998.
29. Zong WX and Thompson CB: Necrotic death as a cell fate. Genes Dev 20: 1-15, 2006.

30. Gerain J, Lienard D, Pampallona S, Baumgartner M, Ruegg C, Buurman WA, Eggermont A and Lejeune F: Systemic release of soluble TNF receptors after high-dose TNF in isolated limb perfusion. Cytokine 9: 1034-1042, 1997.
31. Mundt AJ, Vijayakumar S, Nemunaitis J, Sandler A, Schwartz H, Hanna N, Peabody T, Senzer N, Chu K, Rasmussen CS, Kessler PD, Rasmussen HS, Warso M, Kufe DW, Gupta TD and Weichselbaum RR: A Phase I trial of TNFerade biologic in patients with soft tissue sarcoma in the extremities. Clin Cancer Res 10: 5747-5753, 2004.
32. Senzer N, Mani S, Rosemurgy A, Nemunaitis J, Cunningham C, Guha C, Bayol N, Gillen M, Chu K, Rasmussen C, Rasmussen H, Kufe D, Weichselbaum R and Hanna N: TNFerade biologic, an adenovector with a radiation-inducible promoter, carrying the human tumor necrosis factor alpha gene: a phase I study in patients with solid tumors. J Clin Oncol 22: 592-601, 2004.
33. Kufe D and Weichselbaum R: Radiation therapy: activation for gene transcription and the development of genetic radiotherapy-therapeutic strategies in oncology. Cancer Biol Ther 2: 326-329, 2003.
34 Lans TE, Ten Hagen TL, van Horssen R, Wu PC, van Tiel ST, Libutti SK, Alexander HR and Eggermont AM: Improved antitumor response to isolated limb perfusion with tumor necrosis factor after upregulation of endothelial monocyte-activating polypeptide II in soft tissue sarcoma. Ann Surg Oncol 9: 812-819, 2002.
35. Van Horssen R, Ten Hagen TL and Eggermont AM: TNF-alpha in cancer treatment: molecular insights, antitumor effects, and clinical utility. Oncologist 11: 397-408, 2006.
36. Schmelz K, Wieder T, Tamm I, Muller A, Essmann F, Geilen CC, Schulze-Osthoff K, Dorken B and Daniel PT: Tumor necrosis factor alpha sensitizes malignant cells to chemotherapeutic drugs via the mitochondrial apoptosis pathway independently of caspase-8 and NF-kappaB. Oncogene 23: 6743-6759, 2004.
37. Shepard HM and Lewis GD: Resistance of tumor cells to tumor necrosis factor. J Clin Immunol 8: 333-341, 1988.
38. Sugarman BJ, Aggarwal BB, Hass PE, Figari IS, Palladino MA, Jr and Shepard HM: Recombinant human tumor necrosis factor-alpha: effects on proliferation of normal and transformed cells *in vitro*. Science 230: 943-945, 1985.
39. Kroemer G, El-Deiry WS, Golstein P, Peter ME, Vaux D, Vandenabeele P, Zhivotovsky B, Blagosklonny MV, Malorni W, Knight RA, Piacentini M, Nagata S and Melino G: Classification of cell death: recommendations of the Nomenclature Committee on Cell Death. Cell Death Differ 12 (Suppl 2): 1463-1467, 2005.
40. Chang BD, Xuan Y, Broude EV, Zhu H, Schott B, Fang J and Roninson IB: Role of p53 and p21waf1/cip1 in senescence-like terminal proliferation arrest induced in human tumor cells by chemotherapeutic drugs. Oncogene 18: 4808-4818, 1999.
41. Dimri GP and Campisi J: Molecular and cell biology of replicative senescence. Cold Spring Harb Symp Quant Biol 59: 67-73, 1994.
42. Shay JW and Roninson IB: Hallmarks of senescence in carcinogenesis and cancer therapy. Oncogene 23: 2919-2933, 2004.
43. De Smaele E, Zazzeroni F, Papa S, Nguyen DU, Jin R, Jones J, Cong R and Franzoso G: Induction of gadd45beta by NF-kappaB downregulates pro-apoptotic JNK signalling. Nature 414: 308-313, 2001.
44. Papa S, Zazzeroni F, Bubici C, Jayawardena S, Alvarez K, Matsuda S, Nguyen DU, Pham CG, Nelsbach AH, Melis T, De Smaele E, Tang WJ, D'Adamio L and Franzoso G: Gadd45 beta mediates the NF-kappa B suppression of JNK signalling by targeting MKK7/JNKK2. Nat Cell Biol 6: 146-153, 2004.
45. Van Antwerp DJ, Martin SJ, Kafri T, Green DR and Verma IM: Suppression of TNF-alpha-induced apoptosis by NF-kappaB. Science 274: 787-789, 1996.
46. Wang CY, Mayo MW and Baldwin AS Jr: TNF- and cancer therapy-induced apoptosis: potentiation by inhibition of NF-kappaB. Science 274: 784-787, 1996.
47. Wang CY, Mayo MW, Korneluk RG, Goeddel DV and Baldwin AS Jr: NF-kappaB antiapoptosis: induction of TRAF1 and TRAF2 and c-IAP1 and c-IAP2 to suppress caspase-8 activation. Science 281: 1680-1683, 1998.
48. Jeoung DI, Tang B and Sonenberg M: Effects of tumor necrosis factor-alpha on antimitogenicity and cell cycle-related proteins in MCF-7 cells. J Biol Chem 270: 18367-18373, 1995.
49. Beyne-Rauzy O, Recher C, Dastugue N, Demur C, Pottier G, Laurent G, Sabatier L and Mansat-De Mas: V Tumor necrosis factor alpha induces senescence and chromosomal instability in human leukemic cells. Oncogene 23: 7507-7516, 2004.