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Inhibition of c-Myc Expression Sensitizes Hepatocytes to Tumor Necrosis Factor-induced Apoptosis and Necrosis*

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Hailing Liu‡, Chau R. Lo‡, Brett E. Jones‡, Zehra Pradhan‡, Anu Srinivasan§, Karen L. Valentino§, Richard J. Stockert‡, and Mark J. Czaja‡¶

From the ‡Department of Medicine and Marion Bessin Liver Research Center, Albert Einstein College of Medicine, Bronx, New York 10461 and §IDUN Pharmaceuticals, Inc., La Jolla, California 92037

The typical proliferative response of hepatocytes to tumor necrosis factor (TNF) can be converted to a cytotoxic one by transcriptional arrest. Although NF-κB activation is critical for hepatocyte resistance to TNF toxicity, the contribution of other TNF-inducible transcription factors remains unknown. To determine the function of c-Myc in hepatocyte sensitivity to TNF, stable transfectants of the rat hepatocyte cell line RALA255-10G containing sense and antisense *c-myc* expression vectors were isolated with increased (S-Myc cells) and decreased (AN-Myc cells) c-Myc transcriptional activity. While S-Myc cells proliferated in response to TNF treatment, AN-Myc cells underwent 32% cell death within 6 h. Fluorescent microscopic studies indicated that TNF induced apoptosis and necrosis in AN-Myc cells. Cell death was associated with DNA hypoploidy and poly(ADP-ribose) polymerase cleavage but occurred in the absence of detectable caspase-3, -7, or -8 activation. TNF-induced, AN-Myc cell death was dependent on Fas-associated protein with death domain and partially blocked by caspase inhibitors. AN-Myc cells had decreased levels of NF-κB transcriptional activity, but S-Myc cells maintained resistance to TNF despite NF-κB inactivation, suggesting that c-Myc and NF-κB independently mediate TNF resistance. Thus, in the absence of sufficient c-Myc expression, hepatocytes are sensitized to TNF-induced apoptosis and necrosis. These findings demonstrate that hepatocyte resistance to TNF is regulated by multiple transcriptional activators.

Tumor necrosis factor (TNF)[1] is a pleiotrophic cytokine that can induce either proliferative or cytotoxic responses in a variety of cultured cells including hepatocytes (1). The biological effects of TNF in cultured hepatocytes are relevant to the liver *in vivo*, since TNF also acts as a hepatic mitogen (2, 3) or cytotoxin (4–6) *in vivo*, depending on the pathophysiological setting. TNF has been implicated as a mediator of hepatocyte death following injury from toxins, ischemia/reperfusion, and hepatitis virus (for a review, see Ref. 7). In the absence of an injurious cofactor such as a toxin, hepatocytes are resistant to TNF cytotoxicity, and the mechanism by which they become sensitized to TNF-induced death in the setting of cell injury remains unknown.

The pathway from TNF stimulation to cell death has been well described (for a review, see Ref. 8). TNF binding to the type 1 TNF receptor (TNFR-1) causes receptor trimerization and the recruitment and binding of a series of intracellular proteins including TNFR-associated death domain protein and Fas-associated protein with death domain (FADD). FADD binding leads initially to activation of caspase-8, and subsequently to activation of caspase-3, resulting in apoptosis (8). While the steps in the TNF death pathway leading to apoptosis are known, the mechanism by which cells inactivate this caspase cascade and maintain resistance to TNF toxicity is unclear. A recent advance in our understanding of cellular TNF resistance has come from the demonstration that activation of the transcription factor NF-κB is critical for the induction of cellular resistance to TNF toxicity (9–12). Inhibition of NF-κB activation in cultured hepatocytes (13, 14) or in the liver *in vivo* (15) converts the hepatocellular TNF response from one of proliferation to one of apoptosis. This finding fits well with the fact that *in vitro* resistance to TNF-induced cytotoxicity requires RNA and protein synthesis (16), suggesting that TNF signaling up-regulates a protective cellular gene(s). NF-κB inactivation may sensitize cells to TNF toxicity by preventing the transcriptional up-regulation of an NF-κB-dependent protective gene(s). However, TNF activates other transcriptional activators, including c-Myc and AP-1, and their potential contribution to the transcriptional regulation of hepatocyte resistance to TNF toxicity is unknown.

c-Myc is a transcription factor that regulates cell proliferation, differentiation, and apoptosis (for a review, see Ref. 17). c-Myc expression not only promotes proliferation but also can induce or sensitize cells to apoptosis (18, 19). Overexpression of c-*myc* under circumstances in which this gene is usually down regulated such as serum deprivation, results in apoptotic cell death in nonhepatic cells (20) and in a hepatoma cell line (21). c-Myc expression has been reported to be induced by TNF alone (22, 23) or in combination with cycloheximide (24). Previous investigations in nonhepatic cells have consistently reported that increased c-Myc expression initiates or promotes TNF-induced apoptosis (24–27). However, in TNF-dependent liver injury *in vivo* induced by the toxin galactosamine (6), TNF induces hepatocyte injury and death associated with a block in the up-regulation of *c-myc* mRNA expression that normally

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¶ To whom correspondence should be addressed: Marion Bessin Liver Research Center, Albert Einstein College of Medicine, 1300 Morris Park Ave., Bronx, NY 10461. Tel.: 718-430-4255; Fax: 718-430-8975; E-mail: czaja@aecom.yu.edu.

[1] The abbreviations used are: TNF, tumor necrosis factor; TNFR, TNF receptor; FADD, Fas-associated protein with death domain; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; FACS, fluorescence-activated cell sorting; PARP, poly(ADP-ribose) polymerase; IAP, inhibitor of apoptosis; PAGE, polyacrylamide gel electrophoresis.

occurs during a hepatic proliferative response (28). These findings suggested that hepatocytes may undergo TNF-induced death in the absence of c-Myc expression or even become sensitized to TNF toxicity by a failure to up-regulate c-Myc. We therefore tested the hypothesis that c-Myc expression promotes hepatocyte resistance to TNF toxicity by examining the sensitivity of rat hepatocyte cell lines with differential c-Myc expression to TNF toxicity.

EXPERIMENTAL PROCEDURES

Cell Lines and Culture Conditions—Cells lines with differential c-Myc expression were derived from the wild-type RALA255–10G rat hepatocyte cell line (29). These cells are conditionally transformed with a temperature-sensitive T antigen. Cells were grown at the permissive temperature of 33 °C and then maintained at 37 °C to allow suppression of T antigen expression and development of a differentiated hepatocyte phenotype as described previously (29). All experiments were performed in cells cultured at 37 °C.

The *c-myc* cDNA subcloned into the expression vector pMEP4 (Invitrogen, San Diego, CA) as described previously (21) was transfected into RALA hepatocytes using LipofectAMINE Plus (Life Technologies, Inc.) according to the manufacturer's instructions. Stable transfectants were selected by resistance to 200 μg/ml hygromycin (Calbiochem). The subsequent experiments employed pooled transfectants expressing sense (S-Myc cells) and antisense (AN-Myc cells) *c-myc* constructs. All cells were cultured in 50 μM zinc for 4 days prior to the start of experiments in order to induce transgene expression from pMEP4, which contains a zinc-inducible human MT IIa promoter.

In some experiments, cells were treated with rat recombinant TNF (TNF-α, R & D Systems, Minneapolis, MN) at a concentration of 10 ng/ml, 50 μM C_2 ceramide (Biomol, Plymouth Meeting, PA), or 1.25 μmol/10^6 cells of hydrogen peroxide (H_2O_2) (Sigma). To inhibit caspase activity, cells were pretreated for 1 h before the addition of TNF with the following caspase inhibitors dissolved in dimethyl sulfoxide: 100 μM Val-Ala-Asp-fluoromethylketone (BACHEM, Torrance, CA), 50 μM *N*-[(indole-2-carbonyl)-alaninyl]-3-amino-4-oxo-5-fluoropentanoic acid (IDN-1529), or *N*-[(1,3-dimethylindol-2-carbonyl)-valinyl]-3-amino-4-oxo-5-fluoropentanoic acid (IDN-1965) (IDUN Pharmaceuticals, La Jolla, CA). IDN-1529 and IDN-1965 have broad anti-caspase activity, inhibiting caspase-1, -3, -6, and -8.[2]

Transient Transfections and Reporter Gene Assays—RALA hepatocytes were transiently transfected with luciferase reporter genes using LipofectAMINE Plus. Cells were transfected with NF-κB-Luc (30), which contains three NF-κB binding sites, or pMyc 3E1b-Luc (31), which contains three c-Myc binding sites, driving firefly luciferase reporter genes. Cells were cotransfected with pRL-TK (Promega, Madison, WI) a *Renilla* luciferase vector driven by a Herpes simplex virus thymidine kinase promoter, which served as a control for transfection efficiency. To assay luciferase activity, cells were washed in phosphate-buffered saline and lysed in 1% Triton X-100, and the cell extract was assayed for firefly luciferase activity in a luminometer. *Renilla* luciferase was assayed in the same sample according to the manufacturer's instructions. Firefly luciferase activity was then normalized to *Renilla* luciferase activity.

RNA Isolation and Northern Blot Hybridization—RNA was extracted from cells as described previously (32). Steady-state mRNA levels were determined by Northern blot hybridizations using samples of 20 μg of total RNA (32). The membranes were hybridized with [^{32}P]dCTP (PerkinElmer Life Sciences)-labeled cDNA clones for lactate dehydrogenase A (33) and glyceraldehyde-3-phosphate dehydrogenase (34). The hybridized filters were washed under stringent conditions (32).

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium Bromide (MTT) Assay—Relative cell number was determined by the MTT assay, as described previously (14). Cell survival was calculated as a percentage of control cells by taking the optical density reading of cells given a particular treatment, dividing that number by the optical density reading for the untreated, control cells, and then multiplying by 100.

Microscopic Determination of Apoptosis and Necrosis—The numbers of apoptotic and necrotic cells were determined by examining cells under fluorescence microscopy following costaining with acridine orange and ethidium bromide (14). The percentage of cells with apoptotic morphology (nuclear and cytoplasmic condensation, nuclear fragmentation, membrane blebbing, and apoptotic body formation) under acridine orange staining was determined by examining >400 cells/dish. Necrosis was determined by the presence of ethidium bromide staining in the same cell population.

FACS Analysis of DNA Hypoploidy—The identification of hypoploid cells by FACS detection of DNA loss after controlled extraction of low molecular weight DNA was performed as described previously (35). Cells were trypsinized and centrifuged, and the cell pellets were fixed in 70% ethanol and placed at −20 °C for a minimum of 17 h. The cells were washed and resuspended in Hanks' buffered saline solution and incubated in phosphate-citric acid buffer (0.2 M Na_2HPO_4, 0.1 M citric acid, pH 7.8) for 5 min. The cells were then centrifuged, and the pellet was resuspended in Hanks' buffered saline solution containing propidium iodide (20 μg/μl) and RNase (100 μg/ml). Following a 30-min incubation at room temperature, the cells were analyzed on a FACScan (Becton Dickinson Immunocytometry Systems, San Jose, CA) at an excitation of 488 nm. DNA fluorescence pulse processing was used to discriminate between single cells and aggregates of cells (Doublet Discrimination) by evaluating the FL2-Width *versus* FL2-Area scatter plot. Light scatter gating was used to eliminate smaller debris from analysis. An analysis gate was set to limit the measurement of hypoploidy to an area of 10-fold loss of DNA content.

Protein Isolation and Western Blot Analysis—For protein isolation for Western immunoblots of c-Myc and protein-disulfide isomerase, cells were washed in phosphate-buffered saline, centrifuged, and resuspended in lysis buffer composed of 50 mM Tris, pH 7.5, 150 mM sodium chloride, 0.1% SDS, 1% Nonidet P-40, 0.5% sodium deoxycholate, 1 mM dithiothreitol, 1 mM EDTA, 1 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, and 2 μg/ml of pepstatin A, leupeptin, and aprotinin. Cells were then mixed at 4 °C for 30 min. After centrifugation, the supernatant was collected, and the protein concentration was determined by the Bio-Rad protein assay. Fifty micrograms of protein were resolved on 10% SDS-PAGE as described previously (14). Membranes were stained with Ponceau red to ensure equivalent amounts of protein loading and electrophoretic transfer among samples. Membranes were exposed to a rabbit anti-c-Myc polyclonal antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) or protein-disulfide isomerase rabbit antiserum (36), at 1:1000 dilutions followed by a goat anti-rabbit secondary antibody conjugated with horseradish peroxidase (Life Technologies) at a 1:20,000 dilution. Proteins were visualized by chemiluminescence (SuperSignal West Dura Extended; Pierce).

For poly(ADP-ribose) polymerase (PARP) immunoblots, cells were washed in phosphate-buffered saline, centrifuged, and resuspended in lysis buffer containing 20 mM Tris, pH 7.5, 1% SDS, 2 mM EDTA, 2 mM EGTA, 6 mM β-mercaptoethanol, and the protease inhibitors as above. After a 10-min incubation on ice, the cell suspension was sonicated. Fifty micrograms of protein were resolved on 8% SDS-PAGE and immunoblotted with a rabbit anti-PARP polyclonal antibody (Santa Cruz Biotechnology) at a 1:1000 dilution followed by a goat anti-rabbit antibody at a 1:20,000 dilution.

For caspase immunoblots, cells were scraped in medium; centrifuged; resuspended in lysis buffer containing 10 mM HEPES, pH 7.4, 42 mM $MgCl_2$, 1% Triton X-100, and the protease inhibitors listed previously; and mixed at 4 °C for 30 min. Fifty micrograms of protein were resolved on 10% SDS-PAGE and immunoblotted with rabbit polyclonal anti-caspase-3, -7, and -8 antibodies (IDUN Pharmaceuticals) at 1:2000, 1:1000, and 1:4000 dilutions, respectively, followed by a goat anti-rabbit secondary antibody at a 1:10,000 dilution.

To examine mitochondrial cytochrome *c* release, mitochondrial fractions were prepared by differential centrifugation in sucrose as described previously (35). Fifty micrograms of mitochondrial protein were subjected to 15% SDS-PAGE as described above. A mouse anti-cytochrome *c* monoclonal IgG (Pharmingen, San Diego, CA) and a mouse anti-cytochrome oxidase subunit IV monoclonal IgG (Molecular Probes, Inc., Eugene, OR) were used at 1:1000 dilutions together with a goat anti-mouse IgG conjugated to horseradish peroxidase (Life Technologies).

Adenovirus Preparation and Infection—The following adenoviruses were employed: a control virus Ad5LacZ that expresses the *Escherichia coli* β-galactosidase gene; NFD-4 containing a dominant negative FADD; a CrmA-expressing adenovirus; and Ad5IκB, which expresses a mutated IκB that irreversibly binds NF-κB, preventing its activation (13). Viruses were grown in 293 cells; purified by banding twice on CsCl gradients; dialyzed against 5 mM Tris, pH 8.0, 50 mM $MgCl_2$, 3% glycerol, and 0.05% bovine serum albumin; and stored at −80 °C. Cells were infected with 5×10^9 particles of the appropriate virus per 35-mm culture dish (~1.5×10^3 particles/cell or 5–15 plaque-forming units/cell) as described previously (14).

Electrophoretic Mobility Shift Assays—Nuclear proteins were iso-

[2] J. Wu, personal communication.

lated by the method of Schreiber *et al.* (37), modified as described previously (21). Electrophoretic mobility shift assays were performed on 5 μg of protein with a ^{32}P-end-labeled oligonucleotide for the NF-κB consensus sequence (Santa Cruz Biotechnology). The DNA binding reaction was performed as described previously (21); the samples were resolved on a 4% polyacrylamide gel, dried, and subjected to autoradiography.

Statistical Analysis—All numerical results are reported as mean ± S.E. and represent data from a minimum of three independent experiments performed in duplicate.

RESULTS

Establishment of Sense and Antisense c-myc-expressing Cell Lines—RALA hepatocytes were transfected with the pMEP4 expression vector containing the *c-myc* cDNA in either a sense or antisense orientation. Stable transfectants were selected in hygromycin and initially screened for *c-myc* expression by Northern blot analysis. Two polyclonal cell lines were selected in which expression of sense *c-myc* (S-Myc cells) and antisense *c-myc* (AN-Myc cells) constructs resulted in maximally increased and decreased *c-myc* levels, respectively. Western immunoblotting confirmed that S-Myc cells had increased c-Myc levels compared with AN-Myc cells, while the two cell lines had equivalent levels of the constitutively expressed protein-disulfide isomerase (Fig. 1*A*). The relative amounts of c-Myc transcriptional activity in the two cells lines were measured with a transiently transfected c-Myc firefly luciferase reporter, and the results were normalized to a cotransfected *Renilla* luciferase reporter under the control of a minimal reporter. c-Myc transcriptional activity in untreated cells was increased over 14-fold in S-Myc cells as compared with AN-Myc cells (Fig. 1*B*). Although c-Myc-dependent transcriptional activity increased in both cell lines following TNF treatment, the activity in AN-Myc cells was still less than 10% of the activity in S-Myc cells (Fig. 1*B*). As additional evidence of differential c-Myc transcriptional activity in the two cell lines, mRNA levels for the c-Myc-dependent lactate dehydrogenase A gene (33), were determined by Northern blot analysis. S-Myc cells had significantly increased expression of lactate dehydrogenase A relative to AN-Myc cells, while RNA levels of the constitutively expressed glyceraldehyde-3-phosphate dehydrogenase gene were equivalent in the two cell lines (Fig. 1*C*). Thus, as assessed by protein levels, transcriptional activity, and c-Myc-dependent gene expression, c-Myc levels were increased in S-Myc cells relative to AN-Myc cells.

Decreased c-Myc Expression Sensitizes RALA Hepatocytes to TNF Cytotoxicity—TNF treatment of RALA hepatocytes results in a proliferative response (14), similar to the known mitogenic effect of TNF on the liver *in vivo* following partial hepatectomy (2, 3). To convert the TNF response from proliferation to apoptosis requires either cotreatment with the RNA synthesis inhibitor actinomycin D or inhibition of activation of the transcription factor NF-κB (14, 35). Similar to wild-type RALA hepatocytes, S-Myc cells were resistant to TNF toxicity as determined by MTT assays 6 and 24 h after TNF treatment (Fig. 2*A*). Despite the use of highly confluent cultures, S-Myc cell number increased 11% at 24 h, indicating that these cells underwent a proliferative response to TNF, a result identical to previous findings in wild-type cells (14). In contrast, TNF treatment of AN-Myc cells resulted in a 32% decrease in cell number within only 6 h and only a slight further decrease in cell number by 24 h (Fig. 2*A*). In keeping with previously published results (14), TNF at 10 ng/ml resulted in a maximal death response because no further decrease in cell number occurred when AN-Myc cells were treated with a higher TNF concentration of 30 ng/ml (data not shown).

To examine whether AN-Myc cell sensitivity to TNF toxicity represented a nonspecific sensitization to any cell death stimulus, cell survival was determined following treatment with C_2 ceramide and H_2O_2. Ceramide is a known apoptotic stimulus that has been implicated as a downstream mediator of TNF-induced cell death (38). The oxidant H_2O_2 triggers apoptosis in many cell types, including RALA cells (39), and oxidative stress has been implicated as a mechanism of TNF toxicity (1). Iden-

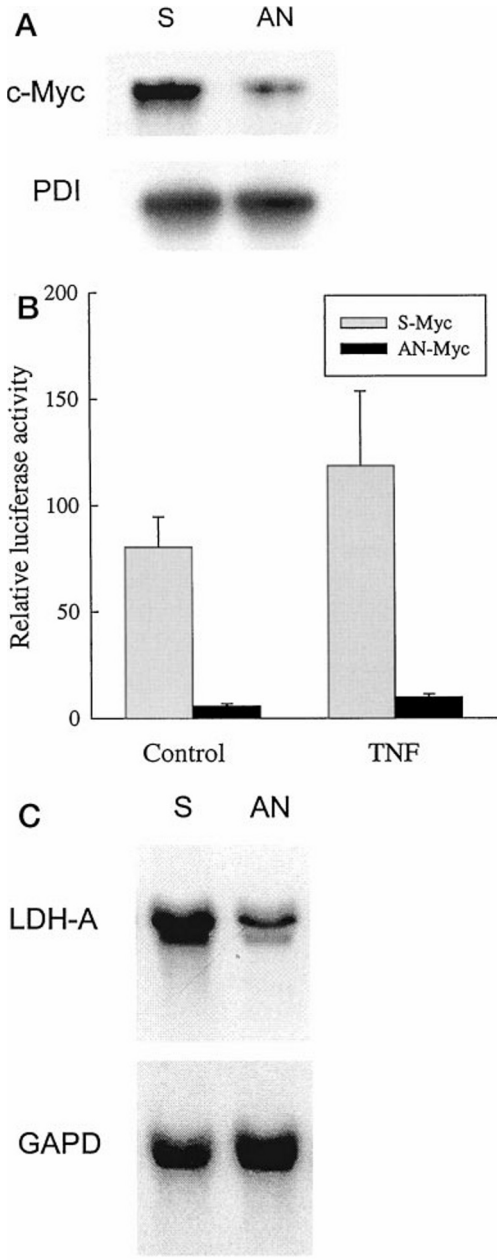

FIG. 1. **S-Myc and AN-Myc cells have differential levels of c-Myc protein and transcriptional activation.** *A*, aliquots of total cell lysates were subjected to SDS-PAGE and immunoblotting with anti-c-Myc and anti-protein-disulfide isomerase (*PDI*) antibodies as described under "Experimental Procedures." Protein was isolated from untreated S-Myc (*S*) and AN-Myc (*AN*) cells. *B*, S-Myc and AN-Myc cell lines were transiently cotransfected with the c-Myc-regulated reporter construct pMyc 3E1b-Luc and the constitutive *Renilla* luciferase vector pRL-TK as described under "Experimental Procedures." Some cells were treated with TNF, and 4 h later untreated control cells and TNF-treated cells were assayed for firefly and *Renilla* luciferase activity. Firefly luciferase activity was then normalized to *Renilla* luciferase activity. The amounts in arbitrary units of firefly luciferase normalized to *Renilla* luciferase are shown. *C*, autoradiograms of Northern blot hybridizations of 20 μg of total RNA isolated from S-Myc (*S*) and AN-Myc (*AN*) cells hybridized with lactate dehydrogenase A (*LDH-A*) and glyceraldehyde-3-phosphate dehydrogenase (*GAPD*) cDNAs as indicated.

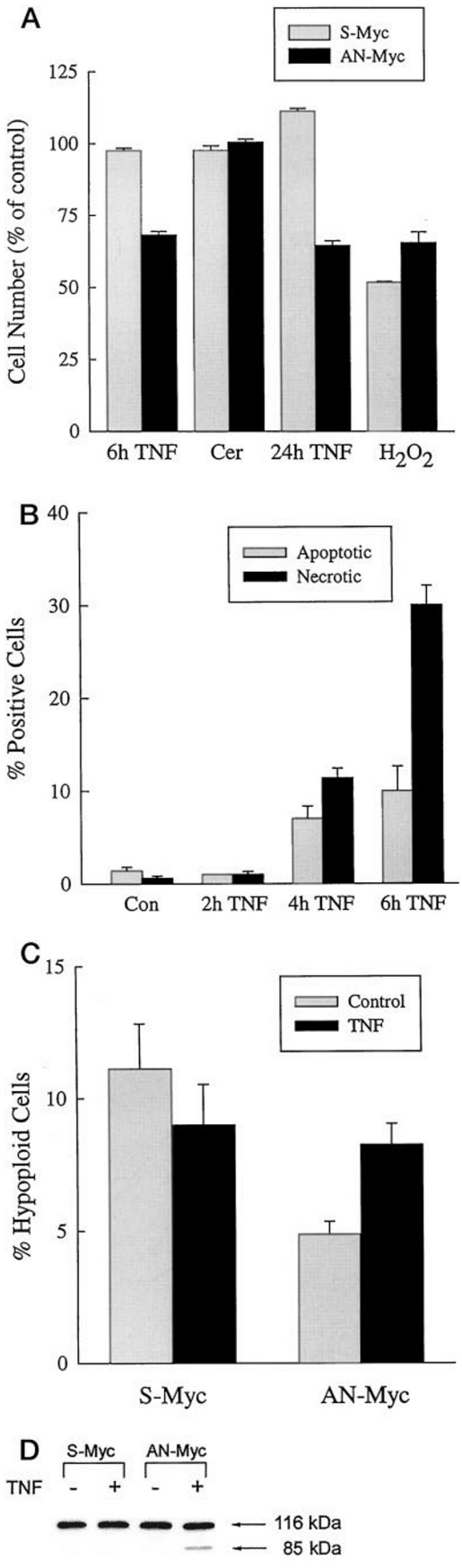

FIG. 2. **AN-Myc cells are sensitized to TNF-induced cell death.** *A*, S-Myc and AN-Myc cells were cultured as described under "Experimental Procedures" and treated with TNF, ceramide (*Cer*), or hydrogen peroxide (H_2O_2). The relative cell number as a percentage of untreated cells was determined at 6 h (*6 h TNF* and *Cer*) and 24 h (*24 h TNF* and H_2O_2) by MTT assay. *B*, the percentages of apoptotic and necrotic cells in AN-Myc cells untreated (*Con*) and treated with TNF for 2, 4, and 6 h were determined under fluorescence microscopy after costaining with acridine orange and ethidium bromide as described under "Experimental Procedures." The levels of apoptosis and necrosis in untreated cells were determined at 2 h after TNF administration. *C*, apoptosis was quantitated by flow cytometric analysis of propidium iodide-stained cells as described under "Experimental Procedures." The percentage of sub-G_1 cells in untreated (*Control*) and 4 h TNF-treated S-Myc and AN-Myc cells are shown. *D*, aliquots of total cell lysates from untreated and 6-h TNF-treated S-Myc and AN-Myc cells were subjected to SDS-PAGE, and immunoblotting was performed with an anti-PARP antibody. The intact 116-kDa PARP and its 85-kDa cleavage product are indicated.

tical to previous reports in wild-type RALA hepatocytes (40), both S-Myc and AN-Myc cells were resistant to ceramide toxicity at the 6-h time point, at which sensitization to TNF toxicity had occurred (Fig. 2*A*). Both S-Myc and AN-Myc cells underwent significant cell death 24 h after H_2O_2 treatment (Fig. 2*A*), indicating no significant alteration in sensitivity to this toxin between the two cell lines. Inhibition of c-Myc expression did not sensitize RALA hepatocytes indiscriminately to any form of cell death but specifically modulated resistance to TNF-induced cell death.

TNF-induced Cell Death in AN-Myc Cells Occurs by Apoptosis and Necrosis—Cell death from TNF may result from apoptosis or necrosis depending on the cell type. To determine which type of cell death occurred in TNF-treated AN-Myc cells, cells were examined for morphological and biochemical evidence of apoptosis. AN-Myc cells were examined under fluorescence microscopy following costaining with acridine orange and ethidium bromide to quantitate the numbers of apoptotic and necrotic cells. Over the 6 h after TNF treatment, AN-Myc cells had marked increases in both the numbers of apoptotic and necrotic cells (Fig. 2*B*).

As additional evidence that inhibition of c-Myc expression sensitized cells to death at least in part from apoptosis, FACS analysis was performed to quantitate the numbers of hypoploid cells as a measure of the presence of DNA fragmentation. Despite 24 h of culture in serum-free medium, S-Myc cells had a low level of hypoploidy that decreased slightly with TNF treatment (Fig. 2*C*). Untreated AN-Myc cells had a lower basal level of hypoploidy that increased 2-fold at 6 h following TNF treatment (Fig. 2*C*).

DNA fragmentation results from the caspase-dependent activation of a DNase, so the induction of DNA hypoploidy in TNF-treated AN-Myc cells implied the presence of caspase activation. For an additional functional indication of caspase activation, cells were examined for the presence of caspase-dependent cleavage of the protein PARP. PARP cleavage was detected in TNF-treated AN-Myc cells but not in untreated AN-Myc cells or in untreated or TNF-treated S-Myc cells (Fig. 2*D*). Inhibition of c-Myc expression sensitized RALA hepatocytes to TNF-induced death associated with the caspase-dependent markers of DNA fragmentation and PARP degradation.

AN-Myc Cell Death Is Dependent on FADD Signaling—The TNF death pathway is dependent on the binding of FADD to the TNFR-bound adaptor protein TNFR-associated death domain protein (8). To ensure that TNF-mediated death in AN-Myc cells proceeded through this pathway, a dominant negative FADD was expressed in these cells. AN-Myc cells were infected with either a control virus, Ad5LacZ, which expresses the β-galactosidase gene, or NFD-4, which expresses a dominant negative FADD. The amount of cell death in AN-Myc cells following TNF treatment was decreased 67% in NFD-4-infected cells as compared with Ad5LacZ-infected cells (Fig. 3),

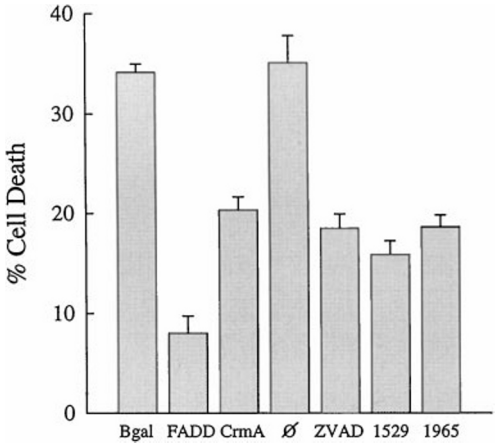

FIG. 3. **TNF-induced AN-Myc cell death is blocked by expression of a FADD dominant negative or CrmA or by chemical caspase inhibitors.** Cells were infected with Ad5LacZ (*Bgal*), NFD-4 (*FADD*), or an adenovirus expressing CrmA (*CrmA*). Other cells were uninfected (Ø) or were uninfected and pretreated with the caspase inhibitors Val-Ala-Asp-fluoromethylketone (*ZVAD*), IDN-1529 (*1529*), or IDN-1965 (*1965*). All cells were treated with TNF, and the percentage cell death 6 h later was determined by MTT assay.

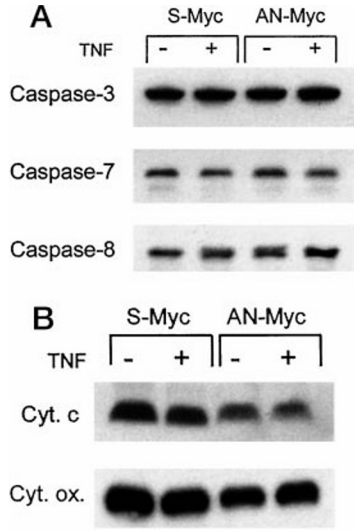

FIG. 4. **TNF treatment of AN-Myc cells does not lead to caspase-3, -7, or -8 activation or mitochondrial cytochrome *c* release.** *A*, aliquots of total cell lysates were subjected to SDS-PAGE, and immunoblotting was performed using anti-caspase-3, -7, and -8 antibodies. Protein was isolated from untreated cells and cells treated with TNF for 6 h. *B*, mitochondrial fractions were prepared from S-Myc and AN-Myc cells that were untreated or treated with TNF for 6 h as indicated. Aliquots of mitochondrial lysates were subjected to SDS-PAGE, and immunoblotting was performed with anti-cytochrome *c* (*Cyt. c*) and anti-cytochrome oxidase (*Cyt. ox.*) antibodies.

indicating that cell death occurred through a FADD-dependent pathway.

Engagement of FADD leads to activation of caspase-8, which then activates downstream caspases such as caspase-3, causing cell death (8). The involvement of caspases in AN-Myc cell death was examined by determining the effect of caspase inhibition on cell death. Adenoviral expression of the viral caspase inhibitor CrmA decreased cell death in AN-Myc cells by 42% following TNF treatment as compared with Ad5LacZ-infected cells (Fig. 3). In addition, pretreatment with the chemical pan-caspase inhibitors Val-Ala-Asp-fluoromethylketone, IDN-1529, or IDN-1965 also inhibited cell death by approximately 50% (Fig. 3). Fluorescence microscopic studies of acridine orange- and ethidium bromide-stained cells treated with IDN-1529 confirmed this inhibition of cell death. IDN-1529 decreased the number of apoptotic AN-Myc cells 6 h after TNF treatment to the level found in untreated, control cells. In addition, the number of necrotic AN-Myc cells at 6 h was decreased 58% by IDN-1529 pretreatment.

TNF-induced AN-Myc Cell Death Is Not Associated with Caspase-3, -7, or -8 Activation—The ability to inhibit TNF-induced cell death in AN-Myc cells by blocking FADD function or inhibiting caspase activation suggested that this form of cell death occurred by the classic TNF death pathway involving caspase-8 and caspase-3. To determine whether caspase activation occurred in this model, levels of caspase-3, -7, and -8 were examined by immunoblotting in TNF-treated cells. None of these caspases were activated in AN-Myc cells undergoing TNF-induced cell death as indicated by stable procaspase levels (Fig. 4*A*) and the absence of processed caspase subunits (data not shown). These data are in sharp contrast to our previous findings of caspase-3, -7, and -8 activation in RALA hepatocytes sensitized to TNF cytotoxicity by inhibition of NF-κB activation or actinomycin D cotreatment (14, 35).

An intermediate step between caspase-8 and caspase-3 activation in TNF-induced and other forms of apoptosis is the mitochondrial release of the caspase activator cytochrome *c* (41). Examination of mitochondrial cytochrome *c* levels by Western immunoblots demonstrated that TNF induced cell death in AN-Myc cells without triggering cytochrome *c* release (Fig. 4*B*). Levels of cytochrome oxidase, a mitochondrial protein not released during apoptosis (42), were also equivalent, indicating that equal amounts of mitochondrial protein had been isolated from each sample (Fig. 4*B*).

AN-Myc Cells Have Reduced Levels of NF-κB Activation—NF-κB activation is known to play a critical role in hepatocyte resistance to TNF toxicity (13, 14), and the effects of c-Myc on hepatocyte sensitivity to TNF-induced cell death may be secondary to changes in NF-κB. The effect of c-Myc expression on the induction of NF-κB activation by TNF was therefore determined by electrophoretic mobility shift assays. TNF induced marked increases in NF-κB DNA binding at 2 and 4 h in both S-Myc and AN-Myc cells (Fig. 5). Increases occurred in two bands previously determined to represent p65/p50 heterodimers and p50/p50 homodimers by supershifts (14).

The effect of DNA-bound NF-κB on transcription can vary depending on the composition of the DNA binding complex, the state of NF-κB phosphorylation, and coactivating factors like p300 (43). Levels of NF-κB-dependent transcription were measured in S-Myc and AN-Myc cells by means of transient transfections with an NF-κB-driven luciferase reporter gene. Levels of NF-κB-dependent transcription normalized to a cotransfected, constitutive reporter were increased 13-fold in untreated S-Myc cells relative to AN-Myc cells (Fig. 6). After TNF treatment, NF-κB transcriptional activity increased 2-fold in AN-Myc cells, while the level in S-Myc cells remained unchanged (Fig. 6). Thus, although NF-κB DNA binding increased in AN-Myc cells after TNF stimulation, NF-κB transcriptional activity was still greatly decreased in AN-Myc cells relative to S-Myc cells.

Sensitization to TNF Cytotoxicity by Inhibition of c-Myc Expression Occurs Independently from Effects on NF-κB—The association of c-Myc inhibition with decreased NF-κB transcriptional activation suggested that the mechanism of c-Myc-induced sensitization to TNF toxicity may result from NF-κB inactivation. To test this possibility, the effects of inhibition of NF-κB activity on S-Myc and AN-Myc cell sensitivity to TNF-induced death were examined. If AN-Myc cell sensitivity to TNF resulted from NF-κB suppression, then attempts to inhibit NF-κB activation should not increase AN-Myc cell death from TNF. In addition, S-Myc and AN-Myc cells should be

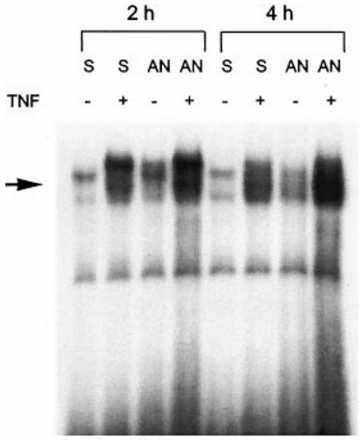

FIG. 5. **c-Myc expression does not affect TNF-induced NF-κB DNA binding.** Nuclear extracts were isolated at the times indicated from untreated and TNF-treated S-Myc (*S*) and AN-Myc (*AN*) cells. The extracts were used for electrophoretic mobility shift assays with an NF-κB consensus oligonucleotide as described under "Experimental Procedures." *Solid arrow*, NF-κB binding complex.

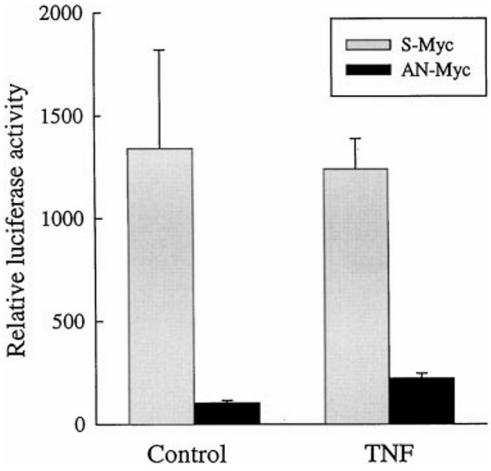

FIG. 6. **AN-Myc cells have decreased NF-κB transcriptional activity.** S-Myc and AN-Myc cells were transiently cotransfected with the NF-κB-driven reporter NF-κB-Luc and pRL-TK. Some cells were subsequently treated with TNF for 4 h, and cell lysates were assayed for firefly and *Renilla* luciferase activity. Data show firefly luciferase normalized to *Renilla* luciferase activity in arbitrary units.

equally sensitive to TNF toxicity after NF-κB inactivation. Wild-type, S-Myc, and AN-Myc cells were infected with the adenoviruses Ad5LacZ or Ad5IκB. Ad5IκB expresses a mutant IκB that cannot be phosphorylated and therefore binds NF-κB irreversibly, preventing its activation. Similar to uninfected cells, Ad5LacZ-infected wild-type and S-Myc cells were resistant to TNF toxicity, while Ad5LacZ-infected AN-Myc cells underwent 24% cell death within 6 h (Fig. 7). After Ad5IκB infection, all three cell types underwent cell death within 6 h of TNF treatment; however, the amount of cell death was significantly decreased in S-Myc cells and increased in AN-Myc cells, relative to wild-type cells (Fig. 7). In the presence of NF-κB inactivation, increased c-Myc expression decreased cell death, while inhibition of c-Myc further increased cell death. These data suggest that c-Myc expression affects hepatocellular sensitivity to TNF toxicity at least partly through a mechanism independent of NF-κB.

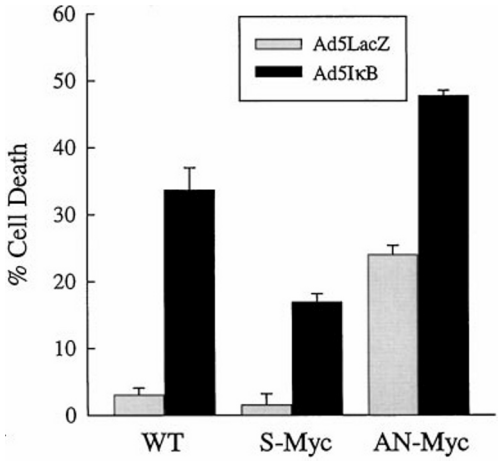

FIG. 7. **S-Myc cells are resistant to TNF-induced cell death following NF-κB inactivation.** Wild-type (*WT*), S-Myc, and AN-Myc cells were infected with Ad5LacZ or Ad5IκB and treated with TNF. The percentage cell death was determined 6 h after TNF treatment by MTT assay.

DISCUSSION

An understanding of the mechanism by which resistant cells convert to sensitivity to TNF cytotoxicity is crucial to preventing the detrimental effects of this cytokine in inflammatory conditions such as toxin-induced liver injury. Recent investigations have demonstrated that NF-κB activation is critical to the maintenance of hepatocyte resistance to TNF cytotoxicity (13, 14). However, the function of other TNF-activated transcriptional regulators such as c-Myc and AP-1 in hepatocyte sensitization to TNF killing has been unknown.

The present studies demonstrate that in RALA hepatocytes c-Myc expression mediates resistance to TNF toxicity. S-Myc hepatocytes were resistant to TNF killing, while AN-Myc cells with reduced levels of c-Myc transcriptional activity underwent rapid, TNF-induced cell death. Inhibition of c-Myc specifically sensitized hepatocytes to the TNF death pathway, because AN-Myc cells were equivalent to S-Myc cells in their resistance to death from ceramide and their sensitivity to death from H_2O_2. The differential susceptibility of AN-Myc cells to TNF killing was not due to an absence of TNF receptor in S-Myc cells, because S-Myc and AN-Myc cells were both TNF-responsive as indicated by their equivalent increases in NF-κB nuclear translocation after TNF treatment. All AN-Myc cells did not undergo cell death from TNF treatment, which may be due to the fact that low levels of c-Myc in this polyclonal cell line were sufficient to maintain resistance to TNF toxicity in some cells. Alternatively, other factors such as cell cycle phase may influence susceptibility to TNF-induced death in RALA hepatocytes. Supporting this possibility is the fact that following NF-κB inactivation and actinomycin D treatment a significant percentage of RALA hepatocytes also fail to undergo TNF-mediated cell death (14, 35).

TNF-induced death in AN-Myc cells occurred at least partially by apoptosis as indicated by apoptotic morphology under fluorescence microscopy, increased hypoploidy, PARP cleavage, and partial caspase dependence. However, fluorescence microscopic studies indicated that a significant portion of TNF-induced AN-Myc cell death occurred by necrosis. While it is impossible to completely rule out that this necrosis was not secondary to apoptosis, several facts support the contention that necrosis was a primary event. The first is that large numbers of necrotic cells were detected at the same time as the initial appearance of apoptotic cells. Second, the increase in the numbers of hypoploid cells was relatively low compared with the overall amount of cell death. These data are in sharp contrast to our previous report of TNF-induced cell death in RALA hepatocytes sensitized by NF-κB inhibition in which a similar amount of overall cell death occurred but with a more marked increase in hypoploidy and no increase in the numbers

of necrotic cells (14).[3] The final data consistent with the coexistence of apoptosis and primary necrosis are provided by the finding of only partial prevention of cell death by caspase inhibition. This result again contrasts with the almost total inhibition of cell death by chemical caspase inhibitors when cells were sensitized by NF-κB inactivation (14, 35). The present study we believe is the first to implicate c-Myc in the regulation of death from necrosis as well as apoptosis. This result, along with prior studies on the involvement of AP-1 in hydrogen peroxide-induced necrosis (44), demonstrates that liver cell necrosis can be regulated by active gene expression.

The cell death pathway induced by TNF treatment of AN-Myc cells proceeded through FADD as adenoviral expression of a dominant negative FADD blocked cell death. The degree of inhibition of cell death by blocking FADD suggests that both apoptosis and necrosis were FADD-dependent. Further evidence for this conclusion is the finding that inhibition of FADD function was more effective in blocking cell death than caspase inhibition, which presumably prevented only apoptotic death. We have previously reported that FADD mediates a caspase-independent TNF death pathway in RALA hepatocytes sensitized by actinomycin D treatment (35). The present data showing that FADD-dependent, TNF-induced necrosis occurs in RALA hepatocytes demonstrate that FADD signaling can initiate a number of forms of cell death depending on the mode of sensitization. Although TNF-induced apoptosis in AN-Myc cells was FADD- and caspase-dependent, there was no evidence of caspase-8 or caspase-3 activation in contradistinction to other forms of TNF-induced apoptosis (8, 14, 35). Cell death also occurred without mitochondrial release of cytochrome *c*, in contrast to the occurrence of cytochrome *c* release in TNF-induced hepatocyte death mediated by NF-κB inactivation (13, 14). However, it has become evident that cytochrome *c* is not critical for TNF killing because hepatocyte actinomycin D/TNF toxicity occurs in the absence of cytochrome *c* release (35), and cytochrome *c*-deficient mice have increased rather than decreased sensitivity to TNF killing (45). The present studies suggest the existence of a hepatocyte TNF death pathway in which FADD triggers a novel, caspase-dependent death pathway that does not involve caspase-8, cytochrome *c* release, or caspase-3 activation.

The finding that c-Myc expression protected RALA hepatocytes from TNF toxicity contradicts the general concept of c-Myc up-regulation as a proapoptotic signal (18, 19). Investigations of TNF toxicity in nonhepatic cell types have demonstrated that increased c-Myc expression sensitized fibroblasts (26, 27), HeLa cells (24), and fibrosarcoma cells (25) to TNF cytotoxicity. The mechanism by which c-Myc promoted TNF-induced death in these cells is unclear, but it may have involved increased cyclin D3 (25), or down-regulation of NF-κB (26). Our results together with these reports indicate that c-Myc expression during the cellular TNF response may act to inhibit or promote cell susceptibility to TNF toxicity in a cell type-specific fashion. One potential reason for the divergent function of c-Myc in hepatocytes is that these cells are not only resistant to TNF toxicity but also normally undergo proliferation in response to TNF stimulation (2, 3, 14). c-Myc expression may be required to complete the TNF-induced cell growth response. In the absence of sufficient c-Myc expression, cell cycle progression may be aborted, causing the hepatocyte to initiate a death pathway. This situation would be analogous to the induction of apoptosis by c-Myc overexpression during culture in serum-free medium because the absence of growth factors fails to allow progression of a cell cycle initiated by c-Myc (20). While our study is the first to report that c-Myc expression protects against TNF toxicity, there is a precedent for c-Myc as an antiapoptotic gene, since c-Myc expression has been reported to inhibit dexamethasone- and immunoglobulin-induced lymphocyte apoptosis (46, 47).

AN-Myc cells also had markedly reduced levels of NF-κB transcriptional activity relative to S-Myc cells. NF-κB is known to up-regulate *c-myc* gene expression (48), but we are unaware of previous reports of c-Myc modulating NF-κB activity. In fact, a previous report of NF-κB inactivation causing cellular susceptibility to TNF toxicity implicated suppression of c-Myc expression as the mechanism (48). AN-Myc hepatocyte sensitivity to TNF killing may result from the fact that in these cells up-regulation of c-Myc increases rather than decreases NF-κB transcriptional activation. While direct regulation of NF-κB by c-Myc remains to be proven, several facts suggest that c-Myc had an effect on RALA hepatocyte TNF resistance distinct from NF-κB. The first is that there were differences in the form of cell death resulting from NF-κB and c-Myc repression. NF-κB inhibition sensitized RALA hepatocytes to a purely apoptotic cell death, involving caspase-8 and caspase-3 activation, that could be almost completely blocked by caspase inhibition (14, 35). In contrast, decreased c-Myc expression induced cell sensitivity to a TNF-induced cell death with a significant necrotic component, no caspase-8 or caspase-3 activation, and only partial caspase dependence. The second finding supportive of independent effects of c-Myc and NF-κB is that c-Myc expression decreased the amount of death in cells sensitized to TNF toxicity by NF-κB inactivation. If NF-κB is the downstream effector of upstream changes in c-Myc expression, then NF-κB inactivation should sensitize all cells equally to TNF toxicity irrespective of their c-Myc expression. However, S-Myc cells with Ad5IκB-induced NF-κB inactivation still had increased resistance to TNF toxicity relative to AN-Myc cells, suggesting that c-Myc induced resistance independently of effects on NF-κB.

NF-κB activation is thought to lead to cellular resistance to TNF toxicity through the transcriptional up-regulation of a protective gene(s). Since c-Myc is also a transcriptional activator, its expression may similarly increase levels of a protective factor. A possible target gene could be a member of the inhibitor of apoptosis (IAP) gene family (49). However, by Western immunoblot analysis, S-Myc and AN-Myc cells have equivalent levels of both c-IAP1 and c-IAP2.[4] Alternatively, c-Myc can repress gene transcription (17) and may therefore decrease expression of a proapoptotic gene. While the mechanisms by which c-Myc and NF-κB regulate hepatocyte TNF resistance remain to be determined, the coexistence of two independent transcriptional pathways of resistance to TNF toxicity points to the importance in liver homeostasis of preventing the harmful effects of this frequently expressed cytokine.

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