The EMBO Journal Vol.16 No.24 pp.7382–7392, 1997

Induction of TNF-sensitive cellular phenotype by c-Myc involves p53 and impaired NF-κB activation

Juha Klefstrom, Elena Arighi, Trevor Littlewood[1], Marja Jäättelä[2], Eero Saksela, Gerard I.Evan[1] and Kari Alitalo[3]

Molecular/Cancer Biology Laboratory, Haartman Institute, PO Box 21, 00014 University of Helsinki, Finland, [1]Biochemistry of the Cell Nucleus Laboratory, Imperial Cancer Research Fund, PO Box 123, 44 Lincoln's Inn Fields, London WC2A 3PX, UK and [2]Apoptosis Laboratory, Danish Cancer Society, Strandboulevarden 49, DK-2100 Copenhagen, Denmark

[3]Corresponding author
e-mail: Kari.Alitalo@Helsinki.FI

J.Klefstrom and E.Arighi contributed equally to this work

Normal fibroblasts are resistant to the cytotoxic action of tumor necrosis factor (TNF), but are rendered TNF-sensitive upon deregulation of c-Myc. To assess if oncoproteins induce the cytotoxic TNF activity by modulating TNF signaling, we investigated the TNF-elicited signaling responses in fibroblasts containing a conditionally active c-Myc protein. In association with cell death, c-Myc impaired TNF-induced activation of phospholipase A_2, JNK protein kinase and cell survival-signaling-associated NF-κB transcription factor complex. The TNF-induced death of mouse primary fibroblasts expressing deregulated c-Myc was inhibited by transient overexpression of the p65 subunit of NF-κB, which increased NF-κB activity in the cells. Unlike other TNF-induced signals, TNF-induced accumulation of the wild-type p53 mRNA and protein was not inhibited by c-Myc. TNF, with c-Myc, induced apoptosis in mouse primary fibroblasts but only weakly in *p53*-deficient primary fibroblasts. The C-terminal domain of p53, which is a transacting dominant inhibitor of wild-type p53, failed to inhibit apoptosis by c-Myc and TNF, suggesting that the cell death was not dependent on the transcription-activating function of p53. Taken together, the present findings show that the cytotoxic activity of TNF towards oncoprotein-expressing cells involves p53 and an impaired signaling for survival in such cells.

Keywords: apoptosis/Myc/NF-κB/p53/tumor necrosis factor

Introduction

Tumor necrosis factor-α (TNF) is a cytokine capable of cell death induction. The ligand-bound, active p55 TNF receptor can interact with caspase proteases via cytoplasmic adaptor proteins TRADD and FADD (Boldin *et al*., 1996; Muzio *et al*., 1996). The action of caspases is thought to execute most if not all forms of apoptotic cell death. The active p55 kDa TNF receptor complexes also interact via TRADD with RIP and TRAF2 proteins, which are involved in the activation of c-Jun amino-terminal kinases (JNKs, also known as stress-activated protein kinases SAPKs) and NF-κB (Hsu *et al*., 1996; Liu *et al*., 1996b). The NF-κB transcription factor complex mediates part of the pleiotropic biological effects of TNF by binding to and activating promoter regions of genes encoding growth factors, chemokines and leukocyte adhesion molecules (reviewed in Verma *et al*., 1995). Various stress conditions, especially genotoxic stress, also activate JNKs and NF-κB by as yet uncharacterized mechanisms (Kyriakis *et al*., 1994; Liu *et al*., 1996a).

In primary cell cultures, TNF initiates a wide variety of cellular responses other than death, whereas the induction of death occurs mainly in cells derived from tumors or infected with viruses (Carswell *et al*., 1975; Aderka *et al*., 1985). However, TNF sensitivity can be induced in virtually all types of cells by chemically blocking *de novo* protein synthesis. This effect may be due to an inhibition of the synthesis of proteins which protect cells against the cytotoxic activity of TNF. The pre-treatment of cells with TNF confers resistance to subsequent TNF cytotoxicity, which suggests that a subset of such proteins is TNF-inducible (Wallach, 1984). In this context, it has been shown that if the expression of the TNF-inducible manganese superoxide dismutase (MnSOD) is inhibited concomitantly with TNF stimulation, cells which otherwise are resistant to TNF die. Similarly, inactivation of the TNF-inducible NF-κB transcription factor complex potentiates the cytotoxic action of TNF or genotoxic agents and can render normal cells TNF-sensitive (Beg and Baltimore, 1996; Van Antwerp *et al*., 1996; Wang *et al*., 1996). Thus, some of the TNF signals emanating from the activated receptor apparently via the TRAF2 pathway increase cellular resistance against death, and these signals have a crucial role in the survival of cells exposed to TNF.

Given that TNF exerts its cytotoxic effects towards cells derived from tumors or infected with viruses, the cellular sensitivity to TNF-induced death may be a consequence of the expression of viral or endogenous oncoproteins. We and others have shown that both deregulated c-Myc (Janicke *et al*., 1994; Klefstrom *et al*., 1994) and adenoviral E1A (Chen *et al*., 1987; Duerksen-Hughes *et al*., 1991) can render fibroblasts sensitive to the cytotoxic effects of TNF. It is not yet clear, however, how oncoproteins activate cell death signaling by TNF. In order to elucidate these mechanisms, we studied the TNF-induced signaling pathways in fibroblasts containing a conditionally active form of c-Myc (MycER, Eilers *et al*., 1989). While TNF stimulation increased the cellular activities of phospholipase A_2 (PLA_2), JNK and NF-κB transcription factor complex in fibroblasts, these responses were inhibited upon c-Myc activation. The c-Myc-induced TNF sensitivity was inhibited but not completely abrogated by

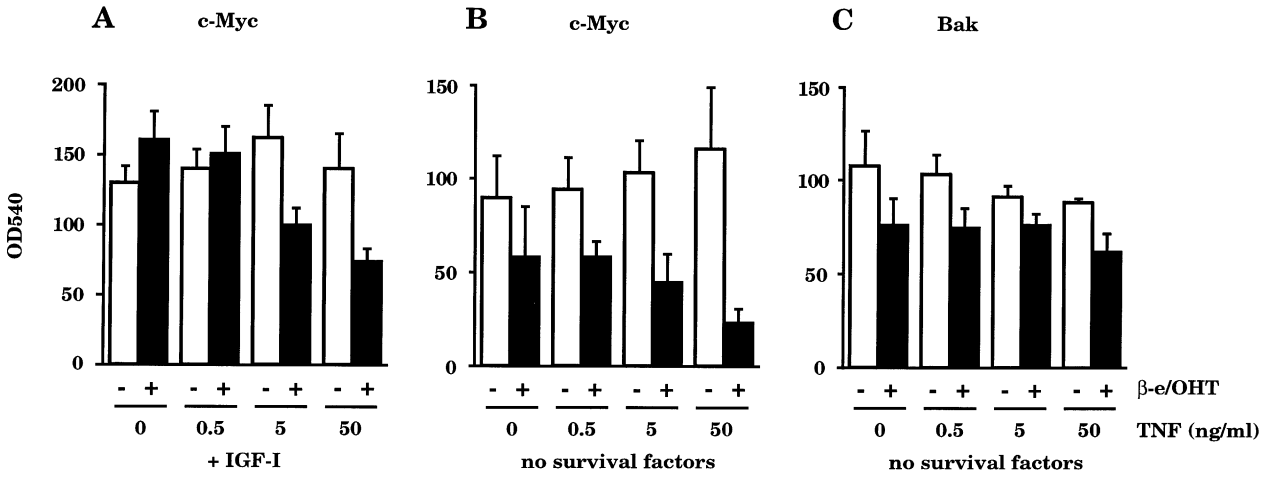

Fig. 1. Survival of Rat1 cells following activation of c-Myc or Bak in the presence of various concentrations of TNF. (**A**) Induction of TNF sensitivity by c-Myc in the presence of IGF-I. Rat1-MycER cells serum starved for 2 days were incubated in serum-free medium containing 100 ng/ml IGF-I and equivalent amounts of either ethanol carrier, β-estradiol (β-e) or OHT, and various concentrations of TNF. After 2 days of incubation with the drugs, the viability of the cells was quantitated by the MTT assay. The results represent means ± SD of five independent experiments. (**B**) and (**C**) TNF sensitivity of apoptosis-prone cells. Rat1-MycER and Rat1-Bak cells were serum starved for 2 days, after which the medium was replaced with serum-free medium containing ethanol carrier, β-e or OHT, and TNF at the concentrations indicated. After incubation for 2 (Rat1-MycER) or 6 days (Rat1-Bak) with the drugs, the viability of the cells was quantitated as above. The results represent means ± SD of four to five independent experiments. Note that in serum-deprived cells, both c-Myc and Bak decrease cell survival but only cells with active c-Myc are killed by TNF.

the overexpression of the p65 subunit of NF-κB, which substantially increased cellular NF-κB activity. Thus, part of the c-Myc-induced TNF sensitivity can be attributed to an inhibition of signaling for cell survival. The TNF-induced accumulation of p53 mRNA and protein was not inhibited by c-Myc. The p53 protein was necessary for TNF-induced cell death, suggesting that, in addition to the impaired signaling for survival, the TNF-induced p53 dictates the cytotoxic effect of TNF towards oncogene-expressing cells.

Results

Induction of TNF sensitivity by c-Myc but not by the pro-apoptotic protein Bak

The deregulated expression of c-Myc induces cellular TNF sensitivity, but it has been unclear whether this is a specific function of c-Myc or a result of c-Myc-induced secondary cellular changes. The activation of c-Myc increases the susceptibility of fibroblasts to undergo apoptosis (Evan *et al*., 1992), which makes it possible that TNF sensitivity is associated with a low cellular threshold to apoptosis.

Serum and its component, insulin-like growth factor-I (IGF-I), counteract the apoptotic functions of c-Myc (Harrington *et al*., 1994). As shown in Figure 1A, the addition of IGF-I completely blocked Rat1 cell apoptosis upon c-Myc activation, as quantitated by the MTT assay. When both TNF and IGF-I were added to the cell cultures containing activated c-Myc, cell viability decreased in a TNF dose-dependent manner. Without c-Myc activation, TNF did not cause a decrease in cell survival. Similar results were obtained when the cells were grown in the presence of high concentrations of insulin or in 10% fetal calf serum (FCS) (data not shown). These experiments demonstrate that the cells expressing deregulated c-Myc are not protected against the cytotoxic effects of TNF by the presence of IGF-I or other serum survival factors.

Bak is a protein of the Bcl2 family, which, like c-Myc, induces apoptosis in serum-deprived fibroblasts, but unlike c-Myc, Bak does not stimulate entry into the cell cycle (Chittenden *et al*., 1995). We tested whether the pro-apoptotic Bak induces TNF sensitivity in Rat1 cells. Experimental induction of Bak in serum-starved Rat1 cells decreased cell survival (Figure 1C). However, whereas TNF treatment in the serum-free conditions caused a further dose-dependent decrease in the survival of c-Myc-overexpressing cells (Figure 1B), it did not significantly change, in the same conditions, the survival of Bak-overexpressing cells (Figure 1C). The failure of Bak to induce TNF sensitivity was not due to an absence of TNF receptors, since a rapid cell death resulted if TNF was added to the cultures together with sublethal concentrations of the protein synthesis inhibitor cycloheximide (data not shown). These results show that the activation of the apoptotic machinery is not sufficient to induce TNF sensitivity. Thus, the induction of TNF sensitivity is a specific function of c-Myc.

Regions of c-Myc required for the induction of TNF sensitivity

c-Myc is a basic region helix–loop–helix leucine zipper (bHLHZip) transcription factor which can either stimulate or repress transcription. In a heterodimer with Max, c-Myc binds specific DNA sequences and activates transcription from promoters containing its target sequences. These functions of c-Myc are required for c-Myc-induced cell cycle progression, cell transformation and apoptosis (Amati *et al*., 1993). In contrast, c-Myc can also repress transcription without an absolute need for Max (Roy *et al*., 1993; Philipp *et al*., 1994). The integrity of a small amino-terminal region encompassing amino acids 92–106 of c-Myc is critical for repression of cyclin D1, but is dispensable for transformation. Conversely, the amino-terminal residues 128–143 of c-Myc are critical for transformation, but not for repression of cyclin D1. By using

Table I. N-terminal regions in c-Myc involved in the induction of TNF sensitivity

Myc mutant	Cyclin D1 repression[a]	Transformation[a]	TNF sensitivity
MYC	+	+	+
MYCΔ92–106	−	+	+
MYCΔ128–143	+	−	−
MYCΔ104–136	−	−	−

[a]Philipp *et al*. (1994).

the amino-terminal mutant forms of c-Myc differing in their effects on cell transformation and cyclin D1 repression (Philipp *et al*., 1994), we wished to learn which functions of c-Myc are associated with the induction of TNF sensitivity. The mutant forms of c-Myc were introduced by retroviral infection into NIH-3T3 cells, followed by selection of drug-resistant clones, which were pooled. Equal expression of c-Myc mutants was confirmed in the pools by Western blot analysis (data not shown). In repeated experiments, equivalent numbers of cells from each clone were seeded on duplicate culture dishes. After overnight adherence, TNF was added to one of the duplicate cultures and cell death in the cultures was examined microscopically at various times thereafter. The results from these experiments are summarized in Table I. They indicate that only those c-Myc mutants having transforming activity enhance cellular TNF sensitivity as does the wild-type c-Myc. Thus, like other known c-Myc-mediated biological responses, the induction of TNF sensitivity also requires c-Myc interaction with Max (Klefstrom *et al*., 1994) and a competence of c-Myc for transcriptional activation.

Deregulated c-Myc inhibits TNF-induced stress signals

To elucidate mechanisms whereby deregulated c-Myc induces TNF sensitivity, we examined whether c-Myc modulates TNF-induced signal transduction in the target cells. TNF-induced activation of PLA_2, JNK and NF-κB was assessed in Rat1 cells containing the conditionally active form of c-Myc (MycER) or, as a control, in Rat1 cells expressing a similar form of c-MycΔ (MycΔER is devoid of transcriptional activity due to a deletion encompassing c-Myc residues 106–143).

TNF increases PLA_2 activity in association with its cytotoxic and mitogenic responses (Palombella and Vilcek, 1989; Jäättelä, 1993). This leads to the enzymatic release of arachidonic acid (AA) from membrane phospholipids. PLA_2 activity in TNF-treated cells was examined by measuring the release of [^{3}H]arachidonic acid into the cell culture supernatants (Jäättelä, 1993). TNF stimulation reproducibly caused the release of AA metabolites from the tested cells (Table II). AA release from the TNF-treated cells was ~1.2- to 1.4-fold higher than the spontaneous release from cells incubated in medium alone. However, if c-Myc was activated with 4-hydroxytamoxifen (OHT) 6 h prior to the addition of TNF, the TNF-induced AA release was inhibited (Table II). The activation of c-Myc as such did not affect the release of AA metabolites in these conditions. The inhibition of the TNF-induced AA release was a specific effect of the transcription factor activity of c-Myc, since the TNF-induced AA release was not impaired by c-MycΔ.

Table II. Effect of c-Myc deregulation on the TNF-induced PLA_2 acivity

Cells	Relative release of arachidonic acid			
	–OHT/ –TNF	–OHT/ +TNF	+OHT/ –TNF	+OHT/ +TNF
Rat1	1.00	1.33	1.03	1.38
Rat1-MycER	1.00	1.22	1.01	1.08
Rat1-MycΔER	1.00	1.41	0.98	1.46

Cells were treated with 100 nM OHT to activate c-Myc and with 50 ng/ml TNF as indicated. The release of arachidonic acid after 20 h of treatment is reported relative to the spontaneous release from untreated cells (1.0). The values represent means of triplicate experiments repeated three times with similar results.

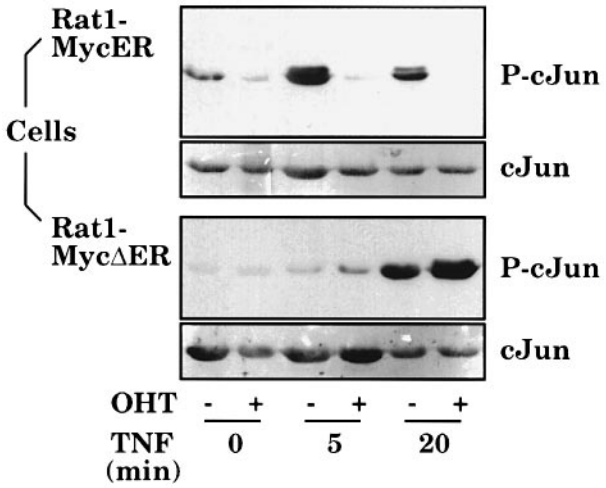

Fig. 2. Deregulation of c-Myc inhibits TNF-induced JNK activity. Cells were pre-treated in normal growth medium with ethanol carrier or with OHT for 6 h before addition of 50 ng/ml TNF. TNF-stimulated cells were lysed at the indicated time points and the N-terminal fragment of c-Jun (1–89) was incubated with the lysates, isolated with the bound JNK isoforms and subjected to kinase assay. The amount of phosphorylation at the Ser63 residue of c-Jun was determined from immunoblots by using phospho-c-Jun-specific antibody. After antibody detection, the immunoblots were stained with colloidal gold to confirm equivalent amounts of N-terminal c-Jun in the samples (the lowermost panels of two immunoblots). The assay was repeated three times with similar results.

We also determined whether c-Myc inhibits the early TNF-induced signals, such as JNK activation. TNF was added to cells containing either active or inactive c-Myc. Cells were lysed after 5 or 20 min TNF treatment, and the JNK isoforms were bound to an amino-terminal c-Jun fragment. The JNK–c-Jun complexes were subjected to kinase assays and the amount of phosphorylated c-Jun protein was determined using specific anti-phospho-c-Jun antibody. As shown in Figure 2, TNF treatment caused a transient activation of JNKs in Rat1 fibroblasts. Maximal JNK activity was observed variably at 5–20 min. However, if c-Myc was activated prior to TNF stimulation, no activation of JNKs was observed. In parallel assays, c-MycΔ did not impair the TNF-induced JNK activation.

The NF-κB transcription factor complex is implicated in counteracting the cytotoxic activity of TNF. We used a reporter construct assay to examine the transcriptional activity of NF-κB in the TNF-treated cells. This reporter contained binding sites for NF-κB in front of the minimal c-*fos* promoter and the luciferase gene. One or two days after transfection, TNF was added to the cultures and luciferase activity was measured from the cell lysates. As shown in Figure 3, TNF treatment stimulated the NF-κB activity in Rat1 fibroblasts. In comparison, c-Myc caused an ~50% inhibition of the transcriptional activation by NF-κB (Figure 3A); c-MycΔ did not cause such an inhibition (Figure 3B). The effect of c-Myc on NF-κB activation by TNF was also analyzed in mouse embryonic fibroblasts (MEFs). The cells were infected by high-titer MycERtm retroviruses as described in Materials and methods. As with Rat1 cells, the activation of c-Myc also inhibited the TNF-induced NF-κB activation in early passage MEFs (Figure 3C).

Overexpression of p65 increases NF-κB activity in cells containing deregulated c-Myc and inhibits TNF-induced cell death

To test if the level of cellular NF-κB activity can affect the c-Myc-induced TNF sensitivity, the NF-κB subunits, p50 or p65, or the negative regulator of NF-κB activity, IκBα, were transiently overexpressed in the low passage MEF-MycERtm cells. To identify the transfected cells, p50, p65, IκBα or control vector was co-transfected into the cells with a β-galactosidase-encoding reporter vector. Aliquots of each transfected cell culture were seeded to duplicate wells. The cells in one well were treated with ethanol carrier (untreated cells) and in the other well with OHT (to activate c-Myc) and TNF. After 24 or 48 h incubation with the drugs, cells were fixed and stained with X-gal to identify the β-galactosidase-expressing cells. The total number of adherent blue cells in each well was scored by microscopy. The NF-κB activity in the transfected cells was monitored in two separate transfection experiments by including the NF-κB luciferase reporter in the co-transfection mixture.

TNF treatment decreased the viability of the cells containing deregulated c-Myc and control vector (pGD); the number of blue cells in the wells treated for 24 h with OHT and TNF was ~55% of the number in the untreated wells (Figure 4A). In comparison, 80% of the cells co-expressing deregulated c-Myc and transfected p65 survived a 24 h TNF treatment. The corresponding figures after 48 h were 30 and 50%, respectively ($P < 0.05$, paired *t*-test). The NF-κB activity was ~6-fold higher in the cells transfected with p65 than in the control cells (Figure 4B). Thus, the overexpression of p65 increased both the NF-κB activity and the viability of TNF-treated cells containing deregulated c-Myc. In contrast to p65, the overexpression of p50 did not significantly increase the NF-κB activity or the survival of treated cells (Figure 4A and B). The overexpression of IκBα caused a weak decrease of NF-κB activity and of cell viability (Figure 4A and B).

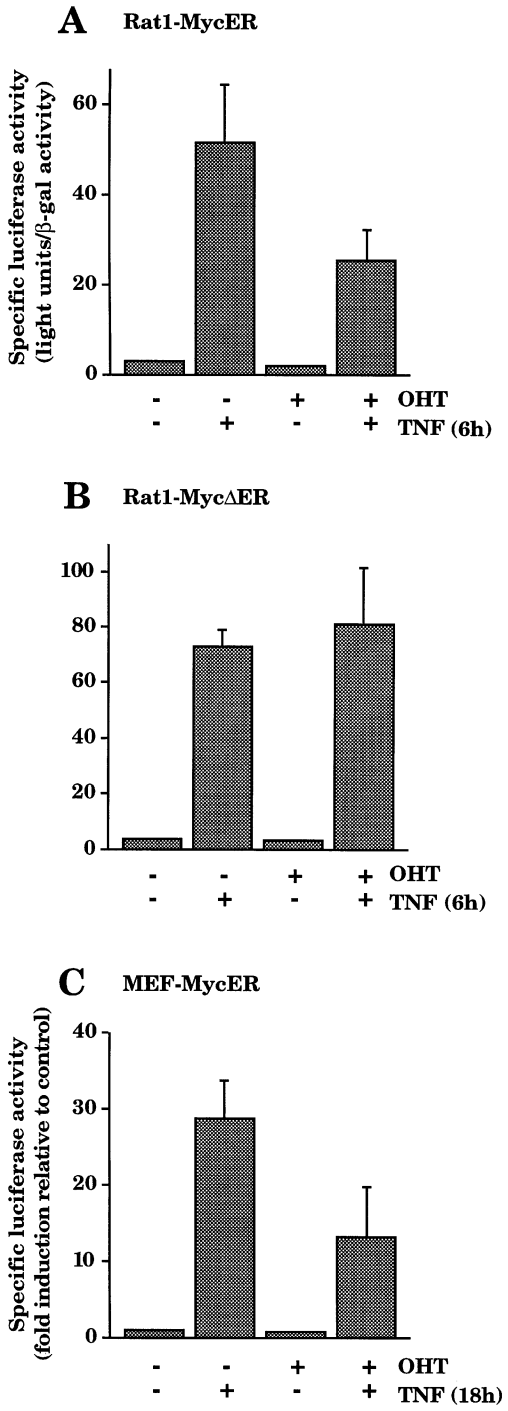

Fig. 3. Deregulation of c-Myc inhibits TNF-induced transcriptional activity of NF-κB. Cells were transfected with 5 μg of CMV-βgal and pBIIX-Luc plasmid and assayed 1 or 2 days after transfection. Transfected cells were pre-incubated for 6 h with ethanol carrier or OHT prior to addition of 50 ng/ml TNF. After a further incubation for 6 or 18 h with TNF, the cells were lysed. The luciferase activities were measured from the lysates and normalized to β-galactosidase activities. The normalized values are shown in (**A**) and (**B**). In (**C**) the values are presented as fold induction relative to control (–OHT, –TNF). The results represent mean ± SD values of six (Rat1-MycER cells), two (Rat1-MycΔER cells) or four (MEF-MycERtm cells) separate transfection experiments.

c-Myc does not inhibit the TNF-induced accumulation of p53

Cellular p53 protein accumulates when cells are subjected to genotoxic stress, but less is known about the effects of death-inducing cytokines on p53 expression. We observed that TNF stimulation of Rat1-MycER cells induced a marked increase in cellular p53 protein levels (Figure 5A,

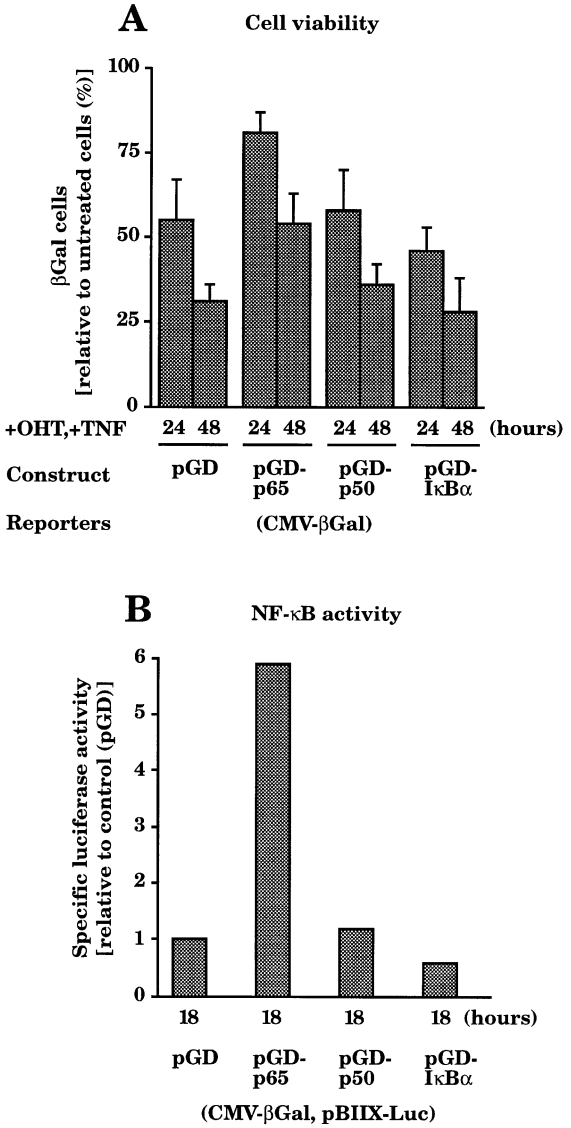

Fig. 4. Effect of NF-κB subunits on the viability and NF-κB activity of TNF-treated mouse embryonic fibroblasts with deregulated c-Myc. (**A**) Cells were co-transfected with 1 μg of CMV-βGal and 10 μg of one of the pGD expression vectors indicated in the figure. Aliquots of the transfected cell cultures were seeded on duplicate wells; one well was treated with ethanol carrier (untreated) and the other with OHT and 50 ng/ml TNF. After 24 or 48 h incubation with the drugs, cells were fixed and stained with X-gal. Each bar in the figure represents the ratio of transfected cells in the well treated with OHT and TNF to transfected cells in the untreated well. The results represent mean ± SD values of three separate transfection experiments. (**B**) Cells were co-transfected with 0.5 μg of CMV-βGal, 0.5 μg of pBIIX-Luc and 5 μg of one of the pGD expression vectors. Transfected cells were treated with OHT and 50 ng/ml TNF and, after 18 h, lysed and analyzed as in Figure 3. The results represent mean values of two separate transfection experiments.

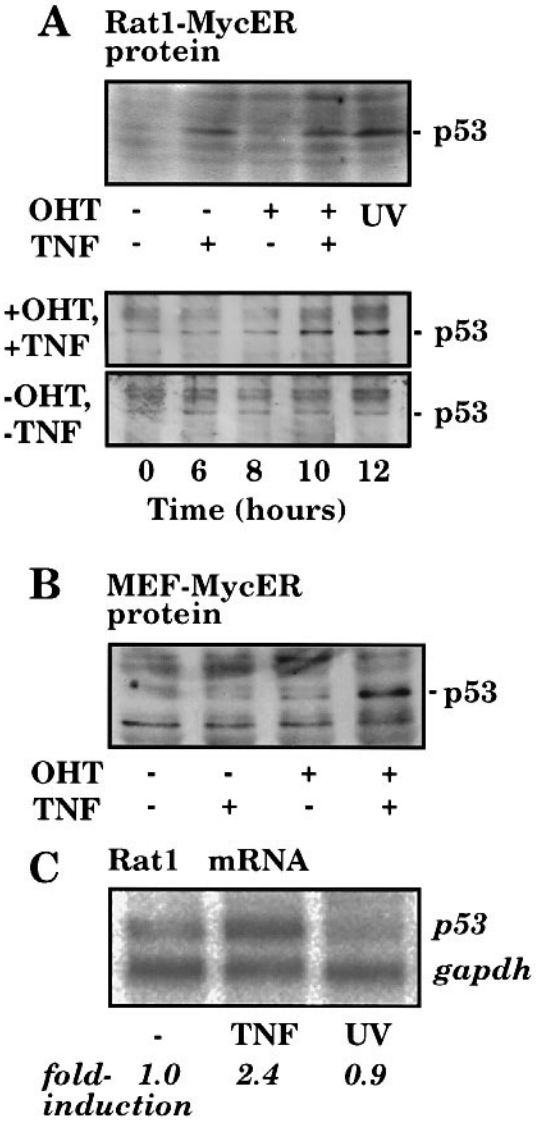

Fig. 5. Analysis of p53 protein and mRNA levels in TNF-stimulated cells. (**A**) and (**B**) Induction of p53 protein by TNF or UV-C. Rat1-MycER cells were incubated for 0–12 h (12 h in the upper panel of A) and MEF-MycERtm cells for 24 h (B) in the presence or absence of 50 ng/ml TNF. OHT or an equivalent volume of ethanol carrier was added to cells 6 h prior to TNF. The last lane in the upper panel of (A) represents cells incubated for 6 h after exposure to 50 J/m^2 UV-C light (Stratalinker, Stratagen). The cells were lysed, and equivalent amounts of total protein (60 μg) were analyzed by Western immunoblotting using anti-p53 Pab421 antibody. (**C**) Induction of p53 mRNA by TNF or UV-C. Northern blotting and hybridization analysis of p53 and GAPDH mRNAs in control Rat1 cells, cells treated for 12 h with 50 ng/ml TNF or incubated for 6 h after exposure to 50 J/m^2 UV-C. The relative level of *p53* induction in comparison with untreated Rat1 cells (1.0) was quantitated by phosphoimager (Fuji) analysis and normalized to *gapdh* signals.

upper panel). In Rat1 cells with deregulated c-Myc, the p53 level gradually increased after 10 h incubation of cells with TNF (Figure 5A, lower panel). Furthermore, immunoprecipitation analyses using p53 conformation-specific antibodies showed that Rat1 cells expressed wild-type p53 (data not shown). In MEFs, TNF induced p53 protein only if c-Myc was activated prior to TNF-stimulation (Figure 5B). While TNF stimulation induced both p53 protein and mRNA, only p53 protein was induced by UV-C treatment (Figure 5A and C). It has been noted previously that genotoxic stress induces p53 by post-translational mechanisms (Maltzman and Czyzyk, 1984; Kastan *et al.*, 1991). The present data indicate that p53 induction by TNF involved changes also at the steady-state mRNA level. Notably, in contrast to the other tested TNF responses, the TNF-induced p53 accumulation in fibroblasts was not inhibited by deregulated c-Myc.

Table III. c-Myc-induced TNF sensitivity in normal and *p53*-deficient mouse embryonic fibroblasts

Day	MEF p53+/+				MEF p53–/–			
	–Myc		+Myc		–Myc		+Myc	
	–TNF	+TNF	–TNF	+TNF	–TNF	+TNF	–TNF	+TNF
1	1.4	0.7	1.8	3.0	0.4	0.5	0.6	1.9
2	0.4	1.2	3.9	8.8	0.0	0.5	0.3	2.5
3	1.2	1.3	3.2	11.8	0.1	0.6	0.2	3.6

Cells were stained with propidium iodide and the condensed and fragmented nuclei were scored by immunofluorescence microscopic detection. The values represent means of two separate retrovirus infection experiments.

TNF, with c-Myc, induces apoptosis in mouse primary fibroblasts but only weakly in p53-deficient primary fibroblasts

The main cellular responses to p53 accumulation are cell cycle inhibition and apoptosis, the latter occurring predominantly in cells expressing growth-deregulating oncoproteins. This prompted us to assess the role of TNF-induced p53 in the cell death. The conditionally active form of c-Myc was introduced into embryonic fibroblasts derived from normal mice (MEFs p53+/+) or from mice having a targeted disruption of the *p53* gene (MEFs p53–/–). Since *p53*-deficient cells acquire genetic abnormalities during continuous culture (Harvey *et al*., 1993), MycERtm was introduced into MEFs by high-titer retroviruses. MEFs of passage 1–4 were infected and then selected in puromycin. An ~90% efficiency of infection was obtained, which allowed us to harvest the MycERtm-expressing MEFs for assays within a week of the infection. An equivalent expression level of MycERtm protein in the retrovirally infected MEF p53+/+ and MEF p53–/– cells was confirmed by Western blot analysis (data not shown).

For assays of TNF sensitivity, infected and uninfected MEF p53+/+ and MEF p53–/– cells of the same passage number were seeded on coverslips. c-Myc was activated with OHT, and 50 ng/ml of TNF was added to the cultures as indicated in Table III. Control MEFs were treated with TNF in parallel assays. At defined time points, cells on coverslips were scored for apoptotic nuclei. The results from these experiments are summarized in Table III. Both MEF p53+/+ and MEF p53–/– cells were resistant to the cytotoxic activity of TNF. However, if c-Myc was activated in the MEF p53+/+ cells, the rate of apoptosis increased substantially in the TNF-treated cultures. In contrast, when c-Myc was activated in the MEF p53–/– cells, the rate of apoptosis in the presence of TNF remained low. After a 3 day incubation with TNF, only ~4% of MEF p53–/– cells with active c-Myc were undergoing apoptosis, in contrast to 12% of MEF p53+/+ cells with active c-Myc. We conclude that TNF-induced p53 is involved in the TNF-induced apoptosis.

In contrast to MEF p53+/+ cells, which enter a non-growing senescent phase after prolonged culture, the MEFp53–/– cells exhibit a high spontaneous immortalization rate (Harvey *et al*., 1993). This allowed us to establish from the MycERtm-expressing MEFp53–/– cells clonal cell lines whose TNF sensitivity was then assessed by the MTT assay. The viability of the five clones examined did not decrease when c-Myc was activated in the presence of TNF (data not shown). This was not due to a lack of functional TNF receptors, since the TNF treatment combined with sublethal amounts of the protein synthesis inhibitor cycloheximide caused a rapid cytotoxic response in these cells.

The expression of the carboxy-terminal domain of p53 inhibits X-irradiation-induced apoptosis but not the c-Myc-mediated cell death

About half of the tumor cells express transforming mutant p53 (Hollstein *et al*., 1996), which is devoid of its wild-type function as a transcription-activating sequence-specific DNA-binding protein. Both p53 alleles commonly are mutated in the tumor cells, but mutation also of a single allele can produce a transforming protein, which dominantly, by forming heteromeric complexes, inhibits the transcriptional activation by wild-type p53 (Kern *et al*., 1992). To mimick this situation, Rat1 and Rat1-MycER cells were infected with retroviruses encoding the C-terminal transforming domain of p53 (p53Cter, p53 residues 302–390, Shaulian *et al*., 1992). p53Cter effectively oligomerizes with the endogenous wild-type p53 and stabilizes its expression. The formation of these heteromeric complexes inhibits transcriptional activation by wild-type p53 (Shaulian *et al*., 1992, 1995). The introduction of p53Cter into Rat1 and Rat1-MycER fibroblasts led to the stabilization of the endogenous wild-type p53, indicating that heteromeric complexes were formed (Figure 6).

The apoptosis of these cells was induced either by X-irradiation or by activating c-Myc in the presence of TNF. The X-irradiation resulted in a slowly progressing apoptosis in Rat1 cells, but this was clearly inhibited in the clones expressing p53Cter (Figure 7). In contrast, no inhibition of cell death was observed when Rat1 cells co-expressing activated c-Myc and p53Cter were exposed to TNF (Figure 8). Three independent cell clones were assayed with similar results. p53Cter also failed to inhibit apoptosis caused by the activation of c-Myc in low serum conditions (data not shown). The data suggest that the transcriptional activity of p53 is necessary for X-irradiation-induced apoptosis but not for apoptosis mediated by c-Myc.

Discussion

Induction of TNF sensitivity is a specific function of c-Myc

Deregulation of c-Myc rendered Rat1 cells and primary fibroblasts TNF sensitive, yet c-Myc alone, without complementation by other oncogenes, does not transform these cells (Mougneau *et al*., 1984; Sawyers *et al*., 1992). In contrast to c-Myc, Bak failed to induce TNF sensitivity, yet it induced sensitivity to apoptosis. These results indicate that induction of TNF sensitivity is a specific function of deregulated c-Myc, which does not depend on cell transformation or cellular sensitivity to apoptosis. Experiments with c-Myc mutants indicated that the induction of TNF sensitivity is a function of c-Myc competent for transcriptional activation.

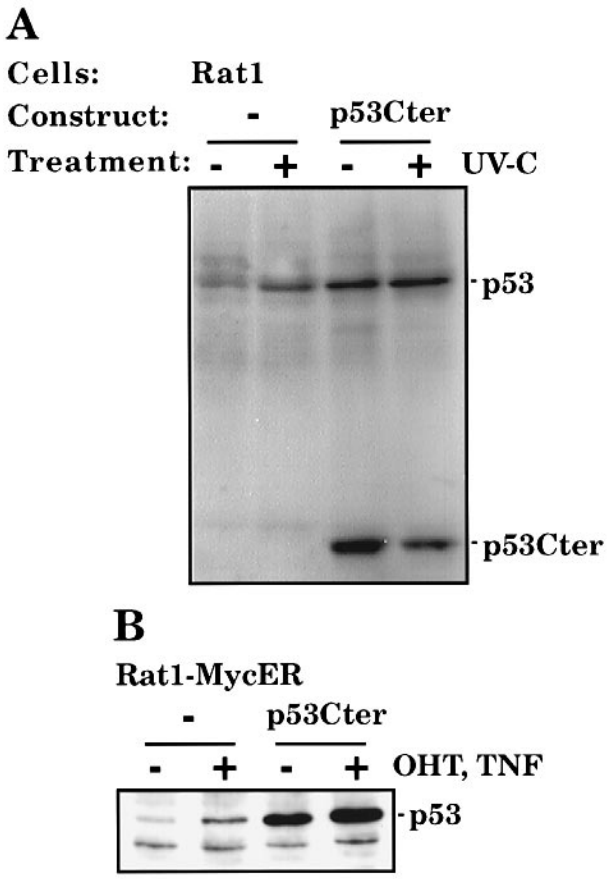

Fig. 6. Stabilization of endogenous p53 in Rat1 and Rat1-MycER cells expressing the C-terminal domain of p53 (p53Cter). To visualize the endogenous p53 and to demonstrate its stabilization, the cells were exposed to 50 J/m^2 of UV-C light (**A**) or treated with OHT and TNF (**B**). Western immunoblotting was performed as in Figure 5. The positions of p53Cter and the endogenous cellular p53 are indicated.

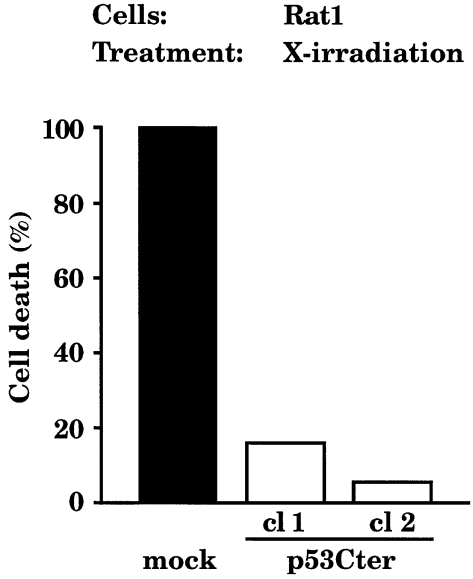

Fig. 7. The C-terminal domain of p53 inhibits X-irradiation-induced apoptosis. Rat1 cells containing empty vector (mock) or two independent clones expressing p53Cter were exposed to 6 Gy of X-irradiation, and cell death in the cultures was determined after 10 days of incubation by trypan blue staining.

c-Myc inhibits TNF-induced stress signals

To gain insight into mechanisms whereby c-Myc induces cellular sensitivity to TNF-induced cell death, we investigated if c-Myc modulated TNF-induced signal transduction. TNF-induced activation of JNKs and NF-κB was examined. The JNK and NF-κB activities are also induced by various stress signals and, in this context, the TNF inducibility of p53 and PLA_2 was also examined. JNKs, NF-κB and p53 are typical stress response proteins, but PLA_2 can also be assigned to the same group, as its activation has been described in response to UV light, oxidative stress and heat shock (Calderwood and Stevenson, 1993; Chen *et al*., 1996). We found that the TNF-induced PLA_2, JNK and NF-κB activation, but not the p53 accumulation, was impaired in cells expressing deregulated c-Myc.

JNKs have been implicated in apoptosis induced by synthetic ceramide analogs or oxidative stress (Verheij *et al*., 1996). In the case of the p55 TNF receptor, however, JNKs are activated via the non-cytotoxic TRAF2 pathway (Liu *et al*., 1996b). JNKs are activated early during stress signal transduction, and constitute a distinct MAP kinase pathway. NF-κB, on the other hand, is activated upon phosphorylation of the inhibitory IκB proteins by the IκB kinase complexes (DiDonato *et al*., 1997). The recent data indicate that the IκBα kinase complex is phosphorylated and activated directly by the upstream kinase MEKK1 of the JNK pathway (Lee *et al*., 1997). This makes it tempting to speculate that c-Myc impairs the activities of both JNKs and NF-κB by inhibiting upstream kinase(s) such as MEKK1. TNF also stimulates the mitogen-activated protein (MAP) kinase pathway in fibroblasts (Vietor *et al*., 1993), which has been implicated in the activation of PLA_2 (Lin *et al*., 1993). Thus, the c-Myc inhibition of upstream kinases of the MAP kinase pathway could play a role in the deficient induction of PLA_2 by TNF. Clearly, c-Myc inhibition of TNF signaling was not due to a down-regulation of active TNF receptors, since c-Myc did not impair the induction of p53 protein by TNF. The deregulation of c-Myc also did not impair the TNF induction of p65 and p105 subunits of NF-κB at the mRNA level (data not shown). Thus, some of the TNF signaling pathways from the receptor to the nucleus remain intact, whereas others are inhibited in cells expressing deregulated c-Myc.

A role for c-Myc inhibition of NF-κB in TNF-induced cell death

TNF-stimulated transcriptional activation by NF-κB was inhibited in both Rat1 and MEF cells by deregulated c-Myc. This impairment occurred already at the earliest assayed time points (2 h, data not shown), which means that NF-κB activity was inhibited prior to the TNF-induced cell death. Recent data indicate that a deficiency of the NF-κB pathway can render TNF-resistant cells sensitive to the cytotoxic effects of TNF (Beg and Baltimore, 1996; Van Antwerp *et al*., 1996; Wang *et al*., 1996). This prompted us to study if NF-κB activity in the cells containing deregulated c-Myc affected the TNF cytotoxicity. The c-Myc-induced TNF sensitivity was inhibited by the overexpression of the p65 subunit of NF-κB, which also increased NF-κB activity. This indicated that the level of TNF cytotoxicity towards cells with active

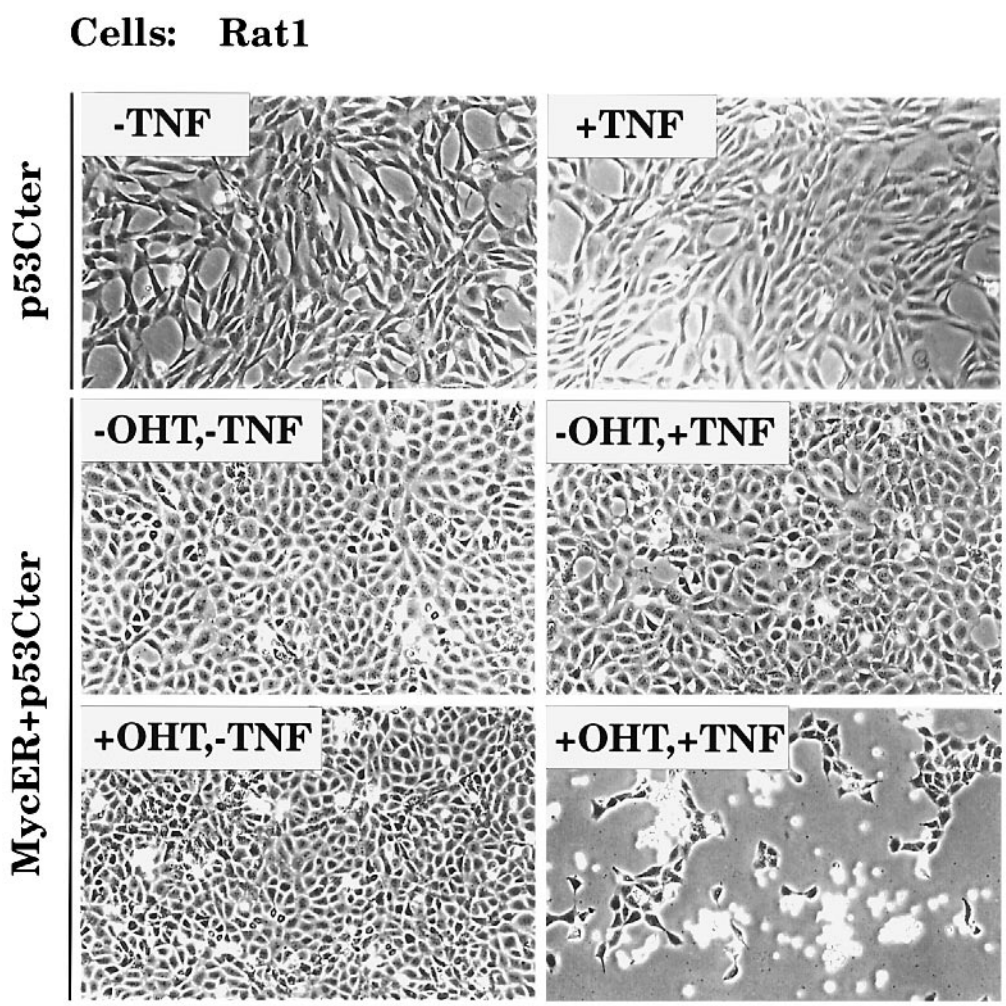

Fig. 8. The C-terminal domain of p53 does not inhibit the c-Myc-induced TNF sensitivity. About 10^6 cells were seeded on 100 mm culture plates and cultured overnight. Then normal growth medium was renewed and ethanol carrier or OHT and 50 ng/ml TNF were added, as indicated in the figure. The uppermost panels show photomicrographs of the Rat1 cells expressing p53Cter after culturing for 3 days in the presence or absence of TNF. The lower panels show Rat1 cells co-expressing MycER and p53Cter after 1 day incubation with the drugs.

c-Myc was dependent on the cellular NF-κB activity. Therefore, c-Myc-mediated inhibition of NF-κB activity is a mechanism which can lead to an increased cellular TNF sensitivity. The TNF-induced NF-κB activity at 18 h was ~3-fold higher in the cells expressing transfected p65 and deregulated c-Myc than in the cells containing control vector and c-Myc in inactive form (data not shown). Thus, even though the NF-κB activity in cells with active c-Myc was increased above the levels normally induced by TNF in cells, it was not sufficient to rescue cells completely from TNF cytotoxicity. Therefore, other mechanisms must have contributed to the c-Myc-induced TNF sensitivity.

A role for TNF-induced p53 in cell death

c-Myc has a diminished potential to induce apoptosis in *p53*-deficient cells upon growth factor-withdrawal. This indicates that endogenous p53 is necessary for the apoptosis by c-Myc (Hermeking and Eick, 1994; Wagner *et al*., 1994). While the activation of c-Myc in Rat1 cells or in MEFs growing in the presence of serum did not increase the cellular p53 level, the addition of TNF to these cells led to the accumulation of p53 in association with cell death. The diminished potential of TNF to kill *p53*-deficient MEFs expressing deregulated c-Myc indicated a role for the TNF-induced p53 in the apoptosis. Notably, TNF also induced p53 in the TNF-resistant Rat1 cells, which means that the TNF-up-regulated p53 was not sufficient, as such, to induce cell death. Similar to our findings with TNF, previous reports show that certain anticancer drugs or hypoxia induce p53 without cell death in normal cells but kill cells containing E1A or deregulated c-Myc in a p53-dependent manner (Lowe *et al*., 1993; Graeber *et al*., 1996). It is possible that the up-regulation of p53 by an extrinsic factor triggers c-Myc-induced apoptosis in normal growth conditions even when growth factors are present. The selective cytotoxicity of TNF towards oncogene-expressing cells may thus be attributable to the capacity of TNF to induce p53.

Transcription-activating function of p53 is not necessary for the c-Myc-mediated cell death

p53Cter, which is an inhibitor of the transcriptional activity of wild-type p53, had different effects on fibroblast apoptosis depending on the nature of the apoptotic stimulus. While inhibiting X-irradiation induced apoptosis, expression of p53Cter failed to inhibit apoptosis mediated by deregulated c-Myc in low serum or in the presence of TNF. This suggests that the transcriptional activity of p53,

which is necessary for p53-dependent apoptosis in certain cases (Levine, 1997), mediated the X-irradiation-induced apoptosis. In contrast, the c-Myc-mediated apoptosis of serum-deprived or TNF-treated cells was not dependent on the transcription-activating function of p53. In agreement with our data, it has been documented previously that primary epithelial cell lines co-expressing c-Myc and a dominant inhibitory point-mutant allele of p53 undergo apoptosis upon serum deprivation (Sakamuro *et al*., 1995). In this context, it has also been noted that p53 in co-operation with c-Myc induces apoptosis even in the absence of new protein synthesis (Wagner *et al*., 1994).

In summary, we propose that deregulated c-Myc sensitizes cells to the cytotoxic effects of TNF through two arms: (i) by inhibiting cell survival signaling via NF-κB and (ii) through the apoptotic effects of c-Myc unleashed by TNF-induced p53. The present data suggest that the transcription-activating function of p53 is not required for the collaboration of c-Myc and p53 in cell death induction.

Materials and methods

Established cell lines and gene transfer

Rat1 and NIH-3T3 fibroblasts were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FCS and antibiotics, except for cells expressing estrogen-inducible constructs which were grown in phenol red-free DMEM supplemented with charcoal-stripped 10% FCS and antibiotics. The cells containing expression constructs were grown periodically in selection medium. We used the following expression vectors and gene transfer procedures. Wild-type and N-terminal mutant forms of c-Myc were cloned into pMV7-Neo retrovirus vector. The c-Myc vectors and empty vector as a mock control were transfected by calcium phosphate precipitation into the BOSC23 retrovirus packaging cell line engineered to produce high-titer viral stocks during transient transfection (Pear *et al*., 1994). The NIH-3T3 cells were infected with the retroviruses and the infected cells were selected in 500 μg/ml G418. The resulting clones in each case were pooled for the assays. The p53Cter DNA fragment encoding the carboxy-terminal amino acid residues 302–390 of murine p53 was cloned into pBabe-Puro and pBabe-Hygro retroviral vectors (Land and Morgenstern, 1990), and transferred by retroviral infection into Rat1 cells or Rat1 cells containing MycER (Evan *et al*., 1992). Rat1 cells expressing hormone-inducible GAL4ER-VP16 and Bak cDNA under the control of a synthetic promoter containing GAL4 DNA-binding sites have been described in Chittenden *et al.* (1995).

Retroviral infection and cell death analyses of mouse embryonic fibroblasts

High-titer retroviral supernatants were harvested from GP+E packaging cell lines stably transfected with OHT-specific pBabe-Puro MycERtm virus construct (Littlewood *et al*., 1995). The third to fourth passage MEF p53–/– cells or first to second passage MEF p53+/+ cells were infected with the retroviruses and, after 2 days, cells were subjected to 2.5 μg/ml puromycin selection. All uninfected cells in control plates died and detached within 2–4 days, whereas ~90% of the infected cells survived the selection. The expression of MycERtm in these cells was analyzed by Western immunoblotting. To analyze cell death by propidium iodide (PI) staining, cells were grown on 0.05% poly-L-lysine-coated coverslips in 35 mm dishes for 2 days prior to the addition of drugs. At the indicated time points, coverslips were fixed in 4% formaldehyde in phosphate-buffered saline (PBS). To score apoptotic cells on the coverslips, the fixed cells were incubated for 20 min in PI solution (50 μg/ml PI and 1 mg/ml RNase A in PBS), washed with PBS and mounted with glycerol-PBS. The condensed and fragmented apoptotic nuclei formed bright images in immunofluorescence microscopy and were easily distinguishable from the round and dim normal nuclei. The ratio of apoptotic to non-apoptotic nuclei was scored by counting 200–1000 nuclei on each coverslip. To score TNF-induced deaths of the transfected cells, cell cultures were co-transfected with a mixture containing CMV-βGal vector which expresses β-galactosidase and the pGD, pGD-p65, pGD-p50 or pGD-IκBα (Scott *et al*., 1993) construct in 10-fold excess. The plasmids were co-transfected with Lipofectamine reagent (Life Technologies) into cultures containing 1–2×10^6 MEF-MycER fibroblasts per 100 mm dish. After overnight transfection, cells were seeded to 6-well plates (50 000 cells/well) and 1 day later treated with drugs as indicated in the text. At defined time periods, cells were washed with PBS, fixed with 0.05% glutaraldehyde, washed again with PBS and stained with a solution containing 0.05% X-gal, 0.2 mg/ml spermidine, 10 mg/ml potassium ferrocyanide, 8 mg/ml potassium ferricyanide and 3 mM $MgCl_2$ in PBS. The blue cells (usually 30–300/well) were scored with the microscope after overnight incubation at 37°C.

TNF and drugs

Recombinant mouse TNF-α (sp. act. 1.2×10^7 U/mg) was kindly provided by Dr Günther R.Adolf (Ernst Boehringer Institute, Vienna). For the activation of MycER constructs, we used either 2 μM β-estradiol (Sigma) or 100 nM OHT (RBI), and for Bak induction 100 nM OHT.

MTT assay

Cell survival was quantitated by colorimetric MTT assay which measures mitochondrial activity in viable cells. For the assays, ~3000 cells/well were seeded on 96-well plates (Nunc). Cells were treated as indicated in the text; all treatments were tested in triplicate. After defined incubation periods, 10 μl of MTT from a 5 mg/ml stock solution was added into each well and the plates were kept for 4 h at 37°C. The reaction was terminated by adding 100 μl of lysis buffer (10% SDS, 10 mM HCl). The resulting formazan products were solubilized overnight at 37°C in a humid atmosphere, after which the absorbance at 540 nm was measured with a Multiscan microtiter plate reader (Labsystems).

JNK assay

The assay was performed with a commercial kit from New England Biolabs (NEB) by using the manufacturer's reagents and protocol. In short, the drug-treated cell cultures were washed twice with cold PBS and solubilized on ice in non-denaturating lysis buffer. The cells were scraped, sonicated and clarified by centrifugation. The supernatants (2 mg total protein) were incubated with 2 μg of GST–c-Jun (1–89) Sepharose beads at 4°C overnight. Beads were washed twice with lysis buffer and twice with kinase buffer (25 mM Tris, pH 7.5, 5 mM β-glycerolphosphate, 2 mM dithiothreitol, 0.1 mM Na_3VO_4, 10 mM $MgCl_2$ and 100 μM ATP). The kinase assays for JNK isoforms complexed with GST–c-Jun beads were performed at 30°C for 30 min using GST–c-Jun as a substrate. Reactions were stopped in Laemmli sample buffer, and samples were electrophoresed in 12% SDS–PAGE. The gels were immunoblotted, and c-Jun phosphorylation at Ser63 was detected with phospho-c-Jun-specific antibody (NEB). Immunoreactive bands were visualized using secondary antibody conjugated with horseradish peroxidase and enhanced chemiluminescence detection.

NF-κB luciferase assay

The NF-κB-driven plasmid pBIIX-Luc contains two copies of the immunoglobulin kappa NF-κB-binding sequence ACAGAGGGACTTTCCGAGAG upstream of the minimal murine *fos* promoter in pfLuc luciferase plasmid (Saksela and Baltimore, 1993). To measure the NF-κB activity, cells were transfected with pBIIX-Luc, and to monitor the transfection efficiency, with the CMV-βGal plasmid. The plasmids were co-transfected with Lipofectamine into cultures containing 5×10^6 fibroblasts per 100 mm dish. At 24–48 h after transfection, cells were split and, after adhering to dishes, were treated with drugs as indicated in the text. Cells were lysed after defined time periods, and the luciferase activity was measured from the cell lysates with the manufacturer's reagents (Promega) and a luminometer (Bioorbit). The β-galactosidase activities were measured from the same lysates by using *o*-nitrophenyl-β-D-galactopyranoside (ONPG; Sigma) as a chromogenic substrate.

Acknowledgements

We wish to warmly thank Dr Larry Donehower for MEF p53–/– cells, Dr Martin Eilers for N-terminal mutant forms of c-Myc, Dr David Lane for Pab421 antibody, Dr Kalle Saksela for pBIIX-Luc and both Dr Kalle Saksela and Dr Amer Beg for pGD constructs. Ms Mari Helanterä and Mrs Paula Hyvärinen are acknowledged for expert technical assistance, and Drs Imre Västrik and Stella Pelengaris for help and many useful discussions. This work was supported by the Finnish Cancer Organizations, the Finnish Academy, Maud Kuistila, Sigrid Juselius, Farmos, Finnish Cultural Foundations and European Molecular Biology

Organization. E.A. is a recipient of a fellowship from the Associazione Italiana per la Ricerca sul Cancro (AIRC).

References

Aderka,D., Novick,D., Hahn,T., Fischer,D.G. and Wallach,D. (1985) Increase of vulnerability to lymphotoxin in cells infected by vesicular stomatitis virus and its further augmentation by interferon. *Cell. Immunol.*, **92**, 218–225.

Amati,B., Littlewood,T.D., Evan,G.I. and Land,H. (1993) The c-Myc protein induces cell cycle progression and apoptosis through dimerization with Max. *EMBO J.*, **12**, 5083–5087.

Beg,A.A. and Baltimore,D. (1996) An essential role for NF-κB in preventing TNF-α-induced cell death. *Science*, **274**, 782–784.

Boldin,M., Goncharov,T., Goltsev,Y. and Wallach,D. (1996) Involvement of MACH, a novel MORT1/FADD-interacting protease, in Fas/APO-1- and TNF receptor-induced cell death. *Cell*, **85**, 803–815.

Calderwood,S.K. and Stevenson,M.A. (1993) Inducers of the heat shock response stimulate phospholipase C and phospholipase A2 activity in mammalian cells. *J. Cell Physiol.*, **155**, 248–256.

Carswell,E.A., Old,L.J., Kassel,R.L., Green,S., Fiore,N. and Williamson,B. (1975) An endotoxin-induced serum factor that causes necrosis of tumors. *Proc. Natl Acad. Sci. USA*, **72**, 3666–3670.

Chen,M.J., Holskin,B., Strickler,J., Gorniak,J., Clark,M.A., Johnson,P.J., Mitcho,M. and Shalloway,D. (1987) Induction by E1A oncogene expression of cellular susceptibility to lysis by TNF. *Nature*, **330**, 581–583.

Chen,X., Gresham,A., Morrison,A. and Pentland,A.P. (1996) Oxidative stress mediates synthesis of cytosolic phospholipase A2 after UVB injury. *Biochim. Biophys. Acta*, **1299**, 23–33.

Chittenden,T., Harrington,E.A., O'Connor,R., Flemington,C., Lutz,R.J., Evan,G.I. and Guild,B.C. (1995) Induction of apoptosis by the Bcl-2 homologue Bak. *Nature*, **374**, 733–736.

DiDonato,J.A., Hayakawa,M., Rothwarf,D.M., Zandi,E. and Karin,M. (1997) A cytokine-responsive IκB kinase that activates the transcription factor NF-κB. *Nature*, **388**, 548–554.

Duerksen-Hughes,P.J., Hermiston,T.W., Wold,W.S.M. and Gooding,L.R. (1991) The amino-terminal portion of CD1 of the adenovirus E1A proteins is required to induce susceptibility to tumor necrosis factor cytolysis in adenovirus-infected mouse cells. *J. Virol.*, **65**, 1236–1244.

Eilers,M., Picard,D., Yamamoto,K.R. and Bishop,J.M. (1989) Chimaeras of Myc oncoprotein and steroid receptors cause hormone-dependent transformation of cells. *Nature*, **340**, 66–68.

Evan,G.I., Wyllie,A.H., Gilbert,C.S., Littlewood,T.D., Land,H., Brooks,M., Waters,C.M., Penn,L.Z. and Hancock,D.C. (1992) Induction of apoptosis in fibroblasts by c-myc protein. *Cell*, **69**, 119–128.

Graeber,T.G., Osmanian,C., Jacks,T., Housman,D.E., Koch,C.J., Lowe,S.W. and Giaccia,A.J. (1996) Hypoxia-mediated selection of cells with diminished apoptotic potential in solid tumors. *Nature*, **379**, 88–91.

Harrington,E.A., Bennett,M.R., Fanidi,A. and Evan,G.I. (1994) c-Myc-induced apoptosis in fibroblasts is inhibited by specific cytokines. *EMBO J.*, **13**, 3286–3295.

Harvey,M. *et al*. (1993) *In vitro* growth characteristics of embryo fibroblasts isolated from p53-deficient mice. *Oncogene*, **8**, 2457–2467.

Hermeking,H. and Eick,D. (1994) Mediation of c-Myc-induced apoptosis by p53. *Science*, **265**, 2091–2093.

Hollstein,M., Shomer,B., Greenblatt,M., Soussi,T., Hovig,E., Montesano,R. and Harris,C.C. (1996) Somatic point mutations in the p53 gene of human tumors and cell lines: updated compilation. *Nucleic Acids Res.*, **24**, 141–146.

Hsu,H., Shu,H.B., Pan,M.G. and Goeddel,D.V. (1996) TRADD–TRAF2 and TRADD–FADD interactions define two distinct TNF receptor 1 signal transduction pathways. *Cell*, **84**, 299–308.

Jäättelä,M. (1993) Overexpression of major heat shock protein hsp70 inhibits tumor necrosis factor-induced activation of phospholipase A2. *J. Immunol.*, **151**, 4286–4294.

Janicke,R.U., Lee,F.H. and Porter,A.G. (1994) Nuclear c-Myc plays an important role in the cytotoxicity of tumor necrosis factor alpha in tumor cells. *Mol. Cell. Biol.*, **14**, 5661–5670.

Kastan,M.B., Onyekwere,O., Sidransky,D., Vogelstein,B. and Craig,R.W. (1991) Participation of p53 protein in the cellular response to DNA damage. *Cancer Res.*, **51**, 6304–6311.

Kern,S.E., Pietenpol,J.A., Thiagalingam,S., Seymour,A., Kinzler,K.W. and Vogelstein,B. (1992) Oncogenic forms of p53 inhibit p53-regulated gene expression. *Science*, **256**, 827–830.

Klefstrom,J., Vastrik,I., Saksela,E., Valle,J., Eilers,M. and Alitalo,K. (1994) c-Myc induces cellular susceptibility to the cytotoxic action of TNF-α. *EMBO J.*, **13**, 5442–5450.

Kyriakis,J.M., Banerjee,P., Nikolakaki,E., Dai,T., Rubie,E.A., Ahmad,M.F., Avruch,J. and Woodgett,J.R. (1994) The stress-activated protein kinase subfamily of c-Jun kinases. *Nature*, **369**, 156–160.

Land,H. and Morgenstern,J.P. (1990) Advanced mammalian gene transfer: high titre retroviral vectors with multiple drug selection markers and a complementary helper-free packaging cell line. *Nucleic Acids Res.*, **18**, 3587–3596.

Lee,F.S., Hagler,J., Chen,Z.J. and Maniatis,T. (1997) Activation of the IκBα kinase complex by MEKK1, a kinase of the JNK pathway. *Cell*, **88**, 213–222.

Levine,A.J. (1997) p53, the cellular gatekeeper for growth and division. *Cell*, **88**, 323–331.

Lin,L.L., Wartmann,M., Lin,A.Y., Knopf,J.L., Seth,A. and Davis,R.J. (1993) cPLA2 is phosphorylated and activated by MAP kinase. *Cell*, **72**, 269–278.

Littlewood,T., Hancock,D., Danielian,P., Parker,M. and Evan,G. (1995) A modified oestrogen receptor ligand-binding domain as an improved switch for the regulation of heterologous proteins. *Nucleic Acids Res.*, **23**, 1686–1690.

Liu,Z.G., Baskaran,R., Lea-Chou,E.T., Wood,L.D., Chen,Y., Karin,M. and Wang,J.Y. (1996a) Three distinct signalling responses by murine fibroblasts to genotoxic stress. *Nature*, **384**, 273–276.

Liu,Z.G., Hsu,H., Goeddel,D.V. and Karin,M. (1996b) 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.

Lowe,S.W., Ruley,H.E., Jacks,T. and Housman,D.E. (1993) p53-dependent apoptosis modulates the cytotoxicity of anticancer agents. *Cell*, **74**, 957–967.

Maltzman,W. and Czyzyk,L. (1984) UV irradiation stimulates levels of p53 cellular tumor antigen in nontransformed mouse cells. *Mol. Cell. Biol.*, **4**, 1689–1694.

Mougneau,E., Lemieux,L., Rassoulzadegan,M. and Cuzin,F. (1984) Biological activities of v-myc and rearranged c-myc oncogenes in rat fibroblast cells in culture. *Proc. Natl Acad. Sci. USA*, **81**, 5758–5762.

Muzio,M. *et al*. (1996) FLICE, a novel FADD homologous ICE/CED-3-like protease, is recruited to the CD95 (Fas/Apo-1) death-inducing signaling complex. *Cell*, **85**, 817–827.

Palombella,V.J. and Vilcek,J. (1989) Mitogenic and cytotoxic actions of tumor necrosis factor in BALB/c 3T3 cells. Role of phospholipase activation. *J. Biol. Chem.*, **264**, 18128–18136.

Pear,W.S., Nolan,G.P., Scott,M.L. and Baltimore,D. (1994) Production of high titer helper-free retroviruses by transient transfection. *Proc. Natl Acad. Sci. USA*, **90**, 8392–8396.

Philipp,A., Schneider,A., Vástrik,I., Finke,K., Xiong,Y., Beach,D., Alitalo,K. and Eilers,M. (1994) Repression of cyclin D1: a novel function of Myc. *Mol. Cell. Biol.*, **14**, 4032–4043.

Roy,A.L., Carruthers,C., Gutjahr,T. and Roeder,R.G. (1993) Direct role for Myc in transcription initiation mediated by interactions with TFII-I. *Nature*, **365**, 359–361.

Sakamuro,D., Eviner,V., Elliott,K.J., Showe,L., White,E. and Prendergast,G.C. (1995) c-Myc induces apoptosis in epithelial cells by both p53-dependent and p53-independent mechanisms. *Oncogene*, **11**, 2411–2418.

Saksela,K. and Baltimore,D. (1993) Negative regulation of immunoglobulin kappa light-chain gene transcription by a short sequence homologous to the murine B1 repetitive element. *Mol. Cell. Biol.*, **13**, 3698–3705.

Sawyers,C.L., Callahan,W. and Witte,O.N. (1992) Dominant negative Myc blocks transformation by ABL oncogenes. *Cell*, **70**, 901–910.

Scott,M.L., Fujita,T., Liou,H.C., Nolan,G.P. and Baltimore,D. (1993) The p65 subunit of NF-kappa B regulates I kappa B by two distinct mechanisms. *Genes Dev.*, **7**, 1266–1276.

Shaulian,E., Zauberman,A., Ginsberg,D. and Oren,M. (1992) Identification of a minimal transforming domain of p53: negative dominance through abrogation of sequence-specific DNA binding. *Mol. Cell. Biol.*, **12**, 5581–5592.

Shaulian,E., Haviv,I., Shaul,Y. and Oren,M. (1995) Transcriptional repression by the C-terminal domain of p53. *Oncogene*, **10**, 671–680.

Van Antwerp,D.J., Martin,S.J., Kafri,T., Green,D.R. and Verma,I.M. (1996) Suppression of TNF-α-induced apoptosis by NF-κB. *Science*, **274**, 787–789.

Verheij,M. *et al*. (1996) Requirement for ceramide-initiated SAPK/JNK signalling in stress-induced apoptosis. *Nature*, **380**, 75–79.

Verma,I.M., Stevenson,J.K., Schwarz,E.M., Van Antwerp,D. and Miyamoto,S. (1995) Rel/NF-kappa B/I kappa B family: intimate tales of association and dissociation. *Genes Dev.*, **9**, 2723–2735.

Vietor,I., Schwenger,P., Li,W., Schlessinger,J. and Vilcek,J. (1993) Tumor necrosis factor-induced activation and increased tyrosine phosphorylation of mitogen-activated protein (MAP) kinase in human fibroblasts. *J. Biol. Chem.*, **268**, 18994–18999.

Wagner,A.J., Kokontis,J.M. and Hay,N. (1994) Myc-mediated apoptosis requires wild-type p53 in a manner independent of cell cycle arrest and the ability of p53 to induce p21*waf1/cip1*. *Genes Dev.*, **8**, 2817–2830.

Wallach,D. (1984) Preparations of lymphotoxin induce resistance to their own cytotoxic effect. *J. Immunol.*, **132**, 2464–2469.

Wang,C.U., Mayo,M.W. and Baldwin,A.S.J. (1996) TNF- and cancer therapy-induced apoptosis: potentiation by inhibition of NF-κB. *Science*, **274**, 784–787.

Received on May 16, 1997; revised on August 22, 1997