ACADEMIC PRESS

Biochemical and Biophysical Research Communications 295 (2002) 24–30

BBRC

www.academicpress.com

# Insights into the molecular mechanism of apoptosis induced by TNF-α in mouse epidermal JB6-derived RT-101 cells

Neeta Singh,* Neeru Khanna, Himani Sharma, Siddhartha Kundu, and Sameena Azmi[1]

*Department of Biochemistry, All India Institute of Medical Sciences, New Delhi 110 029, India*

Received 21 May 2002

**Abstract**

The mammalian response to stress is complex, often involving multiple signaling pathways that act in concert to influence cell fate. To examine potential interaction between the signaling cascade, we have focused on the effects of a model apoptotic system in a single cell type sensitive to TNF-α induced apoptosis through an examination of the relative influences of MAPKs as well as transcription factors AP-1, NF-κB, and various survival genes in determining apoptosis. Our results show that ERKs decreased transiently or remain unchanged, JNK decreased robustly, whereas c-Jun increased transiently, thereby indicating that members of MAPK family are differentially regulated in response to TNF-α induced apoptosis, whereas NF-κB protein expression decreased transiently and activity decreased at 24 h post-treatment. The survival genes Bcl-2, Bcl-XL, and survivin act independently and downstream of ERK and JNK to decrease the survival of TNF-α treated RT-101 cells. The results also suggest the involvement of the mitochondria and cytochrome *c*. Caspase-3 appears to be a part of a downstream event. © 2002 Elsevier Science (USA). All rights reserved.

*Keywords:* Apoptosis; TNF-α; JB-6 cells; MAPK; Transcription factors; Survival genes

Tumor necrosis factor-alpha (TNF-α) is a 17 kDa non-glycosylated potent multifunctional cytokine which has been shown to elicit a wide spectrum of biological responses including apoptosis [1,2], by binding to its receptors and triggering signal transduction events that regulate gene expression [3]. TNF-α has been shown to elevate reactive oxygen species (ROS), to produce DNA damage, to induce expression of c-Jun, c-Fos, and c-Myc genes, and to increase AP-1 mediated transcription [4,5]. The effect of TNF-α is mediated by a distinct TNF receptor [6] which contains a death domain that binds TRADD [7] which is capable of activating the downstream events observed following TNF stimulation. Binding to TNF receptor results in oligomerization, activation of PKC, and generation of ROS but many elements of which remain unknown. Clearly additional studies are required to determine how these events are interrelated.

Apoptosis is an orderly process by which cells disintegrate in a controlled and programmed manner in response to extrinsic and intrinsic signals. The process of apoptosis which normally functions to maintain homeostasis in cell population can be initiated by a variety of stimuli including growth factor withdrawal, cytokines, and cytotoxic drugs [8]. Apoptosis can be induced in many model systems by receptor engagement. Among the cytokines which are known to be capable of triggering apoptosis, most attention has been devoted to TNF-α [5]. A clear understanding of the interrelationship between apoptosis and signal transduction will help elucidate the underlying mechanism involved in apoptosis. We have earlier [9] tried to provide evidence for a signaling mechanism that integrates TNF-α and stress-activated apoptosis. Sensitivity to cell killing by TNF-α was seen in mouse epidermal JB6 derived RT-101 cells. Morphological and biochemical changes characteristic of apoptosis were found to precede TNF-α induced cell death in TNF sensitive RT-101 cells.

The transcription factors NF-κB and activator protein AP-1 mediate signal transduction and modulate the expression of genes involved in cellular proliferation and

* Corresponding author. Fax: +91-11-6862663.
*E-mail address:* singh_neeta@hotmail.com (N. Singh).
[1] Present address: Department of Biochemistry and Molecular Biology, Mount Sinai School of Medicine, New York, NY 10029-6574, USA.

0006-291X/02/$ - see front matter © 2002 Elsevier Science (USA). All rights reserved.
PII: S0006-291X(02)00627-7

apoptosis. Jun and Fos dimers compose the AP-1 complex. Several mitogen-activated protein kinases (MAPKs) phosphorylate c-Jun and c-Fos and are postulated to control AP-1 activity. There is limited information available about the role(s) of MAPKs in TNF action and apoptosis. In this study, we used a clonal genetic variant of JB-6 mouse skin cells that is sensitive to TNF-α induced apoptosis, tried to study the involvement of MAPKs (ERKs, JNK), and sought evidence for interaction between AP-1, NF-κB, and MAPKs, and genes differentially expressed during apoptosis, as well as focused on whether caspases are part of a common downstream apoptotic death pathway.

## Materials and methods

*Materials*. TNF-α and Western blot kit were purchased from Promega, USA. c-Jun/AP-1, c-Fos, ERKs, JNK, NF-κB, Bcl-2, Bcl-$X_L$, Bax, survivin, apoptosis inducing factor (AIF), and cytochrome *c* antibodies were obtained from Santa Cruz, USA. HABP1 antibody was a gift from Dr. K. Dutta of Jawaharlal Nehru University, New Delhi, India. Caspase-3 Assay Kit was from Pharmingen, Germany. PCR–ELISA Kit for telomerase and the DIG Gel Shift Kit were from Roche Biochemicals, USA.

*Cell culture and treatment*. The mouse epidermal JB6 derived TNF sensitive (TNF) transformed RT-101 cells were obtained from Dr. Nancy Colburn, National Cancer Institute, Frederick, USA. They were grown in EMEM containing 5% FBS, at 37 °C in a humidified atmosphere of 5% $CO_2$ in air. Cell viability was measured by trypan blue dye exclusion test. The cells were exposed to a previously standardized dose of TNF-α (150 U/ml) for different time periods ranging from 1 to 24 h.

*Flow cytometry*. The cells were pelleted, resuspended in 100 μl PBS, and fixed by adding 3 ml of 70% ethanol followed by incubation at 4 °C for 1 h. The cells were washed twice with 2 ml PBS each time, then resuspended in 100 μl PBS, with 100 μl RNase solution (1 in 10 dilution of stock solution containing 10 mM Tris, 15 mM NaCl, and RNase 10 mg/ml) and 200 μl propidium iodide solution (100 μg/ml in PBS), and incubated at 4 °C in the dark for 30 min. The cells were then analyzed by flow cytometry using 488 nm for excitation and red fluorescence measured >600 nm using the Coulter epics XL flow cytometer. The data were analyzed using Win MD1 2.8 software [10].

*Estimation of superoxide anions*. The mitochondria were isolated and suspended in a buffer containing 250 mM sucrose, 50 mM Tris, and 1 mM EDTA. Oxidized cytochrome *c* was used as the substrate which was reduced by the generated superoxide anions in the test system. The color was measured at 550 nm as described earlier [11]. The results are expressed as nanomolar of superoxide anions/milligram protein. Phorbol-myristate-acetate (PMA) was used as a positive control and as a negative control superoxide dismutase which acts as a scavenger of superoxide anions was used.

*Caspase-3 assay*. Caspase-3 was measured by the direct assay of caspase enzyme activity in cell lysates using synthetic fluorogenic substrate (Ac-DEVD-AMC; substrate for caspase-3/7; Pharmingen, Germany) as described by the manufacturer. Briefly, cells were washed with PBS and lysed in 10 mM Tris–HCl; pH 7.5, 130 mM NaCl; 1% Triton X-100; 10 mM $Na_4P_2O_7$; and 10 mM $Na_2HPO_4$ on ice for 20 min. Aliquots of cell lysates (50–100 μl) were then added to reaction buffer (20 mM HEPES; 10% glycerol; 2 mM DTT; and 50 μM fluorogenic substrate) and reactions were performed for 1 h at 37 °C. Amounts of fluorogenic AMC moiety released were measured using a spectrofluorimeter (ex. 380 nm, em. 420–460 nm).

*Western blot analysis*. The levels of expression of various proteins were determined by Western blotting. Control and TNF-α treated cells were washed twice in PBS and lysed in RIPA lysis buffer containing protease and phosphatase inhibitors. Equal amounts of protein extracts were then electrophoresed on 12% SDS–polyacrylamide gel, transferred to nitrocellulose membrane, and non-specific binding was blocked with 5% BSA and 5% FCS in Tris-buffered saline (TBS) for 2.5 h at 37 °C. The blot was washed with 0.05% Tween 20 in TBS and then in TBS only. The blot was incubated with primary antibodies against the protein of interest for 3 h at 37 °C. The blot was then incubated with secondary antibody conjugated to alkaline phosphatase for 2 h at 37 °C, rinsed with 0.05% Tween in TBS, and then with TBS only, followed by addition of AP buffer and the bands were visualized by adding BCIP and NBT using the Promega Western blot kit. The bands were analyzed and quantitated using a 'Fotodyne' scanning densitometer. The protein expression is expressed in relative units (RU). One relative unit is the ratio obtained by taking the density between a positive control and a negative control [12].

*PCR–ELISA for telomerase*. The telomerase activity in the cells was assayed using the PCR–ELISA Kit from Roche Biochemicals, USA. Briefly, the cells were lysed using the lysis solution and telomeric repeat sequences were amplified by PCR using the biotin labeled synthetic P1–Ts and P2 primers. Five microliter of the amplified PCR product was then denatured and hybridized to digoxigenin (DIG) labeled telomeric repeat specific detection probe. The resulting product was immobilized via biotin labeled primer to a streptavidin coated microtiter plate. The immobilized PCR product was then detected using an antibody against digoxigenin that is conjugated to peroxidase. Finally, the probe was visualized by adding 5,5′,3,3′-tetramethylbenzidine, a peroxidase substrate, which is metabolized by peroxidase to a blue colored product. After 10–20 min the reaction was stopped with sulfuric acid and the absorbance of the samples was measured at 450 nm in an ELISA reader. Comparison of absorbance with the positive control (telomerase expressing human embryonic kidney cells) and the negative control containing no cell extract gave the relative telomerase activity that was expressed as relative units (RU) [13].

*Electrophoretic mobility shift assay (EMSA)*. EMSA was performed using the DIG Gel Shift Kit obtained from Roche Biochemicals, USA. Nuclear proteins were isolated. Protein–DNA binding was carried out in a mixture containing 4 μl of a 5× binding buffer (1× concentration of 5% glycerol, 1 mM $MgCl_2$, 0.5 mM EDTA, 40 mM KCl, 40 mM Tris, pH 8.0) along with dI-dC and 20 μg nuclear proteins. For specific and non-specific competitor reactions, 100-fold excess of the appropriate unlabeled double-strand DNA was added: specific, GATCGAGGGGACTTTCCCTAGC (NF-κB) and non-specific GTACGGAGTATCCAGCTCCGTAGCATGCAAATCCTGC (Oct 2A). Two microliter of DIG labeled NF-κB probe was added to each reaction and incubated for 20 min at room temperature. Five microliter of loading buffer was added to each reaction and samples were placed on ice until loading. Samples were resolved on a 6% acrylamide gel in 0.5× Tris–borate–EDTA (TBE) buffer. The DNA was then electrotransferred to a positively charged nylon membrane. Anti-DIG antibody conjugated to alkaline phosphatase (1:10,000) was then added to the membrane. CSPD was used as a chemiluminescent substrate for alkaline phosphatase. The membrane was then exposed to an X-ray film for 15–25 min at room temperature. The bands were then analyzed on a 'Fotodyne' densitometer.

## Results

### *Flow cytometric analysis of apoptosis*

Besides morphological analysis, the percentage apoptosis induced by TNF-α was measured by flow

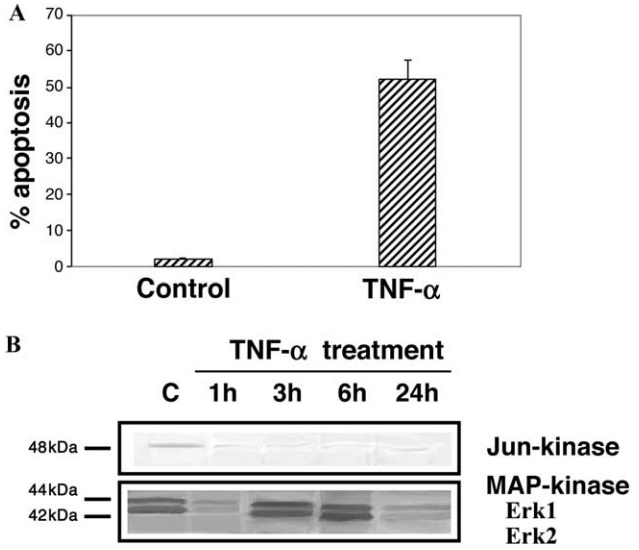


Fig. 1. (A) Bar diagram showing the percentage apoptosis in control and TNF-α treated RT-101 cells 24 h post-treatment as assessed by flow cytometry. The results are means ± SD of three separate experiments. (B) Western blot analysis of Erk1, Erk2, and Jun-kinase for control and TNF-α treated RT-101 cells at 1, 3, 6, and 24 h post-treatment. The results shown are for a representative experiment. Three separate experiments were done which showed similar findings.

cytometry for control and TNF-α treated cells. Maximum apoptosis was observed at 24 h post-TNF-α treatment. The percentage apoptosis was 60% on TNF-α treatment against 5% observed for the control cells (Fig. 1A).

*Regulation of MAPKs and JNK in apoptosis induced by TNF-α*

We addressed the question whether the protein expression of MAPK, i.e., ERK 1, 2, and JNK was differentially altered by TNF-α in TNF RT-101 cells. The basal level of JNK was high but it dropped persistently from 1 h post-TNF-α treatment and remained thus till 24 h. Whereas the basal levels of ERK 1 and 2 were high but they dropped transiently at 1 and 24 h post-TNF-α treatment (Fig. 1B). The results suggest that TNF-α might be exerting its effect by simultaneously inhibiting JNK persistently and MAPK transiently.

*MAPK regulation of AP-1*

MAPK signaling pathway influences AP-1 activity by both increasing the abundance of AP-1 components and by stimulating their activity directly. Because the onset of apoptosis generally requires the induction of a genetic program, it is thought that certain nuclear oncoproteins c-Fos, c-Jun, components of AP-1 complex with transcriptional regulatory activity would also play a role in TNF-α induced apoptosis. Thus, the role of AP-1 along with MAPK signaling cascade and apoptosis was studied by Western blotting. We investigated whether ERK dependent event is needed for AP-1 transactivation focusing essentially on expression of c-Jun/AP-1 and c-Fos components of AP-1 complex. The basal level of c-Jun/AP-1 protein was high, but it decreased by 2-fold at 1 h and the decrease was more marked at 3 h after start of TNF-α treatment. However, it increased transiently by 5-fold at 6 h and returned to basal level at 24 h. c-Fos also showed similar trend as c-Jun. The basal level of c-Fos was less than c-Jun. It dropped at 1 h post-TNF-α treatment, was lowest at 3 h and thereafter increased at 6 h but unlike c-Jun/AP-1, it remained thus till 24 h and did not return to the basal level (Fig. 2A).

*MAPK regulation of NF-κB*

NF-κB is a redox sensitive/stress responsive transcription factor that is activated by oxidative insult and generally its activation can protect cells from

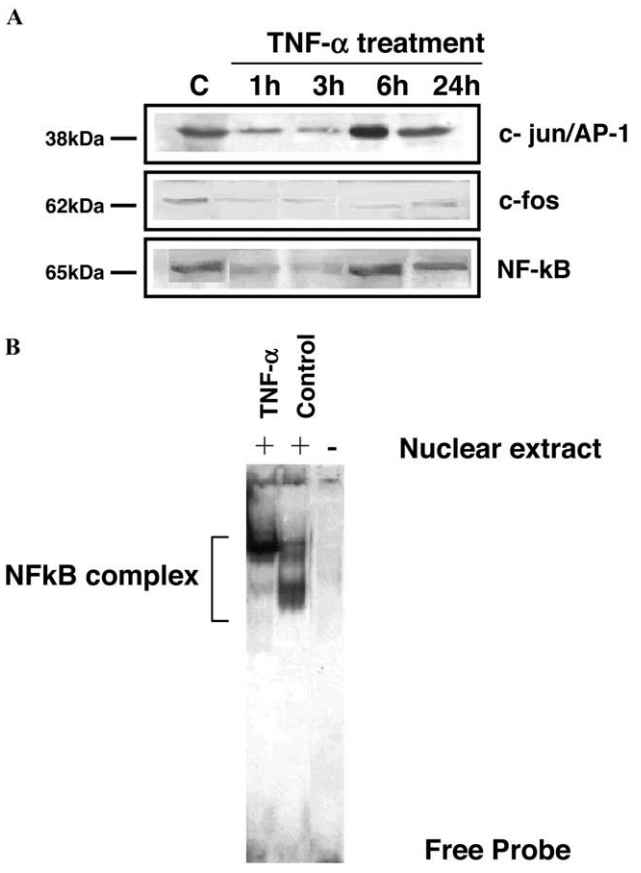


Fig. 2. (A) Western blot analysis for c-jun, c-fos, and NF-κB in control RT-101 cells and TNF-α treated cells for 1, 3, 6, and 24 h. The experiment was repeated three times with similar findings. (B) Electrophoretic mobility shift assay (EMSA) of the transcription factor NF-κB in control RT-101 cells and TNF-α treated cells for 24 h. Shown are the results of a representative experiment which was repeated twice.

apoptosis. It functions to intensify the transcription of various genes and its activation is linked to MAPK signaling pathway. It has been shown to play an important role in such diverse processes such as proliferation, differentiation, and inhibition of apoptosis. To test if NF-κB can protect from apoptotic cell death caused by TNF-α, we exposed RT-101 cells to TNF-α. Even though these cells are sensitive to TNF-α induced apoptosis, the basal protein level of NF-κB was high in these cells, but on TNF-α treatment it decreased at 1 h, with the maximum decrease occurring at 3 h, but thereafter returned to basal levels, suggesting that apoptosis perhaps commences by inhibiting NF-κB in TNF RT-101 cells (Fig. 2A). NF-κB DNA binding was measured by electrophoretic mobility shift assay (EMSA). The NF-κB DNA binding complex was composed of p50 and p65 subunits. A 50% decrease in NF-κB DNA binding was seen on TNF-α treatment for 24 h (Fig. 2B).

*Signal transduction pathway involving genes differentially expressed in apoptosis*

To seek candidate mediator and suppressor/protective genes which might be differentially induced by TNF-α in these cells, we explored the role of Bcl-2 family. Interestingly, TNF-α treatment increased the BCl-2 level by 1.5-fold at 1 h post-TNF-α treatment, which remained thus till 6 h, thereafter it decreased to less than basal levels at 24 h, when maximum apoptosis was seen. Similarly, the protein expression of anti-apoptotic Bcl-XL and survivin also showed maximum decrease at 24 h. However, the pro-apoptotic Bax showed lower basal levels as compared to Bcl-2, and other anti-apoptotic members, but its levels increased 3-fold at 1 h onwards and peaked at 6 h, and though it dropped at 24 h, it was still higher than the basal level (Fig. 3A). A transient decrease at 1 and 24 h post-treatment in the protein expression of apoptosis inducing factor (AIF) was seen. The results show that survival factors/inhibitor of apoptosis are decreased when apoptosis takes place whereas pro-apoptotic Bax increases. Basal levels of pro-apoptotic hyaluronic acid binding protein 1 (HABP1) were maintained till 3 h after start of TNF-α treatment but were markedly elevated 2-fold at 6 h when sufficient number of cells start to undergo apoptosis and remained thus till 24 h, suggesting its involvement in the morphological changes accompanying apoptosis (Fig. 3A).

*TNF-α increases superoxide anions in mitochondria as well as cytochrome c release*

We also investigated the protein expression of cytochrome *c* in mitochondria and cytosol by Western blotting. Though the mitochondria showed higher basal level of cytochrome *c*, its expression was increased in cytosol on TNF-α treatment for 24 h, thereby suggesting its release from the mitochondria by TNF-α (Fig. 3B).

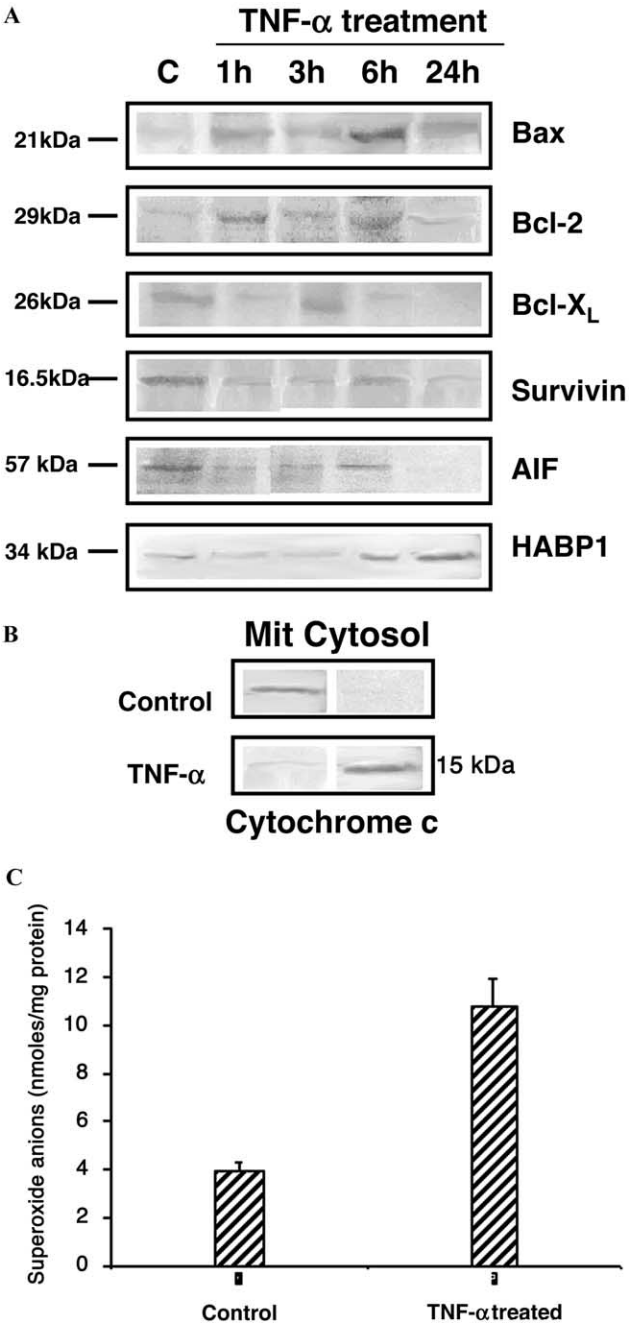


Fig. 3. (A) Western blot analysis for Bax, Bcl-2, Bcl-$X_L$, survivin, apoptosis inducing factor (AIF), and hyaluronic acid binding protein 1 (HABP1) in control and TNF-α treated RT-101 cells for 1, 3, 6, and 24 h. The experiment was repeated thrice and similar findings were obtained. (B) Western blot analysis of cytochrome *c* in the mitochondrial and cytosolic fractions of control and RT-101 cells treated for 24 h with TNF-α. The experiment was repeated thrice and similar results were obtained. (C) Bar diagram showing the levels of superoxide anions (nmoles/mg protein ± SD) in control and RT-101 cells treated for 24 h with TNF-α. The experiment was repeated thrice and similar findings were obtained.

TNF-α also led to an increase in superoxide anions in the mitochondria (Fig. 3C).

*Caspase-3 plays a role in TNF-α induced apoptosis in RT-101 cells*

Caspases are cysteine proteases that exist in cells as inactive proenzymes and are activated by apoptotic signals. Caspase-3 is an executioner caspase and is responsible for cell disassembly occurring as the end result of apoptosis. To seek evidence whether caspase-3 is required for TNF-α induced apoptosis as part of a common downstream apoptotic death pathway, we studied the activity of caspase-3. A maximum increase of 25-fold in caspase-3 activity was seen at 24 h when maximum apoptosis was also observed, suggesting its involvement in TNF-α induced apoptosis in TNF RT-101 cells (Fig. 4A).

*TNF-α decreases telomerase activity*

We also studied the effect of TNF-α on telomerase activity in TNF RT-101 cells and found that it was decreased by 10-fold post-TNF-α treatment for 24 h, thereby allowing apoptosis to occur (Fig. 4B).

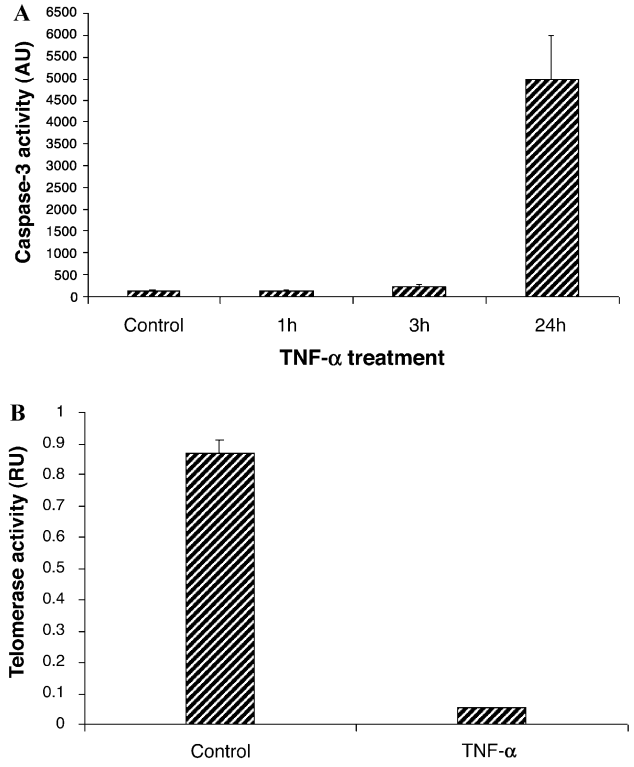


Fig. 4. (A) Caspase-3 activity in control and TNF-α treated RT-101 cells at 1, 3, and 24 h post-treatment. The results are expressed in terms of fluorescence arbitrary units (AU) and are means ± SD of three separate experiments. (B) Telomerase activity in control and TNF-α treated RT-101 (24 h post-TNF-α treatment) as estimated by PCR–ELISA ± SD. The results are expressed in terms of relative units (RU). One relative unit is defined as the ratio of the optical density obtained for the positive and the negative controls.

## Discussion

To characterize the molecular mechanism(s) that regulate TNF-α induced apoptosis in mouse epidermal JB6 derived transformed RT-101 cells, the contribution to cell death of MAPK family members, transcription factors as well as pro- and anti-apoptotic genes was investigated. Mitogen-activated protein kinase cascade plays an important role in transducing environmental/extracellular stimuli signals through both protein kinases and protein phosphatases to the transcriptional machinery in the nucleus by virtue of their ability to phosphorylate and regulate the activity of various transcription factors. ERKs are associated with proliferation and differentiation whereas JNK is implicated in cell cycle arrest and cell death [14,15]. Sensitivity to cell killing by TNF-α was seen in TNF RT-101 cell variant. Morphological and biochemical changes characteristic of apoptosis were found to precede TNF-α induced cell death in these cells [9]. TNF-α has been shown to induce apoptotic cell death and DNA fragmentation in several mammalian cell lines. In murine fibrosarcoma cell line L929, it has been shown to activate ERK-1 and ERK-2 MAPKs [14]. JNK has been shown to be activated by TNF-α and its activation is correlated with enhanced apoptosis in rat pheochromocytoma PC 12 in response to trophic hormone deprivation [15], and JNK but not ERK-2 was found to be persistently activated in apoptosis induced by radiation and UV-C [16]. However, we observed a decrease in JNK in this study. It is thought that MAPK is more involved in mitogenic responses in some cell types with a possible role in cell death in other cells [17]. Thus, one of the mechanisms by which TNF-α induces apoptosis could at least in part involve the reduction of the ERK and JNK activity.

Because the onset of apoptosis generally requires the induction of a genetic program, it is thought that certain nuclear oncoproteins c-Fos, c-Jun, components of AP-1, as well as NF-κB with transcriptional regulatory activity would also play a role in apoptosis. Studies have shown that expression of transcription factors such as AP-1 and NF-κB may either be associated or necessary for induction of apoptosis and that they are known to control the expression of genes associated with apoptosis [18]. The role of these transcription factors along with the MAPK signaling cascade and apoptosis was thus investigated. AP-1 is composed of members of the Jun and Fos family which interact with each other through the leucine zipper motif. They exist as either homo- or heterodimers. AP-1/c-Jun may act as common agents for responses, leading to cell death, neoplastic transformation, mitogenic responses, or differentiation

depending on cell type and stimuli. Inducible phosphorylation and dephosphorylation of c-Jun appear to modulate its transcriptional activation potential [19]. MAPK signaling influences AP-1 activation by both, increasing the abundance of AP-1 components and by stimulating their activity directly. Substrate for MAPKs includes c-Myc, c-Jun, c-Fos, as well as certain extracellular matrix associated proteins such as HABP1. In the TNF RT-101 cells, there is no lack of MAPK, JNK, NF-κB, or AP-1 and its components. The observed findings of transient increase in c-Jun/AP-1 by TNF-α may be suggesting that MAPK transduces its signals not through protein kinases but through protein phosphatase as we had earlier seen in the transient appearance of a dephosphorylated species of c-Jun at 3 h [9]. Use of inhibitors of phosphatase such as okadaic acid/calyculin abolished the apoptosis, thereby further supporting the involvement of a protein phosphatase in TNF-α induced apoptosis in TNF RT-101 cells (data not shown).

The coordinate activation of multiple MAPK pathways may, at the same time, transduce both positive and negative signals towards NF-κB activation and the extent and duration of activation of these pathways likely dictate whether or not NF-κB activation ensues [20]. Whether NF-κB promotes or inhibits apoptosis also appears to depend on the specific cell type and the type of inducer. Stimulation of NF-κB in response to various stimuli including viral infection, UV, cytokine TNF-α, and IL-1 serves to block the process of apoptosis. However, we found a decrease in NF-κB at 1 and 3 h post-TNF-α treatment which then returned to basal levels at 24 h. Cells deficient in NF-κB function display increased sensitivity to the cytocidal effect of TNF, suggesting that some protein regulated by NF-κB serves to protect cells against undue killing by TNF [1]. In cells where death is induced, NF-κB action is prevented by inhibition of TRADD recruitment and activation of the ERKs, although not of JNK. The latter is prevented through action of some caspases [21]. Once NF-κB is activated it elicits the transcription of proteins with anti-apoptotic function. TNF-α is an interesting example of the action of a single cytokine of two different pathways with opposite effects [22]. It triggers the caspase-8 route to apoptosis if NF-κB pathway is inactivated [23]. Other anti-apoptotic genes have been shown to be activated by NF-κB such as Bcl-2 [24]. But there is also evidence to suggest that NF-κB prevents oncogenesis and promotes apoptosis [25]. These observations suggest that secondary events may occur to upregulate NF-κB independent survival pathways. Another study [26] has shown that in hypoxia, NF-κB regulation is independent of MAPK whereas after hydrogen peroxide exposure at a concentration where it induces both MAPK and widespread apoptosis there was no increase in expression of NF-κB or its activation. The most common form of NF-κB is a heterodimer consisting of p50 and p65 protein subunits [20]. To determine whether TNF-α caused increased NF-κB binding to DNA we performed EMSA. Treatment of JB6 RT-101 cells with TNF-α for 24 h caused decreased binding. Abrogation of NF-κB transcription activity is sufficient to abrogate survival and this may explain why TNF-α is sufficient by itself to induce apoptosis in these cells. Similar to our findings, another study has shown that TGF-β induced matrix proteins inhibits MAPK and JNK activation and suppresses TNF-mediated Iκβ α degradation and NF-κB nuclear translocation [14], or perhaps TNF induced apoptosis is not linked to JNK and NF-κB [18].

The function of Bcl-2 is to increase cell survival and it contributes to tumorigenesis by extending cell survival. Bcl-2 might be interfering with the signal transduction or metabolic requirement for death, e.g., oxidative phosphorylation/electron transport chain or import of proteins and metabolites. Since in gene response, multiple genes are involved, looking at the expression of one gene is not enough. Hence, we have looked at gene cooperation during TNF induced apoptosis by investigating the protein expression of Bcl-2, Bcl-XL, Bax, survivin, AIF, and HABP1. The observed increase of Bax and decrease of Bcl-2, Bcl-XL, and survivin perhaps confer sensitivity to apoptosis/favor the onset of apoptosis induced by TNF-α. Perhaps, there is no involvement of AIF in TNF-α induced apoptosis. TNF-α also led to increase in generation of RoS in the mitochondria as well as increased the release of cytochrome *c* from the mitochondria. It appears that TNF-α binds to its receptors, perhaps uncouples electron transport chain, leading to decreased mitochondrial membrane potential and production of reactive oxygen species [9]. Mitochondrial membrane potential collapse in conjunction with oxidative stress appears to release cytochrome *c* and trigger proteases and endonuclease activation and cell death. Hyaluronan and its binding proteins serve important structural and apoptotic regulatory functions. Perhaps this explains the increase observed in HABP1 at 24 h when maximum apoptosis was seen in TNF-α treated RT-101 cells. The observed increase in caspase-3 activity was commensurate with increased apoptosis at 24 h, suggesting its involvement in TNF-α induced apoptosis. Interestingly, the telomerase activity decreased when there was increased apoptosis.

In conclusion, it appears that TNF-α affects the mitochondria and increases free radical production and cytochrome *c* release. Following this oxidative stress, it persistently downregulates JNKs (but increases a phosphatase), but only transiently affects ERKs. The progressed transformed RT-101 cells exhibit higher basal levels of AP-1 and NF-κB, MAPKs and JNK, but the induced levels of AP-1 and NF-κB perhaps are required only in the promotion/progression stages of carcinogenesis but not in apoptosis. The final step of apoptosis is proved to be due to a decrease of anti-apoptotic Bcl-2,

Bcl-XL, and survivin whereas pro-apoptotic Bax, HABP1 are increased followed by caspase-3 activation.

## Acknowledgments

This work was supported by a research grant from Department of Science and Technology, India. Ms. Himani Sharma acknowledges the Junior Research Fellowship awarded to her by Council of Scientific and Industrial Research, India.

## References

[1] D. Wallach, Cell death induction by TNF: a matter of self control, Trends Biochem. Sci. 22 (1997) 107–146.
[2] S.C. Wright, P. Kumar, A.W. Tan, N. Shen, M. Verma, J.W. Larrick, Apoptosis and DNA fragmentation precede TNF-α induced cytolysis in U937 cells, J. Cell Biochem. 48 (1992) 344–355.
[3] M. Tsujimoto, Y.K. Yip, J. Vilcek, Tumor necrosis factor exerts endocrine, paracrine, and autocrine control of inflammatory responses, J. Cell Biol. 107 (1988) 1269–1275.
[4] M. Hoffman, J.B. Weinberg, Tumor necrosis factor-α induces increased hydrogen peroxide production and Fc receptor expression but not increased Ia antigen expression by peritoneal macrophages, J. Leukocyte Biol. 42 (1987) 704–707.
[5] K.G. Leong, A. Karsan, Signaling pathways mediated by tumor necrosis factor alpha, Histol. Histopathol. 15 (2000) 1303–1325.
[6] Z.G. Liu, H. Hsu, D.V. Goeddel, M. Karin, Dissection of TNF receptor I effector functions: JNK activation is not linked to apoptosis while NF-κB activation prevents cell death, Cell 87 (1996) 565–576.
[7] H. Hsu, H.B. Shu, M.G. Pan, D.V. Goeddel, TRADD TRAF2 and TRADD–FADD interactions define two distinct TNF receptor 1 signal transduction pathways, Cell 84 (1996) 299–308.
[8] A. Ashkenazi, V.M. Dixit, Death receptors: signaling and modulation, Science 281 (1998) 1305–1308.
[9] N. Singh, Y. Sun, K. Nakamura, M.R. Smith, N.H. Colburn, c-Jun/AP-1 as possible mediators of tumor necrosis factor-alpha induced apoptotic response in mouse JB6 tumor cells, Oncol. Res. 7 (1995) 353–362.
[10] V.G. Reddy, N. Khanna, N. Singh, Vitamin C augments chemotherapeutic response of cervical carcinoma HeLa cells by stabilizing P53, Biochem. Biophys. Res. Commun. 282 (2001) 409–415.
[11] E. Pick, D. Mizel, Rapid microassays for the measurement of superoxide and hydrogen peroxide production by macrophages in culture using an automatic enzyme immunoassay reader, J. Immunol. Methods 46 (1981) 211–216.
[12] N. Khanna, V.G Reddy, N. Tuteja, N. Singh, Differential gene expression in apoptosis: identification of ribosomal protein S29 as an apoptotic inducer, Biochem. Biophys. Res. Commun. 277 (2000) 476–486.
[13] S. Sen, V.G. Reddy, N. Khanna, R. Guleria, K. Kapila, N. Singh, A comparative study of telomerase activity in sputum, bronchial washing and biopsy specimens of lung cancer, Lung Cancer 1 (2001) 41–49.
[14] N.S. Chang, TGF-beta-induced matrix proteins inhibit p42/44 MAPK and JNK activation and suppress TNF-mediated IkappaB alpha degradation and NF-kappaB nuclear translocation in L929 fibroblasts, Biochem. Biophys. Res. Commun. 267 (2000) 194–200.
[15] K. Umezawa, Induction of cellular differentiation and apoptosis by signal transduction inhibitors, Adv. Enzyme Regul. 37 (1997) 393–401.
[16] Y.R. Chen, X. Wang, D. Templeton, R.J. Davis, T.H. Tan, The role of c-jun N-terminal kinase (JNK) in apoptosis induced by ultraviolet C and gamma radiation. Duration of JNK activation may determine cell death and proliferation, J. Biol. Chem. 271 (1996) 31929–31936.
[17] M.J. Helms, A.A. Mohamed, D.J. MacEwan, Modulated kinase activities in cells undergoing tumour necrosis factor-induced apoptotic cell death, FEBS Lett. 505 (2001) 68–74.
[18] A. Shrivastava, B.B. Aggarwal, Antioxidants differentially regulate activation of nuclear factor-kappaB activator protein-1, c-jun amino-terminal kinases, and apoptosis induced by tumor necrosis factor: evidence that JNK and NF-kappaB activation are not linked to apoptosis, Antioxid. Redox. Signal. 1 (1999) 181–191.
[19] A. Berry, M. Goodwin, C.L. Moran, T.C. Chambers, AP-1 activation and altered AP-1 composition in association with increased phosphorylation and expression of specific Jun and Fos family proteins induced by vinblastine in KB-3 cells, Biochem. Pharmacol. 62 (2001) 581–591.
[20] Y.M. Janssen-Heininger, M.E. Poynter, P.A. Baeuerle, Recent advances towards understanding redox mechanisms in the activation of nuclear factor kappaB, Free Radic. Biol. Med. 28 (2000) 1317–1327.
[21] C. Widman, S. Gibson, G.L. Johnson, Caspase dependent cleavage of signaling protein during apoptosis, a turn of mechanism for antiapoptotic signals, J. Biol. Chem. 273 (1998) 7141–7147.
[22] A.S. Baldwin, The NF-kappaB and I kappaB proteins: new discoveries and insights, Ann. Rev. Immunol. 14 (1996) 649–683.
[23] C.Y. Wang, M.W. Mayo, A.S Baldwin Jr., TNF- and cancer therapy-induced apoptosis: potentiation by inhibition of NF-kappaB, Science 274 (1996) 784–787.
[24] J.L. Herrmann, A.W. Beham, M. Sarkiss, P.J. Chiao, M.T. Rands, E.M. Bruckheimer, S. Brisbay, T.J. Mc Honnell, Bcl-2 suppresses apoptosis resulting from disruption of the NF-κB survival pathway, Exp. Cell Res. 237 (1997) 101–109.
[25] M. Van Hogerlinden, B.L. Rozell, L. Ahrlund-Richter, R. Toftgard, Squamous cell carcinomas and increased apoptosis in skin with inhibited Rel/nuclear factor-kappaB signaling, Cancer Res. 59 (1999) 3299–3303.
[26] Y. Li, W. Zhand, L.L. Mantell, J.A. Kazzaz, A.M. Fein, S. Horowitz, Nuclear factor-kappaB is activated by hyperoxia but does not protect from cell death, J. Biol. Chem. 272 (1997) 20646–20649.