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Activation of two distinct anti-proliferative pathways, apoptosis and p38 MAP kinase-dependent cell cycle arrest, by tumor necrosis factor in human melanoma cell line A375

Abstract

The proliferation of human melanoma cell line A375-6 cells is inhibited by several cytokines, including interleukin-1 (IL-1). A375-R8 cells, a subclone of A375-6, are resistant to IL-1-induced growth inhibition. The proliferation of both cell lines is inhibitable by tumor necrosis factor (TNF). In this study, we characterized the

mechanisms of TNF-induced growth inhibition. TNF-induced growth inhibition in both cell lines was partially suppressed by a selective p38 mitogen-activated protein kinase (MAPK) inhibitor (SB203580), whereas a combination of SB203580 and Z-VAD-fmk, an inhibitor for a wide range of caspases, completely blocked TNF-induced growth inhibition, indicating that TNF-induced growth inhibition is mediated by both p38 MAPK and caspases. However, Z-VAD-fmk alone suppressed TNF-induced growth inhibition in A375-R8, but not A375-6, cells, suggesting that there may exist a TNF-induced anti-apoptotic mechanism in A375-6 cells which is lost or mutated in A375-R8 cells. Evidence in support of this notion includes (1) TNF-induced apoptosis only in A375-R8, but not A375-6 cells; (2) cycloheximide enabled TNF to induce apoptosis even in A375-6 cells; and (3) somatic hybrid cells between A375-6 and A375-R8 cells are resistant to TNF-induced apoptosis. Since TNF-induced NF-kB activation, cell cycle arrest, RB dephosphorylation, and E2F downregulation are indistinguishable in both cell lines, none of these factors is likely to be involved in the TNF-induced anti-apoptotic mechanism in A375-6 cells. Our results indicate that TNF activates two distinct anti-proliferative pathways including p38 MAPK-dependent cell cycle arrest and caspase-mediated apoptosis, as well as an anti-apoptotic mechanism in melanoma cells.

Full text

INTRODUCTION

TNF is a pleiotropic cytokine that is mainly produced by macrophages and monocytes, but also by many other cell types including lymphoid cells and fibroblasts, in response to inflammation, infection, and other environmental attacks [1]. TNF has a large number of effects ranging from inducing other cytokines and immunoregulatory molecules, regulating cell proliferation and differentiation, and causing apoptosis [1, 2]. The multifunctionality of TNF in immune and inflammatory processes implicates it as a critical mediator in many diseases. TNF mediates its diverse activities by triggering trimerization of two distinct cell surface receptors, TNF receptor-1 (TNFR1/p55) and TNFR2 (p75), at least one of which is present in almost every cell type [2-4]. The structural similarity between the two TNF receptors is limited to their extracellular domains [3]. Although most of the biological activities of TNF appear to be transduced by TNFR1, many can also be mediated by TNFR2 [2-4]. TNFR2, however, is a poor inducer of apoptosis [3].

A consensus signaling pathway for TNFR1-mediated apoptosis has been proposed and recently reviewed [5]. Upon ligand binding, the intracellular portion of the receptor containing the death domain (DD) [6], recruits other DD-containing proteins. DD-signaling effectors, including primarily, TNFR-associated death domain protein (TRADD) and receptor-interacting protein (RIP), form a multiprotein complex around the intracellular part of the receptor [7-10]. DD proteins, such as Fas-associated death domain (FADD) [11, 12] and RIP-associated Ich/CED-3 homologous protein with a death domain (RAIDD) [13], are also recruited to the signaling complex. FADD and RAIDD in turn interact with caspases to activate their proteolytic activity and induce apoptosis [14].

The human melanoma cell line A375-6 was initially subcloned from A375 melanoma cells as sensitive to IL-1-induced growth inhibition [15]. We have previously reported that A375-6 cells became resistant to the anti-proliferative effect of IL-1 after a long period of *in vitro* passage [16]. Two IL-1-resistant subclones, A375-R8 and -R19 were obtained, and their characteristics were studied [16]. These two subclones express functional IL-1 receptor regardless of their resistance to IL-1-induced growth inhibition and produce IL-1alpha and IL-6 constitutively. These cells are also resistant to the anti-proliferative effect of IL-6. Although these cells were resistant to IL-1 and IL-6, their proliferation is inhibited by TNF at a comparable level as IL-1-sensitive A375-6 cells.

In this study, we demonstrated that TNF inhibited A375 cell proliferation through two pathways, includ-ing apoptosis induction and p38 MAPK-dependent cell cycle arrest. We also observed that TNF also induced in the IL-1-sensitive A375-6 cell line an anti-apoptotic mechanism that is lacking in the IL-1-resistant A375-R8 cells.

MATERIALS AND METHODS

Reagents

RPMI 1640 was purchased from Sigma Chemical Co. (St. Louis, MO, USA); fetal bovine serum (FBS) was from JRH Bioscience (Lenexa, KS, USA); human recombinant TNF-alpha (1.9×10^6 U/mg) and IL-1alpha (2×10^7 U/mg) were provided by Dr. M. Yamada (Dainippon Pharmaceutical Co. Ltd., Osaka, Japan). Z-VAD-fmk and Z-EK(bio)D-CH2-DMB were purchased from Peptide Institute

(Osaka, Japan). SB203580 was synthesized according to the method of Gallagher *et al.* [17].

Cell culture

A375 is a human melanoma cell line which was originally provided by Dr. R. Ruddon (NCI, Bethesda, MD, USA). By limiting dilution, the IL-1-sensitive cell line A375-6 was subcloned from A375. The IL-1-resistant cell line A375-R8 was established by limiting dilution of A375-6 cells after routine passage for 3 months [16]. These cells were cultured in RPMI 1640, 100 U/ml of penicillin G, 100 mug/ml of streptomycin, 15 mM HEPES and 5% heat-inactivated FBS at 37° C in air containing 5% CO_2.

Assay for proliferation

Hundred mul of cell suspension (2×10^4 cells/ml) were added to each flat-bottomed well of a 96-well micro-titer plate (Falcon, Lincoln, NJ, USA). After 24 hours cultivation, 100 mul of medium containing TNF-alpha were added, and then the cells were cultured for another 72 hours. Proliferation of the cells was determined by staining with crystal violet [18]. After solubilization of the dye-stained cells with 0.1% SDS, the dye-uptake was calculated by measuring the absorbance at 595 nm by an ELISA autoreader (BIO-RAD, Richmond, CA, USA).

Western blot analysis

For p38 MAPK, A375-R8 cells were lysed with SDS sample buffer containing 62.5 mM Tris-HCl (pH 6.8), 2% SDS, 10% glycerol, 50 mM dithiothreitol, and 0.1% bromphenol blue. The lysates were fractionated on SDS- 12.5% polyacrylamide gel, transferred to a PVDF membrane, and then blotted with anti-p38 MAPK polyclonal antibody (Santa Cruz, Delaware Ave, CA, USA) or anti-phospho-p38 MAPK polyclonal antibody (New England Biolabs, Beverly, MA, USA). For caspase-3, -8 and DFF45, A375 cells were lysed with lysis buffer (1% NP-40, 0.5 mM phenylmethylsulfonylfluoride (PMSF), 10 mug/ml pepstatin A, 2 mug/ml leupeptin, 20 mug/ml benzamidine and 10 mug/ml aprotinin in PBS). An equal amount of protein was applied to 12.5% SDS-PAGE, transferred to PVDF membrane, and blotted with anti-caspase-3 monoclonal antibody (Transduction Laboratories, Lexington, KY, USA), anti-caspase-8 monoclonal antibody (Medical & Biological Laboratories, Nagoya, Japan) or anti-DFF45 rabbit antiserum (from

Dr. H. Keino, Aichi Human Service Center Institute For Development Research, Japan). For RB, A375 cells were lysed with lysis buffer containing 50 mM HEPES (pH 7.0), 250 mM NaCl, 0.1% NP-40, 10 mM sodium pyrophosphate, 10 mM sodium fluoride, 2 mM sodium vanadate, 0.5 mM PMSF, 10 mug/ml pepstatin A, 2 mug/ml leupeptin, 20 mug/ml benzamidine and 10 mug/ml aprotinin. An equal amount of protein was applied to 8% SDS-PAGE and blotted with anti-RB polyclonal antibody (C-15; Santa Cruz) or anti-beta-actin (Sigma) as a loading control. The immunoreactive proteins were visualized by ECL Western blotting detection reagents (Amersham, Buckinghamshire, UK), and the light emission was quantified by a lumino-image analyzer LAS-1000 (Fuji, Japan).

Detection of apoptotic cells

A375 cells were fixed with 1% glutaraldehyde in PBS. After washing with PBS, the cells were stained with 0.2 mug/ml 4', 6-diamidino-2-phenylindole (DAPI). The cells with fluorescence were counted under a fluorescence microscope.

Labeling of caspases and affinity blotting

Activated caspases in A375 cells were labeled with biotinylated substrate according to the method of Faleiro *et al.* [19]. The cells (3×10^6) were resuspended in 1 ml of KPM buffer containing 50 mM KCl, 50 mM PIPES, 10 mM EGTA, 1.92 mM $MgCl_2$, pH 7.0, 1 mM dithiothreitol, 0.1 mM PMSF 10 mug/ml cytochalasin B, 10 mug/ml pepstatin A, 2 mug/ml leupeptin, 20 mug/ml benzamidine, 10 mug/ml aprotinin, pelleted and frozen in liquid nitrogen. An equal volume of MDB (50 mM NaCl, 2 mM $MgCl_2$, 5 mM EGTA, 10 mM HEPES, 1 mM dithiothreitol, pH 7.0) was added to the cell pellets in the presence or absence of 20 muM Z-EK(bio)D-CH_2-DMB and cells were lysed by 3 cycles of freezing and thawing. The lysates were incubated at 37° C for 15 min and mixed with an identical volume of 2 x SDS sample buffer, fractionated on SDS - 12.5% polyacrylamide gel, and transferred to a PVDF membrane. The PVDF membrane was incubated with HRP-conjugated avidin (Medical & Biological Laboratories, Nagoya, Japan), and the biotinylated proteins were detected using an ECL kit as Western blot.

*Measurement of NF-*kappa*B and E2F activation*

NF-kappaB and E2F activation was determined using a reporter plasmid pGL3-kappaB wt (from Dr. T. Okamoto, Nagoya City University Medical School, Japan) and pE2F-Luc (CLONETECH, Palo Alto, CA, USA), respectively. A375 cells were transiently transfected with reporter plasmid and pCMV-beta-gal by lipofection in a 12-well plate. After 24 hours, the supernatant was replaced with fresh medium. After another 24 hours, cells were treated as specified, washed with PBS, and then lysed. Luciferase activity was measured with a luciferase reporter gene assay kit (Boehringer, Mannheim, Germany) according to the manufacturer's instructions, and normalized with beta-galactosidase activity.

RESULTS

TNF inhibits A375 cell proliferation through two distinct pathways

As shown in Figure 1A, the proliferation of both IL-1 sensitive A375-6 and resistant A375-R8 cells were inhibited by TNF in a dose-dependent manner and the inhibition was partially attenuated by simultaneous treatment with SB203580, a selective inhibitor of p38 MAPK. In contrast, Z-VAD-fmk, a broad spectrum inhibitor of caspases, blocked TNF-induced growth inhibition only in A375-R8 cells (Figure 1A). Since TNF-induced activation of p38 MAPK was observed, even in Z-VAD-fmk pretreated cells, Z-VAD-fmk did not appear to affect the p38 MAPK cascade (Figure 1B). Z-VAD-fmk did not affect IL-1- or sorbitol-induced p38 MAPK activation either. Combination of SB203580 and Z-VAD-fmk completely blocked TNF-induced growth inhibition in A375-R8 cells. Surprisingly, in A375-6 cells, although treatment with Z-VAD-fmk alone did not influence TNF-induced growth inhibition, TNF-induced growth inhibition was completely suppressed by treatment with SB203580 in the presence of Z-VAD-fmk. Consequently, TNF inhibits A375 cell proliferation through at least two distinct pathways, one is p38 MAPK-dependent and the other is caspase-dependent.

TNF induces caspases-dependent apoptosis in IL-1-resistant A375-R8 cells but not in IL-1-sensitive A375-6 cells

We next investigated whether TNF-induced growth inhibition in A375 cells was mediated by apoptosis. TNF caused apoptosis characterized by nuclei change in A375-R8 cells (Figure 4B) but not in A375-6 cells (data not shown). TNF-induced apoptosis was completely inhibited by the caspases inhibitor, Z-VAD-fmk. In

contrast, p38 MAPK inhibitor SB203580 did not inhibit TNF-induced apoptosis (Figure 2A).

Compared to A375-R8 cells, A375-6 cells were also less sensitive to apoptosis induced by stimulation of Fas (Figure 2B), another member of the TNF receptor superfamily, that induces apoptosis in several cell lines [20]. The Fas-mediated apoptosis was completely inhibited by Z-VAD-fmk, while SB203580 exhibited no inhibitory effect (Figure 2B). Z-VAD-fmk completely blocked the growth inhibition caused by the Fas antibody in A375-R8 cells, but incompletely in A375-6 cells (Figure 2C). Conversely, SB203580 partially reversed the growth inhibition only in A375-6 cells (Figure 2C). Thus, activation of TNF receptor and Fas may result in the induction of not only apoptosis but also of an anti-apoptotic mechanism in A375-6 cells, whereas in A375-R8 cells, the anti-apoptotic mechanism is not simultaneously induced.

TNF induces apoptosis through the activation of caspases in A375 cells

As TNF-induced apoptosis was completely suppressed by treatment with a caspases inhibitor, Z-VAD-fmk, we next examined whether TNF activates caspases in A375 cells. The lysates from A375 cells treated with or without TNF were incubated with biotin-labeled Z-EKD-CH_2-DMB, another broad spectrum inhibitor of caspases capable of suppressing TNF-induced growth inhibition in A375-R8 cells (data not shown). As shown in Figure 3A, three activated caspase-like proteins that could bind irreversibly to Z-EK(bio)D-CH_2-DMB were detected in the lysate from staurosporine-treated A375 cells. As expected, TNF induced the activation of caspase-like activities in A375-R8 cells (lane 5 of Figure 3A), but not in A375-6 cells (lane 2 of Figure 3A). In addition, we investigated which caspases were activated in TNF-treated A375 cells. First, activation of caspase-3 which plays an important role in the execution of apoptosis [14] was studied. TNF-dependent degradation of pro-caspase-3 was observed only in A375-R8 cells (Figure 3B). Next, we examined caspase-8 which is activated close to the death receptor [5, 14]. TNF induced cleavage of pro-caspase-8 resulting in an accumulation of activated subunits, only in A375-R8 cells (Figure 3C). Similar results were observed using fluorescent substrates MOCAc-DEVDPAK(Dnp)-NH_2 and Ac-IETD-MCA for caspase-3 and -8, respectively (data not shown). Furthermore, similarly to caspase activity, TNF-dependent degradation of DNA fragmentation factor 45 (DFF45/ICAD), which is an inhibitor of caspase-activated DNase (CAD) and which is cleaved to activate CAD by caspase-3 [21,

22], was observed in only A375-R8 cells (Figure 3D). In addition to the nuclei change, these observations indicate that TNF causes apoptosis by activating caspases in A375-R8 cells.

De novo *synthesized protein(s), but not NF*-kappa*B activation protects A375-6 cells from TNF-induced apoptosis*

In many tumor cells, induction of apoptosis by TNF requires inhibition of on-going protein synthesis [23]. It was reported that TNF activates cell survival signals as well as cell death pathway through NF-kappaB activation [24-27]. Generally, apoptosis signals are transduced without any *de novo* protein synthesis. To eliminate *de novo* protein synthesis that contributes to preventing apoptosis, we used cycloheximide simultaneously with TNF treatment. No apoptotic cells were observed with cycloheximide treatment alone, however, cycloheximide enhanced TNF-induced apoptosis in A375-R8 cells. Interestingly, even in A375-6 cells, TNF induced apoptosis in the presence of cycloheximide (Figure 4A). This is compatible with the fact that TNF treatment in the presence of cycloheximide resulted in the degradation of pro-caspase-3 (lane 4 of Figure 3B), pro-caspase-8 (lane 4 of Figure 3C), and DFF45 (lane 4 of Figure 3D) in A375-6 cells.

Furthermore, A375-6 x R8 cells, somatic hybrid cells between A375-6 and A375-R8 cells [28], appeared to be completely resistant to TNF-induced apoptosis (Figure 4B). These observations suggest that newly synthesized anti-apoptotic factor(s) is induced by TNF in A375-6 cells, but is not induced or is deficient in A375-R8 cells. Our previous study demonstrated that A375-R8 cells constitutively produced IL-1alpha [16], and IL-1alpha stimulated its own gene expression in an autocrine manner through activation of NF-alphaB [29]. However, constitutive IL-1alpha production did not contribute to TNF-induced apoptosis because TNF treatment in the presence of IL-1alpha failed to induce apoptosis in A375-6 cells (data not shown). NF-kappaB is known to contribute to cell survival against TNF-induced apoptosis [24-27]. NF-kappaB was constitutively activated by constitutive IL-1alpha production in A375-R8 cells [29] and was further augmented by TNF treatment. However, TNF-stimulated NF-kappaB activation in A375-R8 cells was comparable to that in A375-6 cells (Figure 4C). Thus, NF-kappaB-mediated signaling can be transduced in both cell lines.

TNF induces cell cycle arrest in A375 cells

TNF-treated A375-6 cells looked normal under a microscope. Muthukkumar *et al.* have demonstrated that TNF induces cell cycle arrest in the G0/G1 phase in A375-C6 cells [30]. Indeed, a decrease of the S phase population and an increase of the G0/G1 phase population were observed in TNF-treated A375-6 and A375-R8 cells (data not shown). Therefore, we examined whether TNF affects cell cycle regulating molecules in A375 cells. As the hypophosphorylated form of RB is linked to cell cycle arrest in the G0/G1 phase [31, 32], the phosphorylation level of RB protein in response to TNF treatment in A375 cells was analyzed by Western blotting. As shown in Figure 5A, RB protein was phosphorylated at a steady level and dephosphorylated by TNF-treatment in both A375-6 and A375-R8 cells. The amount of RB protein was decreased in TNF-treated cells. Simultaneous treatment with TNF and SB203580 resulted in a suppression of RB protein dephosphorylation. On the other hand, Z-VAD-fmk inhibited TNF-induced dephosphorylation of RB only in A375-R8 cells. These observations were consistent with the effects of the inhibitors on TNF-induced growth inhibition in two A375 subclones (Figure 1A). To further confirm cell cycle arrest by TNF, we measured the activity of E2F, a transcriptional factor that plays an important role in the control of cell proliferation, in A375 cells. E2F is regulated in a cell cycle-dependent manner, and the fluctuation of E2F activity changes the expression of an array of genes closely coupled with the cell cycle stage [33]. E2F activity in both A375-6 and A375-R8 cells was downregulated by TNF treatment (Figure 5B). SB203580, but not Z-VAD-fmk, moderately reversed the downregulation of E2F activity caused by TNF treatment. These findings suggest that TNF induces p38 MAPK-dependent cell cycle arrest in both A375-6 and A375-R8 cells.

DISCUSSION

In this study, we demonstrate that two anti-proliferative pathways are activated by TNF in A375 cells. One is transduced by p38 MAPK, resulting in cell cycle arrest, the other is a caspases-dependent pathway leading to apoptosis. The first pathway was operative in both A375-6 and A375-R8 cells, whereas the latter pathway was activated by TNF only in A375-R8 cells. As the p38 MAPK inhibitor SB203580 exhibited reversal effect on the growth inhibition induced by Fas stimulation only in A375-6 cells, the cell cycle arresting pathway may be dominant in A375-6 cells in response to the growth inhibition induced by TNFR or

Fas. The apoptotic pathway in A375-6 cells is intact since staurosporine could induce apoptosis in these cells (data not shown), and cycloheximide treatment rendered A375-6 cells sensitive to TNF-induced apoptosis, probably by elimination of anti-apoptotic protein. However, the finding of resistance to TNF-induced apoptosis in A375-6 x R8 hybrid cells indicates that an anti-apoptotic factor(s) is present or induced in A375-6 cells but is lost or not induced by TNF in A375-R8 cells. This anti-apoptotic factor(s) may suppress the common molecule utilized by TNF and Fas stimulation because A375-6 cells were also less sensitive than A375-R8 cells to the apoptotic effect of Fas stimulation.

It is well known that NF-kappaB activity contributes to cell survival against apoptosis by inducing transcription of anti-apoptotic factors [24-27]. TNF could upregulate NF-kappaB activity in both A375-6 and A375-R8 cells. Furthermore, in a steady state, higher NF-kappaB activity was observed in A375-R8 cells through a constitutive production of IL-1alpha [29]. Therefore, it is unlikely that the resistance against apoptosis in A375-6 cells was due to the difference in NF-kappaB activation.

Several molecules acting as anti-apoptotic factors have been reported. We examined the expression at mRNA level of anti- and pro-apoptotic molecules known to participate in TNF-induced apoptosis, such as Bcl-2 family members (Bcl-2, Bcl-xL, Bax, and Bid), inhibitor of apoptosis (IAP) family members (c-IAP1, c-IAP2, and survivin), FLICE-inhibitory protein (FLIP), p21/Waf1, p27, p53, RAIDD/CRADD, TNFR-associated factor-2 (TRAF2), silencer of death domains (SODD), and Akt-1 using gene expression array techniques. However, none of them was significantly different between A375-6 and A375-R8 cells (data not shown). We further examined mRNA expression level of TNFR1 and TNFR2 by reverse transcriptase-polymerase chain reaction (RT-PCR) in both subclones. TNFR1 mRNA was comparably expressed in these cells, while expression of TNFR2 mRNA was significantly lower in A375-6 cells than A375-R8 cells (data not shown). However, since apoptosis is mainly induced through TNFR1 [3] and A375-6 cells were relatively resistant to Fas-induced apoptosis, low expression level of TNFR2 could not account for the differential sensitivity to TNF between these two cell lines. It has been reported that TRAF2, an adapter protein for a large number of TNFR super family members, mediated anti-apoptotic signaling independently of NF-kappaB activation [34, 35]. However, it is unlikely that TRAF2 is responsible for the differential TNF sensitivity, because both subclones expressed comparable level of TRAF2 (data not shown) and A375-6 cells were

relatively resistant to apoptosis induced by Fas stimulation that does not recruit TRAF2. In addition to p38 MAPK, TNF activates, *via* TRAF2, c-Jun N-terminal kinase (JNK) that has been reported to promote apoptosis by phosphorylating and inactivating Bcl-2 anti-apoptotic protein [36, 37]. However, because JNK was activated by TNF treatment even in A375-6 cells (data not shown), it is also unlikely that the JNK pathway contributes to the differential sensitivity to TNF. FLIP directly inhibits caspase-8 activity and high level FLIP protein expression has been observed in melanoma cell lines and malignant melanoma tumors [38]. We examined protein level expression of FLIP as well as transcriptional levels in A375-6 and A375-R8 cells. However, there was no significant difference in terms of FLIP expression between these two subclones (data not shown). Akt-1, one of the well known cell survival factors [39], in addition to its mRNA expression level, was phosphorylated even in a steady state in both A375-6 and A375-R8 cells, and TNF treatment did not affect its activity (data not shown). It is possible, however, that an anti-apoptotic molecule(s) is mutated with loss of function in A375-R8 cells. Because TNF failed to activate caspase-8 in A375-6 cells, the anti-apoptotic factor may operate relative up-stream of the TNF signaling pathway, between TNFR and caspase-8 (Figure 6).

TNF induced dephosphorylation of RB protein and downregulation of E2F activity in both A375-6 and A375-R8 cells. These proteins strictly regulate cell cycle progression, and dephosphorylation of RB protein and downregulation of E2F activity are closely linked to cell cycle arrest in the G0/G1 phase [31-33]. TNF inhibits A375 cell proliferation, in addition to inducing apoptosis, by arresting cell cycle in the G0/G1 phase. Although Z-VAD-fmk markedly reversed TNF-induced growth inhibition (up to 60% recovery) in A375-R8 cells, the degree of apoptosis was less (about 20% of the population). The inhibitory effect of Z-VAD-fmk on TNF-induced dephosphorylation of RB protein may explain this inconsistent observation. SB203580 inhibited TNF-induced dephosphorylation of RB protein in both subclones, however, Z-VAD-fmk influenced only A375-R8 cells, in which caspases were activated by TNF treatment. Therefore, in addition to p38 MAPK-dependent regulation, the cell cycle arrest subsequent to RB protein dephosphorylation might be affected by caspases (Figure 6). It is of note that the combination of SB203580 and Z-VAD-fmk treatment blocked TNF-induced growth inhibition more than SB203580 treatment alone in A375-6 cells, whereas treatment with Z-VAD-fmk alone did not affect the growth inhibition at all. The finding supports the hypothesis that cell cycle progression is regulated by both p38 MAPK- and caspases-dependent pathways. Our findings also indicate that

TNF-induced cell cycle arrest is not explained by RB protein dephosphorylation alone, because TNF decreased the amount of RB protein, and SB203580 only moderately reversed TNF-induced downregulation of E2F activity. It is possible that other molecules engaged in cell cycle progression are affected by TNF as well.

CONCLUSION

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Figures

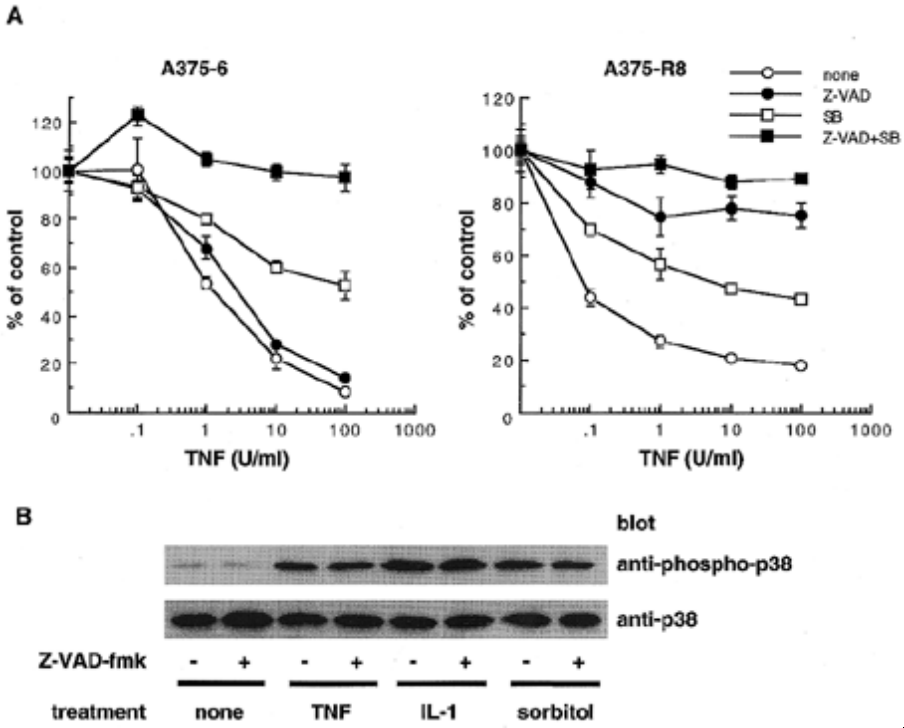


Figure 1

Caspases and/or p38 MAPK activity are crucial for the anti-proliferative effect of TNF in A375-R8 cells.

A: A375-6 (left) and A375-R8 (right) cells were cultured for 3 days with different doses of TNF-alpha alone (open circles) or together with 50 muM Z-VAD-fmk (Z-VAD, filled circles), 10 muM SB203580 (SB, open squares), and Z-

VAD-fmk plus SB203580 (Z-VAD + SB, filled squares). After culture, cell proliferation was determined by staining with crystal violet. The average (mean ± SD) of triplicate wells is shown. **B:** A375-R8 cells were treated with 100 U/ml TNF-alpha, 1,000 U/ml IL-1alpha, or 400 mM sorbitol for 15 min in the presence or absence of 50 muM Z-VAD-fmk. Z-VAD-fmk was added 1 hour before than TNF. Subsequently the cells were lysed and the lysates were fractionated on SDS-PAGE (12.5%), transferred onto a PVDF membrane, and blotted with anti-phospho-p38 MAPK or anti-p38 MAPK antibodies as described in the Materials and Methods section.

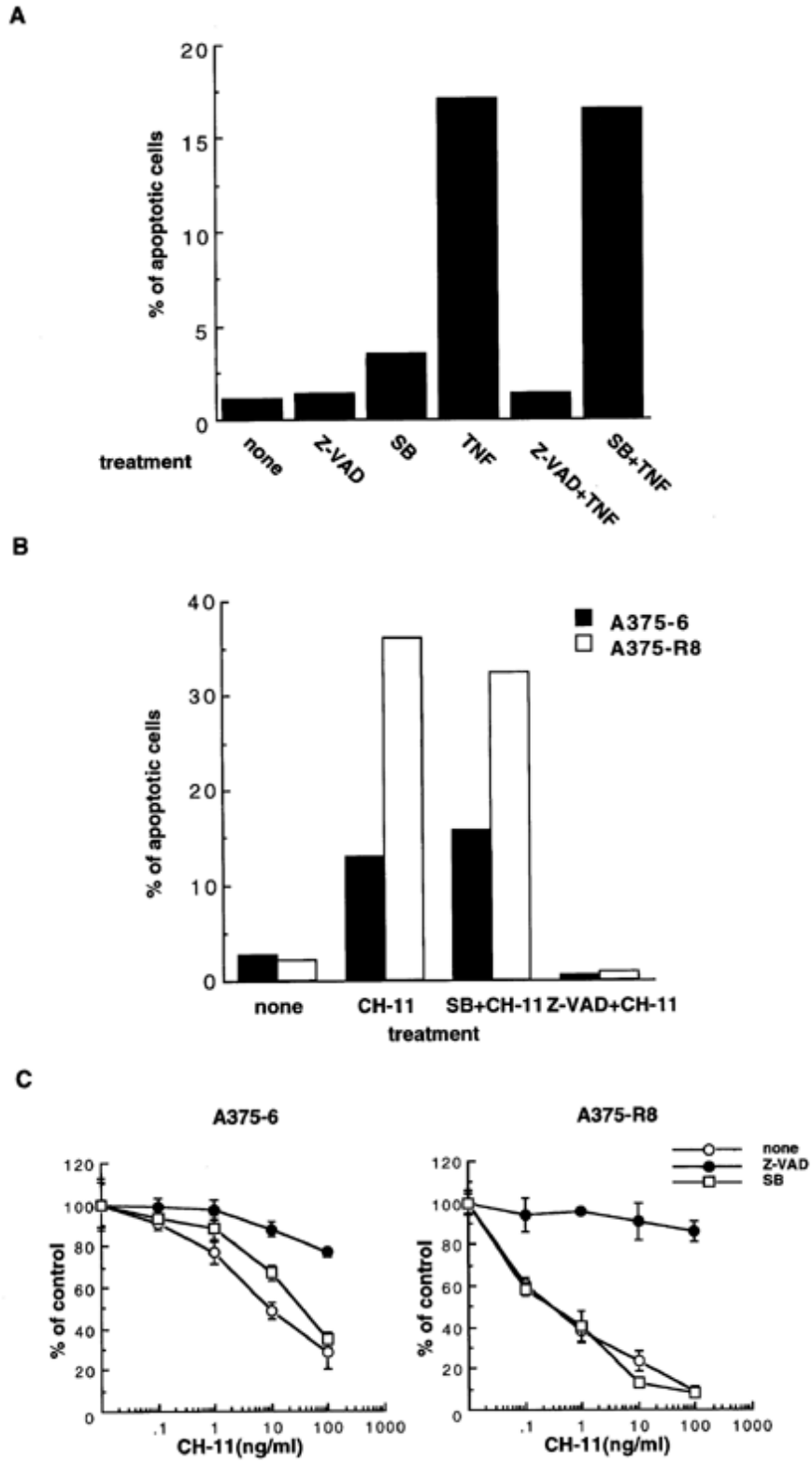


Figure 2

TNF induces apoptosis in A375-R8 but not in A375-6 cells.

A: Blockade of TNF-induced apoptosis in A375-R8 cells by Z-VAD-fmk. A375-R8 cells were treated with 100 U/ml TNF-alpha for 24 hours in the presence or absence of 50 muM Z-VAD-fmk (Z-VAD) or 10 muM SB203580 (SB). **B:** Apoptosis induced by Fas stimulation. A375-6 (filled bars) and A375-R8 (open bars) cells were treated with 100 ng/ml anti-Fas monoclonal antibody (CH-11) for 24 hours in the presence or absence of 50 muM Z-VAD-fmk (Z-VAD) or 10 muM SB203580 (SB). The apoptotic cells were counted a using fluorescence microscope **(A, B)**. **C:** A375-6 (left) and A375-R8 (right) cells were cultured for 3 days with various doses of CH-11 (open circles) with or without 50 muM Z-VAD-fmk (Z-VAD, filled circles), 10 muM SB203580 (SB, open squares). After culture, cell proliferation was determined by staining with crystal violet. The average (mean ± SD) of triplicate wells is shown.

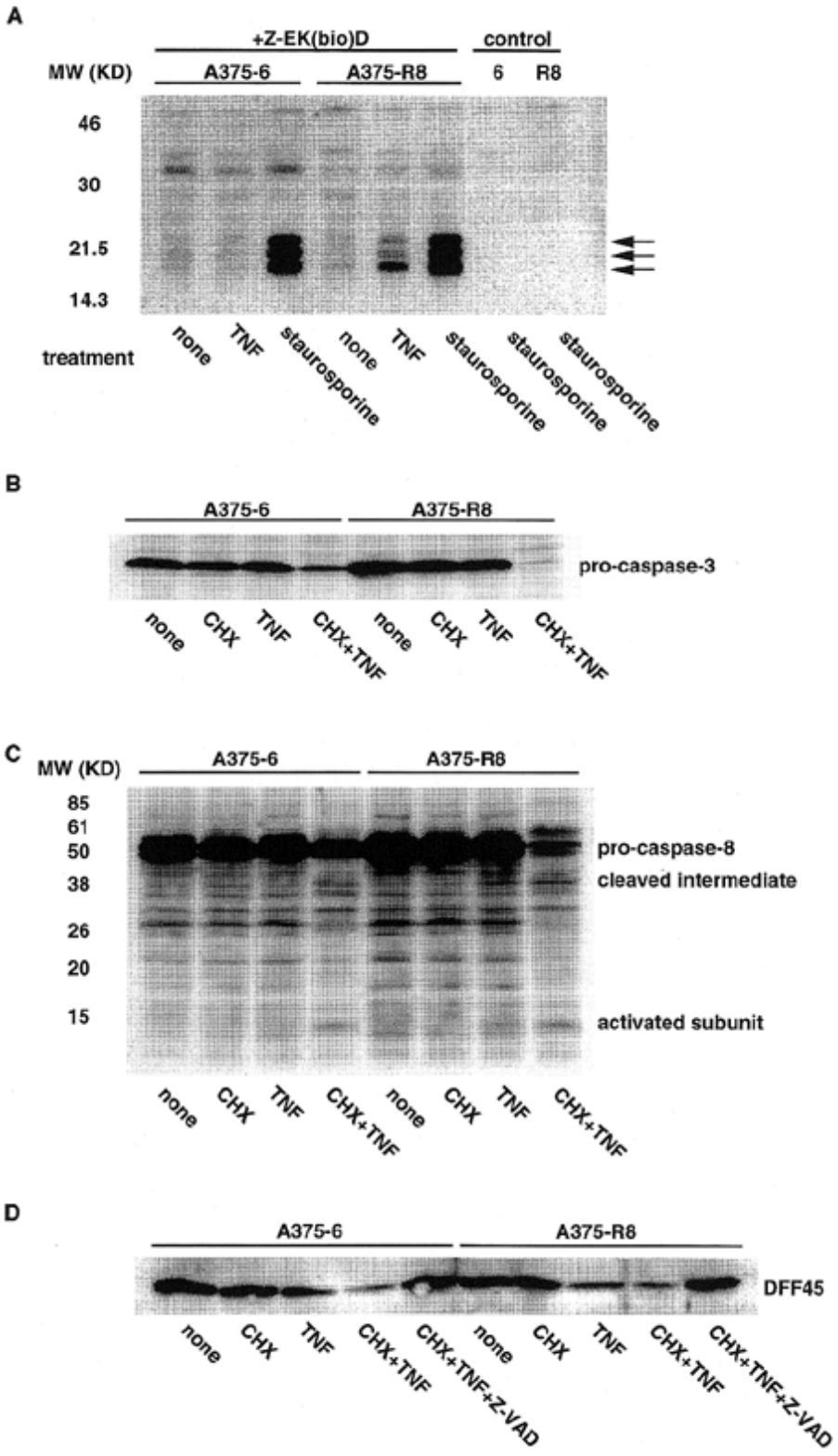


Figure 3

Activities of caspases in TNF-treated A375 cells.

A: Activation of caspases by TNF in A375-R8 cells. A375 cells were treated with 100 U/ml TNF-alpha for 24 hours or

1 muM staurosporine for 6 hours. Then, cells were lysed and the lysates were incubated with Z-EK(bio)D-CH_2-DMB for 15 min at 37° C. The mixture was applied to SDS-PAGE and transferred to PVDF membrane. The activated caspases associated with biotinylated substrate were affinity blotted with HRP-conjugated avidin and detected with an ECL kit. The lysates incubated without biotinylated substrate were applied as controls. Arrows indicate activated caspase-like proteins. **B, C, D:** Activation of caspase-3, -8 and DFF45 in TNF-treated A375 cells. A375 cells were treated with 100 U/ml of TNF-alpha in the presence or absence of 1 muM cycloheximide (CHX) or 50 muM Z-VAD-fmk (Z-VAD) for 24 hours. After lysis, an equal amount of protein was applied to SDS-PAGE. Western blotting was performed with the monoclonal antibody against caspase-3 **(B)** or -8 **(C)** or rabbit antiserum against DFF45 **(D)**.

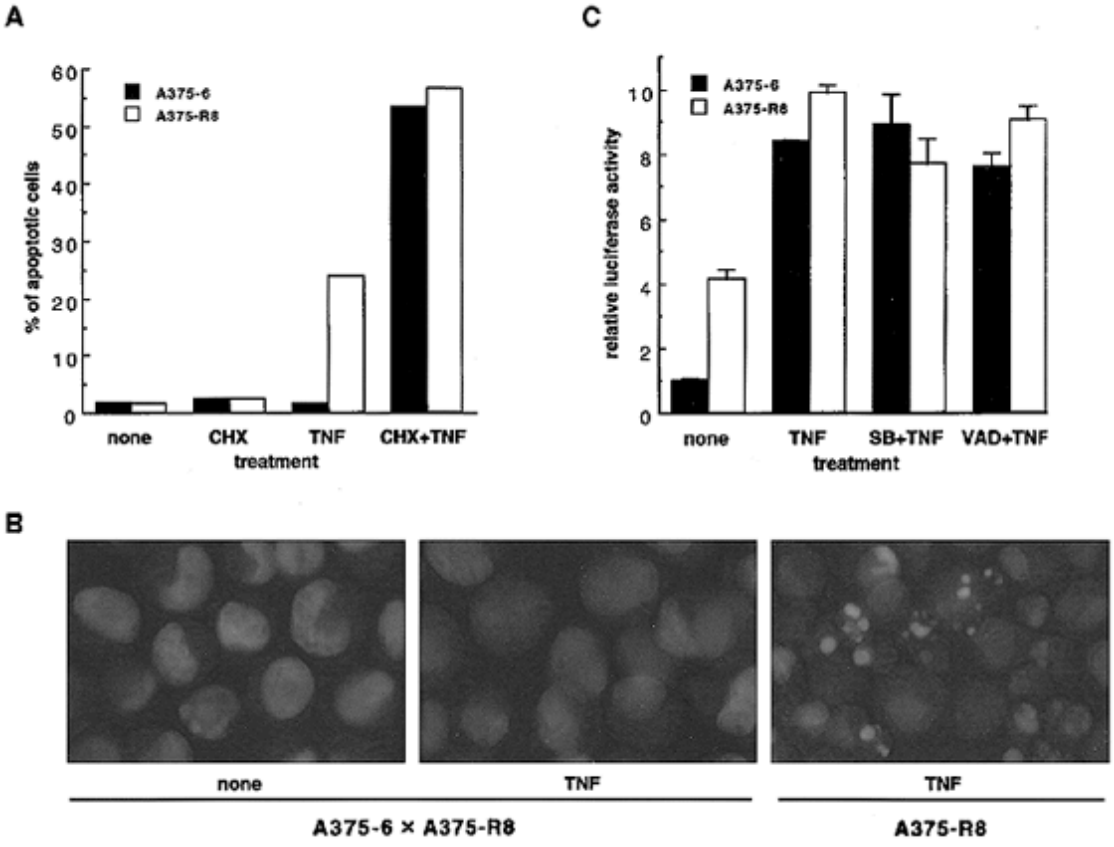


Figure 4

***De novo* protein synthesis but not NF-kappaB activity is necessary for the resistance against TNF-induced apoptosis in A375-6 cells.**

A: A375-6 (filled bars) and A375-R8 (open bars) cells were treated with 100 U/ml TNF-alpha for 24 hours in the presence or absence of 1 muM cycloheximide (CHX). The apoptotic cells were counted using a fluorescence microscope. **B:** A375-6 x A375-R8 hybrid cells [28] were treated with (middle) or without (left) 100 U/ml TNF-alpha for 24 hours. A375-R8 cells were treated with TNF-alpha in the same manner (right). The fluorescence was observed by a fluorescence microscope. **C:** A375-6 (filled bars) and A375-R8 (open bars) cells were co-transfected with pGL3-kappaB wt and pCMV-beta-gal. After 2 days' culture, cells were pretreated with or without 10 muM SB203580 (SB) or 50 muM Z-VAD-fmk (VAD) for 1 hour. Subsequently, the cells were treated with 100 U/ml TNF-alpha for 6 hours. The luciferase activity was normalized with beta-galactosidase activity in the same well. Shown is the relative promoter activity obtained by dividing the luciferase activity of TNF-treated cells by that of untreated A375-6 cells. The average (mean ± SD) of triplicate wells is shown.

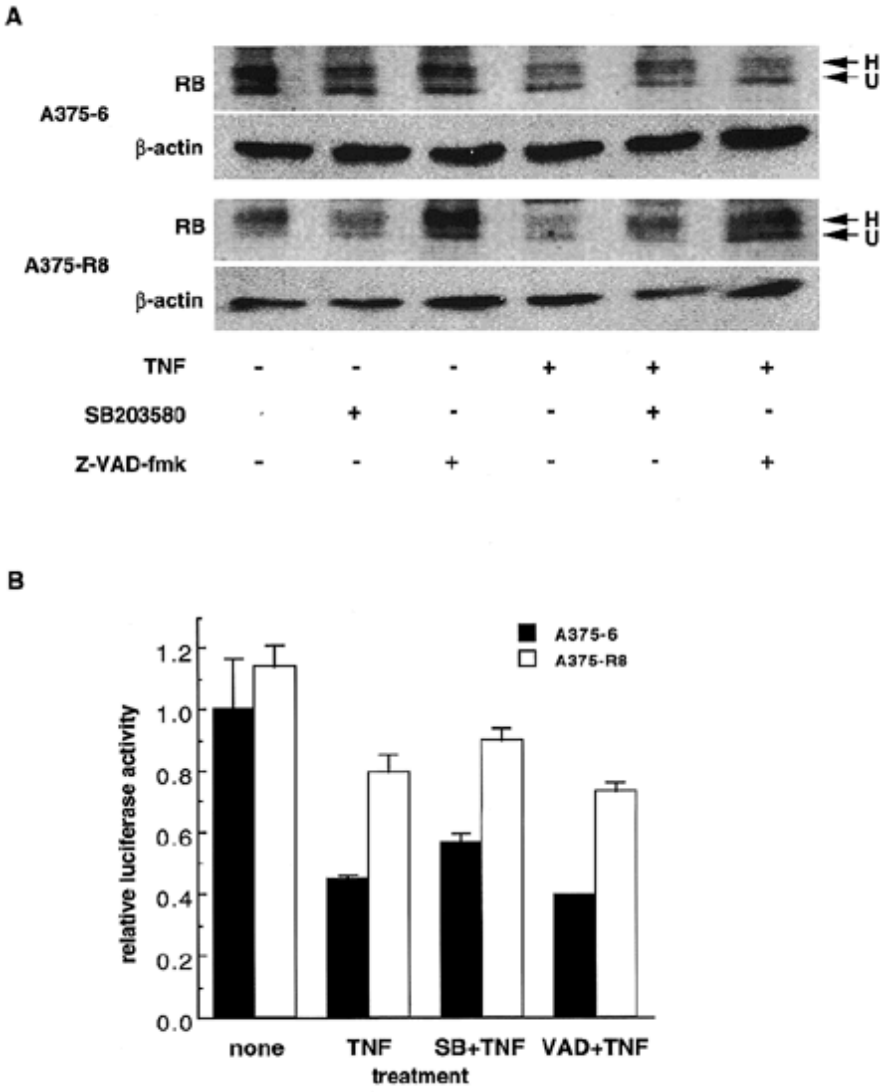


Figure 5

TNF causes the cell cycle arrest in A375 cells.

A: A375-6 (upper) and A375-R8 (lower) cells were treated with or without 100 U/ml of TNF-alpha in the presence or absence of 10 muM SB203580 or 50 muM Z-VAD-fmk for 72 hours. After lysis, an equal amount of protein was applied to SDS-PAGE. Western blotting was performed with the polyclonal antibody against RB or beta-actin. The arrows indicate hyperphosphorylated (H) and hypophosphorylated (U) forms of RB protein. **B:** A375-6 (filled bars) and A375-R8 (open bars) cells were co-transfected with pE2F-Luc and pCMV-beta-gal. After one day of culture, cells were treated with or without 100 U/ml

of TNF-alpha in the presence or absence of 10 muM SB203580 (SB) or 50 muM Z-VAD-fmk (Z-VAD) for 24 hours. The luciferase activity is normalized with beta-galactosidase activity in the same well. Shown is the relative promoter activity obtained by dividing the luciferase activity of TNF-treated cells by that of untreated A375-6 cells. The average (mean ± SD) of triplicate wells is shown.

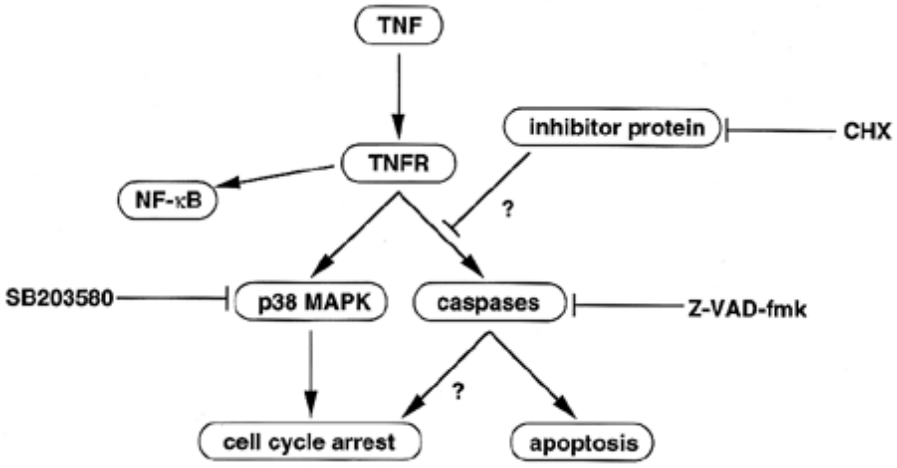


Figure 6

Summary of the TNF-mediated anti-proliferative signal in A375 cells.

TNF activates both caspases and p38 MAPK in A375-R8 cells. While apoptosis is mediated by a caspases-dependent pathway, activation of p38 MAPK pathway results in cell cycle arrest. Activation of caspases may also contribute to the cell cycle arrest in a p38 MAPK-independent manner. In A375-R8 cells, an anti-apoptotic factor(s) whose transcription is independent on NF-kappaB activation does not function or is not induced in response to TNF.

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