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Modulation by caspases of tumor necrosis factor-stimulated c-Jun N-terminal kinase activation but not nuclear factor-κB signaling

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Abstract

Tumour necrosis factor-α (TNF) is capable of activating many downstream signaling molecules *via* its two receptors TNFR1 and TNFR2. TNF can stimulate the proinflammatory transcription factor nuclear factor-κB (NF-κB) as well as the stress induced kinase c-Jun N-terminal kinase (JNK) through mechanisms that are not fully delineated. NF-κB becomes activated mainly through TNFR1 while JNK can be stimulated by either TNF receptor subtype. TNF can also induce apoptosis within cells due to its ability to recruit procaspase-8 to TNFR1, which in turn induces the caspase proteolytic cascade. We provide evidence here in human cells, that TNF-induced JNK activation is under the influence of caspases while NF-κB activity is not. By using pharmacological inhibitors of caspases, we have shown that JNK activity is reduced following caspase inhibition, especially when caspase-3 is targeted. NF-κB activity, as assessed by IκBα or IκBβ degradation, electrophoretic mobility shift assay and NF-κB gene reporter assays, is shown to be unaffected by caspase inhibition. Therefore, downstream TNF receptor signaling events are differentially influenced by caspases.

Keywords: Human; Signal transduction; Apoptosis; Inflammation; Cytokine receptors; Transcription factor

1. Introduction

Since its biochemical isolation in 1984, tumour necrosis factor-α (TNF) has been shown to be a pleiotropic agent produced mainly by activated macrophages and monocytes but also by other cell types such as B lymphocytes, T lymphocytes and fibroblasts. TNF is expressed as a 24 kDa membrane bound protein that is proteolytically cleaved into its 17 kDa soluble form [1]. TNF in its soluble, trimerized form binds to two receptors, TNFR1 (p55) and TNFR2 (p75). Both receptors have an extracellular ligand binding domain that shows 28% homology, a transmembrane region and unrelated intracellular domains. The extracellular domains are characterized by four conserved, cysteine rich repeats that are 38–42 amino acids in length. Each repeat has four to six cysteines organized as disulfide bridges that is characteristic of the TNF/nerve growth factor family of receptors [2].

Both TNFR1 and TNFR2 contain an extracellular pre-ligand binding assembly domain (PLAD) that is distinct from ligand binding regions. The PLADs complex the receptors and encourage them to trimerize upon activation by TNF [3]. The main distinction between the receptors is that TNFR1 has an 80 amino acid ''death domain'' (DD) sequence near the cytoplasmic tail of the receptor that is crucial for its death-inducing properties [4]. This DD allows TNFR1 to recruit downstream signaling molecules to the receptor complex thereby bringing about one branch of the TNF response within the cell (see [5] for review). Whilst TNFR2 lacks a DD, it is still capable of recruiting adapter proteins such as TNF receptor-associated factor 2 (TRAF-2). TNFR2 is proposed to signal *via* two mechan-

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Abbreviations: Caspase, cysteine–aspartate-directed proteases; ERK, extracellular signal-regulated kinase; fmk, fluoromethyl ketone; FITC, fluorescene isothiocyanate; IκB, inhibitor of κB; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; NF-κB, nuclear factor-κB; R1-TNF, R32WS86T TNFR1-specific TNF; R2-TNF, D143NA145R TNFR2-specific TNF; TNFR1, type I 55 kDa TNFR; TNFR2, type II 75 kDa TNFR; TNF, tumour necrosis factor-α; TNFR, TNF receptor; TRAF, TNFR-associating factor.

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isms. Firstly, TNFR2 has been implicated directly in apoptotic signaling [6,7]. Secondly, TNFR2 has a proposed role in "ligand passing" [8] whereby TNFR2 serves to increase the local concentration of TNF by way of it having a greater affinity and half-life for TNF. The bound TNF is then accepted by TNFR1 which then signals for apoptotic cell death.

While TNFR1 has a well-established role in apoptosis, it is also implicated in cell survival mainly through its ability to activate the transcription factor nuclear factor-κB (NF-κB). NF-κB is sequestered in the cytoplasm of cells due to its association with the IκB family proteins that includes IκBα, IκBβ and IκBε. Upon TNF stimulation, the IκB proteins become phosphorylated at the N-terminal by their corresponding IκB kinase proteins. In the case of IκBα this occurs at Ser 32 and Ser 36 whereas the corresponding residues in IκBβ are Ser 19 and Ser 23 [9,10]. This phosphorylation targets IκBα and IκBβ for ubiquitination and proteosome-mediated degradation thereby releasing NF-κB allowing it to translocate to the nucleus and activate a series of genes [11]. TRAF-2 was the first signaling adapter protein identified as an activator of NF-κB [12] through its association with NIK (NF-κB-inducing kinase). However, it has now been demonstrated that receptor interacting protein (RIP) another DD-containing signaling intermediate that binds to TNFR1, plays a role in NF-κB activation [13,14]. Recent evidence suggests that TRAF-2 is required for IκK recruitment to the signaling complex whereas it is actually RIP that is responsible for IκK activation [15].

As well as NF-κB activation, TNF receptors can activate three distinct groups of kinases namely; p42/44MAP kinases (MAPK), p38MAP kinase (p38MAPK) and the JNK. JNK is termed a stress kinase as it is activated in response to a variety of stress stimuli including cytokines (TNF), heat/osmotic stress and UV irradiation [16]. JNK is mainly activated by MEK-4 (SEK-1) but also by MEK-7 [17]. Both TNFR1 and TNFR2 are capable of activating JNK [18–20] through various mechanisms but the majority seem to involve TRAF-2. JNK is proposed to have a possible role in cellular apoptosis [21–25].

Caspases (cysteine–aspartate-directed proteases) are a group of 14 different enzymes that induce apoptotic cell death. Caspase-8 is involved in TNFR1 signaling due to the interaction of its "death effector domain" (DED) with the DD of the TRADD/FADD complex [26]. Caspase-2, -8, -9 and -10 are initiator caspases that serve to activate the quiescent executioner procaspases (-3, -6, and -7) by a process of cleavage, oligomerization and autoactivation [27]. Cell-permeable pharmacological inhibitors of caspases such as zVAD-fmk have been shown to block cell death induced by several ligands [28]. Many of the TNFR1 signaling molecules appear to be a target of caspases. For example, it has been proposed that RIP is a putative target for caspase-8 that results in RIP degradation upon TNF stimulation [29]. This cleavage inhibits NF-κB activation therefore allowing the cell to progress through apoptotic cell death.

The aim of this present study is to demonstrate the involvement of caspases in the activation of NF-κB and JNK by both TNFR1 and TNFR2.

2. Materials and methods

2.1. Cells

HeLa cells that stably express TNFR2 (HeLa-TNFR2) were generated by injecting human TNFR2 (provided by Werner Lesslauer, Yale University) and pBABE hygromycin resistant cDNAs into the HeLa cells using an Eppendorf Injectman microinjection and micromanipulation system [20]. Stable colonies were selected in DMEM containing 100 μg/mL hygromycin-B (Boehringer Mannheim). Following selection, stably transfected cell lines were maintained in a culture of Dulbecco's modified Eagles's medium (DMEM, 100 mg/mL glucose/no sodium pyruvate) with 10% fetal calf serum (Helena Biosciences), 4 mM L-glutamine, 50 U/mL penicillin and 50 μg/mL streptomycin) using standard sterile culture techniques.

2.2. JNK activity

JNK activity was assessed by measuring the phosphorylation of its substrate c-Jun (5–89) linked to a glutathione-*S*-transferase (GST) fusion protein [20]. Briefly, HeLa-TNFR2 cells were grown to a subconfluent state then treated for 15 min with the agents shown. Cell extracts were then prepared and conjugated to GST beads. [^{32}P]-γ-ATP was then incorporated at 2 μCi per tube and the reaction stopped after 30 min by the addition of 6× Laemmli sample buffer. Samples were boiled for 3 min then run on 12% SDS–PAGE. Gels were then dried at 80° for 90 min on a vacuum gel drier. Phosphorylation of c-Jun was determined by exposure of the gels to X-ray film then quantified using a BioRad densitometer.

2.3. Western analysis

Cells were treated with the various stimulants as shown then lysed using RIPA buffer (1× PBS, 1% Nonidet-P40, 0.5% sodium deoxycholate, 0.1% SDS) supplemented with 0.1 mg/mL PMSF, 10 μg/mL aprotinin, 1 mM $NaVO_4$. Following a 60 min incubation on ice, cell debris was pelleted (20 min, 15,000 *g*, 4°) and discarded. Cell protein concentrations were determined by Lowry protein assay (BioRad) then supplemented with 2× Laemmli loading buffer (20% glycerol, 4% β-mercaptoethanol, 0.3% bromophenyl blue) before boiling for 5 min. Western blotting then proceeded as described previously [30]. Primary antibodies (1:200) were incubated overnight at 4° in PBS + 0.1% Tween-20. Secondary antibodies conjugated

to horseradish peroxidase (1:1000) (Autogen Bioclear) were incubated for 45 min at room temperature. Specific protein antibody interactions were detected using enhanced chemiluminescence (Amersham/Pharmacia).

2.4. NF-κB-luciferase activity

HeLa-TNFR2 stably cells were grown in 24-well culture plates reaching 50–80% confluency before use. Cells were transiently transfected with 0.2 μg/well of NF-κB-luciferase reporter construct [31] using the Lipofectamine method [32]. About 48 hr posttransfection the cells were stimulated with TNF, R1-TNF, or R2-TNF + 1 μg/mL MR2-1 antibody [33] for 6 hr. Cells were washed twice with ice-cold PBS then 200 μL of ice-cold lysis buffer (25 mM Tris-phosphate (pH 7.8), 8 mM $MgCl_2$, 1 mM DTT, 1% Triton X-100, 15% glycerol) was added to each well. Following 5 min on ice, cells were scraped into the lysis buffer and transferred to an Eppendorf tube. Cell debris was pelleted (3000 *g*, 2 min) and 100 μL of supernatant was used to determine luciferase activity by injecting it with 100 μL of luciferase buffer (1 mM ATP, 0.25 mM luciferin (Molecular Probes), 1% BSA) in a Berthold LB9501 Lumat Luminometer. Lysis buffer (100 μL) was used as a blank.

2.5. Electrophorectic mobility shift assay (EMSA)

NF-κB oligonucliotide (5′-AGTTGAGGGGACTTTCC-CAGGCGCCTGGGAAAGTCCCCT CAACT-3′) obtained from Oswel Laboratories, was end labeled with [^{32}P]-γ-ATP in the presence of T4 polynucleotide kinase, 10× buffer, [^{32}P]-γ-ATP (specific activity of 3000 Ci/mmol, used at 10 mCi/mL), nuclease free water and T4 polynucleotide kinase to make a final volume of 10 μL. Following incubation at 37° for 10 min, the reaction was stopped by adding 1 μL 0.5 M EDTA. Final volume was made up to 100 μL by the addition of TE buffer. Unincorporated [^{32}P]-γ-ATP was cleared from the probe by centrifugation at 15,000 *g* for 5 min. HeLa-TNFR2 stably expressing cells were seeded into 6-well culture dishes and grown to 80% confluency before stimulation with the agents described. Cells were scraped into the culture medium then centrifuged (4000 *g*, 5 min). The supernatant was discarded and the pellet resuspended in 500 μL of ice-cold PBS, pelleted by centrifugation (6000 rpm, 10 s) then resuspended in 100 μL of Dignam buffer A (10 mM HEPES (pH 7.9), 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, 0.2% NP40) with freshly added inhibitors (5 μL 100 mM AEBSF, 10 μL 18.4 mg/mL sodium orthovanadate, 10 μL 42 mg/mL sodium fluoride, 1 μL 2.2 mg/mL aprotonin). Following centrifugation (15,000 *g*, 10 s) the cytoplasmic extract was discarded and the pellet resuspended in 25 μL of Dignam buffer C (20 mM HEPES (pH 7.9), 25% glycerol, 0.42 mM NaCl, 1.5 mM $MgCl_2$, 5 mM DTT, 0.2 mM EDTA) with freshly added inhibitors, mixed and incubated on ice for 10 min. Cell debris was pelleted (15,000 *g*, 30 s) and the supernatant retained (nuclear extract) then tested for protein concentration as mentioned earlier. The NF-κB labelled probe was then incubated with the nuclear extract to a final volume of 10 μL. Samples were then separated on 5% PAGE in TBE buffer, gels were dried for 1 hr at 80° in a vacuum gel drier and exposed overnight at −70° to X-ray film.

2.6. NF-κB-hrGFP

HeLa-TNFR2 stably expressing cells were plated out at a density of 0.5×10^6 cells/well 24 hr prior to transfection. Cells were transfected with the appropriate cDNAs (enhanced green fluorescent protein; peGFP, kindly provided by Prof. Gwyn Gould, University of Glasgow) positive control, negative control (Tris-EDTA buffer), or pNF-κB-humanized renilla green fluorescent protein (hrGFP, Stratagene) at 1 μg DNA/well using the Lipofectamine method as described earlier. About 24 hr posttransfection cells were preincubated for 1 hr with the caspase inhibitors described then treated with TNF at a final concentration of 50 ng/mL. Cells were then observed over a 36 hr period and the percentage fluorescence measured as described previously [32].

3. Results

3.1. Caspase sensitivity of TNF-induced JNK activity

HeLa human cervical epithelial carcinoma cells express predominantly TNFR1 therefore it was necessary to engineer a cell line (HeLa-TNFR2) to be able to better assess the involvement of TNFR2 in TNF signaling. HeLa-TNFR2 cells express 3000–4000 TNFR1 receptors/cell and 60,000 TNFR2 receptors/cell. Using TNF mutant proteins (“muteins” [33]) it is possible to evaluate the individual contributions of TNFR1 and TNFR2 to TNF signaling. Soluble TNFs are inefficient at stimulating the TNFR2 receptor [34], however, by using agonistic monoclonal antibodies (MR2-1) against TNFR2 we are able to stimulate TNFR2 effectively [20].

We used these tools to assess the capability of both TNFRs to activate JNK. As shown in Fig. 1A both TNFR1 and TNFR2 were capable of stimulating JNK activity in HeLa-TNFR2 cells over a 120 min time period. Maximal JNK activity occurred at 10–30 min with TNFR1 following which JNK activity declined but did not return completely to basal levels. TNFR2-induced JNK activity saw maximum activity again occurring at 10–30 min. However, JNK activity through TNFR2 did not lessen to the same extent as with TNFR1 over the 120 min time course. When samples were preincubated with the broad spectrum caspase inhibitor zVAD-fmk, JNK activation through TNFR1 was inhibited but not as markedly as through TNFR2, which was more sensitive to zVAD inhibition (Fig. 1A).

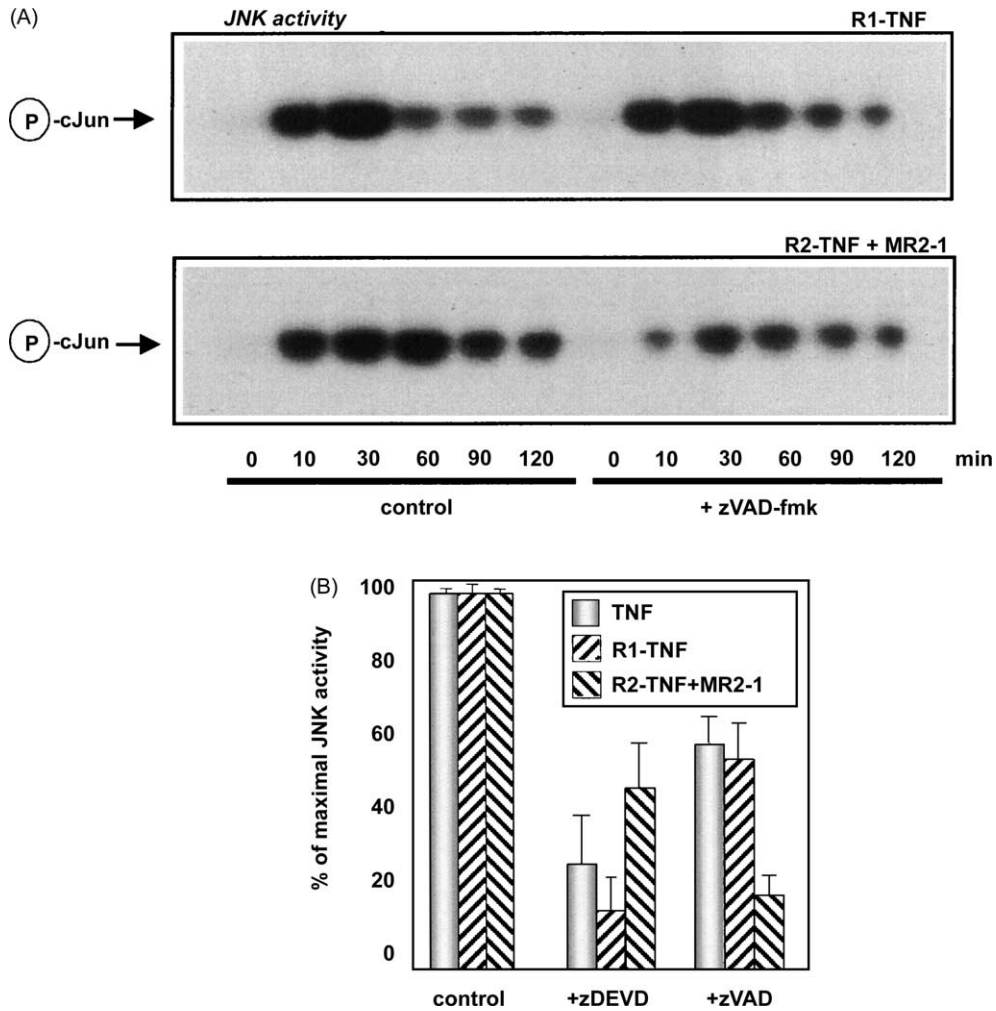

Fig. 1. TNF-induced JNK activity and its caspase-sensitivity. (A) Stably expressing HeLa-TNFR2 cells were treated with R1-TNF or R2-TNF + MR2-1 (antibody specific for TNFR2 activation) at 50 ng/mL for 15 min as indicated, either with or without pretreatment of the broad caspase inhibitor zVAD-fmk (20 μM) for 1 hr. JNK activation was assessed as described in Section 2. (B) The inhibition of JNK activity using the caspase inhibitors zDEVD-fmk or zVAD-fmk (20 μM) was assessed using the protocol outlined earlier and in Section 2. The results represent the mean ± SEM from three individual experiments.

This indicates that perhaps caspases have an alternative role in JNK activation that is mediated through TNFR2.

Fig. 1B assesses the individual contribution of caspase inhibitors to the inhibition of TNFR-induced JNK activity by the caspase inhibitors zDEVD-fmk and zVAD-fmk. zYVAD-fmk, zVEID-fmk, zVDVAD-fmk all have mixed effects on TNF-stimulated JNK activation, resulting in 41 ± 9, 50 ± 11 and $59 \pm 12\%$ of control JNK activation, respectively (means ± SEM, $N = 3$). Other work on HeLa-TNFR2 cells indicates that this profile of inhibition by these pharmacological agents suggests a role for caspase-3 and/or caspase-6 in the observed modulation of JNK activation [35].

3.2. Caspase sensitivity of TNF-induced NF-κB activation

NF-κB is held quiescent in the cytoplasm by the IκB proteins (IκBα and IκBβ) which upon TNF stimulation become phosphorylated, which then targets them for ubiquitination and proteosome degradation. We investigated the ability of TNF to degrade these proteins in the HeLa-TNFR2 over-expressing cell line. It can be seen that TNF (50 ng/mL) in this system is effective at degrading these proteins (Fig. 2). From Fig. 2A it can be seen that IκBα becomes degraded more rapidly than IκBβ. IκBα shows maximal breakdown at 10–30 min and then recovers after 120–180 min of TNF stimulation. IκBβ on the other hand shows slower degradation with maximal breakdown occurring after 60–180 min with no appreciable recovery in the time course investigated. Fig. 2B demonstrates that IκBα and IκBβ degradation is also concentration-dependent with a dose in the range of 5–50 ng/mL TNF being most effective at causing IκBα and IκBβ degradation and therefore NF-κB activation.

Following from the inhibition of JNK activation by caspases, in particular zDEVD-fmk, we were interested to discover whether NF-κB activity was effected in the

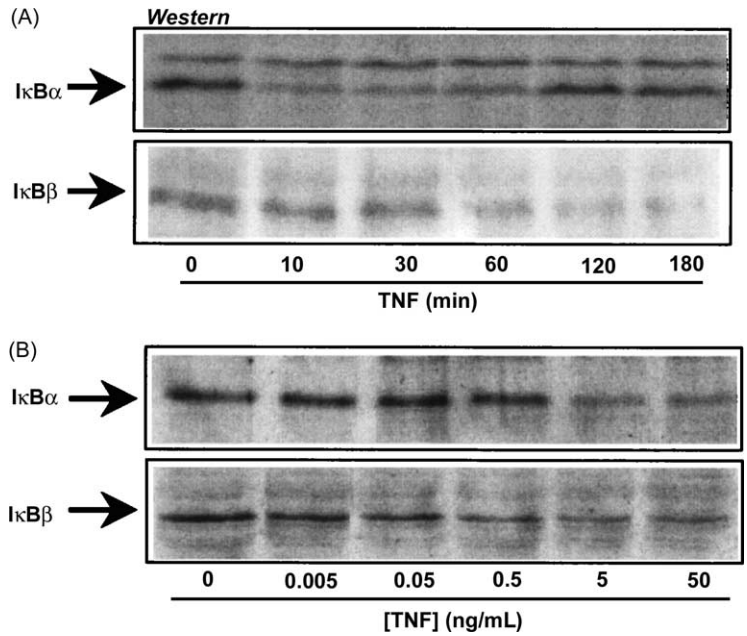

Fig. 2. (A) Time-course and (B) concentration-response relationship of TNF-induced IκBα and IκBβ degradation in HeLa-TNFR2 cells investigated using Western blotting as described in Section 2. HeLa-TNFR2 cells were exposed to 50 ng/mL TNF for the indicated times, or for 30 min to the indicated concentrations of TNF. Results are from a representative experiment repeated at least three other times with essentially the same findings.

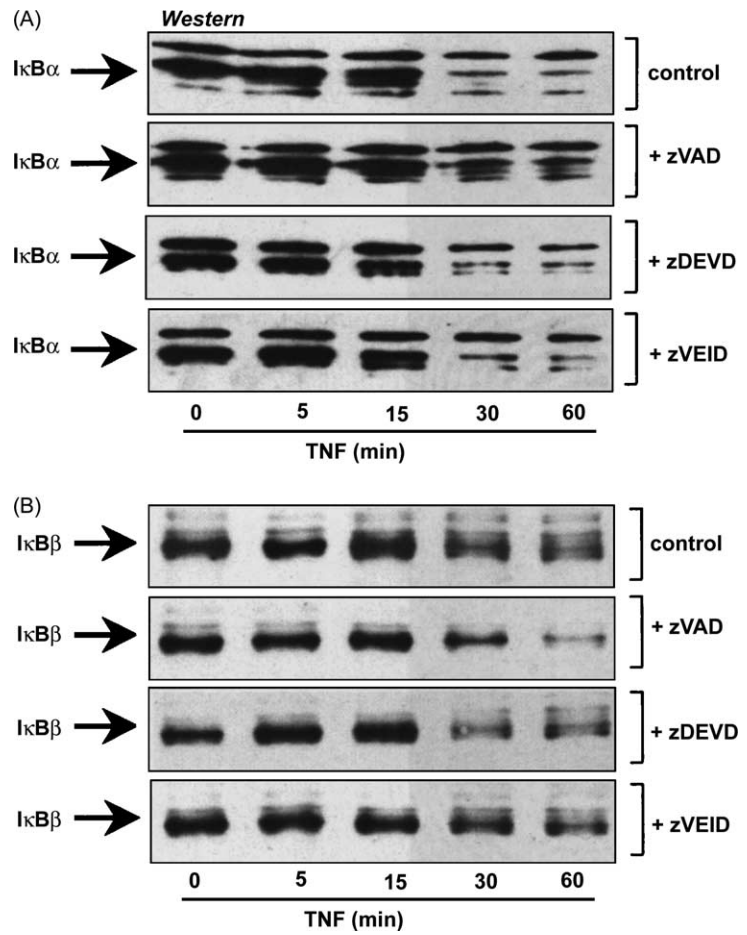

Fig. 3. Caspase-sensitivity of TNF-induced (A) IκBα or (B) IκBβ degradation in HeLa-TNFR2 cells. Pretreatment for 1 hr with 20 μM zVAD-fmk, zDEVD-fmk or zVEID-fmk where indicated, prior to cells exposed to 50 ng/mL TNF for the indicated times before Western analysis. Results are from a representative experiment repeated at least three other times with essentially similar findings.

same way. Fig. 3A clearly demonstrates that IκBα degradation over a 60 min period was not affected either by zVAD-fmk, zDEVD-fmk or zVEID-fmk. This is again the case for IκBβ (Fig. 3B). Although an indirect method of assessing NF-κB activation it appears clear that caspases are not involved at this level of TNF signaling towards NF-κB. These results were confirmed measuring IκBβ degradation in HeLa-TNFR2 cells, by means of flow cytometry (data not shown).

EMSA is an actual indication of NF-κB protein binding to its DNA binding sites. In a natural situation, the translocated NF-κB binds to the 10 bp DNA consensus binding site of a variety of genes including IL-2, IL-6, IL-8, VCAM-1, ICAM-1, RB1, GM-CSF and α-interferon [36]. Under experimental conditions, Fig. 4 demonstrates that following TNF stimulation, NF-κB does become activated (due to its presence in the nuclear extract) and can bind to radiolabeled NF-κB sequence DNA (lanes 1 and 2). However, preincubation with the caspase inhibitors (zVAD-fmk, zVEID-fmk, zDEVD-fmk, zVDVAD-fmk lanes 3–6) does not reduce the level of NF-κB activation to any significant degree. If anything, there may be a slight increase in binding by the caspase inhibitors, but this observation was not consistently observed. Therefore, not only do caspases have no effect at the IκBα and IκBβ degradation level of NF-κB activation, they appear to have no effect on the NF-κB DNA binding ability.

In order to validate this response, it was necessary to assess the involvement of caspase inhibitors in NF-κB activation using a NF-κB-luciferase reporter construct. Although it was apparent that NF-κB was binding DNA, it had been impossible to tell if the transcription factor was still active. By measuring the light production generated by the "switching on" of a luciferase reporter construct we were able to discover if NF-κB was still functioning in the presence of the various caspase inhibitors. Firstly, the activation of the NF-κB reporter construct by TNFR1 and TNFR2 was investigated in the HeLa-TNFR2 stably expressing cells (Fig. 5A). As expected, TNF stimulation activated NF-κB approximately 6-fold in comparison to endogenous NF-κB activity. Similarly, TNF-R1 caused a 5- to 6-fold increase in NF-κB activity. However, TNFR2 stimulation failed to increase the activity of NF-κB to any significant extent [32]. This is in contrast to JNK activation that was similar through TNFR1 and TNFR2 (Fig. 1A). When NF-κB-luciferase activity was studied in the presence of various caspase inhibitors (Fig. 5B) no inhibition of NF-κB activity was seen. This is in accordance with the results presented so far. Again these data highlight that caspases appear not to be involved in NF-κB activation as inhibition of these enzymes does not affect the level of NF-κB activity.

NF-κB-hrGFP is another useful tool for investigating the direct activation of NF-κB in living cells. In the same way

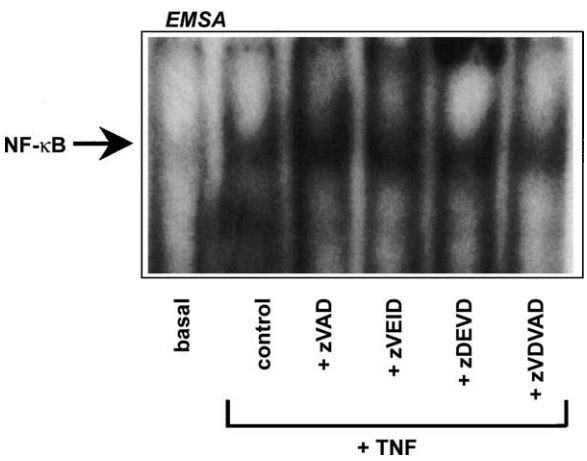

Fig. 4. NF-κB activity was investigated using the EMSA as outlined in Section 2. HeLa-TNFR2 cells were prestimulated with the caspase inhibitors (20 μM) for 1 hr then stimulated with 50 ng/mL TNF for 1 hr. The amount of NF-κB activity is highlighted by the intensity of the band which is an indication of the degree of binding of [^{32}P]-tagged NF-κB oligonucleotide in cell nuclear extract. Results are from a representative experiment repeated at least two other times with essentially similar findings.

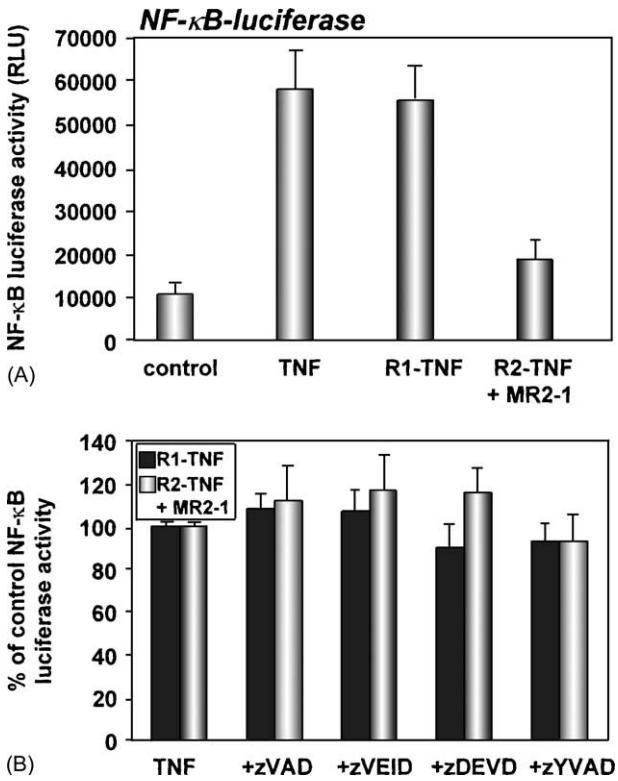

Fig. 5. Activation of NF-κB-stimulated transcription in HeLa-TNFR2 cells investigated using a NF-κB-luciferase reporter construct. (A) Following transfection of the cells with the reporter construct, cells were stimulated with TNF, R1-TNF or R2-TNF (all at 50 ng/mL) plus MR2-1 (2 μg/mL) for 6 hr. Cell extracts were injected with an equal volume of luciferase buffer (see Section 2) and the degree of light production measured. The data are the mean ± SD of quadruplicate measurements from a representative experiment repeated at least four other times with the same findings. (B) The effect caspase inhibition on R1-TNF or R2-TNF plus MR2-1 stimulated NF-κB reporter construct activity, investigated as in (A) except cells were preincubated for 1 hr with the indicated caspase inhibitors (20 μM) before TNFR stimulation. Results represent the mean ± SEM of at least three independent determinations.

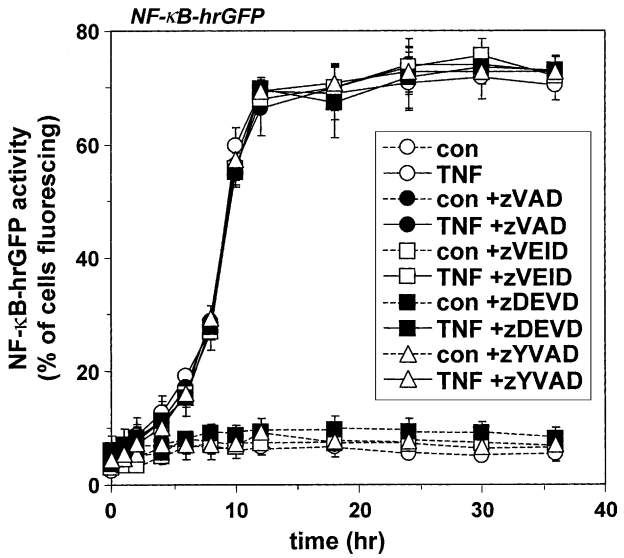

Fig. 6. NF-κB-stimulated gene transcription was investigated using a NF-κB-hrGFP reporter construct. Following transfection of the NF-κB-hrGFP construct, HeLa-TNFR2 cells were treated with TNF (50 ng/mL) for the indicated times in the presence or absence of the caspase inhibitors zVAD-fmk, zVEID-fmk, zDEVD-fmk or zYVAD-fmk (20 μM). Caspase inhibitors were administered 1 hr prior to TNF stimulation at time zero. The activity of NF-κB was assessed by the percentage of cells fluorescing green when visualized under a fluorescence microscope. The data represent the mean ± SEM from three independent evaluations.

as NF-κB-luciferase, the NF-κB-hrGFP reporter construct becomes activated when NF-κB binds to response elements within the promoter region of the cDNA gene. Instead of measuring light production, NF-κB activity in this assay is assessed by the number of cells fluorescing green when visualized under a fluorescent microscope. NF-κB activity is maximal after 10 hr with fluorescence being maintained at around 60–70% for 36 hr. It is apparent from Fig. 6 that basal or TNF-stimulated NF-κB activity is not inhibited by the presence of the caspase inhibitors zVAD-fmk, zVEID-fmk, zDEVD-fmk and zYVAD-fmk. The pattern of NF-κB activation does not alter with the inclusion of the various caspase inhibitors.

4. Discussion

This study has shown that TNF-stimulated JNK activity is sensitive to caspase inhibition while NF-κB activation is not. JNK activation appears to be downstream of caspase activation. Recent evidence also showed that JNK activity was zVAD-fmk sensitive, especially when stimulated *via* TNFR2 [20]. This is consistent with findings here (Fig. 1). This pattern of JNK activation is echoed in several other studies. For example, McFarlane *et al.* [37] demonstrated that TRAIL/CD95 (TNFR family members) induced apoptosis resulted in the activation of caspase-8 and -9 as well as JNK. The broad spectrum caspase inhibitor, zVAD-fmk, was able to inhibit apoptosis in this system along with a concurrent decrease in JNK activity implying that JNK activation is caspase dependent. Similarly, Muhlenbeck *et al.* [38] found that zVAD-fmk could inhibit TRAIL induced JNK activity in HeLa cells, but not in KYM-1 cells. This may imply then that JNK dependence on caspase inhibition is a cell type specific event.

The particular caspases involved and the part of the cysteine protease responsible for JNK activation has still to be established. Chaudhary and coworkers [39–41], suggest that caspases-8 and -9 are critical and that the prodomain along with the DED of the caspase are the crucial segments of the caspase molecule, resulting in the modulation of NF-kB and JNK activities. Our study has implicated caspase-3 as the main caspase involved in JNK activation. This is highlighted by the fact that zDEVD-fmk (most effective against caspase-3) has the greatest inhibitory effect on JNK activation (Fig. 1B), although it is not possible to rule out the involvement of the other caspases, such as caspase-8 which is also activated by TNFR and is efficiently inhibited by zDEVD.

In contrast to the JNK activity regulated through TNFR1 and TNFR2, it is evident from this study that NF-κB activation is not influenced by the inhibition of several caspases. Other studies have been shown that caspases have an influence on NF-κB activation. Caspase-8 has been shown to have a stimulatory effect on NF-κB [39]. In their study, over-expression of caspase-8 led to activation of NF-κB. This is in contrast to the data presented here where we employed TNF to stimulate NF-κB. The activation of NF-κB by caspase-8 may be a consequence of over-expression of caspase-8 whereas in a more physiological situation as presented here, caspase-8 has little influence on the activation of NF-κB. Other groups have studied the activation of NF-κB by over-expression of caspase-8 [42]. This study differed slightly in that, in the presence of caspase inhibitors, over-expression of caspase-8 still had the ability to activate NF-κB. An inactive mutant of caspase-8 was also able to activate NF-κB. Taken together, this implies that caspase induced apoptosis and activation of NF-κB are uncoupled. This may help to explain the results seen here as our protocol may only stimulate the caspase-8/apoptosis pathway, without having an effect on the pathway where caspase-8 aids in the activation of NF-κB. Alternatively, TNF may stimulate the apoptotic pathway involving NF-κB in a dominant fashion, therefore by inhibiting caspases this pathway is favored whilst masking the ability of caspases to stimulate NF-κB [43].

Further evidence supports the involvement of caspases in NF-κB activation. TRAIL has been shown to increase NF-κB activity in HeLa cells following treatment with zVAD-fmk [44]. However, in the same study no increase in NF-κB activity could be detected following TNF treatment, in agreement with our study. This may indicate that caspase involvement in NF-κB activity is dependent on the subset of TNF family members stimulated.

As well as having no direct effect on NF-κB, it is evident that caspases have no significant impact on the degradation

of IκBα and IκBβ. It is well known that IκBα and IκBβ become phosphorylated that makes them a target for ubiquitination and subsequent degradation in order to allow NF-κB to translocate to the nucleus [11]. None of the caspase inhibitors employed here had any significant effect on the degradation of IκBα and IκBβ suggesting that caspases are not crucial in the process of IκBα and IκBβ breakdown in response to TNF. Reuther and Baldwin [45], demonstrated the ability of TNF stimulation to cleave IκBα, supposedly mediated by caspases. Therefore it would be logical to predict that inhibition of caspases would prevent IκBα breakdown, however this is not the case. The version of IκBα cleaved was IκBα-SR a ''super-repressor'' protein that constitutively sequesters NF-κB in the cytosol of cells [45]. This ensures that upon TNF treatment these cells are pushed down the apoptotic pathway due to the permanent inactivation of NF-κB. When IκBα and IκBβ degradation is studied in a more physiological sense as described here, caspases have little influence on the degradation process suggesting that IκBα and IκBβ are not targets of caspases. The same study [45] also saw no accumulation of cleaved IκBα in cells expressing conventional IκBα in response to TNF which they argued was the result of the cells being able to activate NF-κB. This is in agreement with our findings and may represent the true situation that occurs within a cell in response to TNF stimulation. Other studies have also reported the ability of caspase-3 to cleave IκBα *in vitro* at a conserved Asp–Ser sequence [46]. However, when IκBα was induced to undergo signal (e.g. TNF)-induced phosphorylation, it could no longer undergo cleavage by CPP32-like caspases. This highlights that *in vivo*, signal-induced phosphorylation of IκBα would render it unsusceptible to caspase cleavage. This would also support the results presented here where TNF induced IκBα and IκBβ degradation is not under the influence of caspases. It appears that methods employed to study the interaction of caspases in IκBα, IκBβ and NF-κB stimulation, by ligands such as TNF, may result in the discovery of signaling pathways that may not exist *in vivo*. Conversely, if these signaling interactions are in place they may become easily masked by a more dominant signaling event.

Other suggested targets of caspases in TNF signaling have recently been proposed. The main contender seems to be RIP [29]. RIP cleavage by caspases is reported to result in the inhibition of NF-κB activation thereby sensitizing cells to death pathways. Our studies did not highlight this to any extent however, a more specific study may reveal that this is the level of NF-κB signaling where caspases have an influence.

In conclusion, we have provided evidence that downstream signaling pathways of TNF show different sensitivities to caspases. JNK activity appears to require the upstream activation of caspases, possibly caspase-3. On the other hand NF-κB, IκBα and IκBβ are insensitive to caspase activity in the HeLa-TNFR2 over-expressing cells. Perhaps the distinction lies in the fact that JNK is activated more dominantly through TNFR2 receptors while NF-κB stimulation is mainly mediated by TNFR1 receptors. More work needs to be done to characterize the differences as well as discover if any part of the NF-κB pathway is indeed a target for caspases.

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