Tumour necrosis factor-induced activation of c-Jun N-terminal kinase is sensitive to caspase-dependent modulation while activation of mitogen-activated protein kinase (MAPK) or p38 MAPK is not

Ahmed A. A. MOHAMED*, Orla J. JUPP*, Helen M. ANDERSON*, Alison F. LITTLEJOHN*, Peter VANDENABEELE† and David J. MACEWAN*¹

*Department of Biomedical Sciences, Institute of Medical Sciences, University of Aberdeen, Foresterhill, Aberdeen AB25 2ZD, U.K., and †Molecular Signalling and Cell Death Unit, Department of Molecular Biology, Flanders Interuniversity Institute for Biotechnology, University of Gent, Ledeganckstraat 35, B-9000 Gent, Belgium

The activation of the extracellular signal-regulated kinases (ERKs) by tumour necrosis factor- α (TNF) receptors (TNFRs) is an integral part of the cytokine's pleiotropic cellular responses. Here we report differences in the caspase sensitivity and TNFR subtype activation of members of the ERK family. Inhibition in HeLa cells of caspase function by pharmacological inhibitors or the expression of CrmA (cytokine response modifier A), a viral modifier protein, blocks TNF-induced apoptosis or caspase-dependent protein kinase C δ and poly(ADP-ribose) polymerase protein degradation. TNFR1- or TNFR2-stimulated c-Jun N-terminal kinase (JNK) activity was attenuated in cells in which caspase activity was inhibited either by pharmacological blockers or CrmA expression. Both TNFR1- and TNFR2-stimulated JNK activity was caspase-sensitive; however, only TNFR1 was capable of stimulating p42/44 mitogen-activated

protein kinase (MAPK) and p38 MAPK activities. TNFR1-stimulated p42/44 MAPK and p38 MAPK activities were insensitive to pharmacological caspase inhibition or CrmA. These findings were supported when measuring TNF-induced cytosolic phospholipase A₂ activation, which is a downstream target for MAPK and p38 MAPK. Profiling caspase enzymes activated by TNF in HeLa cells showed sequential caspase-8, -3, -7, -6 and -9 activation, with their inhibition characteristics suggesting a role for caspase-3 and/or caspase-6 in modulating JNK activity. Taken together these results show delineated ERK-activation pathways employed by TNFR subtypes.

Key words: apoptosis, cytokine receptor, human, protein kinase/phosphatase, signal transduction.

INTRODUCTION

Tumour necrosis factor- α (TNF) is a cytokine produced mainly by macrophages, T-lymphocytes and monocytes. It is expressed as a 26 kDa type II membrane protein which is cleaved by metalloproteinases to a 17 kDa soluble protein [1,2]. It has a broad spectrum of activities ranging from inflammation and differentiation to cell proliferation or cell death. It is a member of a superfamily of ligands (e.g. LT- β , Fas, CD40, NGF) which bind to a family of receptors [TNFR1 (type I 55 kDa TNF receptor), TNFR2 (type II 75 kDa TNFR), Fas, CD40] that share cysteine-rich domains on their extracellular surfaces, allowing them to recognize their respective ligands [3]. Their intracellular domains, however, appear to be largely unrelated [4].

TNF binds to two distinctive receptors, a 55 kDa and a 75 kDa receptor. Signal transduction through the first receptor, TNFR1, has been widely studied over the past few years, whereas TNFR2 has proved to be far more elusive in revealing its signal transduction pathways [5]. The activation of TNFRs takes place when the ligand forms a homo-trimer resulting in the oligomerization of the receptors and the recruitment of intracellular adaptor proteins [6]. TNFR1 mainly recruits a TNFR1-associated death domain protein (TRADD) directly through a conserved death domain on its intracellular face, which in turn

recruits other proteins with death domains such as Fas-associated death domain protein (FADD). FADD recruits procaspase-8 through homotypic interaction of death effector domains constituting the molecular link to apoptotic cell death. Receptor-interacting protein (RIP) and TNFR-associated factors (TRAF-2) are recruited to this TNFR1/TRADD receptor complex. Signalling through the TNFR2 has been difficult to establish, mainly due to the extremely fast association/dissociation kinetics of the receptor. TRAF-2 and RIP, as well as the kinases p80TRAK and casein kinase, have been demonstrated to directly bind to the TNFR2 [7]. Initially, there was a proposal that the more abundantly expressed TNFR2 acts in a ligand-passing model, whereby it acts as a reservoir for TNF, passing it on to the TNFR1, since both receptors seem to elicit very similar responses [8]. More recently, however, it has been shown that the TNFR2 is not efficiently activated in response to soluble TNF, which is the most commonly used form of TNF experimentally, but only responds fully to the membrane-bound form of the cytokine [9,10]. The development of mutant TNF proteins (muteins), which selectively bind one receptor over the other, coupled with the development of receptor-specific agonistic antisera, has provided a valuable tool for the study of each of the two TNFRs.

Extracellular signal-regulated kinases (ERKs) are a family of serine/threonine kinases important for the control of crucial

Abbreviations used: ATF-2, activating transcription factor 2; cPLA₂, cytosolic phospholipase A₂; CrmA, cytokine response modifier A; DMEM, Dulbecco's modified Eagle's medium; ERK, extracellular signal-regulated kinase; FADD, Fas-associated death domain protein; fmk, fluoromethylketone; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; MKK, MAPK kinase; PARP, poly(ADP-ribose) polymerase; PKC, protein kinase C; RIP, receptor-interacting protein; SEK, stress-activated protein kinase kinase; TNF, tumour necrosis factor- α ; TNFR, TNF receptor; TNFR1, type I 55 kDa TNFR; TNFR2, type II 75 kDa TNFR; R1-TNF, R32WS86T TNFR1-specific TNF; R2-TNF, D143NA145R TNFR2-specific TNF; TRAF, TNFR-associated factor; TRADD, TNFR1-associated death domain protein; zVAD-fmk, benzylloxycarbonyl-Val-Ala-DL-Asp-fluoromethylketone.

¹ To whom correspondence should be addressed (e-mail david.macewan@abdn.ac.uk).

cellular functions and which mediate many cytokine intracellular actions [11]. Mitogen-activated protein kinases (MAPKs) and stress-activated protein kinases, such as c-Jun N-terminal kinase (JNK) and p38 MAPK, have been shown to play pivotal roles in TNF signalling; however, their exact biological functions remain to be fully elucidated. The activation of JNK and p38 MAPK is reported to be important in the modulation of apoptotic cell death whereas p42/44 MAPK (ERKs 1 and 2) are reported to play important roles in cell survival [12]. Members of the ERK family are activated by upstream kinases such as MEKK1 (MAPK kinase 1), which has been shown to be crucial in JNK activation [13]. Their activation leads to the phosphorylation of several transcription factors and other intracellular targets. MAPK/stress-activated protein kinase activation leads to the phosphorylation of c-Myc, AP-1, nuclear factor for interleukin 6, activating transcription factor 2 (ATF-2), Elk-1 and the enzyme cytosolic phospholipase A₂ (cPLA₂). Phosphorylation of JNK by MAPK kinase (MKK) 4/stress-activated protein kinase kinase (SEK) 1 and MKK7 leads to its activation and the subsequent phosphorylation of c-Jun, ATF-2 and Elk-1. The activation of p38 MAPK by MKK3/6 leads to the phosphorylation of ATF-2 and Elk-1, but also results in the activation of MAPK-activated protein kinases 2/3 [11]. The exact role that TNFRs play in the activation of these crucial signalling cascades is not fully delineated.

Caspases are a family of cysteinyl/aspartate-directed proteases that are homologous to the *ced-3* gene product in *Caenorhabditis elegans*, and are the main mediators of programmed cell death [14]. They are all produced as pro-enzymes and are activated only after their cleavage at an aspartic acid residue. The initiator caspases, such as caspase-8, are believed to link the signalling cascade to receptors such as TNFR or Fas through the interaction of procaspase-8 and FADD. The executioner caspases such as caspase-3 and caspase-7 are believed to be responsible for the cleavage of poly(ADP-ribose) polymerase (PARP), DNA-protein kinase, lamins, U1-70 kDa, actin and fodrin, resulting in the characteristic DNA laddering, cell shrinkage and membrane blebbing observed during apoptotic cell death [15,16]. Several peptide inhibitors of the caspases have been developed, including the broad spectrum inhibitor zVAD-fmk (benzyloxycarbonyl-Val-Ala-DL-Asp-fluoromethylketone), that binds irreversibly with the catalytic cysteine through fluoromethylketone (fmk). zVAD-fmk that has proved most useful in studying the actions of caspases, but which at higher concentrations also blocks cathepsin B [17]. In order to confirm our findings with more specific inhibitors we included the cowpox virus protein cytokine response modifier A (CrmA), which produces a serpin containing the recognition motif LVAD, shown to be a very effective and a specific inhibitor of the apical caspases-1 and -8 [18–21].

The aims of this study were to determine the caspase dependency of TNFR-induced ERK activation. In order to ascertain the contribution of caspase activation in TNFR-induced ERK signalling, we used synthetic peptide inhibitors as well as CrmA protein to inhibit various caspase groups which are believed to act at different points in TNFR signalling cascades.

MATERIALS AND METHODS

Cells

KYM-1 cells were a gift from Terje Espevik, Institute of Cancer Research and Molecular Biology, Norwegian University of Science and Technology, Trondheim, Norway. Stably expressing HeLa-TNFR2 cells were generated by injecting human TNFR2 (provided by Werner Lesslauer, Department of Epidemiology and Public Health, Yale University, New Haven, CT,

U.S.A.) and pBABE hygromycin-resistant cDNAs into HeLa cells with an Eppendorf InjectMan microinjection and micro-manipulation system. Colonies of stable transfectants were selected in Dulbecco's modified Eagle's medium (DMEM) containing 100 µg/ml hygromycin-B (Boehringer Mannheim). Stably transfected cell lines were maintained in a culture of DMEM (1000 mg/ml glucose/no sodium pyruvate), with 10% foetal calf serum (Helena Biosciences), 4 mM L-glutamine and 50 units/ml penicillin and 50 µg/ml streptomycin, using standard sterile techniques. KYM-1 cells were grown in RPMI 1640 supplemented with 4 mM L-glutamine, 50 units/ml penicillin, and 50 µg/ml streptomycin. A similar approach was taken for the stable HeLa cell line expressing CrmA protein (HeLa-CrmA), with the cDNA being introduced by Lipofectamine (Boehringer Mannheim) transfection. Positive colonies were hygromycin-resistant and displayed stable CrmA protein expression (results not shown). HeLa cells stably transfected with hygromycin-resistant cDNA alone (HeLa-Hygro) were used as control cells for both HeLa-CrmA and HeLa-TNFR2 hygromycin-resistant cell lines.

Cell death measurements

Cell death measurements were performed as described previously [22] using a 96-well Crystal Violet staining protocol.

In-cell caspase activity measurements

Treated cells were incubated for 1 h in the dark (37 °C) with 1 µM PhiPhiLux cell-permeable fluorogenic substrate (Onco-Immunin, Gaithersburg, MD, U.S.A.) that changes spectral characteristics with the appearance of an excitation peak at 505 nm and an emission peak at 450 nm upon caspase-3, or caspase-3-like, cleavage. Fluorescence microscopy images of UV-excited cells measured PhiPhiLux caspase-dependent cleavage. Transmission light was used to count the total number of cells to assess the percentage of the total cell population displaying positive TNF-induced caspase activity.

FACS

Cells were grown to approx. 70% confluency and dissociated from their culture vessels with 2 ml of trypsin-free cell dissociation solution (Sigma). Cells were washed once in serum-free DMEM and resuspended in serum-free DMEM to give a cell population of 5×10^6 cells. After a saponin-permeabilization step, a 200 µl aliquot of cells was incubated on ice for 1 h in a 1:200 dilution of primary antibody (mouse monoclonal 85 kDa PARP fragment-specific antiserum linked to FITC; BioSource). Prior to FACS analysis, cells were washed three times and resuspended in 1 ml of PBS+2% foetal bovine serum (v/v). FACS analysis was performed in a Becton-Dickson FACScalibur according to the manufacturer's standard protocols.

Confocal fluorescence microscopy

All cells were treated for the indicated time with either 50 ng/ml recombinant human TNF, R32WS86T mutated TNF (a TNFR1-specific 'mutein' termed R1-TNF [23]), D143NA145R TNF (a TNFR2-specific mutein termed R2-TNF) or with TNFR2-specific agonistic MR2-1 monoclonal antibody (1 µg/ml; kindly provided by Wim Buurman, Department of General Surgery, University of Maastricht, Maastricht, Netherlands [24]). Cells were fixed in ice-cold methanol for 20 s and washed with 2 ml of PBS (pH 7.2) after treatment of the cells for the prescribed time period. Cells were then labelled with 1:200 dilution of FITC-

labelled 85 kDa PARP fragment-specific primary antibody in Krebs solution (NaCl 137.4 mM, KCl 5.9 mM, $\text{CaCl}_2 \cdot 6\text{H}_2\text{O}$ 1.2 mM, MgCl_2 1 mM, Hepes 11.6 mM and glucose 11.5 mM) for 1 h. Fixed cells were incubated on a rocking platform for 1 h after which time the primary antibody was removed and plates were washed three times with Krebs solution. Confocal laser microscopy was measured on a Bio-Rad μ radiance system [25] measuring at the green/blue wavelength (480–520 nm) according to the manufacturer's protocol.

[^{125}I]TNF-binding analysis

Specific [^{125}I]TNF-binding experiments were performed essentially as described previously [23] using wild-type TNF, R1-TNF and R2-TNF as a competitive measurement of total, TNFR2- and TNFR1-specific binding respectively.

JNK activity

JNK activity was measured by assessing phosphorylation of its substrate c-Jun (5-89) linked to a glutathione S-transferase fusion protein (kindly provided by Robin Plevin, Strathclyde University, Strathclyde, U.K., and Jim Woodget, Toronto, Canada). Subconfluent cells were treated for 15 min with the indicated stimulus. Cell extracts were then prepared on ice by lysing cells in solubilization buffer with protease and phosphatase inhibitors [20 mM Hepes (pH 7.7), 50 mM NaCl, 0.1 mM EDTA, 1% Triton X-100, 0.2 mM PMSF, 2 $\mu\text{g}/\text{ml}$ leupeptin, 1 mM benzamide, 25 mM β -glycerophosphate and 0.2 mg/ml Na_3VO_4]. The cellular extracts were then affinity-precipitated by rotating with a slurry of c-Jun–glutathione S-transferase conjugated to glutathione–Sephadex beads at 4 °C for 2 h. The beads were recovered by centrifugation at 10000 *g* for 1 min, and washed once with 0.5 ml of solubilization buffer and once with 0.5 ml of kinase buffer [25 mM Hepes (pH 7.6), 20 mM MgCl_2 , 5 mM β -glycerophosphate, 0.1 mM Na_3VO_4 and 2 mM dithiothreitol]. Kinase reactions (performed at 30 °C) were initiated by the addition of 30 μl of kinase buffer with 2.5 mM ATP + 2 μCi per tube of [γ - ^{32}P]ATP. Reactions were stopped after 30 min by the addition of 6 $\times$ Laemmli sample buffer and boiling for 3 min. After SDS/PAGE (12% gels) the gels were dried on a vacuum gel drier (90 min, 80 °C). Phosphorylated c-Jun protein was visualized by exposure of the gel to autoradiographic film and quantified on a Bio-Rad densitometer.

Western analysis

Cells were treated with stimuli for the indicated times before cell monolayers were lysed using RIPA buffer (1 $\times$ PBS, 1% Nonidet-P40, 0.5% sodium deoxycholate and 0.1% SDS) supplemented with 0.1 mg/ml PMSF, 10 $\mu\text{g}/\text{ml}$ aprotinin and 1 mM Na_3VO_4 . After a 30 min incubation on ice, insoluble debris was pelleted (5 min, 10000 *g*, 4 °C) and discarded. Cell protein concentrations were determined by Lowry protein assay (Bio-Rad) then supplemented with 2 $\times$ Laemmli loading buffer (20% glycerol, 4% β -mercaptoethanol and 0.3% Bromophenol Blue) prior to SDS/PAGE (10% gels). Proteins were transferred to nitrocellulose (Costar) and then stained with the reversible protein-staining dye [0.1% Ponceau S (Sigma) in 3% trichloroacetic acid (w/v)] to determine even transfer and equal loading of protein samples. Nitrocellulose filters were blocked for 1 h with 5% fat-free skimmed milk in PBS + 0.5% Tween-20 before incubation with a 1:1000 dilution of primary antibody in PBS + 0.5% Tween-20 for 2 h. Three 5 min washes in PBS + 0.5% Tween-20 preceded incubation of the samples with a 1:5000 dilution of horseradish peroxidase-conjugated secondary antibody (Santa Cruz Bio-

technology) in PBS + 0.5% Tween-20 for 1 h. After a further three washes, specific protein–antibody interactions were detected by enhanced chemiluminescence (Amersham Bioscience) or by colorimetric staining with PBS containing 0.25 mg/ml *o*-dianisidine and 1 $\mu\text{l}/\text{ml}$ 30% H_2O_2 solution. Antiserum against cPLA $_2$ was procured from The Binding Site (Birmingham, U.K.). Phospho-specific MAPK and p38 MAPK antisera were acquired from New England Biolabs. All other antisera were obtained from Santa Cruz Biotechnology.

RESULTS

TNFR expression

Strains of the HeLa human cervical epithelial cell line, such as the HeLa-Hygro and HeLa-CrmA lines, contain almost exclusively TNFR1 subtype (3770 ± 902 specific [^{125}I]TNF receptors/cell; mean $\pm$ S.D. from triplicate determinations), with extremely low levels of TNFR2 (311 ± 398 receptors/cell). To efficiently measure TNFR2 actions, the HeLa-TNFR2 stable cell line was created [26] with similar levels of TNFR1 subtype expression (8064 ± 1188 receptors/cell) but with enhanced TNFR2 protein expression (68436 ± 5869 receptors/cell). By comparison, the KYM-1 human rhabdomyosarcoma cell line endogenously expresses good levels of both TNFR1 (8170 ± 1620 receptors/cell) and TNFR2 (11628 ± 1242 receptors/cell).

Role of caspases in TNF-induced cell death

In HeLa cells (with control hygromycin resistance), treatment with TNF (in the presence of 1 $\mu\text{g}/\text{ml}$ cycloheximide) leads to a concentration-dependent cell death through an apoptotic phenotype (Figure 1). This TNF-induced response is exclusively mediated by TNFR1, but not TNFR2, as judged by the TNFR1-specific mutant TNF, R1-TNF, being able to fully mimic the actions of wild-type TNF. It is not surprising that R2-TNF had no cell death effect, as TNFR2 is mostly absent from HeLa cells. The additional expression of CrmA completely abrogates any TNF-induced cell death response. Additionally, pharmacological inhibition of caspases by zVAD-fmk results in the complete inhibition of TNF-induced cell death. This is true of parental HeLa cells and of the HeLa-TNFR2 cells, which display much greater TNF-induced death responses [27–31], which is also seen in other cell types [32]. None of the other cell-permeable peptide inhibitors were able by themselves to block TNF-induced cell death. This was also true of KYM-1 cells in which only zVAD-fmk could block TNF-induced apoptotic cell death (results not shown).

TNFR-induced caspase activities

In-cell measurement of TNF-induced caspase-3-like activation was performed using the cell-permeable fluorogenic substrate PhiPhiLux (Figure 2). This allowed us an in-cell measurement of caspase activation through an independent protocol, helped to confirm the stimulation of caspase activity caused by TNF in our cells, and also had the advantage of experimentally confirming the effectiveness of the CrmA caspase-blocking protein. TNF-induced caspase activation could clearly be seen in HeLa-Hygro and KYM-1 cells. There was no such TNF-induced caspase activity in the HeLa cells expressing CrmA inhibitor, indicating that the downstream target of TNFR-associated caspase-8 (i.e. caspase-3) was functionally blocked by CrmA cowpox protein expression. The effectiveness of CrmA in blocking TNF-induced caspase activities was further demonstrated when measuring known caspase-dependent protein degradations. As seen in Figure 3, TNF-induced protein kinase C (PKC) δ degradation occurs in HeLa-Hygro cells (apparently through

Figure 1 Blockade of TNF-induced cell death by CrmA and caspase peptide inhibitors

Concentration–response curves for TNF-, R1-TNF- or R2-TNF-induced cell death in HeLa-Hygro (A) and HeLa-CrmA (B) cells. (C) Attenuation of TNF-induced cell death in HeLa-TNFR2 cells pre-incubated with the indicated peptide caspase inhibitors for 1 h before addition of TNF. Cell death was measured 24 h later as described in the Materials and methods section. Data are the means $\pm$ S.D. of 16 replicates from a single experiment representative of at least two other separate determinations which gave similar results.

TNFR1) but not in cells expressing CrmA protein. By comparison, a small amount of TNF-induced PKC ϵ degradation is observable, but again is not evident in CrmA-expressing cells.

PARP cleavage is one of the earliest signs of apoptotic cell death [33]. In order to assess the role of each TNFR in the caspase-dependent cleavage of PARP we used the TNFR-specific mutant TNFs in HeLa-TNFR2 cells (Figure 3). We see that in HeLa-Hygro cells, but not HeLa-CrmA cells, TNFR1 mediates the cleavage of 116 kDa PARP into a 85 kDa fragment. Using an antibody that specifically recognizes the degraded 85 kDa fragment of PARP, we see in HeLa-TNFR2 cells that PARP degradation is mediated by TNFR1 stimulation (seen using R1-TNF), which again was not observed in CrmA-expressing cells. Stimulation of TNFR2 is most efficiently observed using both R2-TNF and an agonistic monoclonal antiserum, MR2-1

[26]. Stimulation of TNFR2 was capable of inducing a minor amount of PARP cleavage as judged by Western, confocal and FACS analyses using the 85 kDa PARP fragment antiserum (Figures 3B–3D). The cleavage of PARP enzyme however could not be attributed to a single receptor and models such as the TNFR-ligand-passing model [8] could not be excluded. What is clear from these analyses is that CrmA expression not only obliterates TNF-induced cell death responses, but also effectively blocks TNF-induced caspase activity.

Activation of ERKs by TNFRs and the role of caspases

JNK activity can be stimulated by both TNFR1 and TNFR2 [5,26,30,31], an effect we also observe here (Figure 4). However, the additional presence of CrmA protein consistently reduced TNF-stimulated JNK activity in HeLa cells, particularly if compared with the anisomycin-positive control stimulus (Figure 4A). This is despite the anisomycin (a cell stress stimulus which directly activates stress kinases through a non-receptor-dependent mechanism) concentration–response relationship for JNK activation being very similar in HeLa-Hygro and HeLa-CrmA cells (Figure 4B). Therefore, CrmA appears to inhibit TNF stimulation of JNK activity to a significant degree, but without completely blocking cytokine-induced JNK activity. Similarly, inhibition of caspases by zVAD-fmk or zDEVD-fmk resulted in a concentration-dependent inhibition of TNF-, R1-TNF- and R2-TNF-stimulated JNK activity in HeLa-TNFR2 cells (Figure 4C). Once again, although zVAD-fmk could inhibit the TNFR response, the stimulated JNK activity was never fully blocked. The action of other peptide inhibitors to abrogate TNF-stimulated JNK activity showed that each of the peptide inhibitors was capable of markedly reducing the stimulated response, with zDEVD-fmk, the group III caspase inhibitor, appearing to inhibit the JNK response most effectively (Figure 4D).

In direct contrast to the caspase sensitivity of TNF-stimulated JNK activity, TNF-stimulated p42/44 MAPK and p38 MAPK activities showed no caspase sensitivity (Figure 5). MAPK and p38 MAPK activation, unlike JNK activation, occurs through only TNFR1 [26]. Therefore only R1-TNF is capable of stimulating p42/44 MAPK and p38 MAPK in HeLa-Hygro cells (also seen in HeLa-TNFR2 cells [26]). The activation profiles of p42/44 MAPK and p38 MAPK were identical in HeLa-CrmA cells, with CrmA expression resulting in no inhibitory effects of CrmA whatsoever (Figure 5A). Preincubation with the pharmacological group caspase inhibitors did not alter total protein levels of p42/44 MAPK or p38 MAPK, as judged using the pan-ERK antiserum. In HeLa-TNFR2 cells none of the cell-permeable peptide pharmacological caspase blockers resulted in an attenuation of TNF- or R1-TNF-stimulated activation of p42/44 MAPK or p38 MAPK. This is in direct contrast to the TNFR-induced caspase sensitivity observed in HeLa-TNFR2 cells seen in Figure 4.

Caspase dependency of TNF-induced cPLA₂ activation

The lack of inhibition of caspase sensitivity of TNF-stimulated MAPK or p38 MAPK activities was also seen when measuring TNF-stimulated cPLA₂ activation, a process known to be mediated through MAPK and p38 MAPK [34,35]. We see here that TNF-induced cPLA₂ phosphorylation, mediated by TNFR1, occurs equally well in HeLa-Hygro and HeLa-CrmA cells (Figure 6A). Similarly, in HeLa-TNFR2 cells, TNF-stimulated cPLA₂ phosphorylation occurs whether the cells have been pretreated with zVAD-fmk caspase inhibitor or not (Figure 6B).

Figure 2 TNF-induced in-cell caspase measurement is blocked in CrmA-expressing cells

Where indicated, HeLa-Hygro, HeLa-CrmA or KYM-1 cells were treated with 50 ng/ml TNF for 6 h before a 1 h incubation with 1 μ M PhiPhiLux cell-permeable caspase-sensitive fluorogenic indicator dye. In-cell caspase activity and assessment of positively fluorescent cell number was measured as described in the Materials and methods section.

These findings support our evidence for TNF-stimulated MAPK and p38 MAPK being a caspase-insensitive signalling process, and suggests that TNF-stimulated JNK activity, which is caspase-sensitive, does not contribute towards cPLA₂ phosphorylation.

TNF-induced caspases and their pharmacological profile

To try and understand which of the caspases may be involved in the modulation of the JNK activity observed, we determined the TNF-induced caspase activation profile in HeLa cells (Figure 7). As can be seen using Western analysis, caspase-8 and caspase-3 are activated rapidly after TNF treatment. Curiously, caspase-8 activation showed a biphasic response with repeated stimulation 4 h after the initial TNF stimulus (Figures 7A and 4B). This may be due to the induction of other factors by TNF that stimulate secondary caspase-8 activity. As expected, a more delayed

but sustained activation of caspase-6, caspase-7 and caspase-9 was detected, suggesting these caspases are downstream of the caspases 8 and 3. The pharmacological profile of these TNF-activated caspases was ascertained using the range of inhibitors used throughout this study (Figure 7B). Interestingly, we did not observe the inhibition of caspases that is often claimed by their manufacturers. Caspase-3 was inhibited least by zVEID, more by zVAD, and was blocked by zYVAD, zVDVAD and zDEVD (which showed the greatest inhibition). This pharmacological profile is the same as that observed when inhibiting JNK activation (Figure 4), and suggests that caspase-3 is the protease that is responsible for the modulation of the JNK activity that we observe. JNK activity was markedly inhibited by zVEID (Figure 4), which poorly inhibits TNF-induced caspase-3 activation. However, a role for caspase-6 could not be excluded in TNF-induced JNK activation, as both JNK activity and caspase-6 stimulation were clearly inhibited by zVEID

Figure 3 TNF-induced caspase actions in HeLa-Hygro, HeLa-CrmA and HeLa-TNFR2 cells

(A) Western analysis of 50 ng/ml TNF-, R1-TNF- or R2-TNF-induced PKC δ , PKC ϵ and PARP degradation in HeLa-Hygro and HeLa-CrmA cells. Cell were preincubated for 1 h with 1 μ g/ml cycloheximide before addition of TNF stimuli for 4 h. (B) Western analysis of 50 ng/ml TNF, R1-TNF or R2-TNF ($\pm$ 1 μ g/ml MR2-1 monoclonal antibody)-induced PARP fragment generation in HeLa-CrmA and HeLa-TNFR2 cells. Where indicated, cells were preincubated for 1 h with 1 μ g/ml cycloheximide (CHX) before addition of TNF stimuli for 4 h. (C) Confocal microscopy demonstrating the production of 85 kDa PARP fragment immunoreactivity in HeLa-CrmA and HeLa-TNFR cells, in response to TNF, TNFR1-specific or TNFR2-specific stimuli as indicated above. (D) FACS analysis of 85 kDa PARP fragment immunoreactivity in HeLa-CrmA and HeLa-TNFR cells, in response to TNF, TNFR1-specific or TNFR2-specific stimuli as indicated above. Data are from a representative experiment repeated at least two other times with similar findings.

(Figure 7B). Apart from caspase-3 and caspase-6, other caspases did not show the corresponding profile of inhibition that would indicate their direct involvement in modulating JNK activation.

DISCUSSION

TNF is an important and highly conserved cytokine with a role in the regulation of life and death processes in many cell types. In HeLa cells, TNF-induced cell death (mediated by TNFR1) is

completely inhibited by CrmA. This serpin-like protein produced by cowpox virus functions to stop cell death in its infected host cell in order to ensure continuous viral propagation. It has been shown to achieve these anti-apoptotic effects by inhibiting mainly caspases 1 and 8 [18,19]. Although CrmA has dramatic effects here on cell death it was completely unable to alter TNF-induced MAPK and p38 MAPK activation. This suggests that the upstream kinases from MAPK and p38 MAPK (MKKs 1 and 2, and MKKs 3 and 6 respectively) are activated in a mechanism

Figure 4 TNF-induced JNK activity in HeLa cells is caspase-sensitive

(A) JNK activation assay in HeLa-Hygro or HeLa-CrmA cells treated for 20 min with 50 ng/ml TNF (from a commercial source), wild-type TNF (wt-TNF), R1-TNF or R2-TNF ($\pm 1 \mu\text{g/ml}$ MR2-1 mAb) or with 200 nM anisomycin as a positive control. (B) Anisomycin concentration-response relationship in HeLa-Hygro and HeLa-CrmA cells. The indicated concentration of anisomycin was incubated on the cells for 20 min before JNK activity was determined as described in the Materials and methods section. (C) JNK activation by 50 ng/ml TNF, R1-TNF or R2-TNF in HeLa-TNFR2 cells. Cells were preincubated for 1 h with the indicated concentration of zVAD-fmk or zDEVD-fmk before TNF stimuli. The lower concentration-response panels indicate the means $\pm$ S.E.M. of at least three independent repeat experiments. (D) JNK activation by 50 ng/ml TNF in HeLa-TNFR2 cells preincubated for 1 h with 30 μM of the indicated caspase peptide inhibitor before TNF stimuli. The right-hand panel indicates the means $\pm$ S.E.M. of at least three independent repeat experiments.

that does not require caspase function. This is contrary to findings using other stimuli which suggested that upstream kinases of MAPK and p38 MAPK were dependent on caspase activity and were inhibitable by CrmA [36–38]. Clearly, there may be more complex factors which contribute towards the caspase-dependency of ERKs, as stimulation of JNK and p38 MAPK

activity in HeLa cells by photodynamic therapy was not affected by caspase inhibition through pharmacological inhibitors or by CrmA [39].

We find a role for caspases in the TNF activation of JNK, suggesting its upstream kinases MKK4/SEK1 and/or MKK7 are modulated by a TNF-inducible caspase activity. However,

Figure 5 TNF-induced p42/44 MAPK and p38 MAPK activity in HeLa cells is caspase-insensitive

(A) Western analysis of p42/44 MAPK and p38 MAPK activation in HeLa-Hygro or HeLa-CrmA cells treated for 30 min with 50 ng/ml TNF, R1-TNF or R2-TNF. ERK activation was assessed with phospho-specific antisera as described in the Materials and methods section. (B) p42/44 MAPK and p38 MAPK activation in HeLa-TNFR2 cells treated for 30 min with 50 ng/ml TNF or R1-TNF. Cells were preincubated for 1 h with 30 μ M of the indicated caspase peptide inhibitor before TNF stimuli. ERK activation was assessed with phospho-specific antisera and total ERK protein levels were determined by a pan-MAPK or pan-p38 MAPK antibody. Data are from a representative experiment repeated at least two other times with similar findings.

although zVAD-fmk and CrmA were able to attenuate TNF-induced JNK activity, we were unable to see a complete block of TNF-induced JNK activity either by pharmacological inhibition or CrmA inhibition of caspases. This suggests that a caspase-dependent and a caspase-independent pathway exists for the TNF induction of JNK activity. Similar caspase-dependent and -independent pathways have been revealed in TNF-stimulated hepatocytes [40]. The ability of CrmA and zVAD-fmk to completely block TNF-induced cell death, but still allow some (albeit reduced) JNK activity, implies that JNK is not crucial to the death processes, but may in some way contribute or be involved [41]. Likewise the excellent inhibition of TNF-stimulated JNK activity by zDEVD-fmk (Figure 4), but complete lack of inhibition of TNF-induced death, suggests that group III caspases (inhibited by zDEVD-fmk) contribute towards TNF/JNK signalling, but are insufficient on their own to control cell death (a process achieved only by the broader-spectrum caspase inhibitor zVAD-fmk). This would imply that there is more than one pathway responsible for the activation of JNK in response to TNF. Indeed, other groups have observed similar findings [42].

Figure 6 TNF-induced cPLA₂ activation in HeLa cells is caspase-insensitive

(A) Western analysis of cPLA₂ activation in HeLa-Hygro or HeLa-CrmA cells treated for 30 min with 50 ng/ml TNF, R1-TNF or R2-TNF as described in the Materials and methods section. 100 nM phorbol 12,13-dibutyrate (PDBu) incubation is used as a positive control. (B) 50 ng/ml TNF-induced cPLA₂ activation in HeLa-TNFR2 preincubated for 1 h where indicated, with 30 μ M zVAD-fmk before 30 min TNF stimuli. Data are from a representative experiment repeated at least one other time with similar findings.

A possible explanation for these findings could be found at the intracellular level. The TNFR1, with its death domain, can associate with TRADD/FADD/caspase-8, but as TNFR2 does not possess a death domain it can only associate with TRAFs. TRAF-2 has been shown to associate with TRADD and RIP in the activation of nuclear factor- κ B. TRAF-2 has also been shown to be partially required for the activation of JNK. This evidence, along with our findings, would seem to imply that TNF can activate JNK through two different cascades, and that there is potentially cross-talk between TNFR1 and TNFR2 mediated via TRAF-2. Roulston and co-workers [43] have also suggested that kinetics of JNK activation are bi-phasic and that there is an early caspase-independent phase, and a late caspase-dependent stage. The role of JNK in a protective role or in an apoptotic role has yet to be clearly established. There have been some who suggested that it is protective [13], and others who have suggested that it antagonizes the action of other anti-apoptotic molecules [44], such as Bcl-2 [45].

Here we have shown that PARP cleavage in response to TNF can only take place in the presence of a protein-translation inhibitor, cycloheximide, in order to remove the dominant effect that the production of protective proteins has on TNF-induced apoptosis [46] and to inhibit production of *de novo* PARP protein synthesis. Secondly we observed that PARP cleavage is a CrmA-inhibitable process, which is consistent with previous findings [33,39]. The process by which PARP is cleaved is believed to take place through an apoptotic cascade initiated by the association of TNFR1 with intracellular proteins which possess a death domain, FADD and TRADD. These in turn associate with procaspase-8, an apical caspase, which triggers its cascade, eventually leading to the cleavage of PARP by the effector caspases 3 and 7. Interestingly, PARP cleavage was detected in cells which were stimulated by TNFR2 activation (Figure 3), a process which was still CrmA-inhibitable. This would appear to suggest that the apoptotic pathways involved in PARP cleavage are the same for both TNFR subtypes. Haridas et al. [31] also observed TNFR2-mediated PARP cleavage, but had ascribed this response to an overexpression artifact of transfected TNFR2 protein. Obviously, TNFR2 as well as TNFR1 is capable of

Figure 7 TNF-induced caspase activation in HeLa-TNFR2 cells and inhibition by peptidic fmks

(A) Western analysis of caspase activation in HeLa-TNFR2 cells treated for the indicated time with 50 ng/ml TNF. Activation is indicated by increased band intensity with active fragment caspase-3, -8 and -7 antisera, or reduced band intensity of full-length caspase-6 and -9 antisera. (B) HeLa-TNFR2 cells were preincubated with the indicated caspase inhibitor for 1 h prior to the addition of 50 ng/ml TNF (time point 0 h), then caspase activation was assessed as in (A). Data are from a representative experiment repeated at least two other times with similar findings.

caspase cascade activation, either by a direct mechanisms or indirectly, and the list of procaspase-interacting receptor-associated molecules is growing: RIP2 and procaspase-1, RAIDD (RIP-associated ICH-1/CED-3-homologous protein with a death domain) and procaspase-2, TRAF-2 and procaspase-12.

The PKC family of isoenzymes have also emerged as prospective downstream signals for TNFRs. Here we decided to study the effect of TNF on the levels of two members of the novel family

of PKC δ and PKC ϵ . TNF had little effect on PKC ϵ levels in both HeLa-CrmA and HeLa-Hygro cell lines. However, in HeLa-Hygro cells, there was a reduction in the levels of PKC δ in response to treatment with TNF. The ability of PKC δ to phosphorylate TNFR1 has been demonstrated by Kilpatrick et al. [47], who were also able to show that it co-localizes with TNFR1. Other PKC isoenzymes have also been demonstrated to play a role in TNF signalling. The atypical PKCs λ/ι have been

shown to activate nuclear factor- κ B through the RIP/NIK (nuclear factor κ B-inhibitor kinase)/IKK β (inhibitor of κ B kinase) pathway but only in a cell-type-specific manner [48,49]. The classical PKCs β/α have been implicated in both cell death [50] and cell survival [51].

The use of pharmacological inhibitors of caspases to investigate the role of these proteases is common practice in the field. Here we found that the range of cell-permeable caspase inhibitors such as zVAD-fmk and zDEVD-fmk are not broad-spectrum caspase blockers. The caspase inhibitors are, to varying degrees, able to reduce most caspase activation, with caspase-8 being particularly resistant to inhibition with all compounds except zYVAD-fmk (Figure 7). The pharmacological profiles of inhibition of caspase activation (Figure 7) and JNK inhibition (Figure 4) indicates that the most likely candidates for caspases that modulate JNK activity are caspase-3 and caspase-6. Multiple stimuli are capable of regulating caspase-3 activation and there exist probably more signalling means by which this protease is regulated. Interestingly, we can never fully block TNF-induced JNK activation by inhibition of caspases, suggesting that caspase-3 modulates JNK activation processes but is not critical in the signalling through this stress kinase, and that there are likely to be other steps that input into JNK activation signalling pathways.

Both TNFR1 and TNFR2 activate JNK, whereas TNFR1 (but not TNFR2) activates MAPK and p38 MAPK pathways [26]. This implies that there may be distinct ERK signalling processes between TNFR1 and TNFR2. The findings here further support a dichotomy between TNFR subtype signalling mechanisms, with JNK activation being caspase-sensitive, unlike MAPK and p38 MAPK cascades (which are TNFR1-activatable only). An enzyme reported to be phosphorylated and activated by MAPK and p38 MAPK is cPLA₂ [34,52], which has also been observed to be important in TNF-induced cell death mechanisms [53]. Inhibition of caspase function in CrmA cells or zVAD-fmk-treated cells still resulted in TNFR1-mediated phosphorylation of cPLA₂. These findings tell us that caspase-insensitive cPLA₂ activation occurs through MAPK and p38 MAPK, but not JNK. This further supports a functional dichotomy in TNFR-induced ERK signalling, and may diminish the importance of cPLA₂ activation in TNF-induced HeLa cell death.

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