

Type II tumour necrosis factor- α receptor (TNFR2) activates c-Jun N-terminal kinase (JNK) but not mitogen-activated protein kinase (MAPK) or p38 MAPK pathways

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The pleiotropic actions of tumour necrosis factor- α (TNF) are transmitted by the type I 55 kDa TNF receptor (TNFR1) and type II 75 kDa TNF receptor (TNFR2), but the signalling mechanisms elicited by these two receptors are not fully understood. In the present study, we report for the first time subtype-specific differential kinase activation in cell models that respond to TNF by undergoing apoptotic cell death. KYM-1 human rhabdomyosarcoma cells and HeLa human cervical epithelial cells, engineered to overexpress TNFR2, displayed c-Jun N-terminal kinase (JNK) activation by wild-type TNF, a TNFR1-specific TNF mutant and a TNFR2-specific mutant TNF in combination with an agonistic TNFR2-specific monoclonal antiserum. A combination of the TNFR2-specific mutant and agonistic antiserum elicited maximal endogenous or exogenous TNFR2 responsiveness. Moreover, alternative expression of a TNFR2 deletion mutant lacking its cytoplasmic domain rendered the cells unable to activate JNK activity through this receptor subtype. The profile of JNK activation by TNFR1 was

more transient than that of TNFR2, with TNFR2-induced JNK activity also being more sensitive to the caspase inhibitor, benzyloxycarbonyl-Val-Ala-DL-Asp-fluoromethylketone. Conversely, only activation of the TNFR1 could stimulate mitogen-activated protein kinase (MAPK) or p38 MAPK activities in a time-dependent manner. The role of TNFR2 activation in enhanced apoptotic cell death was confirmed with agonistic monoclonal antisera in cells expressing high levels of TNFR2. Activation of TNFR2 alone elicited cell death, but full TNF-induced death required stimulation of both receptor types. These findings indicate that efficient activation of TNFR2 by soluble TNFs is achievable with co-stimulation by antisera, and that both receptors differentially modulate extracellular signal-regulated kinases contributing to the cytokine's cytotoxic response.

Key words: apoptosis, cytokine receptors, protein kinases/phosphatases, signal transduction.

INTRODUCTION

Tumour necrosis factor- α (TNF) is a member of a unique superfamily of ligands whose members also include lymphotoxin (LT)- α , Apo-1/Fas ligand, CD40 ligand, CD30 ligand, CD27 ligand, OX40 ligand, CD137 ligand, nerve growth factor, LT- β and several viral gene products [1,2]. This TNF superfamily of cytokines is expressed mainly as biologically active type-II membrane proteins from which soluble forms are derived by proteolytic cleavage. This ligand family activates a corresponding family of structurally related receptors. All members of the TNF receptor (TNFR) family recognize their ligands through a common sequence motif, a cysteine-rich structure of about 40 amino acids, that is found in different numbers (three to six copies) on their extracellular domains. In contrast, the amino acid sequences of the intracellular domains of the TNFR family appear largely unrelated. These receptors initiate signals that regulate cellular actions, including gene induction for immune responses and inflammatory reactions, cell death mechanisms, proliferation, nuclear factor κ B ('NF- κ B') activation and, through excessive signalling, the pathology of a variety of diseases [3].

TNF is one of the main regulators of inflammation and immunity, capable of eliciting a broad spectrum of biological effects, the nature of which depend on the type and growth state of the target cell [4]. TNF has been shown to modulate the proliferation, differentiation and the apoptotic or necrotic cell death in a number of different cell lines. Within a particular cell line, TNF can initiate two distinct pathways, one leading to cell death and the other to the production of 'protective' proteins that are anti-apoptotic. The result of TNF stimulation depends on the weighting of these two pathways [5,6]. This disparity in TNF-induced responses is due, in part, to the presence of two distinct TNF-specific plasma membrane-localized receptors, type I 55 kDa TNFR (TNFR1) and type II 75 kDa TNFR (TNFR2), which are expressed on the surface of most cells, although in different amounts [7]. TNFR1 is expressed ubiquitously, whereas TNFR2 expression is tightly regulated and found predominantly on haemopoietic and endothelial cells.

The role of TNFR1 and TNFR2 in mediating the signals for cell death is widely debated. It is likely that both TNFRs participate in cell death, with TNFR1 being more potent than TNFR2 in this action [8]. A ligand-passing model, in which TNFR2 may facilitate the association of TNF with

Abbreviations used: ATF-2, activating transcription factor-2; ATA, aurintricarboxylic acid; DMEM, Dulbecco's modified Eagle's medium; ERK, extracellular signal-regulated kinase; FADD/MORT, Fas-associated protein with death domain; FBS, foetal bovine serum; FLICE, Fas-like interleukin 1 β -converting enzyme; IAP, inhibitor of apoptosis; LT, lymphotoxin; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; MAPKAPK, MAPK-activated protein kinase; MEK, MAPK/ERK kinase; MKK, MAPK kinase; PI, propidium iodide; TRADD, TNF-associated protein with a death domain; TNF, tumour necrosis factor- α ; TNFR, TNF receptor; TNFR1, type I 55 kDa TNFR; TNFR2, type II 75 kDa TNFR; TRAF, TNFR-associating factor; Z-VAD-FMK, benzyloxycarbonyl-Val-Ala-DL-Asp-fluoromethylketone.

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TNFR1, can explain the contribution of TNFR2 to cytotoxicity [9]. The rapid association/dissociation kinetics of TNFR2 would enable this receptor to serve as a ligand source for TNFR1, which by itself displays much slower ligand association/dissociation kinetics [10]. The actions of TNFR2 may have been experimentally underestimated since membrane-bound TNF completely activates both receptor subtypes, whereas soluble forms of TNF (as most commonly used) are extremely inefficient activators of TNFR2 [11]. Therefore most evidence in which receptors are activated by soluble TNF will be a reflection of mainly TNFR1 activity. There is evidence that TNFR2 is capable of signal transduction to induce cellular responses, in particular when triggered by agonistic receptor-specific antibodies [12–16]. These responses include stimulation of proliferation in mitogen-activated thymocytes and T cells as well as production of granulocyte/macrophage colony-stimulating factor ('GM-CSF').

Signalling of TNFR superfamily members depends on ligand-induced oligomerization and recruitment of adapter proteins that lead to either gene induction, via activation of transcription factors, or apoptosis, via the activation of a proteolytic caspase cascade. What precisely determines the balance between both major pathways is currently not known. In the case of TNF, the signalling outcome is complicated by the complex interplay of its two receptors [7,17]. Distinct receptor-associating adapter proteins have been demonstrated to interact differentially with each receptor family member, indicating distinct cascade pathways for each receptor. In this context, it is important to understand the molecular mechanisms of TNFR2-induced signalling and its possible interplay with signalling induced by TNFR1. TNFR1 has been shown to recruit a variety of proteins, including TNFR-associated protein with a death domain (TRADD), Fas-like interleukin 1 β -converting enzyme (FLICE; also known as caspase-8), receptor interacting protein ('RIP'), Fas-associated protein with death domain (FADD/MORT), TNFR-associated factor (TRAF) 2, *Ice* and *Ced* homologue-1 ('ICH-1'; also known as caspase-2), mitogen-activating protein kinase (MAPK)-activating death domain ('MADD'), TRAF-interacting protein ('TRIP'), FLICE inhibitor (I-FLICE), A-29 and TRAF-interacting protein (I-TRAF). TRADD functions as a platform adapter that recruits several signalling molecules to the activated receptor [18]. The Apo-1/Fas receptor recruits an adapter protein called FADD/MORT. The death domains in FADD/MORT and TRADD can bind to each other, suggesting that TNFR1-induced cell death not only recruits TRADD but also associates FADD/MORT. TNFR2, like TNFR1, can also recruit TRAF2, but also utilizes TRAF1, inhibitor of apoptosis (IAP)-1 and IAP-2. The role of these factors in TNF signalling and cell death mechanisms have yet to be fully elucidated.

Extracellular signal-regulated kinases (ERKs) are central mediators that propagate extracellular signals inside the cell and are activated by diverse stimuli, including cytokines [19]. The three mammalian ERK families shown to be activated by TNF binding are the MAPKs, the c-Jun N-terminal kinases (JNKs) and p38 MAPK. The p44/p42 MAPK group (also termed ERK1 and ERK2, respectively) are activated by phosphorylation via MAPK/ERK kinase (MEK). Once activated, MAPK mediates biological responses involved in cell proliferation and differentiation by phosphorylating a number of substrates, including cytosolic phospholipase A₂ ('cPLA₂') and the transcription factors c-Myc, activating protein-1 ('AP-1'), nuclear factor-interleukin 6 ('NF-IL6'), activating transcription factor-2 (ATF-2) and Elk-1. The dual specificity kinases MAPK kinase (MKK) 4 (also known as stress-activated protein kinase/ERK kinase 1, 'SEK1') and MKK7 activate JNKs, whereas p38 MAPKs are

activated by the MKK3/6 homologues. Once activated, JNKs mediate the phosphorylation and activation of the transcription factors c-Jun, ATF-2 and Elk-1. The p38MAPK cascade is likewise involved in the transcriptional regulation of ATF-2 and Elk-1 as well as CHOP ('CCAAT-enhancer-binding protein homologous protein') and the activation of MAPK-activated protein kinases (MAPKAPK) 2/3, which in turn phosphorylate small heat-shock proteins.

As the MAPK, JNK, and p38 MAPK pathways have been proven to be important in TNF-mediated signalling, the aim of the present study was to investigate the role of both TNFR subtypes in the activation of the ERKs and the modulation of the subsequent cell death responses by using various human cell lines, which express differing relative amounts of TNFR2 and respond to TNF by undergoing apoptotic cell death.

MATERIALS AND METHODS

Materials

Recombinant human TNF was purchased from R&D Systems (Abingdon, Oxon., U.K.). The cytokine's biological activity was confirmed by measurement in the L929 cytotoxicity assay by comparing with the cytotoxic activity of TNF standards (kindly provided by Dr Meenu Wadhwa, National Institute of Biological Standards and Controls, Potters Bar, Herts., U.K.). Each batch of TNF was confirmed to have at least 2×10^7 WHO units/mg. Subtype-specific polyclonal and monoclonal agonistic antisera were a gift from Wim Buurman (Department of Surgery, University of Maastricht, Netherlands). Phospho-specific MAPK and p38 MAPK antisera were purchased from New England Biolabs (Hitchin, Herts., U.K.). Pan MAPK and p38 MAPK antisera used as a control were obtained from Santa Cruz Biotechnology (Heidelberg, Germany). ¹²⁵I-labelled TNF (specific radioactivity 500–100 Ci/mmol), [γ -³²P]ATP (specific radioactivity > 3000 Ci/mmol) and glutathione-Sephadex 4B beads were purchased from Amersham Pharmacia Biotech (Little Chalfont, Bucks., U.K.). Compounds PD98059, SB203580 and Z-Val-Ala-DL-Asp-fluoromethylketone (Z-VAD-FMK) were bought from Calbiochem (Nottingham, U.K.). c-Jun (5–89)-glutathione-S-transferase (GST) and MAPKAPK 2-GST chimeric-expressing bacteria were generously provided by Professor James Woodgett (Ontario Cancer Institute, University of Toronto, Canada), Professor Chris Marshall (CRC Institute of Cancer Research, London, U.K.) and Dr Robin Plevin (Department of Physiology and Pharmacology, University of Strathclyde, U.K.). All other materials were from BDH (Poole, Dorset, U.K.) or from the Sigma (Poole, Dorset, U.K.) and were of the highest grade obtainable.

Cell culture

Human rhabdomyosarcoma cells KYM-1 (a gift from Professor Terje Espevik, Institute of Cancer Research and Molecular Biology, University of Trondheim, Norway) were grown in RPMI 1640 medium supplemented with 10% (v/v) foetal bovine serum (FBS; Helena Biosciences, Sunderland, U.K.), 100 μ g/ml penicillin and 100 μ g/ml streptomycin. HeLa cells were co-transfected on an Eppendorf Transjector 5246/InjectMan micro-manipulator and microinjection system with cDNAs encoding the human TNFR2 (with permission from Professor Werner Lesslauer, Hoffmann La Roche, Basel, Switzerland) and pBABE Hygromycin-resistance. Positive colonies were selected by growth in Dulbecco's modified Eagle's medium (DMEM) containing

10% (v/v) FBS, 1 mM L-glutamine, 100 µg/ml penicillin, 100 µg/ml streptomycin and supplemented with 100 µg/ml Hygromycin B (Boehringer Mannheim, Roche Diagnostics Ltd., Lewes, East Sussex, U.K.). Positive colonies were expanded and characterized for TNFR2-expression levels. A similar TNFR2-overexpressing HeLa clone (HeLa-TNFR2 clone #2) and HeLa cells expressing comparable levels of a TNFR2 deletion mutant lacking the cytoplasmic region (TNFR2^{cyt-}) were gifts from Dr Harald Wajant (Institute of Cell Biology and Immunology, University of Stuttgart, Germany).

FACS analysis

Cells were grown to approx. 80% confluency and dissociated from their culture vessel with 2 ml of trypsin-free cell dissociation solution (Sigma). Cells were washed once in 5 ml of serum-free DMEM and resuspended in serum-free DMEM to give a cell population of 5×10^6 cells. An aliquot (200 µl) of the cells was incubated on ice for 1 h in a 1:200 dilution of primary antibody (mouse anti-human TNFR1 or TNFR2 monoclonal sera: antisera htr9 and utr1, respectively; BMA Biomedicals, Augst, Switzerland). Cells were centrifuged at 1000 g for 2 min at 25 °C, the supernatant was removed and the pellet resuspended in 200 µl of serum-free DMEM before the addition of a 1:50 dilution of secondary antibody (FITC-labelled anti-mouse IgG antibody; Scottish Antibody Production Unit, Law Hospital, Carlisle, Lanarkshire, Scotland, U.K.) and incubation on ice for a further 1 h. Prior to FACS analysis, the cells were washed three times and resuspended in 1 ml of PBS [137 mM NaCl/2.68 mM KCl/4 mM Na₂HPO₄/1.76 mM KH₂PO₄/ (pH 7.2–7.4)] containing 2% (v/v) FBS. FACS analysis was performed on a Becton Dickinson FACSort (Becton Dickinson U.K. Ltd, Oxford, U.K.) according to the manufacturer's protocols.

Confocal laser scanning fluorescence microscopy

Cells were fixed with ice-cold methanol for 20 s and washed with 2 ml of PBS (pH 7.2) prior to labelling with anti-TNFR monoclonal antibody at a 1:100 dilution in Krebs solution (137.4 mM NaCl/5.9 mM KCl/1.2 mM CaCl₂·6H₂O/1 mM MgCl₂·6H₂O/11.6 mM Hepes/11.5 mM glucose) on a rocking platform for 60 min. Primary antibody was removed and replaced by a 1:100 dilution of FITC-labelled anti-mouse antibody. Cells were incubated in the dark at 25 °C for a further 60 min on a rocking platform and washed three times in Krebs solution prior to visualization of the labelled antibody. Confocal laser microscopy was performed on a Bio-Rad µradiance system (Hemel Hempstead, Herts., U.K.) measuring fluorescence at green/blue wavelengths (480–520 nm).

¹²⁵I-labelled TNF binding analysis

Mutational analysis of human TNF revealed that certain mutations of the wild-type sequence could enable the mutated protein to bind selectively to either of the TNFR subtypes. The specific double mutation of R32W,S86T (here termed R1-TNF) allows selective activation of TNFR1 only by this mutant protein ('mutein'), whereas the D143N,A145R (termed R2-TNF) double mutation allows selective activation of the TNFR2 subtype only [20,21]. Specific binding analysis was performed essentially as previously described [16] using ¹²⁵I-labelled TNF and a 200-fold excess of non-radiolabelled TNF for total and non-specific

binding determinations, respectively. TNFR1-specific binding was determined with a 200-fold excess of R2-TNF; conversely, TNFR2-specific binding was determined in the presence of R1-TNF [16]. Binding to confluent cells in serum-containing medium [DMEM containing 10% (v/v) FBS, 1 mM L-glutamine, 100 µg/ml penicillin and 100 µg/ml streptomycin] (100 µl/well) was performed on ice in 96-well plates, preincubated for 40 min with unlabelled TNF, R1-TNF or R2-TNF before addition of 2×10^5 c.p.m. of ¹²⁵I-labelled TNF/well. Plates were incubated on ice for a further 1 h, then washed three times with 200 µl of PBS containing 0.1% BSA/well and counted for radioactivity on a Wallac 96-well counter (EG & G Wallac/PerkinElmer Instruments, Bracknell, Berks., U.K.).

JNK activity

JNK activity was measured by assessing phosphorylation of its substrate c-Jun (5–89) linked to a GST fusion protein essentially as previously described [16]. Subconfluent cells were treated for 15 min with the indicated stimulus. Cell extracts were then prepared on ice by lysing the cells in solubilization buffer containing 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 µg/ml leupeptin/1 mM benzamidine/25 mM β-glycerophosphate/0.2 mg/ml Na₃VO₄]. The cellular extracts were then affinity-precipitated by rotating with a slurry of c-Jun–GST conjugated to glutathione–Sepharose beads at 4 °C for 2 h. The beads were recovered by centrifugation at 10000 g for 1 min at 25 °C, 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 MgCl₂/5 mM β-glycerophosphate/0.1 mM Na₃VO₄/2 mM dithiothreitol]. Kinase reactions (performed at 30 °C) were initiated by the addition of 30 µl of kinase buffer with 2.5 mM ATP and 2 µCi of [γ-³²P]ATP per tube. Reactions were stopped after 30 min by the addition of 6 × Laemmli sample buffer (12% SDS/60% glycerol/12.5% β-mercaptoethanol/1% Bromophenol Blue) and boiling for 3 min. Samples were separated by SDS/PAGE using 12% (w/v) acrylamide gels, and gels were dried on a vacuum gel drier for 90 min at 80 °C. Phosphorylated c-Jun protein was visualized by exposure of the gel to X-ray film and quantified on a Bio-Rad densitometer.

Western blotting

Cells were grown in six-well plates up to > 90% confluency and were serum-starved for 4 h by incubation in RPMI supplemented with 0.1% BSA. After the addition of various stimuli, cell monolayers were directly lysed with 70 mM Tris/HCl (pH 6.8)/2% SDS and scraped into Eppendorf tubes. The nucleic acids were sheared by passing through a 22-gauge needle, and lysates were boiled for 15 min to completely denature the proteins and nucleic acids. Whole cell-sample-protein concentrations were determined by a detergent-compatible Lowry protein assay (Bio-Rad) and 6 × loading buffer (60% glycerol/12.5% β-mercaptoethanol/1% Bromophenol Blue) was added to each sample. Equal amounts of total protein in each sample were separated by SDS/PAGE [12% (w/v) acrylamide gel] and transferred on to a 0.45 µm nitrocellulose membrane (Costar, Poole, Dorset, U.K.). Equal loading and transfer of proteins were determined by staining the membrane with reversible protein stain solution [0.1% Ponceau S in 3% (v/v) trichloroacetic acid]. After washing thoroughly in transfer buffer (25 mM Tris/250 mM glycine/20% methanol) to remove all trace of Ponceau

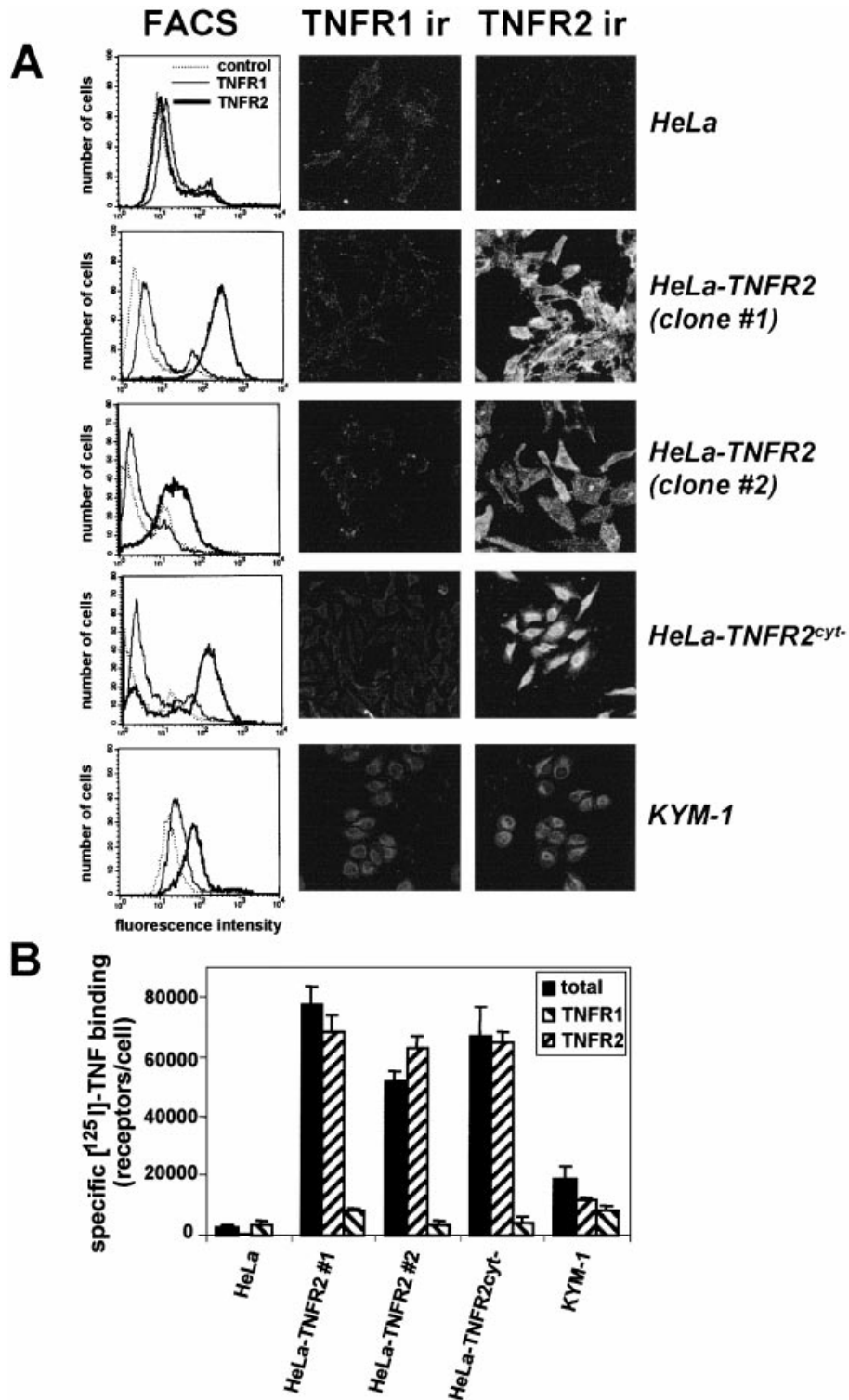


Figure 1 TNFR expression and ligand binding on HeLa and KYM-1 cells

(A) The expression of TNFR1 and TNFR2 was analysed using receptor-subtype-specific monoclonal antisera in HeLa clones and KYM-1 cells as described in the Materials and methods section. FACS analysis was performed with control and FITC-labelled secondary antibody or anti-TNFR1 or anti-TNFR2 and FITC-labelled secondary antibody as indicated. Ungated, entire populations are shown. Laser confocal microscopy of TNFR immunoreactivity (ir) is shown with exactly the same laser power and lens aperture settings to reflect relative antisera binding. The results are from a representative experiment from at least two other independent determinations with essentially the same findings. (B) Functional TNFR1 and TNFR2 expression was analysed in the indicated cell lines using ¹²⁵I-labelled TNF binding and receptor-subtype-specific mutant protein TNFs. Confluent whole cell analysis was performed in 96-well plates. Non-specific binding was determined with a 200-fold excess of wild-type TNF and was subtracted from the data. Total TNFR binding was determined using ¹²⁵I-labelled TNF, whereas functional TNFR2 and TNFR1 were measured using a 200-fold excess of R1-TNF and R2-TNF, respectively. The results are means \pm S.D. of quadruplicate determinations from a single representative experiment repeated at least once with similar findings.

stain, non-specific binding sites were blocked with PBS containing 0.5% Tween-20 (PBST) and 5% (w/v) non-fat dry milk for 1 h at 25 °C. Primary antibody (diluted 1:100 in PBST) was incubated with the membrane for 2 h at 25 °C, the membrane was washed (three times for 5 min in PBST) and then incubated for 1 h with a species-specific secondary antibody (diluted 1:2000 in PBST) conjugated to horseradish peroxidase (Santa Cruz Biotechnology, Heidelberg, Germany). After further washing (three times for 5 min in PBST and then twice for 2 min in PBS), specific protein-antibody interactions were detected with enhanced chemiluminescence detection agents (Amersham Pharmacia Biotech) or by colorimetric staining with PBS containing 0.25 mg/ml *o*-dianisidine and 10 μ l of 30% (v/v) H₂O₂ [22,23]. Phosphorylated p42/p44 MAPK and p38 MAPK protein were quantified on a Bio-Rad densitometer.

Cell death measurements

Cells were aliquoted into 96-well culture plates at a density of 1×10^5 cells/ml in serum containing medium (100 μ l/well) as previously described [22,23]. After growth for 24 h, the plates were treated with the required combination of agents and incubated for a further 24 h. Colorimetric determination of the attached cell number was performed by washing the wells with PBS and then fixing the remaining attached cells with PBS containing 10% formaldehyde for 20 min. After another wash with PBS, fixed cells were stained with 0.1% Crystal Violet/18% (v/v) ethanol in PBS for 30 min. Plates were washed four times in water and dried overnight. To each well, 100 μ l of 0.1 M sodium citrate, pH 4.2/ethanol (1:1, v/v) was added for 30 min before colorimetric intensity was read at 570 nm in a 96-well microplate reader (Molecular Devices, Crawley, Sussex, U.K.). Wells without any cells in each plate defined background staining, which was subsequently subtracted from all readings. Apoptosis versus necrosis measurements were performed using an Annexin V/propidium iodide (PI) staining kit (Bender MedSystems, Vienna, Austria) with FACS analysis of the percentage of positively stained cells determined according to the manufacturer's instructions.

RESULTS

Relative expression of TNFRs

In order to measure aspects of signalling by TNFR2, we have characterized various cell lines that possess differing amounts of this type II TNFR subtype. The cervical epithelial cell line HeLa is routinely used in studies with TNF. However, HeLa cells possess mainly TNFR1 subtype with < 5% of the TNFRs being TNFR2 [10]. Our initial observations suggested no activity of TNFR2 endogenously expressed on HeLa cells (results not shown). To ensure that TNFR2 activation was possible, we created HeLa cell lines possessing increased amounts of exogenous TNFR2. The highest expressing clone was used in this study and is termed HeLa-TNFR2 clone #1 (Figure 1). In order to ensure that results from any genetically modified cells were correct and not a spurious anomaly particular to that clone, a concern previously raised in other overexpressing HeLa cells [24–26], we compared this clone with HeLa-TNFR2 clone #2 (an independently derived clone; [14]), HeLa-TNFR2^{cyt-} cells (cells with comparable amounts of TNFR2 overexpression, but with a mutated TNFR2 receptor devoid of its cytoplasmic domain) and the KYM-1 cell line (which expresses proportionately high levels of endogenous TNFR2 compared with endogenous TNFR2 levels).

FACS analysis of the TNFR composition, by anti-human subtype-specific monoclonal sera, revealed approx. equal amounts of TNFR1 expression in HeLa, HeLa-TNFR2 (both clones), HeLa-TNFR2^{cyt-} and KYM-1 cell lines. Relative expression of TNFR2 among the cell lines varied considerably (Figure 1A). Specific anti-TNFR2 sera binding was very low in non-transfected parental HeLa cells, but much greater in all other cell lines. Confocal microscopy of fixed adherent cells revealed a similar pattern of TNFR expression, with the majority of receptor expression localized to the outer, non-nuclear regions of the cell; a pattern of TNFR localization observed by other workers [27,28]. To give an indication of the relative amounts of receptor expression, confocal data are shown with identical excitation laser power and fluorescence intensity observing conditions for each of the cells and antisera tested. Clearly, there is a large variation in relative immunoreactive TNFR expression levels amongst the cell lines tested.

The functional TNF-binding capability of the cells was determined by radiolabelled TNF binding studies (Figure 1B) using subtype-specific mutein TNFs that bind to either TNFR1 (R1-TNF) or TNFR2 (R2-TNF) as described previously [29]. Replacement of excess wild-type TNF, which competes out radiolabelled TNF to assess non-specific binding levels, with excess R1-TNF or R2-TNF revealed the amount of functional TNFR2 or TNFR1 present on the cells, respectively. These determinations confirmed that, of the approx. 3000 TNFRs on HeLa cells, functional endogenously expressed TNFR1 made up the vast majority of these receptors, with negligible amounts of TNFR2 being present. KYM-1 cells express approx. 19000 TNFRs/cell, with less than 40% of these receptors being the TNFR1 subtype. These parental cell lines expressing endogenous TNFRs are in marked contrast with the HeLa-TNFR2 clones and HeLa-TNFR2^{cyt-}, which express between 50000 and 75000 functional TNFR2 receptors/cell; levels comparable with those reported from other similar clones [30]. KYM-1 cells also show high levels of endogenous TNFR2 expression, with more than 11000 TNFR2s/cell; similar to observations from other workers [31].

Selective kinase activation by TNFRs

The ability of TNFR1- and TNFR2-specific stimuli to activate JNK, MAPK and p38MAPK kinase activities in these cells was measured and the results shown in Figure 2. Treatment of HeLa, HeLa-TNFR2 and KYM-1 cells with R1-TNF revealed the ability of the TNFR1 subtype to maximally activate cellular JNK, MAPK and p38MAPK activities. No significant stimulation of JNK activity in parental HeLa or HeLa-TNFR2^{cyt-} cells by any of the TNFR2 stimuli were observed; percentage of wild-type TNF-stimulated JNK activity after treatment with R1-TNF, R2-TNF, MR2-1 and a combination of R2-TNF and MR2-1 (R2-TNF + MR2-1) was 89%, -2%, -1% and 0% respectively in HeLa cells, and 86%, 8%, 3% and 13% respectively in HeLa-TNFR2^{cyt-} cells (data from an experiment representative of at least one other independent determination with similar findings). In contrast, TNFR2 activation in HeLa-TNFR2 and KYM-1 cells by the R2-TNF resulted in submaximal JNK activity. As soluble TNFs are poor activators of TNFR2, we also tested the ability of agonistic TNFR2-specific antisera to stimulate TNFR2 efficiently. The MR2-1 monoclonal antiserum, known to activate human TNFR2 [32], was able to activate JNK activity more efficiently in HeLa-TNFR2 and KYM-1 cells than parental HeLa cells. Other agonistic polyclonal TNFR2-specific antisera [13] were able to activate JNK in cells expressing high levels of TNFR2 receptor (results not shown).

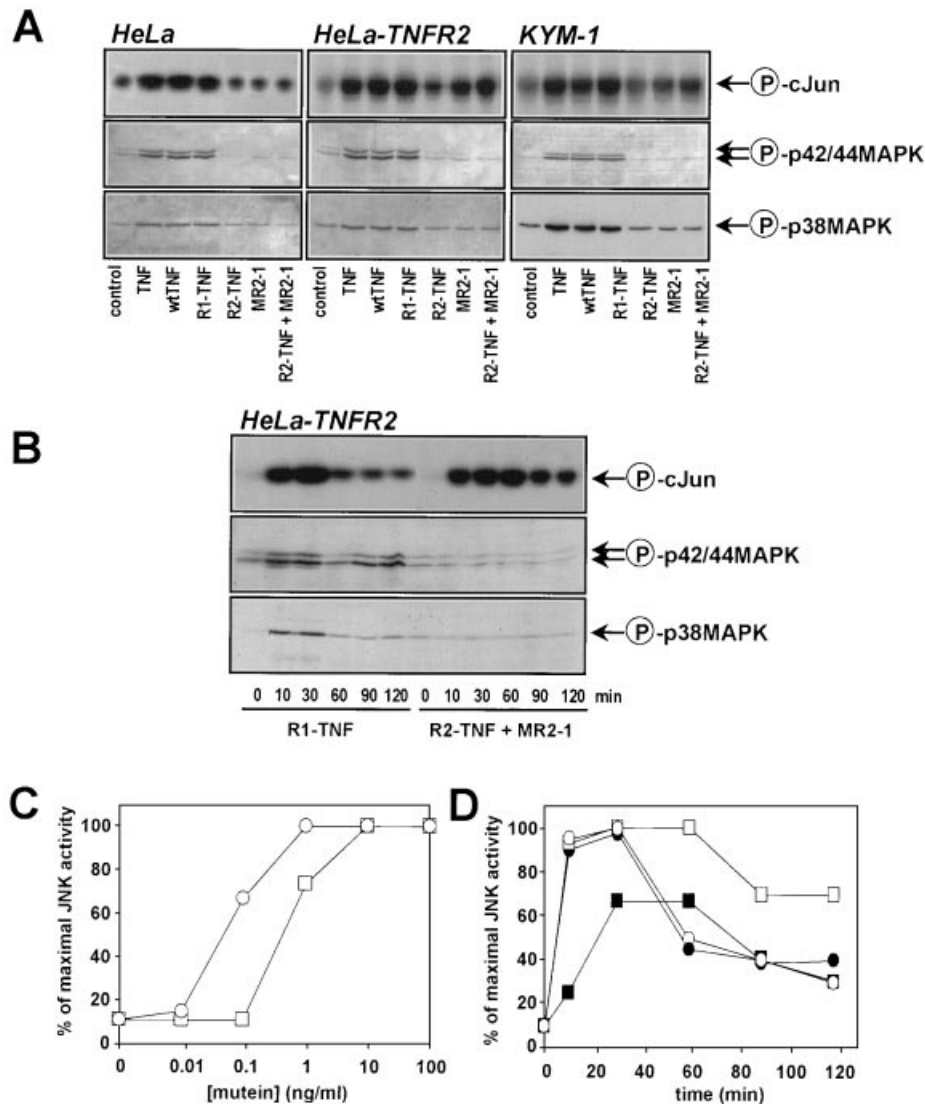


Figure 2 TNFR stimulation of JNK, MAPK and p38 MAPK activities in HeLa and KYM-1 cells

(A) Subtype-specific activation of JNK, MAPK and p38 MAPK was determined in HeLa clones and KYM-1 cells. Cells were treated for either 15 min for JNK activation (c-Jun phosphorylation) or 30 min for MAPK (p42/44 MAPK phosphorylation) and p38 MAPK activation with TNF (from a commercial source), wild-type TNF (wtTNF), mutein TNFs (50 ng/ml), MR2-1 agonistic TNFR2-specific monoclonal antiserum (2 μ g/ml) and a combination of R2-TNF and MR2-1 (R2-TNF + MR2-1; 50 ng/ml and 2 μ g/ml, respectively) as described in the Materials and methods section. (B) Time-course of JNK, MAPK and p38 MAPK activation in HeLa-TNFR2 cells was determined following treatment with R1-TNF (50 ng/ml) or a combination of R2-TNF and MR2-1 (R2-TNF + MR2-1; 50 ng/ml and 2 μ g/ml, respectively). Results are from an experiment representative of at least two other independent determinations with the same findings. (C) The concentration-response relationship of JNK activation in HeLa-TNFR2 cells stimulated for 15 min with the indicated concentrations of R1-TNF (\circ) or a combination of R2-TNF and MR2-1 (\square). For R2-TNF and MR2-1, MR2-1 antiserum, at a concentration of 1 μ g/ml, was combined with 100 ng/ml R2-TNF and then serially diluted 10-fold to give the indicated concentrations of R2-TNF and MR2-1. (D) Time-course of JNK activation in HeLa-TNFR2 cells following treatment with R1-TNF or a combination of R2-TNF and MR2-1 in the absence (\circ , R1-TNF; \square , R2-TNF + MR2-1) or presence (\bullet , R1-TNF; \blacksquare , R2-TNF + MR2-1) of Z-VAD-FMK (30 μ M). Cells were pretreated for 1 h in the absence or presence of Z-VAD-FMK (30 μ M) prior to the addition of R1-TNF (50 ng/ml) and R2-TNF + MR2-1 (50 ng/ml and 2 μ g/ml, respectively).

Agonistic polyclonal and monoclonal (MR1-2) TNFR1-specific antisera stimulated JNK activity in all cell lines tested (results not shown). Treatment with a combination of both R2-TNF and MR2-1 resulted in maximal activation of JNK activity in HeLa-TNFR2 and KYM-1 cells. In complete contrast, MAPK or p38 MAPK activities were stimulated by TNFR1 only, with none of the TNFR2 stimuli activating MAPK or p38 MAPK. TNFR1-stimulated p38 MAPK activity was transient and monophasic, whereas TNFR1-stimulated MAPK activity was biphasic in nature, with activity returning to basal levels after treatment times of 90 min or longer. These findings were confirmed using a

radioactive enzyme-activity assay (similar to the JNK assay) in which we measured MAPK and p38 MAPK activity using a MAPKAPK 2-GST fusion protein as the substrate (results not shown). It was noted in HeLa-TNFR2 cells that TNFR2-stimulated JNK activity was more sustained over longer time periods than TNFR1-induced JNK activity, which showed a more transient response. Figure 2(C) demonstrates the concentration-response relationship of TNFR1- and TNFR2-stimulated JNK activity, indicating that the concentrations of TNFR-specific stimuli used in our experiments were maximal, and therefore were not a factor in the difference observed for ERK

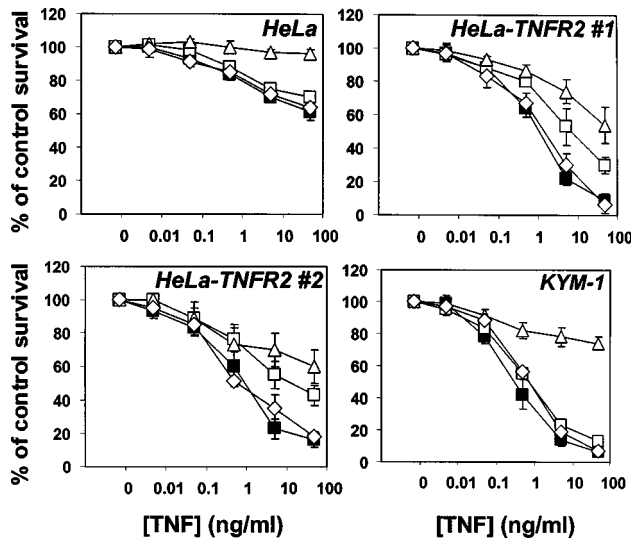


Figure 3 TNFRs in TNF-stimulated cell death in HeLa and KYM-1 cells

Subtype-specific stimulation of cell death in the HeLa clones and KYM-1 cells was determined following treatment for 24 h with wild-type TNF (wt-TNF; ■), R1-TNF (□), R2-TNF (△) or a combination of R1-TNF and R2-TNF (R1 + R2; ◇). Cytotoxicity was analysed as described in the Materials and methods section. The results are means ± S.E.M. from five to eight independent experiments.

activity. Time-course studies using maximal concentrations of TNFR-specific stimuli revealed that both TNFR1- and TNFR2-stimulated JNK activation was transient. Treatment of HeLa-TNFR2 cells with the cell-permeant type I caspase inhibitor Z-VAD-FMK, pretreated for 1 h before TNF stimulation, resulted in the marked reduction of TNFR2-induced JNK activity. Although caspase inhibition reduced TNFR2-stimulated JNK activity by approx. 50 %, the inhibitor did not completely block this receptor-stimulated kinase action. (Figure 2D, and results not shown). TNFR1-stimulated JNK activity in HeLa-TNFR2 cells was not significantly modulated by caspase inhibition with Z-VAD-FMK.

TNFRs and cell death

To assess the role of the TNFR subtypes and their stimulated kinases in cell death, a variety of cell death measurements were performed. Figure 3 reveals that TNF was moderately cytotoxic in parental HeLa cells, with a maximal death response of < 40 % at supramaximal TNF concentrations (50 ng/ml); an effect that occurred via the stimulation of the endogenous TNFR1, as demonstrated by the similar effect of R1-TNF. TNF-stimulated cell death in HeLa-TNFR2 and KYM-1 cells displayed much greater sensitivity towards TNF, with complete cytotoxicity of these cell types occurring at supramaximal TNF concentrations. KYM-1 cells died with a similar sensitivity when treated with R1-TNF. However, R2-TNF treatment alone stimulated a modest amount (< 30 %) of cell death in these cells. Greater amounts of R2-TNF-mediated cell death were observed in the HeLa-TNFR2 clones, with approx. 50 % of cells dying at concentrations of 50 ng/ml. Curiously, R1-TNF was unable to activate the cell death response fully in HeLa-TNFR2 clones, it being less sensitive than wild-type TNF. In these circumstances stimulation of both TNFR subtypes by combined treatment with R1-TNF and R2-TNF was required to accomplish the full cytotoxic TNF response.

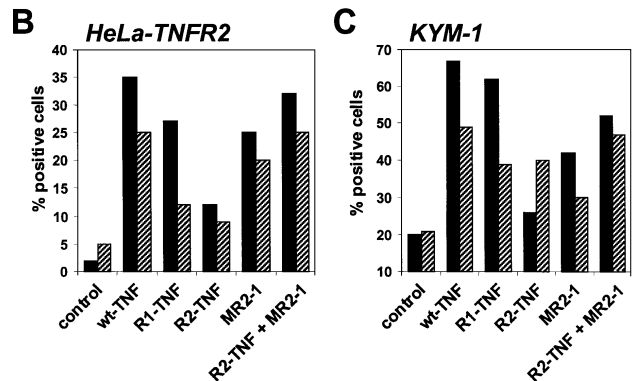
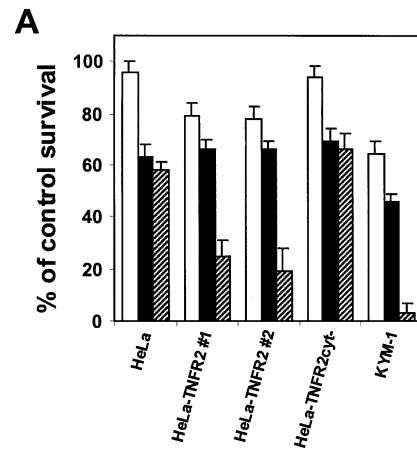


Figure 4 Role of TNFRs in cell death mechanisms in HeLa and KYM-1 cells

(A) HeLa clones and KYM-1 cells were analysed for cytotoxicity after 24 h treatment with TNF (filled bars), MR2-1 agonistic antiserum (open bars) or a combination of TNF and MR2-1 (TNF + MR2-1; hatched bars) by Crystal Violet staining as described in the Materials and methods section. Parental HeLa and HeLa-TNFR2^{cyt} cells were treated with 50 ng/ml TNF, whereas other cell lines received 0.5 ng/ml TNF. MR2-1 agonistic antiserum was used at a concentration of 2 µg/ml. Results are means ± S.E.M. from three to five separate experiments. (B) and (C) Measurement of apoptotic and necrotic characteristics in HeLa-TNFR2 (B) and KYM-1 (C) cells following treatment for 16 h with wild-type TNF (wt-TNF), R1-TNF, R2-TNF, MR2-1 and a combination of R2-TNF and MR2-1 (R2-TNF + MR2-1) as determined by Annexin V (filled bars; apoptosis) and propidium iodide (hatched bars; necrosis). TNF and TNF mutants were used at a concentration of 50 ng/ml and MR2-1 at 2 µg/ml. The results are means from a representative experiment repeated at least once with essentially the same findings.

To clarify further the role of TNFR2 in the cell death mechanisms, we utilized the agonistic properties of MR2-1 monoclonal antiserum in our cell death assays (Figure 4). MR2-1 antiserum treatment either alone or in combination with TNF was unable to elicit cytotoxic activity in HeLa and HeLa-TNFR2^{cyt} cell lines. This was in contrast with HeLa-TNFR2 and KYM-1 cell lines, which express large amounts of TNFR2 and display enhanced cell death responses to MR2-1 stimulation. We also determined the effectiveness of TNF, mutants and agonistic MR2-1 antiserum on the levels of apoptotic and necrotic cell death processes in HeLa-TNFR2 and KYM-1 cells. FACS analysis of the Annexin V-binding capability of cells is a measure of the phosphatidylserine presentation by those cells and is a measure chiefly of apoptotic cell death mechanisms. Co-measurement of the fluorescence intensity of PI, a non-permeant DNA stain, determines the permeability of cells and is a measure of necrotic cells, as well as very late apoptotically destroyed cells. Treatment of HeLa-TNFR2 or KYM-1 cells with TNF resulted in increased Annexin V and PI staining, with both death

Table 1 Inhibitors and activators of ERKs influence cell viability and kinase activity

HeLa cells were plated into 96-well culture dishes, cultured for 24 h and then treated with the indicated combination of agents for 24 h. Cell cytotoxicity measurements were performed using Crystal Violet staining as described in the Materials and methods section. The results represent means \pm S.E.M. of four to six independent experiments. Kinase activity assays on immunoprecipitated MAPK and p38 MAPK from treated HeLa-TNFR2 cells (15 min pretreatment with agent followed by 30 min TNF treatment) are shown. Measurements are from the incorporation of [γ - 32 P]ATP into MAPKAPK2-GST substrate, followed by SDS/PAGE, autoradiography and densitometry.

Treatment	Cytotoxicity (% of control cell survival) [MAPK/p38 MAPK activities (arbitrary units)]	
	-TNF	+ TNF (50 ng/ml)
Control	101 \pm 4 [4.1/2.7]	6 \pm 3 [21.1/35.0]
ATA (50 μ M)	187 \pm 9 [44.6/10.9]	153 \pm 10 [51.2/28.7]
PD98059 (25 μ M)	35 \pm 5 [3.8/3.3]	8 \pm 7 [5.1/29.0]
Anisomycin (25 μ M)	49 \pm 12 [6.7/41.4]	10 \pm 8 [25.2/41.3]
SB203580 (5 μ M)	46 \pm 7 [13.8/0.5]	42 \pm 8 [24.6/4.9]

parameters displaying similar profiles. Once again in HeLa-TNFR2, and to a lesser extent in KYM-1 cells, the full TNF death response was only partially mimicked by R1-TNF, with R2-TNF being markedly less effective. Surprisingly, combined treatment of HeLa-TNFR2 and KYM-1 cells with MR2-1 and R2-TNF displayed almost full TNF-mediated cell death responses. Treatment with the caspase inhibitor Z-VAD-FMK (30 μ M) completely blocked cell death induced by TNF (50 ng/ml) in HeLa-TNFR2 and KYM-1 cells, resulting in 87 \pm 4% and 112 \pm 7% of control cell survival respectively (means \pm S.E.M., $n = 2$), rather than nearly 0% of control cell survival seen in cells without Z-VAD-FMK.

Since exposure of cells to TNF differentially activated MAPK, p38 MAPK, and JNK, the ultimate cellular response to TNF will be a combination of the effects of activating all three kinases. A number of pharmacological tools have been discovered and developed to allow manipulation of the ERK signal transduction pathway. We have used a number of these to determine the fate of cells following TNF exposure when one or more of these protein kinases is inhibited or further activated. As can be seen from Table 1, a stimulus that activates MAPK [19], aurintricarboxylic acid (ATA), was able to induce proliferation in HeLa cells, with an approx. doubling of cell number in only 24 h. Co-treatment of ATA with TNF counteracted this increase in cell proliferation. In contrast, inhibition of the MAPK pathway by treatment with the MEK1 inhibitor PD98059 resulted in considerable cell death, that was further enhanced by the co-addition of TNF. Anisomycin is known to selectively stimulate stress kinases such as JNK and p38 MAPK. Treatment of HeLa cells with anisomycin resulted in an increase in cell death; an effect that was again augmented by TNF. Unfortunately, no pharmacological JNK inhibitors are available which would have been useful to test the role of JNK in death mechanisms. The pyridinyl imidazole compound SB203580 specifically blocks the activation of the p38 MAPK pathway. The results in Table 1 showed that this compound was cytotoxic. Dramatic cell death occurred after treatment with SB203580 for 24 h, as confirmed by light and laser confocal microscopy. The effectiveness of these pharmacological agents in HeLa-TNFR2 cells was determined in specific MAPK and p38 MAPK activity assays in which the kinases were immunoprecipitated using the pan-MAPK and pan-p38 MAPK antisera and the incorporation of [γ - 32 P]ATP into the MAPKAPK 2-GST substrate was measured (Table 1). SB203580

in our cells, at least, and at the concentrations used was toxic and unable to be used in examining the long-term role of p38 MAPK in TNF-induced cell death responses. Indeed, SB203580 significantly stimulated MAPK activity as well as reducing p38 MAPK activity. The use of these pharmacological tools, although limited, does provide evidence that stimulation of the MAPK pathway results in a mitogenic response, in this case proliferation, and opposes TNF-mediated cell death.

DISCUSSION

Although initially recognized and named for its ability to cause necrotization of tumour masses, TNF has subsequently been shown to also have dramatic systemic effects playing a primary role in certain inflammatory diseases, and at extreme physiological concentrations is responsible for septic shock. Understanding how TNF can have such extreme physiological consequences requires an understanding of its mechanistic actions. At the cellular level, TNF has been shown to modulate the fundamental processes associated with development, including cell proliferation, differentiation and cell death. However, the molecular mechanisms by which TNF induces these events are only now beginning to be fully understood. Although both TNFRs are believed to be involved in TNF-mediated signalling, it has been widely thought that the TNFR1 subtype mediates the majority of its responses [5-7]. The aim of this study was to investigate the functional role of TNFR2 in mediating TNF-induced signalling. To examine the role of endogenous TNFR2, we used KYM-1 cells that express high levels of TNFR2 and TNFR1 (Figure 1). We also used HeLa cells (which mainly express TNFR1) that had been transfected with TNFR2 cDNA and thus engineered to express higher numbers of receptors than KYM-1 cells. To distinguish TNFR2 from that of TNFR1 in TNF-mediated signalling, we used TNF mutants made by site-specific mutagenesis that bind to either TNFR1 or TNFR2. We also took into account the fact that soluble TNFs are poor activators of TNFR2 and tested the ability of an agonistic TNFR2-specific monoclonal antiserum to stimulate TNFR2 efficiently.

Concerns have been raised that signalling through TNFR2 occurs only when this receptor subtype is exogenously overexpressed in cells [16], and that these individual clones may represent peculiar systems which do not relate to endogenous TNFR2 expression [24]. We show in the present study that our findings not only occur in clones of HeLa cells which overexpress TNFR2 engineered from separate sources [14], but are entirely consistent with our observations in KYM-1 cells which express endogenous TNFR2 [33,34]. A contributing factor towards underestimating the role of TNFR2 are the findings that soluble TNF (as is most widely used experimentally) poorly activates TNFR2, but fully activates TNFR1 [11]. The limitation can be overcome by the use of agonistic antisera that are capable of activating TNFR2 more fully. We have shown that the soluble TNFR2-specific mutant TNF (R2-TNF) behaves as a partial agonist in that it can bind to TNFR2, but poorly activates its receptor. Increasing the expression levels of TNFR2 is likely to lead to a greater effect of such a partial agonist in stimulating TNFR2 activation [35-38]. The agonistic TNFR2-specific antiserum was more capable of activating TNFR2 than R2-TNF, but a combination of both stimuli resulted in efficacious TNFR2 action (Figure 2). It is not clear why R2-TNF and MR2-1 monoclonal antiserum have an additive effect on TNFR2 stimulation of JNK activity. It may be that each stimulus is accessing a different site on the receptor to encourage oligomerization and

activation; however, the precise reason remains to be ascertained. Uncleaved membrane-expressed TNF (the form of TNF most cells are probably presented with physiologically) has the ability to activate both TNFR subtypes effectively. Thus *in vitro* the role of TNFR2 is commonly underestimated when judging the function of both receptor subtypes.

TNF and R1-TNF activated JNK activity in all cell lines, a finding consistent with previous observations demonstrating that JNK is activated through TNFR1 [39]. However, we show in the present study that TNFR2, when sufficiently activated, was also a potent activator of JNK in the cell lines expressing TNFR2. The ability of TNFR1 to activate MAPK and p38 MAPK is clear [39–41]. However, the ability of TNFR2 to activate these pathways remains to be determined. For example, in transgenic mice lacking TNFRs, MAPK activation could be attributed to both receptor subtypes, although the kinetics of activation appeared to be different in each receptor-deficient system [42]. In the present study we found that MAPK or p38 MAPK was not activated by TNFR2, although this receptor is clearly functional. Our findings imply that the associating factors that bind to TNFR2 do not result in activation of the MAPK and p38 MAPK signalling cascades. TRAF2 is a candidate protein that binds strongly to TNFR2 and may be responsible for the differential activation of the ERK pathways observed here, although other evidence suggests this may be true only in our cell systems [43]. Our observations presented here that TNFR1 efficiently leads to p38 MAPK, MAPK and JNK activation, but TNFR2 propagates only JNK activity, lead to more questions as to the role of TRAFs. As overexpression of TRAF2 can also initiate the activation of these kinases and TRAF2 dominant-negative overexpression blocks it [44], there may be differences in the threshold of triggering initiated by TRAF2 in a TNFR1 or TNFR2 context. Somehow, the intrinsic signalling activity of TNFR1 is much higher than TNFR2. This might also be due to qualitative differences in the composition of both complexes and the absence of TRADD in a TNFR2 complex. The threshold for JNK signalling might be lower than that for p38 MAPK or MAPK, explaining the observation that only the former is clearly propagated by TNFR2 interaction.

TRAF2 is also degraded in a proteasome-dependent manner when interacting with some receptors [45], which may also explain the higher threshold of TNFR2 signalling, or the greater ability seen here of the Z-VAD-FMK caspase inhibitor to reduce TNFR2-induced JNK activity. Thus TNFR2 would operate to dampen signalling via MAPK and p38 MAPK pathways, allowing greater interaction of TNFR2 with TRAF2, and leading to subsequent TRAF2 degradation. It should also be noted that Z-VAD-FMK maximally inhibited only approx. 50% of the TNFR2-stimulated JNK activity. Clearly a more complex mechanism of JNK activation by TNFR2 may exist, with part of this response being through a non-caspase-dependent pathway. This is particularly evident in view of recent findings in embryonic fibroblast cell lines derived from caspase-8 $+/+$ and caspase-8 $-/-$ embryos, which revealed a clear difference in the kinetics of JNK activation between the two groups of cells [46]. JNK activity was observed after 10 min of TNF treatment in both cells. However, while JNK activation was sustained in caspase-8 $+/+$ cells for at least 30 min, there was a rapid decline in the JNK activation in caspase-8 $-/-$ cells, with a return to basal activity by the end of this time period. Taken together with our observations, these results indicate that caspase-8 may play a role in sustaining as well as initiating JNK activity in these cell lines. As seen in our studies presented here, different temporal profiles of JNK activation by the two TNFRs also suggests that non-identical signalling mechanisms for JNK activation exist between

the TNFR subtypes. Moreover, at least two upstream signalling components are involved in JNK activation [19]. MKK4 and MKK7 are both capable of JNK isoform activation. The ability of TNFR subtypes to activate these kinases and their caspase-sensitivity may help to explain the differential kinetics of JNK activation that we see here with TNFR1 and TNFR2 stimulation.

The data presented in this study also suggest that the greater JNK activation observed in TNFR2-expressing cells may be contributing towards the enhanced TNF-induced cell death seen in these cells. It could be that the increased JNK activity is involved directly with death responses mediated through TNFR2. However, the role of JNK in the apoptotic process is not straightforward [47]. Although TNF is a potent activator of JNK, in most cases it does not cause apoptosis unless cells are first treated with cycloheximide or actinomycin D [15]. These considerations indicate that JNK activation cannot be a simple trigger for apoptosis. Other evidence suggests the role of TNFR2 in enhancing cell death is to stimulate the gene induction of interleukins and *de novo* TNF, which, once they are expressed, are responsible for the greater levels of cell death [15,48–50]. None of our observations in the present study in TNFR2-expressing cells can discount such a '*de novo*' role for TNFR2 in the cell death mechanisms. Indeed, it may be that JNK is not solely responsible for cell death, but is responsible for gene induction of other factors, such as endogenous TNF, which are ultimately responsible for killing the cell.

The role of TNFR2 has been brought into question by a ligand-passing model. This suggests that the function of TNFR2 is to bind TNF and present it to TNFR1, which is the receptor that signals for cellular responses, including apoptosis [9]. TNFR1 has an extremely high affinity for TNF, whereas TNFR2 has a 10-fold lower affinity and fast association/dissociation kinetics [10]. The use of a cytoplasmic domain-deficient TNFR2 (HeLa-TNFR2^{cyt-}) in the present study demonstrated that the cytoplasmic face of the receptor, and presumably its associated signalling machinery, is crucial in TNFR2-mediated JNK activation and enhanced cell death. Since this TNFR2 deletion mutant can still bind TNF and present it to TNFR1, it is therefore not possible that a ligand-passing co-operation between the two receptors accounts for the TNFR2 actions observed here. However, we cannot discount the possibility of co-operation between the two receptors, as this may also occur at the level of signal transduction. It has been observed that TNFR1- and TNFR2-associated proteins can bind to each other [47]. This 'cross-talk' between members of the TRAF family, possibly TRAF2, which binds both TNFR1 and TNFR2 and has been shown capable of activating p38 MAPK [44], may account for increased JNK activation through TNFR1-signalling pathways upon TNFR2 activation. Supporting this theory, a recent study has shown a requirement of the TRAF2-association site of TNFR2 for TNFR2-dependent enhancement of TNFR1-mediated cytotoxicity [15]. Another recent study has shown that TNFRs are capable of pre-assembly into complexes that contain characteristics of activated receptors, but exist in the absence of ligand [51]. Indeed others have reported overexpressed TNFR2 signalling in the absence of TNF-ligand stimulus [52,53], but our data presented here indicate a role for ligand stimulation in TNFR2-activation processes.

A contentious point is whether TNFR2 can signal for cell death on its own or whether co-stimulation of TNFR1 is necessary. We have shown here that stimuli that specifically activate TNFR2 have by themselves the ability to stimulate cell death. Similar findings were also seen in other cell lines engineered to express varying amounts of TNFR2, where stimuli acting through this receptor only were capable of inducing apoptotic

cell death [54–56]. It may be that the relative abundance of the two TNFR subtypes can predetermine the amount of cell death observed upon TNF treatment. For example, endogenous TNFR2 expression levels in a human leukaemia cell line varied throughout the cell cycle, and this variation could result in dramatically opposing effects of TNF (i.e. proliferation or apoptotic cell death in response to TNF treatment) [29]. The medical importance of such variations in TNFR2 levels may be underestimated and still to be fully appreciated.

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