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Modulated kinase activities in cells undergoing tumour necrosis factor-induced apoptotic cell death

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Abstract Tumour necrosis factor-α (TNF) has a variety of cellular effects including apoptotic and necrotic cytotoxicity. TNF activates a range of kinases, but their role in cytotoxic mechanisms is unclear. HeLa cells expressing elevated type II 75 kDa TNF receptor (TNFR2) protein, analysed by flow cytometry and Western analysis, showed altered c-Jun N-terminal kinase (JNK) and p38 mitogen-activated protein kinase (p38MAPK; but not MAPK) protein content and activation. There was greater JNK activation, but reduced p38MAPK activation in dying cells compared to those still to enter TNF-induced apoptosis. Moreover, cells displaying more rapid apoptosis possess higher levels of type I 55 kDa TNFR1 receptor isoform, but less TNFR2. These findings reveal differential kinase activation in TNF-induced apoptotic death.

Key words: Cytokine; Receptor; Subtype; Signal transduction; Kinase; Tumor

1. Introduction

Tumour necrosis factor-α (TNF) is a member of a family of ligands whose members also include LT-α, Apo-1/Fas ligand and CD40 ligand [1,2]. This TNF superfamily of cytokines are expressed mainly as type II membrane proteins which can be cleaved proteolytically to produce a soluble form. This ligand family activates a corresponding family of structurally related receptors, with members of the TNF receptor (TNFR) superfamily containing common extracellular cysteine-rich repeat structures (three to six copies). In contrast, the intracellular domains of the TNFR superfamily are largely unrelated. These receptors initiate signals that regulate cellular actions including gene induction for immune responses and inflammatory reactions, cell death mechanisms, proliferation, and through excessive signalling, the pathology of a variety of diseases [3]. 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, with the cellular response often resulting from the balance of these opposing effects [4]. This disparity of TNF-induced responses is due in part to the existence of two distinct TNFR subtypes. Type I 55 kDa TNFR (TNFR1; also known as p55TNFR, TNFRSF1a, and CD120a) has a molecular mass of 50–60 kDa, whereas type II 75 kDa TNFR (TNFR2; also known as p75TNFR, TNFRSF1b, and CD120b) has a molecular mass of 70–80 kDa [5,6]. These receptor subtypes are expressed on the surface of most cells, although in different amounts. TNFR1 is expressed ubiquitously, whereas TNFR2 is found predominantly on haemopoietic and endothelial cells, showing a tightly regulated expression [7].

Signalling of the TNFR superfamily members depends on ligand-induced oligomerisation and recruitment of adapter proteins that lead either to gene induction via activation of transcription factors or to apoptosis via the activation of a proteolytic caspase cascade. What precisely determines the balance between both major pathways is currently not known. Distinct receptor-associating adapter proteins have been demonstrated to differentially interact with each receptor family member [8,9]. TNFR1 has been shown to recruit TRADD, FLICE, RIP, FADD, TNFR-associating factor (TRAF)-2, ICH-1, MADD, TRIP, I-FLICE, A-29, and I-TRAF. TRADD functions as a platform adapter that recruits several signalling molecules to the activated receptor [8]. TNFR2, like the TNFR1, can also recruit TRAF-2, but also utilises TRAF-1, IAP-1, and IAP-2. Others have also observed the direct interaction of p80TRAK and casein kinases with activated TNFR2 [9]. 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 such as TNF [10]. The three mammalian ERK families activated by TNF are: the mitogen-activated protein kinases (MAPKs), the c-Jun N-terminal kinases (JNKs), and p38MAPK. The p44/p42 MAPK group (also termed ERK1 and ERK2 respectively) are activated by dual threonine/tyrosine phosphorylation via MAPK kinase (MEK). Once activated, MAPK mediates biological responses involved in cell proliferation and differentiation by phosphorylating a number of substrates including cytosolic phospholipase A_2 ($cPLA_2$) [11–13] and the transcription factors c-Myc, AP-1, NF-IL6, ATF-2, and Elk-1. The dual specificity kinases MKK4/SEK1 and MKK7 activate JNKs, while p38MAPKs are activated by the MKK3/6 homologues [10]. Once activated, JNKs mediate the phosphorylation and activation of the transcription factors c-Jun,

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Abbreviations: ERK, extracellular signal-regulated kinase; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; PI, propidium iodide; TNF, tumour necrosis factor-α; TNFR, TNF receptor; TNFR1, type I 55 kDa TNFR; TNFR2, type II 75 kDa TNFR; TRAF, TNFR-associating factor

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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 and the activation of MAPK-activated protein kinases (MAPKAPK) 2/3, which in turn phosphorylate small heat shock proteins [14].

Generally, although known to influence mitogenic responses or stress-related activities such as inflammation or ultimately cell death, the precise role of ERKs in these cellular functions is not well understood [14]. As MAPK, p38MAPK and JNK pathways are important in TNF-mediated signalling, the aim of our study was to assess the activation state of these kinases in human cell lines which are undergoing TNF-induced apoptotic cell death.

2. Materials and methods

2.1. Materials

Recombinant human TNF was purchased from R&D Systems (Abingdon, UK). Its biological activity was confirmed to have at least 2×10^7 WHO U/mg in an L929 cytotoxicity assay when comparing TNF standards (kindly provided by Dr Meenu Wadhwa, National Institute of Biological Standards and Controls, Potters Bar, UK). 85 kDa poly[ADP-ribose]polymerase (PARP) fragment polyclonal antisera were purchased from BioSource International (Nivelles, Belgium). Htr-9 (TNFR1) and utr-1 (TNFR2) monoclonal antisera were bought from Bachem (Saffron Walden, UK). Phospho-specific MAPK and p38MAPK antisera were from New England Biolabs (Hitchin, UK). Phospho-specific JNK antisera were from Calbiochem (CNBiosciences, Nottingham, UK). Non-phospho-specific (pan) MAPK, p38MAPK, JNK and the pan-PARP antisera were obtained from Santa Cruz Biotechnology (Heidelberg, Germany). Annexin-V propidium iodide (PI) cell death measurement kit was obtained from Caltag Med Systems (Towcester, UK). p38MAPK inhibitor, SB203580, and MEK inhibitor, PD98059, were procured from Calbiochem. All other materials were from BDH/Merck (Poole, UK) or from Sigma Chemical (Poole, UK) and were of the highest grade obtainable.

2.2. Methods

2.2.1. Cell culture. HeLa cells were co-transfected on an Eppendorf Transjector 5246/InjectMan micromanipulator and microinjection system with cDNAs encoding the human TNFR2 (with permission from 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) with 10% foetal bovine serum (Helena Biosciences), 1 mM L-glutamine, 100 μg/ml penicillin, 100 μg/ml streptomycin supplemented with 100 μg/ml hygromycin B (Boehringer, Mannheim). Positive colonies were expanded and characterised for TNFR2-expression levels by FACS analysis with utr-1 monoclonal antiserum, or by [^{125}I]TNF binding analysis [15] (not shown). HeLa-TNFR2 stable expression cells contain approximately 6000 TNFR1 receptors/cell and 68 000 TNFR2 receptors/cell.

2.2.2. Cell death measurements. Cells death measurements in 96-well culture plates using cresyl violet as described [16]. Treatment with the indicated combination of agents was for 24 h before fixation and colorimetric determination of cell number.

2.2.3. Fluorescence-activated cell sorting (FACS) analysis. HeLa-TNFR2 cells were grown to approximately 80% confluence and dissociated from their culture vessel with 2 ml of trypsin-free cell dissociation solution (Sigma). Cells were washed once in 5 ml serum-free DMEM and resuspended in serum-free DMEM to give a cell population of 5×10^6 cells. Where necessary, cells were given two pre-permeabilisation washes in permeabilisation buffer (1×PBS (pH 7.3), 1% (v/v) foetal calf serum, 0.1% (w/v) sodium azide, 0.1% (w/v) saponin), so as to allow antisera access to intracellular locations. A 200 μl aliquot of cells was incubated on ice for 1 h in a 1:200 dilution of primary antibody. Cells were centrifuged at 5000×*g* for 2 min, supernatant discarded then cell pellet resuspended in 200 μl serum-free DMEM before addition of a 1:50 dilution of secondary antibody (FITC-labelled IgGs, Scottish Antibody Production Unit, Carluke, UK) 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+2% foetal bovine serum (v/v). For PI samples, nuclear staining was performed on freshly-dissociated HeLa-TNFR2 cells that were fixed with 70% ethanol before staining with PI according to the manufacturer's instructions (Bender MedSystems, Vienna, Austria). FACS analysis was performed in a Becton-Dickenson FACSCalibur machine under standard manufacturer's protocols [17].

2.2.4. Western blotting. Cells were treated with stimuli for the indicated times before cell monolayers were lysed using RIPA buffer (1×PBS, 1% (v/v) Nonidet P-40, 0.5% (w/v) sodium deoxycholate, 0.1% (w/v) SDS) supplemented with 0.1 mg/ml phenylmethylsulphonyl fluoride, 10 μg/ml aprotinin, 1 mM $NaVO_4$. After a 30 min incubation on ice, insoluble debris was pelleted (5 min, 10–000×*g*, 4°C) and discarded. Cell protein concentrations were determined by Lowry protein assay (Bio-Rad), then supplemented with 2×Laemmli loading buffer (20% glycerol, 4% β-mercaptoethanol, 0.3% bromophenyl blue) prior to 10% SDS–PAGE. Western analysis and autoradiographic detection by enhanced chemiluminescence were performed essentially as described [18,19].

3. Results

3.1. TNFR-induced cell death

Our studies used a model of TNF-induced cytotoxicity that displays enhanced apoptotic cell death in response to low concentrations of recombinant wild-type TNF. HeLa-TNFR2 cells express endogenous TNFR1 and stably express some ten-fold more exogenous TNFR2 protein. These cells respond to TNF treatment by undergoing relatively rapid apoptotic cell death [20], with near complete cell death seen in 24 h with 5 ng/ml TNF treatment (Fig. 1A). FACS analysis allows us to observe the death of HeLa-TNFR2 cells and distinguish the more granular dying cells, from the less granular, less opaque healthy cells which have still to progress into apoptotic cell death. Comparison of the cells which are healthy (gated region R1, Fig. 1B) to those which are dying after 16 h TNF treatment (gated region R2, Fig. 1B) allows us to distinguish the biochemical and cellular changes which occur in the induction of TNF-stimulated apoptotic death in HeLa-TNFR2 cells. Phosphatidylserine presentation on the extracellular surface of a cell is a characteristic of apoptotic cell death [21–23]. We see greater expression of phosphatidylserine in dying HeLa-TNFR2 cells, as judged by the greater amount of annexin-V binding (which binds to expressed phosphatidylserine) in dying cells as compared to healthy, unapoptotic cells (Fig. 1C). This is also true of PI DNA stain which binds better to DNA in leaky late apoptotic cells, and which shows greater fluorescence in dying HeLa-TNFR2 cells as compared to healthy cells (Fig. 1C). Furthermore, quantitation of the cell cycle profile with PI revealed the number of cells with hypodiploid characteristics (M1 region, Fig. 1C) indicative of apoptotic DNA fragmentation. 0, 0.5 and 16 h TNF treatment resulted in 4.1, 7.7 and 21.7% of R1-gated (healthy) cells being hypodiploid, whereas R2-gated (dying) cells showed 22.9, 21.6 and 62.5% cells being hypodiploid respectively. TNF-induced caspase protease activation is a mediator of cell damage and death [24]. The caspase family of proteases function to cleave key proteins, including the DNA repair enzyme PARP from its 116 kDa holoenzyme into an inactive 85 kDa fragment (Fig. 1D). An antiserum specific for the cleaved 85 kDa fragment also confirmed the greater presence of cleaved PARP enzyme in dying HeLa-TNFR2 cells compared to healthy cells (Fig. 1C,D).

3.2. TNFR activation and cell death

Our data revealed there to be a significant alteration in

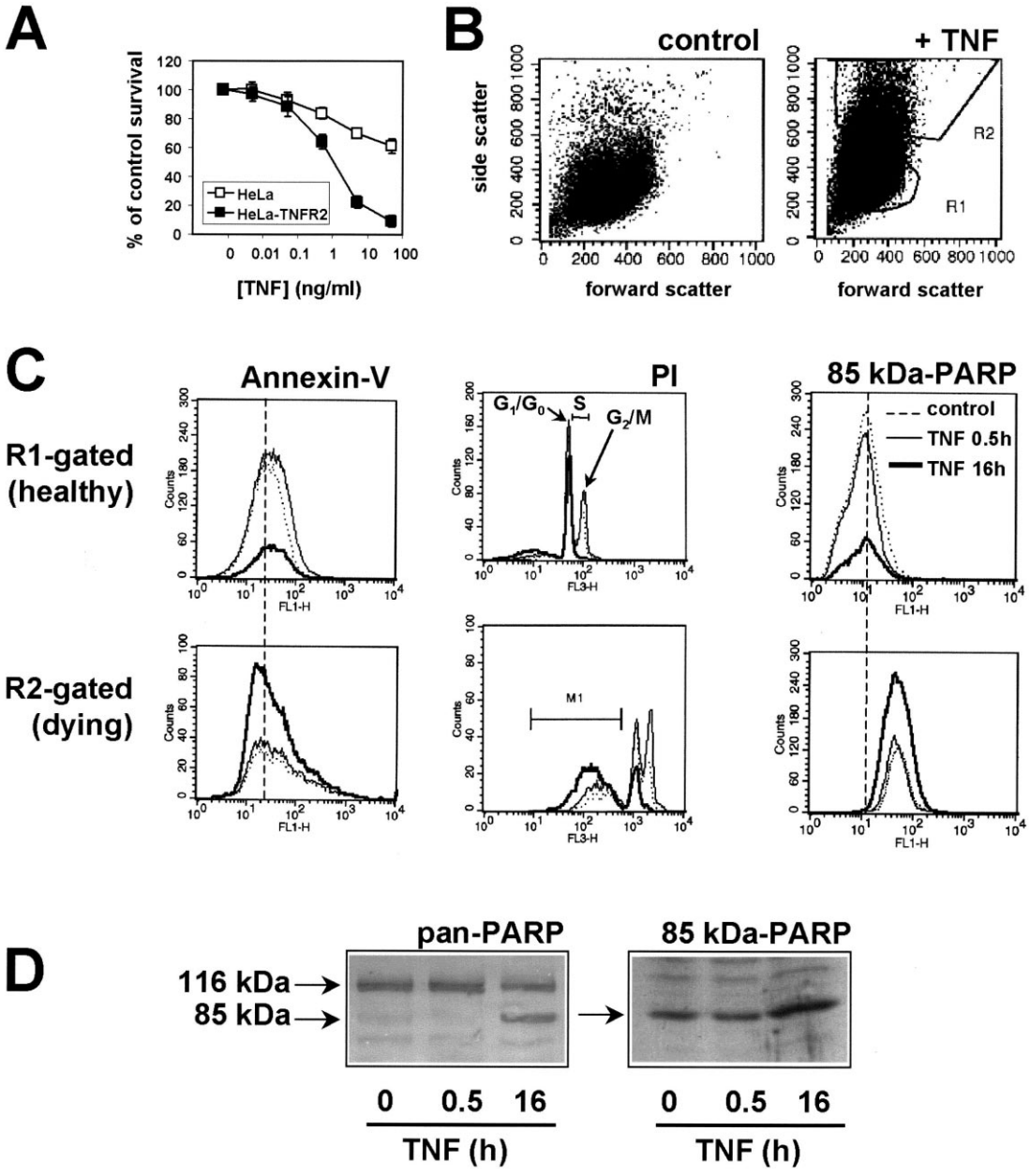

Fig. 1. Analysis of apoptotic cell death markers in TNF-treated HeLa-TNFR2 cells. A: Concentration–response cell death relationship of parental HeLa and HeLa-TNFR2 cells measured after 24 h TNF treatment. The data are the mean ± S.E.M. of at least four determinations in the presence of 1 μg/ml cycloheximide. B: Flow cytometry measurement of HeLa-TNFR2 cells ± 50 ng/ml TNF treatment for 18 h before FACS analysis of side scatter (∼granularity) and forward scatter (∼size), including a typical indication of an R1-gated region (healthy) and R2-gated region (dying). C: Comparison of HeLa-TNFR2 cells treated for the indicated times with 50 ng/ml TNF then histogram measurement of annexin-V, PI, or 85 kDa PARP fragment fluorescence intensities in R1- and R2-gated subsets of cells. M1 region indicates apoptotic cells with hypodiploid characteristics which are to the left of the sharp G1/G0 then G2/M peaks with their intervening S-phase portion. D: Western analysis of PARP degradation in HeLa-TNFR2 cells treated for the indicated times with 50 ng/ml TNF in the presence of 1 μg/ml cycloheximide to inhibit de novo protein synthesis. Data are from a representative experiment similar to at least two other determinations with essentially the same findings.

TNFR levels upon TNF treatment (Fig. 2). Treatment of HeLa-TNFR2 cells with TNF resulted in a time-dependent reduction in the level of TNFR1 and TNFR2 protein, as measured by FACS and Western analyses. TNFR1 levels were reduced after an 16 h treatment with TNF, with minimal effect of a 30 min TNF treatment. However, TNFR2 levels were reduced markedly after only a 30 min TNF treatment period (Fig. 2). This rapid reduction in cell surface TNFR2 protein levels suggests that TNFR2 protein is proteolytically cleaved upon stimulation by TNF. This finding was borne out in FACS experiments in which TNF-treated HeLa-TNFR2 cells were pre-permeabilised before TNFR2 antiserum addition. It was found that TNFR2 receptor was not internalised or rapidly processed into an intracellular store, as most cellular TNFR2 antiserum binding was lost from the cell completely with 30 min TNF treatment (data not shown). This finding was also true of endogenous TNFR2 protein, with comparable studies in KYM-1 human rhabdomyosarcoma

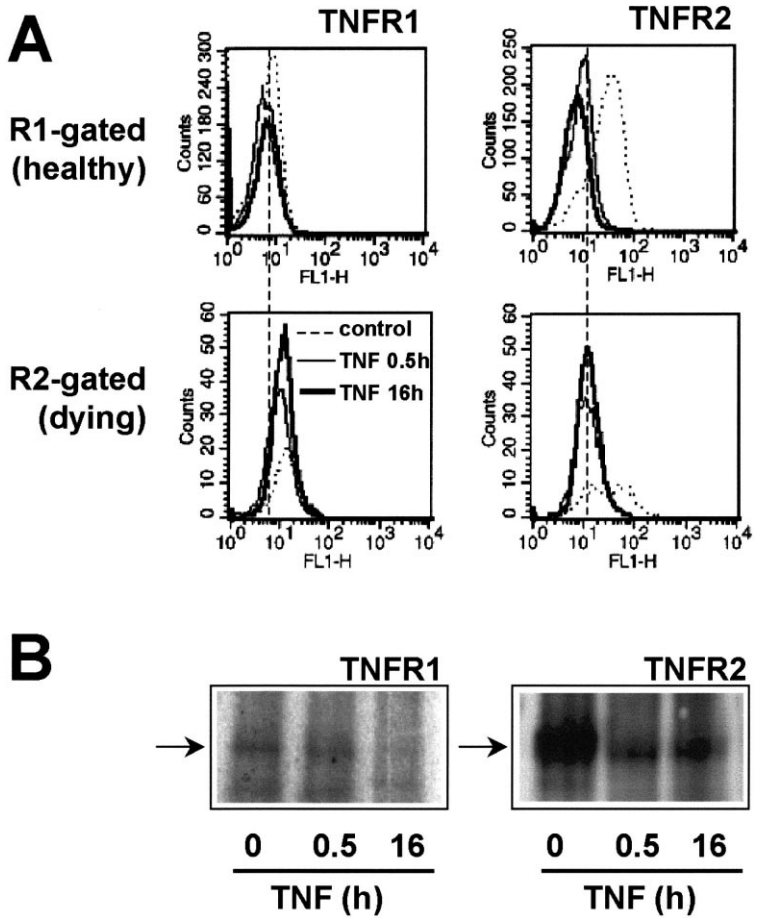

Fig. 2. Analysis of TNFRs in TNF-treated HeLa-TNFR2 cells. A: Flow cytometry measurement of HeLa-TNFR2 cells treated for the indicated times with 50 ng/ml TNF then histogram measurement of TNFR subtype levels in R1- and R2-gated subsets of cells. B: Western analysis of TNFR1 and TNFR2 protein in HeLa-TNFR2 cells treated for the indicated times with 50 ng/ml TNF. Data are from a representative experiment similar to at least two other determinations with essentially the same findings.

cells showing similar findings in TNF-stimulated TNFR2 protein loss (not shown). Not surprisingly, TNFR2 levels were low in healthy or dying cells that were treated with TNF, and did not appear to alter appreciably comparing FACS intensities or in Western blots of 30 min (healthy) or 16 h TNF-treated (dying) cells (Fig. 2). The level of TNFR1 protein was reduced significantly in the total cell population by 16 h TNF treatment (thought to be through a non-shedding internalisation process [6]), however comparison of healthy and dying cells by FACS showed that an appreciably higher level of TNFR1 was present in HeLa-TNFR2 cells that were undergoing apoptotic cell death.

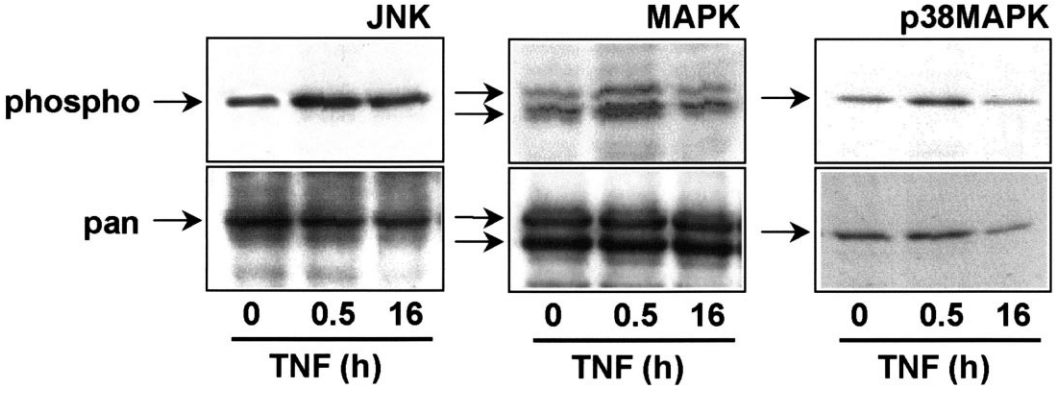

Fig. 3. Western analysis of JNK, MAPK and p38MAPK activation and levels in TNF-treated HeLa-TNFR2 cells. Western analysis of JNK, MAPK and p38MAPK activation using phospho-specific antisera, or total JNK, MAPK and p38MAPK protein levels using the indicated pan-ERK antisera. HeLa-TNFR2 cells were treated for the indicated times with 50 ng/ml TNF. Data are from a representative experiment similar to at least two other determinations with essentially the same findings.

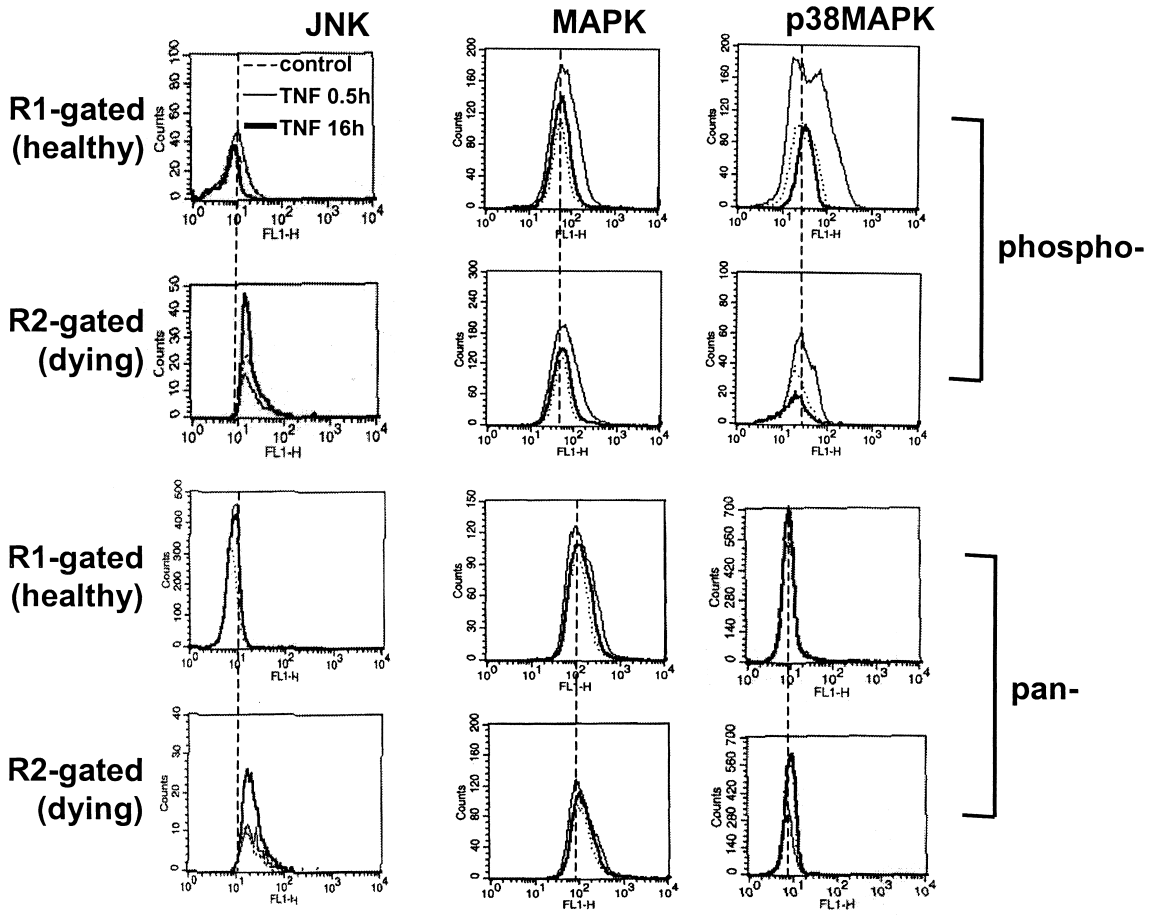

Fig. 4. FACS analysis of JNK, MAPK and p38MAPK activation and levels in TNF-treated HeLa-TNFR2 cells. Flow cytometry analysis of JNK, MAPK and p38MAPK activation using phospho-specific antisera, or total JNK, MAPK and p38MAPK protein levels using the indicated pan-ERK antisera. HeLa-TNFR2 cells were treated for the indicated times with 50 ng/ml TNF before FACS analysis of antisera fluorescence intensities in R1- and R2-gated subsets of cells. Data are from a representative experiment similar to at least two other determinations with essentially the same findings.

3.3. TNF activation of ERKs and cell death

We see that HeLa-TNFR2 cells treated for 30 min by TNF results in the activation of JNK, MAPK and p38MAPK kinase pathways, as assessed in Western blots using phospho-specific activated ERK antisera (Fig. 3). A 30 min TNF treatment did not influence the total levels of ERKs as judged by the pan-ERK antibodies. Longer term (16 h) treatment with TNF markedly reduced the total levels of JNK and p38MAPK stress kinases. However, although MAPK and p38MAPK activation status had returned to or reduced below basal levels, it was seen that JNK activation in 16 h TNF-treated cells was still raised, despite there being a reduction in total JNK protein levels in the entire cell population.

Generally, we observed by FACS that comparing dying HeLa-TNFR2 cells to healthy cells, MAPK could be activated by TNF, but the levels of MAPK activation and protein levels were unchanged in dying cells (Fig. 4). Once again, as seen in the Western analysis, JNK was activated on a short- and long-term basis by TNF plus it was observed that dying cells had significantly greater amounts of JNK activity and protein than healthy cells. A direct measurement of JNK activity (rather than phosphorylation-dependent activation that is actually measured by the phospho-specific antisera) would be helpful, however only total populations (healthy and dying combined) can be measured in conventional kinase activity assays. To this end, we also measured phosphorylation of a downstream target of JNK, c-Jun, using a phospho-specific c-Jun antiserum (serine 63-specific, New England Biolabs) and found that dying TNF-treated HeLa-TNFR2 cells also contained greater levels of phosphorylated c-Jun compared to healthy cells (0, 0.5 and 16 h TNF treatment resulted in 17.8, 19.7 and 54.4% of R1-gated (healthy) cells with high phospho-c-Jun binding, whereas R2-gated (dying) cells showed 33.0, 29.8 and 78.4% cells with high phospho-c-Jun binding respectively). These findings of elevated JNK and c-Jun activation were in direct contrast to the activation and total protein levels of p38MAPK in TNF-treated HeLa-TNFR2 cells, which although activated by TNF treatment, were found to be significantly reduced in dying HeLa-TNFR2 cells as assessed by FACS analysis (Fig. 4). Unfortunately, phospho-specific antisera for downstream targets of MAPK and p38MAPK are not currently available and kinase activity cannot presently be assessed for these ERK members.

The above findings clearly indicate a role for increased JNK activity and reduced p38MAPK activity in HeLa-TNFR2 cells that are more rapidly undergoing TNF-induced apoptosis. Unfortunately, pharmacological experiments were inconclusive mainly due to the lack of any commercially available JNK inhibitors, plus the fact that a selective p38MAPK inhibitor, SB203580 (5 μM), was by itself toxic to HeLa-

TNFR2 over a prolonged period of exposure (54 ± 7% of cells dead after 24 h treatment, mean ± S.E.M., $n = 4$). However, it was clear that activation of JNK by the metabolic cell stressor, anisomycin (25 μM) could enhance HeLa-TNFR2 cell death (51 ± 12 and 90 ± 8% of cells dead without and with 24 h exposure to 50 ng/ml TNF respectively, means ± S.E.M., $n = 4$–6). Moreover, whereas MAPK activation by aurintricarboxylic acid (50 μM) resulted in mitogenesis of HeLa cells (187 ± 9% of control cell number, $n = 6$), inhibition of MAPK activation with the compound PD98059 (25 μM), achieved greater TNF-induced apoptotic cell death in HeLa-TNFR2 cells (65 ± 5% and 92 ± 7% of cells dead without and with 24 h exposure to 50 ng/ml TNF respectively, means ± S.E.M., $n = 4$–6). These pharmacological experiments suggest that MAPK may have a role in mitogenesis, and that JNK may be involved in apoptotic death processes.

4. Discussion

TNF is able to control a wide variety of cellular responses from proliferation and differentiation, through to inflammation and death [3]. TNF achieves these different effects by acting on two TNFRs which are differentially expressed throughout each cell type. It is becoming apparent that the signalling mechanisms employed by these TNFRs may be different depending on the cell type in which they are activated and the intracellular machinery that the TNFRs access, which again is dependent on the proteins present in each individual cell type. In addition, each of the protein components accessed by the TNF signalling events may be regulated themselves in a way which is reliant on the physiological status or environmental experience of that particular cell which encounters TNF ligand. One such signalling component is the ERK family of protein kinases which can direct a variety of biochemical and cellular processes [10,14,25]. Here we have shown that there is regulation of each of the different ERK kinase families and that there may be differential activation processes occurring in TNF-induced apoptotic cell death.

Use of flow cytometry allows us to isolate subpopulations of cells and analyse a parameter within that cell subset. Here we distinguish between healthy regular low granularity cells (gated as region R1) and those cells which have altered side scatter properties to a more opaque granular appearance (gated as region R2) which is characteristic of TNF-induced apoptotic cell death morphology. We confirm the greater apoptotic characteristics in the dying subset of cells by measuring greater phosphatidylserine presentation (as measured by annexin-V binding), cell permeability and nuclear condensation (as measured by propidium iodide fluorescence) and caspase-dependent cell protein destruction (as measured by the cleavage of PARP holoenzyme into an 85 kDa inert fragment). Curiously, whilst measuring the TNFR subtypes in these cells we observed long-term destruction of TNFR1 (probably through internalisation and digestion [6]), and short-term removal of TNFR2, through a mechanisms which is probably proteolytic shedding of the receptor into a soluble, non-membrane-associated form [7], that is seen with both the exogenous TNFR2 protein in HeLa-TNFR2 cells, and with endogenous TNFR2 protein in KYM-1 cells. Given that the presence of TNFR2 in HeLa cells allows greater and more rapid TNF-induced apoptosis, the abrupt loss of this receptor isoform seems at odds with its cellular actions. However, its rapid activation and long-term effectiveness of TNFR2 is seen when HeLa-TNFR2 cells are treated for 5 min with TNF (which is then removed and antagonistic antisera added) and then still die 24 h later, hence the TNFR2-signalling cascades are already initiated before shedding of the receptor occurs.

The role that ERKs play in proliferation and apoptotic death processes is unresolved [14,25]. It is thought that MAPK is more involved in mitogenic responses [26–30] in some cell types with a possible role in cell death in other cells [31]. Although activated by TNF, we found no role for MAPK in the TNF-induced apoptosis we investigated. We did see marked stimulation of mitogenesis with a MAPK activator, and enhancement of TNF-induced cell death with the specific MEK inhibitor, PD98059. Others found that p38MAPK may have a role in proliferation [4,32–34], or in cell death [35–38]. Our findings support a role for p38MAPK in proliferative processes judged on the reduction of p38MAPK activity and total protein content in TNF-treated apoptotic cells. Unfortunately our results using a p38MAPK inhibitor were inconclusive as the compound was significantly toxic to the cells. More of a role for JNK in apoptosis has been reported [39–43] than a role for the kinase in proliferative responses [37,44,45]. Our data heavily indicate a role for JNK in the mediation of apoptotic cell death. Not only do we see prolonged JNK activation (as was reported to be crucial elsewhere [46]), but the pharmacological data support JNK not being protective against TNF-stimulated cell death. Unfortunately, no commercial inhibitors of JNK are currently available to help us support our contention of a role for JNK in TNFR-mediated apoptotic cell signalling.

These findings indicate that the regulation of different members of the ERK superfamily occurs by TNFRs. Moreover, the kinase regulation was seen in individual subsets of dying cells and their observed regulation supports a role for the regulation of these kinases in apoptotic cell death cellular processes.

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