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TRAIL activates JNK and NF-κB through RIP1-dependent and -independent pathways

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ABSTRACT

The death receptor (DR) ligand TRAIL is being evaluated in clinical trials as an anti-cancer agent; however, many studies have found that TRAIL also enhances tumor progression by activating the NF-κB pathway in apoptosis-resistant cells. Although RIP1, cFLIP and caspase-8 have been implicated in TRAIL-induced JNK and NF-κB activation, underlying mechanisms are unclear. By examining the kinetics of pathway activation in TRAIL-sensitive lymphoma cells wild-type or deficient for RIP1, TRAF2, cIAP1/2 or HOIP, we report here that TRAIL induces two phases of JNK and NF-κB activation. The early phase is activated by TRAF2- and cIAP1-mediated ubiquitination of RIP1, whereas the delayed phase is induced by caspase-dependent activation of MEKK1 independent of RIP1 and TRAF2 expression. cFLIP overexpression promotes the early phase but completely suppresses the delayed phase of pathway activation in lymphoma cells, whereas Bcl-2 overexpression promotes both the early and delayed phases of the pathways. In addition, stable overexpression of cFLIP in RIP1- or TRAF2-deficient cells confers resistance to apoptosis, but fails to mediate NF-κB activation. HOIP is not essential for, but contributes to, TRAIL-induced NF-κB activation in cFLIP-overexpressing cells. These findings not only elucidate details of the mechanisms underlying TRAIL-induced JNK and NF-κB activation, but also clarify conflicting reports in the field.

1. Introduction

Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) and agonistic antibodies against TRAIL receptor 1 and 2 [TRAILR1/2; also known as death receptor 4 and 5 (DR4/5)] are considered a potential anti-cancer agents, as they show selective high cytotoxicity toward tumor cells with little or no toxicity against normal cells [1]. Consequently, an optimized version of recombinant human TRAIL and humanized agonistic monoclonal antibodies directed at TRAILR1 and TRAILR2 are currently being tested in clinical trials [2]. However, TRAIL has also been found to act as a tumor promoter in certain contexts, increasing cancer cell proliferation and metastasis by activating the NF-κB and c-Jun N-terminal kinase (JNK) pathways in apoptosis-resistant cells [3,4]. Therefore, defining the mechanisms that permit TRAIL to activate JNK and NF-κB is critical for the development of strategies that maximize the potential effectiveness of TRAIL in clinical applications.

A subset of the TNF receptor (TNFR) superfamily members, such as TNFR1, Fas and TRAILR1/2, contain a death domain (DD) in their cytoplasmic tails [5]. Ligation of TNFR1 by TNFα leads to the recruitment of TNFR-associated death domain (TRADD), receptor interacting protein 1 (RIP1), TNFR-associated factor 2 (TRAF2) and cellular inhibitor of apoptosis 1 and 2 (cIAP1/2) to trigger activation of the pro-survival NF-κB and JNK pathways. On the other hand, activated TRAILR1/2 and Fas directly recruit Fas-associated death domain (FADD) and caspase-8 to activate the pro-apoptotic pathways in many types of cancer cells [1]. However, in apoptosis-resistant cancer cells, TRAIL and FasL can induce NF-κB and JNK activation and cell proliferation [1,6]. Although the factors that determine which of the opposing responses (apoptosis or proliferation) predominates are not fully understood, overexpression of cellular FLICE-like inhibitory protein (cFLIP) has been shown to

Abbreviations: cIAP1, cellular inhibitor of apoptosis 1; cFLIP, cellular caspase-8 (FLICE)-like inhibitory protein; DR, death receptor; FADD, Fas-associated death domain; IκB, inhibitor of κB; IKK, IκB kinase; JNK, c-Jun N-terminal kinase; KO, knockout; MEKK1, mitogen-activated kinase kinase (MEK) kinase 1; NF-κB, nuclear factor κB; RIP1, receptor interacting protein 1; siRNA, small interfering RNA; SM, Smac mimetic; TAK1, transforming growth factor β-activated kinase 1; TBK1, TANK-binding kinase 1; TNFα, tumor necrosis factor α; TNFR, TNF receptor; TRADD, TNFR associated death domain; TRAF2, TNFR associated factor 2; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; WT, wild-type.

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inhibit apoptosis and mediate JNK and NF-κB activation following TRAILR crosslinking [7,8].

The long form of cFLIP (cFLIP$_L$; hereafter referred to as cFLIP) resembles caspase-8 structurally; however, it lacks caspase activity owing to the substitution of critical amino acids in its caspase-like domain [1]. cFLIP can form a heterodimer with caspase-8, and is able to trigger limited caspase-8 activation while inhibiting its complete cleavage to the fully active p20/p10 dimer. Concomitantly, partially activated caspase-8 cleaves cFLIP at D376 to generate a p43cFLIP fragment, and this p43cFLIP has been reported to be essential for the recruitment of TRAF2 and RIP1 to TRAILR and activation of MAPK and NF-κB [7]. However, several independent studies have demonstrated that cFLIP inhibits TRAIL- and FasL-induced JNK and NF-κB activation [8–10]. In addition, caspase-8 inhibitors have been shown to inhibit or promote JNK and NF-κB activation following TRAIL or FasL treatment, depending on cell type [3,11–13]. For example, Muhlenbeck et al. reported that zVAD-fmk inhibits TRAIL-induced JNK activation in HeLa cells but promotes this activation in Kym-1 cells [11]. Moreover, Grunert et al. reported that neither RIP1 nor cFLIP is required for TRAIL-induced NF-κB activation [14]. One of the reasons for the accumulation of these conflicting conclusions in the field is that most studies examined JNK and NF-κB activation at limited (either early or late time) time points following TRAIL or FasL stimulation.

In this study, we stably transduced cFLIP or an empty vector in lymphoma cell lines wild-type or deficient for RIP1, TRAF2, cIAP1/2 or HOIP, and analyzed the kinetics of JNK and NF-κB activation following TRAIL stimulation. Our data demonstrate that TRAIL activates two phases of JNK and NF-κB activation; the early phase is dependent on RIP1, TRAF2, cIAP1/2 and HOIP, and the delayed phase is dependent on caspase-8 and MEKK1. Notably, cFLIP plays dual roles in TRAIL signaling, promoting the early phase while suppressing the delayed phase of JNK and NF-κB activation. These findings not only provide detailed mechanisms underlying TRAIL-induced JNK and NF-κB activation, but also clarify conflicting data in the field.

2. Material and methods

2.1. Cell lines, plasmids and reagents

RIP1$^{+/+}$ and RIP1$^{-/-}$ Jurkat lymphoma lines were cultured in IMDM supplemented with 10% FCS and antibiotics, and the A20.2J and multiple myeloma cell lines were cultured in RPMI 1640 supplemented with 10% FCS and antibiotics. Antibodies (Abs) and reagents were purchased as follows: anti-TRAF2 (C-20) and anti-DR5 (N-19) Abs and an siRNA pool targeting human MEKK1 from Santa Cruz Biotechnology (Santa Cruz, CA); anti-cFLIP (NF6) Ab from Alexis (San Diego, CA); pan-specific anti-cIAP1/2 (MAB3400) Ab from R&D System (Minneapolis, MN); anti-phospho-IκBα (14D4, #2859), anti-IκBα (#9242), and anti-caspase-8 (1C12) Abs from Cell Signaling (Danvers, MA); anti-RIP1 (#610459) Ab from BD Bioscience (San Jose, CA); recombinant human TRAIL from Roche (Indianapolis, IN); anti-phospho JNK Ab from Promega (Madison, WI); inhibitors of TBK1 (BX795), TAK1 (5Z-7-oxozeaenol) and AKT (124005) from Calbiochem (San Diego, CA); N-ethylmaleimide (NEM) from Sigma-Aldrich (St Louis, MO, USA); zVAD-fmk from BioMol (Plymouth Meeting, PA, USA); and cocktail inhibitors of proteases and phosphatases from Pierce (Rockford, IL). The pBabe-puro-cFLIP retroviral plasmid was generated by PCR amplification and insertion of the amplicons into the pBabe-puro vector. Retroviral pMSCV-IRES-GFP and -Bcl-2 plasmids were kindly provided by Dr. Michael Knudson (University of Iowa). All plasmids were sequenced from both sites to confirm the ligations and mutations.

2.2. Protein extraction and Western blotting

The cells were mock treated or treated with TRAIL, harvested in ice-cold PBS, and then lysed in TNE lysis buffer (20 mM HEPES pH 7.4, 1.0 % Triton X-100, 350 mM NaCl, 2 mM EDTA, 30 % glycerol, 1 mM DTT, 0.2 mM PMSF, 1X cocktail inhibitors of protease and phosphatases) on ice for 30 min with gentle agitation, followed by centrifugation at 12,500 ×g for 15 min at 4 °C. 20 μg of protein samples was separated by SDS-PAGE and transferred onto nitrocellulose membranes. For analysis of IκBα and JNK phosphorylation, blots were blocked in 3% BSA/T/TBS for 4 h before incubation with phosphoantibodies overnight at 4 °C. For analysis of the expression of other proteins, blots were blocked in 5% fat-free Milk/T/TBS for 2 h, and then incubated with the indicated antibodies overnight at 4 °C. Protein expression was then detected using ECL solution. IκBα phosphorylation blots were quantified by densitometry using integrated intensity values (ImageJ program), and the ratios of IκBα phospho-signal to the total protein were calculated and normalized to untreated 0 min signal. The values from three independent experiments were presented as mean ± SE.

2.3. Preparation of retroviral supernatants and infection of Jurkat T and MDA-MB-231 cells

293T cells at 60-70% confluency were co-transfected with 2 μg of pMD.OGP (encoding gag-pol), 2 μg of pMD.G (encoding vesicular stomatitis virus G protein) and 2 μg of pBabe-puro-cFLIP, pMSCV-IRES-GFP or -Bcl-2 by the standard calcium phosphate precipitation method. 48 h after transfection, the viral supernatant was collected and filtered through a 0.45 μm filter. The retroviral supernatants were then used immediately for the infection of the cells in the presence of 4 μg/ml polybrene for 24 h (Jurkat T cells) or 6 h (MDA-MB-231 cells). 48 h after infection, pBabe-puro-cFLIP transduced Jurkat T cells were selected with puromycin (2.0 μg/ml) for 14 days, and resistant cells were pooled. In the case of pMCSV-IRES-GFP/Bcl-2 transduced MDA-MB-231 cells, the cells were cultured for five days after infection, and then sorted for GFP positive cells using Becton Dickinson FACS DiVa. After an additional five days of culture, the GFP positive cells were FACS sorted again, and then used for the experiments within three weeks.

2.4. Real time RT-PCR

Real time RT-PCR was carried out as described previously [15,16]. Briefly, the cells were untreated or treated with TRAIL as indicated, and total RNA was extracted using the RNeasy Mini Kit (QIAGEN). 5 μg of total RNA was treated with RQ1 RNase-free DNase for 30 min at 37 °C, and then reverse transcribed using an oligo dT-primer. 5% of resulting cDNA was then subjected to quantitative real-time PCR using the Power SYBR Green AB Master Mix and an ABI Prism 7700 Sequence Detector (Applied Biosystems). GAPDH-specific primers were used to generate an internal control, and the average threshold cycle (C_T) for samples in triplicate was used in the subsequent calculations. Relative expression level of IP-10 was calculated as a ratio relative to the GAPDH expression level. The mean ± S.E. of four independent experiments was considered to be statistically significant at $p < 0.05$.

2.5. Cell viability assay

Cells (5.0×10^4/well in 100 μl) were plated on 96-well plates in 2% FBS/phenol red-free RPMI, incubated for 24 h, and then treated with TRAIL as indicated. At 24 h after treatment, MTT at 0.25 mg/ml was added to the plates, and incubation continued for another 4 h at 37 °C. After which, the 96-well plates were spun down at 1,500 rpm for 10 min, the supernatants (80 μl from each well) were carefully removed, and then 100 μl of DMSO was added to dissolve the formazan crystals. The absorbance of the solubilized product at 570 nm was measured with a 96-well plate reader. All determinations were confirmed in at least three identical experiments.