EXPERIMENTAL CELL RESEARCH 317 (2011) 1841–1850

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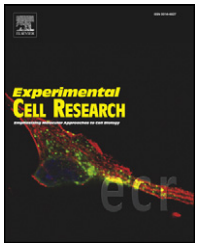

Research Article

Regulation of death complexes formation in tumor necrosis factor receptor signaling

Hien Chau[a], *Christine Mirtsos*[a], *Huey-Lan Huang*[a,b,*]

[a]*Department of Medical Biophysics/Advanced Medical Discovery Institute/The Campbell Family Institute for Breast Cancer Research, University Health Network, and University of Toronto, 620 University Avenue, Suite 706, Toronto, Ontario, Canada, M5G 2C1*
[b]*Department of Bioscience Technology, College of Health Science, Chang Jung Christian University, 396 Chang Jung Rd., Sec.1, Tainan 71101, Taiwan, ROC*

ARTICLE INFORMATION

Article Chronology:
Received 27 November 2010
Revised version received 8 April 2011
Accepted 13 May 2011
Available online 20 May 2011

Keywords:
TNFR
cFLIP
Caspase-8
PDGF-B

ABSTRACT

TNFα stimulation triggers both cell death and survival programs. Since dysregulated apoptosis or cell growth can cause inflammatory diseases, cancer, or autoimmune disorders, it is important to understand the molecular mechanism of controlling cell death and survival by TNFR downstream signaling molecules. In this study, we used normal diploid cells, mouse embryonic fibroblasts (MEFs), to mimic the general TNFα-resistant phenomenon seen under physiological conditions. We elucidated the TNFα-induced death signaling complexes in TNF α-resistant WT MEFs and TNFα-sensitive MEFs that were cFLIP-, RelA-, TRAF2- or RIP1-deficient. Consistent with TNFα-mediated killing, we detected TNFα-induced high molecular weight complexes containing caspase-8 and FADD by gel filtration in the deficient MEFs, especially in those devoid of cFLIP. In addition to the presence of caspase-8-FADD in the TNFα-induced-death complex in the deficient MEFs, we also detected an intermediate protein complex containing RIP1, TRAF2 and caspase-8. Moreover, we demonstrated a correlation between TNFα-sensitivity and death-inducing complex ability in two transformed cell lines, E1A- and Ras- transformed MEFs and PDGF-B-transformed NIH-3T3 cells with PDGF-B signaling inhibited by the tyrosine kinase inhibitor STI571. Taken together, our results suggest the involvement of cFLIP-, RelA-, RIP1-, or TRAF2-related mechanisms for preventing FADD-caspase-8 interaction in wild-type MEFs.

Introduction

TNFα is an important cytokine with pivotal functions in the immune system. TNFα not only induces apoptosis (or programmed cell death) [1], but it is also a pro-inflammatory cytokine in innate immunity [2], such as the immediate early induction of TNFα in the downstream signaling of toll-like receptors. TNFα-mediated signaling cascades turn on systematic responses through

* *Corresponding author at:* Department of Bioscience Technology, College of Health Science, Chang Jung Christian University, 396 Chang Jung Rd., Sec.1, Tainan 71101, Taiwan, ROC. Fax: +886 6 278 5010.
E-mail address: hhuang@mail.cjcu.edu.tw (H.-L. Huang).

Abbreviations: CHX, cycloheximide; cFLIP, cellular FLICE-inhibitory protein; cIAP-1, inhibitors-of-apoptosis 1; DD, death domain; DISC, death-inducing signaling complex; FADD, Fas-associated death domain protein; IP, immunoprecipitation; KOSR, knockout serum replacement; MEFs, mouse embryonic fibroblasts; MW, molecular weight; PDGF-B, platelet-derived growth factor B; RIP1, receptor-interacting protein 1; TNFα, Tumor necrosis factor α; TNFR, TNF receptor; TRADD, TNFR-associated death domain protein; TRAF, TNF-associated factor; WB, western blot; WT, wild-type.

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doi:10.1016/j.yexcr.2011.05.015

induction of other proinflammatory cytokines, changing the fate of immune cells or other cells to proliferate, differentiate or undergo cell death.

Although the intracellular domain of TNF receptor 1 (TNFR1) contains a death domain (DD), one of the most well known downstream features of TNFR1-signaling is its ability to transduce both survival and death signaling. The detailed mechanisms of how these death and survival signals interact to determine cell fate remain ambiguous. TNFR1 differs from several other TNF receptor superfamily members who only elicit cell death via their DD [1,3]. Upon TNFα stimulation, NF-κB activation is thought to play a pivotal role in TNFα-induced survival signals. Cell fate changes from TNFα resistance to TNFα sensitivity when survival factors induced by NF-κB activation are blocked in the presence of I-κB mutant or protein synthesis inhibitor cycloheximide (CHX) [4–7]. Thus, NF-κB turns on the expression of many survival proteins that favor cell survival. One hypothesis is that NF-κB-induced survival proteins collaborate for cell survival. It was found that cells with NF-κB inactivated were almost completely rescued from TNFα-induced cell death by cotransfection with 4 survival proteins: TRAF1 (TNF-associated factor 1), TNF-associated factor 2 (TRAF2), inhibitors-of-apoptosis 1 (cIAP-1) and cIAP-2, in contrast to cells transfected with only one of these proteins [8]. On the other hand, NF-κB-induced target genes, such as PDGF-B oncogene (also a growth factor), provide a pivotal role for TNFα-induced survival signaling [9]. Exogenous PDGF-B treatment effectively rescues TNFα-induced cell death in NF-κB-deficient cells [9]. However, the complete mechanism for protecting cells by NF-κB activation and NF-κB-induced anti-apoptotic proteins remains to be elucidated, since comparable NF-κB activation remains in TNFα-sensitive cells, such as E1A/Ras transformed mouse embryonic fibroblasts (MEFs) [10].

The outcome of cell death or survival is thought to depend on the TNFα-induced protein complex that assembles following TNFα stimulation. TNFα binding initiates protein–protein interactions between TNFR1 and TNFR-associated death domain protein (TRADD). TRADD either recruits receptor-interacting protein (RIP, or also called RIP1) and TRAF2 for NF-κB-mediated survival signals, or it recruits Fas-associated death domain protein (FADD) for death signals [3,11–16]. Interestingly, deficiency in one set of signals results in the dominance of the other pathway [17].

A more detailed model about events downstream of TNFα/TNFR1 has recently been demonstrated as an assembly of two sequential complexes [18]. The first complex, called the survival complex (or complex I) is mainly composed of TNFR1, TRADD, RIP1 and TRAF2. After TNF binding, complex I is rapidly formed around TNFR1 in the lipid rafts of the plasma membrane [19]. Complex I is thought to mediate survival signals via NF-κB activation. Then the TRADD, RIP1 and TRAF2 in complex I dissociate from TNFR1 and the plasma membrane and form a death complex (or complex II) with the apoptotic components, FADD and caspase-8 in the cytosol. Interestingly, TNFR1 is not directly associated with caspase-8 and FADD [18,20], which is different from the death-inducing signaling complex (DISC) formed with other TNFR superfamily members such as Fas or TRAIL receptors that directly bind FADD and caspase-8 [20–22]. Interestingly, it is known that only certain kinds of cell types, such as L929 (a type of mouse fibroblast cells), are sensitive to TNFα-induced cell death [1,17]. However, questions remain for the model of TNFR1 signaling complexes, including whether death complex II formation appears in different cell types or in TNFα-resistant cells [23].

We wondered if a caspase-8- and FADD-containing complex II formed after TNFα stimulation in wild-type cells, as previously seen in Ras-transformed human fibrosarcoma cells [18,19]. In the present study, we investigated whether, in wild-type cells, there exists a distinct mechanism that helps to keep the deadly complex II in check. To determine functions of the downstream molecules involved in the TNFα signaling complexes, we analyzed the signaling complexes formed in normal TNF α-resistant WT MEFs and TNFα-sensitive MEFs that were cFLIP-, RelA-, TRAF2- or RIP1-deficient, as well as TNFα-sensitive transformed cells. Our study reveals the importance of these TNFα downstream mediators in preventing the assembly of cell death-promoting FADD-caspase-8 complexes and protecting cells from TNFα-induced cell death.

Materials and Methods

Cell Culture and TNFα Treatment

MEFs and PDGF-B-transformed NIH3T3 cells [9] were cultured in Dulbecco's modified Eagles' medium supplemented with 10% fetal bovine serum (Gibco) and 50 μM β-mercaptoethanol (Gibco). RIP1−/− and RelA−/− MEFs were kindly provided by Drs. Michelle Kelliher (University of Massachusetts Medical School) [16] and Bruce Horwitz (Brigham and Women's Hospital, Harvard Medical School). For TNFα treatments, cells were left untreated in fresh medium or treated with medium plus recombinant mouse TNFα (R&D Systems) 10 ng/ml (or as indicated in Fig. 1C). TNFα plus CHX (50 μg/ml) was used for Figs. 1D and 3B. E1A/Ras transformed MEFs and PDGF-B-transformed NIH3T3 cells were kindly provided by Samuel Benchimol (York University, Toronto) and Stuart Aaronson (Mount Sinai School of Medicine). E1A/Ras transformed MEFs were treated with TNFα in 10% knockout serum replacement (KOSR) medium (Gibco) in Fig. 5A or cotreated with TNFα and a trace of CHX (0.1 μg/ml) in regular 10% DMEM in Fig. 5C. PDGF-B-transformed NIH3T3 cells were untreated, treated with TNFα alone, 10 μM STI571 alone or both TNFα plus STI571 for the indicated time points [9].

Immunoprecipitation (IP) and Western Blot Analysis (WB)

MEFs from two 150-mm plates were used per treatment for IP-WB experiments or gel filtration assays. After TNFα treatment, MEFs were washed with PBS and resuspended in lysis buffer (50 mM Tris–HCl [pH7.4], 150 mM NaCl, 5 mM EDTA and 10% glycerol) or RIPA buffer (PBS containing 0.1% SDS, 1% NP-40 and 0.5% sodium deoxycholate) with protease inhibitors (Roche Biochemicals). The lysis and RIPA buffers were for TRAF2 and FADD IP reactions, respectively. After centrifugation at 13,000 *g* for 10 min, precleared lysates were immunoprecipitated with TRAF2 or FADD antiserum (kind gifts from D. Goeddel, Z. Cao, and G. Chen, Tularik, South San Francisco) and washed 5 times with the respective buffer. Immunoprecipitates were then subjected to 10% SDS-PAGE, and immunoblotted with RIP1 (BD Biosciences), FADD (Upstate), Caspase-8 (Alexis), and cFLIP (Apotech) antibodies.