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RIP1-mediated mitochondrial dysfunction and ROS production contributed to tumor necrosis factor alpha-induced L929 cell necroptosis and autophagy

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ABSTRACT

Tumor necrosis factor alpha (TNFα) induces necroptosis and autophagy; however, the detailed molecular mechanism is not fully understood. In this study, we found that TNFα administration caused mitochondrial dysfunction and reactive oxygen species (ROS) production, which led to necroptosis and autophagy in murine fibrosarcoma L929 cells. Notably, the RIP1 (serine–threonine kinase receptor-interacting protein 1, a main adaptor protein of necroptosis) specific inhibitor necrostatin-1 (Nec-1) recovered mitochondrial dysfunction and ROS production due to TNFα administration. Moreover, pan-caspase inhibitor z-VAD-fmk (zVAD) increased RIP1 expression and exacerbated TNFα-induced mitochondrial dysfunction and ROS production, indicating that RIP1 led to mitochondrial dysfunction and ROS production. In addition, cytochrome *c* release from mitochondria was accompanied with TNFα administration, and Nec-1 blocked the release of cytochrome *c* upon TNFα administration, while zVAD enhanced the release. These further suggested that RIP1 induced mitochondrial dysfunction accompanied with cytochrome *c* release. Furthermore, autophagy inhibitor 3-methyladenine (3MA) did not affect RIP1 expression as well as mitochondrial dysfunction and ROS production. Together with our previous publication that autophagy was a downstream consequence of necroptosis, we concluded that TNFα induced mitochondrial dysfunction accompanied with ROS production and cytochrome *c* release via RIP1, leading to necroptosis and resulting autophagic cell death.

1. Introduction

Necroptosis, also known as type III programmed cell death, is a basic cell death pathway defined by Degterev et al. [1]. Necrostatin-1 (Nec-1), targeting serine–threonine kinase receptor-interacting protein 1 (RIP1), is a specific inhibitor of necroptosis which is dependent on RIP1/3 complex activation [2,3]. Necroptosis regulates the normal embryonic development, T-cell proliferation and chronic intestinal inflammation [4–7]. Type II programmed cell death, autophagy, plays a pivotal role in degradation and recycling cellular components. During nutrients or growth factor withdrawal; autophagy plays an important role for maintaining cell survival. However, abnormal autophagy may lead to cell death, termed autophagic cell death [8,9]. Macroautophagy (hereafter referred to as autophagy) is the most active form of autophagy and in this process, organelles and cytosolic macromolecules are sequestered into double-membrane structures known as autophagosomes, which are subsequently delivered to the lysosome for degradation [10]. In autophagy induction, LC3 I (the mammalian homolog of Atg8) conjugates with phosphatidylethanolamine to form the autophagosome-associated LC3 II. The accumulation of LC3 II is correlated with the extent of autophagosome numbers [11].

Mitochondria are double membrane-enclosed organelles that play an essential role in cellular metabolism, ATP generation, ROS production and regulation of cell proliferation and death [12]. Due to these multiple roles, mitochondrial dysfunction leads to many pathological processes including diabetes, aging, asthma, neurodegenerative disease, cardiovascular disease and cancer [13]. Reactive oxygen species (ROS) including superoxide, singlet oxygen, hydrogen peroxides, hydroxyl free radical and nitric oxide, mainly generated from the mitochondria, play an important role in cell death [14]. Mitochondrial ROS was reported to exert a crucial role in TNFα-induced necrotic cell death in L929 cells [15]. Our previous study demonstrated that TNFα-induced L929 cell necroptosis and autophagy could be completely inhibited by RIP1 inhibitor Nec-1 [16]. However, the association between RIP1-mediated necroptosis and autophagy with mitochondrial dysfunction remains to be examined in TNFα-treated L929 cells. We also tried to speculate the roles of caspases on induction of necroptosis and autophagy.

2. Materials and methods

2.1. Reagents

Human recombination TNFα was prepared from PMAL-C2-TNF/JM109 (*E. coli*) in our laboratory. Crystal violet, propidium iodide (PI), monodansylcadervarine (MDC), 2′,7′-dichlorodihydrofluorescein

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diacetate (DCFH-DA), Rhodamine-123, necrostatin-1 (Nec-1), cyclosporine A (CsA), pifithrin-α (PFT-α), N-acetyl-L-cysteine (NAC), 3-methyladenine (3MA), pan-caspase inhibitor z-VAD-fmk (zVAD), rotenone and antimycin A were purchased from Sigma Chemical (St. Louis, MO, USA). MitoTracker® Green FM, MitoTracker® Deep Red 633 and MitoSOX™ Red were obtained from Molecular Probes (Eugene, OR, USA). Small interfering RNA (siRNA) against mouse RIP1 and control siRNA were designed by Shanghai GenePharma Co., Ltd (GenePharma, Shanghai, China). Lipofectamine 2000 was purchased from Invitrogen (Carlsbad, CA, USA). Rabbit polyclonal antibodies to RIP1, LC3, Bax, p53, p-p53, mouse polyclonal antibodies against Bcl-2, cytochrome *c* and β-Actin and horseradish peroxidase-conjugated secondary antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

2.2. Cell culture

L929 cells were cultured in RPMI-1640 medium (Gibco, NY, USA) supplemented with 10% fetal bovine serum (FBS) (Beijing Yuanheng Shenyang Research Institution of Biotechnology, Beijing, China), 100 μg/ml streptomycin, 100 U/ml penicillin and 0.03% L-glutamine, and maintained at 37 °C with 5% CO_2 at a humidified atmosphere. All the experiments were performed on logarithmically growing cells.

2.3. Cell viability assay

The cell viability of TNFα on L929 cells was measured by crystal violet staining. The cells were dispensed in 96-well plates with 5×10^4 cells/ml. After 48 h incubation, they were treated with or without the indicated inhibitors at given concentrations 1 h prior to the administration of TNFα, then incubated for 24 h. The cells were washed twice with phosphate buffered saline (PBS) and stained with 0.5% crystal violet solution containing 20% ethanol at room temperature for 30 min. After washing three times with water, the retained dye was dissolved in 120 μl methanol for each well and the absorbance was measured at 620 nm using an enzyme-linked immunosorbent assay (ELISA) reader (Tecan Spectra, Wetzlar, Germany). The cell viability was calculated as follows:

$$\text{Cell viability}(\%) = 100 - (A_{620},\ \text{control} - A_{620};\ \text{experiment})/(A_{620}, \text{control} - A_{620}, \text{blank}) \times 100$$

2.4. Measurement of PI positive ratio (cell death ratio)

The L929 cells were treated with TNFα for the indicated time periods or co-incubated with the given inhibitors for 24 h. The collected cells were washed twice with PBS, after washing the cells were stained for DNA content with PI for 10 min. PI can be inserted into double-stranded DNA, penetrated the membrane of dying cell but rejected by living cell and apoptotic cell without fixing with 70% ethanol at 4 °C overnight. The percentage of PI positive ratio was measured by FACScan flow cytometry (Becton Dickinson, Franklin Lakes, NJ, USA).

2.5. Measurement of ROS level and mitochondrial dysfunction

The L929 cells were treated with 4 ng/ml TNFα or co-incubated with the given inhibitors for 24 h.

DCFH-DA was widely used for ROS detection. DCFH-DA is a stable nonpolar compound that readily diffuses into cells and is hydrolyzed by nonspecific esterases to DCFH. This nonfluorescent molecule is further oxidized by ROS to form fluorescent compound DCF [17]. The cells were incubated with 10 μM DCFH-DA at 37 °C for 30 min, then harvested and the pellets were suspended in 0.5 mL of PBS. The samples were analyzed by flow cytometry.

MitoTracker® Green FM, MitoTracker® Deep Red 633 and MitoSOX™ Red were used for distinguishing total, respiring and ROS-generating mitochondria, respectively [18]. Mitochondria in cells stained with nanomolar concentrations of MitoTracker® Green FM dye exhibit bright green fluorescein-like fluorescence. When this dye accumulates in the lipid environment of mitochondria it becomes fluorescent. MitoTracker® Deep Red 633 is a red fluorescence which is well resolved from the green fluorescence of MitoTracker® Green FM, thus it is suited for multicolor labeling experiments. This probe does not fluoresce until it enters an actively respiring cell, where they are oxidized to the corresponding fluorescent mitochondrion-selective probe and then sequestered in the mitochondria. The treated cells were incubated with 200 nM MitoTracker® Green FM and 500 nM MitoTracker® Deep Red 633 in the dark at 37 °C for 30 min. Next, the cells were harvested and the pellets were suspended in 0.5 mL of PBS. The samples were analyzed by flow cytometry.

MitoSOX™ Red reagent is a fluorogenic dye for highly selective detection of superoxide in the mitochondria. MitoSOX™ Red reagent is live-cell permeant, once in the mitochondria, it is oxidized by superoxide and exhibits red fluorescence. The treated cells were incubated with 5 μM MitoSOX™ Red in the dark at 37 °C for 30 min. Next, the cells were harvested and the pellets were suspended in 0.5 mL of PBS. The samples were analyzed by flow cytometry.

2.6. Measurement of autophagy

The L929 cells were treated with TNFα for the indicated time periods or co-incubated with the given inhibitors for 24 h. After being collected, the cells were cultured with 0.05 mM MDC at 37 °C for 1 h, then the samples were analyzed by flow cytometry.

2.7. siRNA transfection

The cells were transfected with siRNAs using Lipofectamine 2000 according to the manufacturer's instructions. The transfected cells were used for subsequent experiments 24 h later.

2.8. Measurement of mitochondrial membrane potential ($\Delta\psi m$)

Rhodamine 123 as a cationic fluorescent dye enters the mitochondrial matrix dependent on mitochondrial transmembrane potential. If mitochondrial membrane potential is depleted, the rhodamine 123 is released from the mitochondria. The mean fluorescence intensity of rhodamine 123 was measured to determine the loss of mitochondrial membrane potential. The treated cells were incubated with 5 μM rhodamine 123 in the dark at 37 °C for 30 min. Next, the cells were harvested and the pellets were suspended in 0.5 mL of PBS. The samples were analyzed by flow cytometry.

2.9. Western blot analysis

The cells were treated with TNFα for 0, 6, 12, 24 and 36 h or co-incubated with the given inhibitors for 24 h. The cells were lysed in lysis buffer (20 mM Tris–HCl, pH 7.4, 1% Triton X-100, 10% glycerol, 150 mM NaCl, 1 mM phenylmenthanesulfonyl fluoride (PMSF), and 1 μg/ml each leupeptin, antipain, chymostatin, and pepstatin A) on ice for 1 h and centrifuged (9500×g, 15 min). Equivalent amounts of total proteins were separated by SDS polyacrylamide gel electrophoresis (SDS–PAGE) and transferred to nitrocellulose membrane. The membrane was blocked with 5% skim milk powder in 0.1% Tween 20 in Tris-buffered saline (TBS) for 2 h and incubated with the primary antibodies at 4 °C overnight. Membranes were washed three times with 0.1% Tween 20 in TBS for 10 min and incubated with the respective peroxidase-conjugated secondary antibodies for