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The mitochondrial pathway and reactive oxygen species are critical contributors to interferon-α/β-mediated apoptosis in Ubp43-deficient hematopoietic cells

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ABSTRACT

UBP43 (also known as USP18) plays a role in the negative regulation of interferon-α/β signaling, and bone marrow cells in Ubp43-deficient mice exhibited hypersensitivity to interferon-α/β-mediated apoptosis. Here, we show that the mitochondrial apoptotic pathway and reactive oxygen species are major contributors to the elevated interferon-α/β-mediated apoptosis in Ubp43-deficient mouse bone marrow cells and in UBP43-knockdown THP-1 cells. Furthermore, TRAIL and FASL, which were proposed as apoptosis inducers upon interferon-α/β treatment in UBP43-knockdown adherent cancer cells, did not cause apoptosis in these hematopoietic cells. Therefore, although UBP43 depletion can cause hypersensitivity to interferon-α/β-mediated apoptosis in a broad range of cell types, the downstream pathway may vary depending on the cell type.

1. Introduction

Type I interferons (IFNs), represented by IFN-α/β, are pleiotropic cytokines that play a variety of biological roles in anti-viral, immunomodulatory, anti-proliferatory, anti-tumor, and apoptotic pathways [1–4]. IFN-α/β induces the expression of several hundred genes called interferon-stimulated genes (ISGs) via the JAK-STAT signaling pathway [5,6]. Among the ISGs, UBP43 was identified as a deconjugating protease for a ubiquitin-like protein ISG15 [7,8], and the expression of *UBP43* was specifically induced by IFN-α/β [9]. A knockout mouse model for the *Ubp43* gene revealed a negative regulatory role for UBP43 in IFN-α/β-mediated JAK-STAT signaling [10]. Ubp43-deficient cells were found to be hypersensitive to IFN-α/β through the increased and prolonged activation of the JAK-STAT signaling pathway, leading to a much higher expression of ISGs compared to the normal cells. Along with the hypersensitivity to IFN-α/β, Ubp43-deficient mice are more resistant to viral and bacterial infections [9,11]. Furthermore, Ubp43 deficiency increased the resistance to oncogenic transformation by BCR-ABL, the causative agent of chronic myeloid leukemia [12]. Detailed analyses for the cause of the hypersensitivity to IFN-α/β in Ubp43-deficient mice and cells have revealed that UBP43 negatively regulates JAK-STAT signaling independent of its deISGylating enzyme activity [13]. Regardless of its enzymatic activity, UBP43 directly interacts with the IFNAR2 subunit of the IFN-α/β receptor such that UBP43 inhibits the activation of receptor-associated JAK1 by blocking the interaction between JAK1 and IFNAR2 [13].

It has been shown that IFN-α/β induces apoptosis in many types of malignant cells [14] and in hematopoietic cancer cells [15–17]. IFN-α/β induces the extrinsic apoptotic pathway through FADD/caspase-8 signaling and the mitochondrial pathway [3]. One interesting phenotype of the Ubp43-deficient mice that is in agreement with the hypersensitivity to IFN-α/β is increased apoptosis in hematopoietic cells [10]. The administration of polyI:C or LPS, which in turn induces IFN-α/β production, is more lethal to Ubp43-deficient mice than their wild-type counterparts owing to the extensive apoptosis especially in hematopoietic cells [9,10]. Another group also reported elevated apoptosis in UBP43-knockdown cells upon IFN-α/β administration. The depletion of UBP43 from adherent types of cells, such as E1A-transformed IMR90 fibroblasts (IMR90-E1A) and MCF7, promoted the activation of the extrinsic apoptotic pathway by IFN-α, in accordance with an increased TRAIL production and upregulated expression of transcription factors IRF-1, IRF-7, and IRF-9 [18]. In spite of the obvious apoptotic phenotype in Ubp43-deficient hematopoietic cells, the exact downstream mechanism that causes the increased apoptotic cell death was not clearly defined.

Here we show that, as in Ubp43-deficient mouse bone marrow cells, UBP43 depletion dramatically increases IFN-α/β sensitivity in

Abbreviations: ROS, reactive oxygen species; IFN, interferon; ISGs, interferon stimulated genes; NAC, N-acetylcysteine; FACS, fluorescence activated cell sorting.

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UBP43-knockdown THP-1 cells, as exemplified by enhanced and prolonged STAT1 phosphorylation and several-fold increases in apoptosis. A detailed analysis of the apoptotic pathway revealed that the mitochondrial pathway rather than the extrinsic pathway plays the major role in the IFN-α/β-mediated apoptotic cell death in both Ubp43-deficient mouse bone marrow cells and UBP43-knockdown THP-1 cells. Furthermore, the elevated generation of ROS upon IFN-α treatment and the reduction of IFN-α-mediated apoptosis by the elimination of ROS in the UBP43-knockdown THP-1 cells indicated that ROS is also a major contributor to the elevated IFN-α/β-mediated apoptosis in the UBP43-depleted hematopoietic cells.

2. Materials and methods

2.1. Plasmid construction and transfection

The shRNA targeting the human *UBP43* gene, pLKO.1-shUBP43 (TRCN0000004194), and control shRNA, pLKO.1-TRcontrol, were purchased from Open Biosystems (USA). pLKO.1-shUBP43 and pLKO.1-TRcontrol were transfected into THP-1 cells using an Amaxa nucleofector (Amaxa Biosystems, USA). The transfected cells were selected in the presence of puromycin (0.5 μg/ml) for 2 weeks.

2.2. Cell culture and treatment

The mouse bone marrow cells were cultured in RPMI 1640 medium (Invitrogen, USA) containing 10% FBS (Invitrogen, USA), 10 ng/ml IL-3, 10 ng/ml IL-6, and 100 ng/ml stem cell factor (PeproTech, USA), and the THP-1 cells were cultured in RIPM 1640 medium containing 10% FBS and 2 mM L-glutamine (Invitrogen, USA). Recombinant human IFN-α and mouse IFN-β (PBL Interferon Source, USA) were used at 1000 units/ml and 500 units/ml, respectively. Recombinant human or mouse FASL (R&D Systems, USA) were used at two concentrations, 100 or 300 ng/ml. Recombinant human TRAIL (R&D Systems, USA) or recombinant mouse TRAIL (PeproTech, USA) were used at 300 or 500 ng/ml. An ROS antagonist, N-acetylcysteine (NAC; Sigma–Aldrich, USA), was used at 10 mM.

2.3. Antibodies

The anti-Bid, anti-caspase-3, anti-cleaved caspase-3, anti-cytochrome c, anti-STAT1, and anti-phosphoSTAT1^{Tyr701} antibodies were purchased from Cell Signaling (USA). The anti-Bax and anti-caspase-8 antibodies were purchased from Santa Cruz (USA). The antibody generated against human UBP43 was described previously [13].

2.4. Immunocytochemistry

THP-1 cells (2×10^5 cells/slide) were attached onto slides using Shandon Cytospin 4 (Thermo Scientific, USA). After fixing with 3.7% paraformaldehyde, the cells were permeabilized with 0.5% Triton X-100 in PBS and blocked with PBST containing 10% FBS and 1% BSA. The cells were incubated with primary antibodies diluted in 3% BSA at room temperature (RT) and then incubated with fluorescein isothiocyanate (FITC)-conjugated secondary antibody (Zhongshan Goldenbridge Biotech, China) in the dark. After being washed four times with PBST, the cells were mounted using a Vectorshield mounting medium containing DAPI (Vector Labs, USA) and observed with a fluorescence microscope (Olympus, Japan).

2.5. Measurement of apoptotic cell death

The apoptotic cell death was analyzed by fluorescence activated cell sorting (FACS) after staining using the Annexin V-FITC Apoptosis Detection Kit I (BD Biosciences, USA). Briefly, the THP-1 cells were harvested and washed twice with cold PBS. The cells (1×10^6 cells) were resuspended in 100 μl of the kit's binding buffer and incubated with Annexin V-FITC for 15 min at RT. Flow cytometry was performed using the FACS Canto II (BD Bioscience, USA).

2.6. Measurement of ROS

Intracellular ROS production was determined by incubating the cells (1×10^6 cells) with 10 μM H_2DCFDA (Sigma–Aldrich, USA) for 30 min at 37 °C in the dark. The cells were washed twice in PBS and analyzed by flow cytometry or observed using a fluorescence microscope (Olympus, Japan).

2.7. Measurement of the mitochondrial membrane potential

The mitochondrial membrane potential was evaluated by staining the cells (1×10^6 cells) using the JC-1 Mitochondrial Membrane Potential Detection Kit (Biotium, USA). The cells were then analyzed by flow cytometry.

2.8. Caspase activity assay

Cells (5×10^6 cells) were harvested and resuspended in extraction buffer (40 mM HEPES [pH 7.5], 0.1% Triton X-l00, 20% glycerol, and 4 mM DTT) on ice for 10 min. The extracts were then incubated with 50 μM Ac-LEHD-AFC (Alexis Corp., USA) at 37 °C for 2 h. The caspase activity was measured (excitation 400 nm/emission 505 nm) using the SpectraMax M5e (Molecular Devices, USA).

3. Results

3.1. Increased IFN-α/β-mediated apoptosis in Ubp43-deficient mouse bone marrow cells and UBP43-knockdown THP-1 cells

Previously we had shown that the loss of Ubp43 in mice resulted in an enhanced and prolonged STAT1 phosphorylation upon IFN-α/β stimulation and increased apoptosis of hematopoietic cells upon polyI:C or LPS administration [10]. We confirmed whether the elevated death of the Ubp43-deficient hematopoietic cells by polyI:C or LPS *in vivo* is in fact mediated by IFN-α/β. Bone marrow cells from *Ubp43*$^{+/+}$ and *Ubp43*$^{-/-}$ mice were cultured *in vitro* and treated with IFN-β. Both cells showed similar basal levels of spontaneous cell death in the absence of IFN-β. In contrast, the addition of IFN-β increased cell death by approximately 40% in the Ubp43-deficient cells, whereas the wild-type cells showed only approximately 10% increases in cell death (Fig. 1A). Thus, the Ubp43-deficient mouse bone marrow cells are more susceptible to IFN-α/β-mediated apoptosis.

To test whether the elevated susceptibility to apoptosis is also applied to human cells, we generated stable UBP43 knockdown cells using human monocytic leukemia cell line, THP-1. We first examined whether UBP43-knockdown THP-1 cells showed similar hypersensitivity to IFN-α/β with UBP43-deficient bone marrow cells. Since UBP43 functions as a negative feedback regulator against JAK/STAT signaling after its expression by IFN-α/β, we challenged the cells with IFN-α twice. As shown in Fig. 1B, the cells were pre-treated with IFN-α for 2 h and cultured in the same medium but without IFN-α for 24 h. The cells were then treated with IFN-α again and analyzed for the phosphorylation of STAT1 as an indication of signal activation at various time points. Two independent lines of UBP43-knockdown THP-1 cells showed a significant elevation and extended duration of STAT1 phosphorylation when compared to the control cells (Fig. 1B). With the elevated STAT