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Reactive oxygen species-regulated glycogen synthase kinase-3β activation contributes to all-*trans* retinoic acid-induced apoptosis in granulocyte-differentiated HL60 cells

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Chemical compounds studied in this article:
All-*trans*-retinoic acid (PubChem CID: 444795)
Phorbol 12-myristate 13-acetate (PubChem CID: 27924)
6-bromoindirubin-3′-oxime (PubChem CID: 5287844)
Lithium chloride (PubChem CID: 433294)
SB415286 (PubChem CID: 4210951)
Diphenylene iodonium (PubChem CID: 3101)
LY294002 (PubChem CID: 3974)

ABSTRACT

All-*trans* retionic acid (ATRA) treatment confers disease remission in acute promyelocytic leukemia (APL) patients by inducing granulocytic differentiation, which is followed by cell apoptosis. Although glycogen synthase kinase (GSK)-3β is known to be required for spontaneous cell death in neutrophils, the requirement of GSK-3β activation for the apoptotic effects remains unknown. This question is addressed in the present study using a model of ATRA-induced granulocytic differentiation and apoptosis in APL HL60 cells. ATRA at a therapeutic concentration (1 μM) induced granulocytic differentiation, followed by apoptosis. ATRA treatment caused decreased Mcl-1, caspase-3 activation, and PARP cleavage following the inactivation of phosphatidylinositol 3-kinase/AKT and the activation of GSK-3β. Pharmacologically and genetically inhibiting GSK-3β effectively retarded ATRA-induced Mcl-1 degradation and apoptosis. Additional differentiation inducers, phorbol 12-myristate 13-acetate and dimethyl sulfoxide, also triggered GSK-3β-dependent apoptosis. Mechanistically, ATRA caused the generation of reactive oxygen species (ROS) through increased expression of NADPH oxidase subunits ($p47^{phox}$ and $p67^{phox}$) to facilitate ATRA-induced GSK-3β activation and cell apoptosis. This study indicates that ROS initiate GSK-3β-dependent apoptosis in granulocyte-differentiated cells after long-term ATRA treatment.

1. Introduction

Following differentiation from bone marrow, polymorphonuclear neutrophils have the shortest lifespan regarding both homeostasis and inflammation resolution with respect to its potential effects on microbial defense and inflammatory responses [1]. Indeed, the appropriate modulation of neutrophil survival and apoptosis constitute strategies to manipulate the resolution of inflammation [2,3]. A type of cell death initiated in neutrophils is spontaneous or constitutive apoptosis that is independent of extracellular apoptotic stimuli, and studies demonstrate that the activation of caspase-8 in a non-death receptor manner mediates spontaneous apoptosis in neutrophils [4]. Additionally, the intrinsic pathway of Bax activation and loss of Mcl-1, which results in mitochondrial injury, are typically required for apoptotic processing [5,6], and the generation of reactive oxygen species (ROS) through NADPH oxidase is also crucial in the execution of spontaneous neutrophil apoptosis [6,7]. Although the molecular mechanisms have been investigated in primary human neutrophils, genetic approaches are difficult to undertake without the use of animal models [8,9]. However, an alternative method of

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triggering granulocytic differentiation in human myeloid cell lines affords the possibility of examining the crosstalk signaling among apoptotic processes [10–12].

Acute promyelocytic leukemia (APL), classified as the M3 subtype of acute myeloid leukemia (AML) characterized by an increase in differentiation-blocked myeloblasts [13], is specifically associated with a t(15; 17)(q22; q12) translocation that generates a promyelocytic myeloid leukemia/retinoic acid receptor α (PML/RARα) fusion transcript. The fusion protein possesses a high binding affinity for DNA that results in the blockage of transcription and granulocytic differentiation. The universal treatment of patients with APL is achieved with the promising drug all-*trans* retinoic acid (ATRA), the carboxylic acid form of vitamin A as the substrate of PML/RARα [14–16]. Studies have demonstrated that ATRA treatment prompts human APL HL60 cells to undergo granulocytic differentiation, followed by apoptosis [10–12]. Further studies have shown that ATRA induces the intrinsic apoptotic pathway, which involves Bax activation and Bcl-2 downregulation, accompanied by mitochondrial dysfunction [17–19]. Thus, the model of ATRA-based granulocytic differentiation followed by apoptosis is suitable for investigating the mechanisms underlying spontaneous neutrophil apoptosis.

Current studies show that a variety of stimuli cause neutrophilia by sustaining cell survival against spontaneous apoptosis [2,20–23]. A common mechanism via the inactivation of glycogen synthase kinase (GSK)-3β followed by Mcl-1 stabilization contributes to neutrophil survival [24,25]. Essentially, anti-apoptotic Mcl-1 functions by inhibiting Bax activation prior to mitochondrial damage [26], and the pro-apoptotic role of GSK-3β is due to its direct phosphorylation of Mcl-1, followed by the latter's degradation via an ubiquitin–proteasome-dependent system [27]. Furthermore, the activation of GSK-3β also phosphorylates Bax at Ser^{163} to promote Bax activation, resulting in mitochondrial destabilization and apoptosis [28]. As phosphatidylinositol 3-kinase (PI3K)/AKT signaling is the major process for GSK-3β inactivation through the phosphorylation at Ser^{9} [29], PI3K/AKT activation and GSK-3β inhibition are therefore able to reverse constitutive neutrophil apoptosis [24,25]. In the present study, we investigated the molecular mechanism underlying ATRA-induced granulocytic differentiation followed by apoptosis in APL HL60 cells. The results showed that an increase in NADPH oxidase triggers ROS-regulated PI3K/AKT/GSK-3β/Mcl-1 signaling to facilitate apoptosis in granulocyte-differentiated cells.

2. Materials and methods

2.1. Cells, culture condition, and reagents

Human APL HL60 cells were kindly provided by Dr. Chi-Chang Shieh, Institute of Clinical Medicine, National Cheng Kung University, Taiwan. The cells were cultured in RPMI-1640 medium (Invitrogen Life Technologies, Carlsbad, CA, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Invitrogen Life Technologies) and maintained at 37 °C with 5% CO_2. ATRA, phorbol 12-myristate 13-acetate (PMA), GSK-3 inhibitors 6-bromoindirubin-3′-oxime (BIO), lithium chloride (LiCl), and SB415286, and NADPH oxidase inhibitor diphenylene iodonium (DPI) were purchased from Sigma-Aldrich (St. Louis, MO, USA) and dissolved in dimethyl sulfoxide (DMSO, Sigma-Aldrich). The PI3-kinase inhibitor LY294002 was obtained from Cayman (Ann Arbor, MI, USA) and dissolved in DMSO.

2.2. Western blot analysis

Forty micrograms of proteins from each sample were separated by SDS-PAGE and then transferred to a polyvinylidene difluoride membrane (Millipore, Billerica, MA, USA). After blocking, the blots were developed with a series of antibodies, as indicated. Goat antibodies specific for human $p67^{phox}$ and mouse antibodies against enhanced green fluorescent protein (EGFP) and $p47^{phox}$ (Santa Cruz Biotechnology, Santa Cruz, CA, USA) were used. Rabbit antibodies specific for human AKT and phosphorylated AKT (Ser^{473}), GSK-3β and phosphorylated GSK-3β (Ser^{9}), and Mcl-1 (Cell Signaling Technology, Beverly, MA, USA), phosphorylated GSK-3β (Tyr^{216}) (Abcam, Cambridge, UK), and β-actin (Sigma-Aldrich) were used. The blots were incubated with horseradish peroxidase-conjugated anti-goat, rabbit or mouse IgG (Cell Signaling Technology) and developed using an ECL development kit (Millipore). The relative signal intensity was quantified using ImageJ software (version 1.41o) from W. Rasband (National Institutes of Health, Bethesda, MD). The quantitative results of optimal band density were used for labeling the changes by the ratio of phosphorylated protein compared to total protein and the ratio of protein compared to β-actin.

2.3. Transfection

For human GSK-3β and Mcl-1 overexpression, GSK-3β dominant-negative mutant (EGFP-GSK-$3\beta^{R96A}$) and its control (EGFP-C2) and pcDNA3-HA-hMcl-1 and its control (pcDNA3-HA) were kindly provided by Dr. Pei-Jung Lu (Institute of Clinical Medicine, National Cheng Kung University, Taiwan) and Dr. Hsin-Fang Yang-Yen (Institute of Molecular Biology, Academia Sinica, Taiwan), respectively. The transfection was performed by electroporation using a pipette-type microporator (Microporator system; Digital Bio Technology, Suwon, Korea). Transfections were carried out using 5 μl of lipofectamine transfection reagent (Invitrogen Life Technologies) and 0.5 μg of each construct in serum-free medium and transfection condition was set following 1400 V/30 ms/2 pulse for 5×10^6 cells. The transfected cells were then cultured in RPMI 1640 growth medium for 24 h.

2.4. Lentiviral-based short hairpin RNA transfection

Non-targeting shRNA control vector (shLuc; TRCN0000072247) and shRNA constructs targeting human GSK-3β (shGSK-3β; TRCN0000040001, containing the target sequence 5′-GCTGAGCTGTTACTAGGACAA-3′), human $p47^{phox}$ no.1 (shNCF1; TRCN0000256331 containing 5′-CCATTGCCAACTACGAGAAGA-3′), and human $p47^{phox}$ no.2 (shNCF1; TRCN0000256333 containing 5′-AGGGCACACTTACCGAGTACT-3′) were purchased from National RNAi Core Facility (Institute of Molecular Biology/Genomic Research Center, Academia Sinica, Taipei, Taiwan). Lentivirus was prepared as in our previous study [30].

2.5. Liu's staining

The morphological assessment of differentiated cells was performed using Liu's staining method, a modified method originally from Romanowsky stain. Cells (5×10^4) were fixed on slides using a cytocentrifuge (Cytospin 4, Thermo Scientific, Runcorn, Cheshire, UK), and the samples were processed with Liu's stain (TONYAR Biotech, Taoyuan, Taiwan). First, we added 0.5 ml LiuA reagent containing Eosin Y (for cytoplasm stain) to the slides for 40 s at room temperature and then added 1 ml LiuB reagent containing Azur I and methylene azure (for nuclei stain) to the slides for 2 min at room temperature, followed by gent mixing of the reagents by blowing on the slides. The slides were washed with running water for 2 min and then air dried. The morphology of the cells was examined under a light microscope.