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STAT3 contributes to lysosomal-mediated cell death in a novel derivative of riccardin D-treated breast cancer cells in association with TFEB

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ABSTRACT

RDD648, a novel derivative of a natural molecule riccardin D, exhibited potent anticancer activity by targeting lysosomes *in vitro* and *in vivo*. Mechanistic studies revealed that RDD648 facilitated STAT3 to translocate into the nucleus, and this activity was involved in lysosome-mediated cell death as evidenced by our finding that inhibition of STAT3 alleviated lysosomal membrane permeabilization. Further investigation indicated that nuclear STAT3 directly interacted with transcription factor TFEB, leading to the partial loss of function of TFEB, which is essential for lysosome turnover. The present study first uncovers that STAT3 contributes to lysosomal-mediated cell death in RDD648-treated breast cancer cells though interacting with TFEB, and the findings may be significant in the design of treatments for breast cancers where STAT3 is constitutively expressed.

1. Introduction

Signal transducers and activators of transcription (STAT) proteins are a family with seven cytoplasmic members that translocate to the nucleus on activation by cytokines and growth factors. Activated STATs regulate gene expression to affect cell communication and induce diverse biological responses [1]. Among of these factors, STAT3, originally discovered as a transcription factor activated during inflammatory cytokine signalling, is critical in cancer development and progression because it is constitutively expressed in approximately 70% of human cancer cell lines, including breast, prostate, lung, brain, and pancreas [2,3]. Therefore, STAT3 is considered an oncogene, that regulates downstream target genes involved in cell proliferation, survival, metastasis, inflammation, and DNA damage repair [4]. However, in contrast to numerous reports in cancers, STAT3 is involved in lysosome-mediated cell death during mammary gland involution as evidenced by up regulation of the expression of lysosomal proteases cathepsins, which results in lysosomal membrane permeabilization (LMP) [5,6]. This study is the first report to demonstrate that STAT3 contributes to a lysosomal-mediated programme of cell death (LM-PCD) under physiological conditions. The role of STAT3 in mediating LMP in cancer has not been elucidated.

As key subcellular organelles, with numerous digestive enzymes, lysosomes are involved in multiple cellular processes such as autophagy, receptor degradation, apoptosis, and release of nutrients into the cytoplasm [7,8]. Lysosomes are increasingly becoming an area of interest in oncology [9], because the number of these acidic organelles in cancer cells is increased, exhibiting increased volume and cathepsin activity. However, these lysosomes are less stable than those in normal cells [10,11]. LMP actively triggers cancer cell death by releasing cathepsins into the cytoplasm where they induce mitochondrial apoptosis or apoptosis-like pathways [12]. Most recently, LMP-mediated non-apoptotic cell death is demonstrated in mammary gland epithelial cells and characterized by hypercondensed nuclei, swollen cells, and caspase independence [6]. This process is controlled by STAT3 via stimulation of the expression of cathepsins B and L [6,13]. These findings constitute a mechanism for STAT3-mediated cell death and reveal an association among STAT3, cysteine cathepsins and LM-PCD. Whether highly expressed STAT3 in cancer cells is also able to induce LMP-mediated cell death remains largely unknown. In addition, most of cancer-associated

Abbreviations: STAT3, signal transducers and activators of transcription 3; RD-N, Riccardin D-N; CTSB, cathepsin B; LMP, lysosomal membrane permeabilization; LAMP1, lysosome associated membrane protein 1; LM-PCD, lysosomal-mediated programme of cell death; AO, acridine orange; MTT, 3-(4,5-dimethylthiazol)-2,5-diphenyltetrazolium bromide; TFEB, transcription factor EB; CLEAR, coordinated lysosomal expression and regulation; SQSTM1, sequestrome 1; NBR1, Neighbor of BRCA1 Gene 1; LC3, Microtubule-associated proteins light chain 3; ATM, ataxia telangiectasia mutated; ATR, ataxia telangiectasia and Rad3 related; GAPDH, Glyceraldehyde-3-phosphate dehydrogenase; GRP78, glucose-regulated protein 78; PARP, poly(ADP-ribose)polymerase; PERK, RNA-dependent protein kinase-like ER kinase

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lysosomal changes promote invasive growth, however, these same changes also sensitize cancer cells to LMP. Therefore, these results provide a rationale for utilizing chemotherapeutic agents that destabilize lysosomal membranes in cancer therapy [14].

Weakly basic drugs accumulate more efficiently in lysosomes due to the acidic lysosomal conditions. Previously, we have demonstrated that the natural alkaloidal steroid solamargine and the natural ent-kurane diterpenoidal derivative that involved the introduction of a basic group exhibited potent anticancer effects by targeting the lysosomes [11,15]. Riccardin D-N (RD-N), the aminomethylated derivative of riccardin D, exhibits promising efficiency in anticancer activity through lysosomal rupture [16]. Currently, we further modified the riccardin D with 4-methylpiperazinyl groups to evaluate the anticancer activity of the compounds. Cell-based assays resulted in the identification of the bis-Mannich modification of riccardin D, RDD648 as a potential agent for further mechanical investigation [17]. RDD648 significantly induced LMP *in vitro* and *in vivo,* accompanied increased STAT3 expression levels in breast cancer cells after prolonged treatments. Upon STAT3 depletion, lysosomes could relatively load more lysosomotropic fluorescence probe in RDD648-treated cells. Encouraged by the previous studies and these results of RDD648 treatment, we further researched the mechanism of RDD648 and the association between STAT3 and lysosomes. RDD648 facilitated STAT3 to translocate into nucleus where it directly interacted with transcription factor TFEB, leading to the partial loss of TFEB function, which is essential for lysosomal turnover. The present study is the first to demonstrate that STAT3 contributes to lysosomal-mediated cell death in RDD648-treated breast cancer cells though interaction with TFEB.

2. Materials and methods

2.1. Chemicals and reagents

RDD648 was prepared from riccardin D, 1-methylpiperazine and aqueous formaldehyde by the Mannich reaction in moderate yield (Fig. 1A). The final product was more than 98% pure and dissolved in dimethyl sulfoxide (DMSO) at a stock concentration of 10 mM, and was stored at −20 °C. LC3B and β-Actin antibodies, MTT, DMSO, Hoechst 33342 and Z-VAD-fmk were purchased from Sigma Co. (USA). Primary antibodies for Beclin-1, STAT3, p-STAT3 (Tyr705), p-STAT3 (Ser727), p-ATM, p-Chk-2, p-ATR, p-Chk-1, p-BRCA-1, p-cdc2, p-p53, TFEB, γH2AX and PCNA were obtained from Cell Signaling Technology (Boston, MA). CTSB was obtained from Abcam (Cambridge, UK). GAPDH, SQSTM1, CTSD, CTSL, p53, p21, LAMP2, CyclinB1, PERK, p-PERK antibody and secondary biotin-conjugated goat anti-mouse IgG or anti-rabbit IgG were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). GRP78/BIP polyclonal antibody were purchased from Proteintech (USA). DAPI, DCFH-DA, JC-1, PI, Lyso-Tracker Red and acridine orange (AO) were purchased from Beyotime Institute of Biotechnology (China). AnnexinV-FITC Apoptosis Detection Kit was brought from BD Biosciences (USA). Lipofectamine 2000 was purchased from Invitrogen (USA). S3I201 and leukemia inhibitory factor (LIF) were purchased from Selleckchem (USA) and Peprotech (USA), respectively. Z-RR-AMC, E64d, CA074Me and pepstatin A were purchased from Enzo Biochem Inc. (New York, USA).

2.2. Cell culture and treatments

Human breast adenocarcinoma cell lines MCF-7 and HCC1428 were purchased from Shanghai Institute for Biological Sciences (SIBS), China Academy of Sciences (China). These cells were cultured in RPMI-1640 (HyClone) medium containing 10% FBS, 100 units/mL of penicillin G, and 100 μg/mL of streptomycin in a stable environment with 37 °C and 5% CO_2. After cell confluence reached 50–80%, the cells were treated with RDD648 as indicated. Control cells were subjected to DMSO treatment.

2.3. MTT assay

Cells were seeded into 96-well plates at (3–5) × 10^3/well and incubated with RDD648 for indicated time points. After incubation, cells were supplied with MTT (5.0 mg/mL) solution, and the plates were incubated for an additional 4 h at 37 °C. Cell growth response to the chemical was determined at 570 nm on a microplate reader (Bio-Rad 680). All experiments were performed in triplicate in three independent experiments. The cell viability inhibitory ratio was calculated by comparing to vehicle control through the formula:

$$\text{Cell viability ratio}(\%) = \frac{\text{A570sample}-\text{A570blank}}{\text{A570control}-\text{A570blank}} \times 100\%$$

2.4. Immunofluorescence staining

Cells were seeded onto 12-mm round, glass cover slips in 24-well plates. After indicated treatment, cells were fixed with cold methanol: acetone (1:1) for 5 min and washed twice with cold PBS. Then cells were incubated with 3% goat serum (in 0.1% Triton X-100) 20 min to prevent nonspecific antibody binding. After removing the liquid, cover slips were incubated with the antibody overnight, washed in PBS thrice, and incubated with TRITC or FITC-conjugated goat anti-rabbit or anti-mouse secondary antibodies for 1 h. Then, cells were washed in PBS thrice and counter-stained with DAPI for 15 min. Fluorescence images were captured under an LSM 700 confocal microscope using a Plan-Apochromat 63 × /1.40 Oil lens and ZEN 2009 software (Carl Zeiss).

2.5. Flow cytometry

We used Annexin V- FITC (0.1 mg/mL) for the assessment of phosphatidylserine exposure, PI (0.5 mg/mL) for cell viability, Lyso-Tracker Red (100 nM) or AO (5 μM) for lysosomal integrity assays, JC-1 (10 μg/mL) for the measurement of mitochondrial membrane potential. Flow cytometry was performed on a FACScan cytometry (FACSCalibur, Becton Dickinson, USA). Data were analysed using MODFIT or CELLQUEST software (Verity Software House, Topsham, Maine, USA).

2.6. Transient transfection assay

Chemically synthesized siRNAs were purchased from Shanghai Integrated Biotech Solutions Co., Ltd. Non-targeting control siRNA sequence was 5′-UUC UCC GAA CGU GUC ACG UTT-3′. TFEB siRNA sequences were used as follows: 5′-AGA CGA AGG UUC AAC AUC A-3′. STAT3 siRNA sequences were used as follows: 5′-UUG AGA UUC UGC UAA UGA CGU UAU C-3′ [18]. ATG5 siRNA and LC3 siRNA were kindly provided by Professor Yuan [19,20]. Cells were grown to 60% confluence in 6-well plates prior to transfect with siRNA oligos using lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. After transfection for 24 h, cells were exposed to RDD648 for an additional 12 h. Cell lysates were prepared for Western blotting assay and so on.

2.7. RNA extraction and relative quantification by real-time PCR

Total mRNA was extracted using the TRNzol-A$^+$ Reagent (Tiangen Biotech, Co., Ltd.) according to the manufacturer's protocol. cDNA was synthesized through reverse transcription using ReverTra Ace qPCR RT Kit (TOYOBO, Japan) according to the manufacturer's protocol. Genes expression were detected by real-time PCR. PCR amplification was performed in an 8-tube strip format (Axygen, Union City, CA) in triplicate. Each reaction contained 1 × SYBR Green PCR Master mix, 1 μL forward primer and reverse primer and 1 μL template cDNA in a final volume of 10 μL. PCR was performed on a Mastercycler ep realplex apparatus (Eppendorf, Germany). Following primers were used: LAMP1 gene (forward: 5′-ACG TTA CAG CGT CCA GCT CAT-3′ and reverse: 5′-