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Ferroptosis is a lysosomal cell death process

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ABSTRACT

Ferroptosis is a form of regulated cell death resulting from iron accumulation and lipid peroxidation. While impaired ferroptosis is tightly linked to human diseases and conditions, the mechanism and regulation of ferroptosis remain largely unknown. Here, we demonstrate that STAT3 is a positive regulator of ferroptosis in human pancreatic ductal adenocarcinoma (PDAC) cell lines. Activation of the MAPK/ERK pathway, but not inhibition of system Xc^-, was required for STAT3 activation during erastin-induced ferroptosis. Importantly, pharmacological inhibition and genetic silencing of STAT3 through small molecules (e.g., cryptotanshinone and S3I-201) or siRNA blocked erastin-induced ferroptosis in PDAC cells. Mechanically, STAT3-mediated cathepsin B expression was required for ferroptosis. Consequently, inhibition of lysosome-dependent cell death by pharmacological blockade of cathepsin activity (using CA-074Me) or vacuolar type H+-ATPase (using bafilomycin A1) limited erastin-induced ferroptosis. These studies indicate that ferroptosis is a lysosomal cell death process.

1. Introduction

Pancreatic ductal adenocarcinoma (PDAC) is the most common type of pancreatic cancer and has the universal mutations in the proto-oncogene K-RAS [1]. In the USA, PDAC accounts for about 3% of all cancers and about 7% of all cancer deaths [2]. The five-year and one-year survival rates for PDAC patients are 5–7% and less than 20%, respectively [2]. For PDAC that has not spread to distant sites, surgery is considered the primary treatment. Most PDAC patients are diagnosed at a late stage and radical pancreatic resection is not possible. Gemcitabine-based chemotherapy is currently the leading therapy for all stages of PDAC [3]. However, cell signaling pathway alterations, metabolic reprogramming, and an immunosuppressive microenvironment result in resistance to conventional chemotherapy, including gemcitabine [4–7].

STAT3 (signal transducer and activator of transcription 3) is a signaling molecule for many cytokines and growth factor receptors and plays an important role in tumor biology [8]. Basic and clinical studies have found that STAT3 is constitutively activated in PDAC and possesses oncogenic potential in tumorigenesis and anti-apoptotic functions in therapy [9–12]. After activation, STAT3 translocates from the cytosol to the nucleus where it controls expression of several genes involved in the regulation of multiple cellular processes, including cell survival and cell death [13–17]. Thus, STAT3 is an important therapeutic target for human cancers, including PDAC [8].

Ferroptosis is a multi-step regulated cell death process from iron accumulation and lipid peroxidation [18,19]. This non-apoptotic form of cell death was first observed in RAS mutated cells in response to the small molecule erastin, which can inhibit the cystine/glutamate transporter system Xc^-, leading to cysteine starvation, glutathione depletion, and consequently, oxidative death [20]. In addition to inhibition of system Xc^- activity, impaired antioxidant GPX4 (glutathione peroxidase 4) enzyme functions [21,22] or NRF2 (nuclear factor erythroid 2-related factor 2)-antioxidant response [23,24] also can induce ferroptosis *in vitro* or

Abbreviations: PDAC, pancreatic ductal adenocarcinoma; STAT3, signal transducer and activator of transcription 3; GPX4, glutathione peroxidase 4; NRF2, nuclear factor erythroid 2-related factor 2; Q-PCR, quantitative real-time polymerase chain reaction; CCK-8, cell counting kit-8; MDA, malondialdehyde; MAPK, mitogen-activated protein kinase; JNK, c-JUN N-terminal kinase; ERK, extracellular signal-regulated kinase; VDAC, voltage-dependent anion channel; MEK1/2, mitogen-activated protein kinase kinase1/2.

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in vivo. However, the molecular mechanisms of signal transduction in ferroptosis remain obscure.

In this study, we demonstrated that STAT3 promotes erastin-induced ferroptosis through activation of lysosomal cell death in human PDAC cell lines. Our findings uncover an important function of STAT3 in regulated cell death and provide a new strategy for the treatment of pancreatic cancer.

2. Methods

2.1. Regents

The antibodies to phospho-STAT3 (Tyr705) (Cat#9145), STAT3 (Cat#9139), ERK1/2 (Cat#4696), MEK1 (Cat#9124), MEK2 (Cat#9147), cathepsin B (Cat#31718), LAMP1 (Cat#9091), and actin (Cat#3700) were purchased from Cell Signaling Technology (Danvers, MA, USA). Erastin (Cat#S7242), SP600125 (Cat#S1460), SB203580 (Cat#S1076), SCH772984 (Cat#S7101), cryptotanshinone (Cat#S2285), S3I-201 (Cat#S1155), liproxstatin-1 (Cat#S7699), Z-VAD-FMK (Cat#S7023), necrosulfonamide (Cat#S8251), CA-074Me (Cat#S7420), and bafilomycin A1 (Cat#S1413) were purchased from Selleck Chemicals (Houston, TX, USA). Oncostatin-M (Cat# 295-OM-010) and IL-6 (Cat# 206-IL-010) were purchased from R&D Systems (Minneapolis, MN, USA).

2.2. Cell cultures

PANC1 (Cat#CRL-1469) and CFPAC1 (Cat#CRL-1918) were obtained from American Type Culture Collection (ATCC, USA). These cells were grown in Dulbecco's Modified Eagle's Medium or Iscove's Modified Dulbecco's Medium with 10% fetal bovine serum, 2 mM L-glutamine, and 100 U/ml of penicillin and streptomycin. All cells were mycoplasma free and authenticated by Short Tandem Repeat DNA Profiling Analysis.

2.3. RNAi

SLC7A11-siRNA (Cat#AM16708) was purchased from Thermo Fisher Scientific (Pittsburgh, PA, USA). ERK1/2-siRNA (Cat#6560), MEK1-siRNA (Cat#6426), MEK2-siRNA (Cat#6431), and STAT3-siRNA (Cat#6582) were purchased from Cell Signaling Technology (Danvers, MA, USA). Transfections were performed with Lipofectamine® RNAiMAX (Cat#13778, Thermo Fisher Scientific) according to the manufacturer's instructions.

2.4. Quantitative real time polymerase chain reaction (Q-PCR) analysis

First-strand cDNA synthesis was carried out using a Reverse Transcription System Kit according to the manufacturer's instructions (Cat#11801-025, OriGene Technologies, Rockville, MD, USA). cDNA from various cell samples was amplified with specific primers (cathepsin B: 5′-TACCTTCGAGGTACTGGTCCCT-3′ and 5′-GGTGGAGAAAGTCCAGCAACTG -3′) and the data was normalized to actin RNA (5′-CACCATTGGCAATGAGCGGTTC -3′ and 5′-AGGTCTTTGCGGATGTCCACGT -3′).

2.5. Western blot

Western blot was performed as previously described [25]. In brief, proteins in the cell lysate or supernatants were resolved on 4%–12% Criterion XT Bis-Tris gels (Cat#3450124, Bio-Rad, Hercules, CA, USA) and transferred to a nitrocellulose membrane. After blocking with 5% milk, the membrane was incubated for 2 h at 25 °C or overnight at 4 °C with various primary antibodies. After incubation with peroxidase-conjugated secondary antibodies for 1 h at routine temperature, the signals were visualized using enhanced or super chemiluminescence (Pierce, Rockford, IL, USA) and by exposure to X-ray films.

2.6. Cell viability assay

Cell viability was evaluated using the Cell Counting Kit-8 (CCK-8) (Cat#96992, Sigma) according to the manufacturer's instructions. The assay is based on utilizing the highly water-soluble tetrazolium salt WST-8 [2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium,monosodium salt] to produce a water-soluble formazan dye upon reduction in the presence of an electron carrier. Absorbance at 450 nm is proportional to the number of living cells in the culture.

2.7. Glutamate release assay

The release of glutamate from cells into the extracellular medium was detected using an Amplex Red glutamate release assay kit (Cat#A12221, Thermo Fisher Scientific). Glutamate release was first normalized to the total cell number determined with the CCK-8 Kit at the end of the experiment, then values were expressed as a percentage of no-treatment controls.

2.8. Malondialdehyde (MDA) assay

The relative MDA concentration in cell lysates was assessed using a Lipid Peroxidation Assay Kit (Cat#ab118970, Abcam, Cambridge, MA, USA) according to the manufacturer's instructions [26]. Briefly, the MDA in the sample reacted with thiobarbituric acid (TBA) to generate a MDA-TBA adduct. The MDA-TBA adducts were quantified colorimetrically (OD = 532 nm) or fluorometrically (Ex/Em = 532/553 nm).

2.9. Lysosome isolation

Lysosome isolation was performed using a Lysosome Isolation Kit (Cat#LYSISO1-1KT, Sigma) according to the manufacturer's instructions.

2.10. Statistical analysis

Statistics were calculated with GraphPad Prism 7. A standard two-tailed unpaired Student's *t*-test or one-way ANOVA was used for statistical analysis. A P value of less than 0.05 was considered statistically significant.

3. Results

3.1. Erastin induces STAT3 activation in ferroptosis

To determine the impact of system Xc^- on STAT3 phosphorylation during ferroptosis, we treated human PDAC cell lines (PANC1 and CFPAC1) with erastin and sorafenib. As expected, a glutamate release assay revealed that system Xc^- activity was reduced in PDAC cell lines in response to erastin and sorafenib (Fig. 1A). In contrast, erastin promoted whereas sorafenib inhibited STAT3 phosphorylation at Tyr705 in these PDAC cell lines (Fig. 1B), indicating that erastin and sorafenib play different roles in the regulation of STAT1 activation.

We next investigated whether the expression of SLC7A11, a core component of system Xc^-, is required for erastin-induced STAT1 activation. Similar to previous studies [20], erastin induced SLC7A11 upregulation in PANC1 cells. However, knockdown of

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