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Original Article

MLKL-PITPα signaling-mediated necroptosis contributes to cisplatin-triggered cell death in lung cancer A549 cells

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ABSTRACT

Necroptosis has been reported to be involved in cisplatin-induced cell death, but the mechanisms underlying the occurrence of necroptosis are not fully elucidated. In this study, we show that apart from apoptosis, cisplatin induces necroptosis in A549 cells. The alleviation of cell death by two necroptosis inhibitors—necrostatin-1 (Nec-1) and necrosulfonamide (NSA), and the phosphorylation of mixed lineage kinase domain-like protein (MLKL) at serine 358, suggest the involvement of receptor-interacting protein kinase 1 (RIPK1)-RIPK3-MLKL signaling in cisplatin-treated A549 cells. Additionally, the initiation of cisplatin-induced necroptosis relies on autocrine tumor necrosis factor alpha (TNF-α). Furthermore, we present the first evidence that phosphatidylinositol transfer protein alpha (PITPα) is involved in MLKL-mediated necroptosis by interacting with the N terminal MLKL on its sixth helix and the preceding loop, which facilitates MLKL oligomerization and plasma membrane translocation in necroptosis. Silencing of PITPα expression interferes with MLKL function and reduces cell death. Our data elucidate that cisplatin-treated lung cancer cells undergo a new type of programmed cell death called necroptosis and shed new light on how MLKL translocates to the plasma membrane.

1. Introduction

The core of cancer therapy is to remove cancer cells from normal tissue. Apart from surgery, chemotherapy is the strategy that best manifests this idea. Cisplatin (*cis*-diamminedichloroplatinum(II), DDP), a chemotherapeutic drug targeting DNA, is still incorporated in a broad range of combined antineoplastic chemotherapy regimens for various solid tumors. Inasmuch as cisplatin remains a first-line agent in lung cancer chemotherapy, it is pivotal to fully understand its cytotoxic mechanisms in lung cancer cells. Generally, apoptosis is widely believed to be the fate of the cisplatin-treated cells. Recent researches have shown that cisplatin may cause a new type of programmed cell death known as necroptosis [1–3]. Necroptosis is substantially involved in pathological states such as organ inflammation [4,5], arteriosclerosis [6], cerebral ischemia [7], and antiviral responses [8]. Mechanisms that initiate necroptosis differ. Chemical compounds (MNNG, shikonin), protein ligands (TNF-α, TRAIL), and analog of nucleic acids (poly (I:C)) are suggested to trigger necroptosis under certain circumstances [9–13]. Several antineoplastic drugs (5-fluorouracil, Obatoclax, and cisplatin) are demonstrated to kill tumor cells through necroptosis as well [14–16].

Interactions between RIPK1-RIPK3 or RIPK3-RIPK3 and the ensuing recruitment and phosphorylation of MLKL are widely acknowledged necroptosis pathways [17–19]. Phosphorylated MLKL then forms oligomers and translocates to the plasma membrane [20,21]. It has been demonstrated that to kill cells, MLKL needs to bind on some species of phosphatidylinositol phosphates (PIPs) at the plasma membrane, and interfering with the synthesis of PIPs inhibits its killing ability [22–24]. However, the underlying mechanisms are not fully explored. PITPα belongs to the family of PITPs. It participates in the transfer of phosphatidylinositol (PI) between membranes, as well as in the regulation of PIPs synthesis [25]. Thus, we hypothesized that PITPα might assist in the function of MLKL.

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In this study, we show that apart from apoptosis, cisplatin-treated A549 cells undergo necroptosis. We show that MLKL forms oligomers and shifts from the cytosol to the plasma membrane during necroptosis, and its oligomerization and membrane translocation partially depend on PITPα. Additionally, autocrine TNF-α signaling contributes to the initiation of cisplatin-induced necroptosis.

2. Materials and methods

2.1. Reagents

Cisplatin was obtained from Qilu Pharmaceutical (Jinan, China). z-VAD(OME)-FMK was purchased from Santa Cruz Biotechnology (sc-311561; Dallas, TX, USA). Necrostatin-1 was obtained from Sigma-Aldrich (N9037; St. Louis, MO, USA). Necrosulfonamide was purchased from Abcam (ab143839; Cambridge, UK). Cholera Toxin Subunit B (Recombinant), Alexa Fluor® 594 Conjugate (CT-B) was obtained from Molecular Probes (C34777; Eugene, OR, USA). The human TNF-alpha ELISA Kit was obtained from Dakewe Biotech (DKW12-1720-096; Shenzhen, China).

2.2. Cell culture, cell death induction and inhibition

A549 and HEK-293T cells were purchased from American Type Culture Collection (Manassas, VA, USA) and cultured in RPMI-1640 supplemented with 10% FBS, 2 mM L-glutamine, and 100 U/ml penicillin/streptomycin, and maintained at 37 °C in a 5% CO_2 atmosphere. To induce death, cells were treated with cisplatin (A549 cells: 4 μg/ml) for 48 h. In the immunoprecipitation analysis, the drug treatment lasted for 48 h. In some experiments, the cells were pre-incubated with zVAD-FMK (80 μM), necrostatin-1 (40 μM), or necrosulfonamide (0.5 μM) for 1 h before cisplatin treatment.

2.3. MTT assay and lactate dehydrogenase (LDH) release assay

Cell viability was measured by MTT assay as previously described [26]. Cell death was estimated by determining LDH released into the culture medium as previously described [27].

2.4. Flow cytometry analysis

A FITC Annexin V Apoptosis Detection Kit with Propidium iodide was purchased from BioLegend (640914; San Diego, CA). Cell death was recorded on a FACSCalibur flow cytometer (Becton, Dickinson and Company, Franklin Lakes, NJ, USA) in the total population (10,000 cells), and the data were analyzed using FlowJo software (Version 7.6.1; Tree Star, Ashland, OR, USA).

2.5. Construction of recombinant plasmids and transfection

All sequences were cloned from cDNAs and amplified via PCR. Sequences encoding full-length human MLKL and truncated versions of MLKL (1–180 aa and 181–471 aa) were cloned into pEGFP-C2 (Clontech, Mountain View, CA, USA). The mutation of full-length MLKL was made by site directed mutagenesis using the QuickChange site-directed mutagenesis kit (200518; Stratagene, La Jolla, CA, USA). The sequence encoding human full-length PITPα was cloned into pDsred2-N1 (Clontech). For transfection, cells were seeded in a 6-well plate or in a 96-well plate on day 0 and transfected with DNA on day 1. Both A549 cells and HEK-293T cells were transfected with Lipofectamine 2000 reagent (Invitrogen, Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions.

2.6. Molecular docking

Protein-protein docking based on the solution structure of human MLKL N-terminal domain (PDB: 2MSV) and the structure of human PITPα (PDB: 1UW5) was conducted via RosettaDock [28,29].

2.7. RNA interference

The siRNA for human PITPα and the non-target siRNA were both designed by and obtained from GenePharma (Suzhou, China). Sequences of PITPα siRNA and non-target siRNA are described in the Supplementary Materials and Methods.

2.8. Western blotting, fraction and immunoprecipitation

Protein extraction from cells and western blotting were done as described previously [26]. To analyze the non-reducing gels, the cells were lysed in RIPA lysis buffer (P0013D; Beyotime Institute of Biotechnology) and separated via SDS-PAGE without β–mercaptoethanol or SDS. Cytosolic and membrane fractions were obtained according to the manufacturer's instructions (KGP350; KeyGEN BioTECH, Nanjing, China). For immunoprecipitation, the Pierce™ Co-Immunoprecipitation Kit (26149; Thermo Fisher Scientific, Waltham, MA, USA) was used, and all of the experimental procedures were performed according to the manufacturer's instructions. Primary antibodies were human anti-MLKL (Biorbyt, orb95482), anti-phosphorylated MLKL (S358) (Abcam, Ab187091), human anti-RIPK1 (BD Biosciences, 610458), human anti-RIPK3 (Abcam, ab56164), anti-GFP (Santa Cruz, sc-9996), anti-PITPα (Proteintech, 16613-1-AP and Santa Cruz Biotechnology, sc-13569), anti-TNF-α and anti-ATP1A1 (Proteintech, 60291-1-Ig and Proteintech, 14418-1-AP).

2.9. Transmission electron microscopy

A549 cells were dissociated from the culture plates, washed once with PBS, and then fixed in PBS containing 2% paraformaldehyde/2% glutaraldehyde for 3 h. Then we obtained the samples as previously described [30]. Thin sections were examined with a JEM-1230 transmission electron microscope (JEOL, Tokyo, Japan).

2.10. Detection of cell death via propidium iodide

Propidium iodide staining was performed as previously described [27]. To quantify the propidium iodide-positive cells, photographs were taken from three randomly selected × 100 fields per well. The propidium iodide -positive cells were expressed as a percentage of the Hoechst-positive cells.

2.11. Immunocytochemistry

For MLKL staining, A549 cells were seeded in 35 mm culture dishes. After cisplatin treatment, cells were treated as previously described [27]. The samples were examined under an Olympus FV10i-LIV microscope (Olympus, Tokyo, Japan). Photographs were taken from three randomly selected fields for each sample. In the untreated cells, MLKL was scattered and showed better overlap with the nuclei. However, MLKL aggregated near plasma membrane in the cisplatin-treated cells, which influenced the overlap. The intensity of the scattered MLKL staining was quantified by analyzing the co-localization of the MLKL protein and the nuclei using Image-Pro Plus 6.0 (Media Cybernetics, Rockville, MD USA), and the differences were analyzed by using Pearson's correlation coefficient.

2.12. Quantitative PCR (q–PCR) analysis

Total RNA was isolated with the E.Z.N.A.® Total RNA Kit II (R6934-01; Omega Biotek, USA) according to the manufacturer's protocol. cDNAs were synthesized using PrimeScript™ RT reagent Kit (RR037A; Takara, Japan). Quantitative PCR analysis was performed using the SYBR® Premix Ex Taq™ II (Tli RNaseH Plus) (RR820A; Takara, Japan). Primer sequences are described in the Supplementary Materials and Methods.

2.13. FRET measurement

HEK-293T cells were seeded in 35 mm culture dishes. After transfection, fluorescent images were captured using an A1 confocal microscopy (Nikon, Tokyo, Japan). The FRET efficiency was calculated with NIS-Elements software (Nikon, Tokyo, Japan) as previously described [31,32].

2.14. Immunohistochemistry

Immunohistochemistry was performed as described in the Supplementary Materials and Methods.

2.15. Semi-quantitative and statistical analysis

Analysis was performed as described in the Supplementary Materials and Methods.

3. Results

3.1. Cisplatin induces both apoptosis and necroptotic-like cell death in lung cancer A549 cells

To test our hypothesis that necroptosis might contribute to cisplatin-triggered lung cancer cell death, we firstly pretreated A549 cells with zVAD-FMK, a pan-caspase inhibitor of apoptosis, before cisplatin treatment to see whether it could rescue all cells from cisplatin-induced cell death. Unexpectedly, we still noticed dead cells in A549 cells with zVAD-FMK pretreatment and the dead cells in supernatant were round and appeared to be identical, whereas, the morphology of the dead cells varied when treated with cisplatin alone (Fig. 1A). The existence of death in cells with apoptosis inhibition was also verified by flow cytometry and MTT assay (Fig. 1C and Fig. S1A). Transmission electron microscopy (TEM) observations were then conducted on A549 cells treated with cisplatin or cisplatin/zVAD-FMK. The majority of dead cells in cisplatin/zVAD-FMK-treated sample showed swollen nuclei with integrated nuclear membrane, translucent cytoplasm and ruptured