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Drp1 mediates compression-induced programmed necrosis of rat nucleus pulposus cells by promoting mitochondrial translocation of p53 and nuclear translocation of AIF

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A B S T R A C T

Compression-induced programmed cell death of nucleus pulposus (NP) cells is an important contributor to intervertebral disc degeneration (IDD). Dynamin-related protein 1 (Drp1), a crucial mitochondrial fission protein, triggers programmed necrosis upon cellular injury. However, limited information is available about the role of Drp1 in compression-induced programmed necrosis of NP cells. In the present study, we found that compression resulted in upregulation and mitochondrial translocation of Drp1. Inhibition of Drp1 by siRNA or mitochondrial division inhibitor 1 (mdivi-1) effectively prevented the programmed necrosis of NP cells treated with compression. Furthermore, Drp1 promoted mitochondrial translocation of p53 and nuclear translocation of apoptosis-inducing factor (AIF) in compression-treated NP cells. Inhibition of p53 mitochondrial translocation by pifithrin-μ (PFT-μ) and silencing of AIF expression by siRNA significantly alleviated compression-induced NP cell programmed necrosis. These data indicates that Drp1 mediates compression-induced programmed necrosis of NP cells by promoting mitochondrial translocation of p53 and nuclear translocation of AIF.

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1. Introduction

Low back pain (LBP), one of the most common musculoskeletal disorders, is associated with high economic loss and disability in patients [1]. In the United States, this acute condition is associated with a healthcare cost of more than USD 100 billion per year [2]. Previous studies have confirmed that intervertebral disc degeneration (IDD) is an important cause of LBP [3,4]. To date, compressive force stimulus have been identified as a main etiological factor in IDD [5]. Programmed death of nucleus pulposus (NP) cells result from excessive compression may play a crucial role in the pathogenesis of IDD [6]. Despite accumulating evidence, mechanisms underlying compression-induced cell death are not completely understood. Therefore, an understanding of molecular events regulating programmed death of NP cells under compression is necessary to effectively treat IDD-associated disorders.

Necrosis was previously described as a passive and uncontrolled type of cell death. Recent studies indicate that necrosis can be modulated by different signal transduction pathways and programmed necrosis is now regarded as a new type of programmed cell death besides apoptosis and autophagic cell death [7,8]. Morphological changes in necrotic cells include swelling of cell and rupture of plasma membrane and organelles, which are different from those observed in apoptotic cells. More than 50% NP cells in adult human intervertebral discs undergo necrotic cell death and this percentage is approximately 80% in the elderly [9]. We previously found that compression-induced NP cell programmed necrosis was mediated by RIPK1/RIPK3/MLKL [10]. However, molecular regulation mechanisms of compression-induced NP cell programmed necrosis remain largely unexplored.

Abbreviations: LBP, low back pain; IDD, intervertebral disc degeneration; NP, nucleus pulposus; Drp1, dynamin-related protein 1; AIF, apoptosis-inducing factor; CCK-8, cell counting kit-8; TEM, transmission electron microscopy; Mdivi-1, mitochondrial division inhibitor 1; LDH, lactate dehydrogenase; HMGB1, high mobility group box 1; PFT-μ, pifithrin-μ; MPTP, mitochondrial permeability transition pore.

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Mitochondria are dynamic organelles that continuously undergo fission and fusion in response to different cellular stimuli. Dynamin-related protein 1 (Drp1) is a large GTPase protein that mediates this fission in mammalian cells [11]. Drp1 has been reported to mediate cell apoptosis by promoting release of cytochrome c and activation of caspases [12]. Recent studies indicate that Drp1 plays a crucial role in the process of programmed necrosis by promoting mitochondrial dysfunction and membrane damage [13–15]. Inhibition of Drp1 reduces acetaminophen-induced programmed necrosis of hepatocytes [14]. However, the role of Drp1 in compression-induced programmed NP cell death has not been investigated to date.

In the present study, we examined the role of Drp1 in the programmed necrosis of rat NP cells under compression. NP cells under mechanical stress showed Drp1 overexpression and mitochondrial translocation. Drp1 inhibition protected NP cells against compression-induced programmed necrosis. We also found that Drp1 mediated NP cell programmed necrosis by inhibiting mitochondrial translocation of p53 and nuclear translocation of apoptosis-inducing factor (AIF) from mitochondria.

2. Materials and methods

2.1. NP cell culture and treatment

The Huazhong University of Science and Technology animal experimentation committee approved all experiments. Primary rat NP cells were isolated and cultured as previously described by Risbud et al. [16]. Lumbar intervertebral discs from Sprague–Dawley rats (aged 11 months, 250–300 g) were harvested immediately after intraperitoneal injection of chloride hydrate anesthesia (350–400 mg/kg). Rat lumbar discs were horizontally cut open, and the nucleus pulposus (NP) was extracted with tweezers and placed in 0.25% type II collagenase (Gibco, USA) for 15 min digestion. The digested NP was explanted in complete culture medium (Dulbecco's modified Eagle's medium/ham's F-12 [DMEM/F-12, Gibco, USA] and 10% fetal bovine serum [FBS, Gibco, USA]) supplemented with 1% penicillin/streptomycin (Beyotime, China). Cells were passaged using 0.25% trypsin (Beyotime, China) when they reached 80–90% confluence and the second generation was used for subsequent experiments.

2.2. Application of a compression apparatus on rat NP cells

Rat NP cells were cultured in a pressure apparatus to mimic in vivo conditions, as described previously [17]. NP cells grown on plates were placed at the bottom of the pressure apparatus and were exposed to 1 MPa mechanical stress for 12, 24 and 48 h. Control cells (0 h) were culture in the absence of compression under the same culture conditions.

2.3. Cell viability assay

Cell viability was assessed using the Cell Counting Kit-8 (CCK-8) (Dojindo, Japan) according to the manufacturer's instructions. In brief, cells were seeded at a density of 5×10^4 cells/well in 96-well plates in 100 ml cell culture medium. At the end of the indicated treatments, 90 μl medium and 10 μl CCK-8 solution were added to each well and the plate was maintained at 37 °C for 2 h in the dark. The absorption at 450 nm was measured using a spectrophotometer (Biotek, USA).

2.4. Transmission electron microscopy

Transmission electron microscopy (TEM) was performed to examine changes in the ultrastructure of NP cells, as described previously [18]. Briefly, NP cells were trypsinized, centrifuged, and washed twice with phosphate-buffered saline (PBS). The cells were then pelleted by centrifugation at 1000*g* for 15 min, and supernatant obtained was discarded. Next, the cells were fixed with 2.5% glutaraldehyde for 2 h, followed by treatment with 1% osmium tetroxide for 2 h. The cells were then dehydrated using an ascending ethanol series and were embedded in Epon 812. Ultrathin sections of Epon 812-embedded cells were stained with uranyl acetate and lead citrate and were examined using Tecnai $G^2$12 transmission electron microscope (FEI Company, Holland).

2.5. Lactate dehydrogenase release assay

Release of lactate dehydrogenase (LDH) in culture medium was used to determine NP cell damage under compression, according to the manufacturer's (Beyotime, China) instructions. LDH release activity is presented as the ratio of LDH in the culture medium to total cellular LDH.

2.6. Propidium iodide and Hoechst 33258 double staining

The cells were directly stained with 5 mg/ml propidium iodide (PI; Beyotime) and 10 mg/ml Hoechst 33258 (Beyotime, China). After incubation for 1 h at 37 °C, the cells were washed twice with PBS and were examined using Fluoview FV100 laser scanning confocal microscope (LSM; Olympus).

2.7. Flow cytometric analysis of PI uptake

NP cells seeded in 6-well plates were detached by trypsinization and were collected by centrifugation. Next, the cells were resuspended in 500 μl binding buffer and were stained with PI at a final concentration of 5 mg/ml. The number of PI-positive cells was measured by flow cytometry (BD LSR II, Becton Dickinson, USA). Moreover, rat NP cells were treated with 20 μm mitochondrial division inhibitor 1 (mdivi-1, Selleck, USA) and 10 μm pifithrin-μ (PFT-μ, Selleck) to investigate the role of Drp1 and p53, respectively, in PI uptake by NP cells under compression.

2.8. Immunofluorescence staining

Cells grown on cover slips were fixed with 4% paraformaldehyde for 20 min at room temperature and were permeabilized with 0.5% Triton X-100 in PBS for 15 min. Next, the cells were incubated with primary antibodies against Drp1 (1:250; Abcam, USA), TOM20 (1:250; Abcam, USA), or AIF (1:500; Abcam, USA) for 1 h at room temperature, followed by incubation with Alexa Fluor-conjugated secondary antibodies (1:100; Proteintech, China) for 1 h. Finally, the cells were examined under the LSM.

2.9. Small interfering RNAs

Small interfering RNAs (siRNAs) against rat Drp1 (Drp1-siRNA) and AIF (AIF-siRNA) were designed and manufactured by Biomics (Biomics Biotechnologies Co. Ltd, China). The sequences of Drp1-siRNAs are as follows: 5′-GGAGCCAACUGGACAUUAAdTdT-3′, 5′-UUAAUGUCCAGUUGGCUCCdTdT-3'; 5′-GUGGGCUAAUGAACAAUAAdTdT-3′, 5′-UUAUUGUUCAUUAGCCCACdTdT-3'; 5′-GCUGAUCCCGGUCAUCAAUdTdT-3′, 5′-AUUGAUGACCGGGAUCAGCdTdT-3′ respectively. The sequence of AIF-siRNA is 5′-GCAAUGGCGUGUUCCUCUAdTdT-3′, 5′-UAGAGGAACACGCCAUUGCdTdT-3'. NP cells were transfected with the respective siRNA oligonucleotides at a concentration of 100 pmol/10^5 cell by Lipofectamine RNAiMAX (Invitrogen, USA). Next, the

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