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Role of the mitochondrial pathway in serum deprivation-induced apoptosis of rat endplate cells

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ABSTRACT

The apoptosis of cartilage endplates (CEPs), acting as an initiating factor, plays a vital role in the pathogenesis of intervertebral disc degenerative diseases, the underlying molecular mechanism of the apoptotic process in CEPs is still not clear. The present study aimed to investigate the mechanism of CEP cell apoptosis. We found that low levels of fetal bovine serum (FBS) can induce cell apoptosis. Serum deprivation led to high expression levels of caspase-9, caspase-3, PARP, cytochrome-c and Bax. Flow cytometric analysis showed that inhibition of the intrinsic pathway by a caspase-9 inhibitor (z-LEHD-fmk) significantly suppressed serum deprivation-induced apoptosis. However, a caspase-8 inhibitor (z-IETD-fmk) did not reduce apoptotic cell death. These data suggest that serum deprivation induces apoptosis in rat CEP cells via the activation of the intrinsic apoptotic pathway. The efficacy of a caspase-9 inhibitor in attenuating or preventing apoptosis of serum deprivation-induced disc cell apoptosis suggests that targeting the intrinsic apoptotic pathway may be used as a potential therapy for the treatment of disc degeneration.

1. Introduction

Intervertebral disc (IVD) degeneration plays a critical role in the pathogenesis of spinal disorders and is a major cause of low back pain (LBP), which occurs frequently in adult humans. LBP is often accompanied by various neurological symptoms, and it has high social and economic costs. Current treatment strategies for IVD degeneration typically address the symptom of LBP without treating the underlying cause or repairing the structural and biological deterioration because the mechanism of IVD degeneration has not been elucidated [1].

Because the intervertebral disc is an avascular organ, its nutrient-supply depends primarily on diffusion [2,3]. Recent studies found that CEPs undergo pathological histological changes with age and degeneration, including thinning and calcification, decreased cell density, and cartilage cracks as well as microfractures. The CEP alterations evidently precede the Nucleus pulposus changes, whereas the outer annulus fibrosis was significantly affected only in elderly individuals [4]. Once the porosity and permeability of a CEP decreased with these pathological histological changes, nutrient diffusion would decrease and metabolites would accumulate. This response would have a detrimental effect on matrix metabolism and ultimately affect disc composition, including the loss of proteoglycan organization and concentration, a decline in cell density and synthetic activity, and an increase in degradative enzyme activity relative to matrix synthesis, which may lead to the loss of disc structure and function recognized as disc degeneration [5]. The degeneration and calcification of CEPs, acting as initiating factor, play vital roles in the pathogenesis of intervertebral disc degenerative diseases [6,2].

However, the mechanism of CEP degeneration with age remains unclear. Recently, researchers considered that cellular loss due to excessive apoptosis of disc cells contributed to the development of IVD degeneration [6–9]. In the signaling pathways of apoptosis, there are two main caspase-dependent pathways, intrinsic and extrinsic, which are mediated by mitochondria and death-recep-

Abbreviations: CEP, cartilage endplate; FBS, fetal bovine serum; IVD, intervertebral disc; LBP, low back pain; Aparf-1, apoptotic protease activating factor-1; PBS, phosphate buffered saline; DAPI, 4′,6-diamidino-2-phenylindole; FITC, fluorescein isothiocyanate; PI, propidium iodide; PVDF, polyvinylidene difluoride.

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tors, respectively. The extrinsic pathway is chiefly activated by the Fas-receptor, which activates the procaspase-8, an apical caspase that directly activates effector caspases, including caspase-3, and leads to cell apoptosis [10,11]. The mitochondrial intrinsic pathway is mainly activated by various cellular stresses and numerous apoptotic signals with the participation of Bcl-2 family proteins. The pro-apoptotic (Bax, Bak) proteins and the anti-apoptotic (Bcl-2, Bcl-xl) proteins can cause the release of mitochondrial cytochrome c, which combines with apoptotic protease activating factor-1 (Aparf-1), procaspase-9 and ATP to form the apoptosome [12,13]. The apoptosome induces the activation of caspase-3 to trigger a cascade of caspases leading to apoptosis [14].

To date, it has been demonstrated that cellular loss due to excessive apoptosis contributes to the pathological histological changes of CEP, but the apoptosis pathway of endplate cells has not been elucidated. Therefore, with the help of caspase inhibitors, the present investigation was performed to determine which of the two apoptotic pathways is predominant in CEP cell apoptosis.

2. Materials and methods

2.1. Isolation and culture of primary CEP cells

All animal experiments were approved by the Ethics Committee on Animal Experiments of Fudan University. Two male Sprague–Dawley rats, aged 3 months, were euthanized by excessive anesthesia. Lumbar spines were removed under aseptic conditions. The surrounding soft tissues were completely removed to ensure the identification of the IVDs. Cartilage endplate tissues were dissected carefully under the microscope, washed three times in sterile phosphate buffered saline (PBS, Beyotime, Nantong, Jiangsu, China) and minced into small pieces. The CEP tissues were subjected to sequential digestion. Briefly, CEP tissues were agitated at 37 °C for 40 min in an enzyme solution of 0.25% trypsin (Beyotime), followed by digestion with 0.2% collagenase type II (Sigma, St. Louis, MO, USA) dissolved in DMEM with 10% FBS at 37 °C in a gyratory shaker (220 rev/min), the procedure was repeated once, until the cells could be seen in a hemocytometer with a microscope. After enzymatic digestion, the suspension was filtered through a 70-μm mesh (Beyotime). The filtered cells were then washed with PBS three times and used as the primary culture in a complete medium [DMEM supplemented with 10% fetal bovine serum (FBS, Gibco, Grand Island, NY, USA), 100 U/mL penicillin and 100 μg/mL streptomycin (Beyotime)] at 37 °C in a 5% CO_2 atmosphere. Cell purification was performed by the differential adhesion method. After three passages, the cells were trypsinized and subcultured in six-well plates at 1×10^5 cells per well.

2.2. Morphology and measurement

The CEP cells were seeded on sterile glass coverslips placed in 6-well plates and divided into six groups: the control group (10% FBS), and 5 low fetal bovine serum groups, in which the cells were exposed to DMEM with 0%, 1%, 3%, 5%, and 8% of FBS for 48 h, respectively. After the cells reached 90% confluence, morphologic changes of the treated cells were observed with a phase contrast microscope (Olympus IX50, TYO, Japan). The medium was removed, and the cells were fixed with 4% paraformaldehyde for 15 min, washed with PBS 3 times and stained with 500 μl/well DAPI (Beyotime) at 37 °C for 5 min. Following 2 time washes with PBS, the coverslips were mounted on slides using anti-fade mounting medium (Beyotime). Morphologic changes in apoptotic nuclei were observed with a fluorescence microscope (Olympus IX50).

The CEP cells were stained overnight in a 70% ethanol solution containing 1% toluidine blue and then rinsed with a 95% ethanol solution to visualize the pericellular matrix accumulation.

2.3. Flow cytometry

The CEP cells were placed in 6-well culture plates at 2×10^5 cells per well and treated with the same procedure described above. Apoptosis was determined by staining cells with both annexin V-FITC and propidium iodide (KeyGen Biotech, Nanjing, Jiangsu, China) according to the manufacturer's instructions. Briefly, floating cells and trypsinized adherent cells were pooled and centrifuged together. The cells were washed with ice-cold PBS twice and resuspended in binding buffer to which 5 μl fluorescein isothiocyanate (FITC)-conjugated Annexin V and 5 μl propidium iodide (PI) were added. The mixtures were incubated in the dark at room temperature for 15 min. Apoptotic incidence was analyzed with FACScan flow cytometry (Becton Dickinson, San Jose, California, USA) in samples of 1×10^5 cells. This assay discriminates between intact (AnnexinV-negative/PI-negative), early apoptotic (AnnexinV-positive/PI-negative) and late apoptotic (AnnexinV-positive/PI-positive) CEP cells [15]. In the present study, "apoptotic cells" included both early and late apoptotic cells.

2.4. Western blot

The expression of caspase-3, PARP, cytochrome-c, Bcl-2, and Bax was determined by Western blot analysis according to the manufacturer's instructions. β-actin was used as an internal control for protein loading. CEP cells were incubated with 1% FBS, which is necessary for basal cell maintenance. The control group was incubated with 10% FBS. Cells were washed with ice-cold PBS and lysed in RIPA buffer (Beyotime) on ice for 15 min. The lysates were centrifuged at 12,000*g* for 15 min, and the protein concentrations were measured with the BCA Protein assay kit (Beyotime). Samples (50 μg total protein) were loaded and electrophoresed on 12% sodium dodecyl sulfate polyacrylamide gels, and transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Bedford, MA, USA). After being blocked with 5% nonfat milk for 1 h at room temperature, the membranes were incubated overnight with primary antibodies (all purchased from Cell Signaling Technology, Danvers, MA, USA) directed against caspase-3 (1:3000), cytochrome-c (1:1000), PARP (1:1000), Bcl-2 (1:500), and Bax (1:500). The membranes were washed 3 times with TBST and further incubated with horseradish peroxidase-conjugated secondary antibody (Cell Signaling Technology, Danvers, MA, USA) for 1 h. The specific proteins were visualized using an enhanced chemiluminescence system (Amersham, Biosciences, UK).

2.5. Treatment with caspase inhibitors

To determine the serum deprivation-induced apoptotic pathways and to elucidate the anti-apoptotic effects of caspase inhibitors on the apoptosis induced by serum deprivation, CEP cells were incubated with 1% FBS, with or without Z-IETD-FMK (caspase-8 inhibitor, 50 μM, R&D Systems, Minneapolis, MN, USA) or Z-LEHD-FMK (caspase-9 inhibitor, 50 μM, R&D Systems) for 48 h. These cells were analyzed by flow cytometric analysis as described above. The mean of the apoptotic frequency of the inhibitor-treated cells was compared with that of the control group (10% FBS) and the group treated with 1% FBS. The cells treated with DMSO were considered a positive control. To confirm the proper inhibition of the caspases by their corresponding caspase inhibitors, active caspases were observed by Western blotting.

2.6. Statistical analysis

All of the experiments were repeated at least three times. The data are expressed as the means ± standard deviation (SD).

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