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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).

Statistical analyses were performed using Stata10.0 software. Data were analyzed by a one-way analysis of variance (ANOVA), followed by the Bonferroni test. A *P* value less than 0.05 was considered statistically significant.

3. Results

3.1. Effect of serum deprivation on apoptotic morphology

With light microscopy, cells from rat cartilage endplates (CEPs) predominantly showed a chondrocyte-like appearance when stained with toluidine blue, which stains the nuclei of chondrocytes and the proteoglycans within the matrix. The small vacuoles reacted positively to toluidine blue stain, confirming the presence of proteoglycans (Fig. 1). Cells incubated with low levels of FBS for 48 h exhibited cell shrinkage or a threadlike morphology, cell junctions disappeared, and some cells detached (Fig. 1). We then identified the apoptotic CEP cells by DAPI staining and fluorescence microscopy. Nuclei with brightly stained, condensed chromatin were observed in cells treated with low concentrations of FBS (Fig. 1), whereas the nuclei of cells cultured in complete medium (10% FBS) had a homogeneous pattern of staining. Chromosomal condensation as well as cell shrinkage was revealed by DAPI staining, suggesting that CEP cells cultured in starvation medium showed apoptosis.

3.2. Effect of serum deprivation on apoptotic incidence

Flow cytometric analysis showed that serum deprivation induced CEP cell apoptosis in a serum concentration-dependent manner. As shown in Fig. 2, a low apoptotic incidence was observed when CEP cells were cultured in complete culture medium for 48 h. In contrast, serum deprivation led to a significant increase in the apoptotic incidence. However, apoptosis was less frequent among cells cultured in DMEM without FBS (0% FBS) than among cells cultured with 1% FBS. Instead, the cell death in DMEM without FBS occurred because of necrosis. Because serum is necessary for basal cell maintenance and IVDs have a nutrient supply, 1% FBS was used as the optimal apoptosis-inducing condition.

3.3. Changes of apoptotic protein levels in CEP cells

The results of Western blots are shown in Figs. 3 and 4. CEP cells in serum-deprived cultures constitutively expressed caspase-3,

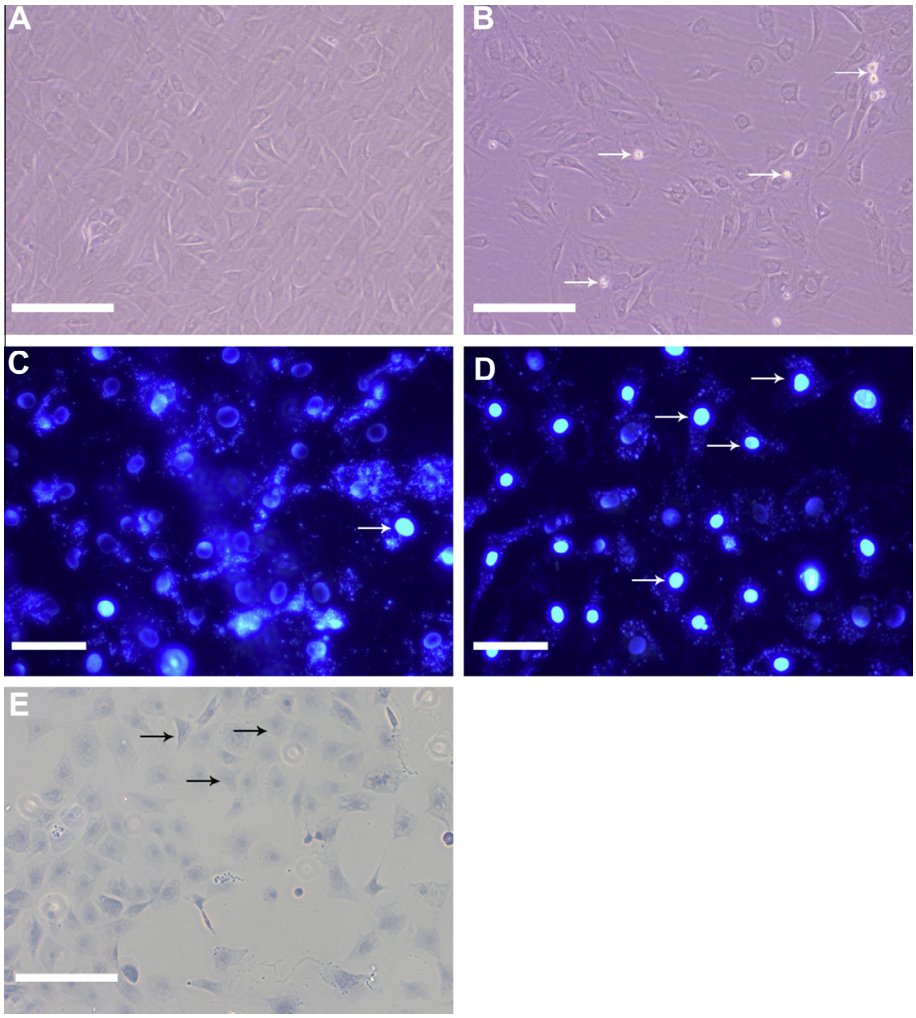

Fig. 1. Morphologic observation of CEP cells. The cells were observed with bright-field microscopy (A&B, original magnification 40×) or with fluorescence microscopy after being fixed with 4% paraformaldehyde and stained with DAPI (C&D, original magnification 100×). Apoptotic cells were characterized by cell shrinkage, shedding of smaller fragments, and partial detachment from the plate (white arrows); nuclei were condensed and brightly stained with DAPI (white arrows). (A, C represent cells grown in 1% FBS, B, D represent cells grown in 1% FBS). Toluidine blue staining (E, original magnification 100×) identified chondrocytes, small cells with negative staining (black arrows). Scale bars represent 50 μm.

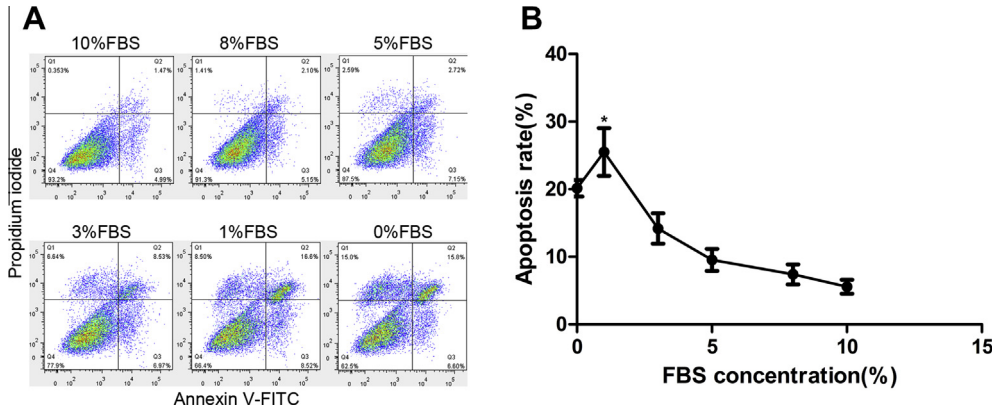

Fig. 2. Effects of low serum on the apoptosis of CEP cells. (A) Representative graphs obtained by flow cytometry analysis after double staining with annexin V-FITC and propidium iodide. (B) The apoptotic incidence of CEP cells cultured with various concentrations of FBS vs. the group cultured with 10% FBS group. $^{*}p < 0.05$. (A) and (B) show the frequency of apoptotic rat CEP cells as a function of serum concentration. 1% FBS is the optimal condition.

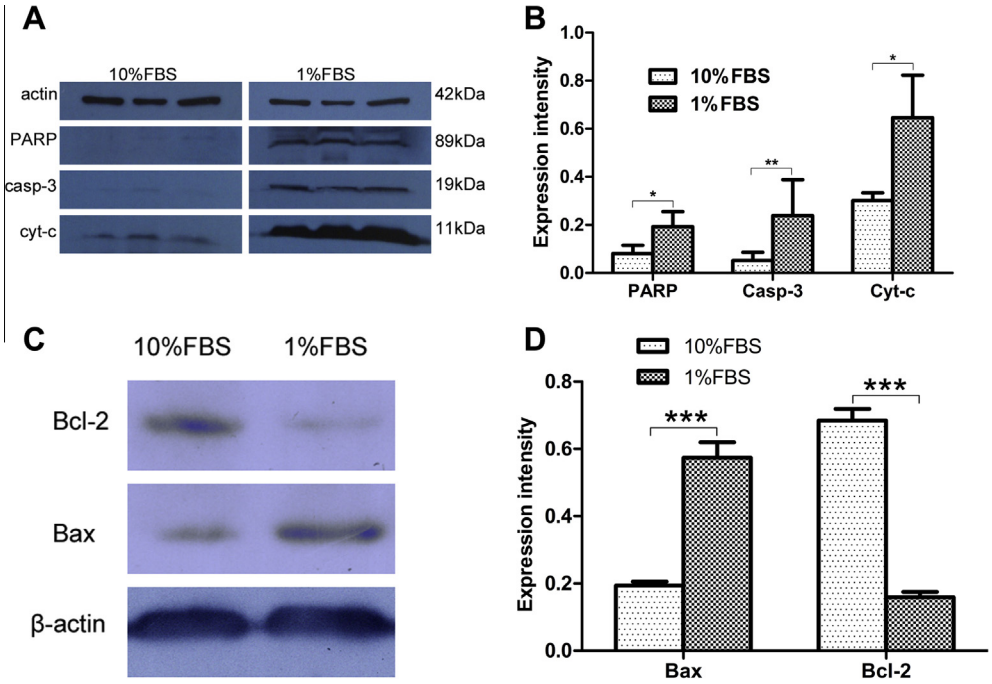

Fig. 3. Expression of apoptosis-related protein in CEP cells grown in 1% or 10% FBS. (A) Blots are representative of three independent experiments and indicate the expression of proteins involved in the caspase apoptotic pathway. Caspase-3, PARP, and cyt-c are abundant when CEP cells are cultured with 1% FBS for 48 h. (B) Densitometry results of caspase-3, PARP, and cyt-c protein expression in cultures grown with 1% FBS or 10% FBS. (C) Western blot analysis shows serum starvation stimulated the levels of Bax but reduced the levels of Bcl-2. (D) Densitometry results of Bcl-2 and Bax protein expression in cultures grown with 1% FBS or 10% FBS. ($^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$).

PARP (poly ADP-ribose polymerase) and cytochrome-c. Compared with the levels in normal cultures, the levels of expression of caspase-3, PARP, and cytochrome-c in serum-deprived cultures were significantly higher ($p < 0.01$, $p < 0.05$ and $p < 0.05$, respectively). The results suggested that the caspase pathway participates in the apoptosis induced by serum deprivation of CEP cells. To clarify the molecular mechanism involved in the apoptosis process, we measured different apoptotic induction markers upstream of caspase-3. The results showed that serum starvation stimulated the levels of cleaved caspase-8, caspase-9, and Bax but reduced the levels of Bcl-2. The cleaved caspase-9 was present in much higher quantities than the cleaved caspase-8. The caspase-9 inhibitor Z-LEHD-fmk and the caspase-8 inhibitor Z-IETD-fmk effectively reduced the activations of procaspases-9 and procaspase-8, respectively (Fig. 4). Based on these results, we concluded that the serum deprivation induced apoptosis of CEP cells may depend predominantly on the intrinsic mitochondrial pathway.

3.4. Caspase-9 inhibitor reduces the serum deprivation-mediated apoptosis of rat CEP cells

To prove that caspase-8 and caspase-9 were actually involved in the apoptosis process, we examined the effects of caspase-8 and caspase-9 inhibitors on serum deprivation-mediated apoptosis. Flow cytometric analysis revealed that the apoptotic rate was significantly reduced by Z-LEHD-fmk (the caspase-9 inhibitor) compared with the 1% FBS group (11.42% ± 2.56% *vs.* 22.16% ± 3.19%, $p < 0.05$). However, Z-IETD-fmk (the caspase-8 inhibitor) did not

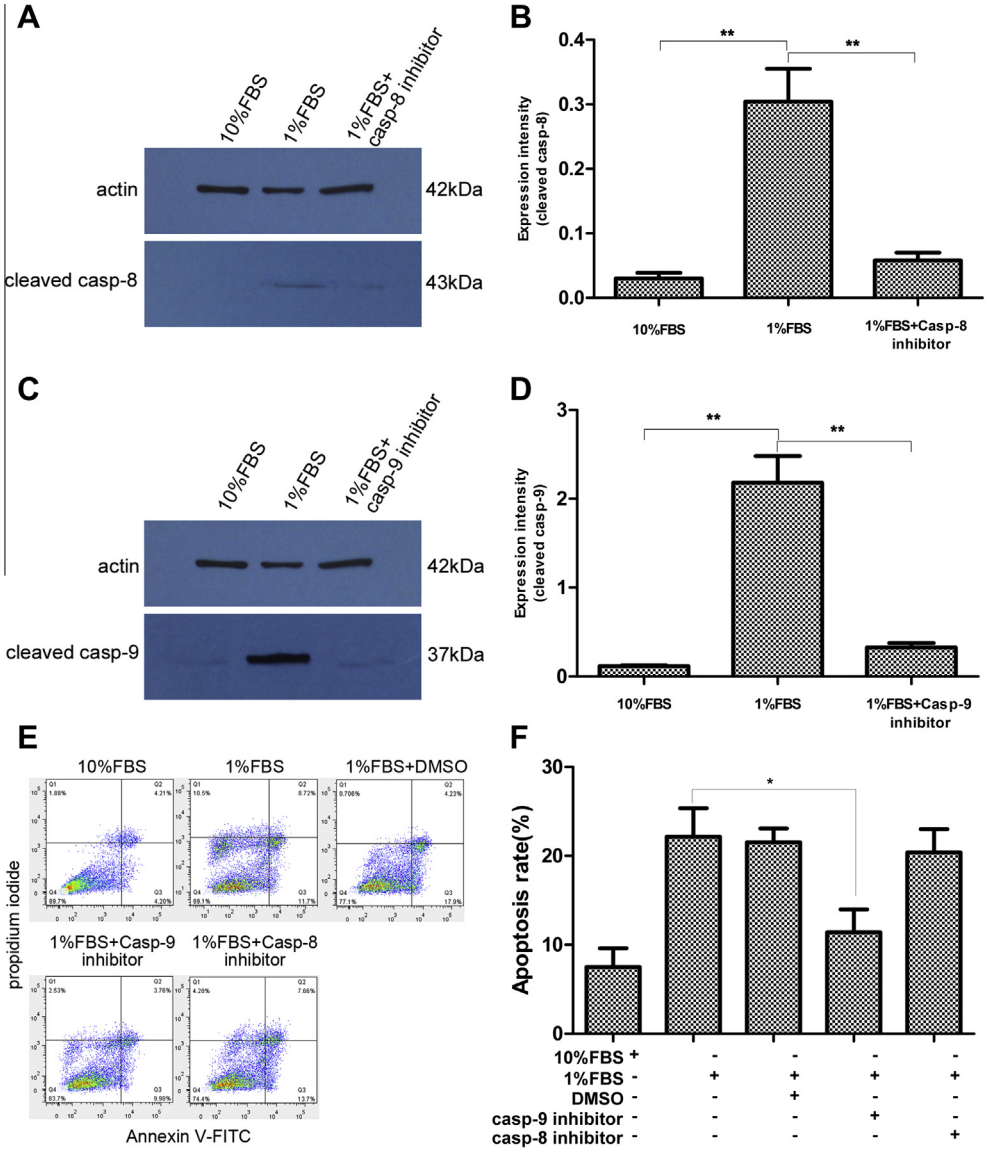

Fig. 4. Effects of caspase-8 and caspase-9 inhibitors on apoptosis induced by serum deprivation. (A and C) Western blot analysis shows that little caspase-8 or 9 was activated in CEP cells cultured in 10% FBS, whereas cleaved caspase-8 and caspase-9 were both detected in cells grown in 1% FBS. The amount of cleaved caspase-9 was greater than that of cleaved caspase-8. The activation of caspase-8 and caspase-9 was inhibited by the corresponding inhibitors. ''1% FBS + casp-8/9 inhibitor'' represents CEP cells cultured with 1% FBS and the caspase-8 or -9 inhibitor for 48 h. (B and D) Densitometry results of cleaved caspase-8 and caspase-9 protein expression in cells grown in medium containing 10% FBS, 1% FBS or 1% FBS and the caspase-8 or -9 inhibitors. (E) Representative dot plot of cell apoptosis by flow cytometry analysis after Annexin V/PI dual staining. (F) Comparison of the apoptosis rate of CEP cells treated with 10% FBS, 1% FBS, DMSO, caspase-9 inhibitor and caspase-8 inhibitor. (E and F) reveal that the inhibition of caspase-9 activation can reduce apoptosis. ($^{*}p < 0.05$ and $^{**}p < 0.01$).

reduce apoptotic cell death significantly (20.39% ± 2.63% *vs.* 22.16% ± 3.19%, $p = 0.15$). We also can excluded the effect of DMSO on cells (21.53% ± 1.56% vs. 22.16% ± 3.19%, $p = 0.84$) because its concentration was too low to cause cell toxicity.

4. Discussion

Because apoptosis plays a vital role in vertebral cell death, a growing number of studies have focused on the mechanism of IVD cell apoptosis. Thus, a large number of culture systems have been used to simulate the degeneration of IVD and to induce cell apoptosis. The *in vitro* apoptotic model used in the present study, apoptosis was induced by serum deprivation, which imitated the low nutrient supply in IVDs. Although this model cannot replicate exactly the *in vivo* situation and the sample size is small, the simplicity of the model makes it ideal as a screening tool to identify the pathways involved in chondrocyte death and to test potential therapeutic agents.

The current study showed that the exposure of CEP cells to serum deprivation induced apoptosis in a concentration-dependent manner. Culturing CEP cells for 48 h in 1% FBS was the optimal condition, and it induced apoptosis of CEP cells that was consistent with previous studies [16,17]. A sequence of cell injury and changes in cell signaling occurred, resulting in morphological changes, including cell shrinkage or threadlike morphology, disappearance of cell junctions and nuclei with brightly staining condensed chromatin. In addition, there were molecular changes, including the release of cytochrome-c, the activation of caspase-3, and an imbalance among anti- and pro-apoptotic proteins in the Bcl-2 family. The Bcl-2 family proteins play an important role in maintaining mitochondrial function. The overexpression of

Bcl-2 has been linked to the protection from apoptosis through the inhibition of cytochrome c release [18], and the depletion of Bax proteins has been shown to reduce cell death in ischemia reperfusion injury in mice [19]. These data indicate an activation of the mitochondrial apoptosis pathway.

This study, using antibodies that specifically recognize the cleaved caspase-9 and cleaved caspase-8, showed that 1% FBS strongly induced the expression of cleaved caspase-9 and resulted in a small amount of cleaved caspase-8. The corresponding inhibitors, Z-LEHD-fmk for caspase-9 and z-IETD-fmk for caspase-8, inhibited the activation of the procaspases. Flow cytometric analysis showed that inhibiting the intrinsic pathway significantly suppressed the apoptosis induced by serum deprivation, but inhibiting the extrinsic pathway with Z-IETD-fmk did not attenuate CEP cell death. In summary, the current study suggests that serum deprivation induced apoptosis of CEP cells primarily via the intrinsic pathways. In the intrinsic pathway, death stimuli directly induce the release of cytochrome c into the cytosol and promote the activation of procaspase-9. In contrast, in the extrinsic pathway, there are two different pathways downstream of Fas depending on the amount of active caspase-8. Strong caspase-8 activation bypasses the mitochondria and leads directly to the activation of procaspase-3 and subsequently to apoptosis; however, a small amount of active caspase-8 cannot propagate the caspase cascade directly, and it must cleave Bid and then be amplified by the mitochondria [20]. Additional research is needed to measure the expression of Bid and truncated Bid (t-Bid) to confirm the process.

Serum deprivation induces cell apoptosis because the serum contains various types of growth factors, some of which are responsible for cell survival, including insulin-like growth factor-1 (IGF-1). It has been recognized that IGF-1 inhibits serum deprivation-induced apoptosis in many cell types including IVD cells. In the study by Gruber [21], AF cells were subjected to serum starvation conditions, which resulted in significantly increased apoptosis. When insulin-like growth factor-1 was added to the culture system containing only 1% fetal bovine serum, the apoptosis rate was significantly reduced in a dose-dependent manner. In this context, serum deprivation is expected to result in the decrease in some growth factors, including IGF-1, which in turn leads not only to a decreased trophism but also to a compromised ability to resist a pro-inflammatory cytokine counterattack of cells. This response was observed by Zhao [17], who revealed that IL-1β significantly increased the effects of serum deprivation in a dose-dependent manner. Some researchers reported that growth factor depletion generates a stress situation within cells and leads to the activation of the mitochondrial pathway of apoptosis [22,23].

Ariga et al. [24] previously reported that mechanical stress induced the apoptosis of endplate chondrocytes in organ-cultured mouse intervertebral discs. They found that chondrocytes underwent apoptosis in the CEP of the mouse intervertebral disc in organ culture conditions via the mitogen-activated protein kinase (MAPK) pathway. Their results are contradictory to those of our study. However, it has been known that apoptotic signal transduction pathways are cell-type and stimulus specific [25,26]. Therefore, multiple apoptotic pathways can be activated in the same cell type in response to different triggering stimuli. Here, the difference may result from the different stimuli (serum deprivation or cyclic stretching) and the culture system (*in vitro* cell cultures or *ex vivo* organ cultures).

In conclusion, our results revealed that serum deprivation induced apoptosis in rat CEP cells, resulting in brightly staining, condensed chromatin by DAPI staining, the increase in apoptotic rate, the activation of caspase-3 and PARP, and the release of cytochrome C. These data indicate that serum deprivation induces the activation of the intrinsic apoptotic pathway, which involves caspase-9. The efficacy of caspase-9 inhibitors in attenuating or preventing apoptosis of serum deprivation-induced disc cell apoptosis suggests that such inhibitors may potentially delay the initiation of disc degeneration.

Conflict of interest

The authors declare that they have no conflict of interest.

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