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# Anti-apoptotic effects of caspase inhibitors on rat intervertebral disc cells

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# ANTI-APOPTOTIC EFFECTS OF CASPASE INHIBITORS ON RAT INTERVERTEBRAL DISC CELLS

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**Background:** Apoptosis is thought to be a critical component of disc degeneration. Two main pathways of Fas-mediated apoptosis have been identified: Type I, which is the death-inducing signaling complex pathway, and Type II, which is the mitochondrial pathway. The apoptotic pathway for anulus fibrosus cells, which is phenotypically different from that of nucleus pulposus cells, has not been elucidated to our knowledge. The ultimate initiators or executioners of apoptosis are caspases. There are also inhibitors of caspases, which have the potential of being used as anti-apoptotic therapeutic agents. We therefore undertook this study to determine (1) the apoptotic pathway of anulus fibrosus cells and (2) the anti-apoptotic potential of caspase inhibitors.

**Methods:** Rat anulus fibrosus cells were isolated, cultured, and placed in either 0% (apoptosis-promoting condition) or 10% (normal control) fetal bovine serum. We identified and quantified the presence of apoptotic cell death, caspase activities, and loss of mitochondrial membrane potential. In addition, we examined the cells for the expression of Fas, procaspases, and cytochrome-c. Finally, we analyzed the degree of anti-apoptotic effects of caspase inhibitors on the cells in 1% fetal bovine serum.

**Results:** The percentage of apoptosis and Fas expression in the cells incubated in 0% fetal bovine serum were increased compared with those in the cells incubated in 10% fetal bovine serum (both  $p < 0.001$ ). Caspase-8, 9, and 3 activities were increased and expression of procaspases was decreased in the 0% fetal bovine serum compared with those in the 10% fetal bovine serum (all  $p < 0.001$ ). In contrast, the loss of mitochondrial membrane potential and cytochrome-c release into the cytosol were unchanged in the 0% fetal bovine serum. Pancaspase and caspase-8 inhibitors reduced apoptotic cell death ( $p < 0.001$  and  $p < 0.05$ , respectively), but caspase-9 inhibitor did not reduce apoptotic cell death.

**Conclusions:** Our results suggest that, unlike nucleus pulposus cells, anulus fibrosus cells are Fas Type-I cells, which undergo apoptosis through the death-inducing signaling complex. We also found that apoptosis of intervertebral disc cells can be attenuated by caspase inhibitors.

**Clinical Relevance:** Caspase inhibitors may play a therapeutic role in slowing disc degeneration that is due to inappropriate or excessive apoptosis of intervertebral disc cells.

Apoptosis, or programmed cell death, is mediated by the activation of caspases (cysteine-containing aspartate-specific proteases, a group of proteolytic enzymes) and is thought to be a critical component of acute and chronic diseases such as myocardial infarction, stroke, neurodegenerative diseases, and disc degeneration<sup>1-3</sup>. There are several caspases, and they act as either initiators (caspase-2, 8, and 9) or executioners (caspase-3, 6, and 7) of apoptosis<sup>4,5</sup>. The initiator caspases make it possible for the executioner caspases to catalyze a series of proteolytic events, resulting in the characteristic biochemical and morphological changes associated with apoptosis. There are also inhibitors of caspases, which have the potential to be

used as anti-apoptotic agents<sup>6,7</sup>. The potential therapeutic use of caspase inhibitors has been explored in animal models for such conditions as cerebral and cardiac ischemia and sepsis<sup>8-13</sup>. To our knowledge, however, therapeutic caspase inhibition to prevent or attenuate apoptosis in intervertebral disc cells has not been previously investigated.

The Fas receptor is a member of the tumor-necrosis factor (TNF) receptor superfamily and is expressed at the plasma membrane in a wide range of tissues, including intervertebral discs<sup>5,14-17</sup>. Ligation of the Fas ligand or a cross-linking antibody to the Fas receptor induces apoptosis in susceptible cells, resulting in the recruitment and autocleavage of the initiator

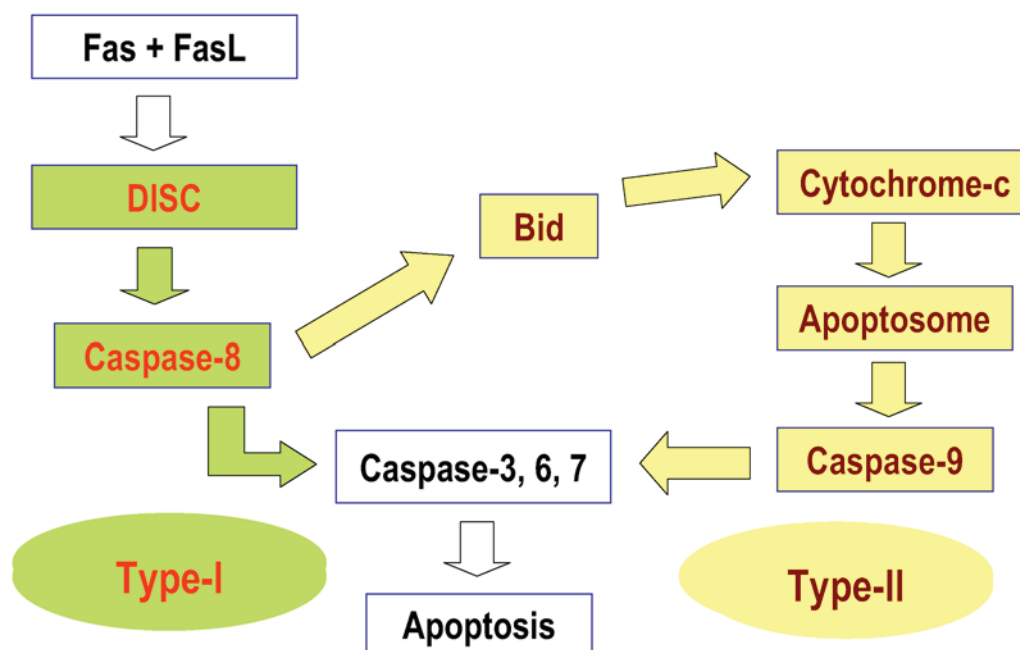


Fig. 1

Fas-mediated apoptotic signaling pathways. In Type-I cells, Fas triggering leads to strong caspase-8 activation at the death-inducing signaling complex (DISC), which bypasses mitochondria, leading directly to the activation of executioner caspases such as caspase-3 and subsequently to apoptosis. In Type-II cells, only a little DISC is formed, leading to the cleavage of Bid, a proapoptotic member of the Bcl-2 family. The cleaved Bid induces the release of cytochrome-c from mitochondria, which then activates caspase-9. Caspase-9 then activates caspase-3. The activated caspase-3 in both Type-I and Type-II cells then cleaves various cellular substrates to cause apoptosis.

caspase, procaspase-8, at the plasma membrane.

Two major pathways of Fas-mediated apoptosis have been identified (Fig. 1)<sup>5,14,17</sup>: the Type-I (death-inducing signaling complex) pathway and the Type-II (mitochondrial) pathway. In Type-I cells, activation of the Fas death receptor on the cell membrane results in the formation of the death-inducing signaling complex, made up of intracellular proteins and caspase-8. This complex activates large quantities of caspase-8, which activates caspase-3 and caspase-7 (executioner caspases). The executioner caspases then destroy the cell's cytoskeletal and reparative proteins. The initiation of cell death occurs at the cell-membrane level rather than at the mitochondria.

In Type-II (mitochondrial) cells, death is initiated at the mitochondria. Unlike in Type-I cells, the quantity of caspase-8 is insufficient to directly activate the executioner caspase-3. Instead, it activates Bid (BH3 interacting domain death agonist), which interferes with mitochondrial function by inhibiting Bcl-2, an apoptosis-inhibiting protein on the outer wall of mitochondria. Blocking Bcl-2 increases the permeability of the mitochondrial membrane, allowing intramitochondrial proteins to escape into the cytosol. One of these proteins is cytochrome-c, which is essential to mitochondrial survival. The extruded cytochrome-c activates caspase-9, which in turn activates caspase-3, the executioner, resulting in cell apoptosis.

Our recent investigations suggested that human lumbar

nucleus pulposus cells undergo Fas Type-II (mitochondrial pathway) apoptotic cell death<sup>18</sup>. However, the apoptotic pathway for annulus fibrosus cells, which is phenotypically different from that of nucleus pulposus cells, has not been clearly determined. Defining the specific pathway for a cell type is the necessary first step to finding a selective therapeutic intervention to modulate apoptosis. We therefore undertook this study to determine the apoptotic pathway of annulus fibrosus cells and the anti-apoptotic potential of caspase inhibitors.

## Materials and Methods

### Cell Culture and Reagents

All lumbar intervertebral discs were harvested from three male Sprague-Dawley rats (four months of age) immediately after they were killed. We carefully dissected the discs under the microscope to obtain only outer annulus fibrosus tissue, and we cultured the tissue in Dulbecco modified Eagle medium supplemented with 10% fetal bovine serum, 100 U/mL of penicillin, and 100 µg/mL of streptomycin under 5% CO<sub>2</sub> in a humidified incubator at 37°C for twelve hours. To isolate the cells, outer annulus fibrosus tissues in Dulbecco modified Eagle medium were digested with 0.2% pronase (Sigma Chemical, St. Louis, Missouri) for four hours. After enzymatic digestion, the suspension was filtered through a 70-µm mesh (Falcon, Franklin Lakes, New Jersey). The filtered cells were then washed with

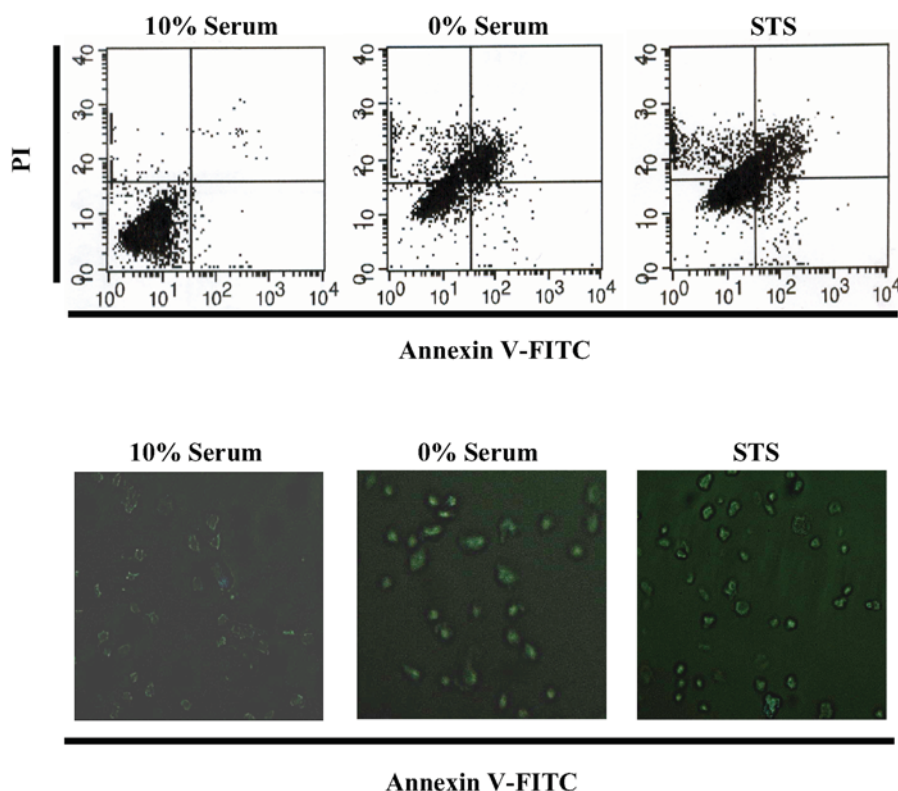


Fig. 2-A

**Figs. 2-A and 2-B** Evaluation of apoptosis. Rat anulus fibrosus cells were treated with 10% or 0% fetal bovine serum for forty-eight hours. Cells treated with 0.5  $\mu$ M staurosporine (STS) for twenty-four hours were used as a positive control for apoptosis. **Fig. 2-A** Cell death was assayed with flow cytometry after double staining with annexin V-FITC and propidium iodide.

Dulbecco modified Eagle medium and used as the primary culture. After three passages, the cells were trypsinized and were subcultured into six-well plates at  $3 \times 10^5$  cells per well.

Z-IETD-FMK (caspase-8 inhibitor), Z-LEHD-FMK (caspase-9 inhibitor), Z-VAD-FMK (pancaspase inhibitor), and Boc-D-FMK (pancaspase inhibitor) were purchased from Calbiochem (San Diego, California) and staurosporine was purchased from Sigma-Aldrich (St. Louis, Missouri). Antibodies against Fas; procaspase-3, 8, and 9; and  $\beta$ -actin were purchased from Santa Cruz Biotechnology (Santa Cruz, California), and antibody against cytochrome-c was obtained from BD Biosciences Pharmingen (San Diego, California).

#### Evaluation of Apoptosis

Apoptosis was determined by staining cells with both annexin V-FITC (PharMingen, San Diego, California) and propidium iodide (PharMingen), according to the manufacturer's instructions. Annexin V-FITC is used to quantitatively determine the percentage of cells undergoing apoptosis. It relies on the property of cells to lose membrane asymmetry in the early phase of apoptosis. In apoptotic cells, the membrane phospholipid phosphatidylserine is translocated from the inner leaflet of the plasma membrane to the outer leaflet, thereby exposing phosphatidylserine to the external environment. Annexin V is a

$\text{Ca}^{2+}$ -dependent phospholipids-binding protein that has a high affinity for phosphatidylserine and is useful for identifying apop-

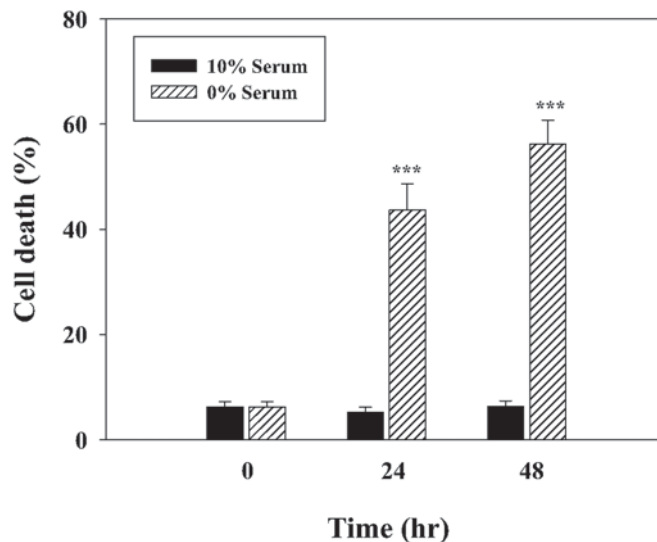


Fig. 2-B

The percentages of apoptotic cell death, expressed as the mean and standard deviation of three independent experiments. \*\*\* $p < 0.001$ .

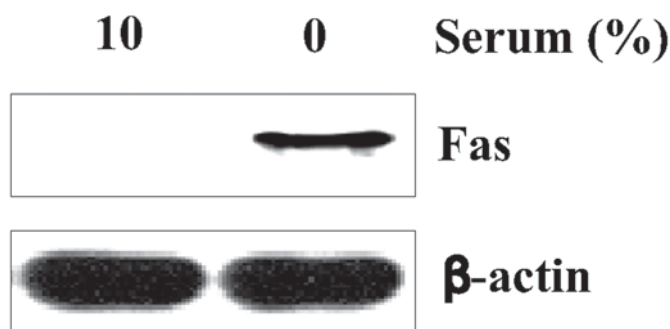


Fig. 3

Western blot analysis showing expression of Fas in cells treated with 0% fetal bovine serum for forty-eight hours but not in cells treated with 10% fetal bovine serum for forty-eight hours.

otic cells with exposed phosphatidylserine. It is typically conjugated to a fluorochrome, such as fluorescein isothiocyanate (FITC), for easy identification of apoptotic cells by flow cytometry. Propidium iodide is a standard flow cytometric viability probe and is used to distinguish viable from nonviable cells. Viable cells with an intact membrane exclude propidium iodide, whereas the membranes of dead and damaged cells are permeable to propidium iodide. Cells that stain positive for annexin V-FITC and negative for propidium iodide are undergoing apoptosis. Cells that stain positive for both annexin V-FITC and propidium iodide are undergoing necrosis<sup>19,20</sup>. To quantitate apoptosis, cells were washed with cold phosphate-buffered saline solution and then suspended in binding buffer (10 mM HEPES/NaOH [pH 7.4], 140 mM NaCl, and 2.5 mM CaCl<sub>2</sub>). The cells were stained with 5  $\mu$ L of annexin V-FITC and 10  $\mu$ L of propidium iodide and then analyzed with FACScan flow cytometry (Becton Dickinson, San Jose, California) at twenty-four and forty-eight hours. The cells treated with 0.5  $\mu$ M staurosporine (Calbiochem) for twenty-four hours were used as positive controls for apoptosis. Staurosporine is a broad-spectrum protein kinase inhibitor that induces apoptosis by markedly increasing cytochrome-c in the cytosol of a variety of cell types<sup>21,22</sup>.

#### Measurement of Caspase Activations

Enzymatic activity of caspase-3, 8, and 9 was determined by the CaspaTag Caspase-3/7, 8, and 9 In Situ Assay Kit (Chemicon, Temecula, California) according to the manufacturer's instructions. Briefly, this kit uses a carboxyfluorescein-labeled fluoromethyl ketone peptide inhibitor of caspase-3/7 (FAM-DEVD-FMK), caspase-8 (FAM-LETD-FMK), and caspase-9 (FAM-LEHD-FMK), which produces a green fluorescence. These reagents enter each cell and covalently bind to a reactive cysteine residue on the large subunit of the active caspase heterodimer, thereby inhibiting further enzymatic activity. Cells that contain the bound labeled reagent were analyzed with FACScan flow cytometry at twenty-four and forty-eight hours.

#### Assay of Mitochondrial Membrane Potential

Mitochondrial membrane potential was measured with a voltage-sensitive lipophilic cationic fluorescent probe, 5,5',6,6'-

tetrachloro-1,1',3,3'-tetraethylbenzimidazolcarbo cyanine iodide (JC-1). Briefly, cells ( $1 \times 10^5$ ) were washed with cold phosphate-buffered saline solution and JC-1 (5  $\mu$ g/mL) and were then added to the incubation buffer. The cells were then incubated in 10% or 0% fetal bovine serum for fifteen minutes, washed with incubation buffer, and analyzed with FACScan flow cytometry at forty-eight hours. Cells treated with 0.5  $\mu$ M staurosporine for twenty-four hours were used as a positive control for apoptosis.

#### Subcellular Fractionation

To analyze the distribution of cytochrome-c, the cells were fractionated into cytosolic and membrane-bound organellar fractions with extraction buffer containing 220 mM mannitol, 70 mM sucrose, 50 mM PIPES-KOH (pH 7.4), 50 mM KCl, 5 mM EGTA, 2 mM MgCl<sub>2</sub>, 1 mM EDTA, 1 mM DTT (dithiothreitol), and protease inhibitor cocktail (Roche, Mannheim, Germany). After thirty minutes of incubation on ice, the cells were homogenized with use of a glass Dounce homogenizer. The cell homogenates were centrifuged at 12,000 g for thirty minutes, and the resulting supernatant was taken as the cytosolic fraction.

#### Western Blot Analysis

Expressions of Fas; procaspase-3, 8, and 9; and cytochrome-c were determined by Western blot analysis according to the manufacturer's instructions.  $\beta$ -actin was used as an internal control for protein-loading. Briefly, the cells were washed with ice-cold phosphate-buffered saline solution and lysed in RIPA (radioimmunoprecipitation assay) buffer. The cell lysates were centrifuged at 12,000 g for fifteen minutes, and protein concen-

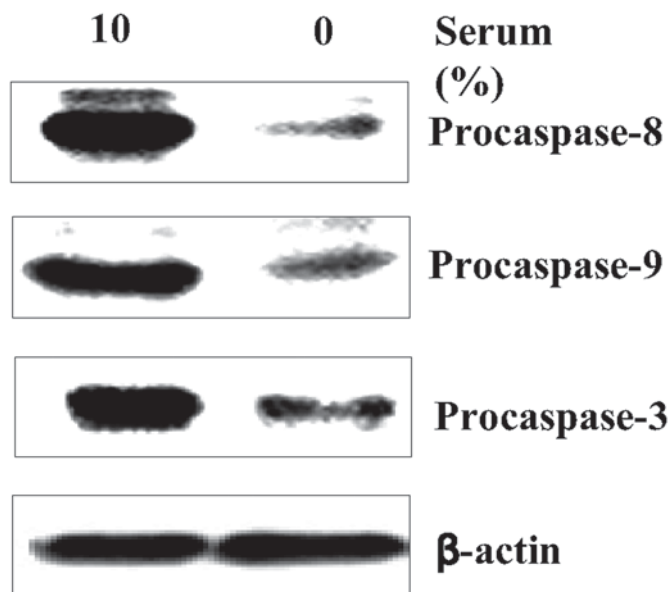


Fig. 4-A

**Figs. 4-A, 4-B, and 4-C** Assay of caspase activations. **Fig. 4-A** Western blot analysis showing clearly decreased expression of procaspase-8, 9, and 3 in the cells treated with 0% fetal bovine serum for forty-eight hours.

trations were measured with the Bradford method<sup>23</sup>. Samples (20 to 40 µg of protein) were electrophoresed on 10% to 15% SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) and transferred to a nitrocellulose membrane. The membranes were incubated with primary antibodies, followed by second antibodies of HRP (horseradish peroxidase)-linked IgG (immunoglobulin G), and immunoreactive bands were visualized with the Amersham ECL detection system (Amersham, Piscataway, New Jersey).

#### Assay of Anti-Apoptotic Effects of Caspase Inhibitors

Anulus fibrosus cells were incubated with 1% fetal bovine serum, which is necessary for basal cell maintenance, with and without Z-IETD-FMK (caspase-8 inhibitor, 100 µM), Z-LEHD-FMK (caspase-9 inhibitor, 100 µM), Z-VAD-FMK (pancaspase inhibitor, 100 µM), and Boc-D-FMK (pancaspase inhibitor, 100 µM) for forty-eight hours. This concentration of caspase inhibitors has been shown to inhibit caspase-3, 8, and 9, respectively. Apoptotic cell death was determined by double staining with 5 µL of annexin V-FITC and 10 µL of propidium iodide followed by analysis with FACScan flow cytometry at forty-eight hours.

#### Statistical Analysis

All experiments were independently conducted three times, and the results were expressed as the mean and standard deviation of the values derived with the three tests. Statistical analysis was done with the paired-samples and independent-samples t test.  $P < 0.05$  was considered to be the level of significance.

#### Results

##### Apoptosis of Rat Anulus Fibrosus Cells Through Fas Upregulation

We first tested the effect of serum deprivation on the apoptosis of rat anulus fibrosus cells. Figures 2-A and 2-B show that rat anulus fibrosus cells treated for twenty-four hours and forty-eight hours solely with 0% fetal bovine serum displayed apoptotic cell death, as determined by double staining with annexin V-FITC and propidium iodide. The mean percentage of apoptotic cell death in 0% fetal bovine serum was significantly increased compared with that in 10% fetal bovine serum at twenty-four hours (44% compared with 5%,  $p < 0.001$ ) and forty-eight hours (56% compared with 6%,  $p < 0.001$ ). The upregulation of Fas expression was identified in 0% fetal bovine serum but not in 10% fetal bovine serum on West-

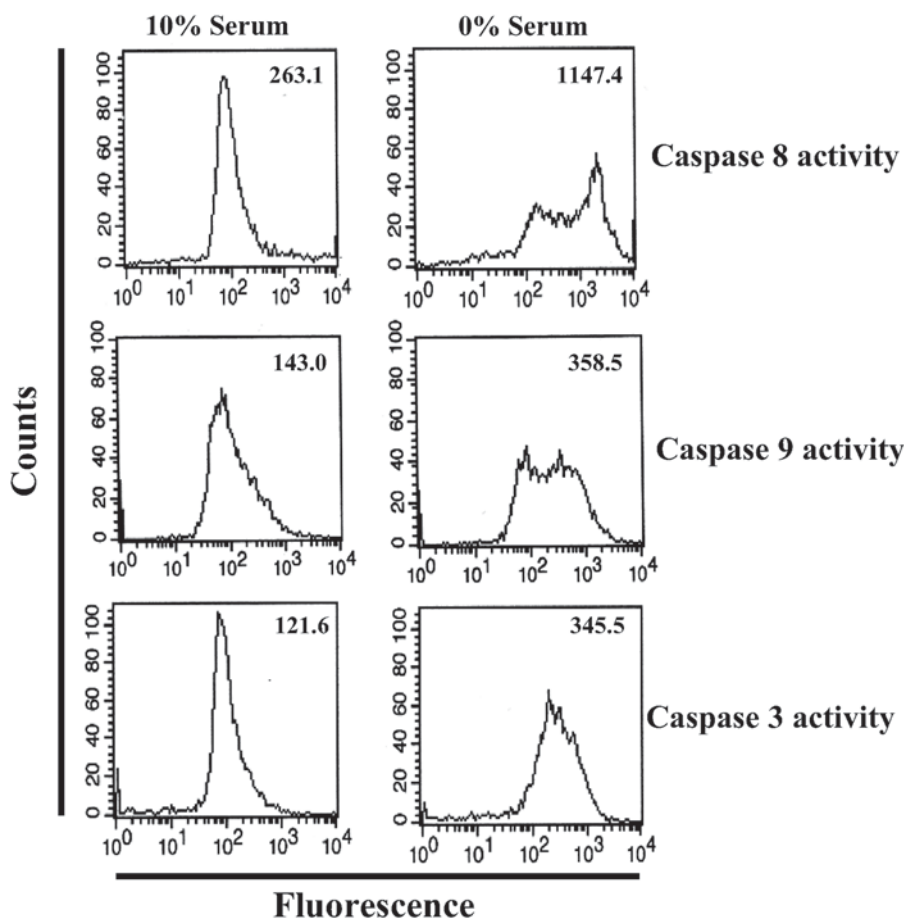


Fig. 4-B

Caspase activations were analyzed by flow cytometry with use of a caspase-3/7, 8, and 9 In Situ Assay Kit.

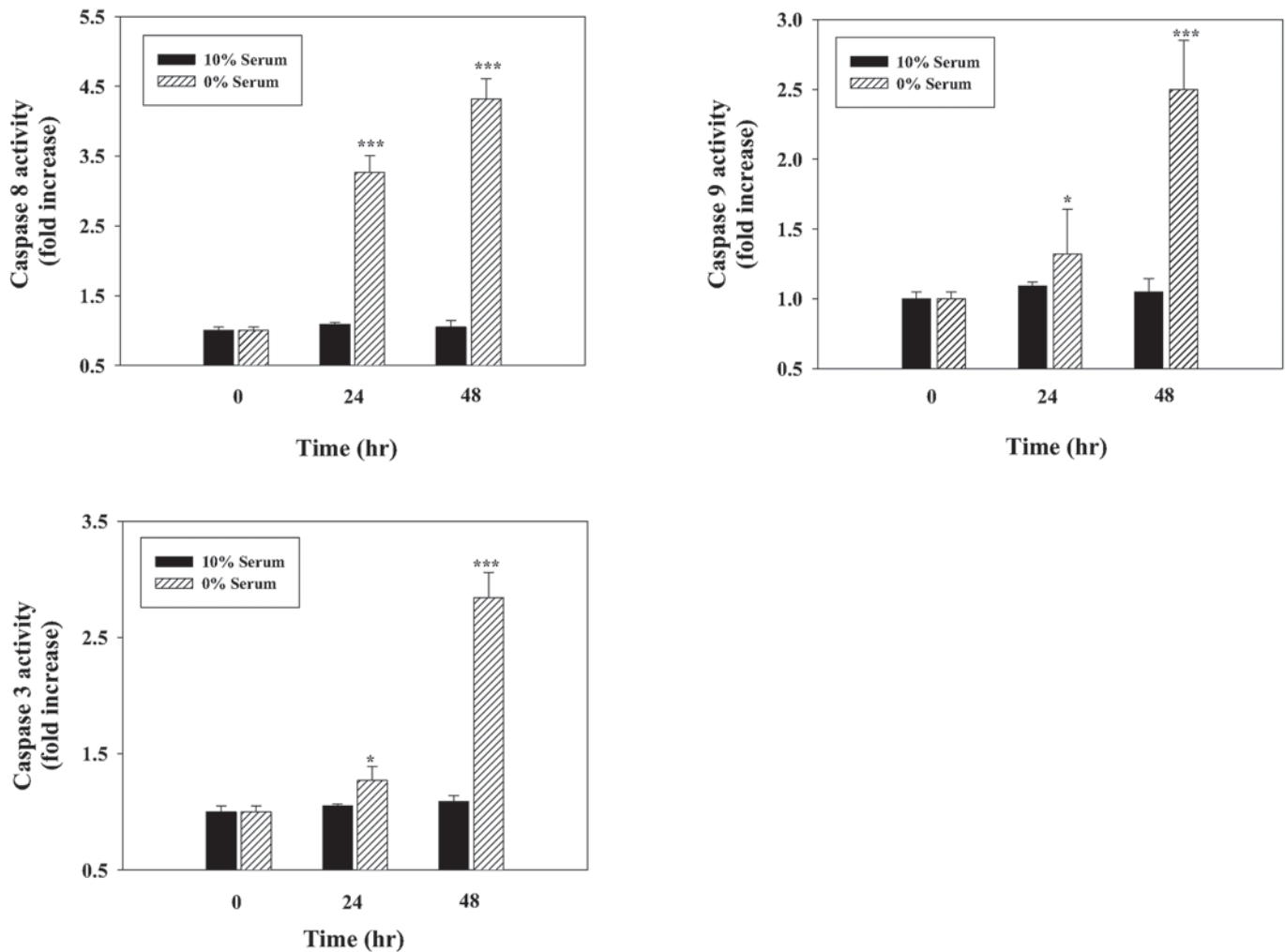


Fig. 4-C

The fold increase in caspase activity. \*\*\*p < 0.001. \*p < 0.05.

ern blot analysis (Fig. 3). These results strongly suggest that up-regulation of Fas expression is responsible for apoptosis of rat anulus fibrosus cells induced by serum deprivation.

#### *Fas-Mediated Apoptosis of Rat Anulus Fibrosus Cells Requires Activation of Caspases*

To characterize the Fas-mediated apoptotic pathway triggered by serum deprivation in rat anulus fibrosus cells, we measured different apoptotic induction markers downstream to Fas, such as caspase-8, 9, and 3 activations. Anulus fibrosus cells treated with 0% fetal bovine serum for forty-eight hours showed clearly decreased expression of procaspase-8, 9, and 3 on Western blot analysis (Fig. 4-A), suggesting that procaspases were converted to active caspases. This was associated with significant increases in the activities of the activations of caspase-8, 9, and 3 of 4.3 times (p < 0.001), 2.5 times (p < 0.001), and 2.8 times (p < 0.001), respectively (Figs. 4-B and 4-C). In anulus fibrosus cells treated with 0% fetal bovine serum for twenty-four hours, caspase-8 activity was increased earlier than was caspase-9 activity.

#### *Fas-Mediated Apoptosis of Rat Anulus Fibrosus Cells Is Not Associated with a Mitochondrial Pathway*

No substantial change in the mitochondrial membrane potential was found in anulus fibrosus cells treated with 0% fetal bovine serum for forty-eight hours. However, when the cells were treated with 0.5  $\mu$ M staurosporine for twenty-four hours, a markedly (3.1 times) increased number of the cells with increased JC-1 fluorescence (diminished mitochondrial membrane potential) was observed compared with that seen in 10% fetal bovine serum for forty-eight hours (p < 0.001) (Fig. 5-A). While cytochrome-c protein levels in the cytosolic fractions of the cells treated with 0% fetal bovine serum for forty-eight hours showed no substantial increase, the protein level was markedly increased in the cells treated with 0.5  $\mu$ M staurosporine for twenty-four hours (Fig. 5-B).

#### *Pancaspase Inhibitors and Caspase-8 Inhibitors Reduce Fas-Mediated Apoptosis of Rat Anulus Fibrosus Cells*

In order to prove that caspase-8, 9, and 3 were actually involved



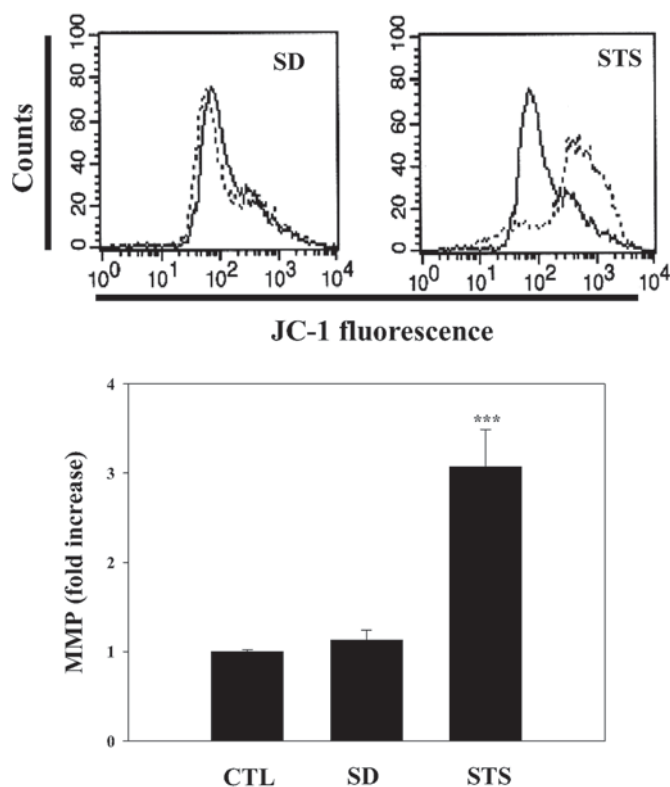


Fig. 5-A

**Figs. 5-A and 5-B** Assay for the mitochondrial pathway. **Fig. 5-A** No substantial change in the mitochondrial membrane potential (MMP) was found in anulus fibrosus cells treated with 0% fetal bovine serum (SD) for forty-eight hours. However, when the cells were treated with 0.5  $\mu$ M staurosporine (STS) for twenty-four hours, a markedly increased number of cells with increased JC-1 fluorescence was observed. CTL = control (10% fetal bovine serum). \*\*\* $p < 0.001$ .

in sensitization of rat anulus fibrosus cells to Fas-mediated apoptosis, we next examined the effects of caspase-8 and 9 and pancaspase inhibitors on Fas-mediated apoptosis. Flow cytometric analysis revealed that Fas-mediated apoptosis of rat anulus fibrosus cells (30% cell death) was significantly reduced by Z-VAD-FMK (to 17%,  $p < 0.001$ ) and Boc-D-FMK (to 10%,  $p < 0.001$ ), which are pancaspase inhibitors, and by Z-IETD-FMK (to 22%,  $p < 0.05$ ), which is a caspase-8 inhibitor. However, Z-LEHD-FMK (a caspase-9 inhibitor) did not reduce apoptotic cell death, which was still 30% ( $p = 1$ ) (Fig. 6). These results indicate that rat anulus fibrosus cells undergo apoptosis through the Fas Type-I pathway in serum deprivation.

### Discussion

Excessive apoptosis of disc cells has been suggested as a potential cause of disc degeneration<sup>3,15,16</sup>. We recently reported that nucleus pulposus cells are Fas Type-II cells, which undergo apoptosis through a mitochondrial pathway, and that caspases are initiators or executioners of apoptosis of nucleus pulposus cells<sup>18</sup>. There are also inhibitors of caspases, which have the potential for being used as anti-apoptotic therapeutic

agents. Therefore, one possible therapeutic approach to modulating the process of disc degeneration may involve the inhibition of apoptosis of disc cells by caspase inhibitors. To our knowledge, therapeutic inhibition to prevent or attenuate apoptosis of intervertebral disc cells has not been previously investigated. Moreover, the apoptotic pathway for anulus fibrosus cells, which is phenotypically different from that for nucleus pulposus cells, has not been clearly determined. Therefore, in the current study, we tried to determine the anti-apoptotic effects of caspase inhibitors as well as the apoptotic pathway of anulus fibrosus cells.

In anulus fibrosus cells treated with serum deprivation (0%), there was a significant increase in Fas expression followed by sequential activation of caspase-8 (initiator) and caspase-3 (executioner), resulting in apoptosis of anulus fibrosus cells. In contrast to the findings in disc cells treated with staurosporine, the loss of mitochondrial membrane potential and the release of cytochrome-c into cytosol were not found in 0% serum. These findings suggest that, unlike nucleus pulposus cells, anulus fibrosus cells subjected to serum deprivation are Fas Type-I cells, which produce large quantities of caspase-8 and do not involve mitochondria.

We also tested the therapeutic effect of caspase inhibitors on apoptosis of anulus fibrosus cells in a serum deprivation model. We found that anulus fibrosus cells treated with pancaspase inhibitors and a caspase-8 inhibitor demonstrated a significant reduction of apoptosis, whereas the cells treated with a caspase-9 inhibitor did not exhibit any inhibition of apoptosis. Pancaspase inhibition more effectively decreased apoptosis than did caspase-8 inhibition. The use of caspase inhibitors has been reported to prevent or attenuate cell death associated with ischemic injury in the brain and heart and to protect against hepatic toxicity<sup>1,8-13</sup>. Our results suggest that pancaspase inhibitors might offer a therapeutic benefit in the treatment of disc degeneration.

The finding that caspase-9 activity, a marker of the Fas Type-II (mitochondrial) pathway, increased simultaneously with an increase in caspase-8 activity was an unexpected and inexplicable result in the current study. However, we also found that the specific caspase-9 inhibitor did not reduce the apop-

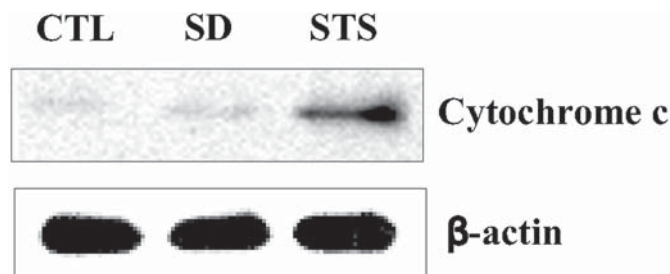


Fig. 5-B

Western blot analysis showing the expression of cytochrome-c in anulus fibrosus cells treated with 0.5  $\mu$ M staurosporine (STS) for twenty-four hours but not in the cells treated with 0% fetal bovine serum (SD) for forty-eight hours. CTL = control (10% fetal bovine serum).

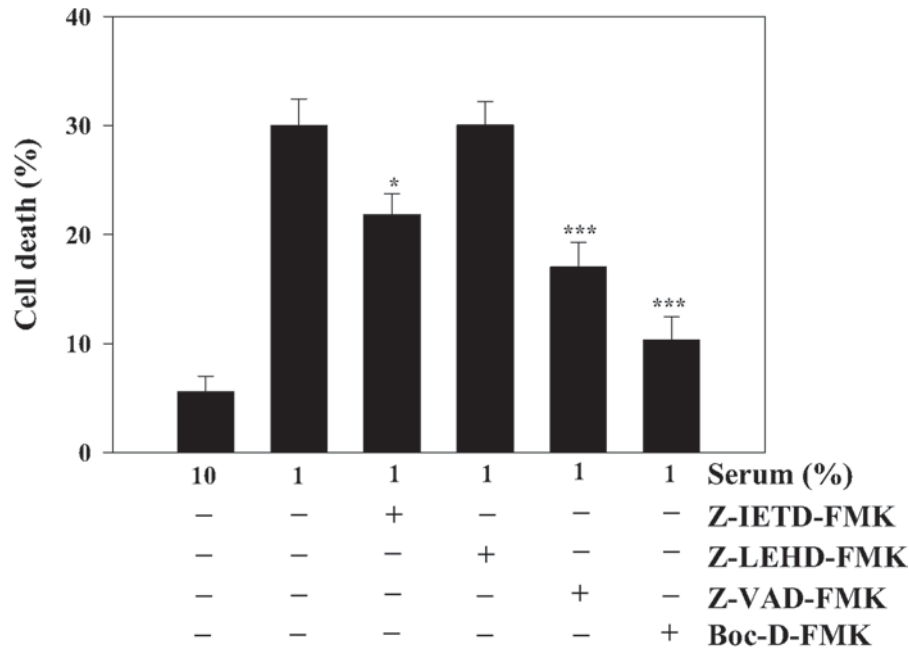


Fig. 6

Assay of anti-apoptotic effects of caspase inhibitors. Anulus fibrosus cells were incubated in 1% fetal bovine serum with and without Z-IETD-FMK (caspase-8 inhibitor, 100  $\mu$ M), Z-LEHD-FMK (caspase-9 inhibitor, 100  $\mu$ M), Z-VAD-FMK (pancaspase inhibitor, 100  $\mu$ M), and Boc-D-FMK (pancaspase inhibitor, 100  $\mu$ M) for forty-eight hours. Apoptotic cell death was determined by double staining with annexin V-FITC and propidium iodide followed by analysis with flow cytometry. The results are presented as the percentage of cell death. \*\*\* $p < 0.001$ . \* $p < 0.05$ .

tosis of anulus fibrosus cells at all. These findings need to be investigated further.

Rannou et al.<sup>24</sup> previously reported that mechanical overload-induced apoptosis of anulus fibrosus cells is mediated through the mitochondrial 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<sup>7,25</sup>. Therefore, multiple apoptotic pathways can be activated in the same cell type in response to different triggering stimuli. In view of the results reported by Rannou et al. and those found in our study, we believe that the activation of multiple apoptotic pathways of anulus fibrosus cells is possible, depending on triggering stimuli such as serum deprivation or cyclic stretching.

In the current study, we used the percentage of cell death as the primary outcome measure, but it is also important to assess cellular function, such as the ability to produce collagens and proteoglycans. It is assumed that apoptosis is undesirable and prevention of cell death should enhance normal structure and function. Programmed cell death or cell turnover might be essential to disc metabolism and might not be detrimental. In fact, cell survival alone without proper function such as matrix synthesis might not be desirable. Even if cells do survive longer with caspase inhibitors, if nutrition is lost, cell death is inevitable.

In conclusion, our results strongly demonstrate a cen-

tral role of caspases in the apoptosis of disc cells. The efficacy of caspase inhibitors in attenuating or preventing apoptosis of disc cells suggests that such inhibitors may potentially represent a novel treatment of disc degeneration due to inappropriate or excessive apoptosis of intervertebral disc cells. ■

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## References

- Guttenplan N, Lee C, Frishman WH. Inhibition of myocardial apoptosis as a therapeutic target in cardiovascular disease prevention: focus on caspase inhibition. *Heart Dis.* 2001;3:313-8.
- Kreuter M, Langer C, Kerkhoff C, Reddanna P, Kania AL, Maddika S, Chlichlia K, Bui TN, Los M. Stroke, myocardial infarction, acute and chronic inflammatory diseases: caspases and other apoptotic molecules as targets for drug development. *Arch Immunol Ther Exp (Warsz).* 2004;52:141-55.
- Gruber HE, Hanley EN Jr. Analysis of aging and degeneration of the human intervertebral disc. Comparison of surgical specimens with normal controls. *Spine.* 1998;23:751-7.
- Creagh EM, Conroy H, Martin SJ. Caspase-activation pathways in apoptosis and immunity. *Immunol Rev.* 2003;193:10-21.
- Krammer PH. CD95(APO-1/Fas)-mediated apoptosis: live and let die. *Adv Immunol.* 1999;71:163-210.
- Concha NO, Abdel-Meguid SS. Controlling apoptosis by inhibition of caspases. *Curr Med Chem.* 2002;9:713-26.
- Philchenkov A. Caspases: potential targets for regulating cell death. *J Cell Mol Med.* 2004;8:432-44.
- Cheng Y, Deshmukh M, D'Costa A, Demaro JA, Gidday JM, Shah A, Sun Y, Jacquelin MF, Johnson EM, Holtzman DM. Caspase inhibitor affords neuroprotection with delayed administration in a rat model of neonatal hypoxic-ischemic brain injury. *J Clin Invest.* 1998;101:1992-9.
- Kunstle G, Leist M, Uhlig S, Revesz L, Feifel R, MacKenzie A, Wendel A. ICE-protease inhibitors block murine liver injury and apoptosis caused by CD95 or TNF-alpha. *Immunol Lett.* 1997;55:5-10.
- Neviere R, Fauvel H, Chopin C, Formstecher P, Marchetti P. Caspase inhibition prevents cardiac dysfunction and heart apoptosis in a rat model of sepsis. *Am J Respir Crit Care Med.* 2001;163:218-25.
- Rouquet N, Pages JC, Molina T, Briand P, Joulin V. Ice inhibitor YVADcmk is a potent therapeutic agent against in vivo liver apoptosis. *Curr Biol.* 1996;6:1192-5.
- Yang B, Johnson TS, Haylor JL, Wagner B, Watson PF, El Kossi MM, Furness PN, El Nahas AM. Effects of caspase inhibition on the progression of experimental glomerulonephritis. *Kidney Int.* 2003;63:2050-64.
- Yaoita H, Ogawa K, Maehara K, Maruyama Y. Attenuation of ischemia/reperfusion injury in rats by a caspase inhibitor. *Circulation.* 1998;97:276-81.
- Scaffidi C, Fulda S, Srinivasan A, Friesen C, Li F, Tomaselli KJ, Debatin KM, Krammer PH, Peter ME. Two CD95 (APO-1/Fas) signaling pathways. *EMBO J.* 1998;17:1675-87.
- Park JB, Kim KW, Han CW, Chang H. Expression of Fas receptor on disc cells in herniated lumbar disc tissue. *Spine.* 2001;26:142-5.
- Park JB, Chang H, Kim KW. Expression of Fas ligand and apoptosis of disc cells in herniated lumbar disc tissue. *Spine.* 2001;26:618-21.
- Scaffidi C, Schmitz I, Zha J, Korsmeyer SJ, Krammer PH, Peter ME. Differential modulation of apoptosis sensitivity in CD95 type I and II cells. *J Biol Chem.* 1999;274:22532-8.
- Park JB, Lee JK, Park SJ, Kim KW, Riew KD. Mitochondrial involvement in fas-mediated apoptosis of human lumbar disc cells. *J Bone Joint Surg Am.* 2005;87:1338-42.
- Koopman G, Reutelingsperger CP, Kuijten GA, Keehnen RM, Pals ST, van Oers MH. Annexin V for flow cytometric detection of phosphatidylserine expression on B cells undergoing apoptosis. *Blood.* 1994;84:1415-20.
- Vermes I, Haanen C, Steffens-Nakken H, Reutelingsperger C. A novel assay for apoptosis. Flow cytometric detection of phosphatidylserine expression on early apoptotic cells using fluorescein labeled Annexin V. *J Immunol Methods.* 1995;184:39-51.
- Kluck RM, Bossy-Wetzel E, Green DR, Newmeyer DD. The release of cytochrome c from mitochondria: a primary site for Bcl-2 regulation of apoptosis. *Science.* 1997;275:1132-6.
- Liu X, Kim CN, Yang J, Jemmerson R, Wang X. Induction of apoptotic program in cell-free extracts: requirement for dATP and cytochrome c. *Cell.* 1996;86:147-57.
- Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem.* 1976;72:248-54.
- Rannou F, Lee TS, Zhou RH, Chin J, Lotz JC, Mayoux-Benhamou MA, Barbet JP, Chevrot A, Shyy JY. Intervertebral disc degeneration: the role of the mitochondrial pathway in annulus fibrosus cell apoptosis induced by overload. *Am J Pathol.* 2004;164:915-24.
- Ekert PG, Silke J, Vaux DL. Caspase inhibitors. *Cell Death Differ.* 1999;6:1081-6.