Effect of nerve growth factor and its transforming tyrosine kinase protein and low-affinity nerve growth factor receptors on apoptosis of notochordal cells

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Abstract

Purpose The disappearance of notochordal cells by apoptosis is thought to be the starting point of intervertebral disc degeneration. The aim of this study was to determine the apoptotic pathway of notochordal cells as well as the anti-apoptotic potential of caspase inhibitors.

Methods Rat notochordal cells were isolated, cultured, and placed in either 0 % (apoptosis-promoting condition) or 10 % (normal control) foetal bovine serum (FBS). We identified and quantified apoptotic cell deaths and caspase activities. In addition, we examined the cells for expression of nerve growth factor (NGF) and its two receptors—TrkA (survival signal) and p75 (apoptotic signal)—and downstream pathways. Finally, we analysed the degree of anti-apoptotic effects of caspase inhibitors on the cells.

Results The apoptotic rate and expressions of caspase-8 (extrinsic pathway), -9 (intrinsic pathway), and -3 (common executioner) of notochordal cells were increased in 0 % FBS compared with those in 10 % FBS. Expressions of NGF, p75 receptor and JNK downstream pathways were also increased in 0 % FBS. In contrast, expressions of the TrkA receptor and Akt and MAPK downstream pathways were decreased in 0 % FBS. Pancaspase, caspase-9 and caspase-8 inhibitors significantly reduced apoptotic cell death.

Conclusions Our results suggest that notochordal cells undergo apoptosis through both the intrinsic and extrinsic pathways by activation of NGF, p75 receptor, and the JNK

downstream pathway. We also found that apoptosis of notochordal cells can be attenuated by caspase inhibitors. Caspase inhibitors may play a therapeutic role in delaying the starting point of disc degeneration that is due to inappropriate or premature excessive apoptosis of notochordal cells.

Introduction

It has been known that notochordal cells form the notochord, which in turn contributes to the formation and maintenance of the nucleus pulposus of the intervertebral disc. After birth, the majority of notochordal cells gradually disappear by apoptosis [1, 2]. In humans, notochordal cells are very rarely present after the age of ten, and the nucleus pulposus transforms with time into a fibrocartilaginous nucleus pulposus. With progress of this chronological transition, intervertebral disc degeneration starts [3]. Therefore, the disappearance of notochordal cells, which is known to be caused by apoptosis, is thought to be the starting point of intervertebral disc degeneration [4]. To date, however, the apoptotic pathway of notochordal cells has not been fully elucidated.

Apoptosis is actually 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 intervertebral disc degeneration [5–7]. There are several caspases, and they act as either initiators (caspase-8 and -9) or a common executioner (caspase-3) of apoptosis. The initiator caspases make it possible

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for the executioner caspases to catalyse a series of proteolytic events, resulting in the characteristic biochemical and morphological changes associated with apoptosis. There are two main pathways of apoptosis, intrinsic and extrinsic, depending on the apoptotic stimuli. While the initiator of the intrinsic pathway of apoptosis is caspase-9, the initiator of the extrinsic pathway of apoptosis is caspase-8. There are also inhibitors of caspases, which have the potential to be used as anti-apoptotic agents. The potential therapeutic use of caspase inhibitors has been explored in animal models for such conditions as cerebral and cardiac ischemia and sepsis [8–11].

Nerve growth factor (NGF) is a member of the neurotrophin family and the biologic effects of NGF on neural cells are mediated by two different receptor classes: the high-affinity tropomyosin-related kinase A (TrkA) of receptor tyrosine kinase and the low-affinity p75 receptor, a member of the tumour necrosis factor receptor superfamily [12–14]. NGF is a unique growth factor because it plays a key role in apoptosis as well as in the differentiation, development and survival of selected neurons. The paradoxical responses to NGF are almost completely dependent on the relative abundance of the two distinct NGF receptors. NGF promotes cell survival through the TrkA receptor. While NGF binding to the p75 receptor triggers apoptosis in the absence of the TrkA receptor, the simultaneous expression of the two receptors signals cell survival by negating the apoptotic signalling of the p75 receptor by the TrkA receptor. While JNK activity is critical for death signalling as a downstream pathway of the p75 receptor signalling pathway, Akt and MAPK activities play a critical role in the survival of cells via the TrkA receptor signalling pathway [15, 16]. Recently, NGF and its two related receptors have been detected on non-neural cells, such as chondrocytes, fibroblasts, lymphocytes, monocytes, osteoblasts, endothelial cells, and keratinocytes [17–19].

Our previous study demonstrated that chordoma and notochordal cells constitutively express NGF and its TrkA and p75 receptors and that overexpression of NGF and the TrkA receptor in chordoma cells might be a possible mechanism of malignant transformation of notochordal cells to chordoma (by negating the apoptotic signal of the p75 receptor) [20]. Thus far, however, notochordal cell survival and death via interaction of NGF and its TrkA and p75 receptors have not been definitely determined. Moreover, the anti-apoptotic effect of caspase inhibitors on the apoptosis of notochordal cells is still unknown. We therefore undertook this study to investigate the apoptotic pathway of notochordal cells as well as the anti-apoptotic potential of caspase inhibitors under serum deprivation conditions.

Materials and methods

Cell culture and reagents

All lumbar intervertebral discs (L1–6) were harvested from four-week-old male Wistar rats immediately after they were killed. We carefully dissected the discs under a microscope to obtain only the gelatinous notochordal nucleus pulposus tissue, and the harvested NP tissue was pooled in [alpha]-minimum essential medium ([alpha]-MEM; Gibco, Grand Island, NY). The notochordal cells were released from the NP tissue (in HBSS; Hyclone, Ottawa, Ontario, Canada with 0.02 % pronase Sigma, St. Louis, MO) by vigorous pipetting. Notochordal cells were cultured in a complete medium [[alpha]-MEM supplemented with 10 % fetal bovine serum (Hyclone), and 1 % penicillin-streptomycin (Gibco)] at 37°C in a humidified atmosphere (95 % air, 5 % CO₂). After the cells grew to confluence, they were split once (passage 1) and grew to confluence again. For use in the experiments, the cells were then trypsinized, washed, and plated in 6-well plates at 1×10^6 cells/well. When notochordal cells reached 80–90 % confluence, the notochordal cells were placed in either 0 % (apoptosis-promoting condition) or 10 % (normal control) FBS for 48 hours. This study was approved by the Institution's Animal Care and Use Committee.

For western blot analysis, antibodies to caspase-9, -3, Akt, MAPK, and poly (ADP) ribose polymerase (PARP) were purchased from Cell Signaling Technology (Danvers, MA); antibody to caspase-8 was from Neomarker (Freemont, CA); antibodies to NGF, TrkA, p75, and JNK were obtained from Santa Cruz Biotechnology (Santa Cruz, CA); and β -actin was obtained from SIGMA (Sigma, St. Louis, MO). Z-IETD-FMK (caspase-8 inhibitor), Z-LEHD-FMK (caspase-9 inhibitor), and Boc-D-FMK (pancaspase inhibitor) were purchased from Calbiochem (San Diego, California).

Western blot analysis

Expressions of NGF, TrkA and p75 receptors, and Akt, MAPK, and JNK downstream pathways were determined by western blot analysis according to the manufacturer's instructions. β -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 15 minutes, and protein concentrations were measured with the Bradford method [21]. Samples (20–40 μ g of protein) were electrophoresed on 10–15 % 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). Blots were quantified using Imaging Densitometer GF670 and Molecular Analyst software (Bio-Rad) three times in each sample, and the average of three densities was used as the final density. The value of the density was presented as the mean $\pm$ SD (arbitrary units).

Evaluation of apoptosis

Apoptosis was determined by staining cells with both annexin V-FITC and propidium iodide (PharMingen, San Diego, California) and APOPercentage (Biocolor Life Science, Carrickfergus, United Kingdom) according to the manufacturer's instructions.

1. Annexin V-FITC is used to quantitatively determine the percentage of cells undergoing apoptosis. It relies on the property of cells to lose their 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 Ca^{2+} -dependent phospholipid-binding protein that has a high affinity for phosphatidylserine and is useful for identifying apoptotic cells with
2. The apoptosis of notochordal cells was also determined by staining cells with 15 μL of APOPercentage dye followed by incubation for 30 min. After syringing off the culture medium and dye mixture, and gently washing the cells twice with 500 μL /well PBS, the cells were transferred to an inverted microscope and photographs were obtained. Notochordal cells that were cultured for 48 hours by themselves were used as the control.

Fig. 1 Notochordal cells were treated with 10 % or 0 % fetal bovine serum (FBS) for 48 hours. Cell death was assayed with flow cytometry after double staining with annexin V-FITC and propidium iodide and APOPercentage

Assay of anti-apoptotic effects of caspase inhibitors

Notochordal cells were incubated with 0 % FBS with and without Z-IETD-FMK (caspase-8 inhibitor, 100 μ M), Z-LEHD-FMK (caspase-9 inhibitor, 100 μ M), Z-VAD-FMK (pancaspase inhibitor, 100 μ M), and Boc-D-FMK (pancaspase inhibitor, 100 μ M) for 48 hours. This concentration of caspase inhibitors has been shown to inhibit caspase-3, 8, and 9, respectively. Apoptotic cell death was also determined by double staining with 5 μ L of annexin V-FITC and 10 μ L of propidium iodide followed by analysis with FACScan flow cytometry and apoptosis assay with APOPercentage at 48 hours according to the manufacturer's instructions, respectively.

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

Apoptotic effect of serum deprivation on notochordal cells

Figure 1 shows that rat notochordal cells treated for 48 hours with 0 % FBS displayed apoptotic cell death, as determined by double staining with annexin V-FITC and propidium iodide and APOPercentage. The mean percentage of apoptotic cell death in 0 % FBS was significantly increased compared with that in 10 % FBS at 48 hours (61.3 ± 12.9 % versus 3.51 ± 1.6 %, $p < 0.001$), respectively (Fig. 2).

Fig. 2 The percentage of apoptotic cell death of notochordal cells, expressed as the mean and standard deviation of three independent experiments. *** $p < 0.001$

Fig. 3 Western blot analysis showing increased expression of NGF, p75 receptor, and JNK downstream pathway but decreased expression of TrkA receptor and Akt and MAPK downstream pathway in notochordal cells treated with 0 % fetal bovine serum (FBS) for 48 hours compared with 10 % FBS

Effect of serum deprivation on expression of NGF and its TrkA and p75 receptors of notochordal cells

The results of western blot analysis are shown in Fig. 3. The expression levels of NGF, p75 receptor (apoptotic signal), and the JNK (apoptotic signal) downstream pathway were significantly increased in 0 % FBS compared with those in 10 % FBS. In contrast, the expression levels of the TrkA receptor (survival signal) and Akt and MAPK (survival signal)

Fig. 4 Western blot analysis shows that notochordal cells treated with 0 % fetal bovine serum (FBS) of serum deprivation for 48 hours caused activations of initiators of the intrinsic (caspase-9) and extrinsic (caspase-8) pathways as well as their common executioner (caspase-3) compared with 10 % FBS

downstream pathways were significantly decreased in 0 % FBS compared with those in 10 % FBS.

Serum deprivation and activation of caspases

The results of western blot analysis are shown in Fig. 4. Notochordal cells in 10 % FBS (normal control) constitutively expressed procaspase-8 (initiator caspase in the extrinsic pathway), procaspase-9 (initiator caspase in intrinsic pathway) and procaspase-3 (common executioner caspase in both intrinsic and extrinsic pathways). 0 % FBS (serum deprivation, apoptosis-promoting condition) led to activations of caspase-8, -9 and -3.

Caspase inhibitors reduce serum deprivation-induced apoptosis of notochordal cells

Flow cytometric analysis and apoptosis assay revealed that apoptosis of notochordal cells was significantly reduced by

Z-IETD-FMK (caspase-8 inhibitor of extrinsic pathway) (61.3 ± 12.9 % versus 49.2 ± 10.5 %, $p < 0.05$) and Z-LEHD-FMK (caspase-9 inhibitor of intrinsic pathway) (61.3 ± 12.9 % versus 48.9 ± 10.9 %, $p < 0.05$). In addition, inhibition of both the intrinsic and extrinsic pathways by Boc-D-FMK (pancaspase inhibitor) more significantly reduced apoptosis of notochordal cells (61.3 ± 12.9 % versus 38.7 ± 9.2 %, $p < 0.01$) (Fig. 5a and b).

Discussion

Recent studies [3, 4] suggested that notochordal cells have a very unique lifespan. The cells reside in the notochordal NP and produce gel-like vacuole-rich nucleus pulposus tissue until they disappear at some point during the individual's lifetime. As notochordal cells disappear by apoptosis, chondrocytes migrating from cartilage-endplates gradually replace notochordal cells and change

Fig. 5 Assay of the antiapoptotic effects of caspase inhibitors. Notochordal cells were incubated with 0 % fetal bovine serum (FBS) with and without Z-IETD-FMK (caspase-8 inhibitor, 100 μ M), Z-LEHD-FMK (caspase-9 inhibitor, 100 μ M), and Boc-D-FMK (pancaspase inhibitor, 100 μ M) for 48 hours (a). 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 (b). * $p < 0.05$; ** $p < 0.01$

the gelatinous notochordal nucleus pulposus into a fibrocartilaginous nucleus pulposus. The disappearance of notochordal cells from the NP is thought to be the starting point of disc degeneration, which may occur at different ages even in the same species. Therefore, it is important to investigate the apoptotic pathway of notochordal cells, but to date, the apoptotic pathway of notochordal cells has not been fully elucidated.

NGF seems to be a unique endogenous ligand needed to cause cell death as well as prevent it. The effects of NGF are mediated by two different receptors: the high affinity TrkA (survival signal) receptor and the low affinity p75 (apoptotic signal) receptor [12, 13]. Recent studies suggest that different responses to NGF are almost completely dependent on the relative amount of the two receptors: the p75 receptor provides an apoptotic signal for various types of cells in the presence or absence of the TrkA receptor, and one of the major ways that the TrkA receptor supports cell survival is by suppressing this death signal. JNK activity is critical for death signaling of the p75 receptor signaling pathway. On the contrary, Akt and MAPK activities play a critical role in the survival of cells [14–16]. The serum deprivation model is one of the reliable methods to investigate events related to the apoptosis of various types of cells [20]. Thus far, however, the apoptotic effect of serum deprivation on notochordal cells is unknown. We therefore undertook the present study to investigate the relationship of NGF and its TrkA and p75 receptors and apoptosis of notochordal cells using serum deprivation.

The present results demonstrate that in notochordal cells treated with serum deprivation (0 % FBS), there was a significant increase in the expressions of NGF, p75 receptor, and JNK downstream pathway followed by sequential activation of caspase-8, -9, and -3, resulting in apoptosis of notochordal cells compared to the normal control (10 % FBS). In contrast, expressions of the TrkA receptor and Akt and MAPK downstream pathways related to the survival signaling pathway were significantly decreased in 0 % FBS compared to those of 10 % FBS. Two major pathways of apoptosis have been identified [18, 21]. In the extrinsic pathway, caspase-8 directly activates caspase-3, an executioner of apoptosis, whereas in the intrinsic pathway, caspase-3 is activated by caspase-9 via mitochondria. Therefore, our present findings suggest that notochordal cells subjected to serum deprivation undergo apoptosis via both the intrinsic and extrinsic pathways induced by the activation of NGF, the p75 receptor, and the JNK downstream pathway.

We also tested the therapeutic effect of caspase inhibitors on apoptosis of notochordal cells in a serum deprivation model. We found that notochordal cells treated with caspase-8 inhibitor and caspase-9 inhibitor demonstrated a significant reduction of apoptosis. Pancaspase inhibition

more effectively decreased apoptosis than did caspase-8 and caspase-9 inhibitors. 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 [1, 8–13]. Our results suggest that caspase inhibitors may play a therapeutic role in delaying the starting point of disc degeneration that is due to inappropriate or premature excessive apoptosis of notochordal cells. In conclusion, this study is the first study to directly demonstrate the effects of NGF and its TrkA and p75 receptors on serum deprivation induced apoptosis of notochordal cells and the antiapoptotic effects of caspase inhibitors.

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