Ceramide accumulation accelerates nucleus pulposus cells degradation by p38MAPK activation

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Abstract. – OBJECTIVE: Ceramide is a lipid molecule that regulates life activities such as cell differentiation, proliferation, apoptosis, and aging. However, whether ceramide plays a role in the intervertebral disc degeneration (IDD) is not clear. The aim of this study is to explore the effect of ceramide during the nucleus pulposus (NP) cells degeneration.

PATIENTS AND METHODS: We used human NP cells and passaged them until the fourth generation to analyze the content of ceramide. Cell-permeable C6-ceramide was used to upregulate ceramide expression, and myriocin was used to inhibit the accumulation of ceramide. To understand the relation between p38MAPK and ceramide, SB203580 was used to inhibit the activation of p38MAPK. We tested the viability of NP cells by the detection of collagen II and p16 expression, the proliferation, and the apoptosis of NP cells.

RESULTS: Ceramide content was increased in NP cells from passage 1 (P1) to P4. The upregulation of ceramide accelerated the P1 NP cell degeneration by the reduction of collagen II production and proliferative cells population, increased p16 expression, and apoptosis cell population. However, the accumulation of ceramide delayed the degenerative aspects. The accumulation of ceramide activated the phosphorylation of p38MAPK, and the inhibition of p38MAPK activation also alleviated the C6-ceramide-induced NP cell degeneration.

CONCLUSIONS: Ceramide accumulates during NP cell degeneration, and the upregulated ceramide contributes to NP cells degeneration by p38MAPK activation.

Key words: Nucleus pulposus cells, Apoptosis, Cell proliferation, p38MAPK.

Introduction

Chronic low back pain is a high-prevalence, difficult-to-treat orthopedic disease, which is one of the most serious diseases associated with chronic aging. There are about 600 million people suffer low back pain worldwide, and 70%-85% of adults will experience different degrees of low back pain throughout their lives, which will greatly affect the patient’s own quality of life, national medical expenses, and social productivity. Most low back pain is tolerable and self-limiting. The acute phase lasts about 4 weeks, and the subacute phase lasts 4 to 12 weeks. However, some patients will develop into the chronic phase lasting up to 12 weeks. Clinically, the disease based on histopathology and imaging indicate that most of the occurrence of low back pain begins with degenerative changes in the nucleus pulposus (NP) of the intervertebral disc. The structure and biochemical composition of the NP changes during the progress of intervertebral disc degeneration (IDD). The decrease in proteoglycan (PG) and water content affects the carrying capacity of the intervertebral disc. In addition, the change in the composition of collagens and the decrease in its content has lost its original flexibility and scalability. Therefore, exploring the mechanism of NP degeneration is one of the important directions of medical research on IDD.

Ceramide is a class of lipid molecules that has important regulatory effects on life activities such as cell differentiation, proliferation, apoptosis, and aging. When cells are stimulated by various external conditions such as ultraviolet radiation, heat shock, and chemotherapy drugs, endogenous ceramides begin to synthesize and may induce a series of physiological responses such as cell aging, growth inhibition, and apoptosis. Dadsena et al reported ceramide triggers mitochondrial apoptosis by binding VDAC2. Boon et al found ceramide promotes inflammation in type 2 diabetes. Jadhav et al elucidated ceramide induces C2C12 myoblasts senescence which can be inhibited by Metformin. Gulbins et al stated ceramide regulates neuronal stem cell proliferation in the hippo-
campus. Ceramide is also proved to have an ability in tumor suppressive action\(^4\). However, the effect of ceramide in the IDD still remains unknown.

In our study, we used the NP cells spontaneous degeneration model by repeated passage to explore the effect of ceramide in the progression of NP cell degradation. The inhibitor of ceramide named myriocin was applied to see whether the suppression of ceramide made sense to prevent the NP cell degeneration. What we found indicated that ceramide accumulated during the repeated passage of NP cells and the intervention of ceramide may be an effective way to alleviate NP cell degeneration.

**Patients and Methods**

**NP Tissue Collection and NP Cells Isolation**

We collected degenerative disc samples from patients who underwent NP discectomy due to disc herniation in Ruijin Hospital, Shanghai Jiaotong University School of Medicine. All of the written informed consents from the patients were signed before the surgeries. We divided the NP from the endplates and conserved them in cold cell culture medium for the following NP cell isolation. Before isolation, we washed the samples with phosphate-buffered saline (PBS) and then cut them into pieces as smaller as possible. The fragments were incubated with 0.15% type II collagenase for digestion at 37°C overnight. The next day, the solution was filtrated using a 100 µm pore strainer and resuspended with Dulbecco's Modified Eagle's Medium/F12 (DMEM/F12; Gibco, Rockville, MD, USA) containing 10% fetal bovine serum (FBS; Gibco, Rockville, MD, USA). The cells were seeded in 6-well plates at 1×10\(^5\) cells/well and passed till the fourth generation. C6-ceramide and the p38MAPK inhibitor SB203580 used for NP cell culture were purchased from Sigma-Aldrich (St. Louis, MO, USA). This investigation has been approved by the Ethics Committee of Ruijin Hospital, Shanghai Jiaotong University School of Medicine.

**Reverse Transcription-Polymerase Chain Reaction (RT-PCR)**

Total RNA was isolated from NP cells with TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s method. It was reverse transcribed to complementary deoxyribonucleic acid (cDNA) by Maxscript Mix (Applied Biosystems, Foster City, CA, USA) and SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA) to detect IL-1β, TNF-α, MMP-3, ADAMTS-5, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as normalization) RNA levels. The primer sequences used for RT-PCR are listed in Table I based on the Primer Bank database. Relative mRNA expression levels were analyzed by the 2-ΔΔCt method.

**Western Blot (WB) Analysis**

NP cells were lysed with radioimmunoprecipitation assay (RIPA) lysate (Beyotime, Shanghai, China), centrifuged at 4°C (12000 G for 5 min), and the supernatant was removed, and the protein concentration was determined by bicinchoninic acid (BCA) method (Beyotime, Shanghai, China) to adjust samples with protein equally. The protein was subjected to vertical electrophoresis on a polyacrylamide gel, and then electrotransferred onto a nitrocellulose membrane. The membrane was blocked with 5% skimmed milk for 2 h and incubated with anti-p38 (ab170099, Abcam, Cambridge, MA, USA), anti-Phospho-p38 (p-p38, Cat#44-684G, Thermo Fisher Scientific, Waltham, MA, USA), anti-β-actin (ab179467, Abcam, Cambridge, MA, USA) antibodies at 4°C overnight. After washing, the membrane was incubated with the secondary antibody for 2 h at room temperature. The gray ratio of the bands of the protein was scanned by Scinn Corporation analysis software corresponding to the relative content of β-actin.

**Immunofluorescence (IF) Staining**

NP cells were fixed with 4% formaldehyde for 15 min at room temperature, washed with PBS, and

### Table I. Primer sequences of the genes for RT-PCR.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward (5’→3’)</th>
<th>Reverse (5’→3’)</th>
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<tr>
<td>TNF-α</td>
<td>CGCCGATGGTACGGTTGTCGAGGACCTGGGAGTAGATGAG</td>
<td>GAGGACCTGGGAGTAGATGAG</td>
</tr>
<tr>
<td>IL-1β</td>
<td>ATGATGCCTTATACAGTGGCACAAGGAGAA</td>
<td>GTCCGGAGATTCTGAGCTGGA</td>
</tr>
<tr>
<td>MMP-3</td>
<td>AGTCTTCAATCTACTTGTGGTCTTTCCCTGA</td>
<td>TCCGGTACCTCCATCTCC</td>
</tr>
<tr>
<td>ADAMTS-5</td>
<td>GAAACATTGCAACACTTACTCTCC</td>
<td>CAATTGCACCCAGAACCATCT</td>
</tr>
<tr>
<td>GAPDH</td>
<td>ACAACTTTGATATCGTTGAGAG</td>
<td>GCCATCAGGCCACAGTTTC</td>
</tr>
</tbody>
</table>

RT-PCR, quantitative reverse-transcription polymerase chain reaction.
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then blocked with 5% bovine serum albumin (BSA) for 30 min. NP cells were following incubated with a primary antibody against p16 (ab51243, Abcam, Cambridge, MA, USA) or collagen II (ab34712, Abcam, Cambridge, MA, USA) overnight at 4°C and then incubated by Cy3-conjugated 488 antibody for 1 h in the dark. 4',6-diamidino-2-phenylindole (DAPI) was used to stain the cell nucleus. Finally, the fluorescence intensity of NP cells was visualized using an inverted microscope.

**Flow Cytometry**

The apoptotic ratio of NP cells was determined by flow cytometry using Annexin V-FITC/PI double-stained apoptosis detection kit (Keygen, Nanjing, China). NP cells were collected, washed with PBS, centrifuged, and labeled with Annexin V-FITC and propidium iodide (PI), and we used the FACS Calibur flow cytometer (BD Corporation, Franklin Lakes, NJ, USA) to detect the distribution of apoptotic rate. The proliferative ratio of NP cells was determined using the EdU assay kit (Keygen, Nanjing, China) according to the manufacturer’s protocol.

**Ceramide Quantitation**

The ceramide content of NP cells was determined by liquid chromatography-mass spectrometry (LC-MS) method. Briefly, cells were harvested and then washed and diluted in 100 μL PBS. Sphingolipid extracts were prepared by Bligh-Dyer method and analyzed as previously described. Quantification of ceramide performance with the extracted ion chromatogram using a triple quadrupole mass spectrometer as reported.

**Statistical Analysis**

All data were presented by the means values ± SD (standard deviation), and graphs were generated by GraphPad 5.0 (La Jolla, CA, USA) software. Differences between two groups were analyzed by using the Student’s ‘t’-test. Comparison of multiple groups was done using One-way ANOVA test followed by Post-Hoc Test (Least Significant Difference). A p-value below 0.05 was considered statistically significant.

**Results**

**Ceramide Accumulated In Spontaneously Regressive NP Cells**

To determine the content of ceramide in the NP cells of different degenerated degrees, we passaged NP cells from the first generation to the fourth to achieve the spontaneously regressive NP cells. As shown in Figure 1A and 1B, the expression of collagen II decreased significantly with the increased generation of NP cells, which meant NP cells in a higher generation were weak in collagen II synthesis. Additionally, the p16 expression was raised as the cells going to a higher generation, indicating a senescent phenotype gradually appeared. Followingly, the result of ceramide content measurement suggested the ceramide level was upregulated from the first generation to the fourth generation (Figure 1C). As with many other cellular aging processes, ceramide accumulated in the NP cells. Apart from this, we also analyzed the proliferative and apoptotic ratio of NP cells by flow cytometry (Figure 1D, 1E). As expected, the proliferative NP cell population was decreased along with the cell continually passaged and the apoptotic cells increased obviously from passage 1 to passage 4, corresponding to a degenerated cell phenotype. The data indicated that the content of ceramide accumulated in the degenerated NP cells, which should be associated with the development of NP cell degeneration.

**Ceramide Overexpression Accelerated NP Cells Degeneration**

To determine whether the upregulation of ceramide affects the phenotype of NP cells, we treated passage 1 NP cells with a cell-permeable analog of naturally occurring ceramides named C6-ceramide (ceramide). Under the stimulation of ceramide, the expression of collagen II decreased compared to the control with a dose-dependent. Besides, the p16 level significantly increased due to the ceramide treatment compared to the control (Figure 2A, 2B). The result of the LC-MS showed that ceramide was upregulated resulting from the ceramide analog treatment, especially in the concentration of 20 µM (Figure 2C). Ceramide also affected the viability of NP cells by increasing the apoptosis (Figure 2D) but decreasing the proliferation (Figure 2F) compared to the control. The overexpression of ceramide was proved to be related to the inflammatory response in many cells. Therefore, we also tested the mRNA levels of some inflammatory factors during the NP cell degeneration, such as IL-1β, TNF-α, MMP-3, and ADAMTS-5, which were all increased after ceramide treatment (Figure 2E). The results indicated that the ceramide overexpression by an analog stimulation accelerated the NP cell degradation by the reduction of collagen II synthesis and cell
viability and the increase of senescent cells and the inflammatory response.

**Ceramide Suppression Alleviated NP Cells Degeneration**

To determine whether the suppression of ceramide protects the NP cells naturally degeneration, we used myriocin to suppress ceramide levels by de novo synthetic pathway. We passaged NP cells from the first generation to the fourth generation with or without myriocin (1 mM or 2 mM). Under the treatment of myriocin, we found the collagen II expression was mainly maintained compared to the cells without myriocin treatment, and 2 mM was effective to alleviate the degenerative progress of NP cells. The marker of senescent cell p16 was inhibited in a dose-dependent compared to the control with no myriocin intervention (Figure 1).
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Figure 2. Ceramide overexpression promoted NP cells degeneration. NP cells in P1 were treated with C6-ceramide (10 µM or 20 µM) for 24 h and changed the medium. A, IF staining of collagen-II, and p16 (magnification: 400×). B, Quantification analysis. C, Total ceramide quantitation by LC-MS. D, Ratio of apoptotic NP cells was determined by flow cytometry. E, mRNA expression levels were assayed by RT-PCR. F, Ratio of proliferative NP cells was determined by flow cytometry. Data are presented as mean ± S.D. of three independent experiments. (*p<0.05, **p<0.01, ***p<0.001).
flow cytometry showed that myriocin meanwhile prevented NP cells apoptosis and protected their proliferation (Figure 3D, 3F). Then, we detected

3A, 3B). As expected, the ceramide content was decreased in passage 4 resulting from the stimulation of myriocin (Figure 3C). The result of

Figure 3. Ceramide inhibition alleviated NP cells degeneration. NP cells were treated with or without myriocin (10 mM or 2 mM) from P1 to P4. A, IF staining of collagen-II, and p16 (magnification: 400×). B, Quantification analysis. C, Total ceramide quantitation by LC-MS. D, Ratio of apoptotic NP cells was determined by flow cytometry. E, mRNA expression levels were assayed by RT-PCR. F, Ratio of proliferative NP cells was determined by flow cytometry. Data are presented as mean ± SD of three independent experiments. (*p<0.05, **p<0.01).
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**Figure 4.** p38 Suppression Rejected Ceramide-Induced NP Cell Degeneration. P1 NP cells were treated with C6-ceramide (20 μM) and then changed the medium. Besides, P1 NP cells were pre-treated with C6-ceramide (20 μM) and then treated with SB (50 nM) for another 24 h. A, Protein expression of p38 and p-p38 was determined by WB and quantification analysis. B, Total ceramide quantitation by LC-MS. C, IF staining of collagen-II, and p16 (magnification: 400×). D, IF staining of collagen-II, and p16 (magnification: 400×). E, Quantification analysis. The ratio of F, apoptotic or G, proliferative NP cells was determined by flow cytometry. Data are presented as mean ± SD of three independent experiments. (*p<0.05, **p<0.01, ***p<0.001).

Like ceramide, the p38MAPK signaling pathway can be activated during extracellular stimulation such as ultraviolet irradiation, heat shock, high osmotic pressure, and inflammatory factors, and regulates various physiological processes, such as cell differentiation, cell cycle, and inflamm-
matory response. Therefore, we wondered whether ceramide affected NP cells metabolism cross-linking with p38 activity. We used SB203580 (SB) to suppress p38MAPK activation. As shown in Figure 4A and 4B, the expression of total p38 was significantly different among these groups. However, the 20 μM ceramide treatments markedly upregulated the p-p38 expression, which was suppressed by SB again. So, the overexpression of ceramide accompanied by the activation of p38 in addition, SB decreased the accumulation of ceramide even a small drop (Figure 4C). The result of IF showed that SB could partly reverse the ceramide-induced collagen II downregulation and p16 upregulation (Figure 4D, 4E). Finally, apoptosis and proliferation were also measured and the result suggested that SB treatment protected the NP cell proliferation and prevented the apoptosis progress compared to the ceramide treated group (Figure 4F, 4G). We found the suppression of p38MAPK phosphorylation could alleviate the upregulation of ceramide-induced NP cell degeneration, indicating that ceramide might accelerate the NP cell degeneration by p38MAPK activation.

Discussion

Ceramide belongs to a class of highly hydrophobic families and is the central substrate of the sphingomyelin signaling pathway. Ceramide acts as a second messenger that participates in the activation of multiple proteases and protein phosphatases, and it regulates signal processes such as apoptosis, proliferation, differentiation, and cell arrest. It is an important physiological response to cell homeostasis. NP degeneration of the intervertebral disc is a multi-factor complex disease, and it is currently difficult to achieve the delay or even reverse the process of degeneration. Since now, the relation between ceramide and IVD is not clear. In our study, we elucidated that the content of ceramide accumulated during the degeneration of NP cells from P1 to P4 generations. Therefore, we held the viewpoint that ceramide played a role in the degeneration of IVD.

We treated NP cells in the P1 generation with exogenous ceramide to make it overexpressed. Ceramide can rapidly induce human umbilical vein endothelial cells senescence. Chan et al.21 found ceramide promoted inflammatory processes and apoptosis in the epithelium of the lung. Ceramide also triggers oxidative stress and nitric oxide synthase on human endothelial cells. Therefore, ceramide accelerates different cell types of degradation with different manners. In our study, the cell-permeable analog effectively increased the ceramide content and the phenotype of P1 NP cells similar to the P4, indicating the accumulation of ceramide promotes the degenerated degree of NP cells. On the contrary, the suppression of ceramide accumulation by myriocin could prevent the cell degeneration containing the aspects of collagen II synthesis, inflammation response, apoptosis, senescence, and proliferation. We proposed to express inflammatory and fungal by inhibiting ceramide de novo synthesis in chronic lung inflammation with compound Ami. Jelek et al.22 stated myriocin destroys ceramide de novo synthesis, inhibiting lipid metabolism in the liver of streptozotocin treated rats.

The mechanism underlines ceramide in the intervention of cell metabolism is complicated. Ceramides can activate c-Jun N-terminal kinase (JNK)23, toll-like receptor (TLR)27 signaling pathway, and NF-κB26, toll-like receptor (TLR)27 signaling pathway regulates various physiological processes such as cell differentiation and apoptosis, cell cycle, and inflammatory response. We suggested that ceramide contributed to NP cell degeneration by the activation of p38MAPK. We found the p-p38 level was upregulated by the treatment of C6-ceramide. Additionally, the suppression of p38MAPK activation partly reversed the pro-degenerated function of ceramide accumulation. This result indicates that ceramide promotes the activation of p38MAPK contributing to the NP cell degeneration. However, the detailed mechanism of p38MAPK crosslinking with ceramide and the relation between other signaling pathways with ceramide need further exploration.

Conclusions

In summary, we elucidated that ceramide accumulated during the development of NP cell degeneration. The overexpressed ceramide accelerates NP cells degeneration in decreasing proliferation, advancing the apoptosis, promoting senescence, and suppressing inflammation of NP cells. Besides, the suppression of ceramide can alleviate NP cells natural degeneration, and these effects underlining ceramide may be associated with the activation of p38MAPK. We believe cer-
amide could be a potential target for the treatment strategy of IDD.

Conflict of Interest
The Authors declare that they have no conflict of interests.

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References


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