Expression and Role of Sphingosine 1-Phosphate Receptors in Intervertebral Disc Degeneration

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Abstract: Background: The mechanism of intervertebral disc degeneration is unclear, inflammation may play an important role in it. Sphingosine 1-phosphate (S1P) is an important lipid mediator, has been confirmed to be implicated in many inflammation processes. Purpose to study the expression and role of S1PRs in the intervertebral disc (IVD) degeneration to enhance understanding of disc degeneration. Methods Degenerated and normal IVD were harvested from patients with IVD degeneration via surgery operation. The degenerated grade of each IVD specimen was assessed by Histological method. Expression of S1P receptor subtypes in each specimen was evaluated using real-time PCR, immunohistochemistry, and western blotting. The effect of S1PR on inflammation induced by interleukin-1β in nucleus pulposus (NP) cells was also assessed by real time PCR and western blot. Results The nucleus pulposus mainly expressed the S1PR1/2/3, and the expression decreased in the severe degenerated nucleus pulposus cells. The ligand, S1P, inhibited the up-regulation of matrix metallopeptidase-3 (MMP-3) and ADAM metallopeptidase with thrombospondin type 1 motif 4 (ADAMTS4) induced by IL-1β. Conclusions The results show that the expression of S1PRs in degenerative discs is down-regulated as degeneration, and S1P can inhibit the inflammation response induced by IL-1β in NP cells, implicating that S1P/S1PR may contribute to IVD degeneration.

Keywords: Phingosine 1-Phosphate Receptors, Nucleus Pulposus Cells, Intervertebral Disc Degeneration, IL-1β

1. Introduction

Chronic low back pain (LBP) is a widespread problem all over the world, which affects about 50 to 80% of adults during their lifetime. Numerous studies have shown that intervertebral (IVD) degeneration is one of the major underlying factors in chronic LBP. Various changes occur with the degeneration, such as fissures in the annulus fibrosus, and dehydration in the nucleus pulposus (NP). Alterations in the extracellular matrix and expression profile of the NP cells, including decreases in proteoglycan and type II collagen synthesis, increases in type I collagen synthesis and aggrecan fragment accumulation, is the major pathogenic characterization of IVD degeneration.

Furthermore, a variety of inflammatory cytokines have been implicated in IVD degeneration. Previous studies have demonstrated that IL-1β and TNF-α are overexpressed in degenerated IVD, and led to matrix degradation in degenerated disc. Thus inhibition of the effect of these cytokines may be a target for therapeutic intervention.

Sphingosine 1-phosphate (S1P) is an important lipid mediator, formed by phosphorylation of sphingosine and catalyzed by sphingosine kinase. It has been confirmed to be implicated in many biological processes, including cell migration, differentiation, inflammation and angiogenesis. S1P exerts its various functions by binding to specific G protein-coupled receptors, and 5 functionally different isoforms (termed S1PR 1-5) have been identified. Strikingly, previous researches have shown that in human chondrocytes, S1P can inhibit the catabolic response induced by via activation of the S1P receptor, thus the activation of the receptor may be a therapeutic target for OA. In the light of similar biological property of nucleus pulposus cells, S1P and its receptors reflect a certain application prospect in the treatment and control of intervertebral degeneration. While, to the extent of our knowledge, the expression of S1P receptors or the effect of S1P in IVD degeneration have not been explored.

In the current study, we investigates the expression of S1P receptors in nucleus pulposus, and compared the expression in
NP cells with different degree of degeneration in order to verify the relation between the S1PRs and IVD degeneration. Finally, we preliminary explored the effects of S1P on IL-1β induced inflammation response in NP cells.

2. Materials and Methods

2.1. Patients and Samples

34 patients with lumbar degeneration (lumbar herniation or Spondylolisthesis) and 5 patients with vertebral fracture were selected for this study. Study protocols were approved by the Ethics Committee of our institution, and the informed consent was signed. Patients with infection, tumor, immunological and endocrine disease were excluded from the current study.

2.2. Histological Grading of IVD Tissues

To histologically assess grade of degenerated IVD, samples obtained from the surgical operation were fixed with Formalin and Embedded with Paraffin. Five microns sections of the tissue were acquired and processed into HE staining and Saf-O staining. The degree of degeneration was graded according to previously published histological scoring system [15]. Two readers independently graded each of the lumbar IVDs. Consensus was reached when the initial reading of the two investigators differs.

2.3. Immunohistochemistry

Paraffin sections (5µm thick) were treated with xylene to remove paraffin and rehydrated in graded alcohol baths followed by three rinses with phosphate-buffered saline (PBS). Slides were immunostained with the streptavidin–biotin peroxidase (SABC) technique. The primary antibodies of S1PR1 (Santa Cruz, sc-48356), S1PR2 (Santa Cruz, sc-25491) and S1PR3 (Santa Cruz, sc-30024) 1:50 dilution were used to stain.

2.4. Nucleus Pulposus (NP) Cell Isolation and Culture

The IVD tissues were harvested from three patients during surgery operation and transported to the laboratory within 30 min. NP tissue were carefully picked and digested in a 0.5% type II collagenase (Sigma Aldrich) solution in serum-free medium. The digested tissue/cell suspension was passed through a 100µm cell strainer to remove tissue debris, and cells were then collect by centrifugation at 1200 rpm for 5 minutes. The supernatant was removed, and cells were resuspended and cultured to confluence in a 25-cm2flask with Dulbecco’s Modified Eagle Medium: Nutrient Mixture F-12 (DMEM-F12), supplemented with 10% Fetal Bovine Serum (Gibco), 1% penicillin–streptomycin (PS), at 37°C in a humid atmosphere containing 5% CO₂, with medium changed every 3 days. The cells were subcultured when confluence 75-80%. The NP cells for subsequent experiment were used at passage 1 or 2.

2.5. Cell Treatment

To explore the role of S1PRs in Human NP cells, the cells were stimulated with ligand, Sphingosine 1-phosphate (S1P), and the effect was assessed by subsequent real-time PCR. Specific information is as follows: firstly, S1P (Sigma-Aldrich) was dissolved in methanol, evaporated, and then resuspended in 0.4% fatty acid-free bovine serum albumin. Recombinant human IL-1β (10 ng/ml; Sigma-Aldrich) was dissolved in the DMEM-F12 culture medium. The cell were treated with the IL-1β solution for to 24 hours with or without 5µmol/ml S1P.

2.6. Real-Time PCR

Total RNA was extracted from the NP cells by Trizol (Invitrogen, Carlsbad, CA) as according to the manufacturer’s instructions. The mRNA was analyzed with real-time polymerase chain reaction using a real-time polymerase chain reaction system (Geneline 9640, Bioer, Hangzhou, China). The cDNA was then reverse transcribed (model R0037A, TaKaRa, Dalian, China) according to the manufacturer’s instructions. Real-time polymerase chain reaction reactions were done in triplicate in 96-well plates in a final volume of 20 µL, including 10µL of Sybr Green, 2µL of cDNA, 1µL of each primer, and 6µL of sterile distilled water. All primers (shown in Table 2; obtained from Sangon, Shanghai, China) were designed on the basis of coding sequences. The cycle threshold values were obtained; and data were normalized to GADPH expression using the 2 − ∆ ∆ Ct method.

2.7. Western-Blot

The total protein was extracted using RIPA supplemented with 1% protease inhibitor cocktail (both from Sigma-Aldrich). Four independent experiments using chondrocytes derived from different patients were performed. The protein concentration was measured with BCA method (Bio-Rad). Proteins were separated by SPS-PAGE electrophoresis using a 10% polyacrylamide gel, and then transferred to a nitrocellulose membrane (Bio-Rad). After blocking in 5% skim milk-Tris buffered saline, the membranes were incubated with primary antibodies overnight. Thereafter, the membranes were rinsed in blocking solution and incubated for 1 hour with a secondary antibody conjugated to horseradish peroxidase. Bands were visualized using an acridan-based substrate detection system (ECL Plus; Amersham). And quantify protein levels relativly by densitometry using the program ImageJ.

2.8. Statistical Analysis

Histological staining and western-blot data are described qualitatively. The data of Real-PCR is shown as means ±standard deviation. In accordance with the data distribution and the homogeneity of variance, one-way ANOVA or Kruskal–Wallis H-test were selectively utilized. The P value of less than 0.05 was considered statistically significant.
3. Results

3.1. Evaluation the Degeneration of IVD Samples

In order to evaluate the grade of degeneration in NP cells, Histological grading was proceeded by HE and Saf-O staining. The scores of Histological grading of the IVD tissues harvested from patients via surgery operation is shown in Table 1. Other information of the patients is also included. The representative HE and SAF-O staining of NP tissue with different level of degeneration is shown in FIG1. Over all, with the progress of the degeneration, the NP cells get together into colonies, and the extracellular matrix lose.

According to the grading system [15], which generates a score between 0 and 12, we define that a grade of 0 to 4 represents a histologically normal (N), grades of 5 to 8 indicate mild degeneration (MD), and grades 9 to 12 severe degeneration (SD). Then all of the samples were divided into three groups, the N group includes 5 samples, the MD group includes 12 samples, and the SD group 22 samples.

![Figure 1](image1.png)

**Figure 1.** HE and Saf-O stain of the intervertebral disc: normal NP shows abundant extracellular matrix and stained red evenly, small-size clonies in mild degenerated NP and less stained red, severe degenerated NP shows huge stained and stained red mainly in the cell clonies, bar 100µm.

3.2. Expression of S1PRs in NP with Different Degree of Degeneration

To investigate the distribution of S1P receptors in IVD tissue, immunohistochemical staining was performed. S1PR1/2/3 was detected on the IVD sections (FIG2). Both cytoplasmic and cytomembrane staining of S1PR1/2/3 immunoreactivity were observed in the nucleus pulposus cells. Comparing the immune-expression with degree of degeneration, it seems down-regulated during disease progression.

![Figure 2](image2.png)

**Figure 2.** The expression of S1PR in NP was shown by Immunohistochemistry. S1PR1, S1PR2, S1PR3 are all positively expressed in NP tissues, and it is down-regulated with degeneration, bar 100µm.
Real-time PCR was used to compare the gene expression of S1PR1/2/3 in human NP cells with different level of degeneration (FIG 3). The results confirmed that human degenerative NP cells demonstrated a significant decrease in S1PR1/2 expression at mRNA level in severe degenerated NP cells, S1PR3 also decreased but not significantly. Western-blot shows that the decrease tendency is same in protein level, which the expression of S1PR1/2/3 decrease in severe degenerated NP cells (Figure 3).

![Figure 3. Expression of 3 type of S1PRs(S1PR1/2/3) in NP cells with different degrees of degeneration was measured by real-time PCR and Western blot. The typical grayscale of western-blot from some samples was also shown. All of them decrease in the severe degenerated cells. The relative expression to the severe degenerated was shown as Mean±SEM(A and C). (N, normal; MD, mild degenerated; SD, severe degenerated),* means P<0.05.](image)

### 3.3. The Effect of the Ligand (S1P) in NP Cells.

Previous studies have confirmed that the in human chondrocytes, S1P can reduces the induction of catabolic genes in the presence of IL-1β via activation of the S1P receptor 2(S1PR2). Here simulating the same pattern (10 ng/ml IL-1β with or without 5µmol/ml S1P) on the NP cell isolated from Mild degenerated IVD, it shows that S1P counteracted the IL-1β induced up-regulation of ADAMTS-4 and MMP-3 after treatment for 24 hours by real-time PCR. (Figure 4). The suppressive effect of S1P on IL-1β is also verified by western-blot in protein level (Figure 4).

![Figure 4. Expression of MMP-3 and ADAMTS4 in NP cells treated with IL-1β in presence or absence of S1P was measured by real-time PCR and western-blot. S1P significantly inhibited the up-regulation of MMP-3 and ADAMTS4 induced by IL-1β. The relative gene expression to the control is shown as Mean ±SEM(A), calculated by the2−ΔΔCt method. * means P<0.05; The grayscale of western-blot was also shown(B).](image)

| Code | Age | IVD segment | diagnosis                  | Histological grading |
|------|-----|-------------|----------------------------|----------------------|
| 1    | 51  | L4/5        | LDH                        | 9                    |
| 2    | 67  | L4/5        | LDH                        | 10                   |
| 3    | 57  | L5/S1       | LDH & Spondylolisthesis    | 10                   |

*Table 1. The scores of Histological grading of the IVD tissues harvested from patients via surgery operation.*
4. Discussion

The results of this study demonstrate for the first time that S1PR1/2/3 are expressed in the human nucleus pulposus, and it is down-regulated with the intervertebral disc degeneration. The activation of the receptor (stimulated by the ligand, S1P) inhibits the up-regulation of MMMP-3 and ADAMTS4 induced by IL-1β in NP cells. Although whether there is S1P in IVD tissue is unknown, the presence in synovial fluid, blood plasma and cerebrospinal fluid has been confirmed[16, 17]. With the development of

| Code | Age | IVD segment | diagnosis | Histological grading |
|------|-----|-------------|-----------|----------------------|
| 4    | 24  | L5/S1       | LDH       | 7                    |
| 5    | 61  | L5/S1       | LDH & Spondylolisthesis | 10 |
| 6    | 61  | L4/5        | LDH       | 10                   |
| 7    | 66  | L4/5        | LDH & Spondylolisthesis | 12 |
| 8    | 49  | L4/5        | LDH       | 8                    |
| 9    | 67  | L4/5        | LDH       | 10                   |
| 10   | 60  | L5/S1       | LDH & Spondylolisthesis | 10 |
| 11   | 44  | L4/5        | LDH       | 8                    |
| 12   | 46  | L5/S1       | LDH       | 7                    |
| 13   | 49  | L4/5        | LDH       | 8                    |
| 14   | 65  | L5/S1       | LDH & Spondylolisthesis | 9  |
| 15   | 68  | L5/S1       | LDH & Spondylolisthesis | 6  |
| 16   | 40  | L4/5        | LDH       | 7                    |
| 17   | 42  | L5/S1       | LDH       | 6                    |
| 18   | 20  | L2/3        | vertebral fracture | 2  |
| 19   | 47  | L4/5        | LDH       | 7                    |
| 20   | 40  | L5/S1       | LDH       | 7                    |
| 21   | 39  | L5/S1       | LDH       | 9                    |
| 22   | 47  | L4/5        | LDH       | 9                    |
| 23   | 21  | L2/3        | vertebral fracture | 2  |
| 24   | 57  | L5/S1       | LDH       | 10                   |
| 25   | 51  | L5/S1       | LDH       | 9                    |
| 26   | 47  | L5/S1       | LDH       | 9                    |
| 27   | 41  | L5/S1       | LDH       | 7                    |
| 28   | 18  | L2/3        | vertebral fracture | 2  |
| 29   | 34  | L4/5        | LDH       | 8                    |
| 30   | 31  | L4/5        | LDH       | 7                    |
| 31   | 64  | L4/5        | LDH       | 10                   |
| 32   | 39  | L4/5        | LDH       | 7                    |
| 33   | 67  | L4/5        | LDH       | 12                   |
| 34   | 41  | L4/5        | LDH       | 6                    |
| 35   | 61  | L4/5        | LDH       | 11                   |
| 36   | 57  | L4/5        | LDH & Spondylolisthesis | 10 |
| 37   | 60  | L4/5        | LDH       | 10                   |
| 38   | 19  | L1/2        | vertebral fracture | 3  |
| 39   | 20  | L2/3        | vertebral fracture | 2  |

Abbreviation: LDH, lumbar disc herniation

Table 2. Primer Sequences for real-time PCR.

| gene        | Primer Sequences                          |
|-------------|-------------------------------------------|
| S1PR1       | Forward TACGCGCGCAGAACAGGAACAG            |
|             | Reverse ATACGCGCACCAGGCAGATGAG            |
| S1PR2       | Forward CATCGTGCAGCGCTTTTA                |
|             | Reverse GGCAAGCCACCAAGGAGGA               |
| S1PR3       | Forward CTGCCTGCACACCTCCTCGACTG           |
|             | Reverse GGCCCACCCGAGATCCTC               |
| MMP-3       | Forward CTCGGAGAGTGGCTGCCTAAT             |
|             | Reverse GACCGAGTGGCTGCTGTGA               |
| ADAMTS4     | Forward TTTGTGGAGACACTGGTGGGT             |
|             | Reverse ACCACAAAGCTGGACAGGATT             |
| GADPH       | Forward TGGTATGCGGAAAGGACTCA              |
|             | Reverse CCAGTGGAGCCAGGGAGATG              |
IVD degeneration, blood vessel infiltrated the nucleus pulposus tissue[18], the plasma component may affect on the metabolism of the NP cells. What’s more, immune cells, such as macrophages, neutrophils and T cells, can be recruited to degenerated IVD[19], thus as an important lipid mediator,SIP probably participate in the inflammatory response.

NP cells showed a different pattern of gene expression with degeneration, [20, 21] and this variations may lead to distinct cells function, and interact with the degeneration. Here we compared the expression of S1PR1/2/3 in NP cells with different level of degeneration, and found that the expression of S1PR1/2/3 all decrease with the development of degeneration. The change of S1PR2 is in agreement with previous study, [13], while S1PR1 and S1PR3 is not. The disagreement maybe due to the difference between NP cells and chondrocytes, or the different experimental method. This result may be helpful to assess the risk of recurrence after operation.

In addition to the change of the expression of S1PRs, we confirmed the anti-inflammatory effect of SIP. This is also in line with previous studies in chondrocytes. [13, 14]. Together with the decrease of S1PRs, this result may explain the accumulation of matrix degrading enzymes, such as MMP-3 and ADAMTS4 in IVD degeneration.

While, other effects of SIP in chondrocyte have also been reported [11, 22]. Notably, Masuko’s study drew the conclusion Sphingosine-1-phosphate attenuates proteoglycan aggrecan expression via production of prostaglandin E2 from human articular chondrocytes, thus SIP may promotes cartilage degradation. What’s more, SIP is also involved in spinal nociceptive processing[17]. Taken account of these, the down-regulation of S1PRs may be advantageous for the nucleus pulposus cells to adapt to the deterioration of internal environment caused by degeneration. While, further studies are needed to fully illuminate the role of SIP and its receptor in IVD degeneration.

The biological action of SIP is largely ascribed to specific S1PRs that may evoke distinct biological responses. The key receptor of the anti-inflammatory effect in NP cells has not been studied. Even this in chondrocytes has been in controversies. Moon’s study shows that SIP1R is involved in the chondrocyte anti-inflammatory effect of SIP [14]; while Stradner’s study drew the Conclusion that SIP reduces the induction of catabolic genes in the presence of IL-1β via S1PR2. The latter conducted not only pharmaceutics Experiment but also siRNA of S1PRS, which is more dependable.

In the current study, we investigated the role of SIP and its receptors in intervertebral degeneration. We drew the Conclusion that human nucleus pulposus cells mainly express S1PR1/2/3, and the expression of decreased when severe degeneration appears; SIP can inhibit the inflammation response induced by IL-1β via the receptors, while further researcher are needed to make clear the key receptor in the effect and the signaling pathway related. These results add new aspects to our understanding of intervertebral degeneration and may have therapeutic implications in light of the novel class of SIP agonist drugs currently being developed.

5. Conclusions

The results show that an the expression of S1PRs in degenerative discs is down-regulated as degeneration, and SIP can inhibit the inflammation response induced by IL-1β in NP cells, implicating that S1P/S1PR may contribute to IVD degeneration.

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