LABORATORY INVESTIGATION

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Role of Matrix Metalloproteinases in Degenerative Lumbar Disc; Molecular and Immunohistochemical Study

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Objective : Little is known about the comprehensive molecular and biological mechanism on the development of the degeneration of the intervertebral disc. Many kinds of matrix metalloproteinase(MMP) initiate the degradation of the extracellular matrix including several kinds of collagens and proteoglycans. We compared molecular and immunohistochemical features of degenerated intervertebral disc and normal counterparts in order to investigate the role of MMP-1, 2, 3, 9.

Methods : We have evaluated MMP-1, 2, 3, 9 expression in 30 surgically resected lumbar disc from degenerative disc disease patients and 5 normal control cases. RT-PCR(reverse transcriptase-polymerase chain reaction) and immunohistochemistry were performed.

Results : By RT-PCR, normal tissue samples showed merely scant expression of MMP-1, 2, 3, 9 mRNA, but degenerated disc samples revealed more pronounced expression. mRNA amplifications were detected in 60%, 63.3%, 70%, 53.3% cases. By immunohistochemistry, normal tissue samples showed minimal protein expression of MMP-1, 2, 3, 9, but degenerated disc samples revealed more pronounced expression. Protein expressions were detected in 73.3%, 63.3%, 76.7%, 63.3% cases. Both the mRNA amplification and protein overexpression rates were significantly higher in degenerated disc than in the normal tissue. Concordance between both the mRNA amplification and protein expressions of MMP-1, 3, 9 were not observed, but there is well correlation in MMP-2 expression.

Conclusion : We concluded that the over-expressions of the MMP-1, 2, 3, 9 may contribute to the development of degeneration of the intervertebral disc.

KEY WORDS : Matrix metalloproteinase(MMP) · Intervertebral disc · RT-PCR · Immunohistochemistry.

Introduction

Degeneration of the intervertebral disc may be due to pathologic denaturation of protein components in the intervertebral disc[12,18]. Considering the denaturation mechanism of rheumatic arthritis and osteoarthritis, it is speculated that the severe destruction of protein components mainly cause disc degeneration and it is thought that various types of enzymes may play a role in substrate destruction[19].

Proteolytic enzymes are essential for life events such as maintaining normal growth, wound healing, bone remodeling, and embryonic implantation, but also they play an important role in pathologic inflammation and tumor growth[1]. Proteolytic enzymes are classified into: serine proteinase, cysteine proteinase, and matrix metalloproteinase(MMP). Among them, MMP is the most important enzyme in destroying collagen, which takes up the largest portion of protein substrate[1,6,11,13].

MMPs are a zinc-dependent proteinase, most of which are released as an inactive precursor to be made active through further degradation[13]. So far, about 20 different types of MMP are revealed, and the main members are collagenase (MMP-1), gelatinase (MMP-2, 9), stromelysin (MMP-3, 10, 11)[11,13].

Recently, some authors have suggested that excessive degradation of extra-cellular substrates by MMPs might be one of the mechanism of disc degeneration[3,10,14,15]. But, these areas are still remain as a new world, and the investigations of MMPs in the disc degeneration are just beginning.

The authors performed molecular and immunohistochemical study to demonstrate the role of MMP-1, 2, 3, 9 in the degenerated intervertebral disc by comparing with normal counterpart.

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Materials and Methods

As subjects of this study, 30 cases of degenerative intervertebral disc tissue obtained from the patients underwent lumbar disc surgery, and 5 cases of normal disc tissue obtained from the autopsy of children.

Fresh tissues were used for molecular biologic experiments. For immunohistochemistry, paraffin-embedded tissue were used after the fixation in 10% neutral formaldehyde and processing the common steps of pathologic diagnosis. For paraffin block, the tissue was stained with hematoxylin and eosin(H&E). A pathologist made microscopic observation and analysis on the tissue.

RT-PCR (reverse transcriptase-polymerase chain reaction)

The fresh tissues were obtained from surgery or autopsy, soaked in liquid nitrogen, and frozen at -80°C for extraction RNA by TRIZOL (monophasic phenol + guanidine isothiocyanate) method. The frozen tissue was washed in PBS (phosphate bufered saline) solution and 50~100mg of 0.5cm^3 fragment was destroyed in liquid nitrogen by Polytron homogenizer (Brinkmann, Westbury, NY, USA), mixed with 1ml TRIZOL reagent (GIBCO BRL, Grand Island, NY), and was kept in room temperature for 5 minutes.

0.2㎖ chloroform was added and the mixture was shaken for 15 seconds and kept in room temperature for 10 minutes. It was centrifuged (12,000g) at 4°C for 15 minutes. Then, the water-soluble portion (upper part) was carefully moved to new test tube and 0.5㎖ isopropanol was added, which was kept in room temperature for 10 minutes following vortexing. It was centrifuged (12,000g) for RNA to be precipitated. After removing the upper part, it was mixed with 1ml of 75% ethanol. It was re-centrifuged to wash RNA pellet. RNA pellet was dried in room temperature and dissolved in distilled water treated with diethyl pyrocarbonate, and kept at -80°C following survey its concentration by spectrophotometer (Hitachi Co, Tokyo, Japan) in 260nm.

After poly-dT primer and annealing of total RNA, first strand cDNA was made using AMV reverse transcriptase. PCR was conducted with this cDNA using an appropriate primer for MMP-1, 2, 3, 9 mRNA. The primer was custom-made by GIBCO (USA) and the base arrangements are shown in Table 1. PCR was performed using thermal cycler (Perkin Elmer Cetus 9700, USA) and by methods suggested by the manufacturer. PCR solution consisted of 1㎕ primer-5' and primer-3' (2mM), 1㎕ dNTP mixture (2mM), 1㎕ 10X buffer (20mM $MgCl_2$, Takara), and 1㎕ Taq polymerase (Takara, 5U/㎕) that were added to 3㎕ template (cDNA solution), to which distilled water was also added to make a 20㎕ solution. The conditions of PCR include: pre-denaturation at 94°C for 5 minutes, denaturation at 94°C for 1 minute, combining reaction at 58°C for 1 minute, prolongation reaction at 72°C for 90 seconds, and the final post-prolongation reaction at 72°C for 10 minutes after 30 cycles. For β-actin the combining reaction was started at 59°C for 1 minute. The experiment was first conducted on β-actin and the quantity of cDNA template was controlled on the basis of its outcome. And then PCR was performed again to make sure the quantity of PCR product of β-actin is fixed. Next, PCR was performed using a controlled amount of cDNA template and each primer. Each PCR product was put on electrophoresis with 1.5% agarose gel and analyzed in band showing molecules of predicted size.

Immnunohistochemistry

The tissue after formaldehyde fixation and paraffin embedding was cut into 3 fragments with 5~6㎛ thickness. It is then washed with xylene to remove paraffin and immunohistochemical staining was performed on each fragment for MMP-1, 2, 3, 9. To maintain the antigenicity of protein, microwave oven method was used to treat it in boiling phosphate buffered saline, hydrogen peroxide was applied to inhibit any activation of intrinsic peroxidase. And diluted Zymed (USA) was applied to inhibit any non-specific binding. After that, the primary antibodies against MMP-1 (Chemicon Int Inc, Temecula, CA, USA), MMP-2 (Oncogene Science, USA), MMP-3 (Santa Cruz Biotechnology, Inc, Santa Cruz, CA), and MMP-9 (Oncogene Research Products, Cambridge, MA, USA), which were diluted at 1:100 ratio, were induced to show reactions at room temperature for 2 hours. The secondary biotinylated link antibody (LSAB kit, DAKO, USA) was induced to show reaction for 20 minutes and then washed in PBS. Another reaction was induced in a solution with Streptavidin (Zymed, USA) and peroxidase put together for 30 minutes. 3,3-diaminobenzidine tetrachloride brought coloration and Meyer;s hematoxylin was used for counterstain. Finally, it was washed in running water, dried in room temperature, and enclosed.

Table 1. Primer DNA sequences used in this RT-PCR study

MMP-1	sense	5'-CTGTTCAGGGACAGAATGTGCT-3'
	anti-sense	5'-TCGATATGCTTCACAGTTCTAGGG-3'
MMP-2	sense	5'-ATTGATGCGGTATACGAGGC-3'
	anti-sense	5'-GGCACCCTTGAAGAAGTAGC-3'
MMP-3	sense	5'-CTCACAGACCTGACTCGGTT-3'
	anti-sense	5'-CACGCCTGAAGGAAGAGATG-3'
MMP-9	sense	5'-TTCTACGGGCCACTACTGTGC-3'
	anti-sense	5'-CGCCCAGAGAAGAAGAAAAG-3'
β-actin	sense	5'-AGGCCAACCGCGAGAAGATGACC-3'
	anti-sense	5'-GAAGTCCAGGGCGACGTAGCAC-3'

Table 2. Raw Data Results of RT-PCR and Immunohistocemical Methods

A. patient group

	RT-PCR				IHC			
	MMP-1	MMP-2	MMP-3	MMP-9	MMP-1	MMP-2	MMP-3	MMP-9
1	+	+	+	+	+	+	−	+
2	−	+	+	−	+	+	+	+
3	−	−	+	+	−	−	−	+
4	+	−	−	+	+	+	+	−
5	+	+	+	−	−	−	+	+
6	+	+	−	−	+	+	+	+
7	−	+	+	−	+	+	+	−
8	+	−	+	+	+	−	−	+
9	−	+	+	−	−	+	+	+
10	+	−	+	−	+	+	+	−
11	−	+	−	+	+	−	+	+
12	+	−	+	+	+	−	+	+
13	+	+	+	−	+	+	+	+
14	−	+	−	−	−	+	+	−
15	+	−	+	+	+	+	−	−
16	+	+	+	+	−	−	+	+
17	+	−	+	−	+	+	+	+
18	−	+	−	+	+	+	+	−
19	+	+	+	+	+	+	+	+
20	+	−	+	−	+	−	+	+
21	−	+	+	+	+	+	−	−
22	+	+	−	−	−	+	+	−
23	−	−	+	+	+	−	+	+
24	+	+	+	−	+	+	+	−
25	−	+	−	+	+	+	−	+
26	+	−	+	−	−	−	+	+
27	+	+	−	+	+	+	+	+
28	−	−	+	−	−	−	−	−
29	+	+	−	+	−	+	+	+
30	−	+	+	+	+	+	+	−

B. control group

	RT-PCR				IHC			
	MMP-1	MMP-2	MMP-3	MMP-9	MMP-1	MMP-2	MMP-3	MMP-9
1	−	−	−	−	−	+	−	−
2	−	+	−	−	−	−	+	−
3	−	−	−	−	+	−	−	−
4	−	−	−	−	−	−	−	−
5	−	−	−	−	−	−	−	+

(RT-PCR : reverse transcriptase polymerase chain reaction, IHC : immunohistochemistry)

Staining was graded from 0 to 3+ according to its intensity and specificity ; (0) for no staining, (1+) for weak staining, (2+) for partial staining only in cell membranes, and (3+) for more than 50% cellular staining. Any expressions more than (2+) were considered positive.

Statistical analysis

The frequency of mRNA and protein expressions of MMP-1, 2, 3, 9 from each cases was analyzed in percentages. All of data were processed and analyzed using SPSS version 10.0 for Windows, and P-value less than 0.05 was considered statistically significant.

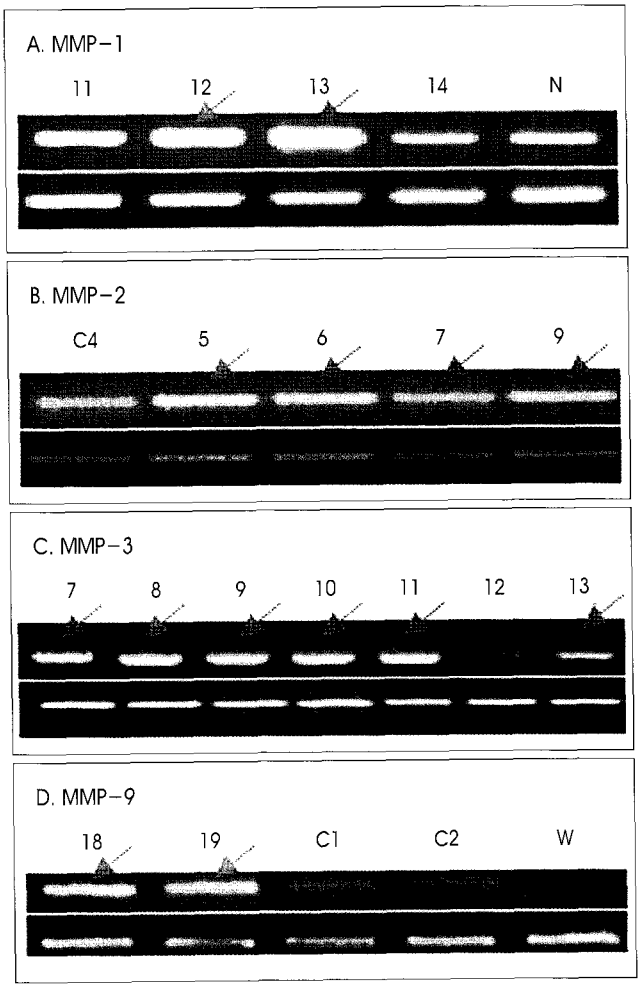

Fig. 1. Representative RT-PCR results of MMPs mRNA expression in intervertebral disc tissue. The numbers above the upper lanes indicate case number. Each lower lanes indicate β-actin. N : lymph node control tissue, W : water, C : cadaver. (A) Cases 12 and 13 show MMP-1 mRNA overexpression(arrows). (B) Cases 5, 6, 7, 9 and one cadaver case all show MMP-2 mRNA overexpression(arrows). (C) All cases except case 12 show MMP-3 mRNA overexpression(arrows). (D) Cases 18 and 19 show MMP-9 mRNA overexpression(arrows). C1 and C2 case show down regulation.

Results

Thirty cases of lumbar disc surgery have shown pathologically varying degrees of degeneration in fibrous tissues and local inflammatory reactions under microscope. Five autopsy cases that are the control group did not exhibit any degeneration nor signs of inflammation.

RT-PCR

The mRNA expressions of MMPs through reverse transcriptions were found in almost all patient and control group. The strength of expression in normal tissues was so little it

was not almost never eye-witnessed, whereas the degenerative disc tissues showed a strong band (Fig. 1). Given the strength of normal lymphoid tissue or control group as 1, the mean strength of MMP-1 mRNA expression in 5 cases of normal control group was 1.22±0.34, that of MMP-2 mRNA expression 1.18±0.27, that of MMP-3 mRNA expression 1.30 ±0.36, and that of MMP-9 mRNA expression was 1.14± 0.23. The mean value added to doubled standard deviation was used as positive standard value.

The measures for excessive expression of MMP-1, 2, 3, 9 mRNA were 1.90, 1.72, 2.02, and 1.50, respectively. According to these measures, MMP-1, 2, 3, 9 mRNA amplifications were found in 18(60%), 19(63.3%), 21(70%), 16(53.3%) of the cases in patient group. These expressions showed a statistical significance compared to the normal control group (p=0.001) (Table 2).

Immunohistochemistry

In patient group, over-expressions of MMP-1, 2, 3, 9 protein were found in 22(73.3%), 19(63.3%), 23(76.7%), and 19 (63.3%) of the cases, respectively (Fig. 2). Whereas positive finding was observed in only one case of the normal control group, which showed a statistical significance (p=0.001) (Table 2).

Correlation between RT-PCR and immunohistochemistry

The relationship between RT-PCR and immunohistochemistry study could be categorized by 4 different representations; simultaneous positive expression, simultaneous negative expression, positive RT-PCR alone, and positive immunohistochemistry alone.

The expression of MMP-2 consists of 15 simultaneous positive expressions(50.0%), 7 simultaneous negative expressions(23.3%), 4 positive RT-PCR alone(13.3%), and 4 positive immunohistochemistry alone(13.3%), and there was a statistically significant correlation (p=0.037). However, in case of MMP-1(14(46.7%), 4(13.3%), 4(13.3%), 8(26.7%), retrospectively), MMP-3(15(50.0%), 1(3.3%), 6(20%), 8(26.7%)) and MMP-9(11(36.7%), 6(20%), 5 (16.7%), 8(26.7%)), there was no statistical significance (Table 3). However, protein expressions were found among mRNA expressions of MMP-1, 2, 3, and 9 with rates 77.7%, 78.9%, 71.4%, and 68.8%, respectively.

Table 3. Correlation between RT-PCR & Immunohistochemical method

MMP-1	simultaneous positive expressions	14
	simultaneous negative expressions	4
	positive RT-PCR alone	4
	positive IHC alone	8
MMP-2	simultaneous positive expressions	15
	simultaneous negative expressions	7
	positive RT-PCR alone	4
	positive IHC alone	4
MMP-3	simultaneous positive expressions	15
	simultaneous negative expressions	1
	positive RT-PCR alone	6
	positive IHC alone	8
MMP-9	simultaneous positive expressions	11
	simultaneous negative expressions	6
	positive RT-PCR alone	5
	positive IHC alone	8

(RT-PCR : reverse transcriptase polymerase chain reaction, IHC : immunohistochemistry

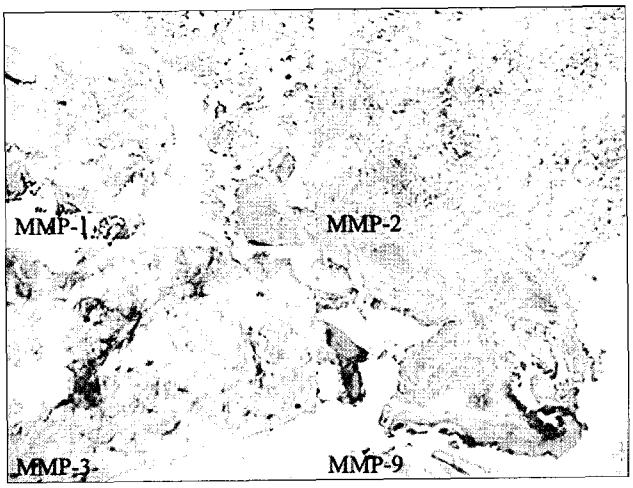

Fig. 2. Representative immunohistochemical stains for MMP-1, 2, 3 and 9 proteins in intervertebral disc tissue.

Discussion

Recently, the alterations in substrate components due to degenerated intervertebral disc have been revealed and there are reports suggesting important roles of substrate destroying proteinase expressions and of increased enzymatic activations in the degenerated disc[3-5,10,14,15].

The components of substrates in intervertebral disc are mainly collagen and proteoglycan. 60% of weight in fibrous ring is composed of collagen, 80% of which is Type 1 collagen. 20% of nucleus pulposus is collagen, most of which is Type 2. Although it is only a little amount, Type 3, 5, 6, 9, 11, and 12 collagens are also part of the structure. Proteoglycan is only a small part in annulus fibrosus; however, it takes up 50% of the nucleus. As aging proceeds and degeneration occurs, the ratio between collagen and proteoglycan changes. Large-sized proteoglycan mass begins to disappear in the nucleus but separated molecules of proteoglycan begin to increase in number, causing a loss of ability to absorb water, as aging proceeds[2,18].

Among these substrate destroying proteinases, the major one to be discussed is MMP, a metal-containing, extracell-

ular proteinase. MMPs are zinc-dependent proteinases consisting of MMP-1 (interstitial collagenase), MMP-2(gelatinase A), MMP-3 (stromelysin-1), MMP-7 (matrilysin), MMP-8 (neutrophil collagenase), MMP-9 (gelatinase B), MMP-10 (stromelysin-2), and MMP-11(stromelysin-3)[1,13].

The subjects of this study were MMP-1, a major enzyme responsible for breaking down Type 1 collagen, found most in annulus fibrosus and for breaking down Type 3 collagen, MMP-2, responsible for break down of Type 5, 9, and 10 collagen, MMP-9, and MMP-3, which is mainly responsible for destroying proteoglycan[11,13]. The expressions of these enzymes were identified not only through RT-PCR but also through immunohistochemistry, which made pos-sible the simultaneous expressions of mRNA.

As a result of this study, excessive expressions of MMP-1, 2, 3, and 9 mRNA were found in degenerated disc with 60%, 63.3%, 70%, and 53.3%, respectively. MMP-1, 2, 3, 9 mRNA expressions in patient group all show statistical significance when compared to normal control group.

Nishida et al.[15] have reported that there was no particular difference of MMP-3 mRNA expressions between the degenerated lumbar disc cases and normal autopsy cases. But Sobajima et al.[17] have demonstrated increased expressions of MMP-3 mRNA and Weiler et al.[20] have showed amplifications of MMP-2 and MMP-3 mRNA in situ hybridizations, which is similar to the result of this study.

As a result of immunohistochemical study, the excessive expression of MMP-1, 2, 3, and 9 protein were shown in 73.3%, 63.3%, 76.7%, and 63.3%, respectively. Only 1 case in normal control group showed a positive finding. A significant difference in expression was shown between the two groups. In addition, the rate of protein expression was somewhat higher than that of mRNA expression in RT-PCR. Matsui et al.[10] and Le Maitre et al.[9] have reported an increased expression of MMP-1 and 3 proteins according to degrees of protein denaturing. Nemoto et al.[14] have reported that of MMP-3, and, Weiler et al.[20] have reported MMP-1, 2, 3, 9, which match the result of this study.

Even though the simultaneous mRNA and protein expressions of MMP-1, 2, 3 and 9 showed in 77.7%, 78.9%, 71.4%, and 68.8%, respectively, no particular correlation was found, except MMP-2. This partial mismatch of results of two methods could suggest following limits of this study. First, RT-PCR method was not able to quantify the expression of mRNA. Second, 30 cases are too small a number for the study of this type. The cases used for control group are only a few. Third, the usual activation of MMP protein is inhibited by tissue inhibitor of metalloproteinase(TIMP). Some reports show that through the interaction between MMP and TIMP, the release and activation of MMP are regulated[6,11,16], however, the expression of TIMP was not considered in this study. Forth, the extent of degeneration of the intervertebral disc was not graded. Fifth, only the immunohistochemical assays and RT-PCR techniques were applied to evaluate MMP expressions.

Therefore, the further studies should be based on adequate number of clinical cases and of control group and should be done using real-time RT-PCR, enzymatic immuno assays, Western blotting, and enzyme activation techniques so that MMP expressions in degenerated intervertebral disc may be quantified. In addition, an approach to inhibitors of MMP expressions is required at the same time. It is thought that not only TIMP but regulatory mechanism of cytokines and other growth factors should also be revealed as in the studies of Kato et al.[7] and Konttinen et al.[8].

Conclusion

Increasing of expressions of MMPs could contribute to the degeneration of intervertebral disc. Further well-designed studies are required in order to reveal the actual roles and patho-physiologic mechanisms of MMP expression in the degenerative disc disease.

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Commentary

This paper describes the possible role of matrix metalloproteinase(MMP) in the development of degenerative lumbar disc by comparing matrix metalloproteinase level in herniated discs with immature normal disc tissue. The authors evaluated MMP-1, 2, 3, 9 expression in 30 surgically resected lumbar disc from degenerative disc disease patients and 5 normal control cases with RT-PCR (reverse transcriptase-polymerase chain reaction) and immunohistochemistry. They found that both the mRNA amplification and protein over-expression rates were significantly higher in degenerated disc than in the normal tissue. There was significant correlation in both mRNA amplification and protein expressions of MMP-2, while concordance between mRNA amplification and protein expressions of MMP-1, 3, 9 were not observed. The authors conclude that the over-expression from the excessive degradation of the matrix metalloproteinase (MMP)-1, 2, 3, and 9 may contribute to the degeneration of the intervertebral disc.

It is well known that MMPs prevalent in herniated discs than in other disc disorders and MMPs have certain role in the disc degeneration and resorption. However, there have been no reports comparing matrix metalloproteinase level in herniated discs with immature disc.

This study was well-designed, conducted, and analyzed without any technical error. However, as the authors pointed out, further studies with adequate number of sample and control group is needed using modern techniques of detection (immunocytochemistry, *in situ* hybridization, and zymography) to quantify MMP expressions in degenerated intervertebral disc. Tissue inhibitor of metalloproteinase(TIMP) should be assessed also to evaluate their role in regulating balance between MMP and TIMP in the following study.

The authors used 5 cases of normal disc tissue obtained from the autopsy of children as a control group without permission. It may give rise to ethical issue.

This study provided valuable insight to the biomolecular basis of the intervertebral disc degeneration mechanism and the present study has important implication, not only in designing pharmacological therapies to block degradative cascade, but also developing biological repair strategies in disc diseases.

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