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The imbalance between TIMP3 and matrix-degrading enzymes plays an important role in intervertebral disc degeneration

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ABSTRACT

It is well-known that one of the most important features of intervertebral disc degeneration (IDD) is the extracellular matrix (ECM) degradation. Collagen and aggrecan are major components of ECM; the degradation of ECM in intervertebral discs (IVDs) is closely related to the activities of collagenase and aggrecanase. TIMP-3 is the most efficient inhibitor of aggrecanase in IVD. However, only few studies focus on the potential relationship between TIMP-3 and IDD. In our study, we found TIMP-3 gene expression was decreased after stimulating with LPS in rat nucleus pulposus (NP) cells. Then we used a lentivirus vector to reconstruct rat NP cells which high expressed TIMP-3 gene (LV-TIMP3). The upregulation of MMPs and ADAMTSs induced by LPS was significantly inhibited in LV-TIMP3 cells. After overexpression of TIMP-3, the aggrecan breakdown caused by LPS was also reduced in both monolayer culture and three-dimension culture model. To further study the relation between TIMP-3 and IDD, we collected human NP tissue samples of different degenerative degrees. Real-time PCR and immunohistochemical staining showed that the expression of TIMP-3 was negatively correlated with the degree of intervertebral disc degeneration, while MMP-1 and ADAMTS-4 were markedly increased in degenerative IVD. Taken together, our results suggest that the imbalance between aggrecanase and TIMP-3 may play an important role in the pathogenesis of IDD and therefore be a potential therapeutic target for treating IDD.

1. Introduction

Low back pain (LBP) is one of the most common musculoskeletal complaints in the world. According to the Global Burden of Disease Study 2014, nearly 29% of the US population age 18 years and older self-reported having had low back pain during the past three months [1]. Intervertebral disc degeneration (IDD) is thought to be a significant contributor to the development of LBP [2,3]. Despite the complete pathogenesis of IDD has not been understood, previously studies have revealed that mechanism of IDD contains a complex biochemical cascade [4]. One of the most important features of IDD is the extracellular matrix (ECM) degradation in intervertebral discs (IVDs). Collagens and proteoglycans (PGs) are major components of ECM; the degradation of ECM in IVDs is closely related to the gene expression and activities of matrix-degrading enzymes which can cleave collagens and proteoglycans [5,6]. The primary matrix-degrading enzymes in IVDs are metalloproteinases (MMPs) and disintegrins and metalloproteinases with thrombospondin motifs (ADAMTSs).

Tissue inhibitors of metalloproteinases (TIMPs) are important regulators of MMPs and ADAMTSs. To date, 3 isoforms (TIMP-1, TIMP-2, TIMP-3) of TIMPs have been found in human IVDs [7]. TIMP-3 is a member of the TIMPs family, which is found in both degenerative and non-degenerative IVDs [8]. Among TIMPs family, TIMP-3 is reported to be the most efficient inhibitor of ADAMTSs

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[9–11]. Prior researches showed that TIMP-1, TIMP-2, MMPs and ADAMTSs expression were all significantly increased in degenerative IVDs [7]. However, unlike other members of TIMPs family and matrix-degrading enzymes, TIMP-3 expression do not change [7,12] or even decrease in degenerative IVDs [8].

To our knowledge, there is no research focus on this unusual imbalanced expression between TIMP-3 and matrix-degrading enzymes in degenerative IVDs. Our research aims to verify this imbalance in degenerative IVDs and study the potential therapy effect of TIMP-3 in IDD.

2. Materials and methods

2.1. Reagents and animal ethics

LPS and dimethylmethylene blue (DMMB) were purchased from Sigma (St. Louis, USA). Animal experiment was approved by the Animal Experimental Ethical Committee of Shanghai Ninth People's Hospital (Approval number: 2013-47).

2.2. NP cell isolation and monolayer culture

6 Weeks old Sprague Dawley rats were euthanized by abdominally injecting a lethal dose of 10% chloral hydrate. Then nucleus pulposus (NP) were isolated from Sprague Dawley rats lumbar spines, after digestion with trypsin and collagenase, NP cells were cultured in complete media (high glucose DMEM with 10% FBS and 1% antibiotic) up to passage 2–3.

2.3. Lentivirus vector and transduction

Lentivirus vector (GV358) loading the coding sequences of hTIMP-3 (Genbank ID:NM_000362) or a scrambled control were purchased from Genechem (Shanghai, China). Vectors were propagated on HEK293 cells (American Type Culture Collection, Manassas, VA), purified, and titered for by end-point dilution and for particle concentration by optical absorbance. NP cells were transfected with TIMP-3 (LV-TIMP3) or scrambled control lentivirus vector (LV-CON) at 50 multiplicity of infection (MOI). All transfected cells were subsequently exposed to 3 μg/ml of puromycin (Sigma) for 4 weeks post-infection to remove untransfected cells.

2.4. Immunofluorescence

LV-TIMP3, LV-CON and NP cells were stimulated with 10 μg/ml LPS in a 24-well plate for 7 days. Then, the cells were fixed with 4% paraformaldehyde for 30 min, treated with 0.1% Triton X-100 for 10 min, and blocked with 2% bovine serum albumin (Sigma) for an hour. All cells were incubated with anti-aggrecan antibody (1:100, Abcam) over night at 4 °C and then exposed to the Alexa Fluor® 594-conjugated secondary antibodies (1:100 dilution; Life Technologies) for 60 min at room temperature. After that, all cells were counterstained with DAPI and phalloidin. A laser confocal microscopy (OLYMPUS) was used for observation and imaging. Integrated optical density (IOD) of each picture was measured using the Image-Pro Plus 6.0 software (Media Cybernetics). The immunofluorescence results were expressed as IOD/cell number per view.

2.5. RNA isolation and real-time PCR

For rat NP cell samples, LV-TIMP3 LV-CON and NP cells were stimulated with 10 μg/ml LPS for 24 h. After that, total RNA were isolated using TRIzol reagent (Invitrogen). For human tissue samples, total RNA were directly isolated using TRIzol reagent. Reverse transcription was carried out from total RNA using 1st Strand cDNA Synthesis Kit (TAKARA) for first-strand complementary DNA (cDNA) synthesis. The relative gene expression was determined by real-time PCR. Real-time PCR was performed using the SYBR Premix Ex Taq kit (TAKARA) with the ABI Prism 7500 Fast Real-Time PCR system (Applied Biosystems) according to manufacturer's instruction. Primers were designed and selected using BLAST. Gene expression was measured using the $2^{-\Delta\Delta Ct}$ method. β-Actin was used as the internal control. The primer sequences are summarized in Table 1.

2.6. Western blot

NP cells were washed twice with cold PBS, after which the total protein was extracted using the RIPA lysis buffer. The protein concentration was quantified using a BCA Protein Assay Kit (Thermo Scientific). The protein was separated by 12.5% SDS-PAGE and transferred onto polyvinylidene fluoride (PVDF) membranes (Millipore). The transfer membranes were blocked with 5% fat-free milk at room temperature for 1 h, and then incubated with primary antibodies against TIMP-3 (1:1000, Cell Signaling Technology) at 4 °C over night. After three washes, the membranes were incubated with appropriate secondary antibodies conjugated with IRDye 800 CW at room temperature for 1 h. Immunoreactive bands were detected using Odyssey infrared imaging system (LI-COR). The β-actin antibody (1:2000, Cell Signaling Technology) was used as a control.

2.7. 3D culture and dimethylmethylene blue assay

Alginate bead 3D culture was performed according to previous report [13]. Briefly, LV-TIMP3 and NP cells were resuspended in 1.2% alginate respectively, and then slowly dropped in a 3.5% $CaCl_2$ solution through a 22-gage needle to form alginate beads. Beads were cultured in a 24-well plate with DMEM medium for 12 days with or without the presence of 10 μg/ml LPS. Culture media was replaced

Table 1
Sequences of the primers used in real-time PCR.

Gene	Primer sequences (5′–3′)	
rMMP-3	Forward	TTTGGCCGTCTCTTCCATCC
	Reverse	GCATCGATCTTCTGGACGGT
rMMP-13	Forward	ACCATCCTGTGACTCTTGCG
	Reverse	TTCACCCACATCAGGCACTC
rADAMTS-4	Forward	ACCGATTACCAGCCTTTGGG
	Reverse	CCGACTCCGGATCTCCATTG
rADAMTS-5	Forward	CCGAACGAGTTTACGGGGAT
	Reverse	TGTGCGTCGCCTAGAACTAC
rTIMP-3	Forward	ACAGACGCCAGAGTCTCCTA
	Reverse	ACCTCAAGTCTGTCCGGGTA
rTIMP-1	Forward	GCTTTCTGCAACTCGGACCT
	Reverse	AACCGGAAACCTGTGGCATT
rβ-actin	Forward	AACCTTCTTGCAGCTCCTCCG
	Reverse	CCATACCCACCATCACACCCT
hMMP-3	Forward	GGTTCCGCCTGTCTCAAGAT
	Reverse	AGGGATTTGCGCCAAAAGTG
hMMP-13	Forward	CATGAGTTCGGCCACTCCTT
	Reverse	CCTCGGAGACTGGTAATGGC
hADAMTS-4	Forward	CACATTCTTGTCCGGCAGCA
	Reverse	CCCCTCCCCACTGAGTCTTA
hADAMTS-5	Forward	ACTTTTGCAAGTGGCAGCAC
	Reverse	AAGTGCATTTGGACCAGGGC
hTIMP-3	Forward	ACAGACGCCAGAGTCTCCTA
	Reverse	ACCTCAAGTCTGTCCGGGTA
hTIMP-1	Forward	GCTTTCTGCAACTCGGACCT
	Reverse	AACCGGAAACCTGTGGCATT
hβ-actin	Forward	CCAACCGCGAGAAGATGA
	Reverse	CCAGAGGCGTACAGGGATAG
rMMP-1	Forward	AGCTCATACAGTTTCCCCGT
	Reverse	GCCTCAGCTTTTCAGCCATC
hMMP-1	Forward	ATGCACAGCTTTCCTCCACTG
	Reverse	ACTGGGCCACTATTTCTCCG

r: rat; h: human.