Overexpression of TIMP3 inhibits discogenic pain by suppressing angiogenesis and the expression of substance P in nucleus pulposus

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Abstract. Approximately 50% of the cases of low back pain (LBP) are attributed to discogenic origin. The causes of discogenic pain are complicated and consist of a complex biochemical cascade. Neovascularization of intervertebral discs (IVDs) is believed to be associated with discogenic pain. The anti-angiogenesis ability of tissue inhibitor of metalloproteinase-3 (TIMP3) has been reported in many tumors, yet whether TIMP3 is associated with neovascularization of IVDs remains unknown. In the present study, both in vitro and in vivo models were used to investigate the association between discogenic pain and TIMP3 expression in nucleus pulposus (NP). PCR results demonstrated that inflammation induced downregulation of TIMP3 expression in NP cells. By using an adenovirus system to upregulate TIMP3 expression, the effect of TIMP3 on angiogenesis was measured by endothelial cell migration and tube formation assays. The results demonstrated that overexpression of TIMP3 suppressed angiogenesis in NP without the regulation of vascular endothelial growth factor (VEGF) expression. TNF-α converting enzyme (TACE) expression was downregulated by TIMP3, thus inhibiting the TACE-induced activation of TNF-α in NP cells. Immunohistochemical staining of IVDs also confirmed that TIMP3 inhibited the expression of substance P in NP. Taken together, the present results indicated the expression of TIMP3 in NP may have a key role in the development of discogenic pain.

Introduction

Low back pain (LBP) is one of the most common complaints worldwide (1). Approximately 70 to 85% of the Western population will develop LBP at least once during their lifetime (2). It has been reported that 40 to 50% of cases of LBP are attributed to discogenic origin (3,4). The mechanisms of discogenic pain are complicated, consisting of a complex biochemical cascade in which proinflammatory cytokines such as interleukin (IL)-1β and tissue necrosis factor (TNF)-α play an important role (5). Cytokines induce discogenic pain by promoting the expression of pain mediators and inducing inflammatory cascades (6). The upregulation of TNF-α in intervertebral discs (IVDs) may not only contribute to catabolic process, but also be related with discogenic pain (7,8), especially following neural and vascular ingrowth (9,10). Tissue inhibitor of metalloproteinase-3 (TIMP3) was reported to be a suppressor of TNF-α which induces inflammation in various tissues and organs (11,12). However, further studies are needed to clarify the association between TIMP3 and discogenic pain.

Healthy IVDs are aneural and avascular, while neural and vascular ingrowth has been frequently found in degenerated IVDs (9,10), and the neovascularization of IVDs is believed to be associated with discogenic pain (13). Nucleus pulposus (NP) cells were found to induce endothelial cell (EC) invasion by expressing vascular endothelial growth factor (VEGF), especially under inflammatory condition (13). ECs further express nerve growth factor (NGF) that accompany ingrowing nerves (13) and the release of pain mediators (10). VEGF expression in NP cells could be induced by degeneration or inflammation (13,14). TIMP3 is an angiogenesis inhibitor, and the anti-angiogenesis ability of TIMP3 has been demonstrated in many types of tumor tissues (15-17). However, whether TIMP3 is associated with the neovascularization of IVDs remains unknown.

Substance P (SP) is a sensory marker related to pain. SP-positive nerve fibers were demonstrated to be related to discogenic pain (18). SP expressed in small nociceptive dorsal root ganglion (DRG) neurons is believed to be the sensory transmitter of nociceptive information (19). Numerous studies have demonstrated an association between the expression of SP in ingrowth nerves and the extent of disc degeneration (13). SP expression is important for pain transmission from the IVD...

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and occurrence of discogenic pain. Considering the potential anti-inflammatory and anti-angiogenesis abilities of TIMP3, the relationship between TIMP3 expression and SP release should be further investigated.

Previous studies indicated that TIMP3 expression decreases with age and IVD degeneration (20-23). In addition, previous research has confirmed that this unbalanced expression of TIMP3 may lead to intervertebral disc degeneration (IDD) (24). Despite the finding that the loss of TIMP3 expression leads to IDD, whether TIMP3 expression is related with discogenic pain still lacks direct evidence. In the present study, we explored the relationship between TIMP3 expression and discogenic pain using in vitro and in vivo models.

Materials and methods

Reagents. The antibodies and reagents used in the present study are as follows: Rat Vascular Endothelial Growth Factor-164 (rVEGF164; cat. no. 5874, Cell Signaling Technology, Inc., Danvers, MA, USA), fetal bovine serum (FBS; Gibco, Thermo Fisher Scientific, Inc., Waltham, MA, USA) and lipopolysaccharides (LPS; L5543, Sigma-Aldrich, Merck KGaA, Darmstadt, Germany). Primary antibodies against TIMP3 (ab155749, Abcam, Cambridge, UK), collagen-2 (ab34712, Abcam), GAPDH (ab181603, Abcam), Substance P (ab14184, Abcam), aggrecan (ab36861, Abcam) and CD34 (50589-r013, Sinai Biological, Beijing, China) were used in the study. Secondary antibodies for western blotting (ab205718, Abcam) and immunohistochemical analysis (ab205719, Abcam) were also used in the study.

Cell culture. According to previously reported methods, primary nucleus pulposus (NP) cells and rat aorta endothelial cells (RAECs) were isolated from Sprague-Dawley (SD) rats (24,25). A total of 34 SD rats were used for the present study. The SD rats (6 weeks of age) were euthanized using an abdominal injection of pentobarbital sodium (150 mg/kg). Briefly, NP cells were isolated from lumbar spines and cultured in complete media (high-glucose DMEM with 10% FBS and 1% antibiotic), RAECs were isolated from aortas of SD rats and cultured with DMEM/F12 media (with 10% FBS and 1% antibiotic). RAECs were also used in the study.

Gene expression assay. The total RNA of the various NP cells was isolated using TRIzol reagent (Invitrogen, Thermo Fisher Scientific, Inc.) following the manufacturer’s instructions. Reverse transcription was carried out using the 1st Strand cDNA Synthesis Kit (Takara Biotechnology co., ltd., Dalian, China). Gene expression was measured using the SYBR Premix ex Taq kit (Takara) followed by real-time PCR. The primers were designed and synthesized by GenePharma (Shanghai, China). Gene expression was measured using the 2−ΔΔcq method (27). The primer sequences are summarized in Table I, and GAPDH was selected as a reference gene.

Western blot analysis. Aggrecan, Coll-2 and TIMP3 protein expression levels were assessed by western blot analysis in NP cells treated with lipopolysaccharide (1 µg/ml) for 2 or 5 days. Briefly, for cell samples, cells were lysed with RIPA buffer and total proteins were isolated. For tissue samples, a tissue homogenate was made and total proteins were isolated. A total of 20 µg of each protein sample was separated by 10% SDS-PAGE, then transferred onto PVDF membranes (EMD Millipore, Billerica, MA, USA). The membranes were blocked with 5% fat-free milk at room temperature for 1 h, incubated with primary antibodies (1:1,000) at 4°C overnight, followed by secondary antibodies (1:5,000) at room temperature for 1 h, and finally incubated with an ECL solution (Millipore, Billerica, MA, USA). The blotted proteins were visualized using an ImageQuant LAS 4000 mini (GE Healthcare, Piscataway, NJ, USA) and analyzed using the ImageQuant TL software (GE Healthcare, Piscataway, NJ, USA). The primary antibodies against TIMP3 (nm_012886) or a scramble control were used at 50 multiplicity of infection (MOI) according to standard procedure. The transfection efficacy was verified by western blotting 3 days after transfection.

Table I. Sequences of the primers used in PCR.

| Gene     | Primer      | Sequence (5’-3’)       |
|----------|-------------|------------------------|
| TACE     | Forward     | CCGAACGAGTGTACGCGATT   |
|          | Reverse     | TGTGGCTGCTTAGAAGACTAC  |
| TIMP3    | Forward     | ACAGACGCGCAAGTCTCCTCA  |
|          | Reverse     | ACCCTAGTCTGTCGCGGTA    |
| Substance P | Forward | TTCTACTTCTATGTTGTCGCCG |
|          | Reverse     | GTCTAGGAGGGTCACCACATT  |
| TNF-α    | Forward     | TCGTAGAAAAACACCAAGCA   |
|          | Reverse     | TCGTAGAAAACACCAAGCA    |
| GAPDH    | Forward     | AACCTTTCTTGACGCTCTCCG  |
|          | Reverse     | CCATACCCACCATCACCCT    |

TACE, TNF-α converting enzyme; TIMP3, tissue inhibitor of metalloproteinase-3; TNF-α, tissue necrosis factor-α.
Immunoreactive bands were detected using the Odyssey infrared imaging system (LI-COR). Densitometrical analysis of the bands was completed using Image-Pro Plus 6.0 software supplied by Media Cybernetics, Inc. GAPDH was used as the internal reference.

**ELISA assessments.** Various NP cells were exposed to LPS (1 µg/ml) for 5 days. The TNF-α level in the culture medium was measured using commercially available enzyme-linked immunosorbent assay (ELISA) kits according to the manufacturer’s instructions (R&D Systems, Inc., Minneapolis, MN, USA).

**Animal experiments.** Twenty-four male SD rats (6-8 weeks weighing 200-250 g), were purchased from the Experimental Animal Center of Beijing Anzhen Hospital. Rats were housed under controlled conditions, including a 12-h light/dark cycle, 21±2°C and 60-70% humidity, and rats were also allowed free access to standard dry rat diet and tap water. The surgical procedure was performed as previously described (28).
Briefly, rats were anesthetized by an abdominal injection of pentobarbital sodium (40 mg/kg), and percutaneously punctured with a 21G needle in the coccygeal vertebra (puncture and TiMP3+puncture group). For the TiMP3+puncture group, rats were injected with adenovirus vector (1x10^9 pfu/level) immediately after puncture. At day 28 after puncture, rats were euthanized by intraperitoneal injection of an overdose of pentobarbital sodium (150 mg/kg) and nucleus pulposus tissues were isolated for western blot assay and histologic analysis.

**Histologic analysis.** Discs from rats were fixed and serial sagittal sections of discs (5-µm thick) were obtained to prepare slides. TIMP3, CD34 and substance P expression levels were determined by immunohistochemical (IHC) staining. All staining procedures were performed following standard histochemical protocols. We analyzed 3 sections from each disc samples, and for each section, we calculated 3 fields and took the average. The positive staining was calculated and analyzed using Image-Pro Plus 6.0 software supplied by Media Cybernetics, Inc.

**Statistical analysis.** All the experiments were repeated at least 3 times. The data are expressed as the mean ± SD. Statistical analysis was performed with a one-way analysis of variance (ANOVA), followed by Duncan's post hoc test using SPSS 22.0 software (IBM, Inc.). A P-value <0.05 was considered to indicate a statistically significant result.

**Results**

**LPS induces TIMP3, aggrecan and collagen-II downregulation in the NP cells.** To study the regulation of TIMP3 expression and the matrix degradation in the presence of inflammation, NP cells were treated with or without LPS for 2 or 5 days. The gene and protein expression of TIMP3, aggrecan and collagen-II were assessed by PCR and western blot analysis (Fig. 1A-G). Our results revealed that gene expression of TIMP3, aggrecan and collagen-II were downregulated from day 2 after exposure to LPS, while significant protein expression reduction was observed only at day 5 except for collagen-II. These results suggest that the downregulation of TIMP3 plays a role in the matrix degradation of NP cells.

**TIMP3 regulates TNF-α and pain mediator expression in NP cells.** Considering that the expression of TIMP3 was found to be downregulated after exposure to LPS, we aimed to increase TIMP3 expression in an inflammatory condition and explore the role of TIMP3 in inflammation. TIMP3 was overexpressed by transfection of an adenovirus vector in NP cells. TIMP3 overexpression was confirmed by western blot analysis.

**Figure 2.** TIMP3 inhibits TNF-α and substance P expression in NP cells. NP cells were transfected with an adenovirus vector loading TIMP3 or a control. The different NP cells were then treated with or without LPS for 2 days. After that, the gene expression of TACE and substance P was determined by PCR. TNF-α secretion was measured by ELISA. (A and B) Overexpression of TIMP3 was confirmed by western blot analysis. (C) Gene expression of TACE was downregulated by TIMP3. (D) Gene expression of TNF-α was not affected by TIMP3. (E) Gene expression of substance P was downregulated by TIMP3. (F) TNF-α secretion was reduced by TIMP3. The data are presented as the mean ± SD. *P<0.05. TIMP3, tissue inhibitor of metalloproteinase-3; TNF-α, tissue necrosis factor-α; NP, nucleus pulposus; TACE, TNF-α converting enzyme; LPS, lipopolysaccharides.
analysis (Fig. 2A and B). After exposure to LPS, the gene expression of TNF-α converting enzyme (TACE), TNF-α and substance P in different NP cells was measured by qPCR. The results showed that TIMP3 overexpression downregulated TACE and substance P expression which were induced by LPS (Fig. 2C and E). Although the gene expression of TNF-α was not affected upon overexpression of TIMP3 (Fig. 2D), the ELISA measurement indicated that the protein level of TNF-α in medium was decreased in the TIMP3+LPS group (Fig. 2F). Taken together, our results suggest that instead of
directly inhibiting TNF-α expression, TIMP3 was more likely to suppress the activation of TNF-α induced by TACE.

**TIMP3 inhibits tube formation and migration of RAECs.** According to ELISA measurement, NP cells secreted VEGF after stimulation with LPS (Fig. 3C). To further investigate the association between angiogenesis and TIMP3, RAECs were treated with different conditioned medium and VEGF or not. Tube formation was observed under a light microscope. As shown in Fig. 3, NP cell medium significantly suppressed tube formation of RAECs, while overexpression of TIMP3 further significantly inhibited tube formation. Treatment with VEGF promoted tube formation of RAECs, however, this promoting effect was blocking by overexpression of TIMP3 (Fig. 3). The results of the cell migration assay were similar to that of the tube formation assay (Fig. 4). TIMP3 significantly
inhibited the migration ability of the RAECs and eliminated the promoting effect of VEGF. According to the above results, TIMP3 inhibits angiogenesis without regulating VEGF expression, indicated that TIMP3 may inhibit angiogenesis by blocking the bonding of VEGF and VEGFR-2.

Overexpression of TIMP3 reduces pain mediator expression in IVD. Substance P is a key pain mediator in IVD, and CD34 is a marker of angiopoiesis. Considering the inhibitory effects of TIMP3 on substance P expression and angiopoiesis, these effects were confirmed in an in vitro model. The inhibitory effect of TIMP3 on discogenic pain was further investigated in an in vivo model by assaying substance P and CD34 expression. IDD rat model was established by puncture of IVD. After injection of an adenovirus vector loading TIMP3, TIMP3 expression was significantly upregulated at day 28 (Fig. 5). The puncture group exhibited more positive CD34 and Substance P staining, which indicated the neovascularization of IVDs after puncture. The positive staining rate of CD34 and Substance P was significantly reduced in the TIMP3+puncture group compared with that in the control group (Fig. 5). These results indicate that TIMP3 may suppress the angiogenesis and pain mediator expression in degenerative discs, thus inhibiting discogenic pain in IVD. On the other hand, the loss of TIMP3 expression may play a role in the development of discogenic pain.

Discussion

As a key matrix-degrading enzyme inhibitor, the imbalance of expression between TIMP3 and matrix-degrading enzymes has been reported to be responsible for the aggrecan breakdown in intervertebral discs (IVDs) (20-22,24). Lipopolysaccharides (LPS) markedly induce inflammation in nucleus pulposus (NP) cells (29). And LPS also induce the gene and protein overexpression of various matrix-degrading enzymes, thus leading to matrix degradation (20,30). In the present study, exposure to LPS induced the downregulation of collagen-II and aggrecan at both the gene and protein levels, and the TIMP3 expression was also inhibited by LPS, which was consistent with a previous study (24). In addition to direct inhibition of enzyme activity, TIMP-3 also provides a feedback gene downregulation of matrix-degrading enzymes (24).

The relationship between TNF-α and discogenic pain has been studied for years. There are two forms of TNF-α in humans, membrane-bound (mTNF-α) and a secreted form (sTNF-α). The transformation from mTNF-α to sTNF-α is processed by TACE. Increased levels of sTNF-α were reported to present in degenerate and herniated IVDs (31,32). TNF-α may trigger discogenic pain in several ways, including sensitization of neurons, induction of inflammation and upregulation of various receptors (33,34). These modifications promote the release of pain mediators, resulting in chronic, persistent pain (33,35). Although there is still controversy concerning the clinical effect, the use of anti-TNF-α has been proven effective in discogenic pain (35-38). TIMP3 was reported to be a suppressor of TNF-α-induced inflammation in various tissues and organs (11,12). Our study also showed that instead of directly inhibited TNF-α expression, TIMP3 suppressed TNF-α secretion by downregulating TACE expression. As a previous study reported, intradiscal administration of TNF-α inhibitor could alleviate discogenic pain for up to 8 weeks (39). Based on our results, overexpression of TIMP3 in NP cells may be able to relieve discogenic pain by the inhibition of TNF-α.

SP has been reported to be a sensory marker which is released by nerve fiber. However, in an inflammatory pain model, SP release was also increased (40). The interplay

![Figure 6. Schematic diagram of the mechanism of TIMP3 in discogenic pain. TIMP3 inhibits discogenic pain by suppressing angiogenesis and the expression of substance P. TIMP3, tissue inhibitor of metalloproteinase-3; TNF-α, tissue necrosis factor-α; TACE, TNF-α converting enzyme.](image-url)
between inflammatory cytokines and neurotrophins, which is produced by disc cells may explain the phenomenon. Neurotrophins such as NGF expression was found to be increased in painful and degenerate discs and are sensitive to TNF-α (13,35). Neurotrophins would further promote nerve ingrowth and the release of SP (13,14). Collectively, in light of the present results and the research mentioned above, we deduced that TIMP3 may suppress SP released by the inhibition of TNF-α expression. Innervation is limited in the normal disc, while abundance of nociceptive nerve endings was found in patients with chronic back pain (9,13). Neural ingrowth in IVDs was found to be accompanied by angiogenesis, and nerve growth factor (NGF) was expressed by vascular tissue, thus promoting neural ingrowth (13). Moreover, NGF was found to be expressed only in blood vessels in painful degenerative IVDs (13), which further confirms the close relationship between angiogenesis and discogenic pain. VEGF is an important growth factor of angiogenesis. Overexpression of VEGF was found in NP cells under inflammatory or degenerated situations, which was considered to be one of the key reasons causing angiogenesis (14). Co-culture model also showed that Fas ligand (FasL) and immune privilege were involved in the process of IVD angiogenesis, indicating a more complicated mechanism of neovascularization of IVDs (41,42). TIMP3 was reported as an effective angiogenesis inhibitor in various tumor tissues, which suppressed vascular ingrowth by interfering with the binding of VEGF and VEGFR-2 (15). TIMP3 also inhibits the inflammation which was proven as a trigger of VEGF release (11,12,14). Moreover, aggrecan from healthy IVDs inhibited neural and vascular ingrowth (43). Downregulation of TIMP3 may cause matrix degradation, thus breaking the defense effect provided by intact aggrecan leading to discogenic pain. Our study further confirmed the anti-angiogenesis effect of TIMP3 and interruption of the positive feedback of neural ingrowth in IVD. However, the tube formation results seemed a bit confusing. The nP group exhibited suppressed tube formation compared with the blank control. We speculated that this occurred due to the influence of notochord cells. The NP cells were collected from young rat IVDs which consist of a certain amount of notochord cells, and notochord cells were reported to be able to inhibit the vascular ingrowth in IVDs (44). We did not separate these notochord cells from NP cells in our study. The mixed state of NP and notochord cells was more in accord with the natural state of an organism.

Combined with the results above, we believe that TIMP3 may reduce the neovascularization of IVDs and suppress the inflammation-related SP release, and ultimately inhibit discogenic pain (Fig. 6). However, there are still some limitations of our study. First, the detailed mechanism of the anti-angiogenesis effect of TIMP3 was not thoroughly studied. The interaction between VEGF and VEGFR-2 was not demonstrated experimentally. Moreover, all of the conclusions in the present study were based on in vitro and in vivo experiments. More data from clinical samples are needed in the future.

In conclusion, our in vitro and in vivo studies indicate that overexpression of TIMP3 inhibits discogenic pain by suppressing angiogenesis and the expression of pain mediator in nucleus pulposus. TIMP-3 may play an important role in the pathogenesis of discogenic pain and therefore be a potential therapeutic target.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

MWH, JLP, WPG and GRZ conceived and designed the study. MWH, JLP and HYS performed the experiments. MWH, JLP and HYS wrote the paper.YL and MWH analyzed the data. GRZ, YL and WPG reviewed and edited the manuscript. All authors read and approved the manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Ethics approval and consent to participate

The primary cell procurement and animal experiments were approved by the Animal Experimental Ethics Committee of the Beijing Anzhen Hospital (approval no. 20170614).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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