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Expression profiles of MMP-1 and TIMP-1 in lumbar intervertebral disc degeneration

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ABSTRACT. Lumbar intervertebral disc degeneration (IDD) is a common clinical pathology and has become a focus for research in recent years. Matrix metalloproteinases (MMPs) are enzymes responsible for the degradation of almost all extracellular matrix proteins (ECM). The over-expression of MMPs or tissue inhibitors of metalloproteinases (TIMPs) may disrupt the dynamic balance of the ECM. Therefore, in the current study, the expression levels of MMP-1 and TIMP-1 in lumbar IDD patients were evaluated in an attempt to elucidate their role in IDD pathogenesis and progression. In total, 60 IDD patients were recruited as the experimental group, along with 20 cases of lumbar vertebral injury without disc degeneration as the control group. Preoperative venous blood samples were collected, and intervertebral disc tissues were collected from the lesion during surgery. Serum and tissue levels

of MMP-1 and TIMP-1 were quantified by enzyme-linked immunosorbent assay and immunohistochemical staining, respectively. Serum and tissue MMP-1 levels in IDD patients were significantly higher than those in the control group ($P < 0.05$). Additionally, sub-group analysis revealed that severe IDD patients had higher MMP-1 levels compared with mild or moderate IDD patients ($P < 0.05$). However, there were no significant differences in TIMP-1 levels in either the serum or tissues of IDD patients compared to patients in the control group ($P > 0.05$). These results demonstrate that MMP-1 expression is increased in IDD, with higher expression observed in more severe cases, whereas TIMP-1 expression was similarly expressed in both normal and degenerated discs.

Key words: Lumbar intervertebral disc degeneration; Matrix metalloproteinase-1; Tissue inhibitor of metalloproteinase-1

INTRODUCTION

Lumbar intervertebral disc herniation is the most common form of intervertebral disc degeneration (IDD), and is manifested by bulging of the central nucleus pulposus granulation of the fibrous ring, and tissue fibrosis (Li et al., 2010; Samartzis et al., 2012). Matrix metalloproteinases (MMPs) participate in both synthesis and degradation of the extracellular matrix (ECM), thereby regulating intervertebral disc dynamics (Ozkanli et al., 2015). Tissue inhibitors of metalloproteinases (TIMPs) are specific inhibitors of MMPs, and are known to be involved in IDD (Leung et al., 2011). Therefore, it is possible that the balance between MMPs and TIMPs is involved in the pathogenesis and progression of IDD. This study investigated the expression profile of MMP-1 and TIMP-1 in lumbar IDD patients as an attempt to elucidate the function of these two ECM regulating factors in IDD pathology.

MATERIAL AND METHODS

Patient information

In total, 60 lumber IDD patients (30 males and 30 females between 22 and 55 years old with an average age of 35.6 ± 1.8 years) were recruited for this study as the experimental group. All patients were diagnosed with IDD via magnetic resonance imaging (MRI) as categorized by the following three stages: stage I, mildly decreased intervertebral disc signals; stage II, moderately decreased intervertebral disc signals; and stage III, severely decreased intervertebral disc signals. Another 20 patients (10 males and 10 females, aged between 21 and 56 years old with an average of 34.7 ± 2.2 years) each with a lumbar spine injury but normal intervertebral disc height and signal as determined by MRI. There were no statistically significant differences regarding gender or age between the experimental and control groups. The experimental protocol was pre-approved by the ethical committee of our hospital and written informed consent was obtained from all patients and healthy volunteers.

Serum protein level assays

Venous blood samples drawn from patients were centrifuged to separate and isolate the

serum, which was then frozen until further use. Before the enzyme-linked immunosorbent assay (ELISA), all samples and reagents were pre-warmed to room temperature. ELISA kits for MMP-1 and TIMP-1 were purchased from Roche (Switzerland) and ELISAs were performed according to manufacturer instructions. Five replicates were added to the ELISA plates for each sample dilution, followed by washing, development, and termination. Optical density (OD) values were measured at 450 nm. The concentrations of the target proteins were determined by a reference curve that was plotted using kit-provided standards.

Immunohistochemical (IHC) staining

Nucleus pulposus tissue samples were collected from patients of two groups, and were cut into 0.5 cm x 0.5 cm x 0.5 cm cubes. After fixation in 10% formalin, dehydration, embedding in wax, de-waxing, and rehydration, antigen retrieval was performed using citric acid buffer. The slices were then processed using 3% hydrogen peroxidase to quench endogenous peroxidase activity. Non-specific binding sites were blocked by the addition of goat serum. Slices were incubated with a primary antibody and secondary antibodies conjugated with horseradish peroxidase for 1 hour and 10 min, respectively. Slices were then dehydrated and mounted with coverslips. Images were captured by an inverted light-field microscope (Olympus, Japan).

Analysis of IHC staining

Negative controls were incubated with PBS buffer instead of the primary antibody in parallel. After image capture, 5 image fields were randomly selected from one slide. Positive signals for MMP-1 or TIMP-1 were observed as dark brown/yellow brown coloring in the cytoplasm or at the cell membrane but not in the nucleus. The percentage of positive cells in one field was calculated and averaged among the five fields. The grade of staining was defined as: 1) negative (-), <10% cells stained positive; 2) weak positive (+), 11-25% cells stained positive; 3) positive (++), 26-50% cells stained positive; and 4) strong positive (+++), >50% cells with positive signals. The overall positive rate was defined as the number of cases with grade (2), (3) or (4) / total number of cases in one group.

Statistical analysis

The SPSS 17.0 software was used for statistical analyses of all collected data, of which numeration data were compared using chi-square tests while measurement data were analyzed via analyses of variance (ANOVA). Between-group comparisons were performed using an LSD test. All measurement data are reported as means ± standard deviation (SD). Statistical significance was defined as $P < 0.05$.

RESULTS

Serum levels of MMP-1 and TIMP-1

ELISA was used to quantify serum MMP-1 and TIMP-1 levels in IDD patients and control individuals. The results (Figure 1A) showed significantly elevated MMP-1 levels in the IDD patient group ($P = 0.015$, $P = 0.011$, $P = 0.001$). A further comparison among three sub-groups of IDD revealed that patients with stage III (severe) IDD had significantly higher MMP-1 serum levels

compared to patients with stage I (mild) or stage II (moderate) (P = 0.014, P = 0.008). However, there were no significant differences in the serum TIMP-1 levels between any of the three IDD stages or control group (P > 0.05, Figure 1B).

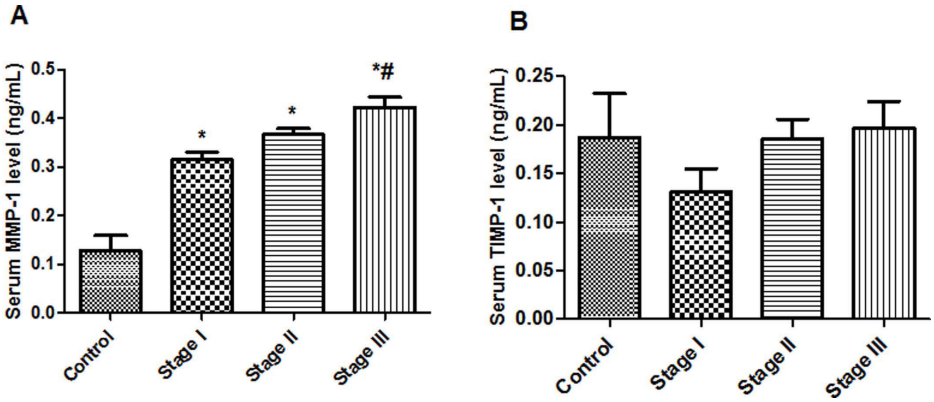

Figure 1. Serum levels of MMP-1 (**A**) and TIMP-1 (**B**). Serum levels were compared among control and all three stages of IDD. MMP-1 concentration was elevated in IDD patients, with the highest level observed in stage III (sever) IDD patients. No significant differences were observed in TIMP-1 expression. Significance was determined by one-way analysis of variance (ANOVA) followed by the *post hoc* LSD test. *P < 0.05 compared to controls; #P < 0.05 compared to stage I and stage II IDD patients.

MMP-1 and TIMP-1 protein expression in tissues

We further used IHC analyses to examine the expression profiles of MMP-1 and TIMP-1 proteins in the patient and control groups. As revealed by the IHC images, control tissues had irregular cell shapes, with blue round nuclei, pink cytoplasm, and evenly distributed ECM. Conversely, IDD patients had round-shaped nuclei, cytoplasmic vacuoles, increased ECM with disarranged fibers, and significantly elevated cell proliferation (Figure 2). Both MMP-1 and TIMP-1 were expressed in the membrane and cytoplasm and were observed as brown and yellow granules. Further analysis revealed that the percent positive rates of MMP-1 in all three IDD stages were significantly higher than that in the control group (Table 1, P < 0.05). Furthermore, stage III patients had the highest (95%) positive rates of MMP-1, which was significantly higher than those of stage I or stage II patients (P < 0.05). TIMP-1 positive cells were identified in the patient and control groups but no significant differences in the positive rates were observed (P > 0.05).

DISCUSSION

As a prominent pathological change associated with lumbar intervertebral disc herniation, IDD is clinically manifested by symptoms including low back pain and fatigue. The major component of the ECM in intervertebral discs is proteoglycan, which can interact with collagen to form a stable matrix to lubricate and decrease pressures in the intervertebral space (Wu et al., 2010; Mavrogonatou et al., 2014). The major types of collagen in the intervertebral discs are type I and type II. For example, in the fibrous ring, collagen fibers mainly consist of type I and type II, in

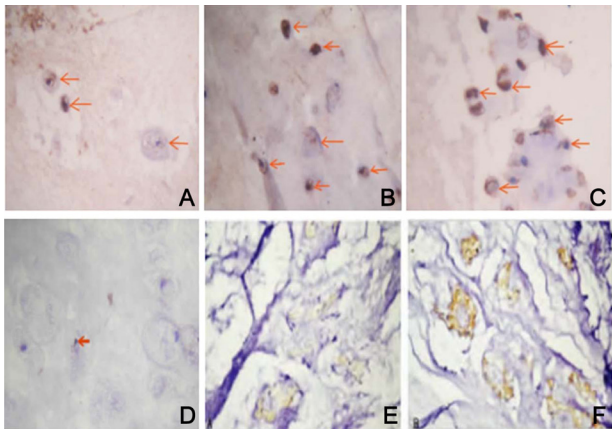

Figure 2. MMP-1 expression in intervertebral disc tissues. (**A**) and (**D**), weak positive (+); (**B**) and (**E**), positive (++); (**C**) and (**F**), strong positive (+++). Images are shown at 400X magnification.

Table 1. MMP-1 and TIMP-1 expression in patient and control tissues.

Group/Stage	N	MMP-1				TIMP-1			
		-	+/++	+++	Positive rate (%)	-	+/++	+++	Positive rate (%)
IDD patients	60								
Stage I	20	10	7	3	50*	14	5	1	30
Stage II	20	6	9	5	70*	13	6	1	35
Stage III	20	1	11	8	95*#	12	6	2	40
Control	20	18	2	0	10	10	10	0	20

*P < 0.05 compared to control; #P < 0.05 compared to stage I and stage II IDD patients.

addition to minor amounts of type III, V, VI, and IX. In the nucleus pulposus, collagens are mainly type II but also include type VI, IX, and XI (Yuan et al., 2011; Canbay et al., 2013).

Recent studies have revealed the important roles of cytokines and inflammatory factors in the pathogenesis of IDD (Gomis-Rüth, 2009). The synthesis and degradation of the ECM in intervertebral disc tissues are known to be regulated by MMPs and are closely related to IDD (Zawilla et al., 2014). The up-regulation of MMPs may lead to elevated ECM degradation, alternative collagen components, breakdown of the fibrous ring, and intervertebral tissue damage, finally leading to the herniation of the nucleus pulposus (Rastogi et al., 2013). Previous reports have attributed the major reason for over-degradation of the intervertebral disc matrix as the imbalance between MMP and TIMP (Carvalho et al., 2009; Kimura et al., 2010). MMP-1, as one kind of collagenase, is able to degrade collagens including type I, II, III, VII, and VIII (Pockert et al., 2009). As the inhibitors of MMPs, TIMPs can suppress the activity of MMPs by the formation of one-to-one complexes (Gruber et al., 2012; Zou et al., 2014).

In the current study, we categorized all recruited IDD patients into three stages of IDD (mild, moderate, and severe) according to MRI signal strength. Preoperative venous blood samples were collected for quantification of serum MMP-1 and TIMP-1 levels. Results showed that IDD patients had significantly higher serum MMP-1 levels compared to those in the control group. Further between-group comparisons revealed that severe IDD patients had higher MMP-1 levels compared to mild or moderate IDD patients. The levels of TIMP, however, were not significantly

different between patients and control individuals. IHC analysis revealed similar results: 1) severe IDD patients had significantly higher MMP-1 positive rates compared to moderate or mild IDD patients; 2) All IDD patients had a greater number of MMP-1 positive cells compared to controls; and 3) TIMP levels were similar across all groups. Taken together, these results suggest that MMP-1 expression is elevated in IDD patients especially in patients with more severe disc degeneration, while TIMP is expressed similarly in both normal and IDD tissues. Our results are supported by other studies that have shown that herniated intervertebral disc tissues had high levels of MMPs, and that expression levels were positively correlated with IDD (Vo et al., 2013). Study with both human and animal model systems has demonstrated that surgical injury to the intervertebral disc leads to elevated MMP-1 expression (Baillet et al., 2013), suggesting the participation of MMP-1 in ECM degradation, which can be suppressed by TIMP to slow the occurrence of IDD. Other studies have attributed IDD to the decrease of cytokines and elevated inflammatory factor release, both of which lead to higher MMP-1 and lower TIMP-1 (Yurube et al., 2012). Although both degenerative and normal intervertebral disc tissues express MMP-1 and TIMP-1, in degenerative intervertebral disc tissues, significantly elevated MMP-1 and decreased TIMP-1 cause an imbalance that leads to ECM degradation. The decreased TIMP-1 levels cannot inhibit the degradation of the ECM by MMP-1, which leads to inelasticity of the intervertebral disc and herniation (Furtwangler et al., 2013). All of these prior studies are consistent with the results herein.

In summary, MMP-1 is prominently expressed during lumbar IDD, implying a positive correlation with IDD severity. TIMP-1, however, is expressed similarly in both normal and degenerative intervertebral disc tissues. The imbalance of MMP-1 and TIMP-1, and resultant degradation of the intervertebral disc ECM, may underlie the pathogenesis of IDD. Therefore, the regulation of the relative expression of MMPs and TIMPs, and subsequent ECM metabolism regulation, may provide new strategies in treating intervertebral disc herniation, although further mechanistic investigations are needed.

Conflicts of interest

The authors declare no conflict of interest.

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