Expression of matrix metalloproteinase–2 and –9 in human ligamentum flavum cells treated with tumor necrosis factor–α and interleukin-1β

Bum-Joon Kim, MD, PhD;1 Junseok W. Hur, MD, PhD;1 Jong Soo Park, MD;1 Joo Han Kim, MD, PhD;1 Taek-Hyun Kwon, MD, PhD;1 Youn-Kwan Park, MD, PhD;1 and Hong Joo Moon, MD, PhD1

1Department of Neurosurgery, Korea University College of Medicine; and 2Department of Neurosurgery, Thejoeun Hospital, Seoul, Korea

OBJECT An in vitro study was performed to understand the potential roles of matrix metalloproteinase (MMP)–2 and MMP-9 in the elastin degradation of human ligamentum flavum (LF) cells via treatment with tumor necrosis factor–α (TNFα) and interleukin-1β (IL-1β). Previous studies have identified a decreased elastin to collagen ratio in hypertrophic LF. Among the extracellular matrix remodeling endopeptidases, MMP-2 and MMP-9 are known to have elastolytic activity. The hypothesis that activated LF cells exposed to inflammation would secrete MMP-2 and MMP-9, thereby resulting in elastin degradation, was examined.

METHODS To examine MMP-2 and MMP-9 expression in human LF, cells were isolated and cultured from LF tissues that were obtained during lumbar disc surgery. Isolated LF cells were equally divided into 3 flasks and subcultured. Upon cellular confluency, the LF cells were treated with TNFα, IL-1β, or none (as a control) and incubated for 48 hours. The conditioned media were collected and assayed for MMP-2 and MMP-9 using gelatin zymography and Western blot analysis. The electrophoresis bands were compared on densitometric scans using ImageJ software.

RESULTS The conditioned media from the isolated human LF cells naturally expressed 72-kD and 92-kD gelatinolytic activities on gelatin zymography. The IL-1β–treated LF cells presented sustained increases in the proenzyme/zymogen forms of MMP-2 and -9 (proMMP-2 and proMMP-9), and activeMMP-9 expression (p = 0.001, 0.022, and 0.036, respectively); the TNFα–treated LF cells showed the most elevated proMMP-9 secretion (p = 0.006), as determined by Western blot analyses. ActiveMMP-2 expression was not observed on zymography or the Western blot analysis.

CONCLUSIONS TNFα and IL-1β promote proMMP-2 and proMMP-9 secretion. IL-1β appears to activate proMMP-9 in human LF cells. Based on these findings, selective MMP-9 blockers or antiinflammatory drugs could be potential treatment options for LF hypertrophy.

http://thejns.org/doi/abs/10.3171/2015.6.SPINE141271

KEY WORDS elastin degradation; ligamentum flavum cells; interleukin-1β; tumor necrosis factor–α; matrix metalloproteinase–2; matrix metalloproteinase–9

Hypertrophied ligamentum flavum (LF) is common in patients with lumbar spinal stenosis. A comparative decrease in the elastin to collagen ratio associated with hypertrophied LF has been described in several research studies. However, the exact mechanism of elastin degradation has not yet been established. The decreased elasticity of LF may result in folding within the spinal canal and contribute to spinal stenosis.

Matrix metalloproteinases (MMPs) are zinc-dependent endopeptidases that are thought to play important roles in extracellular matrix (ECM) remodeling. These proteolytic effectors are involved in in vivo processes such as wound

ABBREVIATIONS ECM = extracellular matrix; EDTA = ethylenediaminetetraacetic acid; FBS = fetal bovine serum; IL-1β = interleukin-1β; HBSS = Hank’s balanced saline solution; LF = ligamentum flavum; MMP = matrix metalloproteinase; proMMP = proenzyme/zymogen form of MMP; P/S = penicillin/streptomycin; SDS-PAGE = sodium dodecyl sulfate–polyacrylamide gel electrophoresis; TIMP = tissue inhibitor of the metalloproteinase; TNFα = tumor necrosis factor–α.


INCLUDE WHEN CITING Published online November 13, 2015; DOI: 10.3171/2015.6.SPINE141271.
MMP-2 and MMP-9 knockout mice did not develop elastase—respectively, have elastolytic activity.26,36 Many studies related to cardiovascular disease have addressed the relationship between MMP-2 and MMP-9 with elastin degradation.1,18 Researchers identified that MMP-2 and MMP-9 knockout mice did not develop elastin degradation when subjected to CaCl₂-mediated aortic injury.1,16 Additionally, doxycycline, a nonspecific MMP inhibitor, has a preventive effect against the development of aortic aneurysm by elastin protection.2,27 Although the exact role of MMPs in inflammatory reactions is not clear, an increase or dysregulation of MMPs is frequently observed during inflammatory response.26,44 Gelatinases, including tumor necrosis factor-α (TNFα) and interleukin-1β (IL-1β), have been identified in herniated intervertebral discs.15,41 Additionally, Sairyo et al. reported the expression of TNFα and IL-1β genes in LF.32

Thus, this study hypothesizes that MMPs play an important role in the elastin degradation of LF during the inflammation of LF cells. The expression and activity of MMP-2 and MMP-9 in cultured human LF cells in response to proinflammatory cytokines were examined using an in vitro model.

Methods

LF Cell Isolation and Culture

We conducted this study in accordance with the regulations of the institutional review board at Korea University Guro Hospital, and appropriate informed consent was obtained from all participating patients. Tissue samples were collected from 7 patients (5 men and 2 women) during their lumbar disc herniation surgeries. Human LF cells were isolated from the LF tissues of patients who did not show any hypertrophy on previous MR images. Patients who did not require a ligamentum flavectomy, patients with a previous history of surgery or steroid injection, patients with a suspected infection, and pediatric patients were excluded.

Tissue specimens were washed 3 times with sterile Hank’s balanced saline solution (HBSS; Gibco-BRL) containing 1% penicillin/streptomycin (P/S; Gibco-BRL) in order to achieve pure isolation of LF cells. After removing contaminants like blood clots, the specimens were minced and digested for 60 minutes in Ham’s F-12 media (Gibco-BRL) containing 1% P/S, 5% heat-inactivated fetal bovine serum (FBS; Gibco-BRL), and 0.2% pronase (Calbiochem); the specimens were then incubated overnight in 0.025% collagenase I (Roche Diagnostics). The LF cells were filtered through a sterile 70-μm pore nylon mesh to remove any remnant tissue debris and centrifuged at 224 g for 5 minutes. Approximately 3.0 × 10⁶ LF cells were resuspended in Ham’s F-12 culture media containing 10% FBS and 1% P/S and placed in a tissue culture flask (VWR Scientific Products). The LF cells were incubated at 37°C in a humidified atmosphere of 5% carbon dioxide, and the culture medium was changed twice per week.

Treating LF Cells With Proinflammatory Cytokines

After an average incubation of 3 weeks, the LF cells were grown to full confluence and removed from the flask by treatment with 0.05% trypsin/EDTA (Gibco-BRL) for subculturing. The pellet aggregates were formed by centrifugation (5 minutes at 224 g) and resuspended in culture medium. At the second passage, the culture medium containing the isolated LF cells was equally divided into 3 flasks and reincubated for 2 more weeks until almost full confluence was reached. The culture flasks were washed 3 times with HBSS. Then, the control LF cells were reincubated for 48 hours in Ham’s F-12 culture medium that contained 1% FBS and 1% P/S in the absence of proinflammatory cytokines. The other 2 flasks of LF cells were incubated for 48 hours in Ham’s F-12 culture media containing 1% FBS and 1% P/S in the presence of 10 ng/ml recombinant human TNFα (R&D Systems) and 1 ng/ml recombinant human interleukin-1β (R&D Systems), respectively. The concentration and exposure time of the proinflammatory cytokines were determined based on previous experimental data.20

Collection of Conditioned Media and Protein Measurement

Following incubation with proinflammatory cytokines for 48 hours, the conditioned media were collected from the 3 LF cell culture flasks. The LF cells were removed from the flasks by treatment with 0.05% trypsin/EDTA. Approximately 5.0 × 10⁶ LF cells were counted per sample. The conditioned media were centrifuged at 1578 g at 4°C for 5 minutes to remove cellular debris. The protein content of each supernatant was determined using the bicinchoninic acid assay (23227; Thermo Scientific). The conditioned media were stored at −80°C for Western blot analysis and gelatin zymography. Because the preliminary assay of the LF cell lysates revealed no notable difference in the expression levels of MMP-2 and MMP-9, further analysis of the cell lysates was not performed.

Gelatin Zymography

MMP-2 and MMP-9 enzymatic activity was measured in the conditioned media using sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) gelatin zymography. Equal amounts of protein from the conditioned media were diluted in nonreducing zymogram sample buffer (161–0764; Bio-Rad). The sample was then electrophoresed in 10% precast polyacrylamide gel with gelatin (161–1113; Bio-Rad) at 4°C. The gels were washed 3 times with 2.5% Triton X-100 and incubated in the presence of a renaturation buffer (161–0765; Bio-Rad) at 37°C for 1 hour with shaking. Subsequently, the gels were transferred to development buffer that contained 50 mmol/L Tris-HCl, 5 mmol/L CaCl₂, and 200 mmol/L NaCl (pH 7.5) and incubated overnight at 37°C. After rinsing in water the next day, each gel was stained with Coomassie blue (0.5% Coomassie blue in 30% ethanol/10% acetic acid) for 1 hour and de-stained for 10 minutes using an aqueous Tris-HCl buffer 7.5) and incubated overnight at 37°C. After rinsing in water the next day, each gel was stained with Coomassie blue (0.5% Coomassie blue in 30% ethanol/10% acetic acid) for 1 hour and de-stained for 10 minutes using an aqueous Tris-HCl buffer.
ous mixture of 30% ethanol and 10% acetic acid. The molecular size of each gelatinolytic band was estimated using known protein standards (Bio-Rad) and a positive control (HT1080 fibrosarcoma cells). MMP-2 and MMP-9 activity were determined by densitometric scanning of the bands using a flatbed photo scanner (Epson 1680 Pro), and analysis was performed using ImageJ 1.48p software (W. Rasband, National Institutes of Health; available at http://rsb.info.nih.gov/ij/).

**Western Blot Analysis**

Equal amounts of protein from the conditioned media (15 μg) were heated, denatured in Laemmli sample buffer (161–0747; Bio-Rad), and subjected to reducing SDS-PAGE in precast gels (456–9034; Bio-Rad). After electrophoresis, the proteins were transferred to polyvinylidene fluoride membranes (Millipore Corp.) for 2 hours at 100 V. The membranes were blocked in 5% skim milk for 1 hour at room temperature and incubated overnight at 4°C with purified rabbit polyclonal antibody specific to MMP-2 (1:5000; ab37150, Abcam) and rabbit monoclonal antibody specific to MMP-9 (1:2000; ab76003, Abcam) in 5% skim milk in TBS-T (Tris-buffered saline with 0.1% Tween 20). After washing, the membranes were incubated with goat anti–rabbit polyclonal secondary antibody (1:20,000; ab6721, Abcam) for 1 hour at room temperature in 5% skim milk in TBS-T with shaking. The bands were visualized using an x-ray film processor (Konica SRX-101A). Each blot was repeated 2 times, and representative scans are presented.

**Quantification and Statistical Analysis**

The bands of the electrophoresed gels were digitized on a flatbed photo scanner and quantified using ImageJ 1.48p software. The integrated band densities were measured and analyzed using IBM SPSS Statistics 20 software (IBM Corp.). The box plots show the median, upper, and lower quartiles, as well as the maximum and minimum values. Repeated measures ANOVA was performed followed by paired t-test and Bonferroni correction to compare the differences between the stimulated and control groups, and p values < 0.05 were considered statistically significant.

**Results**

**Identification of proMMP-2 and proMMP-9 in the Conditioned Media of LF Cells Using Gelatin Zymography**

Gelatin zymography of the LF cell culture supernatants (n = 7) showed a sustained increase in proMMP-2 activity within TNFα- and IL-1β–stimulated LF cells in comparison with the control samples. A representative zymography of LF samples is depicted in Fig. 1. An intense 72-kD digestive band, which is consistent with the proenzyme/zymogen form of MMP-2 (proMMP-2), was identified in every LF sample. The mean gelatinolytic activity of the proMMP-2 band measured by densitometric analysis was highest in IL-1β–stimulated LF cells. LF cells treated with TNFα or IL-1β also presented a faint 92-kD band, whereas the control showed only a single 72-kD band. In some stimulated LF cells, tiny 82-kD bands were visible to the naked eye. However, digitalization with the flatbed scanner failed to detect the weak 82-kD band, and it was therefore not included in quantitative analyses.

The densitometric analysis shown in Fig. 2 shows the statistically significant variation in proMMP-2 expression as determined by repeated measures ANOVA (p = 0.014). However, the Bonferroni post hoc comparison failed to show statistical significance (p = 0.062). In contrast, the proMMP-9 bands were significantly different, and the IL-1β–stimulated LF cells showed higher enzyme activity in comparison with the control cells (p = 0.035).

**Effect of TNFα and IL-1β on MMP-2 on Western Blot Analysis**

Western blot analysis detected a single intense band with a molecular weight of 72 kD, consistent with the proMMP-2, in every LF cell sample. Both the TNFα– and IL-1β–stimulated LF cells presented an overall increase in the expression of the proMMP-2 band in comparison with the control, which is consistent with the results of
MMP-2 and -9 in ligamentum flavum elastin degradation

For the densitometric analysis, the Bonferroni post hoc test revealed a significant difference between the control and IL-1\textsubscript{β}–stimulated LF cells (p = 0.001). However, other comparisons did not show this statistical significance. The active form of MMP-2 was not detected on Western blot analysis.

As shown in Fig. 3, 2 bands corresponding with pro-MMP-9 (92 kD) and activeMMP-9 (67 kD) were clearly identified in the majority of the LF samples. TNF\textalpha–stimulated LF cells showed the most intense proMMP-9 bands. The IL-1\textsubscript{β}–stimulated LF cells presented 2 definite bands, with an activeMMP-9 band that was more intense than both that of the control and that of the TNF\textalpha–stimulated LF cells. In the densitometric analysis, the integrated density of proMMP-9 increased in both the TNF\textalpha– and IL-1\textsubscript{β}–stimulated LF cells in comparison with the control cells (p = 0.006 and 0.022, respectively), and the activeMMP-9 density was distinctly higher in IL-1\textsubscript{β}–stimulated LF cells (p = 0.036), as shown in Fig. 4.

**Discussion**

The normal human LF is predominantly composed of elastic fibers and contains about 70% elastin.\textsuperscript{28,35,48} This elastin-rich tissue is hypertrophied by aging or pathological degeneration. LF hypertrophy exacerbates the tightness of the spinal canals in patients with spinal stenosis and can be partially responsible for sciatica in these patients.\textsuperscript{12,28,31} Currently, the only treatment for LF hypertrophy is resection. However, surgeries for spinal stenosis are a socioeconomic burden on patients and the community.

According to our literature review, MMP-2 and MMP-9 are thought to impact elastin degradation. Yasmin et al. identified a linear correlation between aortic stiffness and MMP-9 levels.\textsuperscript{45} In cardiovascular diseases, many researchers have focused on the causal relationship between aortic aneurysms and MMP-2 and MMP-9 levels.\textsuperscript{10,27,39,45} In chronic obstructive lung diseases, Maclay et al. demonstrated that systemic elastin degradation, combined with the upregulation of MMP-2 and MMP-9, is associated with chronic obstructive lung diseases.\textsuperscript{17} Though human LF cells are composed of various cell types, cells from healthy LF are known to have fibroblast-like features.\textsuperscript{40}
In this study, LF cells presented MMP-2 and MMP-9 activities, with a demonstrated increase in response to inflammatory cytokines. Based on these findings, it can be assumed that MMP-2 and MMP-9 play roles in elastin degradation in LF cells.

Inflammatory cytokines such as TNFα and IL-1β can upregulate various ECM-degrading proteins and growth factors, even though the expression pattern differs for each cell type. Researchers have found that TNFα and IL-1β activate MMP gene expression through the nuclear translocation of NF-κB and mitogen-activated protein kinase stimulation of activator protein–1. Inflammatory reaction is associated with elastin degradation in various in vivo situations. Previous research has demonstrated that TNFα and IL-1β have inhibitory effects on elastin gene expression in lung fibroblasts. Considering that inflammatory cytokines are frequently observed in degenerative discs and LF, it could be inferred that TNFα and IL-1β play roles in inflammatory ECM remodeling via both elastin degradation by inducing MMP-9 secretion and blocking elastin regeneration at the gene transcription level. Recent studies have identified that MMPs are related not only to ECM remodeling, but also angiogenesis and phagocytosis during inflammation, and they regulate cellular functions and growth factors. An MMP from a specific cell has multiple functions, and enzymatic functions may overlap with other MMPs, thereby making it difficult to define their exact roles.

MMPs are regulated in multiple steps as follows: transcription, translation, enzyme trafficking, proMMP activation, inhibition, and degradation. MMPs are secreted as proMMP, which is catalytically inactive. proMMP can be activated by the cleavage or destabilization of the interaction between the thiol group of a prodomain cysteine residue and the zinc ion of the catalytic site (the “cysteine switch”). The activation of proMMPs is regulated via coordination with a tissue inhibitor of the metalloproteinase (TIMP) species. MMP-2 is known to be activated by the interaction between MT1-MMP and TIMP-2. proMMPs can also be activated by other MMPs, various proteases, and chemical agents such as plasmin, amino phenyl mercuric acid, and SDS. According to previous studies, MMP-3 (stromelysin-1) activates proMMP-9, and Le Maitre et al. demonstrated in an in vitro study that IL-1 treatment enhances MMP-3 gene expression in disc cells. In this current study, IL-1β treatment resulted in a marked increase in activeMMP-9. Thus, it is possible that IL-1β activated proMMP-9 by inducing the secretion of MMP-3 in LF cells. In contrast, proMMP-2 expression substantially increased in response to TNFα and IL-1β, however, the active form of MMP-2 was not observed on zymography or Western blot analysis. During proMMP-9 activation, cleavage of the 92-kD enzyme can result in an 82- to 83-kD active product or 64- to 67-kD final active product. In this study, only the 67-kD form was detected by our Western blot analysis. Moreover, other researchers have observed that MMP-2 can activate MMP-9. It is possible that MMP-9 can be completely activated without the activation of MMP-2 in human LF cells.
Initially, we aimed to perform a comparative study between nonhypertrophied LF tissue from patients with lumbar disc herniation and hypertrophied LF tissue from patients with lumbar spinal stenosis. However, in the primary culturing of LF cells, the hypertrophied LF tissue was found to have scanty and unhealthy LF cells. After repeated failed attempts to isolate and culture pure, healthy LF cells from patients with stenosis, we realized that hypertrophied LF is not an appropriate source of LF cells for culturing. Therefore, although we agree that it would have provided interesting findings, the results of the LF cells from patients with stenosis were not included in this study.

In this study, it was determined that human LF cells constitutively secrete MMP-2 and MMP-9, which are upregulated by inflammatory cytokines. Moreover, MMP-9 regulation appeared to be associated with TNFα and IL-1β. TNFα-stimulated LF cells showed the highest expression of proMMP-9, and IL-1β-stimulated LF cells showed the significantly increased expression of activeMMP-9 in comparison with control cells. These results demonstrate that TNFα stimulation can upregulate proMMP-9 secretion in human LF cells during the presecretion step, whereas IL-1β plays a role in the activation cascade of proMMP-9 in LF cells. We coincidentally observed that LF cells obtained from patients who had preoperative symptoms for a relatively longer duration showed weak responses to inflammatory cytokines, although this finding could not be further analyzed because of the small sample size of our study (Table 1). A detailed study with a larger sample size should be conducted to confirm the changes in sensitivity of LF cells to inflammatory cytokines according to the duration of preoperative symptoms.

The results of this experiment must be carefully interpreted in light of several potential limitations, particularly because the function of MMP in vivo is quite complex and not yet fully understood. One explanation for the role of MMP-9 in the elastin degradation of LF cells is direct cleavage and remodeling by activeMMP-9, since MMP-9 itself has the ability to digest elastin. Another possible pathomechanism occurs by means of TGF-β1. Both MMP-2 and MMP-9 enhance TGF-β1 activity.19,24,49 TGF-β1 is known to be associated with fibrosis and LF hypertrophy.21,25,33 Sairyo et al. demonstrated that LF hypertrophy is associated with scarring from inflammatory reactions.31,32 It is important to understand the roles and cascades of MMPs in human LF cells because theoretically MMPs could be potential treatment targets for spinal stenosis. Additional histochemical and genotypic in vivo studies on inflammatory cytokines and MMPs are warranted in order to better understand the role of MMP-9 in elastin degradation.

Conclusions

The effects of TNFα and IL-1β on MMP-2 and MMP-9 expression in human LF cells were evaluated using cell culture methods. The findings of this study provide evidence that human LF cells constitutively secrete MMP-2 and MMP-9 and demonstrate a sustained increase in proMMP-9 secretion in response to TNFα stimulation, whereas treatment using IL-1β showed the distinct activation of MMP-9. This suggests that inflammatory cytokines such as TNFα and IL-1β are involved in the MMP-9 pathway in human LF cells, which is a potential treatment strategy for LF hypertrophy.

References


TABLE 1. Baseline characteristics of patients∗

<table>
<thead>
<tr>
<th>Case No.</th>
<th>Age (yrs), Sex</th>
<th>Level</th>
<th>Smoking</th>
<th>Nonsteroidal Antinflammatory Drugs</th>
<th>Duration of Symptoms</th>
<th>Response to Inflammatory Cytokines</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>36, F</td>
<td>L4–5</td>
<td>No</td>
<td></td>
<td>4 mos</td>
<td>Weak proMMP-2 band in response to TNFα &amp; weak active MMP-9 band in response to IL-1β</td>
</tr>
<tr>
<td>2</td>
<td>54, M</td>
<td>L2–3</td>
<td>No</td>
<td></td>
<td>1 wk</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>33, M</td>
<td>L4–5</td>
<td>3 pack-yrs</td>
<td></td>
<td>3 mos</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>45, M</td>
<td>L4–5</td>
<td>20 pack-yrs</td>
<td></td>
<td>3 mos</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>51, M</td>
<td>L5–S1</td>
<td>20 pack-yrs</td>
<td></td>
<td>2 mos</td>
<td>Weak proMMP-2 band in response to IL-1β &amp; weak active MMP-9 in response to IL-1β</td>
</tr>
<tr>
<td>6</td>
<td>31, F</td>
<td>L4–5</td>
<td>No</td>
<td></td>
<td>2 mos</td>
<td>Weak proMMP-9 in response to TNFα</td>
</tr>
<tr>
<td>7</td>
<td>28, M</td>
<td>L5–S1</td>
<td>No</td>
<td></td>
<td>1 mo</td>
<td></td>
</tr>
</tbody>
</table>

* No patient had experienced trauma, had diabetes, or was taking oral steroids.


**Disclosures**
The authors report no conflict of interest concerning the materials or methods used in this study or the findings specified in this paper.

**Author Contributions**
Conception and design: Moon. Acquisition of data: BJ Kim. Analysis and interpretation of data: BJ Kim. Drafting the article: Hur, JS Park, JH Kim, Kwon, YK Park.

**Correspondence**
Hong Joo Moon, Department of Neurosurgery, Korea University College of Medicine, 148 Gurodong-ro, Guro-gu, Seoul 152-703, Korea. email: vagusmoon@gmail.com.