Basic Science

Experimental chemonucleolysis with recombinant human matrix
metalloproteinase 7 in human herniated discs and dogs

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Abstract

BACKGROUND CONTEXT: Chemonucleolysis has been proposed as a less invasive technique than surgery for patients with lumbar disc herniation. Once chymopapain had been approved as a chemonucleolysis drug, it was withdrawn because of serious complications. A novel agent with fewer complications would be desirable.

PURPOSE: The purpose of this study was to investigate the effects of recombinant human matrix metalloproteinase 7 (rhMMP-7) in experimental chemonucleolysis in vitro and in vivo and examine its effects on tissue damage.

STUDY DESIGN: The study design is the experimental study using human herniated discs and enzyme substrates in vitro and dogs in vivo.

METHODS: The effects of rhMMP-7 on the degradation of human herniated discs were examined by measuring the wet weight in vitro. The correlations between the decrease in wet weight by rhMMP-7 and the conditions associated with herniated discs were also analyzed. The effects of rhMMP-7 on the proteoglycan and water contents were respectively examined with alcian blue staining and T2-weighted magnetic resonance imaging at 7 days after intradiscal injection in dogs. The distribution of [\textsuperscript{125}I]-labeled rhMMP-7 was investigated by autoradioluminography at 7 days after intradiscal injection in dogs. An epidural injection study with rhMMP-7 was performed to evaluate the effects on the tissue damage around the discs at 1 and 13 weeks after the treatment in dogs. The Type 1 and 2 collagen cleavage rates were measured and compared with those of aggrecan in vitro.

RESULTS: Recombinant human matrix metalloproteinase 7 concentration dependently decreased the wet weight of herniated discs in vitro. The decrease in wet weight of the discs by rhMMP-7 did not significantly correlate with the conditions associated with herniated discs. Intradiscal injection of rhMMP-7 reduced the proteoglycan and water contents, with an increase in the serum keratan sulfate levels. Radioactivity of [\textsuperscript{125}I]-labeled rhMMP-7 was detected in the nucleus pulposus and annulus fibrosis but not in the muscle. Epidural injection of rhMMP-7 had no effect on the injection site or the nerve tissues. The Type 1 and 2 collagen cleavage rates of rhMMP-7 were 1,000-fold weaker than those of aggrecan.

CONCLUSIONS: This study demonstrated experimental chemonucleolysis with rhMMP-7 in vitro and in vivo. The effects of rhMMP-7 were not affected by the conditions associated with herniated discs. The epidural injection study together with the autoradioluminography and


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in vitro enzyme assay suggests that intradiscal injection of rhMMP-7 may not induce tissue damage around the discs because of its distribution and substrate selectivity. Recombinant human matrix metalloproteinase 7 may be a novel and promising chemonucleolysis agent. © 2014 Elsevier Inc. All rights reserved.

Keywords: Chemonucleolysis; Matrix metalloproteinase 7; Intervertebral disc; Herniation; Minimally invasive treatment; Herniated disc resorption

Introduction

Lumbar disc herniation commonly occurs through the annular disruption associated with degenerative disc disease. In the United States, 2.8 million patients suffer from lumbar herniated discs each year [1]. Patients with lumbar herniated discs are usually 20 to 40 years old and show acute onset of lower extremity pain and/or low back pain. This can result in absenteeism from work because of extensive limitations of daily activity. Intervertebral disc herniation is currently treated with conservative therapy or surgery, including microdiscectomy. Although microdiscectomy is less invasive than traditional herniated discectomy [2], it remains minimally invasive because a skin incision under general anesthesia is needed, and hospitalization is sometimes required. In addition, unavoidable complications such as dural tear and postoperative hematoma are also reported [3].

Chemonucleolysis, a medical procedure involving enzyme injection into a herniated disc, has been proposed as a simpler and less invasive approach for patients who wish to avoid surgery. The hypothetical mechanism of action of chemonucleolysis by the injected enzyme is as follows. First, the intradiscally injected enzyme degrades aggrecan, a major component of the extracellular matrix in the disc. The degradation of aggrecan also results in the reduction of water content in the herniated disc. These processes lead to a decrease in herniated disc volume and reduce the pressure of the herniated disc against nerve tissues on the spinal cord or nerve root. As a result, pain relief will be achieved. Smith et al. [4] administered chymopapain derived from papaya latex into lumbar herniated discs in 1963. The therapeutic effects have been well documented, but chymopapain was withdrawn because of its serious complications, such as anaphylaxis, paraplegia, and subarachnoid hemorrhage. Thus, no drugs for chemonucleolysis are currently available anywhere in the world, and a novel agent with fewer complications would be desirable.

Matrix metalloproteinases (MMPs) belong to a family of neutral secreted Zn$^{2+}$ proteases, and they degrade various components of the extracellular matrix. In particular, both MMP-3 (stromelysin 1) and MMP-7 (matrilysin), which degrade cartilage proteoglycans [5,6], are strongly expressed in surgical samples of herniated discs, and they play a crucial role in the natural resorption process of herniated discs, especially the noncontained type [7–12]. We previously demonstrated that recombinant human matrix metalloproteinase 7 (rhMMP-7) decreased the wet weight of surgical samples of herniated discs more extensively than MMP-3 and the control not treated with either MMP, and it decreased the protruded mass at 1 week after injection in naturally occurring herniation in dogs [13]. The above results suggested that rhMMP-7 might be an ideal candidate as a chemonucleolysis agent.

In this study, the effects of rhMMP-7 on the degradation of human herniated discs were further investigated in vitro, and the effects of the conditions associated with herniated discs, such as patients’ ages, degeneration grade, or interval between the onset of symptoms and surgery, on the wet weight reduction of the herniated discs by rhMMP-7 were examined. In addition, the in vivo pharmacologic effects and distribution of rhMMP-7 after intradiscal injection were examined in normal dogs because there is no reproducible animal model of intervertebral disc herniation that accurately reflects the clinical condition in vivo. An epidural injection study with rhMMP-7 was performed to evaluate tissue damage around the discs of dogs. The substrate selectivity of rhMMP-7 was also examined in an in vitro enzyme assay in a mechanistic analysis.

Materials and methods

The in vitro study with surgical samples from herniated patients was approved by the Institutional Review Board of our Institute (approval No. 392, HB11-001). The animal experimental protocols were approved by the Animal Care and Use Committee of our Institute (approval No. B08-001, B09-001, B09-004).

In vitro chemonucleolysis in human herniated discs

All subjects provided their informed consent for use of surgically removed samples for the experiments.

Concentration-dependent effects

Ten surgical samples of herniated discs were obtained from patients undergoing primary lumbar herniotomy. These subjects were nine men and one woman, with an average age of 46 years (range, 18–85 years) at the time of surgery.

Surgical samples were sliced into several pieces, and their weights were measured. Each sample was divided into 10- to 100-mg pieces. Each piece was placed in a separate well of a 24-well plate (Nunc; Thermo Fisher Scientific Inc., Waltham, MA, USA) and incubated with or without rhMMP-7 (0.0198, 0.0781, 0.310, 1.24, and 4.96 U/mL;
The Chemo-Sero-Therapeutic Research Institute, Kumamoto, Japan) in 1 mL of OPTI-MEM (Invitrogen, Life Technologies Corp., Carlsbad, CA, USA) containing 50 μg/mL of gentamicin and 0.25 μg/mL of fungizone (Invitrogen, Life Technologies Corp.) for 24 hours in a humidified atmosphere of 5% CO2 at 37°C. Before and after incubation, culture media were removed from surgical samples by patting briefly with a Kimtowel (Nippon Paper Creencia Co. Ltd., Tokyo, Japan), and the wet weights of the surgical samples were measured [13]. In addition, because keratan sulfate is one of the degradation products of the intervertebral discs and is leaked from intervertebral discs as a catabolite of proteoglycans [14–16], concentrations of keratan sulfate in the media were measured using an ELISA system (high sensitive keratan sulfate ELISA kit; Seikagaku Biobusiness Corp., Tokyo, Japan).

The effects of the conditions associated with herniated discs

Nineteen surgical samples of herniated discs were obtained from patients undergoing primary lumbar herniotomy. These subjects were 16 men and 3 women, with an average age of 41 years (range, 18–64 years) at the time of surgery. The types of herniated discs were classified as subligamentous extrusions (14 cases) and transligamentous extrusions (5 cases) [17]. The disc degeneration was graded as Types I to V according to Pfirrmann et al. [18], and the samples were assigned to Grade II (1 case), Grade III (12 cases), Grade IV (4 cases), and Grade V (2 cases). The average interval between the onset of symptoms and the surgery was 4.2 months (range, 1–9 months). As described previously, the wet weight of the herniated disc was measured before and after incubation with rhMMP-7 (3.1 U/mL). The correlations between the decrease in wet weight by rhMMP-7 and the conditions associated with herniated discs were analyzed by Pearson or Spearman correlation coefficients.

In vivo chemonucleolysis in dogs

The effects on the proteoglycan content and serum keratan sulfate levels

Twenty male beagle dogs at the age of 8 or 9 months (Kitayama Labes Co., Ltd., Nagano, Japan) were used. The animals were anesthetized with intravenous injection of propofol (7.5 mg/kg), followed by the inhalation of isoflurane (Abbott Japan Co., Ltd., Tokyo, Japan), and rhMMP-7 solution (0.0155, 0.0620, 0.155, and 0.620 U per disc) or control solution in a volume of 50 μL per disc was injected into lumbar intervertebral discs (L2–L3, L3–L4, and L4–L5) with a 26-ga needle (1710SN 26/4; Hamilton Company, Reno, NV, USA) under X-ray control. Blood samples were collected, and the animals were sacrificed 7 days after the injection. Injected discs were harvested, and alcian blue staining was performed to examine proteoglycan content. Alcian blue staining is a commonly used method, and the staining intensity is regarded as a quantitative indicator of the proteoglycan level. [19] Photographs of alcian blue-stained tissue specimens were taken with a digital imaging microscope. To ensure constant conditions, all specimens were photographed in a single session with light intensity and white balance settings unchanged. These digital images were analyzed with imaging software (analySIS; Soft Imaging System GmbH, Muenster, Germany), and brightness values were calculated (n=12 discs per group). Briefly, square images were selected for analysis from original images so as to include the maximum area of the intervertebral disc. The intensity of each pixel of the square images was converted to a 255-step gray scale. Brightness and number of pixels were measured from the images, and the brightness values were calculated by multiplying brightness and number of pixels.

In addition, concentrations of serum keratan sulfate were measured using an ELISA system for keratan sulfate (high sensitive keratan sulfate ELISA kit; Seikagaku Biobusiness Corp.) in the animals treated with rhMMP-7 solution (15.5, 62.0, and 155 mU per disc) or control solution.

Effects on water content in the magnetic resonance imaging study

Six male beagle dogs at the age of 9 or 10 months (Kitayama Labes Co., Ltd.) were used. The animals were anesthetized with pentobarbital (25.92 mg/kg), and rhMMP-7 solution (0.155 U per disc) or control solution was injected into lumbar intervertebral discs (L2–L3, L3–L4, and L4–L5) with a 26-ga needle under X-ray control. A T2-weighted magnetic resonance imaging (MRI) image (SIGMA Contour/I (0.5T); General Electric Company, Fairfield, CT, USA) was taken at 7 days after the injection of rhMMP-7. To ensure constant conditions, all images were taken at the same instrument settings.

The distribution of [125I]-labeled rhMMP-7 after intradiscal injection in dogs

[125I]-labeled rhMMP-7 ([125I] rhMMP-7) was prepared, and its specific radioactivity was 4.50 MBq/mg. Autoradioluminography (ARLG) of the intervertebral discs and their surrounding tissues was performed in 8- to 11-month-old, male beagle dogs (Kitayama Labes Co., Ltd.) after intradiscal injection (L4–L5) of [125I] rhMMP-7 at a dose of 0.155 U per disc. The animals were sacrificed 7 days after dosing, and the intervertebral disc and its surrounding tissues were sampled for ARLG. The samples were rapidly frozen and coated with 3% sodium carboxymethyl cellulose (CMC·Na) solution, followed by embedding in 5% CMC·Na solution and frozen in a bath of hexane and dry ice. The sections of frozen blocks of intervertebral disc samples, 30-μm thick (setting), were prepared using a cryomicrotome (Leica CM3600; Leica Microsystems GmbH, Wetzlar, Germany). The following sections were prepared: the section in which muscle, 1 cm outside the dosed intervertebral disc, could
be confirmed (injection side and noninjection side); the section in which the annulus fibrosus of the dosed disc could be confirmed (injection side and noninjection side); and the section in which the central region including the nucleus pulposus of the dosed intervertebral disc could be confirmed. The selected sections were lyophilized and exposed to an imaging plate (BAS-MS2040; Fujifilm Corporation, Tokyo, Japan). After exposure, the imaging plate was analyzed using a bioimaging analyzer (BAS-2500; Fujifilm Corporation) to obtain the ARLGMs.

**Epidural injection study in dogs**

Eighteen male beagle dogs at the age of 11 to 13 months (Kitayama Labes Co., Ltd.) were used (three animals per group). The animals were anesthetized with intravenous injection of propofol (AstraZeneca K.K., Osaka, Japan), followed by the inhalation of nitrous oxide (Nissan Chemical Industries, Ltd., Tokyo, Japan) and isoflurane (Abbott Japan Co., Ltd.). After they were fixed in the prone position, the dorsal skin was incised, and a catheter (Perifix; B. Braun Aesculap Japan Co. Ltd., Tokyo, Japan) was inserted through a point between the fourth and the sixth lumbar spinous processes into the epidural space to a depth of approximately 5 cm. Two weeks after catheter implantation, rhMMP-7 solution (3.1 and 12.4 U/mL) or control solution in a volume of 0.1 mL per body was injected into the lumbar epidural space through the catheter under an unanesthetized condition. The animals were sacrificed 1 and 13 weeks after dosing, and the injection site (lumbar spine area, epidural space including posterior longitudinal ligament, nerve root, and spinal cord) and nerve tissues (cerebrum, cerebellum, pons, medulla oblongata, thoracic and lumbar spinal cord [remote from the injection site], and sciatic nerve) were sampled and used for histopathological examination with hematoxylin-eosin (HE) staining. Regarding the injection site, special stains (Masson trichrome stain for collagen fibers and Kluver-Barrera [KB] stain for Nissl bodies or myelin sheaths) and immunohistochemistry of glial fibrillary acidic protein (GFAP) (antiglial fibrillary acidic protein, polyclonal rabbit; Dako Cytomation, Tokyo, Japan), neurofilament (NF) heavy (anti-NF heavy, mouse monoclonal, clone: RNF402; Abcam, Cambridge, UK), and macrophage surface antigen (MS) ( antimacrophage surface antigen, mouse monoclonal, clone; AM-3K; Transgenic Inc., Kumamoto, Japan) were also performed for histopathological examination. Clinical signs, body weight, food consumption, and necropsy findings were also examined.

**Cleavage activity on matrix macromolecules**

**Preparation of the interglobular domain of aggrecan of humans and dogs**

The interglobular domain of aggrecan (aggrecan IGD) of humans and dogs was prepared in an *Escherichia coli* system. Briefly, complementary DNA (cDNAs) of aggrecan IGD of humans and dogs were prepared by polymerase chain reaction, and the templates were the cDNA of human adult normal tissue from the trachea (Cat No. C1234260-10; Biochain Institute Inc., Hayward, CA, USA) and the cDNA of dog normal tissue from the trachea (Cat No. C1734160; Biochain Institute Inc.). The resulting polymerase chain reaction fragments were cloned into pET28 vector (pET28-aggrecan IGD). Plasmid pET28-aggrecan IGD was transfected into *E. coli* strain BL21 (DE3). The transformed cells were grown in Luria broth with 50 μg/mL of kanamycin, and the recombinant proteins were induced by adding 1 mmol/L of isopropyl-1-β-d-galactopyranoside. Cells were collected by centrifugation and suspended in sonication buffer (50 mM potassium phosphate [pH 7.5], 50 mM NaCl, 2 mM dithiothreitol/complete EDTA free, and Bugbuster ×10). After disruption by sonication, cell debris was removed by centrifugation. The supernatants including recombinant aggrecan IGD were purified by a Ni-NTA agarose column and the reverse-phase high performance liquid chromatography (HPLC) method. The purity of these proteins was 97.216% for human aggrecan IGD and 98.870% for dog aggrecan IGD. The sequence of human aggrecan IGD was GSHMASTGED FVDIPENFFG VGGEEDITVQ TVTWPDMELP LPRNITEGEA RGSVI...
Cleavage activity on aggrecan IGD

The cleavage rates of rhMMP-7 on aggrecan IGD of humans and dogs were calculated from the remaining amount of aggrecan IGD after enzyme reaction. The enzyme reactions of rhMMP-7 at a concentration of 3.1 mU/mL were carried out in a volume of 25 μL at a concentration of aggrecan IGD of 250 μg/mL, containing 37.5 mmol/L of Tris, 150 mmol/L of NaCl, 10 mmol/L of CaCl₂, 0.01% BSA, and 0.01% Brij-35 pH 7.5 at 37°C for 60 minutes. The linearity of the enzyme reaction was ensured for 60 minutes under these conditions. The amount of aggrecan IGD was measured by the HPLC method (Protein-RP column; YMC Co., Ltd., Kyoto, Japan). With the reverse phase condition, aggrecan IGD eluted at a concentration of acetonitrile from 55% to 57%.

Cleavage activity on Type 1 and 2 collagens

The examination of cleavage rates on Type 1 and 2 collagens was carried out using assay kits for Type 1 and 2 collagens (Collagen assay kit; Chondrex, Inc., Redmond, WA, USA).

Statistical analysis

All data are expressed as means ± standard error of the mean. For multiple comparisons, William or Shirley-William test was used to compare each dose group versus a control group. Differences with a p value < .05 were considered significant. The above data were analyzed with SAS software (SAS9.2; SAS Institute Japan, Ltd., Tokyo, Japan). Pearson or Spearman correlation coefficients were used as appropriate to assess the association between the decrease in wet weight of the surgical samples by rhMMP-7 and various associated conditions, such as patients’ ages, degeneration grade of herniated discs, and interval between the onset of symptoms and the surgery. The data were analyzed with Prism software (Prism5 for Windows, version5.0.4; GraphPad Software, Inc., La Jolla, CA, USA).

Results

In vitro chemonucleolysis in human herniated discs

The effects of rhMMP-7 on the degradation of human herniated discs were examined in tissue culture at concentrations from 0.0198 to 4.96 U/mL. The wet weight of herniated discs decreased in a concentration-dependent manner, and the effects of rhMMP-7 were significant (p < .05) at 0.0781 U/mL and higher (Fig. 1). The keratan sulfate level increased in a concentration-dependent manner, and the effects of rhMMP-7 were significant (p < .05) at 0.310 U/mL and more (Fig. 1).

Fig. 2. The correlation between the effects of rhMMP-7 and conditions associated with herniated discs. (Top Left) Patients’ age, (Top Right) degeneration grade of herniated disc, and (Bottom Left) interval between the onset of symptoms and the surgery. rhMMP-7, recombinant human matrix metalloproteinase 7.
Whether the decrease in wet weight by a submaximal concentration of rhMMP-7 at 3.1 U/mL was affected by the conditions associated with the herniated discs, such as patients’ ages (Fig. 2, Top Left), degeneration grade of herniated discs (Fig. 2, Top Right), and interval between the onset of symptoms and the surgery (Fig. 2, Bottom Left), was further examined. The correlations between the decrease in wet weight of herniated discs and any of the associated conditions were not significant (patients’ age, \( r = -0.3030, p = .2073 \); degeneration grade, \( r = -0.07712, p = .7537 \); interval between the onset of symptoms and the surgery, \( r = -0.3213, p = .1798 \)) (Fig. 2).

**In vivo chemonucleolysis in dogs**

The dose-dependent effects of rhMMP-7 on the degradation of proteoglycans and the serum keratan sulfate level were examined in beagle dogs after intradiscal injection at doses from 0.0155 to 0.620 U per disc. Recombinant human matrix metalloproteinase 7 decreased alcian blue staining intensity in a dose-dependent manner (Fig. 3, Right), and the effects were significant (\( p < .05 \)) at 0.0620 U per disc and higher (Fig. 3, Top Left). In the 0.0620 and 0.155 U per disc groups, serum keratan sulfate level increased significantly (\( p < .05 \)) (Fig. 3, Bottom Left).

The effect of intradiscal injection of rhMMP-7 at 0.155 U per disc on the water content of intervertebral discs was examined with MRI in beagle dogs. The water content in the rhMMP-7–treated group decreased compared with that in the control group. Typical examples in the control and rhMMP-7–treated groups are shown in Fig. 4.

**The distribution of \(^{125}\text{I} \) rhMMP-7 after intradiscal injection in dogs**

The distribution of radioactivity in the intervertebral discs and their surrounding tissues was evaluated by ARLG after intradiscal injection of \(^{125}\text{I} \) rhMMP-7. Autoradioluminograms, which were overlaid on the photographs of each section 7 days after dosing, are shown in Fig. 5, Top and Bottom. Radioactivity was detected in the nucleus pulposus and the annulus fibrosus but not in the muscle. The results indicated that \(^{125}\text{I} \) rhMMP-7 did not leak into the surrounding tissues of the disc after intradiscal injection into normal dogs.

**Epidural injection study in dogs**

The epidural injection study with rhMMP-7 was performed to evaluate the effects on tissue damage around the discs in dogs. Histopathological examinations showed no rhMMP-7–related changes at the injection site (epidural space including posterior longitudinal ligament, nerve root,

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Fig. 3. The effects of rhMMP-7 in dogs. (Top Left) The effects on the proteoglycan content of intervertebral discs in dogs (N=12). (Bottom Left) The effects on serum keratan sulfate in dogs (N=4). Data are expressed as means±standard error of the mean. *\( p < .05 \), Shirley-Williams test versus control solution. (Right) Representative photographs of alcian blue staining of lumbar vertebral discs in dogs. (a) control solution, (b) rhMMP-7 (0.0620 U per disc), and (c) rhMMP-7 (0.155 U per disc). rhMMP-7, recombinant human matrix metalloproteinase 7.
and spinal cord) or in the nerve tissues (Fig. 6 and Table 1). Representative photographs of HE staining, KB staining, and NF immunohistochemistry are shown in Fig. 6. No rhMMP-7–related changes were noted in the clinical signs, body weight, food consumption, or necropsy findings.

Cleavage activity on matrix macromolecules

The cleavage activities of rhMMP-7 on human and dog aggrecan, a major substrate of MMP-7, were examined by the degradation rate of aggrecan IGDs, which conserve specific cleavage sites among the species. The cleavage rate of human aggrecan IGD was 5.083 μg substrate/min/μg enzyme, whereas the rate of dog aggrecan IGD was 4.570 μg substrate/min/μg enzyme (Table 2). The results indicated that rhMMP-7 cleaved aggrecan IGD of both species at similar rates (0.899-fold).

The cleavage activities of Type 1 and 2 collagens, which are important components of the annulus fibrosus, nucleus pulposus, dura mater, and cartilage endplate, were measured and compared with that of human aggrecan IGD. The cleavage rates of Type 1 and 2 collagens were 0.00248 and 0.00256 μg substrate/min/μg enzyme, respectively. Because the cleavage rate of human aggrecan IGD was 5.083 μg substrate/min/μg enzyme, the relative ratios of Type 1 and 2 collagens to aggrecan IGD were 0.000488 and 0.000504, respectively (Table 2). These results demonstrated that the cleavage rates of rhMMP-7 on Type 1 and 2 collagens were 1000-fold weaker than those on aggrecan IGD.

Discussion

The present study further elucidated the in vitro and in vivo efficacies of rhMMP-7 as a novel chemonucleolysis agent based on the previous findings [13]. The degrading activity of rhMMP-7 on human herniated discs in vitro was not affected by the conditions of the discs. The epidural injection study, as well as the ARLG study and in vitro enzyme assay, suggested that intradiscal injection of rhMMP-7 did not cause tissue damage around the discs because of its distribution and substrate selectivity. Thus, rhMMP-7 may be a novel chemonucleolysis agent with fewer complications.

In vitro chemonucleolysis in human herniated discs

The activity of MMPs is inhibited by α2-macroglobulin or tissue inhibitors of metalloproteinases (TIMPs), which form complexes with MMPs [20]. Because the expression of TIMPs has been reported to increase in the degenerated discs at gene and protein levels [21–24], it might be possible that exogenously applied rhMMP-7 did not exert any pharmacologic effects. In this study, the water content of the herniated discs decreased and the concentration of keratan sulfate increased in the culture media. These effects were due to the degradation of proteoglycans by rhMMP-7. These results indicate that the amount of rhMMP-7 that was injected experimentally is far greater than the normal concentration in vivo and overwhelmed the TIMP regulatory system at the intervertebral discs and that rhMMP-7 could have degrading activity on proteoglycans.

Degenerative changes, which occur in human intervertebral discs from youth [25], are probably among the causes of lumbar disc herniation. Because the composition of extracellular matrix proteins alters during degenerative changes [10,26,27], the degradation activity on a variety of degenerative discs is essential for chemonucleolysis agents. This study demonstrated that the decrease in the water content by rhMMP-7 did not significantly correlate with the conditions associated with herniated discs, such as patients’ ages, degeneration grade, or the interval between the onset of symptoms and the surgery. The results suggest that the effects of rhMMP-7 were not affected by the type of herniated disc. Hence, rhMMP-7 may be clinically effective for a broad range of patients with herniated discs.

In vivo chemonucleolysis in dogs

Chemonucleolysis is expected to degrade components of herniated discs and reduce the water content and volume of herniated discs. This decreases the pressure caused...
by herniated discs on the spinal cord, cauda equina, or nerve root, which finally ameliorates the pain response. Because there is no reproducible animal model of intervertebral disc herniation that accurately reflects the clinical condition in vivo, the present study was designed to confirm that rhMMP-7 actually degrades the disc in vivo. The results showed that rhMMP-7 reduced both the proteoglycan and the water contents detected by alcian blue staining and T2-weighted MRI, respectively. Previously, the effects of chemonucleolysis agents, chymopapain or chondroitinase ABC, on the proteoglycan content, water content, disc height, and pressure inside the disc were examined, and their beneficial effects on each parameter have been shown in various experimental animals [28–32]. It is noteworthy that these effects were confirmed at the same dose range but over a different time...
range. The decrease in the proteoglycan and water contents can be seen from several days after treatment, whereas the disc narrowing may develop relatively slowly for a couple of months until the structural changes of the intervertebral discs are completed. In our preliminary study, rhMMP-7 showed a tendency for disc narrowing at 7 days after injection in dogs (data not shown). We consider that more time is needed to clearly demonstrate the disc narrowing caused by rhMMP-7.

Because the effective concentration of rhMMP-7 on the degradation of herniated discs in vitro ranged from 0.0781 to 4.96 U/mL and the cleavage rate of dog aggrecan IGD is lower than that of human aggrecan IGD (0.899-fold), the estimated effective concentration in dogs is calculated as 0.0869 to 5.52 U/mL. Because the volume of the intervertebral disc of dogs is approximately 0.36 cm³ (unpublished data), the estimated dose is 0.0313 to 1.99 U per disc. Therefore, these dose ranges are close to the effective dose.

Table 1
Histopathology results of the injection sites in the epidural injection study in beagle dogs

<table>
<thead>
<tr>
<th>Histopathologic finding</th>
<th>Control 1 wk</th>
<th>Control 13 wk</th>
<th>rhMMP-7 (3.1 U/mL) 1 wk</th>
<th>rhMMP-7 (3.1 U/mL) 13 wk</th>
<th>rhMMP-7 (12.4 U/mL) 1 wk</th>
<th>rhMMP-7 (12.4 U/mL) 13 wk</th>
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<tbody>
<tr>
<td>Number of animals examined</td>
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<td>No abnormal changes, HE staining*</td>
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<td>No abnormal changes, MTC staining†</td>
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<td>No abnormal changes, KB staining</td>
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<td>No abnormal changes, GFAP immunohistochemistry§</td>
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<td>No abnormal changes, NF immunohistochemistry¶</td>
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</tr>
<tr>
<td>No abnormal changes, MS immunohistochemistry‖</td>
<td>3</td>
<td>3</td>
<td>3</td>
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<td>3</td>
</tr>
</tbody>
</table>

* No abnormal changes were noted on HE staining.
† No abnormalities such as an increase in collagen fibers were noted on MTC staining.
§ No morphological abnormalities in Nissl bodies or myelin sheaths were noted on KB staining.
¶ No morphological abnormalities in astrocytes or increases in astrocytes were noted on GFAP immunohistochemistry.
‖ No morphological abnormalities in axons were noted on NF heavy immunohistochemistry.

Fig. 6. Histology of spinal nerve roots around the injection sites in the epidural injection study. 1, HE staining; 2, KB staining; and 3, NF immunohistochemistry. (Top) The spinal nerve root within the epidural space in the control animal 13 weeks after the injection. (Middle) The dorsal root within the subarachnoid space in the rhMMP-7 (12.4 U/mL)–treated animal 1 week after the injection. (Bottom) The spinal nerve root within the epidural space in the rhMMP-7 (12.4 U/mL)–treated animal 13 weeks after the injection. HE, hematoxylin-eosin; KB, Kluver-Barrera; NF, neurofilament; rhMMP-7, recombinant human matrix metalloproteinase 7.
rhMMP-7 on Type 1 and 2 collagens were 1000-fold weaker than those on aggrecan. Because Type 1 collagen is the main component of dura mater or nerve [36–39], the lack of histologic changes may be explained in part by the substrate selectivity of rhMMP-7. The epidural injection study, as well as the ARLG study and the in vitro enzyme assay, suggests that intradiscal injection of rhMMP-7 may not give rise to tissue damage around the discs because of its distribution and substrate selectivity. In addition, although chymopapain has significant immunogenicity and has been reported to cause anaphylaxis in some cases, rhMMP-7 is expected to cause no anaphylaxis in clinical use because of its human protein. Thus, rhMMP-7 may have fewer complications than chymopapain as a chemonucleolysis agent, although we cannot completely rule out the possibility that it might induce some neurologic complications in clinical use.

Effects on tissue damage around the discs

In rare cases, chymopapain induced subarachnoid hemorrhage in clinical use [33]. However, this raised a serious concern about the tissue damage caused by chemonucleolysis agents. In fact, chymopapain has been reported to damage nervous and ligamentous tissues, as well as cartilaginous tissue, in rabbits [34]. The effects may be caused by the low selectivity of this enzyme because chymopapain is shown to degrade aggrecan into multiple products giving a broad molecular weight distribution [35]. In the present study, it was first confirmed that the radioactivity of \([^{125}\text{I}]\) rhMMP-7 was detected in the nucleus pulposus and the annulus fibrosus, but not in the muscle, at 7 days after intradiscal injection in the ARLG study. This indicated that rhMMP-7 did not degrade tissues across the longitudinal ligament and was not leaked into the surrounding tissues of the discs as long as it was correctly applied to normal discs. Regarding the clearance of rhMMP-7 from the discs, rhMMP-7 might be transferred to the circulation via the capillaries in the cartilage end plate and outside the annulus fibrosus and finally inhibited by \(\alpha_2\)-macroglobulin or TIMPs in the blood. Although rhMMP-7 did not distribute to the epidural space in dogs, the epidural injection study was performed to evaluate the effects of rhMMP-7 on the tissue damage around the discs, assuming that intradiscally injected rhMMP-7 might be leaked into the epidural space because of noncontained disc herniation or unexpected clinical error. Recombinant human matrix metalloproteinase 7 had no effects on the tissue damage at the injection site or in the nerve tissues 1 and 13 weeks after treatment. A variety of histopathologic data indicated that rhMMP-7 did not affect collagen fibers, neurons, axons, myelin sheaths, or astrocytes. In addition, no changes in clinical signs, body weight, food consumption, and necropsy findings in the study suggest that no systemic changes occur even if rhMMP-7 is leaked into the epidural space. The in vitro study showed that the cleavage rates of rhMMP-7 on the interglobular domain of humans.

### Table 2: Cleavage rate of rhMMP-7 on matrix macromolecules

<table>
<thead>
<tr>
<th>Matrix macromolecules</th>
<th>Cleavage rate ((\mu)g substrate/min/(\mu)g enzyme)</th>
<th>Ratio*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aggrecan interglobular domain (human)</td>
<td>5.083</td>
<td>1</td>
</tr>
<tr>
<td>Aggrecan interglobular domain (dog)</td>
<td>4.570</td>
<td>0.899</td>
</tr>
<tr>
<td>Type 1 collagen (bovine)</td>
<td>0.00248</td>
<td>0.000488</td>
</tr>
<tr>
<td>Type 2 collagen (bovine)</td>
<td>0.00256</td>
<td>0.000504</td>
</tr>
</tbody>
</table>

* Relative activity normalized by the cleavage rate on the aggrecan interglobular domain of humans.

(0.062–0.620 U per disc) in dogs. These data and estimates suggest that the in vitro effective concentration of rhMMP-7 would be enough to produce chemonucleolysis in vivo.

Study limitations and perspectives

The present study demonstrated the effects of rhMMP-7 at 7 days after intradiscal injection in dogs. Because the impact of intervertebral disc resorption with chemonucleolysis agents is reported to last for several months [29], further studies will be necessary to confirm the long-term effects on the intervertebral disc and the surrounding tissues after intradiscal injection of rhMMP-7. We are now proceeding with some experiments to elucidate the long-term effects, including disc narrowing, of rhMMP-7.

In general, we should be careful about extrapolating the results seen in experimental animals to humans. First, this study involved the degradation of native disc by rhMMP-7 using normal dogs in vivo. However, the canine native disc has little relevance to the pathologic disc of human patients with disc herniation. Thus, we performed the in vitro study using the surgical sample of human herniated disc to supplement the in vivo canine data. Second, the grade of low back pain expressed as a visual analogue scale score is one of the most important end points of lumbar disc herniation therapy. Because the grade of low back pain cannot be assessed in animal models, the effects of rhMMP-7 on the pain response should be carefully evaluated in the clinical situation. Third, although the amino acid sequences of the main components (eg, Type 1 and 2 collagens) of the intervertebral discs and the surrounding tissues are probably conserved among humans and dogs, the possibility that significant and unforeseen differences might exist between the two species cannot be excluded. Because the Phase 1/2 study of rhMMP-7 is about to start in the United States, patients’ conditions in the clinical trial should be carefully monitored.

To have the option of chemonucleolysis as an alternative to microdiscectomy would allow surgeons and patients to weigh each procedure’s risk versus benefit and determine the best choice. We expect that chemonucleolysis with rhMMP-7 will become a new alternative treatment for
intervertebral disc herniation and contribute to providing surgeons and patients with the best choices.

Conclusions

This study demonstrated experimental chemonucleolysis with rhMMP-7 in vitro and in vivo. The effects of rhMMP-7 were not affected by the conditions associated with herniated discs. The epidural injection study together with the ARLG and in vitro enzyme assay suggests that intradiscal injection of rhMMP-7 may not induce tissue damage around the discs because of its distribution and substrate selectivity. Recombinant human matrix metalloproteinase 7 may be a novel and promising chemonucleolysis agent.

References