A Mouse Intervertebral Disc Degeneration Model by Surgically-Induced Instability

Takeshi Oichi, MD* Yuki Taniguchi, MD, PhD* Kazuhito Soma, MD* Song Ho Chang, MD, PhD*
Fumiko Yano, D.D.S., PhD† Sakae Tanaka, MD, PhD*, Taku Saito, MD, PhD*

*Sensory & Motor System Medicine, Graduate School of Medicine, The University of Tokyo, Tokyo, Japan
†Bone and Cartilage Regenerative Medicine, Graduate School of Medicine, The University of Tokyo, Tokyo, Japan

Correspondence should be addressed to:
Taku Saito, M.D., Ph.D.
Associate Professor
Sensory and Motor System Medicine, Graduate School of Medicine, The University of Tokyo, Hongo 7-3-1, Bunkyo-ku, Tokyo 113-8655, Japan.
Phone: (+81)-3-3815-5411 (ext. 37369); Fax: (+81)-3-3818-4082;
E-mail: tasaitou-tky@umin.ac.jp
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Structured Abstract

Study Design. Experimental study to develop a mouse model of lumbar intervertebral disc degeneration (IDD).

Objective. The aim of this study was to develop a mouse lumbar IDD model using surgically-induced instability and to compare the findings of this model to those in human IDD.

Summary of Background Data. Previously, various kinds of inducers have been used to reproduce IDD in experimental animals; however, there is yet no standard mouse lumbar IDD model without direct injury to intervertebral disc.

Methods. A total number of 59 C57BL/6J male mice at eight weeks old were used. Instability of lumbar spine was induced by surgical resection of posterior elements including facet joints, supra- and interspinous ligaments. We then analyzed time course changes in radiographical (n = 17) and histological analyses (n = 42), and compared these findings with those in human IDD.

Results. Radiographical analyses showed that the disc height began to decrease in the first two weeks after the surgery, and the decrease continued throughout 12 weeks. Bone spurs at the vertebral rims were observed in the late stage of 8 and 12 weeks after the surgery. Histological analyses showed that the disorder of the anterior anulus fibrosus (AF) was initially obvious, followed by posterior shift and degeneration of the nucleus pulposus (NP). Proteoglycan detected in inner layer of AF and periphery of NP was decreased after 8 weeks. Immunohistochemistry displayed the increase of type I and X collagen, and matrix metalloproteinase 13 in the anterior AF.

Conclusions. Surgical resection of posterior elements of mouse lumbar spine resulted in reproducible IDD. Because the present procedure does not employ direct injury to intervertebral disc and the
radiological and histological findings are compatible with those in human IDD, it may contribute to further understanding of the native pathophysiology of IDD in future.

**Key Words:** intervertebral disc degeneration; mouse model; annulus fibrosus; nucleus pulposus

**Level of Evidence:** N/A
INTRODUCTION

Low back pain is a common complaint which over 80% of adults experience during their lifetimes.\textsuperscript{1} The high morbidity of low back pain is associated with lower health-related quality of life\textsuperscript{2} and high medical expenses,\textsuperscript{3} resulting in human suffering and high socioeconomic costs. Intervertebral disc degeneration (IDD) is a major contributor to low back pain,\textsuperscript{4} and also precedes other spinal disorders such as disc herniation, spondylosis, and lumbar spinal stenosis.\textsuperscript{5} Epidemiologic observation revealed that impact, heavy lifting, and vibration exposure are risk factors for IDD.\textsuperscript{6} Furthermore, acute trauma to intervertebral disc was shown to cause IDD using \textit{ex vivo} whole-organ intervertebral disc culture model.\textsuperscript{7,8} Taken together, excessive mechanical loading is clearly associated with IDD development; however, the underlying mechanisms has yet to be clarified.

Development of appropriate animal models of IDD is imperative to elucidate its molecular pathophysiology. So far, various species have been used, including rodents, rabbits, dogs, goats, sheep, and primates.\textsuperscript{9} Larger animals are appropriate for IDD models, since their spines are more analogous to those of human with respect to biomechanics, geometry and structure. However, it takes a longer time to analyze disc development and subsequent degeneration in larger animals, and their housing is cost-prohibitive.\textsuperscript{10} On the other hand, in smaller animals like rats and mice, IDD changes can be analyzed in a shorter period and the housing is less expensive. Furthermore, mouse genomics and gene modification technology have rapidly progressed. In molecular research of knee osteoarthritis (OA), mouse surgically-induced OA models which were established in 2005,\textsuperscript{11,12} have contributed to elucidation of several essential mechanisms in this decade.\textsuperscript{13-15} Mice may have severe drawbacks arised from its small size, e.g. there may be no nutritional problems, collagen disorganization, or cell death, which are often seen in chronic human IDD.\textsuperscript{16} However, considering the successful progress in the knee OA research, mice maybe useful for IDD research as well, in spite of the small number of previous mouse IDD models.\textsuperscript{17-20}
Previously, various kinds of inducers have been used to reproduce IDD in experimental animals, including compression,\textsuperscript{19,21} injury,\textsuperscript{18,22,23} instability,\textsuperscript{24-28} postural bipedality,\textsuperscript{29,30} chemical,\textsuperscript{31} genetic,\textsuperscript{32} vibration,\textsuperscript{33} spontaneous,\textsuperscript{34} and smoking.\textsuperscript{35} In rodents, annulus puncture, compression by external fixation, or looped tail were utilized to induce IDD. To mimic the pathogenesis of human IDD, surgically-induced instability without direct injury to anulus fibrosus (AF) may be appropriate; however, there is yet no standard mouse IDD model.

In the present study, we aimed to develop a mouse lumbar IDD model for a novel tool to analyze involvement of genes in IDD using genetically modified mice. We employed surgical resection of bilateral inferior articular processes to induce supra-physiologic movement. We then analyzed time course changes in radiographical and histological analyses, and compared these findings with those in human IDD.

**MATERIALS AND METHODS**

**Mice**

All experiments were performed according to a protocol approved by the Animal Care and Use Committee of the University of Tokyo. We selected C57BL/6J mice because they are most widely used for genetically modification. Fifty-nine C57BL/6J male mice (18–22 g) were used for all experiments, during which they were placed in plastic cages with sawdust bedding in a specific pathogen free facility, with four to five mice per cage. The room had a 12 h light/12 h dark cycle and was at a constant temperature (18–22°C). The mice were allowed to move freely in the cages and had free access to food pellets and water.
Surgical Procedures

Skeletally matured mice at the age of 8 weeks were divided into two groups: a surgery group (n = 38) and a sham group (n=21). All surgical procedures were performed under general anesthesia using 2-3% isoflurane. In the surgery group, bilateral facet joints at the L4–L5 level were exposed but not transected. Then bilateral inferior articular processes were transected by microscissors using a surgical microscope, followed by transection of supra- and interspinous ligaments. The operative fields were closed with 4-0 nylon sutures. The procedures are shown in Supplemental video 1, http://links.lww.com/BRS/B308. In the sham group, only the bilateral facet joints at the L4–L5 level were exposed.

Radiographical Analyses

The lateral radiographs of the operated spines were taken before the surgery, and at 2, 4, 8, and 12 weeks after the surgery under general anesthesia using a soft X-ray apparatus (CMB-2; Softex Co., Tokyo, Japan). The disc height index (DHI) was calculated using imageJ software (National Institutes of Health, Bethesda, MD), and changes in the DHI were expressed as %DHI and normalized to the DHI obtained before the surgery as described previously (%DHI = postoperative DHI/ DHI before the surgery x 100). We performed radiological analyses in 11 mice in the surgery group and 6 mice in the sham group.

Histological Analyses

Thirty-six mice were used for histological analyses. The mice were sacrificed by cervical dislocation. Those in the surgery group were sacrificed at 2, 4, 8, and 12 weeks (n = 6 at each time point), and those in the sham group were sacrificed at 2, 4, 8, and 12 weeks (n = 3 at each time point).
For H & E staining and Safranin-O staining, we prepared frozen sections according to Kawamoto’s film method. Briefly, the lumbar spine was removed en bloc, immediately placed into hexane with dry ice, and embedded in SCEM compound (Section-lab, Hiroshima, Japan). We prepared 6-μm sections using Cryofilm Type IIC (Section-lab) in the mid-sagittal plane using a Leica cryostat, and stained with H & E or Safranin-O according to the standard protocol. Images were captured by a Biozero BZ-8100 microscope (KEYENCE, Osaka, Japan).

We quantified the severity of IDD using Masuda’s histological scoring system, which focuses on morphological changes of AF, the border between AF and NP, the cellularity of NP, and the matrix of NP. We quantified 3 sections per mouse. The total scores were summed over all 4 features at each time point. The histological scores were compared between the surgery and sham groups at each time point.

**Immunohistochemistry of Marker Proteins**

For immunohistochemistry, we prepared paraffin-embedded sections at 12 weeks after surgery from both the surgical (n = 3) and sham groups (n = 3). The lumbar spine was fixed in 4% paraformaldehyde for 24 hours, decalcified in 10% EDTA at 4°C for 3 weeks, embedded in paraffin wax and sectioned at 6-μm thickness in the mid-sagittal plane. The sections were deparaffinized with xylene, and incubated with 2.5% hyaluronidase (Sigma, St. Louis, MO) for 30 minutes at 37°C. We analyzed type I collagen (Col1) which is abundantly expressed in bone and ligaments, type II collagen (Col2) known as a cartilage marker, type X collagen known as a specific marker for hypertrophic chondrocytes, and matrix metalloproteinase 13 (MMP13) as a representative catabolic enzyme for cartilage matrix protein. After blocking by PBS containing 1% BSA for 1 hour, the sections were incubated with antibodies to Col1 (1:100; LSL, Tokyo, Japan, LB1102), Col2 (1:100; Merck Millipore, Darmstadt, Germany, MAB8887), Col10 (1:100; LSL, LB0092), and Mmp13 (1:100; Merck Millipore, MAB13426) for overnight at 4°C. The sections were subsequently treated with Anti-Mouse (Col2 and Mmp13) or Anti-Rabbit (Col1 and Col10) Envision-Plus System-HRP (DAKO, Glostrup, Denmark).
for 30 min. Peroxidase labeling was visualized using peroxidase substrate 3,3’-diaminobenzide and was counterstained with methyl green. These histological analyses were performed at least three times using 2–3 mice for confirmation of the results.

Statistical Analyses

Radiographical and histological scores between the surgery and sham groups for each time point were analyzed by Student’s t-test and Mann-Whitney U test, respectively. To assess changes in DHI, one-way ANOVA with the Turkey–Kramer post hoc test was used. All tests were two-tailed. Statistical analyses were performed using JMP software, version 9.0 (SAS Institute, Inc., Cary, NC).

RESULTS

Surgical Resection of Posterior Elements of Mouse Lumbar Spine

First, the L4–L5 level was identified with reference to Jacoby’s line [Fig. 1(A)]. After the facet joints were exposed [Fig. 1(B)], bilateral inferior articular processes, supra- and interspinous ligaments were transected by microscissors under a surgical microscope [Fig. 1(C,D)]. All mice were alive until the sacrifice, and wound infection did not occur.

Time Course of Radiographic Changes

We first examined the time course of radiographic changes in lateral radiographs [Fig. 2(A)]. The disc height, indicated as %DHI, began to decrease in the first two weeks in the surgery group, and the decrease continued throughout the experimental period [Fig. 2(B,C)]. Bone spurs at the anterior vertebral rims and posterior bony fusions were frequently observed in the late stage of 8 and 12 weeks after the surgery [Fig. 2(A)]. The local sagittal alignment at L4/5 became more kyphotic with time [Fig. 2(A)].
Time Course of Histological Changes

We next examined the time course of histological changes. AF cells were localized in orderly stratified curves around the nucleus pulposus (NP) before the surgery [Fig. 3(A)]. At 2 weeks after the surgery, the anterior AF began to be disordered, and this continued to progress throughout [Fig. 3(A)]. The NP began to diminish and be shifted to the posterior side after 4 weeks, and thereafter its degeneration progressed and the border between the NP and AF gradually disappeared [Fig. 3(A)]. Safranin-O staining showed that inner layer of AF and periphery of NP were proteoglycan-rich; however, the intensity was decreased after 8 weeks. These findings were not observed in the sham group [Fig. 3(A)]. Notably, in the late stage, hypertrophic chondrocyte-like cells were observed in the inner layer of AF [Fig. 3(B)]. Quantification by Masuda’s histological grading scales indicated that the scores of AF, border between AF and NP, and matrix of NP in the surgical group were increased in the earlier phase, while that the score of cellularity of NP was increased in the later phase [Fig. 3(C)].

Expression of Marker Proteins

Finally, we examined the expression of marker proteins in IDD. In the sham group, Col1 was detected in the outer layer of AF and in the vertebral bones. In the surgery group, it was intensely observed in and around the hypertrophic chondrocyte-like cells at the inner layer of AF [Fig. 4]. There was no apparent difference in the intensity of Col2 staining between the sham and surgery groups [Fig. 4]. Col10, a specific marker for hypertrophic chondrocytes, and Mmp13, a representative protease of cartilage matrix, were scarcely detected in the AF of the sham group [Fig. 4]. However, in the surgery group, Col10 was detected in and around the hypertrophic chondrocyte-like cells in the inner layer of AF, and Mmp13 was observed in the hypertrophic chondrocyte-like cells at the inner layer of AF [Fig. 4].
DISCUSSION

Here we demonstrated the development of IDD over a time course by lumbar facetectomy of standard experimental mice strain C57BL/6. The radiographical analyses showed disc space narrowing and bony spur formation, which are similar to the features of human IDD.\textsuperscript{37-39} Histological analyses displayed disorganization of lamellar structure in the anterior AF, loss of border between the NP and AF, and shrinkage of NP, which are compatible with the features of human IDD.\textsuperscript{40-44} Furthermore, the increased expression of Col1, Col10, and Mmp13 was similar to the previous results in degenerated human intervertebral disc.\textsuperscript{37,45-48} As a limitation of the present model, we should note the facts that intervertebral discs are not the same in mice and human in terms of their size, nutrition, the biomechanical stress that they receive (less axial compression or torsion), and cell composition (notochordal cells throughout life).\textsuperscript{49} These differences should be considered in interpreting the results obtained from the present model. Furthermore, the histological and radiographical changes observed in the late stage at 8 and 12 weeks after the surgery may be the results of unloading due to the posterior fusion.\textsuperscript{50}

In this study, we selected the facetectomy as the trigger to degenerate an intervertebral disc by supra-physiologic movement in the spine in the same manner as previously reported.\textsuperscript{24,25,27,28} In the previous study, the resection of spinous process and inter- and supraspinous ligaments caused cervical spondylosis over a 6- to 12-month-period in mice.\textsuperscript{26} This procedure, in which the facet joints were not resected, did not cause apparent lumbar IDD on radiographs within 12 weeks after surgery in our preliminary experiments (data not shown). Biomechanical study using human or pig spines showed that instability of the motion segment was increased when lumbar facet joints were resected in addition to other posterior lumbar elements.\textsuperscript{51,52} Thus, it is necessary to resect both facet joints and spinous ligaments to induce lumbar IDD within a short period. In contrast to the present data, the disc height increased after lumbar facetectomy in the recent rat IDD model.\textsuperscript{24} The discrepancy between these
rodent models may be due to the difference of actual surgical procedures, or that of species. Meanwhile, in human, instability can be induced by other means including nucleus decompression following endplate fracture. These various factors involved in stability of spine should be considered in future.

In the present model, histological alteration occurred in AF first, followed by posterior shift and degeneration of NP. These histological changes in a time course are similar to those in the previous mouse cervical spondylosis model. Structures subjected to bending incur greatest stress at locations farthest from the neutral axis, which for the disc is within the annulus. When the spine is destabilized by resection of the posterior elements like the cervical spondylosis model and the present model, the spine seems to be subject to bending more frequently. Thereafter, the anterior AF may suffer the greatest stress because it is located the farthest from the neutral axis, resulting in the initial degeneration there, although human disc degeneration is usually more severe in the posterior annulus rather than anterior annulus. The enhanced osteophyte formation at the anterior vertebral rim in the present model may be the result of remodeling to counteract the excessive movement, probably in response to increased radial bulging of the annulus. Considering that the changes in morphology and marker protein expression were observed mainly in the anterior AF, our model is a useful tool to examine the molecular mechanisms of how supra-physiologic movement initiates the degeneration of AF.

Immunohistochemistry showed that marker protein expression in the AF changed with its degeneration. The expression of Col1 was increased in the inner AF, indicating the AF cells were transformed into a more fibrocartilaginous phenotype, similar to the process of human IDD. This change may be caused by the reduced NP and the subsequent increase of compressive force in inner AF. Col10 was highly detected around the hypertrophic chondrocyte-like cells in the degenerated AF, as observed in the previous studies using human IDD samples. Since the hypertrophic
differentiation of chondrocytes is observed in the mouse knee OA model and is associated with the degeneration of articular cartilage,\textsuperscript{12,58} this finding may occur in similar mechanisms. Upregulation of Mmp13 in these hypertrophic chondrocyte-like cells at the inner layer of AF was also similar to the previous results observed in the inner layer of degenerate human AF.\textsuperscript{46} It may lead to degeneration of extracellular matrix protein, and consequent IDD. As a limitation of the present study, we did not quantify expression levels of these markers. For more precious evaluation, it is desirable to quantify tissue-specific mRNA or protein levels of the markers in the following studies.

In conclusion, we established a reproducible mouse IDD model by surgical induction of lumbar instability. Since C57BL/6 is the most standard strain, application of the present model to various genetically modified mice may contribute to elucidation of IDD pathophysiology. Notably, the radiographical and histological analyses displayed the degeneration of AF in the early stage, and the consequent degeneration of NP in the later stage. These findings may provide a clue to mechanisms of the IDD development.

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References


Figure Legends

Figure 1. The surgical resection of mouse lumbar facet joints. **A**, L4–5 level with reference to Jacoby’s line (red line). The yellow curved dash lines indicate bilateral posterior iliac crests. Scale bar, 10 mm. **B**, Exposed L4-5 facet joints. The curved dash lines indicate outer border of the L4 lamina. Scale bar, 2 mm. Before (**C**) and after (**D**) resection of the inferior articular process by microscissors and sharp tweezers under surgical microscope. The curved dash line in the left panel indicates the inferior articular process, and white arrowheads indicate the supra supraspinous ligaments. Scale bar, 2 mm.
Figure 2. Time course of radiographic changes. A, Lateral radiographs of the operated lumbar vertebrae immediately (0), 2, 4, 8 and 12 weeks after the surgery. White and black arrowheads indicate posterior bony fusion and bone spurs, respectively. Scale bar, 1 mm. B, Measurement of vertebral body height and intervertebral disc. The height of intervertebral disc was expressed by disc height index (DHI). C, %DHI in the surgery (n = 11) and sham groups (n = 6). * P < 0.05, one-way ANOVA. † P < 0.05, student’s t test.
Figure 3. Time course of histological changes. A, H&E (top panels) and safranin-O stainings (middle and bottom panels) of the operated intervertebral disc immediately (0), 2, 4, 8 and 12 weeks after the surgery, and of the sham group at 12 weeks. Inset boxes in the middle panels indicate the enlarged images in the bottom panels. Scale bar, 300 μm for top and middle panels, and 100 μm for bottom panels. B, Safranin-O staining after 12 weeks. An inset box in the left panel indicates the enlarged image in the right panel. Blue arrowheads indicate the hypertrophic chondrocyte-like cells. Scale bar, 300 μm and 50 μm for low and high magnification images, respectively. C, The scores of Masuda’s histological grading scales in the surgery (n = 6 at each time point) and sham groups (n = 3 at each time point). † P < 0.05, Mann-Whitney U test.
Figure 4. Immunohistochemistry of Col1, Col2, Col10, and Mmp13 in the intervertebral disc of the surgery and sham mice at 12 weeks after the surgery. Inset boxes in the left panels indicate the enlarged images in the right panels. Scale bar, 200 and 50 μm for low and high magnification images, respectively.