Evaluation of the association between runt-related transcription factor 2 expression and intervertebral disk aging in dogs

Hisanori Itoh, DVM, PhD; Yasushi Hara, DVM, PhD; Masahiro Tagawa, DVM, PhD; Tsuyoshi Kato, MD, PhD; Hiroki Ochi, DVM, PhD; Daisuke Koga, MD, PhD; Atsushi Okawa, MD, PhD; Yoshinori Asou, MD, PhD

Objective—To investigate the relationship between runt-related transcription factor 2 (RUNX2) expression in canine nucleus pulposus (NP) cells and intervertebral disk aging in chondrodystrophoid dogs.

Animals—7 healthy Beagles (mean age, 35.6 months) and 11 Dachshunds with herniated disks (mean age, 61 months).

Procedures—All dogs underwent MRI examination of the thoracic and lumbar vertebral column immediately before sample collection under general anesthesia. The disk center–to–CSF T2-weighted signal intensity ratio was determined for healthy Beagles. Samples of NP were obtained from nonherniated disks in healthy Beagles and from herniated disks during surgical treatment of hospitalized Dachshunds. Samples were evaluated for RUNX2 and matrix metalloproteinase 13 transcript expression via reverse transcriptase PCR assay; RUNX2 protein expression was evaluated via immunohistochemical analysis, and correlation between these variables and age of dogs was evaluated. A 3′ and 5′ rapid amplification of cDNA ends method was used to identify the RUNX2 coding region.

Results—RUNX2 cDNA had >97% conservation with the human cDNA sequence and approximately 95% conservation with the mouse cDNA sequence; RUNX2 and matrix metalloproteinase 13 mRNA expression and RUNX2 protein expression in NP cells were positively correlated with age. The disk center–to–CSF T2-weighted signal intensity ratio was negatively correlated with RUNX2 protein expression in the NP of healthy dogs.

Conclusions and Clinical Relevance—Results indicated that RUNX2 mRNA and protein expression in the NP are enhanced in aging intervertebral disks in dogs. (Am J Vet Res 2012;73:1553–1559)

The pathogenesis of intervertebral disk aging is poorly understood but is known to be associated with a variety of cellular and biochemical changes. As aging progresses, radial fissures of the annulus fibrosus progress outward from the NP. This process is associated with the activation of degradative enzymes, leading to weakening of the annulus fibrosus.1 Most extracellular matrix degradation is mediated by MMPs.2 Collagenase-3 (MMP-13) is expressed in chondrocytes of human adults3 and is overexpressed in chondrocytes of humans with osteoarthritis4 as well in affected disk tissues of rabbits and rats with experimentally induced disk degeneration.5–7 Thus, MMP-13 is a biological marker of, and may contribute to the pathogenesis of, intervertebral disk degeneration. However, the mechanisms that lead to upregulation of MMP-13 expression in degenerated disks are poorly understood.

The characteristic histologic change associated with disk aging or disk degeneration in humans is cell hypertrophy in the NP.8–10 Phenotypically, NP chondrocytes resemble cartilage chondrocytes.11 Previous in vitro research with cell lines and in vivo research in mice have shown that RUNX2 is required for chondro-
cyte hypertrophy12 and for MMP-13 expression in the hypertrophic region of bone growth plates.13,14 Runt-related transcription factor 2 expression is also induced in the articular cartilage of wild-type mice in early stages of osteoarthritis, and this induction occurs prior to MMP-13 expression,13 indicating that RUNX2 has an important role in the osteoarthritis disease process. Considering the phenotypical similarity between chondrocytes and NP cells, RUNX2 may also be implicated in the progression of intervertebral disk aging. However, the expression pattern of RUNX2 in intervertebral disks is unknown.

Human notochordal cells disappear by 10 years of age,16 but some nonhuman species appear to retain their notochordal cells for an extended period after attaining maturity. Chondrocytoidostrophod dogs, such as Dachshunds and Beagles, are among the few species that have intervertebral disk cell populations mimicking those found in adult humans.17 Dachshunds and Beagles are chondrocytidostrophod dog breeds that become prone to disk disorders as early as adolescence.18 Because Dachshunds have the strongest tendency to develop intervertebral disk disease, it is easy to collect naturally occurring herniated disk specimens from dogs of this breed.18 Thus, the intervertebral disk material from these dogs may be useful for the investigation of mechanisms underlying disk aging in humans and chondrocytidostrophod dogs.

A high degree of accuracy in detecting morphological changes in the spinal column attributable to aging and degeneration has been achieved with MRI. Investigators reported significant correlations of T2-weighted signal intensity with decreases in water and proteoglycan content,19,20 typical features of disk aging. Normal intervertebral disks have high signal intensity, whereas degenerating disks have lower signal intensity.20

The purpose of the study reported here was to investigate the relationship between RUNX2 expression patterns and disk aging in chondrocytidostrophod dogs through examination of the spatial and temporal mRNA expression patterns of RUNX2 and MMP-13 (a biological marker of disk degeneration) in NP from intervertebral disks of healthy Beagles of various ages and from herniated intervertebral disks of Dachshunds. We also sought to compare signal intensity in T2-weighted MRI, which is a distinct marker for intervertebral disk aging, with RUNX2 protein expression.

Materials and Methods

Dogs—Nonherniated NP samples were obtained from 7 Beagles that were part of the Nippon Veterinary and Life Science University’s research colony and were determined to be healthy on the basis of results of physical examination (including neurologic evaluation) by a veterinarian, analysis of MRI images, and evaluation of the results of a CBC and serum biochemical analysis. Herniated disk material was collected from 11 client-owned Dachshunds that underwent surgical treatment for intervertebral disk herniation at the Nippon Veterinary and Life Science University Hospital. These patients were determined to have intervertebral disk herniation via neurologic examination and standard MRI evaluations. Ethical approval for the study was obtained from the institutional review board of Nippon Veterinary and Life Science University, and because all samples from Dachshunds were removed for treatment purposes, owner consent was not required for use of samples from client-owned dogs.

MRI and DC:CSF determination—Healthy Beagles and Dachshunds with herniated disks received medetomidine (0.4 µg/kg, IV) prior to MRI. Anesthesia was induced with propofol (5.5 to 7.0 mg/kg, IV) and maintained with isoflurane in oxygen. Thoracic and lumbar vertebral column MRI examinations were conducted with a clinical 1.5-T unit in all dogs; this was done immediately before euthanasia of the Beagles. Signal intensity of intervertebral disks was evaluated in T2-weighted images. Examination included T2-weighted fast spin echo sagittal images with effective echo and repetition times of 120 and 5,000 milliseconds, respectively. A field of view of 22 × 25 cm, acquisition matrix size of 224 × 256, and 2.2-mm sections with a 0.4-mm section gap were used. For DC:CSF determination in healthy Beagles, the plane with the subjectively brightest disks on midsagittal T2-weighted images was chosen, and a region of interest was selected centrally in the high signal area of each disk and in the free CSF region. The regions of interest in the disk and CSF of each individual dog were of the same size. The T2-weighted signal intensity of the disk centers and of the CSF were measured, and DC:CSF was calculated.

Sample collection—Dachshunds treated for herniated disks received medetomidine (0.4 µg/kg, IV), and anesthesia for surgical decompression was induced as described for MRI procedures. The NP samples collected during surgery (1 sample/dog) were divided into 2 pieces for histologic analysis and PCR analysis. The piece for histologic analysis was transferred to 4% paraformaldehyde in PBS solution, and the piece for RT-PCR was frozen in liquid nitrogen immediately after the resection.

After surgery, patients were monitored during recovery from anesthesia, antimicrobials and pain medications were administered as determined by the attending clinician, and routine postoperative care was provided until discharge from the hospital. Healthy Beagles were euthanized via IV administration of an overdose of sodium pentobarbital, and 1 intervertebral disk, which was determined to be intact via MRI examination, was collected from each dog. Nucleus pulposus samples were obtained, and fibrous tissue, cartilaginous end plate, and outer annulus were removed. Each sample was cut into 2 pieces along the sagittal plane; one piece was used for histologic analysis, and the other was used for RT-PCR analysis as described for samples from Dachshunds.

Histologic analysis—All histologic specimens were decalcified in a 20% EDTA solution (pH, 7.4) after fixation in 4% paraformaldehyde in PBS solution and were subsequently embedded in paraffin. Serial sections (5 µm thick) were cut and immunostained as described elsewhere.13 To determine RUNX2 protein expression, polyclonal antibody against human RUNX226 was used for immunohistochemical analysis. First, we established the specificity of anti-RUNX2 anti-

Unauthenticated | Downloaded 09/16/23 04:14 PM UTC
body against canine samples by immunostaining canine growth plate and bone marrow. Immunopositive signals were detected exclusively in osteoblasts and hypertrophic chondrocytes, indicating that the antibody we used specifically detected canine RUNX2 protein. Next, we performed immunohistological analysis for NP samples. The total number of NP cells and the number of immunoreactive cells were counted via light microscopy at 250x magnification for statistical analysis. Cell count was performed by 1 observer who was blinded with regard to the experimental group. More than 100 cells for each sample were analyzed to evaluate the proportion of cells that tested positive for RUNX2.

**Semiquantitative RT-PCR analysis**—Each disk sample was homogenized with a rotor-stator homogenizer, and total RNA was extracted with a commercially available reagent according to the manufacturer’s instructions. Total RNA yield was quantified by measuring spectrometric absorbance at 260 nm. One microgram of total RNA was reverse transcribed with a proprietary RT product as described by the manufacturer. One microliter of each cDNA product was used for PCR amplification in a PCR thermocycler. Primer sequences obtained from GenBank were as follows: RUNX2 (sense strand, 5′-CCAACCTCCTGTGCTCTGT-3′; antisense strand, 5′-GGTAGAACTCTTGCTCGTC-3′; and GenBank accession No. AY738265), MMP-13 (sense strand, 5′-AATGTTTTCCCGGAACTCT-3′; antisense strand, 5′-GGCCGTGTAGTTGATAGTGG-3′; and GenBank accession No. AF201729), and 18S rRNA (sense strand, 5′-ACCGCAGCTAGGAATAATGG-3′; antisense strand, 5′-CGTTTATGGTCGGAAC-TACG-3′; and GenBank accession No. X03205). Amplification conditions were 30 or 35 cycles (30 cycles for 18S rRNA; 35 cycles for other products) of 94°C for 30 seconds, 57° or 60°C for 30 seconds (57°C for RUNX2 and 60°C for other products), and 72°C for 30 seconds, followed by 1 cycle of 72°C for 7 minutes. Expression levels of RUNX2 and MMP-13 were normalized to 18S expression.

**Sequence analysis of RUNX2**—The unknown canine RUNX2 coding region was identified via 3′- and 5′-RACE, followed by direct sequencing as previously described, with cDNA from Beagle bone tissue. The 3′ and 5′-RACE was performed with a commercially available kit according to the manufacturer’s instructions. Briefly, first-strand cDNA synthesis was initiated on poly(A)+RNA by use of an oligo(dT) anchor primer for 3′-RACE and at the known canine RUNX2-specific sequence (GenBank accession No. AY738265) with the SP1 primer (5′-ggtgaaactcttgcctcgtc-3′). This reaction was followed by the addition of a poly(A) site at the 3′ end by use of terminal deoxynucleotidyl transferase and deoxyadenosine triphosphate for 5′-RACE. Polymerase chain reaction amplification for 3′- or 5′-RACE was performed with either the SP2 (5′-accatggtggagatcatcg-3′) and SP3 (5′-ggggccttcaaggtggtagc-3′) primers or SP1 and SP4 (5′-gcccaacattagctcg-3′) primers. The PCR product was directly sequenced by a DNA sequencer. Results were compared with the sequence of human (GenBank accession No. NM_001024630) and mouse (GenBank accession No. NM_001146038) RUNX2 cDNA to evaluate the degree of conservation among species.

**Statistical analysis**—Pearson linear regression was used to determine the degree of association between RUNX2 or MMP13 mRNA expression and age of dogs, between percentage of cells expressing RUNX2 and age of dogs, and between immunoreactivity for RUNX2 and age of dogs.
ing RUNX2 protein and age of dogs, and between percentage of cells expressing RUNX2 protein and the DC:CSF determined via analysis of MRI images. The linear regression coefficient $R$ and 95% CIs were reported. Values of $P < 0.05$ were accepted as significant.

**Results**

The 7 healthy Beagles included 4 females and 3 males with a mean body weight of 10.7 kg (range, 8.2 to 11.7 kg) and mean age of 3 years (individual ages, 4 months [$n = 2$], 1.5 years [$1$], 2.4 years [$1$], 4 years [$1$], and 10 years [$2$]). The 11 Dachshunds with herniated disks included 1 female and 10 males with a mean body weight of 6.1 kg (range, 4.0 to 7.2 kg) and mean age of 5.1 years (individual ages, 3.8 years [$n = 2$], 4.2 years [$1$], 4.8 years [$1$], 5.2 years [$1$], 5.3 years [$2$], 5.5 years [$2$], 5.9 years [$1$], and 7.7 years [$1$]).

Samples from healthy dogs were collected from intervertebral disks in the T7-8 through L4-5 region. Samples from dogs with herniated disks were obtained from disks in the T11-12 through L4-5 region, and most of these (4/11) were from the T11-12 site. Samples from two 10-year-old Beagles and a 4-year-old Beagle were excluded from MMP-13 mRNA analysis, and 1 sample from a 10-year-old Beagle was excluded from analysis of RUNX2 protein expression because of technical problems.

**Histologic appearance of NP cells**—The histologic appearance of intervertebral disk cells evaluated varied with age. Clusters of large cells were distributed throughout the NP in both 4-month-old Beagles (Figure 1). Whereas notochordal cells (characterized by a large amount of cytoplasm, spindle-shaped nuclei, and presence of interconnections among the cells) were detected in the NP from both 4-month-old Beagles, NP from 4- to 5-year-old dogs (healthy Beagles [$1/1$] and Dachshunds with herniated disks [$8/8$]) appeared to lose their notochordal cells, regardless of the presence or absence of herniated disks.

**Figure 2**—The complete canine RUNX2 predicted amino acid sequence encoded by the cDNA sequence determined via 3′- and 5′-RACE in canine bone tissue. The open reading frame encoded by canine RUNX2 cDNA had > 97% identity with human RUNX2 (NM_001024630) and approximately 95% conservation with mouse RUNX2 (NM_001146038). Dashes indicate absence of amino acid residues. Nonmatching residues are highlighted.

**Figure 3**—Correlation between age of dogs and expression of RUNX2 (A) and MMP-13 (B) mRNA (normalized to 18S expression) in NP. Both RUNX2 and MMP-13 mRNA expression were significantly ($P < 0.001$ and $P = 0.011$, respectively) correlated with age. White triangles indicate NP from healthy Beagles ($n = 7$ for RUNX2 and 4 for MMP-13). Gray circles indicate NP from herniated disks of Dachshunds (11).
sence of a pathological condition. Instead, this tissue contained small clusters of unconnected chondrocyte-like cells, characterized by small amounts of cytoplasm and round nuclei in these older dogs.

Sequence analysis of RUNX2 cDNA—The complete sequence of RUNX2 cDNA determined via 3′- and 5′-RACE from canine bone tissue was compared with that of human and mouse RUNX2 orthologs. The open reading frame encoded by canine RUNX2 cDNA had > 97% conservation with human RUNX2 and approximately 95% conservation with mouse RUNX2; the resulting predicted amino acid sequence was summarized (Figure 2).

Correlation analysis—Expression of RUNX2 and MMP-13 mRNA in disk samples (determined via RT-PCR analysis) was positively correlated with age of the dogs. Correlations between age and RUNX2 mRNA expression ($R = 0.774$ [95% CI, 0.481 to 0.911]; $P <$
herniated; by breed or by the condition of disks (nonherniated vs
17), and this association did not appear to be affected
1558   AJVR, Vol 73, No. 10, October 2012

duration and hypertrophy in developing growth plates
changes resemble characteristics of chondrocyte mat-
trix remodeling, proliferation, and apoptosis, are as-

ers of intervertebral disk aging in dogs.

that RUNX2 may be among the candidates for biomark-
with the DC:CSF in MRI images. These data suggest
positively correlated with age and negatively correlated
ing to the percentage of RUNX2-positive NP cells) was
more, RUNX2 protein expression (determined accord-
ly increased in NP with increasing age of dogs. Further-
pressing RUNX2 were not detected in the dogs that
were approximately 4 months old. The percentage of
RUNX2-positive cells in NP was also correlated with age
(R = 0.764 [95% CI, 0.448 to 0.910]; P < 0.001; n =
17), and this association did not appear to be affected
by breed or by the condition of disks (nonherniated vs
herniated; Figure 4).

Whereas NP from a 1.5-year-old Beagle contained
only a few RUNX2–expressing cells as determined via
immunohistochemical analysis, RUNX2–expressing cells
were abundant in NP from 4- to 5-year-old dogs
(9/9), especially in chondrocyte-like cells. Cells ex-
pressing RUNX2 were not detected in the dogs that
were approximately 4 months old. The percentage of
RUNX2–positive cells in NP was also correlated with age
(R = 0.764 [95% CI, 0.448 to 0.910]; P < 0.001; n =
17), and this association did not appear to be affected
by breed or by the condition of disks (nonherniated vs
herniated; Figure 4).

The DC:CSF in MRI images was negatively corre-
related (R = -0.910 [95% CI, -0.987 to -0.499]; P =
0.005; n = 7) with the percentage of RUNX2–positive
cells in NP samples from healthy Beagles (Figure 5).
This finding was in agreement with RT-PCR results indi-
cating that RUNX2 expression in NP was positively correlated with the degree of disk aging.

Discussion

Results of the study reported here suggested that expression of RUNX2 and MMP-13 mRNA was similarly increased in NP with increasing age of dogs. Furthermore, RUNX2 protein expression (determined according to the percentage of RUNX2–positive NP cells) was positively correlated with age and negatively correlated with the DC:CSF in MRI images. These data suggest that RUNX2 may be among the candidates for biomarkers of intervertebral disk aging in dogs.

Changes in NP cell characteristics, including matrix remodeling, proliferation, and apoptosis, are associated with the progression of disk aging, and these changes resemble characteristics of chondrocyte maturation and hypertrophy in developing growth plates or joint cartilage.17 We hypothesize that the enhanced expression of RUNX2 mRNA and protein detected in aging intervertebral disks of dogs in the present study may activate a subsequent program of gene expression

Figure 5—Correlation between DC:CSF and the percentage of cells expressing RUNX2 protein in NP samples from healthy Beagles (n = 7). A strong negative correlation (P = 0.005) was detected.

in NP cells. Overexpression of RUNX2 has been shown to promote collagen type X expression in chondrocytes as well as vascular endothelial growth factor expression in fibroblasts.15 Collagen type X and vascular endothelial growth factor expression are upregulated in osteo-
arthritic cartilage in humans24 and also in herniated or aged disks in humans and dogs.25,26 Thus, increased RUNX2 expression may be among the phenomena of disk aging in multiple species.

Two major classifications of herniated disks have been described: the herniated disk, which includes transligamentous extrusion–type and sequestrated-type herniations, and the nonprolapsed disk, which includes protrusion-type and subligamentous extrusion–type herniations.21 Clinical and basic science evaluations of human patients with disk herniation revealed that extruded disks have more severe inflammation than do nonextruded disks,25,26 most likely because extruded disk material is exposed to the patient’s immune sys-

tem. In the present study, because all samples of the herniated disk group were from extruded disk material, an effect of inflammation on the variables examined could not be excluded. Regardless, chondrocyte-like disk cells expressed RUNX2 in the NP of both nonherniated and herniated disks, suggesting that increased RUNX2 expression should be considered a feature of the natural process of intervertebral disk aging.

The mechanism underlying the enhancement of RUNX2 expression in aging NP remains unknown. Run-related transcription factor 2 has been reported10 to be a target of mechanical signals, mainly in osteo-

bластs, causing anabolic activity in bone. Minor stretch-

ing as well as extracellular nucleotides released in response to mechanical stimuli upregulates the expression of RUNX2 mRNA and protein and increases the DNA binding activity of RUNX2 protein in cultured osteoblasts.31 Thus, we believe it is possible that in-

creased mechanical stress might also be among the trig-
gers for enhancement of RUNX2 expression in inter-
vertebral disks.

The present study had several limitations, most importantly the small number of dogs evaluated in the 2 groups (ie, those with herniated and nonherniated disks) and the inclusion of 2 breeds of dogs (because of ethical reasons). Samples from healthy aged Beagles were excluded from some analyses because of technical problems, which was also a limitation that interfered with evaluation of whether there was an association be-
tween MMP-13 and RUNX2 expression in these disks or whether MMP-13 upregulation may have been attrib-
utable to disk extrusion. However, to our knowl-
edge, the present study is the first to reveal that RUNX2 transcript and protein expression in NP cells are corre-
lated with disk aging in dogs. Further study is required to reveal the mechanisms and function of RUNX2 in the process of disk aging.

Results of the present study suggest that RUNX2 transcript and protein expression, potentially in com-

bination with MMP-13, are enhanced in aged interver-
tebral disks of dogs. Decreasing RUNX2 expression in intervertebral disks may potentially be a novel strategy for reducing changes related to disk aging in future studies.
References


