FULL PAPER *Surgery*

Degenerative changes of the cranial cruciate ligament harvested from dogs with cranial cruciate ligament rupture

Tom ICHINOHE[1)]*, Nobuo KANNO[1)], Yasuji HARADA[1)], Takuya YOGO[1)], Masahiro TAGAWA[1)], Satoshi SOETA[2)], Hajime AMASAKI[2)] and Yasushi HARA[1)]

[1)]*The Laboratory of Veterinary Surgery, Nippon Veterinary and Life Science University, 1-7-1 Kyounan, Musashino, Tokyo 180–8602, Japan*
[2)]*The Laboratory of Veterinary Anatomy, Nippon Veterinary and Life Science University, 1-7-1 Kyounan, Musashino, Tokyo 180–8602, Japan*

(Received 27 July 2014/Accepted 5 February 2015/Published online in J-STAGE 17 February 2015)

ABSTRACT. Degenerative cranial cruciate ligament (CCL) rupture is characterized histologically by degenerating extracellular matrix (ECM) and chondroid metaplasia. Here, we describe the progression of chondroid metaplasia and the changes in the expression of ECM components in canine CCL rupture (CCLR). CCLs from 26 stifle joints with CCLR (CCLR group) and normal CCLs from 12 young beagles (control group) were examined histologically and immunohistochemically for expression of type I (COLI), type II (COLII), type III collagen (COLIII) and Sry-type HMG box 9 (SOX9). Cell density and morphology of CCLs were quantified using hematoxylin–eosin staining. The percentage of round cells was higher in the CCLR group than in controls. COLI-positive areas were seen extensively in the connecting fibers, but weakly represented in the cytoplasm of normal CCLs. In the CCLR group, there were fewer COLI-positive areas, but many COLI-positive cells. The percentages of COLII-, COLIII- and SOX9-positive cells were higher in the CCLR group than in controls. The number of spindle cells with perinuclear halo was high in the CCLR group, and most of these cells were SOX9-positive. Deposition of COLI, the main ECM component of ligaments, decreased with increased COLIII expression in degenerated CCL tissue, which shows that the deposition of the ECM is changed in CCLR. On the contrary, expression of SOX9 increased, which may contribute to the synthesis of cartilage matrix. The expression of COLII and SOX9 in ligamentocytes showed that these cells tend to differentiate into chondrocytes.
KEY WORDS: cranial cruciate ligament disease, SOX9, type I collagen, type II collagen, type III collagen

doi: 10.1292/jvms.14-0383; *J. Vet. Med. Sci*. 77(7): 761–770, 2015

Canine cranial cruciate ligament rupture (CCLR) is one of the most common diseases of the canine stifle joint [28]. The cranial cruciate ligament (CCL) prevents cranial displacement of the tibia on the femur and abnormal internal rotation of the tibia [2]. Cranial tibial thrust, which is the force generated during weight bearing on the hind limb [24], and an abnormal internal rotation of the stifle may cause subsequent development of progressive stifle osteoarthritis and secondary meniscal damage in dogs affected by CCLR [18]. Although anterior cruciate ligament (ACL) rupture can occur acutely with trauma in humans, most canine CCLRs occur secondary to chronic degenerative changes in the CCL [9]. These are collectively referred to as cranial cruciate ligament disease (CCLD) [3, 9].

It is suggested that various factors affect degeneration of the CCL, for example age, breed, sex hormones, body weight and excessive tibial plateau angle (TPA) [8]. Histological changes, such as decreasing cell density, disorganization of collagen fibers and phenotypic changes in ligamentocytes, have been reported in the degenerated CCL [13]. One key histological characteristic is the alteration of the extracellular matrix (ECM) [19], particularly in chondroid metaplasia [7]. Vasseur *et al*. [26] reported that ligaments with histological signs of chondroid metaplasia on hematoxylin–eosin (HE)-stained tissue sections have reduced mechanical properties in dogs >5 years of age and with body weight of >15 kg. The ECM of ligaments is composed of a large amount of type I collagen (COLI), which is the main determinant tensile strength of the ligament [27]. In humans, chondroid metaplasia leads to a decrease of COLI and an increase of cartilage matrix components, such as type II (COLII), III (COLIII) and X collagens, in the ECM of degenerating ligaments [12]. Comerford *et al*. [7] reported that a similar fibrocartilaginous appearance was seen in interfascicular areas in Alcian blue–periodic acid–Schiff-stained sections of the CCLs of normal Labrador retrievers and Greyhounds. The authors proposed that this degenerative change is a physiological, not pathological, adaptation to repetitive stress or response to micro-injury to protect CCLs. However, the underlying mechanisms are not well documented in the degenerated CCL of dogs. Furthermore, regulatory mechanism of differentiation from ligament cells to chondrocytes remained to be unclear. In the process of chondrogenesis, Sry-type HMG box 9 (SOX9), a transcription factor specifically expressed in chondrocyte-lineages, directs mesenchymal stem cells (MSCs) to undergo chondrogenic differentiation and to activate transcription of chondrocyte-specific genes, such as COLII and aggrecan [1, 16, 17]. In dogs, however, no studies

*CORRESPONDENCE TO: ICHINOHE, T., Nippon Veterinary and Life Science University, Department of Veterinary Surgery, 1–7–1 Kyonan-Cho, Musashino-City, Tokyo 180–8602, Japan.
e-mail: Tom.Ichinohe@gmail.com

©2015 The Japanese Society of Veterinary Science

This is an open-access article distributed under the terms of the Creative Commons Attribution Non-Commercial No Derivatives (by-nc-nd) License <http://creativecommons.org/licenses/by-nc-nd/3.0/>.

Table 1. Dogs with ruptured cranial cruciate ligament

Case No.	Affected side of limb	Sex	Age (months)	Body weight (kg)	Breed	Tibial plateau angle (deg)	Period from onset of clinical signs to operation (days)
1	Right	nf	118	33.4	Golden Retriever	28	30
2	Left	nf	120	33.35	Labrador Retriever	25	36
3	Right	nf	64	38.5	Bernese Mountain Dog	31	14
4	Right	nf	75	32	Bernese Mountain Dog	32	17
5	Right	nf	101	34.4	Bernese Mountain Dog	28.5	62
6	Left	nf	87	21.2	Siberian Husky	25	27
7	Right	nm	78	34.5	Golden Retriever	25	120
8	Left	m	43	35	Bernese Mountain Dog	22	112
9	Left	m	124	20.35	Beagle	33	40
10	Left	nm	72	78	Pyrenean Mastiff	34	38
11	Left	nm	39	39.3	Golden Retriever	33.5	70
12	Right	nf	75	30.1	Siberian Husky	26	300
13	Right	m	93	18.18	Shiba Inu	22	48
14	Right	m	120	13.2	Shiba Inu	24	32
15	Left	nm	121	15.3	Beagle	30.5	75
16	Right	nm	68	16.5	Pembroke Welsh Corgi	36	29
17	Left	nf	85	31.05	Labrador Retriever	26	82
	Right		85	31.05			
18	Left	nf	58	26.5	Siberian Husky	28	119
	Right		83	27.1		27	100
19	Left	nm	115	35	Golden Retriever	27	36
	Right		128	31.25		33.5	4
20	Left	nm	137	14.1	Shiba Inu	33	19
	Right		120	14.4		33	58
21	Left	nf	132	10.8	Shiba Inu	22	72
	Right		132	10.8		22	72

m: Male, f: Female, nm: Neutered male, nf: Neutered female

have addressed whether ligament cells in degenerating CCLs induce SOX9. The objective of this study was to describe the changes in the expression of ECM components (COLI, COLII and COLIII) and SOX9 in the CCLs with chondroid metaplasia of dogs affected by CCLR.

MATERIALS AND METHODS

Specimen collection: CCL specimens were collected from 26 stifle joints of 21 dogs with ruptured CCL (CCLR group). Ruptured CCL was diagnosed on physical examination by demonstrating stifle joint instability and confirmed at the time of surgery. Normal CCL specimens were collected from 12 young beagles (9 sexually intact females and 3 sexually intact males) without stifle joint pathology that were euthanatized by intravenous administration of barbiturates for reasons unrelated to this study (control group). Euthanasia of the dogs was performed in accordance with the Guidelines for Care and Use of Laboratory Animals of Nippon Veterinary and Life Science University (approval No. 46J-27). Mean body weight of the control group was 9.6 ± 0.5 kg, age was 15.2 ± 4.9 months, and TPA was 29.3 ± 3.9°. This group of dogs was selected to provide baseline data for comparison. The breed, age, weight, gender, TPA and period from onset of clinical sign to operation for each dog with CCLR were recorded (Table 1).

Sample preparation and histological analysis: Remnants of the ruptured CCL were excised from the femoral and tibial attachment sites, and the tibial attachment sites were used for this study. For the control dogs with normal CCL, the entire ligament was collected. CCL specimens were fixed in 4% paraformaldehyde, embedded in paraffin wax, sectioned longitudinally and stained with HE and Alcian blue (AB) for light microscopy.

Immunohistochemistry: All specimens were used for immunostaining. Paraffin-embedded specimens were first deparaffinized in xylene and ethanol before rehydration in water. Endogenous peroxidase was quenched for 30 min with 3% H_2O_2 in methanol. After a wash with phosphate-buffered saline (PBS), antigen retrieval was performed by incubation in citrate buffer (0.01 M, pH 6.0) for 60 min at 60°C. Specimens were then cooled slowly and washed with PBS. Sections were blocked with BlockAce (BlockAce; DS Pharma Biomedical Co., Ltd., Osaka, Japan.) for 30 min at room temperature before applying COLI, COLII, COLIII and SOX9 antibodies. Sections were incubated overnight at 4°C with primary antibodies against COLI (polyclonal antibody against human-bovine COLI [1:5,000 dilution]; LSL Co., Ltd., Tokyo, Japan), COLII (polyclonal antibody against human-bovine COLII [1:5,000 dilution]; LSL Co., Ltd.), COLIII (polyclonal antibody against human-bovine COLIII [1:5,000 dilution]; LSL Co., Ltd.) and SOX9 (polyclonal antibody against human SOX9 [0.25 μg/m*l*]; Acris Antibodies Inc., San Diego, CA, U.S.A.). The primary antibodies

used in this study for COLI and SOX9 were guaranteed by the manufacturers to have cross-reactivity with canine tissues. To confirm cross-reactivity, appropriate negative and positive controls were included in each immunostaining protocol. The normal canine embryonic bone and cartilage were selected as the positive controls for COLI, COLII and SOX9, and the normal canine mandibular lymph node was selected for COLIII. After washing with PBS, sections were incubated with horseradish peroxidase (HRP)-labeled goat anti-rabbit immunoglobulin antibody (HRP-labeled goat anti-rabbit immunoglobulin antibody; Dako Japan Inc., Tokyo, Japan) for 30 min at room temperature. Finally, sections were stained with LSAB2 kit/HRP (Dako Japan Inc.) for 2 min. Slides were then rinsed in tap water, counterstained with hematoxylin, washed and mounted.

Cell density and morphology: It is reported that after CCLs transected completely, there was some early fibroblastic proliferation of cut ends until 4 weeks after transected, and by 4 weeks, new collagen formation was seen in the cut ends of the CCLs. Then, it is also reported that the changes in the cut ends of the CCLs were little at 10 weeks from that at 4 weeks [23]. Therefore, according to the period from onset of clinical signs to operation, the CCLR group was classified as follows: up to 4 weeks, acute group; 4–10 weeks, subacute group; and >10 weeks, chronic group. For the quantification of cell density and classification of cell morphology after HE staining, and to determine the number of positive cells following immunostaining of COLII, COLIII and SOX9 in the CCL, at least three different macroscopic fields (100 ×) for each sample were randomly chosen and analyzed by one reader (T. I.). Moreover, the number of SOX9-positive cells with periarticular halo (manifestation of cell border with cytoplasmic enlargement) containing fine granules was determined [22]. Cell counts were performed twice. Round cells and spindle cells were defined as follows [15]: cells whose cellular long axis could not be determined were defined as round cells, and all the others were defined as spindle cells. All specimens were analyzed in core regions of the CCLs. The percentage of cell positive rate was calculated as follows: (positive cells number/total cell number) × 100. The percentages of positive spindle or round cells to the total positive cells were calculated as: (number of positive spindle or round cells/total number of positive cells) × 100. Cell density was calculated as the total number of cells from the chosen field divided by the area of analysis. The percentage of spindle or round cell with halo was calculated as follows: (the number of the spindle or round cells with halo/ the number of total spindle or round cells) × 100. The Vasseur scoring system was used to classify the degree of degeneration of the CCL specimens [26]. The areas between bony attachment sites and torn ends were analyzed; bony attachment sites and torn ends were excluded from the analysis.

COLI and COLIII content: To evaluate the COLI and COLIII immunoreactivity of the CCL specimens, slides stained for COLI and COLIII were scanned by using a microscope digital camera (DP72; Olympus Co., Ltd., Tokyo, Japan) at 10× magnification. Three fields were randomly selected and digitally analyzed with Aperio software (Leica Microsystems Inc., Tokyo, Japan). Aperio software generated intensity indices of brown color of each chosen field, which constituted a quantitative indicator of COLI and COLIII content in the CCL [5]. The percentage of COLI- and COLIII-positive pixels in each chose field was calculated as total number of positive pixels divided by total number of pixels × 100.

Statistical analysis: Tukey's honestly significant difference (HSD) test was used to compare the control, acute, subacute and chronic groups. The 2-sample *t*-test and Welch's *t*-test were used to compare round cells and spindle-shaped cells, for parametric and nonparametric data, respectively. Tests for no correlation were performed to correlate independent factors (age, body weight and TPA) with dependent variables. Differences were considered significant at $P<0.05$. Results are reported as the mean ± standard deviation (SD).

RESULTS

All stifles of the CCLR group had palpable instability and were confirmed with complete rupture. Among the 26 stifles with ruptured CCL, 18 had meniscal tears. Synovial fluids showed typical changes of osteoarthritis including mild inflammation with mild to moderate increases in mononuclear cell numbers in all cases. In addition, 15 stifles were confirmed to be chronic synovitis by pathologists during pathological examination of stifle synovial membranes collected at the time of surgical treatment.

Ligament fibers and numerous spindle-shaped and few round-shaped ligament cells showed arrangements and were observed in the control group (Fig. 1A). Conversely, the number of spindle-shaped ligament cells decreased, and the percentage of round ligament cells increased in the CCLR group (Fig. 1B). Cell density was significantly lower in the acute, subacute and chronic groups (305.9 ± 185.8, 322.9 ± 177.5 and 381.6 ± 186.8 cells/mm^2, respectively) than in the control group (719.4 ± 212.6 cells/mm^2). There was no significant difference among each group in the CCLR group (Fig. 1C). However, the percentage of round cells was significantly higher in the acute, subacute and chronic groups (40.0 ± 21.9, 32.1 ± 19.4 and 41.6 ± 24.4%, respectively) than in the control group (1.3 ± 1.9%). There was no significant difference among each group in the CCLR group (Fig. 1D). According to the Vasseur scoring system, in the control group, 7 stifles were classified as grade 0, and 5 stifles were classified as grade 1. In the CCLR group, 2, 9 and 15 stifles were classified as grades 1, 2 and 3, respectively. The grade in the CCLR group tended to be higher than in the control group.

In the AB-stained sections, fewer AB-reactivity was detected in the ligament fibers in all control animals (Fig. 2A), whereas the largest reactivity was seen in the ECM in ligaments of all CCLR animals (Fig. 2B). There is obvious difference in AB-pattern between the control and CCLR groups.

COLI immunoreactivity was detected in the bone matrix, but not in the calcified cartilage matrix in the primary bone trabeculae (Fig. 3A). COLII-positive areas were seen in the cartilage matrix, but not in the bone area (Fig. 3B). COLIII-positive areas were seen in the connective tissues around the blood vessels of the mandibular lymph node (Fig. 3C).

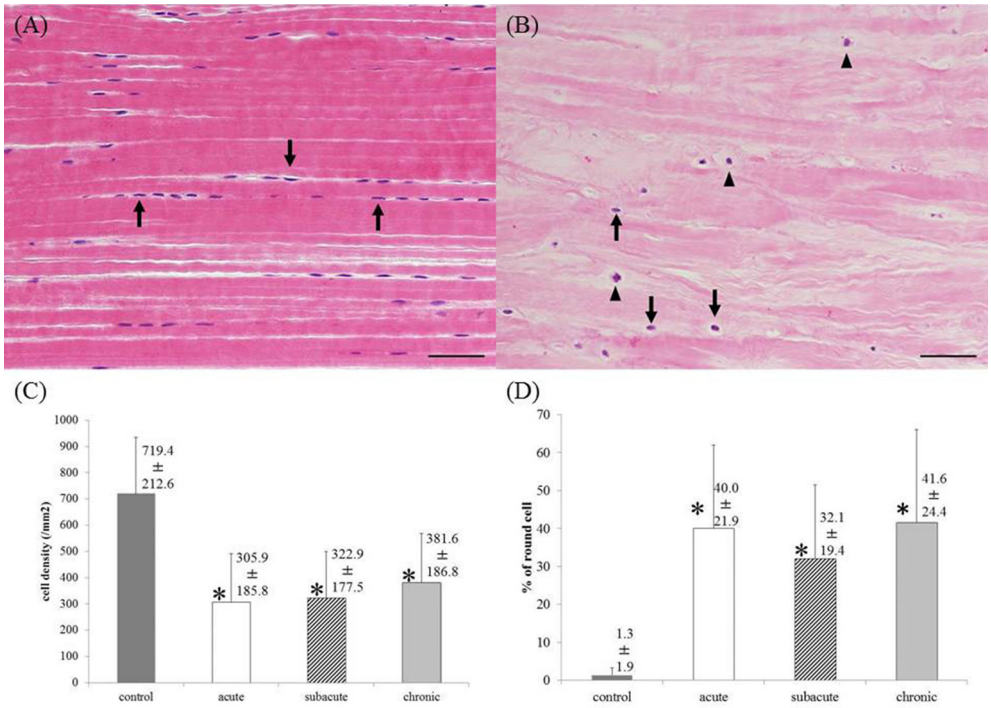


Fig. 1. Cell density and percentage of round cells in CCL specimens. (A) In control CCL specimens, cell density is high, and many spindle cells (arrows) are observed. Scale bar=50 μm. (B) In CCLR, cell density is low, and spindle cells (arrows) and round cells (arrowheads) are observed. HE staining; scale bar=50 μm. (C) Cell density in each group. * P<0.05 by Tukey's HSD test vs. control group. (D) Percentage of round cells in each group. * P<0.05 by Tukey's HSD test vs. control group.

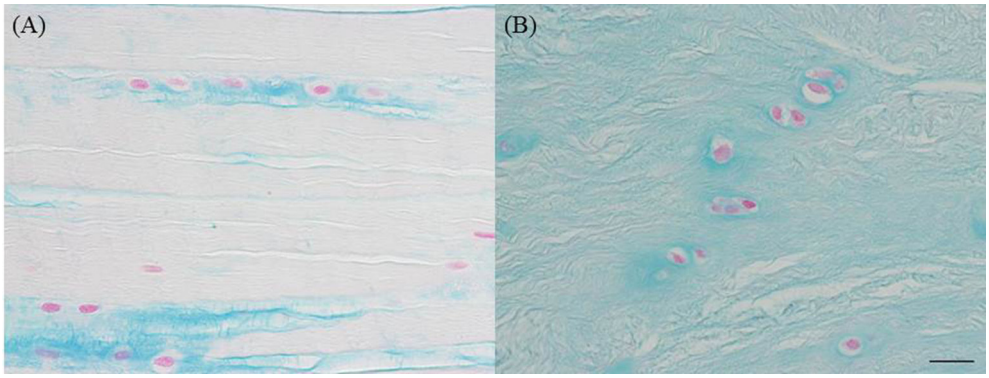


Fig. 2. AB staining of CCL specimens. (A) Little AB reactivity was detected in the ligament fibers of control animals; scale bar=20 μm. (B) AB reactivity was seen in the ECM in ligaments of all CCLR animals; scale bar=20 μm.

Only chondrocytes showed positive reaction against SOX9 antibody (Fig. 3D).

In contrast, no positively stained areas were seen in the negative controls of CCLR and control groups (Fig. 3E and 3F). These findings indicate that the primary antibodies have cross-reactivity with canine tissues.

Extensive COLI-positive areas were seen in the ligament fibers of the control group. However, expression of COLI was low in the cytoplasm of the ligament cells (Fig. 4A). Compared with the control group, COLI-positive areas were fewer in the CCLR group. Conversely, many of the ligament cells expressed COLI in the cytoplasm (Fig. 4B). The ECM was stained larger in the control group (average percentage of COLI-positive pixels, 47.0 ± 20.6%) than in the acute, subacute and chronic groups (16.7 ± 16.9, 15.3 ± 16.7 and 11.1 ± 13.1%, respectively). There was no significant difference among each group in the CCLR group (Fig. 4C).

Only a few COLII-positive cells were observed in the

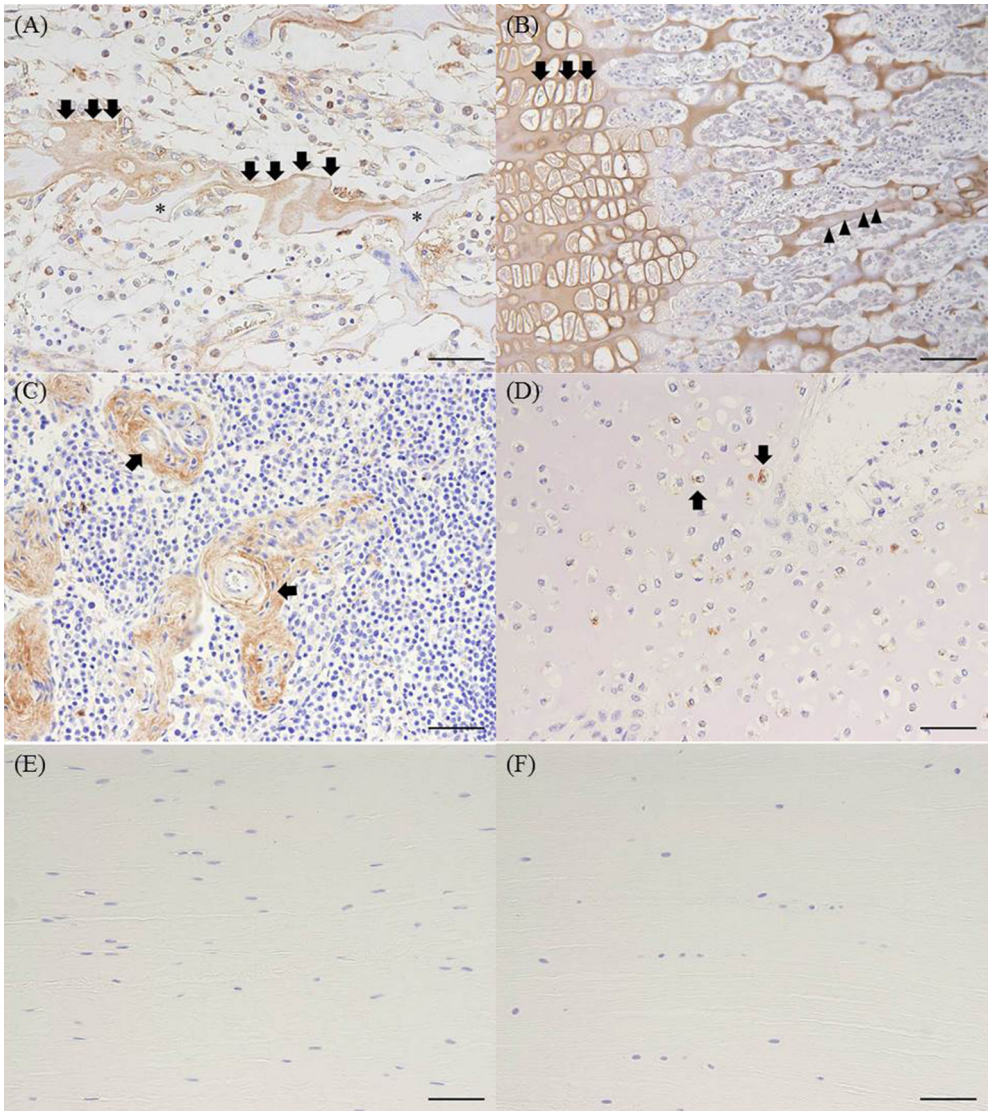


Fig. 3. Positive and negative controls for primary antibodies. (A) Normal canine embryonic bone. COLI immunoreactivity was detected in the bone matrix (arrows), but not in the calcified cartilage matrix in the primary bone trabeculae (asterisks). (B) Normal canine embryonic bone. This image shows the border region between the bone and the cartilage. COLII-positive areas are seen in the cartilage matrix (arrows), but not in the bone area (arrowheads). (C) Normal canine mandibular lymph node. COLIII-positive areas are seen in the connective tissues around the blood vessels of the mandibular lymph node (arrows). (D) Normal canine embryonic cartilage. Only chondrocytes show positive reaction against SOX9 antibody. (E) Negative control of the control group. No positively stained areas are seen. (F) Negative control of the CCLR group. No positively stained areas are seen; scale bar=50 μm.

ligaments of the control group (Fig. 5A), but there were many COLII-positive cells, especially round cells, in the CCLR group (Fig. 5B). The percentage of COLII-positive cells was significantly higher in the acute, subacute and chronic groups (40.8 ± 28.7, 35.5 ± 20.8 and 45.9 ± 28.2%, respectively) than in the control group (12.8 ± 9.1%). There was no significant difference among each group in the CCLR group (Fig. 5C). In the control group, the percentage of COLII-positive round cells (62.5 ± 35.9%) was higher than that of the positive spindle cells (11.9 ± 8.7%, Fig. 5D). Similarly, in each group of the CCLR group, the percentage of COLII-positive round cells was higher than that of the positive spindle cells (Fig. 5D).

Only a few COLIII-positive cells were observed in the ligaments of the control group (Fig. 6A), but there were many COLIII-positive cells, especially round cells, in the CCLR group (Fig. 6B). Moreover, some COLIII-positive areas were seen in the ligament fibers, which were irregular and obscure, of the CCLR group (Fig. 6C). The ECM was stained in larger area in the subacute group significantly (average percentage of COLIII-positive pixels, 0.60 ± 0.62%) than in the control group (0.18 ± 0.21%) and tended to be larger in chronic group (0.50 ± 0.62%) than control group.

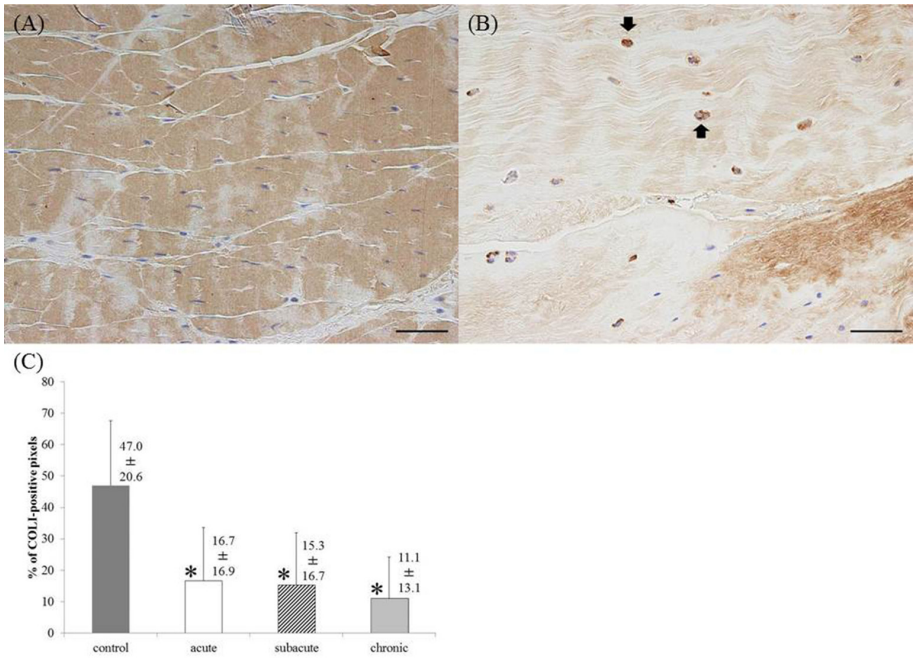


Fig. 4. Immunostaining for COLI. (A) Extensive COLI-positive areas are seen in the ligament fibers of a control CCL. The expression of COLI is low in the cytoplasm of the ligament cells; scale bar=50 μm. (B) COLI-positive areas are sparse in the fibers in the ligament, and many of the ligament cells express COLI in the cytoplasm (arrows) of a CCL in the CCLR group; scale bar=50 μm. (C) The percentage of COLI-positive pixels. The ECM of the control group contained a higher percentage of COLI-stained cells than the ECM of the acute, subacute and chronic groups.

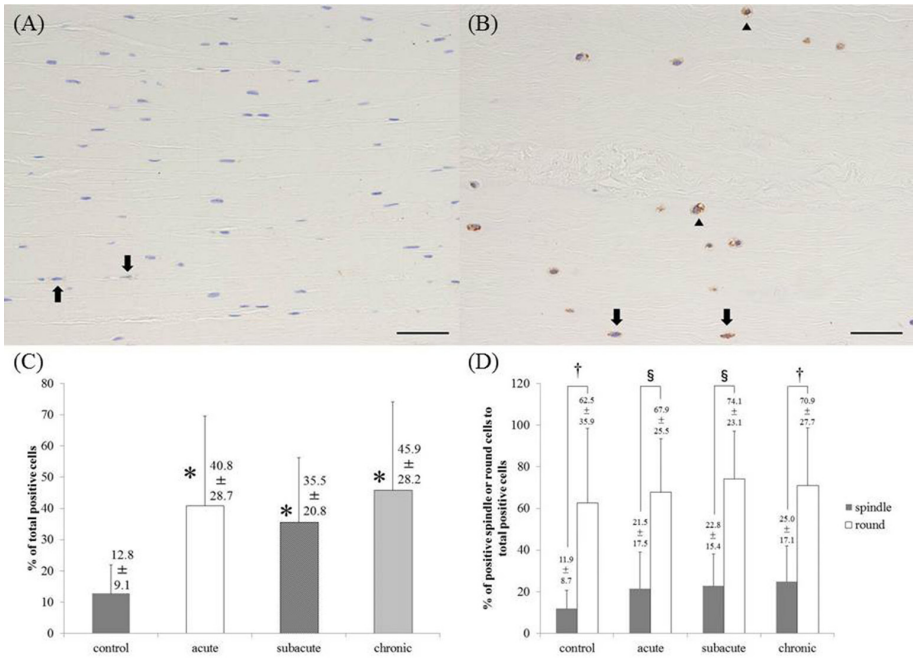


Fig. 5. Immunostaining for COLII. (A) Control group specimen. Only a few spindle COLII-positive cells (arrows) are observed; scale bar=50 μm. (B) CCLR group specimen. Many COLII-positive cells, spindle cells (arrows) and round cells (arrowheads) are observed; scale bar=50 μm.(C) Comparison of the percentage of COLII-positive cells among each group. * P<0.05 by Tukey's HSD test vs. control group.(D) Comparison of the percentage of positive cells between spindle and round cells in each group. § P<0.05 by two-sample t-test. † P<0.05 by Welch's t-test.

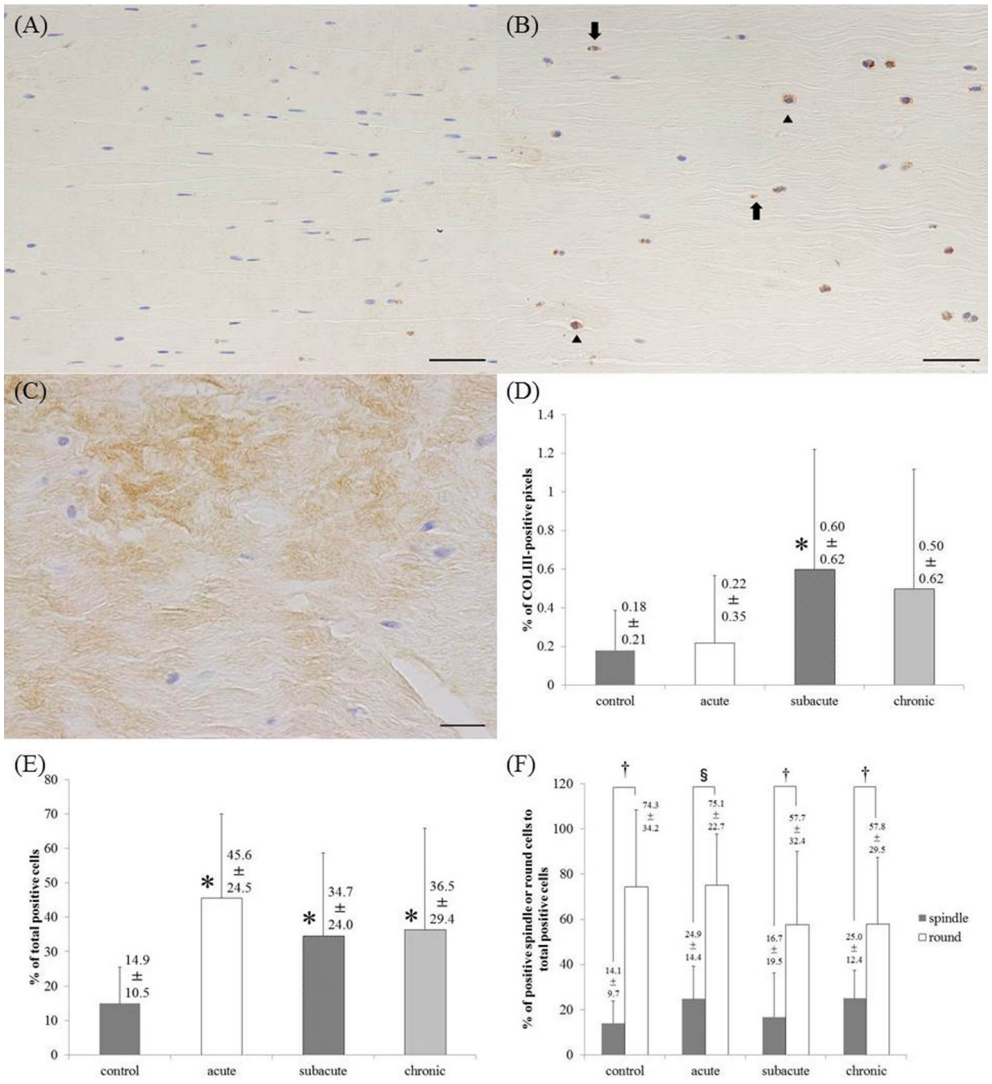


Fig. 6. Immunostaining for COLIII. (A) Control group specimen. Few COLIII-positive cells are observed; scale bar=50 μm. (B) CCLR group specimen. Many spindle (arrows) and especially round (arrowheads) COLIII-positive cells are observed; scale bar=50 μm. (C) Some COLIII-positive areas are seen in the ligament fibers of the CCLR group; scale bar=20 μm. (D) The percentage of COLIII-positive pixels. The ECM of the subacute group contained a significantly higher percentage of COLIII-stained cells than the ECM of the control group, and the percentage tended to be larger in the chronic group than in the control group. There was no difference in the percentage of COLI-positive pixels between the control group and acute group. (E) Comparison of the percentage of COLIII-positive cells among each group. * P<0.05 by Tukey's HSD test vs. control group. (F) Comparison of the percentage of COLIII-positive cells between spindle and round cells in each group. § P<0.05 by two-samples t-test. † P<0.05 by Welch's t-test.

There was no difference in the percentage of COLIII-positive pixels between the control group and acute group (0.22 ± 0.35%, Fig. 6D). The percentage of COLIII-positive cells was significantly higher in the acute, subacute and chronic groups (45.6 ± 24.5, 34.7 ± 24.0 and 36.5 ± 29.4%, respectively) than in the control group (14.9 ± 10.5%). There was no significant difference among each group in the CCLR group (Fig. 6E). In the control group, the percentage of COLIII-positive round cells (74.3 ± 34.2%) was higher than that of the positive spindle cells (14.0 ± 9.7%, Fig. 6F). Similarly, in each group of the CCLR group, the percentage of COLIII-positive round cells was more than that of the positive spindle-shaped cells (Fig. 6F).

Only a few spindle cells with perinuclear halo were observed in the ligaments of the control group, and most of these cells showed the SOX9-positive reaction (Fig. 7A). On the other hand, there were many spindle and round cells with perinuclear halo in the ligaments of the CCLR group, and most of these cells showed the SOX9-positive reaction (Fig. 7B). The percentage of SOX9-positive cells was significantly higher in

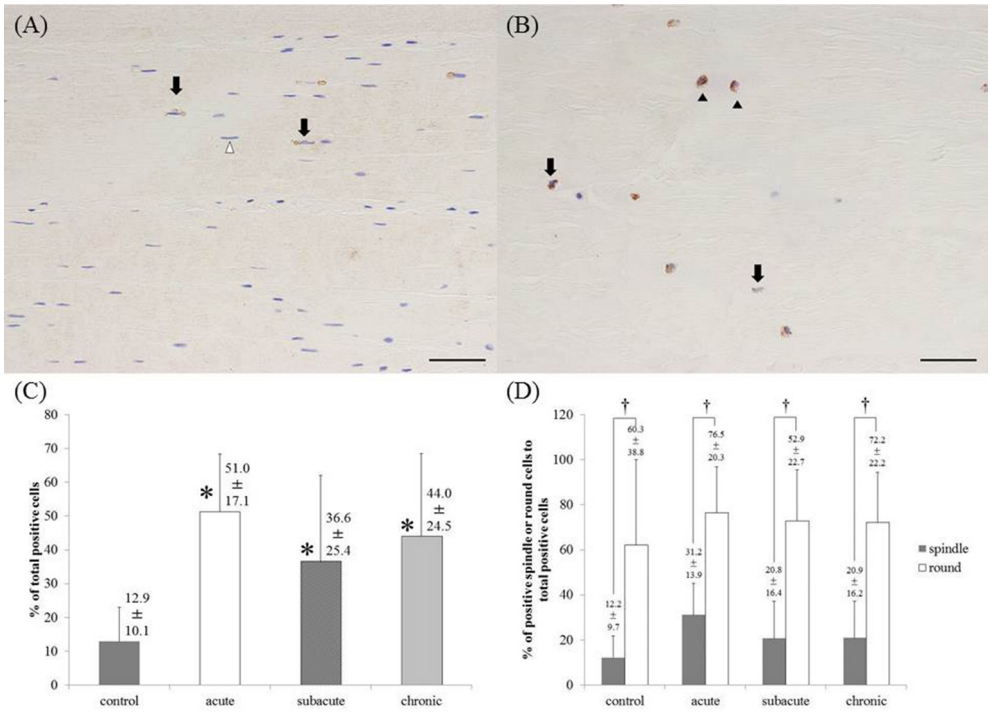


Fig. 7. Immunostaining for SOX9. (A) Control group specimen. Only a few spindle SOX9-positive cells with perinuclear halo (arrows) and many spindle cells without perinuclear halo (white arrowhead) are observed.(B) CCLR group specimen. Many spindle (arrow) and round (arrowheads) SOX9-positive cells with perinuclear halo are observed; scale bar=50 μm. (C) Comparison of the percentage of SOX9-positive cells among each group. * P<0.05 by Tukey's HSD test vs. control group. (D) Comparison of the percentage of SOX9-positive cells between spindle and round cells in each group. † P<0.05 by Welch's t-test.

the acute, subacute and chronic groups (51.0 ± 17.1, 36.6 ± 25.4 and 44.0 ± 24.5%, respectively) than in the control group (12.9 ± 10.1%). There was no significant difference among each group in the CCLR group (Fig. 7C). In the control group, the percentage of SOX9-positive round cells (60.3 ± 38.8%) was higher than that of the positive spindle cells (12.2 ± 9.7%, Fig. 7D). Similarly, in each group of the CCLR group, the percentage of SOX9-positive round cells was higher than that of the positive spindle cells (Fig. 7D).

Moreover, classified according to the cell morphology, the percentages of the spindle cells with halo were 12.7 ± 9.6% in the control group, 31.8 ± 14.9% in the acute group, 24.9 ± 15.9% in the subacute group and 25.9 ± 15.6% in the chronic group. The percentage of the spindle cells with halo was fewer in the control group significantly than that in the other groups, and there was no significant difference in the percentage of the spindle cells with halo among the acute, subacute and chronic groups (Fig. 8). The percentages of the round cells with halo were 88.7 ± 20.1% in the control group, 95.8 ± 3.8% in the acute group, 88.2 ± 14.0% in the subacute group and 92.0 ± 8.0% in the chronic group. There was no significant difference among these groups (Fig. 8). The percentages of the positive spindle cells with halo in the total spindle cells with halo were 95.6 ± 7.2% in the control group, 95.7 ± 6.5% in the acute group, 90.2 ± 17.7% in the subacute group and 87.7 ± 18.9% in the chronic group. The percentages of the positive round cells with halo in the total round cells with halo were 92.4 ± 18.6% in the control group, 77.2 ± 20.5% in the acute group, 81.2 ± 17.3% in the subacute group and 75.8 ± 24.1% in the chronic group. There was no significant difference in the percentages of the positive spindle or round cells with halo among each group.

There was no correlation between the number of positive cells following immunostaining for COLII, COLIII and SOX9 and the various demographic and clinical parameters.

DISCUSSION

In this study, SOX9-expressing ligamentocytes increased remarkably in the degenerative CCLs in the CCLR group compared with non-degenerative CCLs in the control group. SOX9 expression occurs from MSC state through hypertrophic chondrocytes. Bi *et al*. [4] reported that SOX9−/− cells do not express chondrocyte-specific markers, including COLII, and suggested that SOX9—as the first transcription factor—is essential for chondrocyte differentiation and cartilage formation. Cultured ACL-derived cells acquire a chondrogenic phenotype with SOX9 expression under chondrogenic-induction medium [11]. Moreover, Takimoto *et al*. [25] have demonstrated that overexpression of SOX9 induces

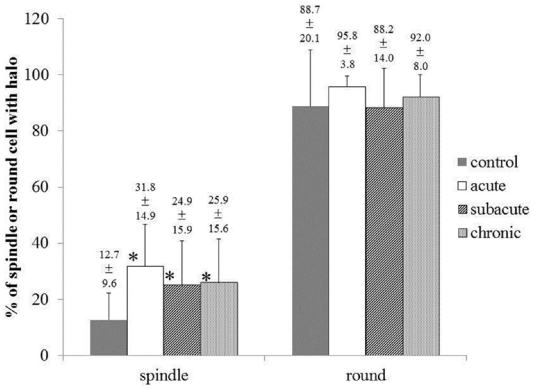


Fig. 8. The percentages of spindle and round cells with halo in each group. * $P<0.05$ by Tukey's HSD test vs. control group.

direct conversion of tenocytes into chondrocytes *in vitro*. To our knowledge, there is no study reporting on the expression of SOX9 in canine degenerative CCL. Narama *et al*. [22] reported that the most frequent and earliest lesion was a nuclear enlargement with perinuclear halo formation in fibrocytes, which was observed in a fairly intact area. The authors also suggested that the nuclear enlargement and perinuclear halo are considered to be caused by the activation of fibrocytes because of the characteristics shared with cells showing proliferating activity. In the present study, the percentage of spindle cells with perinuclear halo was higher in the degenerative CCLs of the CCLR group than in the non-degenerative CCLs of the control group, and most of these cells were SOX9-positive. A few spindle cells with halo were also observed in the non-degenerative CCLs of the control group, and most of these cells also expressed SOX9. Therefore, it is suggested that production of cartilage matrix and transformation of the fibrocytes may be promoted by increased expression of SOX9 in response to some external force, such as micro-injury.

In this study, areas of AB-positive staining, indicating mucopolysaccharides, were seen extensively in the ligaments of degenerated CCLs. Moreover, expression of COLII was increased in the degenerated CCLs. Vasseur *et al*. [26] reported dogs >5 years of age and with a body weight of >15 kg were found to display histological signs of "fibrocartilage" with HE staining. Narama *et al*. [22] have reported that areas of the ECM with AB-positive staining were seen extensively, and many chondrocyte-like cells with oval nuclei were obtained in the ruptured CCL. Comerford *et al*. [7] reported that a similar fibrocartilaginous appearance was seen in interfascicular areas in Alcian blue–periodic acid–Schiff-stained sections of the CCLs of normal Labrador retrievers and Greyhounds. The authors proposed that this degenerative change is a physiological, not pathological, adaptation to repetitive stress. In humans, chondroid phenotypic transformation of ligament fibroblasts and associated changes to the anterior cruciate ligament ECM are also known to be recognized after ligament rupture [20, 21]. From the above results, it is suggested that the expression of cartilage matrix components, such as COLII, may increase in response to stimuli along with an increase in the expression of SOX9 in the degenerated CCLs after CCLR. Whether this degenerative change precedes ligament injury is unclear in this study. Nonetheless, there was no significant difference among the period from onset of clinical sign to operation.

In the present study, we showed that expression of COLIII, which was low in the ligaments of the control group, increased remarkably in the CCLR group. The content of COLIII is typically low in the normal CCL [27] and confined to the zone of unmineralized fibrocartilage [14]. The change in the ratio of COLIII may show the changes to the fibrocartilage of degenerated CCLs. In addition, irregular and obscure ligament fibers were observed in these areas. Increased expression of COLIII is the first step during injury healing, which is then finally replaced with COLI [29]. Therefore, it is suggested that there may be micro-injury of ligament fibers, which promotes the expression of COLIII. It is also suggested that the rupture of CCLs has no influence on the expression of COLIII and that it may take some time to deposit COLIII in the ligament fibers, because the COLIII-positive area was larger in the degenerative CCLs of the subacute group and tended to be larger in the chronic group than the control CCLs, but not larger than the acute group.

In the present study, we showed a reduction of COLI deposition in the degenerated CCL. Most skeletal ligaments contain approximately 60–80% water, and nearly 70–80% of their dry weight is collagen [10]. Up to 90% of the ligamentous collagen is COLI, the principal tensile-resistant fiber [27]. Therefore, it is suggested that tensile strength of the ligament may decrease because of reduction in the content of COLI in degenerative CCLs. Moreover, we found that many ligament cells of degenerated CCLs expressed COLI in their cytoplasm compared to normal CCLs. Comerford *et al*. [6] reported that immature collagen crosslinks are increased in ruptured CCLs, which may contribute to a decrease in tensile strength. Therefore, it is suggested that the high turnover of COLI leads to relaxation of the ligament collagen fibers, because of increased immature collagen crosslinks in the degenerative CCLs.

In this study, expressions of COLII, COLIII and SOX9, which extend in fibrocartilage tissues, were more in cytoplasm of round cells than in spindle cells. The expression of COLI also tended to increase in cytoplasm of round cell. Narama *et al*. [22] have reported that many chondrocyte-like cells with oval nuclei were obtained in the ruptured CCL. Therefore, the expression of COLI, COLII, COLIII and SOX9 in round cells suggests they may be abnormally differentiated cells that express both mature chondrocyte and immature fibroblast markers.

One limitation of this study is that we were unable to investigate the mechanism of CCL degeneration directly, because ruptured CCLs were collected as degenerating CCLs. However, with respect to a wide range of factors, there was no correlation between the expression of COLII, COLIII and SOX9 and demographic and clinical parameters. Therefore, this suggests that these factors may have no effect on the expression of each marker. The beagle is the only experimental canine breed available in Japan. However, we are aware that large breed dogs are more predisposed to CCLR. Another

limitation of this study is that we used young dogs as controls.

In conclusion, in degenerative CCLD, the expression of COLI decreased with increased COLIII expression. The composition of the ECM is changed in degenerative CCL disease. On the contrary, expression of SOX9 increased, which may contribute to the synthesis of cartilage matrix. Further investigations are required to identify the factors that increase expression of SOX9.

ACKNOWLEDGMENT. We thank the students of the Laboratory of Veterinary Surgery, Nippon Veterinary and Life Science University for supporting this study.

REFERENCES

1. Akiyama, H., Chaboissier, M. C., Martin, J. F., Schedl, A. and de Crombrugghe, B. 2002. The transcription factor Sox9 has essential roles in successive steps of the chondrocyte differentiation pathway and is required for expression of Sox5 and Sox6. *Genes Dev.* **16**: 2813–2828. [Medline] [CrossRef]
2. Arnoczky, S. P. and Marshall, J. L. 1977. The cruciate ligaments of the canine stifle: an anatomical and functional analysis. *Am. J. Vet. Res.* **38**: 1807–1814. [Medline]
3. Bennett, D., Tennant, B., Lewis, D. G., Baughan, J., May, C. and Carter, S. 1988. A reappraisal of anterior cruciate ligament disease in the dog. *J. Small Anim. Pract.* **29**: 275–297. [CrossRef]
4. Bi, W., Deng, J. M., Zhang, Z., Behringer, R. R. and de Crombrugghe, B. 1999. Sox9 is required for cartilage formation. *Nat. Genet.* **22**: 85–89. [Medline] [CrossRef]
5. Chen, J., Wang, A., Xu, J. and Zheng, M. 2010. In chronic lateral epicondylitis, apotosis and autophagic cell death occur in the extensor carpi radialis brevis tendon. *J. Shoulder Elbow Surg.* **19**: 355–362. [Medline] [CrossRef]
6. Comerford, E. J., Innes, J. F., Tarlton, J. F. and Bailey, A. J. 2004. Collagens in fibrocartilages at the Achilles tendon insertion—A biochemical, molecular biological and immunohistochemical study. *Am. J. Vet. Res.* **65**: 1136–1141. [Medline] [CrossRef]
7. Comerford, E. J., Tarlton, J. F., Wales, A., Bailey, A. J. and Innes, J. F. 2006. Ultrastructural differences in cranial cruciate ligaments from dogs of two breeds with a differing predisposition to ligament degeneration and rupture. *J. Comp. Pathol.* **134**: 8–16. [Medline] [CrossRef]
8. Comerford, E. J., Smith, K. and Hayashi, K. 2011. Update on the aetiopathogenesis of canine cranial cruciate ligament disease. *Vet. Comp. Orthop. Traumatol.* **24**: 91–98. [Medline] [CrossRef]
9. Duval, J. M., Budsberg, S. C., Flo, G. L. and Sammarco, J. L. 1999. Breed, sex, and body weight as risk factors for rupture of the cranial cruciate ligament in young dogs. *J. Am. Vet. Med. Assoc.* **215**: 811–814. [Medline]
10. Frank, C., Amiel, D., Woo, S. L. and Akeson, W. 1985. Normal ligament properties and ligament healing. *Clin. Orthop. Relat. Res.* 15–25. [Medline]
11. Furumatsu, T., Hachioji, M., Saiga, K., Takata, N., Yokoyama, Y. and Ozaki, T. 2010. Anterior cruciate ligament-derived cells have high chondrogenic potential. *Biochem. Biophys. Res. Commun.* **391**: 1142–1147. [Medline] [CrossRef]
12. Hasegawa, A., Nakahara, H., Kinoshita, M., Asahara, H., Koziol, J. and Lotz, M. K. 2013. Cellular and extracellular matrix changes in anterior cruciate ligaments during human knee aging and osteoarthritis. *Arthritis Res. Ther.* **15**: R29. [Medline] [CrossRef]
13. Hayashi, K., Frank, J. D., Dubinsky, C., Zhengling, H., Markel, M. D., Manley, P. A. and Muir, P. 2003. Histologic changes in ruptured canine cranial cruciate ligament. *Vet. Surg.* **32**: 269–277. [Medline] [CrossRef]
14. Kumagai, J., Sarkar, K., Uhthoff, H. K., Okawara, Y. and Ooshima, A. 1994. Immunohistochemical distribution of type I, II, and III collagens in the rabbit supraspinatus tendon insertion. *J. Anat.* **185**: 279–284. [Medline]
15. Kumagai, K., Sakai, K., Kusayama, Y., Akamatsu, Y., Sakamaki, K., Morita, S., Sasaki, T., Saito, T. and Sakai, T. 2012. The extent of degeneration of cruciate ligament is associated with chondrogenic differentiation in patients with osteoarthritis of the knee. *Osteoarthritis Cartilage* **20**: 1258–1267. [Medline] [CrossRef]
16. Lefebvre, V., Li, P. and de Crombrugghe, B. 1998. A new long form of Sox5 (L-Sox5), Sox6 and Sox9 are coexpressed in chondrogenesis and cooperatively activate the type II collagen gene. *EMBO J.* **17**: 5718–5733. [Medline] [CrossRef]
17. Lui, P. P., Fu, S. C., Chan, L. S., Hung, L. K., and Chan, K. M. 2009. Chondrocyte phenotype and ectopic ossification in collagenase-induced tendon degeneration. *J. Histochem. Cytochem.* **57**: 91–100. [Medline]
18. Marshall, J. L. and Olsson, S. E. 1971. Instability of the knee. A long-term experimental study in dogs. *J. Bone Joint Surg.* **53**: 1561–1570. [Medline]
19. Muir, P., Schamberger, G. M., Manley, P. A. and Hao, Z. 2005. Localization of cathepsin K and tartrate-resistant acid phosphatase in synovium and cranial cruciate ligament in dogs with cruciate disease. *Vet. Surg.* **34**: 239–246. [Medline] [CrossRef]
20. Murray, M. M. and Spector, M. 1999. Fibroblast distribution in the anteromedial bundle of the human anterior cruciate ligament: the presence of alpha-smooth muscle actin-positive cells. *J. Orthop. Res.* **17**: 18–27. [Medline] [CrossRef]
21. Murray, M. M., Martin, S. D., Martin, T. L. and Spector, M. 2000. Histological changes in the human anterior cruciate ligament after rupture. *J. Bone Joint Surg. Am.* **82**: 1387–1397. [Medline]
22. Narama, I., Masuoka, M., Matsuura, T., Ozaki, K., Nagatani, M. and Morishima, T. 1996. Morphogenesis of degenerative changes predisposing dogs to rupture of the cranial cruciate ligament. *J. Vet. Med. Sci.* **58**: 1091–1097. [Medline] [CrossRef]
23. O'Donoghue, D. H., Rockwood, C. A. Jr., Frank, G. R., Jack, S. C. and Kenyon, R. 1966. Repair of the anterior cruciate ligament in dogs. *J. Bone Joint Surg. Am.* **48**: 503–519. [Medline]
24. Slocum, B. and Devine, T. 1983. Cranial tibial thrust: a primary force in the canine stifle. *J. Am. Vet. Med. Assoc.* **183**: 456–459. [Medline]
25. Takimoto, A., Oro, M., Hiraki, Y. and Shukunami, C. 2012. Direct conversion of tenocytes into chondrocytes by Sox9. *Exp. Cell Res.* **318**: 1492–1507. [Medline] [CrossRef]
26. Vasseur, P. B., Pool, R. R., Arnoczky, S. P. and Lau, R. E. 1985. Correlative biomechanical and histologic study of the cranial cruciate ligament in dogs. *Am. J. Vet. Res.* **46**: 1842–1854. [Medline]
27. Waggett, A. D., Kwan, A. P. L., Woodnutt, D. J. and Akeson, W. 1985. Collagens in fibrocartilages at the Achilles tendon insertion—A biochemical, molecular biological and immunohistochemical study. *Trans. Orthop. Res. Soc.* **21**: 25.
28. Wilke, V. L., Robinson, D. A., Evans, R. B., Rothschild, M. F. and Conzemius, M. G. 2005. Estimate of the annual economic impact of treatment of cranial cruciate ligament injury in dogs in the United States. *J. Am. Vet. Med. Assoc.* **227**: 1604–1607. [Medline] [CrossRef]
29. Williams, I. F., McCullagh, K. G. and Silver, I. A. 1984. The distribution of types I and III collagen and fibronectin in the healing equine tendon. *Connect. Tissue Res.* **12**: 211–227. [Medline] [CrossRef]