Cranial cruciate disease in dogs is characterized by progressive degradation of the ligament, leading to its partial and, ultimately, total rupture. Most dogs affected with the disease are lame prior to total rupture, which is associated with an inflammatory arthritis.1,2 A prCCL has been described as a common clinical finding in dogs with CCLD. Biomechanically, a partially ruptured cranial cruciate ligament is defined by a disruption of the CCL in a stable stifle joint. Older reports indicate that 8% to 22% of dogs with CCLD are admitted to the veterinary hospital with prCCLs.3,4 Because arthroscopic evaluation of the stifle joint has become a routine procedure, a prCCL is diagnosed much more commonly. Besides the classic signs of instability, indications to perform arthroscopy include stifle joint effusion, signs of local pain, and progressive unresponsive osteoarthritis. Identification of a prCCL has therefore become much more common in recent years and accounts for a diagnosis in > 80% of all dogs admitted to our veterinary hospital for arthroscopy of the stifle joint. Evidence that an intrinsic mechanism in the ligament is responsible for the gradual degradation of the matrix in CCLD is increasing. Tartrate-resistant acid phosphatase and collagenolytic cathepsin K may influence the integrity of the ligament.2 Results of 1 study5 indicate that overproduction of matrix-degrading metalloproteinases is responsible for ligament destruction. Increases in inflammatory mediators, such as interleukin-1, interleukin-6, tumor necrosis factor, and nitric oxide, might trigger matrix degradation through

### Abbreviations

<table>
<thead>
<tr>
<th>prCCL</th>
<th>Partially ruptured cranial cruciate ligament</th>
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<tr>
<td>CCLD</td>
<td>Cranial cruciate ligament disease</td>
</tr>
<tr>
<td>trCCL</td>
<td>Totally ruptured cranial cruciate ligament</td>
</tr>
<tr>
<td>PARP</td>
<td>Poly (ADP-ribose) polymerase</td>
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From the Divisions of Small Animal Surgery (Krayer, Rytz, Forterre, Spreng) and Clinical Research (Oevermann, Doherr, Zurbriggen), Department of Clinical Veterinary Medicine, Vetsuisse Faculty, University of Bern, 3012 Bern, Switzerland.
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Address correspondence to Dr. Spreng.
direct influence on either metalloproteinase production or cell viability.5,6

We have recently shown the presence of a significant amount of apoptotic cells in ruptured canine CCLs.7 Programmed cell death has a definite role in osteoarthritis, as shown in several clinical and experimental studies2–12 in a variety of species. Generally, apoptosis is a form of cell death that does not produce a pericellular reaction as seen with necrosis.12

Normal tissue homeostasis requires a constant rate of cell death and new cell formation to maintain matrix production in a steady state. Ligamentous cells are surrounded by a comparatively thick layer of matrix, in which replacement of dead cells is uncertain. Indeed, replacement of dying ligamentous cells by new cells in the CCL has thus far not been demonstrated to our knowledge. We have recently shown that trCCLs have significantly more apoptotic cells than ligaments from normal joints.8 It is, however, difficult to study cellular events in trCCLs because biopsy specimens after total rupture of the ligament are influenced by the primary intrinsic event of CCLD as well as by the mechanical trauma resulting from instability and inflammation after ligament rupture, both of which can potentially cause some degree of cell death.

The purpose of the study reported here was to investigate the presence of apoptosis in grossly intact and ruptured parts of prCCLs. On the basis of the fact that increased premature cell death could be responsible for decreased matrix production, we wanted to study the amount of apoptotic cells in the intact parts of prCCLs. Our hypothesis was that significant amounts of dead cells are already present in the still intact part of the ruptured ligament. Biopsy specimens of the CCL from dogs with prCCLs were therefore harvested during routine arthroscopy prior to surgical treatment of CCLD, and the degree and distribution of apoptosis were compared with biopsy specimens of the CCL from dogs with trCCLs.

Materials and Methods

Animals—The study protocol was reviewed and approved by the departmental review board for clinical and experimental animal studies. Client-owned dogs that were admitted to the veterinary hospital for arthroscopy of the stifle joint and subsequent treatment of CCLD were included in this study. Age, weight, sex, breed, and duration of lameness prior to surgery were recorded for each dog.

Biopsy specimen collection—Arthroscopic joint assessment and biopsy specimen collection were done by the same experienced investigator (UR). Dogs were prepared routinely for surgery of the stifle joint. The stifle joint was examined before surgery for instability by detection of a cranial drawer sign. Evidence of CCLD as well as the type of rupture were confirmed during arthroscopy. A 2.4-mm, 25° fore-oblique arthroscope was used to guide a 2.7-mm miniature double-spoon forceps for biopsy specimen collection. The type of rupture (total or partial) and the degree of synovitis and osteophytosis were assessed during arthroscopy. Both variables were graded from 0 to 4, corresponding to normal, mild, moderate, or marked signs of synovitis or osteophytosis.

From prCCLs, biopsy specimens were taken within the macroscopically intact midregion of the ligament (group A) as well as from the ruptured area (group B). Biopsy specimens from trCCLs were taken within the ruptured area of the ligament (group C). Biopsy specimens had an approximate size of 1 to 2 mm.

All biopsy specimens were immediately placed in containers containing 4% paraformaldehyde and fixed overnight at 4°C. Cranial cruciate ligament biopsy specimens were then embedded in paraffin wax, sectioned, and mounted on glass slides. Multiple slides were created from each specimen for immunohistochemical and H&E staining.

Histologic evaluation—Cranial cruciate ligaments were assessed on H&E-stained slides by a board-certified pathologist (AO) who was blinded to the study protocol. Lesions were graded in accordance with the modified protocol described by Vasseur et al.14 as grade 0 (no apparent structural changes), grade 1 (small solitary or multiple areas of degeneration, mild loss of ligamentous cells, small areas associated with a slight proliferation of resident ligamentous cells, loss of collagen fiber bundling, and mild chondroid metaplasia), grade 2 (moderate degenerative changes including ligamentous cell loss affecting large areas, moderate chondroid metaplasia, and loss of collagen fiber bundling), or grade 3 (severe degenerative changes including large areas with metaplastic chondrocytes, mineralization, and fragmented or separated collagen fibers).

Caspase-3 immunohistochemistry—Immunohistochemical staining specific for caspase-3 was performed according to a previously defined protocol.8 Briefly, tissue sections were cut at a thickness of 5 µm and mounted on positive-laden glass slides. Sections were deparaffinized in xylene and rehydrated in graded alcohols. Antigen unmasking was performed by heating slides in a Coplin jar containing 10mM sodium citrate buffer (pH, 6.0). Endogenous peroxidase activity was quenched by incubation with 3% H2O2 in methanol. After blocking with 5% goat serum in PBSS, slides were incubated with the primary antibody (rabbit anti–caspase-3 polyclonal antibody; diluted with PBSS at 1:125) for 75 minutes at room temperature (approx 20°C). After rinsing, the presence of antigen was detected with a commercially available detection kit containing 3-aminobenzidine as chromogen. For each batch of slides, negative control slides were handled and prepared in the same way as the other slides, apart from the omission of the primary antibody. Sections of normal canine lymph nodes were used as positive controls.

The stained tissue slides were assessed under light microscopy by 2 independent blinded observers (MK and AO) who graded the slides on the basis of the number of caspase-positive apoptotic cells as follows: grade 0, < 5% caspase-positive apoptotic cells (negative apoptotic signal); grade 1, 5% to < 10% caspase-positive apoptotic cells (low apoptotic signal); grade 2, 10% to < 25% caspase-positive apoptotic cells (moderate apoptotic signal); grade 3, 25% to < 50% caspase-positive apoptotic cells (high apoptotic signal); or grade
4, 50% to 100% caspase-positive apoptotic cells (very high apoptotic signal).

**PARP immunohistochemistry**—A second immunohistochemical analysis for apoptosis was performed to confirm results obtained with caspase immunohistochemistry. The applied antibody was a polyclonal antibody directed against the 85-kd caspase-cleaved fragment of human PARP. Initially, sections of normal canine lymph nodes known for the presence of positive apoptotic signals were used to demonstrate PARP-positive signals. Briefly, the paraffin-embedding medium was removed in xylene, and the sections were rehydrated in graded alcohols. Slides were placed first in distilled water, then in PBSS. Antigen unmasking was achieved by incubating in 0.2% Triton X-100 in PBSS for 5 minutes at room temperature. Slides were subsequently washed in PBSS. Sections were blocked with 5% goat serum in PBSS for 20 minutes. After removing the blocking solution, but without any washing procedure, the primary antibody was put on the tissue sections and incubated overnight at 4°C. The antibody solution used was a polyclonal anti-PARP p85 fragment antibody diluted 1:50 in PBSS. After rinsing in PBSS, slides were treated with a commercially available detection kit, which uses a biotinylated goat anti-rabbit-mouse as the secondary antibody. After an incubation time of 10 minutes, slides were rinsed with PBSS. Subsequently, tissue sections were incubated for 10 minutes with streptavidin conjugated with horseradish peroxidase and then washed in PBSS. The last incubation step required a 3-amino-9-ethyl carbazole–H$_2$O$_2$ substrate solution, closely monitored under light microscopy until a distinct positive signal appeared on the positive control tissue. Slides were rinsed in distilled water and counter-colored for 30 seconds in hematoxylin. After a final washing step in distilled water, slides were mounted for evaluation. Negative control slides were handled and prepared in the same way as the other slides, but the primary antibody was omitted. Stained slides were graded in the same way as the caspase-labeled slides.

**Statistical analysis**—Descriptive and comparative analyses were performed by use of a software program. For some ligaments, multiple biopsy specimens and thus measurements were available. In those instances, a mean score of the apoptotic cell number (outcome) was calculated. Demographic data by dog (age and weight) and for the outcome measures were tested for normal distribution with the Kolmogorov-Smirnov test and normal probability plots. As most were not normally distributed, demographic data were described as medians and ranges and the outcome measures as medians and 95% confidence intervals (with exact confidence intervals based on the percentiles of the distribution). A nonparametric Wilcoxon rank sum test was used to test for significant differences between the unpaired scores of group A versus C and group B versus C, whereas a Wilcoxon signed rank test was used to compare the (paired within dogs) scores of group A and B. The Spearman rank correlation coefficient ($r_s$) was used to detect significant correlation between the different outcome measures. For all comparisons, values of $P < 0.05$ were considered significant.

**Results**

Twenty dogs with a prCCL and 14 dogs with a trCCL were examined during the study. No significant difference was found between the groups for age, weight, sex, duration of lameness, and arthroscopic degree of synovitis and osteophytosis (Table 1). Osteophytosis correlated with the degree of synovitis ($r = 0.58; P < 0.001$). Dogs with a clearly positive drawer sign before surgery were all confirmed to have a trCCL. A negative drawer sign was observed in dogs with arthroscopically confirmed prCCLs. All dogs were treated by arthroscopic debridement of the joint followed by tibial plateau leveling osteotomy.

Various stages of degenerative changes were observed in the H&E-stained sections in trCCL specimens as well as in intact and ruptured parts of prCCLs. These included loss of ligamentous cells, proliferation of surviving ligamentous cells, chondroid metaplasia, loss of the primary collagen bundling, and tearing of axial fibers. The cell density, amount of chondroid metaplasia, and structural changes in the matrix were similar in all 3 groups. Hemorrhage and hemosiderin deposition were observed in all groups, with more lesions in ruptured areas (Figures 1 and 2). Median values for the histologic grade of degeneration were 3 (range, 1 to 3).

Table 1—Median (range) values of dogs with prCCLs ($n = 20$) and trCCLs ($n = 14$).

<table>
<thead>
<tr>
<th>Variables</th>
<th>prCCL</th>
<th>trCCL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (mo)</td>
<td>56 (23–132)</td>
<td>76 (14–133)</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>37 (28–62)</td>
<td>32 (23–55)</td>
</tr>
<tr>
<td>Sexually intact male*</td>
<td>6</td>
<td>4</td>
</tr>
<tr>
<td>Castrated male*</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>Sexually intact female*</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>Spayed female*</td>
<td>10</td>
<td>6</td>
</tr>
<tr>
<td>Duration of signs (m)</td>
<td>3 (0–30)</td>
<td>1.5 (0–24)</td>
</tr>
<tr>
<td>Synovitis (grades 1–4)*</td>
<td>3 (1–4)</td>
<td>3 (2–4)</td>
</tr>
<tr>
<td>Osteophytosis (grades 1–4)*</td>
<td>1.5 (0–3)</td>
<td>2 (1–3)</td>
</tr>
</tbody>
</table>

*Actual number of dogs. Graded from 0 to 4, corresponding to normal, mild, moderate, or marked signs, respectively, of synovitis or osteophytosis.

![Figure 1](Image)
in the ruptured area and 2 (range, 1 to 3) in the intact area of prCCLs, with no significant (P = 0.18) difference between the 2 groups.

Although the amount of detected apoptotic cells was slightly higher with PARP immunostaining than caspase-3 staining, results of both methods were comparable and correlate well (r = 0.71; P < 0.001). A significant amount of apoptotic cells could be found in 91% of caspase-3 and in 93% of PARP-stained biopsy specimens. No significant differences were observed between groups with regards to apoptotic rate on the basis of caspase-3 (group A vs B, P = 0.36; group A vs C, P = 0.35; and group B vs C, P = 0.76) or PARP (group A vs B, P = 0.41; group A vs C, P = 0.8; and group B vs C, P = 0.68) immunohistochemistry results (Table 2). Fusi-form fibroblasts as well as chondroid cells had evidence of apoptotic activity. Apoptotic cells had a slightly focal (65%) distribution in the examined slides. No significant correlation was found between the degree of synovitis and apoptotic ligamentous cells on the basis of caspase-3 (r = −0.12; P = 0.32) or PARP (r = −0.19; P = 0.13) immunohistochemistry results. Also, no significant correlation was found between osteophyte production and apoptotic ligamentous cells on the basis of caspase-3 (r = −0.01; P = 0.89) or PARP (r = 0.01; P = 0.88) immunohistochemistry results.

**Discussion**

Previous studies on biochemical alterations in ruptured ligaments have often been problematic because specimen collection is usually performed intraoperatively following total rupture of the ligament. Differentiation between alterations resulting from the inciting cause of CCLD and those from the trauma of disruption is therefore often speculative. The novel approach of biopsy specimen collection under arthroscopic guidance allows precise and selective retrieval of specimens in macroscopically nontraumatized areas. Only sparse information is available in the scientific literature on partial ruptures of the CCL. To our knowledge, histologic studies comparing partial and total rupture have not been previously published.

Results of the present study indicate that the grossly intact part of prCCLs is affected by similar degenerative changes as the ruptured part. The grade of degenerative changes is comparable to published results of totally ruptured ligaments. Additionally, in sections from ruptured areas, acute hemorrhage, hemosiderin deposition, and granulation tissue were observed, consistent with acute mechanical trauma. Histologic evaluation, however, was considered difficult because the small sample size precluded stretching and orientation to provide longitudinal sections.

Results of a previous study indicate that inflammation is more severe in stifle joints with prCCLs than in joints with trCCLs on the basis of synovial fluid WBC counts. Other studies, however, could not find an influence of the CCL grade of disruption on interleukin-6, tumor necrosis factor, and nitric oxide concentrations in synovial fluid. We did not evaluate synovial membrane histologically in our study; however, we had no indication of a difference in the degree of inflammation between trCCLs and prCCLs from the intraoperative gross evaluation.

In a previous report, we demonstrated that ruptured CCLs had significantly more apoptotic cells than did intact ligaments by use of the same protocol with caspase-3 immunohistochemistry. In that study, median values of 0.5 (95% confidence interval, 0 to 0.5) and 2.5 (95% confidence interval, 0 to 3) were found for the degree of apoptosis in normal and ruptured ligaments, respectively (P = 0.037). In the present study, we confirmed that apoptosis is an important form of cell death in ligaments of dogs with CCLD by use of PARP as well as caspase-3 immunohistochemistry. Cleavage of PARP has been found to be a sensitive variable to study early
cell death in a number of cell death models. Detection of the cleaved fragment of PARP signifies activation of caspase 3-like activity.

Initially, sections of normal canine lymph nodes known for the presence of positive apoptotic signals were used to demonstrate PARP-positive signals. Because PARP is a nuclear enzyme, its fragments would at least transiently remain in the nucleus, only leaking out in the cytoplasm. Intense nuclear positive signals were found as well as light-positive cytoplasmic signals in stained lymphocytes.

Tissues with high rates of physiologic cell replacement, such as the intestinal mucosa, have an apoptosis rate in the range of 8 cells/crypt or 3% of gastric mucosal cells. Assuming that the physiologic turnover of chondrocytes and ligamentous cells is slower than that of intestinal tissue, the apoptotic rate of up to 50% of all cells in affected ligaments is high. The reason for this high percentage is not yet clear. Furthermore, results of this study indicate that grossly intact areas of prCCLs have a high amount of apoptotic cells. We conclude that apoptosis is present prior to mechanical disruption of the ligament and therefore most probably not a consequence of the acute trauma of the rupture itself.

Apoptosis is a programmed cell death that is induced by a tightly regulated intracellular pathway, concluding in nuclear chromatin condensation and inter-nucleosomal digestion of DNA. It can be distinguished from necrosis morphologically and biochemically.

Apoptosis can be activated mainly by 2 molecular mechanisms. The extrinsic death receptor pathway leads to apoptosis by binding of ligands (tumor necrosis factor-related apoptosis–inducing ligand, Fas ligand, and tumor necrosis factor) to receptors belonging to the tumor necrosis factor family, such as CD95 (ie, Fas). This, in turn, activates the intracellular caspase cascade. The intrinsic or mitochondrial pathway is initiated by an external cytotoxic stress (eg, drugs or irradiation). The critical step in the mitochondrial pathway is the release of cytochrome c from mitochondria into the cytoplasm, leading to activation of the caspase cascade.

Detection and manipulation of programmed cell death in joint disease have been of growing interest in the last few years. Removal of dead cells is usually carried out by phagocytosis, and cells expressing tartrate-resistant acid phosphatases, present in the epitheligenous area and in the core region of ruptured CCLs, are possibly derived from activated macrophages. Further studies, however, are needed to demonstrate whether tartrate-resistant acid phosphate–positive cells have phagocytic activity to clear dead ligamentous cells. Another possible way for removal and transport of waste matter is via pinocytotic vesicles that have been recently identified in canine CCL capillary endothelium. If, however, inadequate cell debridement is present, investigations are warranted to show whether a detrimental effect on ligament functionality results. Inhibition of normal anatomic remodeling or activation of inflammation could be a consequence of increased amounts of dead cells remaining within the ligament.

Information on the induction of cell death in articular tissues is limited and has concentrated on the response to mechanical injury. It has been proposed that cell death in response to wounding is a combination of necrosis and apoptosis. Injury-induced chondrocyte apoptosis can decrease glycosaminoglycan synthesis as well as increase gene expression of matrix metalloproteinases, which link mechanical injury to cartilage matrix degradation. Similar studies have not been done with ligaments. However, results of our study indicate that direct trauma might not be the only cause of ligamentous cell apoptosis in CCLD of dogs; on the contrary, apoptosis might play a role in the pathogenesis of CCL rupture. Whether this role is critical for the rupture or just a by-product or consequence of micro-injury to the ligament matrix remains unclear.

Apoptosis can be chemically inhibited to increase cell viability, which is not the case for necrosis. How cells die is, therefore, of great importance. Prevention of apoptosis by caspase inhibition in cartilage has been shown to significantly affect glycosaminoglycan synthesis, restore matrix production in injured explant cultures, and maintain cell viability close to that observed before injury. This chondroprotective effect was also proven with an in vivo model of posttraumatic arthritis. Similar studies have not yet been performed on ligaments.

Treatment of partial and complete CCL rupture is similar and has concentrated on mechanical correction of joint instability, including recent techniques such as a tibial plateau leveling osteotomy as well as other treatment strategies such as total debridement of the remnant ligament in a prCCL followed by an extra- or intracapsular procedure.

The function of the remaining part of the ligament in dogs is certainly not clear. It has also been proposed that most partial ruptures of the CCL will progress to a total rupture of the ligament, suggesting an ongoing internal process of micro-injury leading to total rupture of the ligament. Results of the present study corroborate this idea in that we could detect no significant differences between intact and ruptured parts of prCCLs. Inflammation, preceding grossly visible ligament disruption in the stifle joint, has been shown to lead to disorganization of the normal cellular pattern as well as loss of normal fiber orientation in experimentally affected rabbits. Increased amounts of inflammatory cells in canine CCLs, compared with human anterior cruciate ligaments, have been demonstrated and indicate that human anterior cruciate ligament rupture commonly seen after trauma has a different etiology than CCLD in dogs. Increased induction of apoptosis through an inflammatory trigger could lead to an imbalance in matrix morphology followed by CCLD rupture. Additional studies to clarify the role of apoptosis in CCLD are justified.

a. CCD-Endocam camera, 2.4 mm, 25° fore-oblique arthroscope, Richard Wolf GmbH, Knittlingen-D, Germany.
c. Anti-active caspase 3 pAB, Promega Corp, Bara, Switzerland.
d. LSAB/HRP ChemMate detection kit, Peroxidase AEC rabbit/mouse, K5003, DakoCytomation, Baar, Switzerland.
e. Anti-PARP p85 fragment pAB, Promega Corp, Dübendorf, Switzerland.
f. Nr 3051.2, Carl Roth GmbH, Karlsruhe, Germany.
g. Bottle A, ChemMate detection kit, Peroxidase/AEC Rabbit/Mouse, K5003, DakoCytomation, Baar, Switzerland.

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References


