Evaluation of tartrate-resistant acid phosphatase and cathepsin K in ruptured cranial cruciate ligaments in dogs

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Objective—To determine localization of tartrate-resistant acid phosphatase (TRAP) and cathepsin K in ruptured and healthy cranial cruciate ligaments (CCL) in dogs.

Animals—30 dogs with ruptured CCL, 8 aged dogs without ruptured CCL, and 9 young dogs without ruptured CCL.

Procedure—The CCL was examined histologically and cells containing TRAP and cathepsin K were identified histochemically and immunohistochemically, respectively.

Results—Cathepsin K and TRAP were detected within the same cells, principally within the epiligamentous region and to a lesser extent in the core region of ruptured CCL. Numbers of cells containing TRAP and cathepsin K were significantly greater in ruptured CCL, compared with CCL from young or aged dogs, and numbers of such cells were greater in CCL from aged dogs, compared with those of young dogs. In aged dogs, small numbers of cells containing TRAP and cathepsin K were seen in intact CCL associated with ligament fascicles in which there was chondroid transformation of ligament fibroblasts and disruption of the extracellular matrix.

Conclusions and Clinical Relevance—Ruptured CCL contain greater numbers of cells with the proteinases TRAP and cathepsin K than CCL from healthy, young, or aged dogs. Results suggest that cell-signaling pathways that regulate expression of these proteinases may form part of the mechanism that leads to upregulation of collagenolytic ligament remodeling and progressive structural failure of the CCL over time. (Am J Vet Res 2002;63:1279–1284).

Rupture of the cranial cruciate ligament (CCL) is a common and crippling problem in dogs, and causes progressive deterioration in limb function over time. Progressive osteoarthritis and persistent lameness are commonplace even with surgical treatment. Furthermore, partial CCL tears that are treated medically with rest and analgesics often progress to complete tears over time. Complete rupture of the CCL occurs because of progressive structural failure over a period of time in most affected dogs, many of which have bilateral disease. Tissue changes that have been identified during progressive CCL rupture in dogs include loss of ligament fibroblasts, transformation of fusiform ligament fibroblasts to an ovoid or spheroid phenotype, and disruption of the normal hierarchical architecture of type-I collagen within the extracellular matrix, including loss of crimp and disruption of ligament fascicles. Dogs with early cruciate disease usually have a stable stifle joint on physical examination, because most of the CCL must be ruptured for joint instability to be detected clinically. Although various risk factors for CCL rupture have been identified, including aging, body weight, and dog phenotype, the disease mechanism resulting in CCL rupture in dogs is poorly understood.

The CCL is a complex structure consisting of extracellular matrix proteins maintained by a diverse population of cells with fibroblasts being the predominant cell type. Type-I collagen within the extracellular matrix provides the CCL with tensile strength. The major part of the CCL extracellular matrix consists of bundles of type-I collagen separated by type-III collagen. After experimental transection of the CCL, approximately 34% of the ligament mass is lost by 10 days after surgery. However, the proteinases that are primarily responsible for collagenolysis in the CCL transection model have not been determined. Furthermore, the proteinases that initiate collagenolysis during progressive CCL failure in dogs with naturally occurring cruciate disease have not been determined.

Although it is generally accepted that musculoskeletal tissues are mechanotransductive and are able to respond to alterations in their loading environment by adaptation of the load-bearing tissue, little is known about the process of ligament modeling and remodeling. Ligaments are required to adapt precisely to joint growth during development and to respond to joint stresses after maturity. Ligaments have more numerous and larger cells, higher DNA content, a larger amount of reducible collagen cross-links, and more type III collagen, compared with tendons, suggesting that ligaments are more metabolically active and have greater adaptive potential than tendons. Normal noninflammatory adaptive remodeling of ligament collagen is thought to occur by intracellular digestion with lysosomal cathepsins, such as cathepsin B and L, after phagocytosis of extracellular matrix collagen by fibroblasts, whereas rapid inflammatory remodeling of collagen appears to be mediated by matrix metalloproteinase enzymes. However, the loss of collagen mass during remodeling of the CCL after
rupture does not appear to be mediated by matrix metalloproteinases.13

Tartrate-resistant acid phosphatase (TRAP) belongs to a group of iron-binding proteins that includes urotensin and purple acid phosphatase and is localized intracellularly in the lysosomal compartment of osteoclasts, macrophages, and dendritic cells.14 Tartrate-resistant acid phosphatase also may be secreted by some cell types, in particular by osteoclasts.15 Tartrate-resistant acid phosphatase is widely expressed in a range of tissues, including bone, spleen, liver, thymus, and colon, where it is associated principally with cells originating from the bone marrow.17 The process of osteoclastic bone resorption, including collagenolysis and cathepsin K has been identified in CD68+ macrophages involved in bone resorption.21 The purpose of the study reported here was to determine whether an association exists between ruptured CCL and localization of TRAP and cathepsin K within CCL tissue.

Materials and Methods

Selection of dogs—Portions of CCL were collected from 30 dogs with CCL disease, which was confirmed at the time of surgical treatment during lateral or medial parapatellar arthroscopy, resection of damaged meniscus, and extracapsular stabilization with nylon suture. Complete CCL rupture was diagnosed if joint instability was detected on physical examination, indicating extensive biomechanical degradation of the CCL. Partial CCL rupture was diagnosed if the affected stifle joint was stable on physical examination. In addition, CCL specimens were collected from 8 aged dogs and 9 young dogs without CCL disease that were humanely euthanatized by use of IV administration of barbiturates for reasons unrelated to our study. The group of aged dogs was selected as controls to account for the degradation in ligament mechanical properties that is known to occur with aging,3 and the group of young dogs was selected as one of the controls used to validate our histochemical and immunohistochemical staining. Age, weight, sex, and duration of lameness for each dog were determined.

Specimen collection and preparation—Remnants of ruptured CCL were excised from the femoral and tibial attachment sites in affected dogs. In dogs with normal CCL ligaments, the entire ligament was collected. Immediately after collection, ligament specimens were placed in tissue cassettes and fixed in Zamboni fixative23 for 1 to 2 days at 4 C. Longitudinal frozen sections, 10 µm thick, were cut and mounted on glass slides for histologic examination. Multiple slides were created from each specimen for histochemical and immunohistochemical staining, in addition to staining with H&E.

Histochemistry—Histochemical staining specific for TRAP was performed on all ligament specimens and was based on established methods.23,24 All reagents for histochemical staining were obtained from a commercial supplier. A solution of naphthol AS-BI phosphate was prepared by dissolving 25 mg of naphthol AS-BI phosphate in 2.5 ml of n-dimethylformamide to which was added 45 ml of 0.05M Tris-maleate buffer (pH 5). A solution of hexaazotized pararosaniline was prepared by dissolving 0.25 g of pararosaniline hydrochloride in 5 ml of distilled water, to which was added 1.25 ml of hydroxyl chloride. This solution was mixed with an equal volume of 4% sodium nitrite immediately before use. The final reaction mixture for histochemical staining was prepared by adding 4 ml of hexaazotized pararosaniline solution to the naphthol AS-BI phosphate solution, together with 50 mM sodium-potassium tartrate. The final reaction mixture was filtered before use. Sections were incubated in the reaction mixture at 37 C for 1 to 2 hours, rinsed in distilled water, counterstained in Mayer hematoxylin, and mounted.

All of the ligament specimens were examined via light microscopy for cells that contained TRAP. A well-defined resorption-modeling surface from the ulna of a young growing rat25 was used as a positive control. For each batch of slides, a negative control was prepared by omission of the naphthol AS-BI phosphate. Apart from this omission, the negative control slides were handled and prepared in the same manner as the other slides. The negative and positive control slides were reviewed before each batch of slides was analyzed.

Immunohistochemistry—Immunohistochemical staining specific for cathepsin K was performed on frozen sections of all ligament specimens. All incubations were performed in a moist chamber. Endogenous peroxidase activity within frozen sections was quenched by incubation of the slide with a commercial peroxidase blocker at approximately 25 C (room temperature) for 5 minutes. The slides were rinsed in 0.1M phosphate-buffered saline solution with 0.1% Tween 20 (PBSS-Tween) at pH 7.3 for 5 minutes. Slides were treated with a protease solution for 5 minutes. After proteinase treatment, the slides were rinsed in PBSS-Tween, blocked with casein for 5 minutes at approximately 25 C, rinsed in PBSS with 0.1% bovine serum albumin, and blocked with 5% goat serum in PBSS-Tween for 30 minutes at approximately 25 C. After blocking and further rinsing with PBSS-Tween, the specimens were treated with a mouse monoclonal antibody for cathepsin K, diluted 1:50 with antibody diluent, and allowed to incubate at 4 C overnight (minimum of 12 hours).

Following rinsing in PBSS-Tween, the slides were flooded with a biotinylated anti-mouse IgG antibody containing 1% canine serum and allowed to incubate for 20 minutes at approximately 25 C. Slides were rinsed in PBSS-Tween and treated with streptavidin-horseradish peroxidase conjugate for 20 minutes at approximately 25 C. After another PBSS-Tween rinse, slides were flooded with an insoluble 3,3’-diaminobenzidine tetrachloride/nickel-cobalt substrate and observed for staining intensity via light microscopy. The slides were rinsed in running water for 5 minutes and counterstained with nuclear fast red.3 Slides were rinsed in running water a final time, dehydrated in increasing concentrations of ethanol, cleared in xylene, and mounted.

All of the ligament specimens were examined via light microscopy for cathepsin K. The resorption-modeling surface from the rat ulna was used as a positive control. For each batch of slides, negative controls were prepared by omission of the primary or secondary antibodies. Apart from these omissions, the negative control slides were handled and prepared in the same manner as the other slides. The negative and positive control slides were viewed via light microscopy before the test slides were reviewed.

Double immunohistochemical-histochemical staining—Ligament specimens also were double-stained for TRAP and cathepsin K. Immunohistochemical staining was performed on all ligament specimens and was based on established methods.25,26 All reagents for histochemical staining were obtained from a commercial supplier. A solution of naphthol AS-BI phosphate was prepared by dissolving 25 mg of naphthol AS-BI phosphate in 2.5 ml of n-dimethylformamide to which was added 45 ml of 0.05M Tris-maleate buffer (pH 5). A solution of hexaazotized pararosaniline was prepared by dissolving 0.25 g of pararosaniline hydrochloride in 5 ml of distilled water, to which was added 1.25 ml of hydroxyl chloride. This solution was mixed with an equal volume of 4% sodium nitrite immediately before use. The final reaction mixture for histochemical staining was prepared by adding 4 ml of hexaazotized pararosaniline solution to the naphthol AS-BI phosphate solution, together with 50 mM sodium-potassium tartrate. The final reaction mixture was filtered before use. Sections were incubated in the reaction mixture at 37 C for 1 to 2 hours, rinsed in distilled water, counterstained in Mayer hematoxylin, and mounted.
formed for cathepsin K as described, except that counter-staining with nuclear fast red was omitted. The same ligament sections then were histochemically stained for TRAP. Negative control sections were prepared as described. Double-stained sections were reviewed qualitatively for cells in which TRAP and cathepsin K were co-localized.

Statistical analyses—The number of cells that contained TRAP or cathepsin K, degree of epiligamentous proliferation, and degree of chondroid metaplasia within the core region of the CCL were scored for each section by use of a 4-category numeric rating scale (negative = 0; slightly positive = 1; moderately positive = 2; strongly positive = 3). The median ANOVA was used to determine whether the number of cells that contained TRAP and cathepsin K in CCL tissue was significantly different in dogs with CCL rupture, compared with aged dogs and young dogs with normal stifle joints. Spearman rank correlations were used to examine associations between lameness duration, epiligamentous proliferation, chondroid metaplasia, localization of TRAP, and localization of cathepsin K in dogs with CCL rupture. Differences were considered significant at $P < 0.05$.

Results
Twenty-eight of 30 dogs with rupture of the CCL had palpably unstable stifle joints on physical examination and were considered to have complete tears of the CCL. Extensive disruption to the CCL was confirmed at surgery in these dogs. Less extensive disruption to the CCL rupture was confirmed at surgery in the 2 dogs with partial rupture and stable stifle joints. Body weight was $35.5 \pm 10.8$ kg (mean $\pm$ SD), and age was $5.2 \pm 2.1$ years. Median duration of lameness was 2 months, and lameness duration ranged from 3 days to 24 months. One dog was a sexually intact female, 15 dogs were ovariohysterectomized females, 2 dogs were sexually intact males, and 12 dogs were castrated males.

Among the young dogs without CCL rupture, body weight was $10.0 \pm 0.7$ kg, age was $1.7 \pm 0.9$ years, and all dogs were sexually intact females. Among the aged dogs without CCL rupture, body weight was $28.8 \pm 14.8$ kg, age was $10.3 \pm 3.7$ years, 5 dogs were ovario-
hysterectomized females, and 3 dogs were castrated males.

Histologic examination of longitudinal sections of CCL from young unaffected dogs revealed dense regularly orientated connective tissue with parallel bundles of crimped collagen fibers and parallel rows of fusiform ligament fibroblasts. Adjacent to the bone-ligament junction, a thin layer of epiligamentous tissue was evident (Fig 1). Mild chondroid metaplasia was seen in the normal CCL from 1 dog. Cells that stained for TRAP cells or cathepsin K were not seen in normal CCL of these dogs or in negative control tissues. In the positive control tissues, cells that contained TRAP and cathepsin K were seen on the caudomedial resorption-modeling surface of the rat ulna.

The CCL specimens from the aged dogs without CCL rupture and dogs with CCL rupture had variable numbers of cells that contained TRAP and cathepsin K, as well as chondroid metaplasia within the core region.

Figure 2—Photomicrographs of a longitudinal section of the epiligamentous region of a ruptured CCL from an 8-year-old dog, stained histochemically for tartrate-resistant acid phosphatase (TRAP). A—Notice that cells containing TRAP are widely distributed through the ligament tissue but are predominantly in the epiligamentous regions, adjacent to ligament fascicles (white arrows). Mayer hematoxylin counterstain; bar = 500 μm. B—Notice that cells containing TRAP (white arrows) typically have a large, rounded phenotype, quite different from the predominantly fusiform ligament fibroblasts. Mayer hematoxylin counterstain; bar = 50 μm.
of the CCL. A variable amount of epiligamentous proliferation was also seen in dogs with CCL rupture. Chondroid metaplasia was significantly \((P = 0.005)\) greater in aged dogs and dogs with CCL rupture, compared with young dogs without CCL rupture. A significant relationship between shorter duration of lameness and the number of cells that contained cathepsin K in ruptured CCL was found.

The number of cells that contained TRAP and cathepsin K within CCL tissue was significantly greater in dogs with CCL rupture, compared with aged and young dogs that did not have ruptured CCL. The CCL tissue of aged dogs also contained greater numbers of cells that contained TRAP and cathepsin K, compared with young dogs. Many cells that contained TRAP and cathepsin K had a large, rounded phenotype quite different from the fusiform phenotype of the ligament fibroblasts (Fig 2). In dogs with rupture of the CCL, the epiligamentous tissue was much larger in volume and had a high cell-number density and blood-vessel density, compared with that of dogs without CCL rupture. Cells that contained TRAP and cathepsin K were principally located in the epiligamentous region of the CCL and ligament fascicles within the core region that were adjacent to epiligamentous tissue. In aged dogs without CCL rupture, localization of TRAP and cathepsin K was associated with regions of chondroid metaplasia (Fig 3). The presence of cells that contained TRAP was positively correlated with the degree of epiligamentous proliferation \((P < 0.001)\) and the presence of cathepsin K+ cells \((P < 0.01); \text{Fig 4}\). Co-localization of cathepsin K in cells that contained TRAP within the CCL was identified in 16 of 25 dogs with CCL rupture (Fig 4). Furthermore, epiligamentous proliferation was negatively correlated with chondroid metaplasia within the core region of the CCL \((P = 0.005); \text{Fig 5}\).
Discussion

Rupture of the CCL is one of the most common and important orthopedic diseases of pet dogs, and a rich body of literature is available that describes the clinical features and surgical treatment of this condition. However, although it is generally accepted that most CCL ruptures are not associated with a primary traumatic injury, the underlying disease mechanism for CCL rupture is poorly understood. It has been hypothesized that expression of proteinase within the CCL during injury may have an important role in CCL resorption and influence the success of reconstructive surgery.11 In the study reported here, we found that localization of the proteinases TRAP and cathepsin K was significantly associated with ruptured CCL. Small numbers of proteinase-positive cells were also seen in the CCL of aged dogs that did not have ruptured CCL, principally in the connective tissue septae surrounding ligament fascicles with chondroid degeneration, and in epiligamentous tissue.

Tartrate-resistant acid phosphatase and cathepsin K are 2 proteins that are necessary for the formation of activated osteoclasts and normal bone resorption.26,27 In knockout mice, deletion of these genes leads to the development of osteopetrosis.26,27 Cathepsin K is a potent proteinase of type-I collagen and is the principal proteinase acting on bone collagen during osteoclastic bone resorption,28,29 and during resorption of bone by CD68+ macrophages.30 Cathepsin K is secreted as a pro-enzyme, and when activated cleaves type-I collagen at the N-terminal end of the triple helix.26,27 Tartrate-resistant acid phosphatase also is secreted as a pro-enzyme and may be activated by cysteine proteinases,31 such as cathepsin K. Although the precise role of TRAP in osteoclastic bone resorption is not well understood, it is thought that TRAP may facilitate resorption of bone by generation of reactive oxygen species that nonspecifically degrade bone collagen.32 Tartrate-resistant acid phosphatase may be inhibited by binding with α2-macroglobulin.33 The precise role of TRAP and cathepsin K expression in remodeling of ligament collagen and the cell signaling pathways regulating their expression within ligament tissue are unknown. Upregulation of these enzymes in ruptured CCL, and to a lesser extent in the CCL of aged dogs without rupture, suggests that their presence within CCL tissue may form part of the mechanism that leads to progressive structural failure of the CCL. Detection of these proteinases in the CCL of aged dogs suggests that it is less likely that localization of these novel proteinases within the CCL may be secondary to the primary rupture mechanism and that they are simply involved in the epiligamentous remodeling response after CCL rupture. Important objectives in future research will be to determine whether these enzymes cause collagenolysis of the extracellular matrix, whether upregulation of these proteinases causes structural weakening of the CCL, and what the signaling pathways are that regulate expression of these enzymes in the CCL.

Dogs with ruptured CCL in this study had signalments that were typical for this condition34 and were generally large, middle-aged dogs. Middle-aged, large-breed dogs often have mild osteoarthritis of the stifle joint and are known to have characteristically changes to the phenotype of CCL fibroblasts, as well as the ligament extracellular matrix,35 as was identified in the aged dogs of the study reported here. The presence of small numbers of cells that contained proteinase in the CCL of aged dogs with chondroid degenerative changes suggests that these changes may represent preclinical disease of the CCL. In this study, we also evaluated localization of TRAP and cathepsin K in the CCL of young, normal dogs without CCL rupture principally as a control for validation of the specificity of our histochemical and immunohistochemical staining. Although all of our clinically normal young dogs were female, sex is not a significant risk factor for CCL rupture.36 The complete absence of cells that contained TRAP or cathepsin K within the CCL of dogs in this population also supports our working hypothesis that localization of these enzymes within ligament tissue is associated with upregulation of collagenolytic remodeling within diseased CCL. Lameness in dogs with CCL disease is likely caused by joint instability and osteoarthritis. Furthermore, the mechanical properties of the ligament are probably degraded over time.37 We believe that these factors are the likely explanation for the lack of correlation between cells that contained TRAP within the ruptured CCL and duration of lameness in the dogs of this study. Greater numbers of cells containing cathepsin K within the CCL of dogs with a short duration of lameness suggests cathepsin K may be associated with late ligament remodeling and final structural failure.

We identified colocalization of cathepsin K in 64% of ruptured CCL that also contained cells with TRAP. This value probably underestimates colocalization because of false-negative double staining in some ligaments that contained only a small quantity of cathepsin K. Furthermore, some sections from dogs with CCL rupture contained tissue predominantly from the core region, which had marked phenotypic transformation of ligament fibroblasts,35 and little epiligamentous tissue. The precise origin of cells containing TRAP and cathepsin K is unclear. Although it is recognized that ligament tissue adapts and remodels to its loading environment,36 this process is poorly understood. In particular, it is not known what type of cell is primarily responsible for collagenolysis during noninflammatory ligament remodeling. Furthermore, the role of the epiligamentous repair tissue, in which cells that contain TRAP and cathepsin K predominate, in mechanisms associated with CCL rupture is also unclear. Although it has been suggested that ligament fibroblasts may be primarily responsible for collagenolysis during remodeling,36 the rounded phenotype of many of the cells that contained TRAP and cathepsin K and the recent discovery that CD68+ macrophages that contain TRAP and cathepsin K29 are capable of osteolysis, suggests that the cells we have identified in diseased CCL may be bone marrow-derived macrophage-like cells that migrate into CCL tissue in a process analogous to the activation of osteoclastic bone resorption.

In the study reported here, we identified cells with a rounded phenotype within CCL tissue from dogs with CCL rupture and aged dogs without CCL rupture that co-express the proteinases TRAP and cathepsin K.
We did not detect these rounded protease-positive cells in CCL tissue from young dogs without CCL rupture. The significant association between the presence of these proteases and ruptured CCL suggests that they may be involved in the cellular events associated with CCL rupture, repair, or both. It seems possible that the cell-signaling pathways that regulate expression of these enzymes may form part of the mechanism that leads to upregulation of collagenolytic ligament remodeling and progressive structural failure of the CCL in dogs over time.

References