Morphogenesis of Degenerative Changes Predisposing Dogs to Rupture of the Cranial Cruciate Ligament

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ABSTRACT. The cranial cruciate ligaments (CCL) of 13 dogs with clinical signs of CCL rupture and those of 22 clinically healthy young beagle dogs for laboratory use were examined histopathologically and immunohistochemically. The most constant changes at an early stage of degenerating ligament tissue in affected dogs were nuclear enlargement and perinuclear halo formation of fibrocytes followed by chondroid metaplasia. These changes were also frequent in apparently healthy young beagles kept under laboratory conditions. PAS and alcian blue positive substance accumulated around activated fibrocytes and within perinuclear halos. S-100 protein was also positive in these cells preceding the morphological change of chondroid metaplasia. Increased mitotic figures and Ki-67 positive cells showed the proliferating nature of these cells at a later stage. Alteration of extracellular matrices from dense collagen fiber type to those of cartilage tissue seemed to predispose dogs to rupture of the CCL along with a degradation in collagen fiber of the primary bundles. Collagen fiber bundles with a parallel fibrillar array never formed in the CCL with degraded primary bundles, whereas activated fibrocytes constantly underwent chondroid metaplasia. The pathogenic mechanism underlying chondroid metaplasia was thought to be nonspecific and attributable to an essential property of activated fibrocytes in the mature tendon tissue. — KEY WORDS: chondroid metaplasia, cranial cruciate ligament, morphogenesis, perinuclear halo of fibrocyte.

Rupture of the cranial cruciate ligament (CCL) is one of the most frequent causes of lameness in the hindlimb of dogs [6, 8]. Chronic degenerative changes in the CCL predisposing dogs to develop CCL rupture have been pointed out by many authors, because severe traumatic anamnesis was absent in the majority of cases [8]. Some authors have studied the mechanical properties of the CCL in dogs of various ages and breeds showing no clinical signs of the ligament failure, and have reported a decrease in material properties such as maximum stress and strain energy with aging [21]. Although clinical and surgical managements of CCL rupture in dogs have been reported by many authors [4–6, 13, 17–19], only a few histopathological changes in the ruptured CCL have been described [9, 13, 20, 21]. Moreover, the early changes and morphogenesis of the degenerative process causing CCL rupture are controversial. In contrast, there is a wide agreement on the pathogenesis and morphogenesis of secondary changes in the joint cartilage and synovial membrane following naturally-occurring and experimentally-induced rupture of the CCL [7, 8, 10, 14, 16, 19].

The present study was undertaken to clarify the morphogenesis of degenerative changes in the ruptured CCL in dogs, comparing them with the morphology of the CCL in apparently healthy young beagles.

MATERIALS AND METHODS

Ruptured CCL tissue: Ruptured CCL tissues were surgically removed from 13 dogs that were treated for injuries at Midori Animal Hospital. All the 13 dogs ranging in age from 2 to 14 years (Table 1) showed claudication without any apparent evidence of trauma, and were diagnosed with rupture of the CCL after confirmation of drawer sign. These animals underwent surgery within 3 days after the onset of clinical signs. Tissues of the CCL were removed as much as possible and immersed in 10% buffered formalin.

Intact CCL tissue: Intact CCL tissues were sampled from 22 clinically healthy beagles that had been euthanized and necropsied as the control animals for two toxicity studies. They were 11 males and 11 females ranging in age from 15 to 16 months. Almost the same tissue part as that of the ruptured CCL was sampled after fixation in 10% buffered formalin.

Tissue preparation: All ligament tissues were defatted, dehydrated in an automated processor, and embedded in paraffin. Sections were cut at 6–9 μm and stained with hematoxylin and eosin (HE), alcian blue with periodic acid-Schiff (alcian blue-PAS) for mucopolysaccharide, and Masson's trichrome for collagen fiber. Selected sections were immunohistochemically stained with S-100 protein (DAKO; California, USA) and Ki-67 antigen (NOVO CASTRA; Newcastle, UK) for cell cycle proliferation marker using the labeled streptavidin biotin method after either pretreatment with 0.4% pepsin solution in 0.01 N HCl or with microwaves for 1 hr in 10 mM citrate buffered solution (pH 6.0).
Table 1. Dogs with ruptured cranial cruciate ligament

<table>
<thead>
<tr>
<th>Case No.</th>
<th>Sex</th>
<th>Onset age of clinical sign (years)</th>
<th>Breed</th>
<th>Body weight (kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>F</td>
<td>5</td>
<td>Yorkshire terrier</td>
<td>4.7</td>
</tr>
<tr>
<td>2</td>
<td>M</td>
<td>7</td>
<td>Beagle</td>
<td>11.0</td>
</tr>
<tr>
<td>3</td>
<td>F</td>
<td>5</td>
<td>Yorkshire terrier</td>
<td>4.7</td>
</tr>
<tr>
<td>4</td>
<td>F</td>
<td>12</td>
<td>Yorkshire terrier</td>
<td>2.9</td>
</tr>
<tr>
<td>5</td>
<td>F</td>
<td>11</td>
<td>Mongrel</td>
<td>10.6</td>
</tr>
<tr>
<td>6</td>
<td>M</td>
<td>2</td>
<td>Pomeranian</td>
<td>3.0</td>
</tr>
<tr>
<td>7</td>
<td>M</td>
<td>4</td>
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</tr>
<tr>
<td>8</td>
<td>F</td>
<td>3</td>
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<tr>
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<td>11</td>
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<td>4.1</td>
</tr>
<tr>
<td>10</td>
<td>M</td>
<td>8</td>
<td>Beagle</td>
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</tr>
<tr>
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</tr>
<tr>
<td>13</td>
<td>F</td>
<td>13</td>
<td>Maltese</td>
<td>2.7</td>
</tr>
</tbody>
</table>

RESULTS

Intact ligament tissue: Tendinous parts of the CCL tissue from apparently healthy beagles were mainly composed of dense collagen tissue subdivided into secondary bundles of parallel arrays of primary collagen bundles by delicate loose connective tissue containing the small-caliber blood vessels. Ovoid or elongated nuclei of fibrocytes were located at the periphery of the primary collagen bundle array like those of skeletal muscle fibers in the longitudinal sections. In the intact ligament tissue, the most frequent changes were nuclear enlargement and perinuclear halo in fibrocytes (Fig. 5) occurring in 12 out of 22 cases. Collagen fibers at the outer margin showed a weak positive reaction to alcin blue and PAS reagent in the affected areas (Fig. 1). Enlarged nuclei increased in width, and clear spaces delimited by a delicate membrane from the surrounding collagen fibers were formed in the perinuclear area (Figs. 1 & 5). Some perinuclear halos contained fine granular or delicate web-like structures and took on an appearance resembling the foamy cytoplasm of phagocytic macrophages (Fig. 2). All the fine granular substances were positive for S-100 protein, while normal fibrocytes were negative for it (Fig. 3). Some perinuclear halos contained alcin blue and PAS positive substances (Fig. 2). The surface area of primary collagen bundle array increased their staining intensity for alcin blue.

These changes were more prominent in the area where the interbundle connective tissue showed edematous loosening or induration. Perivascular induration or fibrous thickening of the wall of the small-caliber vessels running through the septal connective tissue was observed in 6 out of 22 cases. The lumen of the affected vessels was frequently occluded by PAS positive granular or hyalineous substances (Figs. 6 & 7). In one case there were hemosiderin deposits in macrophages migrating into the interfascicular space of primary collagen bundles and perivascular tissue.

No apparent inflammatory changes were seen in the tendon sheath or synovial membrane. Ruptured CCL tissue: In 13 cases with ruptured CCL tissues, perinuclear halos of fibrocytes were observed more frequently and constantly over a widespread area than in the healthy dogs. Increased amount and intensity of basophilia of the fine granular or web-like substance in the perinuclear halo gave these fibrocytes an appearance of chondrocytes, indicating chondroid metaplasia (Figs. 4 & 8). Mitotic figures and binucleated cells were also prominent in these areas (Fig. 9). The cell proliferating marker Ki-67 antigen was positive in these cells by immunohistochemistry. Each collagen fiber appeared loosened and individually was stained with aniline blue in sections stained with Masson’s trichrome, in contrast to the homogeneous pale red appearance of closely packed fibers in the normal primary collagen bundles (Fig. 4). The normal structure of tendon tissue with a parallel array of compact primary collagen bundles was completely broken down in severely affected areas. The loose collagen fibers arranged irregularly and the parallel array of the primary bundle were replaced by an irregular-shaped mass of degraded and deteriorated collagen fibers (Fig. 10). These deteriorated fibers revealed various changes, i.e., loss of fibroblastic nuclei, accumulation of alcin blue positive mucous substance, slits and vacuole formation of swollen bundles, and partial calcification of degenerated collagen bundles or osteoid metaplasia.

Proliferation of the capillary blood vessels was observed in a part of the ligament tissue in 10 cases (Fig. 11). The degrees of vascularization varied from case to case. In mild cases, several capillaries with enlarged endothelial cells were formed in a degenerated mass of collagen fibers. In severe cases, almost the entire width of the ligament tissue was replaced by granulation tissue consisting of newly formed capillaries and fibroblasts surrounding the capillary lumen. Mild hemorrhage or hemosiderosis was also observed in the perivascular area of 3 cases. Fibrin thrombus formed in the capillary lumen in one case with severe hemorrhage and edematous loosening of the perivascular connective tissue.

The small to moderate numbers of inflammatory cells mainly composed of lymphocytes, plasma cells and macrophages infiltrated into subepithelial layer of the tendon sheath (Fig. 12). A small number of inflammatory cells accumulated throughout the synovial tissue. Papillary proliferation of the hypertrophic synovial epithelium was also prominent in cases that showed a relatively severe inflammatory cell infiltration.

DISCUSSION

Most ruptured CCL in the present study showed various stages of degenerative changes and evidence of previous episodes of hemorrhage such as hemosiderosis or extravasation of erythrocytes in the ligament tissue as reported in aged large dog breeds [20]. Degraded collagen fibers and chondroid metaplasia strongly suggest that these
Fig. 1. Cranial cruciate ligament (CCL) of a young beagle (Clinically healthy dog). Weak positive reaction to alcian blue-PAS reagent in the surface of primary collagen bundle and perinuclear area surrounded by halo. Alcian blue-PAS, × 370.

Fig. 2. CCL of a young beagle (Clinically healthy dog). Enlarged nuclei with formation of perinuclear halo in fibrocytes located at the surface of primary collagen bundle. Some fibrocytes contain PAS-positive fine granules in the halo. Alcian blue-PAS, × 370.

Fig. 3. CCL of a young beagle (Clinically healthy dog). Many activated fibrocytes are S-100 positive, while there are negative cells with slender nuclei. Immunohistochemistry for S-100 protein, × 370.

Fig. 4. Ruptured CCL of an affected dog (Case No. 12). Degraded collagen fibers with increased positive reaction to alcian blue-PAS. Alcian blue positive deposit in cytoplasm of fibrocytes under chondroid metaplasia. Alcian blue-PAS, × 370.

Changes may have caused tissue fragility to the mechanical tension of the CCL. According to Vasseur et al. [20], degenerative changes predisposing to CCL rupture consisted of a loss of the nuclei of fibrocytes, degradation in the primary collagen bundles, chondroid metaplasia of surviving fibroblasts, dystrophic calcification and fibrous repair with
vascularization. These changes were similar to those in our cases, including dogs with lighter weight.

Among changes observed in our study, the most frequent and earliest lesion was a nuclear enlargement with perinuclear halo formation in fibrocytes located on the surface of the collagen bundle, which was observed in a fairly intact area. The nuclear enlargement and perinuclear halo (manifestation of cell border with cytoplasmic
enlargement) are considered to be caused by the activation of fibrocytes because of the character shared with cells showing proliferating activity. Such an activity in fibroblasts (activated fibrocytes) was also evident from increased mitotic figures and Ki-67 positive cells in the more advanced stage. Activation of fibrocytes in the CCL of clinically healthy young beagles indicated that such early changes already began appearing at 2 years of age and below.
even under laboratory conditions. The accumulation of PAS and alcian blue positive substances in surrounding collagen fiber suggested an alteration in collagen fiber protein such as increased mucopolysaccharide, and may have attributed to the homogeneous appearance in collagen fiber bundles. Akerson et al. [1-3] reported that experimental immobilization of the knee joint induced biochemical and biomechanical changes including the mucopolysaccharide content in periarticular connective tissue, and showed that immobility had deleterious effects on the joint tissue [15]. The pathogenesis of this alteration was unclear. However, loosening of the primary collagen fiber bundle and its subsequent homogeneous appearance evidently suggested a local edematous process in this area along with fibrosis of the wall of the small-caliber vessels. Altered microenvironment around fibrocyte is thought to be one of the most important factors involved in the activation of fibrocytes and subsequent metaplasia.

The intracytoplasmic accumulation of the PAS and alcian blue positive substance had never been observed in fibrocytes of the normal tendon. It is likely that an accumulation of intracytoplasmic products precedes the morphological evidence of chondroid metaplasia of these cells in the later stage. This is also suggested by the evidence that the tinctorial and histochemical properties of these substances were identical with those of cartilage matrix. Extracellular deposition of PAS and alcian blue positive substance might also have resulted from the secretion of cartilage matrix by these cells quite apart from degenerative changes in collagen fibers involved in the edematous process. A change in the collagen fiber type seems to be another possible predisposing factor for rupture of the CCL in these areas. The positive reaction to S-100 protein also seems to follow from the altered nature of the intracytoplasmic protein of activated fibrocytes. S-100 protein is positive for a wide variety of mesenchymal cells including chondrocytes. Therefore, such an immunohistochemical detection of altered cytoplasmic protein is considered to be further evidence of a submorphological phenotypic change in fibrocytes, especially for chondroid metaplasia. Production of cartilaginous matrix by metastatic chondrocytes was very common in the affected CCL instead of production of collagen fibers for reinforcement or scar formation by activated fibrocytes. Chondroid metaplasia might be an essential precondition for activated fibrocytes after the production and organization of primary collagen bundles with large amounts of collagen fiber in the mature tendon tissue. From this standpoint, chondroid metaplasia may be not due to a peculiar cause, but rather by wide varieties of factors causing the fibroblast activation resulting in the cartilage production.

Some authors pointed out that immunological or inflammatory processes play an important role in the pathogenesis of CCL rupture in dogs [9, 11]. Lymphocytic aggregation in the tendon sheath or synovial tissue was severe in some cases with CCL rupture in the present study. However, early degenerative changes or activation of fibrocytes were frequently seen even in the young beagles without any inflammatory cell infiltration. An increased level and incidence of inflammatory response in the later stage of CCL rupture may explain the close association with secondary damage of the joint cartilage and synovial membrane [12]. The frequent occurrence of early changes in the CCL tissues of young beagles for laboratory use rather suggests that a lack of exercise may be one of the causative factors.

REFERENCES

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