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Nitric oxide and metalloproteinases in canine articular ligaments: A comparison between the cranial cruciate, the medial genual collateral and the femoral head ligament

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Abstract

Osteoarthritis due to cranial cruciate ligament (CCL) rupture or hip dysplasia is one of the most important causes of chronic lameness in dogs. This study aimed at comparing nitric oxide (NO) production by the CCL with that of the femoral head ligament (FHL) and the medial collateral ligament (MCL), and investigating the pathway of NO production and the concomitant metalloproteinase (MMP) activity in the presence or absence of an inflammatory stimulus. Ligaments of normal dogs were subjected to different stimuli, and NO and MMP activity from explant culture supernatants were compared.

The results showed that in explant cultures of the canine CCL more NO was produced than in those of the other two ligaments. A higher level of NO was produced when CCLs were exposed to the inducible nitric oxide synthase (iNOS)-inducing cocktail TNF/IL-1/LPS, and NO synthesis could be inhibited by both L-NMMA, a general nitric oxide synthase (NOS) inhibitor and L-NIL, a specific iNOS inhibitor. However, a correlation between NO synthesis and iNOS expression levels as determined by immunohistochemistry was not observed. In contrast to CCL, no evidence for iNOS-dependent NO synthesis was observed for MCL and FHL. The CCL produced less MMP than MCL and FHL, and no correlation between MMP and NO could be demonstrated. MMP activity in the CCL increased significantly after 48 h of incubation with the inflammatory stimulus. The results suggest that in canine osteoarthritis NO synthesized by canine CCL plays a more important role in the pathogenesis of osteoarthritis of the stifle than that synthesized by FHL and MCL.

Keywords: Cranial cruciate ligament rupture; Degenerative joint disease; Cranial cruciate ligament; Femoral head ligament; Medial collateral ligament; Nitric oxide; Metalloproteinase

1. Introduction

Cranial cruciate ligament (CCL) rupture is a common disease in humans as well as in dogs. Anterior cruciate ligament ruptures occur in humans most often after trauma, while the majority of canine CCL ruptures are secondary to chronic degenerative changes within the ligament (Arnoczky, 1993; Vasseur et al., 1985). CCL rupture results in severe instability and degenerative joint disease in the stifle. Hip joint laxity is one of the possible causes for hip osteoarthritis (OA) but the function of the femoral head ligament (FHL) in unstable hip joints has not been thoroughly evaluated and most of the present information attributes only a minor role to the FHL in hip OA.

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Instability due to rupture of the medial collateral ligament (MCL) can cause OA in the stifle but this plays a minor role compared to CCL rupture in canine stifle OA.

It is widely accepted that nitric oxide (NO) plays an important role in the pathogenesis of degenerative joint disease (Evans et al., 1995; Murrell et al., 1996). In the joints, NO is mainly produced by chondrocytes and acts as a mediator of inflammation and activator of metalloproteinases (MMP) (Murrell et al., 1995), causing increased proteoglycan degradation (Evans et al., 1995). Recent studies have demonstrated measurable amounts of NO in canine CCLs (Riitano et al., 2002; Spreng et al., 2000).

There are ultrastructural differences between the cells of the CCL and cells from other ligaments such as the medial collateral ligament (MCL) of the knee joint (Lyon et al., 1991). The oval CCL cells more closely resemble chondroid cells than fibroblasts. Following an inflammatory stimulus (i.e. a cocktail of interleukin-1 (IL-1), tumor necrosis factor (TNF) and lipopolysaccharide (LPS)), these cells show pronounced expression of inducible NO synthase (iNOS) in vitro (Riitano et al., 2002). Accordingly, higher amounts of NO are detected in ligaments stimulated with the above cocktail than in unstimulated ligaments (Lyon et al., 1991; Riitano et al., 2002). Another study (Cao et al., 2000) showed that in rabbit CCLs more NO was produced than in the caudal cruciate ligament and in the collateral ligament. The authors suggested that NO was responsible for the lower healing capacity of the CCL versus the MCL. All these findings are consistent with a role of NO in the pathogenesis of canine CCL rupture.

Metalloproteinases are degradative enzymes that may have a major role in the breakdown of articular cartilage and therefore in the pathogenesis of osteoarthritis. They are induced by proinflammatory cytokines such as IL-1, IL-6, TNF and probably also by NO (Pelletier et al., 2004; Tetlow et al., 2000). It is possible that not only NO but also MMP's are produced by ligamentocytes in the CCL.

The aims of this study were to compare the production of NO in canine CCL with another intra-articular ligament (FHL) and an extra-articular ligament (MCL), to assess pathways of NO production in these ligaments and the reaction of the different ligaments to an inflammatory stimulus. Further aims were to measure the MMP activity in the ligaments and to evaluate whether MMP activity is correlated to NO production.

2. Material and methods

2.1. Dogs

Material was collected from seven middle to large breed dogs that were euthanased for reasons unrelated to degenerative joint disease (DJD) at our hospital. DJD was excluded as were dogs that had received anti-inflammatory drugs within the previous month. The mean weight of the five female and two male dogs was 29.0 ± 8.8 kg and the mean age was 6.2 years.

2.2. Ligaments

CCLs and MCLs of both knee joints and FHLs of both legs were harvested immediately after euthanasia. A total of fourteen CCLs, fourteen MCLs and twelve FHLs could be collected for examination. The central third of the ligament was used for further analysis.

2.3. Explant culture

Immediately after harvesting, ligaments were divided longitudinally into five equal pieces (A–E) and placed in sterile pyrogen-free phosphate-buffered salt solution (PBS), weighed, transferred to a 24-well tissue culture plate and cultured in 1 mL of sterile nutrient medium (Ham's F-12, Fluka AG) containing streptomycin (100 μg/mL) and penicillin (100 U/mL) (both from Fluka AG). Piece A was incubated in nutrient culture medium alone. A stimulation cocktail of 0.01 μg/mL IL-1 (human recombinant IL-1, 1β, Pepro Tech), 0.1 μg/mL TNF (human recombinant TNF, Cetus) and 1 μg/mL LPS (*E. coli* 055:B5, cat. no. L2637, Sigma Chemical Company) was added to piece B. The same stimulation cocktail and 2.5 mM L-N_6-iminoethyil-L-arginine (L-NIL, Alexis Biochemicals) were added to piece C, 2.5 mM L-NIL to piece D and 2.5 mM NG-monomethyl-L-arginine (L-NMMA, Sigma) to piece E. Specimens were incubated at 37 °C in humidified air containing 5% CO_2. Explant supernatant was aspirated after 24 and 48 h of culture and stored at −20 °C for further examination. The ligament explants were harvested after 48 h and processed for iNOS immunohistochemistry.

2.4. Tissue specimens

All tissue samples were immersion fixed in 4% paraformaldehyde in PBS, embedded in paraffin, cut in 4 μm sections on a microtome and mounted on positively charged glass slides. Additionally haematoxylin and eosin (HE) stains were applied to samples of piece A from all ligaments.

2.5. Nitric oxide metabolite determination

NO production in cell culture supernatants was estimated by measurement of its stable end products, nitrite and nitrate. Nitrate reduction was performed with bacterial nitrate reductase from *Pseudomonas*