Relative expression of matrix metalloproteinase-2 and -9 in synovial fluid from healthy calves and calves with experimentally induced septic arthritis

David Francoz, DVM, MSc; André Desrochers, DVM, MS, Nathalie Simard, MSc; Yves Saint Pierre, PhD; Gilles Fecteau, DVM; Jean Sébastien Latouche, DVM; Mado Fortin, DVM

Objective—To identify changes over time in relative expression of matrix metalloproteinase-2 (MMP-2) and -9 (MMP-9) in synovial fluid from healthy calves and calves with experimentally induced septic arthritis.

Animals—12 Holstein calves.

Procedures—In 7 calves, *Escherichia coli* was injected in the right tarsal joint on day 1. Joint lavage was performed on day 2, and calves were treated with ceftiofur from days 2 through 21. Synovial fluid samples were collected on days 1 (before inoculation), 2 (before joint lavage), 3, 4, 8, 12, 16, 20, and 24. In the remaining 5 calves, joint lavage was performed on day 2 and synovial fluid samples were collected from the left tarsal joint. Relative expression of MMP-2 and MMP-9 was determined by means of gel zymography.

Results—On day 1, MMP-2 was detected in all synovial fluid samples but MMP-9 was not detected. In calves with septic arthritis, values for relative expression of MMP-9 monomer and dimer were significantly increased on days 2 through 20 and days 2 through 24, respectively, and relative expression of MMP-2 was significantly increased on day 2 through 20. There were significant linear associations between relative expression of the monomer and dimer forms of MMP-9 and between neutrophil count and relative expression of the MMP-9 monomer and dimer forms.

Conclusions and Clinical Relevance—Results indicated that relative expression of MMP-9 and MMP-2 increased in synovial fluid from calves with experimentally induced septic arthritis, with relative expression remaining high for several days after infection. (Am J Vet Res 2008;69:1022–1028)

The inflammatory response associated with septic arthritis is characterized by a rapid influx of inflammatory cells, mostly neutrophils, and activation of synoviocytes and chondrocytes. Together, these cells release numerous inflammatory mediators, particularly MMPs. The MMPs are a group of calcium- and zinc-dependent proteinases that play a crucial role in the turnover of the extracellular matrix of articular cartilage. On the basis of their structural characteristics and substrate specificities, the MMPs have been classified as collagenases, gelatinases, stromelysins, matrilysins, membrane-type MMPs, and other MMPs.

Relative expression of 2 gelatinase MMPs—MMP-2 and MMP-9—has been reported to be increased in synovial fluid from people, dogs, horses, and cattle with various articular disorders. The roles of MMP-2 and MMP-9 in these various articular disorders are not clearly understood, although it is known that expression of MMP-9 is stimulated by inflammatory cytokines, whereas MMP-2 is constitutively expressed by joint cells. Both MMP-2 and MMP-9 cleave denatured collagen, basement membrane type IV collagen, and laminin and have been reported to synergistically potentiate the action of interstitial collagenases and to play an important role in the control of tissue inhibitors of MMPs. These MMPs have been proposed as markers of arthritic diseases.
in dogs and horses and are potential therapeutic targets for people with various arthritic diseases. Understanding the changes in relative expression of MMP-2 and MMP-9 that occur over time in cattle with septic arthritis may provide some insight into their potential use as diagnostic or prognostic markers and may improve our understanding of the underlying pathophysiology of this condition. In previous studies, we examined changes in synovial fluid that occur with repeated arthrocentesis and joint lavage in healthy calves and in calves with experimentally induced septic arthritis treated with ceftioufur. However, expression of MMP-2 and MMP-9 was not examined in those studies. The purpose of the study reported here was to identify changes in relative expression of MMP-2 and MMP-9 in synovial fluid that occur with repeated arthrocentesis and joint lavage in healthy calves and in calves with experimentally induced septic arthritis treated with ceftioufur.

Materials and Methods

Study design—Synovial fluid samples obtained during a previous study involving 7 Holstein calves with experimentally induced septic arthritis and during a previous study involving 5 healthy Holstein calves were used in the present study. The 7 calves with experimentally induced septic arthritis were all male and ranged from 14 to 20 days old at the time of the previous study. On day 1 of the previous study, the right tarsal joint of each calf had been inoculated with $10^8$ CFU of a viable Escherichia coli ECL 1018 O116:K7:H9 strain suspended in 1 mL of PBS solution. Calves were evaluated 3 times a day for the first 6 days after inoculation, then once daily until day 24 for evidence of joint heat, swelling, or pain; lameness; or systemic signs of illness. A clinical pain score was assigned, and analgesia was provided as necessary. Treatment with ceftioufur (1 mg/kg, IV, q 12 h) was begun on day 2 and continued through day 21. Through-and-through joint lavage was performed with lactated Ringer's solution (1 L) on day 2. Synovial fluid samples (5 mL) were collected from the right tarsal joint on days 1 (before inoculation), 2 (before joint lavage), 3, 4, 8, 12, 16, 20, and 24 (3 days after treatment with ceftioufur was discontinued).

The 5 healthy calves in the second study consisted of 3 males and 2 females. Calves were between 20 and 36 days old at the time of the study. Synovial fluid samples (5 mL) were collected from the left tarsal joint of all calves on days 1, 2, 3, 4, 8, 12, 16, 20, and 24. Through-and-through joint lavage was performed with lactated Ringer's solution (1 L) on day 2 after collection of the synovial fluid sample.

During the previous 2 studies, a portion of each synovial fluid sample had been submitted for routine cytologic analysis, including determination of total and differential WBC counts. In addition, portions of the day 1 samples collected from the 5 healthy calves and portions of all samples collected from the calves with experimentally induced septic arthritis were submitted for bacterial culture. For the present study, the remaining portions of the synovial fluid samples were submitted for determination of relative expression of MMP-2 and MMP-9. Samples had been centrifuged at 5,000 × g for 5 minutes immediately after collection and stored at −70°C until analyzed.

Relative expression of MMP-2 and MMP-9—Relative expression of MMP-2 and MMP-9 in synovial fluid samples was determined by means of gelatin zymography. Zymography was performed in 8% SDS polyacrylamide gels that had been cast in the presence of gelatin (0.1 mg/mL), as described. Samples (5 μL) were diluted 1:10 in loading buffer without prior denaturation and loaded on the gels. After electrophoresis, the gels were washed to remove the SDS and incubated for 18 hours at 37°C in a renaturing buffer (30 mM Tris, 5 mM CaCl$_2$, 0.02% NaN$_3$, and 1% Triton X-100). Gels were subsequently stained with Coomassie brilliant blue G-250 and destained in 30% methanol–10% acetic acid (vol/vol) to detect gelatinase expression. Aliquots of supernatants of HT 1080 human cells containing pro-MMP-9 and pro–MMP-2 were included as positive controls and as standards for determination of molecular weight of observed bands.

Computer-assisted image analysis was used to quantify relative expression on the zymography gels. Images were captured with a video camera linked to a computer and analyzed with appropriate software. For each observed band, the area of substrate clearing and mean intensity of the band were determined and expression was calculated by multiplying area of the band by mean intensity. To allow comparisons between gels, this value was divided by expression of the positive control sample (ie, supernatant from HT 1080 human cells) to obtain relative expression.

Statistical analysis—Mean values for relative expression of MMP-2 and of the monomer and dimer forms of MMP-9 were calculated for each sample time. A linear model for repeated measures was used to evaluate the effect of sample time on relative expression. When sample time had a significant effect, the Dunnett post hoc test was used to compare values for each sample time with the value for day 1. Mixed linear model analysis with calf as a random factor was used to evaluate the association between relative expression of MMP-2 and MMP-9 and neutrophil and monocyte counts as well as the association between relative expression of the monomer and dimer forms of MMP-9. The effect of joint lavage on relative expression of the monomer form of MMP-9 was evaluated by comparing the slope between days 2 and 3 with the slope between days 3 and 4 with the Wilcoxon signed rank test. All analyses were performed with standard software. Values of $P < 0.05$ were considered significant.

Results

Details of clinical signs and results of cytologic examination and bacterial culture of synovial fluid samples have been reported. In brief, all 7 calves in which the right tarsal joint was inoculated with E coli developed typical clinical signs of septic arthritis (ie, lameness and joint pain and swelling) that were evident until day 9. None of the 5 healthy calves had any clinical signs of joint disease during the study. For all 12 calves, results of bacterial culture of synovial fluid samples collected on day 1 were negative. For the 7 calves with septic ar-
thritis, results of bacterial culture for samples collected on day 2 were positive and results for samples collected on day 8 were negative. Cytologic examination of synovial fluid samples revealed that some samples had slight to moderate blood contamination. Total and differential WBC counts have been reported for the 5 healthy calves. In these calves, WBC, neutrophil, and monocyte counts on day 2 were significantly increased, compared with day 1 values, and decreased thereafter. Mean monocyte count on day 3 (7.49 × 10⁹ monocytes/L; range, 0.91 to 22.2 × 10⁹ monocytes/L) was significantly increased, compared with mean count on day 1 (0.22 × 10⁹ monocytes/L; range, 0.08 to 0.59 × 10⁹ monocytes/L), and decreased thereafter.

Relative expression of MMP-2 and MMP-9—In all 12 calves, latent pro–MMP-2 (72 kDa) was detected in synovial fluid samples obtained on day 1, but pro–MMP-9 (98 kDa) was not detected (Figures 1 and 2). In calves with experimentally induced septic arthritis, latent pro–MMP-2 (72 kDa) with an additional, partially proteolyzed, activated lower molecular weight form was detected in subsequent synovial fluid samples, along with pro–MMP-9 monomer (98 kDa) with an additional, partially proteolyzed, activated lower molecular weight form, and MMP-9 dimer (225 kDa). In the healthy calves, pro–MMP-2 and pro–MMP-9 monomers were detected in subsequent synovial fluid samples, but MMP-9 dimer was not detected in any samples.

For the 7 calves with experimentally induced septic arthritis, mean values for relative expression of the monomer (Figure 3) and dimer (Figure 4) forms of MMP-9 were highest on day 2 and decreased consistently thereafter. Relative expression of MMP-9 monomer was significantly (\( P < 0.03 \)) increased on days 2 through 20, compared with relative expression on day 1, but relative expression on day 24 was not significantly (\( P = 0.2 \)) different from relative expression on day 1. Relative expression of MMP-9 dimer was significantly (\( P < 0.03 \)) increased on days 2 through 24, compared with relative expression on day 1. There was a significant (\( P < 0.001 \)) linear relationship between relative expression of MMP-9 monomer and relative expression of MMP-9 dimer. Relative expression of MMP-2 was significantly (\( P < 0.01 \)) increased on days 8 through 24, compared with relative expression on day 1 (Figure 5).

For the 5 healthy calves, relative expression of MMP-9 monomer was highest on days 2 and 3 (Figure 6), and relative expression was significantly (\( P < 0.02 \)) increased on days 2, 3, and 4, compared with relative expression on day 1. Relative expression of MMP-2 was significantly (\( P < 0.03 \)) increased on days 8 through 24, compared with relative expression on day 1 (Figure 7).

Relative expression of MMP-9 monomer was not significantly different between the 2 groups on days 1.
Relative expression of MMP-2 was significantly lower in the healthy calves than in the calves with septic arthritis on days 4 and 8 ($P = 0.007$ and $P = 0.02$, respectively) but was not significantly different ($P > 0.07$) between groups on any other day.

For the calves with septic arthritis, mixed linear model analysis revealed significant linear associations between neutrophil count and relative expression of the monomer and dimer forms of MMP-9. However, significant linear associations were not detected between neutrophil count and relative expression of MMP-9 in the healthy calves ($P = 0.32$) or between neutrophil count and relative expression of MMP-2 in either group.

Mixed linear model analysis revealed significant ($P < 0.001$) linear associations between monocyte count and relative expression of MMP-9 monomer for calves in both groups and between monocyte count and relative expression of MMP-9 dimer for calves with septic arthritis ($P < 0.001$). A significant ($P = 0.01$) linear association was detected between monocyte count and relative expression of MMP-2 for calves with septic arthritis but not between monocyte count and relative expression of MMP-2 for healthy calves ($P = 0.42$).

Joint lavage did not appear to have a significant effect on relative expression of MMP-9 monomer in either group, in that the slope of the relative expression of MMP-9 between days 2 and 3 was not significantly different from the slope between days 3 and 4.

**Discussion**

Results of the present study indicated that relative expression of MMP-9 and MMP-2 increased in synovial fluid from calves with experimentally induced septic arthritis, with relative expression remaining high for several days after infection. Additional study is needed to determine whether measuring relative expression of these MMPs can be useful in the diagnosis of septic arthritis and whether these MMPs are useful as potential therapeutic targets in affected animals.

Previous studies involving tracheobronchial lavage fluid from calves and synovial fluid from cattle, other species, and humans have revealed that the 98- and 225-kDa bands obtained by means of gelatin zymography represent the monomer and dimer forms of MMP-9. Therefore, on the basis of these studies, the 98- and 225-kDa bands observed in the present study were considered to be the monomer and dimer forms of MMP-9. The strong linear association between relative expression of the 98-kDa band and relative expression of the 225-kDa band supported this hypothesis. The thin bands of gelatinase expression observed at approximately 86 kDa could be considered an active form of MMP-9. Additional, partially proteolyzed, activated lower molecular weight forms have also been reported in synovial fluid from infected joints in horses and humans.

Matrix metalloproteinase-9 has been reported to be secreted by neutrophils, monocytes, macrophages, synovial cells, and chondrocytes. In diseased joints, however, MMP-9 is reportedly produced mainly by neutrophils and macrophages, and in previous studies, expression of MMP-9 in synovial fluid from septic joints has been correlated with neutrophil count. In the present study, the strong associations between relative expression of the monomer and dimer forms of MMP-9 and neutrophil monocyte counts in synovial fluid from the calves with experimentally induced septic arthritis suggested that MMP-9 mainly originated from infiltrating neutrophils and monocytes. In contrast, neutrophil count was not significantly associated with relative expression of MMP-9 monomer in synovial fluid from the calves with experimentally induced septic arthritis on days 2 through 16.
vial fluid from the healthy calves, although monocyte count was significantly associated with relative expression of MMP-9 monomer in these calves. In our previous study, we found that arthrocentesis significantly increased neutrophil and monocyte counts in synovial fluid from the healthy calves. Together, these results suggest that monocytes were the main source of MMP-9 in synovial fluid from the healthy calves, but the possibility that infiltrating neutrophils played a role cannot be excluded.

As previously reported in cattle and other species, a single 72-kDa gelatinolytic band was identified in synovial fluid obtained from the calves on day 1, and this band was identified as pro–MMP-2 on the basis of results of these previous studies and studies involving tracheobronchial lavage fluid from calves. This band was considered to represent constitutive expression of MMP-2 in synovial fluid. The additional band identified at approximately 62 kDa following infection in the calves with septic arthritis was considered to be active MMP-2 on the basis of results of a previous study. In the present study, relative expression of MMP-2 increased significantly over time both in the calves with septic arthritis and in the healthy calves, although it was significantly lower in the healthy calves on days 4 and 8. Relative expression of MMP-2 has previously been reported to be increased in synovial fluid from horses with septic joint diseases.

Most MMPs are secreted or expressed from cells as proenzymes that require further activation. Inflammatory cytokines, hormones, and growth factors are among the numerous factors that have been reported to regulate MMP gene expression. The reasons for the delay in MMP-2 overexpression (2 days following infection), compared with MMP-9 overexpression (1 day following infection), in the calves with septic arthritis remain unclear, but this delay could be a reflection of the cascade of events that occurs following joint infection. For instance, it is possible that MMP-2 is produced by cells that are not present at the time of joint infection and require time to reach the infected site. Alternatively, it is possible that MMP-9 originates from circulating neutrophils and is liberated in large amounts in the synovial fluid during the initial phase of infection, whereas MMP-2 originates from joint cells, such as chondrocytes, osteoblasts, and synovial fibroblasts, that require activation by cytokines or factors produced by cells involved in the initial phase of infection.

In general, MMP-2 is not constitutively expressed by monocytes. However, MMP-2 expression by monocytes has been reported in response to stroke, cancer, inflammatory cytokine stimulation, or use of polymeric meshes for wound healing. The linear association between relative expression of MMP-2 and monocyte count in synovial fluid from calves with septic arthritis was suggestive of a relationship between MMP-2 expression and monocytes. Unfortunately, the present study was not designed to allow us to evaluate this relationship. Thus, we could not determine whether there was a direct association between MMP-2 expression and monocytes or an indirect association related to the multicellular sources of MMP-2.

Joint lavage is considered an important step in the treatment of septic arthritis, as it is thought to remove potential deleterious enzymes and their cellular sources. In the present study, however, joint lavage did not appear to significantly reduce expression of MMP-9 in synovial fluid from calves with septic arthritis, in that the decrease in MMP-9 relative expression between days 2 and 3 (ie, immediately before and 1 day after joint lavage) was not significantly different from the decrease between days 3 and 4. In our previous study, we found that differential WBC counts in synovial fluid from these calves were also not significantly affected by joint lavage. On the other hand, the present study was not specifically designed to evaluate the effects of joint lavage and further studies are needed to determine its effect on MMP expression.

In humans with certain forms of arthritis, MMP-9 expression has been reported to correlate with local clinical signs and to reflect the severity of joint inflammation. In the present study, relative expression of MMP-9 was high during the acute phase of infection, when local clinical signs were most obvious, and decreased rapidly until day 8. In contrast, relative expression of MMP-2 did not appear to be associated with severity of local clinical signs.

The need for repeated arthrocentesis has been an important confounding factor when studying changes in the relative expression of MMPs. In human, MMP expression did not seem to be affected when a 1-week interval was allowed between arthrocentesis attempts, whereas in horses, a 2-week interval was necessary. However, these studies involved general MMP expression, including expression of MMP-1 and MMP-3, and little is known about the effects of repeated arthrocentesis on expression of MMP-9 and MMP-2. For the healthy calves in the present study, relative expression of MMP-2 was significantly increased, compared with relative expression on day 1, from days 8 through 24, suggesting that repeated arthrocentesis and joint lavage induced an increase in MMP-2 expression. We suspect that arthrocentesis induced moderate inflammation of the synovial membrane that resulted in the release or activation of MMP-2 and that a 4-day interval between arthrocentesis episodes was not sufficient to stop this process and allow MMP-2 expression to return to baseline. In contrast, relative expression of MMP-9 monomer in synovial fluid from the healthy calves was significantly increased only on days 2, 3, and 4, and values for relative expression of MMP-9 monomer on days 8 through 24 were not significantly different from the day 1 value. We speculate that arthrocentesis induced moderate inflammation of the joint that resulted in an immediate release or activation of MMP-9 but that a 4-day interval between arthrocentesis episodes was adequate to allow MMP-9 expression to return to baseline.

In the present study, only young animals were used and only the tarsal joint was evaluated. Calves with septic arthritis were all males, whereas the healthy calves were a mix of males and females. However, it seems unlikely that sex affected MMP expression, in that sex has not been reported to affect MMP expression in humans or mice. Previous studies in horses have demonstrated that MMP expression is higher in sy-
novial fluid from young animals than from old ones, secondary to increased metabolic activity and cartilage turnover. The impact of infection or inflammation on synovial fluid MMP expression could also be different in large joints compared with smaller ones.32 Activated cells and their products are probably more diluted in large joints, than in small ones. Consequently, results of the present study should be extrapolated to other joints and other age groups with caution.

Another important limitation of the present study was that we were not able to determine the effect treatment with ceftiofur had on MMP expression. However, cephalothin36 and other β-lactam antimicrobials37 have been reported to have inhibitory effects on MMP activity.

The effects of blood contamination on relative expression of MMP-2 and MMP-9 in synovial fluid were also not investigated in the present study. Blood cells such as neutrophils, lymphocytes, and monocytes may express MMP-2 and MMP-9, which may have artificially increased relative expression of these MMPs in contaminated samples. However, because results for contaminated samples in the present study were similar to results for uncontaminated samples obtained at the same time, it seems unlikely that blood contamination had a significant effect on our data.

Both MMP-2 and MMP-9 are believed to be important in the progression of joint diseases. However, their precise roles in the pathogenesis of joint disease are not completely understood. These enzymes have a high affinity for denatured collagen; they potentiate the action of interstitial collagenase5 and play a role in the control of tissue inhibitors of MMPs.15 A previous study38 of mice with antibody-induced arthritis suggested that MMP-2 decreased and MMP-9 enhanced the development of arthritis. In a study21 of mice with Staphylococcus aureus–induced arthritis, MMP-9 deficiency led to an increase in the severity of joint inflammation secondary to impaired bacterial clearance. Persistence of MMP-2 and MMP-9 expression secondary to infection has also been identified in humans.39 In the present study, MMP-2 and MMP-9 overexpression persisted for several days after results of bacterial culture of synovial fluid were negative, suggesting that these enzymes could play a role in the development and progression of joint lesions and inflammation and could be targets for therapeutic treatment of septic arthritis.

References


