Synovial fluid gelatinase concentrations and matrix metalloproteinase and cytokine expression in naturally occurring joint disease in horses

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Objectives—To determine concentrations of matrix metalloproteinase (MMP)-2 and -9 in synovial fluid; and mRNA expression of MMP-1, -13, and -3; interleukin[IL]-1α and β; and tumor necrosis factor(TNF)-α in synovial membrane and articular cartilage from horses with naturally occurring joint disease.

Sample Population—Synovial fluid (n = 76), synovial membrane (59), and articular cartilage (45) from 5 clinically normal horses and 55 horses with joint disease categorized as traumatic (acute [AT] or chronic [CT]), osteochondritis dissecans (OCD), or septic (S).

Procedure—Synovial fluid gelatinase concentrations were analyzed, using zymography. Synovial membrane and articular cartilage mRNA expression for MMP-1, -3, and -13, IL-1α and β, TNF-α, type-II collagen, and aggrecan were analyzed, using quantitative reverse transcriptase-polymerase chain reaction.

Results—Synovial fluid pro-MMP-2 concentration was significantly higher in diseased joints than normal joints. Septic joints had significantly higher concentrations of pro and active MMP-9. Stromelysin-1 was expressed in ≥ 80% of synovial membrane and articular cartilage samples and was strongly influenced by age. Collagenases were rarely expressed, with MMP-13 expressed only in diseased joints. Interleukin-1β expression was significantly higher in all OCD samples and was influenced by age. Tumor necrosis factor-α expression was significantly higher in cartilage from joints with AT and OCD. There was no correlation between MMP or cytokines and type-II collagen or aggrecan expression.

Conclusions and Clinical Relevance—Matrix metalloproteinase-2 and -3 are abundant in naturally occurring joint disease and normal joints. Interleukin-1β and TNF-α may be important in the pathogenesis of OCD. Age affects MMP and IL-1β concentrations. (*Am J Vet Res* 2001;62:1467–1477)

Lameness resulting from joint injury and joint disease is a major cause of economic loss from poor performance and early retirement from athletic activity in horses.[1,2] Joint disease often leads to **osteoarthritis (OA)**, which can be classified as primary or secondary. Primary OA is a result of cumulative stress, whereas secondary OA is a result of a preexisting structural abnormality.[3] In horses, OA affecting the proximal interphalangeal joint (pastern) and distal tarsal joints are examples of primary OA that develops over time because of repetitive trauma. Secondary OA in horses is similar to traumatic arthritis and is probably best represented by osteochondral fragmentation.[3] Regardless of the cause, OA is a complex disease process in which alterations in normal biomechanical and biochemical mechanisms lead to soft tissue inflammation (synovitis) and deterioration of articular cartilage.[3,4]

Many of the inflammatory mediators and degradative enzymes associated with normal matrix turnover have also been associated with and identified in osteoarthritic joints.[3,4] The presumed scenario is that joint insult results in synovitis from the release of proinflammatory agents such as **interleukin (IL)-1α** and -β and **tumor necrosis factor (TNF)-α** from the synovial membrane and chondrocytes.[3-7] These cytokines are secreted in excess of the concentrations required for normal metabolic homeostasis in the joint, and they work synergistically.[3,4] Both IL-1 and TNF induce proteoglycan depletion in articular cartilage by increasing the rate of proteoglycan degradation, decreasing synthesis by the chondrocytes, or both.[3,4,8,9] This is accomplished, in part, through the stimulation of **matrix metalloproteinases (MMP)** and **prostaglandin (PG)** E_2 production.[3,4]

The MMP are a family of zinc-dependent endopeptidases that are crucial to normal connective tissue remodeling and commonly involved in pathologic joint destruction.[3] In joint tissue remodeling, the most important of these enzymes are collagenase-1 (MMP-1),[10,11] collagenase-3 (MMP-13),[10,12-14] stromelysin-1 (MMP-3),[15-17] and the gelatinases (MMP-2 and -9).[18-20] Expression of MMP seems to be highly regulated and

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tissue specific,[21,22] and synovial membrane and chondrocytes are sources for many of these enzymes.[10,18] The MMP are synthesized as latent proenzymes that are believed to be activated through extracellular proteolytic action to become fully active metalloenzymes.[3,20,23] Once activated, these enzymes have the potential to destroy all the matrix components of cartilage.[3] The activity of MMP may, therefore, be controlled at multiple levels, including synthesis-secretion, activation, and inhibition.[20] Inhibition of MMP is regulated by **tissue inhibitors of metalloproteinases (TIMP)**.[24] Most MMP (> 95%) in articular tissues are present in an inactive latent or inhibited form.[15] However, during OA, MMP are present in higher concentrations than the TIMP, and the latent forms become activated by other MMP or cytokines.[3,23] Therefore, each MMP has a relative role in the development of OA that may vary according to the species and the stage of joint disease.

The purpose of the study reported here was to measure degradative MMP in synovial fluid and the expression of these MMP and pro-inflammatory cytokines in synovial membrane and articular cartilage from joints of horses with naturally occurring joint disease and compare those values with those obtained from samples obtained from normal joints. Our hypotheses were that the concentrations of MMP-2 and -9 would be higher in synovial fluid in affected joints than in normal joints; tissue mRNA expression of MMP-1, MMP-3, MMP-13, IL-1α, IL-1β; TNF-α would be higher in synovial membrane and articular cartilage from affected joints than from normal joints; and mRNA expression of type-II collagen and aggrecan would be lower in cartilage from affected joints than from normal joints.

Materials and Methods

Experimental design—Horses with joint diseases were chosen from horses evaluated at Colorado State University or the Equine Medical Center in California. Horses were classified into 1 of 4 categories on the basis of history and results of clinical examination, radiographic examination, surgical exploration, and synovial fluid assessment. These categories included **acute trauma (AT)**, **chronic trauma (CT)**, **osteochondritis dissecans (OCD)**, and **sepsis (S)**. Duration of injury was estimated on the basis of historical findings reported by the owner or trainer. Pharmacologic treatment prior to sampling was limited to analgesics and nonsteroidal anti-inflammatory drugs. Horses were not included in the study if there was a known history of intra-articular corticosteroid injection since the time of injury. All horses had clinical signs at the time of sampling. Samples were also obtained from normal joints of 5 horses euthanized for problems unrelated to the musculoskeletal system.

Acute traumatic joint disease was defined as any joint injury or disease that had occurred within 60 days of the initial examination and was characterized by localized radiographic and arthroscopic findings consistent with osteochondral fragmentation or acute intra-articular fracture, without evidence of osteophytosis or joint narrowing. Chronic traumatic joint disease was defined as having occurred > 60 days prior to examination and characterized by localized radiographic and arthroscopic findings consistent with osteochondral fragmentation, intra-articular fractures, or primary OA (pastern joint) combined with signs of osteophytosis, joint narrowing, bony sclerosis, or articular cartilage degeneration. Horses with traumatic injuries that included manifestations of OCD or septic arthritis were excluded from the study. Horses were not included in the study if the surgical and radiographic findings were not consistent with the reported duration of disease. Horses with OCD had joint effusion and radiographic and arthroscopic findings localized to common sites of osteochondrosis (eg, distal portion of the intermediate ridge of the tibia or lateral trochlear ridge of the femur). Septic joint disease was defined by results of physical examination (severe joint effusion and lameness grade > 3 [scale, 1 to 5]) and synovial fluid analysis (WBC count > 30,000 cells/µl).

Treatment of all horses required surgical intervention, with most (51/55) undergoing arthroscopy. Biopsy samples were collected aseptically from the joint during surgery and included synovial fluid, synovial membrane, and articular cartilage. Articular cartilage biopsy specimens were taken directly from the weight-bearing surface of the osteochondral fragments or the area of injury or from local areas of fibrillation directly adjacent to the fragment or injury. Synovial membrane samples were obtained from local areas of synovitis, if present, or from synovium directly adjacent to the osteochondral fragment or injury. Samples of affected synovium and articular cartilage were obtained from horses that underwent arthroscopy, but only articular cartilage samples were collected from horses that underwent arthrotomy; these were collected from local areas of fibrillation. No tissue samples were collected from horses with septic arthritis. Samples collected from normal joints were taken from regions prone to osteochondral fragmentation, to mimic collection from affected horses. Samples were collected from the carpal joints (n = 60) as well as metacarpophalangeal (19), tibiotarsal (10), proximal interphalangeal (4), femoropatellar (10), medial femorotibial (1), distal interphalangeal (1), and shoulder (1) joints.

Synovial fluid analysis—Approximately 4 ml of synovial fluid was collected from the joints during surgery, and 1 ml for synovial fluid analysis was placed into a tube that contained EDTA. Analysis included determination of the WBC or nucleated cell count (cells per microliter) by use of a hemocytometer as well as **total protein (TP)** concentration (grams per decaliter) by use of a refractometer. The remaining 3 ml was centrifuged at 800 × g for 30 minutes to remove any cellular debris, and the supernatant was stored at –70 C until analyzed for MMP content.

Gelatin zymographic techniques—Gelatin zymography was performed on synovial fluid samples to determine enzyme-specific protein content. Zymography allows for gelatin digestion of the gels by gelatinases MMP-2 and -9. Confirmation of the gelatinase in a sample was based on the position of the bands of digested substrate after electrophoresis.[a] As positive controls, 1 ng of each human standard[b] for pro-MMP-2 (72 kd), active MMP-2 (66 kd), pro-MMP-9 (92 kd), and active MMP-9 (86 kd) were loaded into separate wells of each gel (**Fig 1**). A standard volume load of 15 µl of diluted (1:10) synovial fluid (50 µl of synovial fluid to 450 µl of sample buffer) was loaded into each sample well with a 2× sample buffer containing 0.5*M* Tris-HCl (pH 7), 20% glycerol, 4% SDS (wt/vol), and 0.005% bromophenol blue. The samples were electrophoresed on 10% Tris-glycine polyacrylamide gels embedded with 0.1% gelatin.[a] The gels were run under nonreducing conditions at 125 V for approximately 90 minutes at 20 C. The gels were washed in a renaturing buffer (Triton X-100, 2.5% [vol/vol] in water) with gentle agitation for 30 minutes at 20 C and incubated at 37 C overnight in developing buffer (50 m*M* Tris, 0.2*M* NaCl, 5 m*M* $CaCl_2$, 0.02% Brij 35 [wt/vol], pH 7.6). The gels were stained with 0.5% (wt/vol) Coomassie blue R250[c] in 40%

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ethanol-10% glacial acetic acid solution for 3 to 6 hours and destained in deionized water for 6 to 8 hours. Enzymatic activity was viewed as clear bands (Fig 1). Inhibition of gelatinolytic activity was demonstrated by adding 50 m*M* EDTA at a concentration of 1 µg/100 ml to the developing buffer of 2 gels. To obtain semiquantitative data, all gels were analyzed wet, using a densitometer[d] and associated software.[e] The optical assessment value of the sample band was compared with the optical assessment value of each respective standard as a ratio. This allowed an arbitrary unit value to be assigned to each synovial fluid sample in respect to its standard for comparisons between and within gels. Finally, the gels were dried and archived.

Western blot analysis—The western blot technique was performed to definitively identify the protein bands in the sample lanes of the gelatin gels as MMP-2 and -9. This was accomplished by use of purified mouse (monoclonal IgG_1) primary antibodies specific for MMP-2 and -9.[b] The synovial fluid was pretreated with chondroitinase ABC[f] (0.02 U/µl of synovial fluid) and hyaluronidase[c] (0.5 U/ml of synovial fluid). Two Tris-glycine (10%) polyacrylamide gels were loaded and electrophoresed as described. One gel was used for identification of MMP-2 and the other for identification of MMP-9. Blots were obtained according to an established protocol[a] by use of transfer buffer that did not contain methanol. Briefly, transfer of the electrophoretically separated proteins to nitrocellulose was performed for 90 minutes, and the blots were allowed to dry at 20 C overnight. The membrane was presoaked in **phosphate-buffered saline solution (PBSS)** and blocked for 45 minutes on a shaker in 3% **bovine serum albumin (BSA)**-PBSS-Tween. Primary antibody was added at the proper dilution (1:10) overnight at 4 C. The membrane was washed 3 times for 10 minutes each in 1% BSA-PBSS-Tween. The secondary antibody, which was conjugated with alkaline phosphatase (goat anti-mouse IgG),[b] was then added at a 1:1,000 dilution in the 3% BSA-PBS-Tween solution for 1 hour on a shaker. The membrane was washed as described, with a final wash in distilled water. A substrate solution (nitro blue tetrazolium 5-bromo-4-chloro-3-indolyl phosphate)[c] was then added to the membrane until the staining of the specific bands was optimal, and background staining was minimal. After 3 washes in distilled water, blots were dried on filter paper and archived.

Quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) techniques—The qRT-PCR technique was used to semiquantify the mRNA expression of MMP-1,

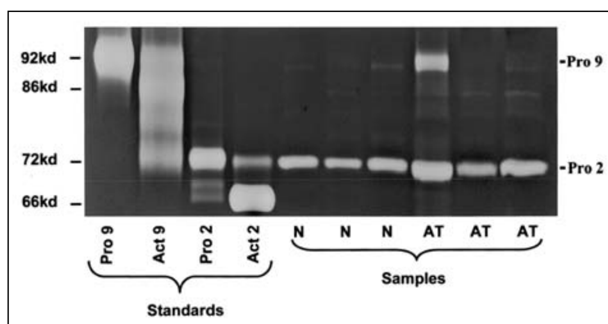

Figure 1—Zymographic analysis of equine synovial fluid for matrix metalloproteinase (MMP)-2 and -9. Standards for the latent (Pro) and active (Act) forms of MMP-2 and -9 were loaded into the first 4 lanes; remaining lanes were loaded with synovial fluid samples from normal (N) joints, and joints affected by acute trauma (AT). Positions and molecular weights of the enzyme standards are indicated on the left side of the figure. Notice that all sample lanes contain pro-MMP-2 (Pro 2) protein at 72 kd, and 1 AT sample contains pro-MMP-9 (Pro 9) at 92 kd.

-3, and -13, IL-1α and -1β, TNF-α, and the primary molecular components of cartilage (type-II collagen and aggrecan). Synovium and cartilage (5 to 10 mg each) were aseptically collected during surgery and immediately placed in 1 ml of monophasic acid phenol solution.[g] Samples were frozen at –70 C until analysis. Samples were thawed at 20 C and homogenized by use of a homogenizer[h] for small tissue samples. To lyse the cells directly, 1 ml of monophasic acid phenol reagent[g] for each 100 mg of tissue was added to the homogenized sample. Samples were centrifuged at 8,000 × g for 5 to 10 minutes at 2 to 8 C. The supernatant was removed, and the homogenate was incubated at 20 C for 5 minutes to allow complete dissociation of the nucleoprotein complexes. Phase separation was accomplished by adding chloroform (0.2 µl/ml of monophasic acid phenol solution) and mixing thoroughly. A 2- to 3-minute incubation at 20 C was followed by centrifugation at 12,000 × g for 15 minutes at 2 to 8 C. Isopropanol (0.5 ml/ml of monophasic acid phenol solution) was added to precipitate the RNA from the aqueous phase. Samples were incubated at 15 to 30 C for 10 minutes followed by centrifugation at 15,000 × g for 30 to 45 minutes at 2 to 8 C. The RNA pellet was evaluated for con tamination with glycosaminoglycans or low yield of RNA by use of spectrophotometric methods. If contamination was present, the pellet was resuspended in 4*M* LiCl to differentially solubilize glycosaminoglycans and RNA. If RNA yield was low, 10 µg of glycogen was added, and precipitation was repeated overnight at –70 C. A visible pellet was obtained on subsequent centrifugation. The supernatant was removed, and the RNA pellet was washed with 1 ml of 75% ethanol/ml of monophasic acid phenol reagent.[g] Centrifugation was performed at 16,000 × g for 15 minutes at 2 to 8 C. Following removal of the supernatant, the RNA pellet was dried for 3 minutes at 65 C. The RNA was dissolved by use of RNase-free water and incubated for 10 minutes at 55 to 60 C.

Samples were then analyzed by use of RT-PCR. One microgram of RNA in 10.5 µl of **diethyl pyrocarbonate (DEPC)**-treated water, based on the 260-to-280 ratio, was used for reverse transcription. The RNA was added to a mixture containing 2 µl of 25 m*M* $MgCl_2$, 2 µl of 10 m*M* dNTP, 0.5 µl of RNAse inhibitor,[i] 2 µl of 10× PCR buffer with $MgCl_2$, 2 µl of random hexamer primers, and 1 µl of Moloney murine leukemia virus reverse transcriptase.[i] This cDNA mixture was allowed to incubate at 37 C for 1 hour. Two microliters of the cDNA mixture was combined with 8 µl of the following master mix: 10 µl of upstream (5') primers, 10 µl of downstream (3') primers, 8 µl of 10× PCR buffer with $MgCl_2$, 51.5 ml of DEPC-treated water, 1 ml of Triton X-100, and 0.5 ml of *Taq* DNA polymerase.[j] The cDNA solution was processed in a thermocycler at the proper annealing temperature to allow for annealing and extension of the primers (**Table 1**). The number of cycles depended on the original amount of RNA in each sample. If the sample contained 1 mg of RNA, 30 cycles were run; any sample containing < 1 µg of RNA was run for 55 cycles. To control nonspecific amplification, controls with no template were run, and each gel was checked for the presence of primer dimers. The cDNA samples were stored at –20 C for further analysis. A RT-PCR for **glyceraldehyde 3-phosphate dehydrogenase (GAPDH)** was performed on each sample as an internal control. All samples that had adequate GAPDH were considered to have adequate RNA for amplification. Various primers for genes of interest were then applied (Table 1). Equine primers used for the articular cartilage and synovial membrane samples were MMP-1, -3,[k] and -13,[l] type-II collagen,[m] and aggrecan.[k] Equine-specific primers were developed for IL-1α, IL-1β, and TNF-α by use of regions of highly conserved sequence in other species. Primers were designed to optimize annealing temperature and minimize internal secondary structure and

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the propensity to form primer dimers in subsequent PCR reactions. The primer sequences were unique to horses, as determined by use of a homology search in GenBank,[m] and oligonucleotides were synthesized by the Macromolecular Resource Center.[n] The PCR products were electrophoresed on a 3.5% agarose gel (**Fig 2**), and intensity of ethidium bromide stained bands was quantified by use of software,[o] comparing the sample to the internal GAPDH control. Signal intensity of the band was normalized to the message level for GAPDH to give a relative level of expression for each sample. Gels were photographed and archived. Two independently isolated clones of each amplified cDNA fragment were sequenced to verify the identity of the cDNA product.

Statistical analyses—Statistical analysis was performed by use of a 2-factor ANOVA, with $P \leq 0.05$ considered significant.[p] Dependent variables that were analyzed included WBC and TP concentrations, MMP-2 and -9 concentrations, and expression of mRNA for MMP-1, -3, and -13, IL-1α, IL-1β, and TNF-α, and type-II collagen aggrecan. These variables were compared with the main effects of age and classification of joint disease. Horses were allocated into 4 age groups for comparisons: ≤ 1 year, 2 to 3 years, 4 to 10 years, and > 10 years. If no significant interaction between age and disease category was determined by use of the ANOVA table, dependent variables were analyzed by disease category only. Individual comparisons were performed by use of least-squares mean analysis. Linear regression was also performed to compare dependent variables and identify linear correlations between these variables.

Table 1—Conditions used for polymerase chain reaction (PCR) analysis of various endopeptidases, cytokines, and cartilage components in samples obtained from the joints of horses

Primers	Primer sequence (5'–3')	PCR product (bp)	Anneal temperature (C)
MMP-1	1. GATGTGGGGTGCCCGATGTG 2. TACATCAGAGCCCCGATGTCA	441	60
MMP-13	1. CTTAGAGGTGACTGGCAAAC 2. AGGGAACTACGGTAATGGTC	672	50
MMP-3	1. CATGGACCTTCTTCAGGACTA 2. CTCCATGTTCTCGAACTCC	420	60
Type II	1. CTGCTCGTCGCTTGT 2. GCCTCCTCATCAAATCCTCCAGC	326	70
Aggrecan	1. CTTAGAGGACAGAAAGCGAC 2. ACTTTGGGCGGAAGAAGG	325	58
IL-1α	1. ACCAATGATGACCTGGAAGC 2. TTTAATGCAGCAGTCGCAAG	200	58
IL-1β	1. CTTCAAAGACCTGAACCTCA 2. GCCACAATGATTGACACGAC	108	59
TNF-α	1. GATCATCTTCTCGAACCCCA 2. TGTCCCCTGTCTGTCTTTCC	266	63

MMP = Matrix metalloproteinase. Type II = Type-II collagen. IL = Interleukin. TNF = Tumor necrosis factor. bp = Base pairs.

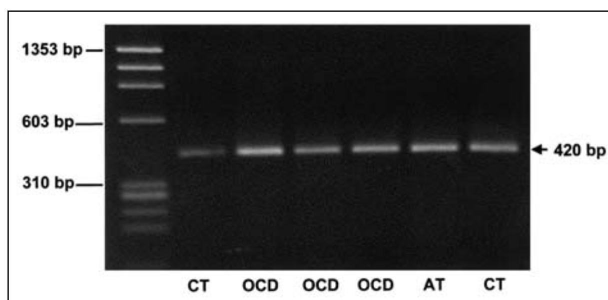

Figure 2—Ethidium bromide-stained agarose gel electrophoretogram of synovial membrane samples from horses with joints affected by chronic trauma (CT), osteochondritis dissecans (OCD), or Acute trauma (AT). A molecular weight marker was loaded into the left lane. The 420-base pair (bp) amplimer represents expression of stromelysin (MMP-3) mRNA. Notice the variability of amplimer intensity among samples.

Results

Horses and samples—One hundred eighty joint samples (76 synovial fluid, 59 synovial membrane, and 45 articular cartilage) were collected from 106 joints of 55 clinically affected horses and 5 clinically normal horses. Of these 60 horses, breeds included Quarter Horse (n = 35), Thoroughbred (14), Warmblood (6), Arabian (3), American Trotter (1), and Appaloosa (1). Twenty-seven horses were geldings, 19 were female, and 14 were sexually intact males. Mean ± SD age of horses was 4.99 ± 4.61 years, and median age was 2 years. Of horses ≤ 1 year of age, 8 of 12 had OCD, whereas 13 of 19 of the 2- and 3-year-old horses had joints affected by AT. For horses > 4 years of age, 14 of 21 had joints affected by CT. Most horses from which samples were collected had traumatic lesions (40/60; AT [n = 19], CT [21]), whereas 12 horses had OCD, 3 had joints affected by S, and 5 control horses had normal joints.

Synovial fluid analysis—Cytologic examination was performed on all 76 synovial fluid samples. Mean WBC concentrations were significantly ($P = 0.001$) higher in joints affected by S (mean ± SD 81,567 ± 41,293 cells/µl) than those affected by AT (529 ± 466 cells/µl), CT (964 ± 852 cells/µl), OCD (200 ± 110 cells/µl), or in normal joints (864 ± 581 cells/µl). Mean TP concentrations in synovial fluid were also significantly ($P = 0.001$) higher in horses with joints affected by S (5.8 ± 0.89 g/dl) than those with joints affected by AT (2.8 ± 0.5 g/dl), CT (2.5 ± 1.0 g/dl), or OCD (2.1 ± 0.9 g/dl) or those with normal joints (2.2 ± 0.5 g/dl). Mean TP concentration of synovial fluid of horses with joints affected by AT was also significantly ($P = 0.003$) higher than that of horses with joints affected by CT or OCD or with normal joints.

Zymography results—Gelatin zymography for MMP-2 and -9 was performed on all 76 synovial fluid samples (**Table 2**). Ethylenediaminetetraacetic acid (50 m*M*) successfully inhibited gelatin digestion, confirming that the proteins detected were metalloenzymes. Between-run coefficient of variation was < 10% for the standards.

All 76 synovial fluid samples contained pro-MMP-2 (72 kd), whereas active MMP-2 (66 kd) was detected

Table 2—Distribution (No. of samples with positive results) of various forms of endopeptidases (pro-, active, and dimer forms) in synovial fluid samples obtained from normal (N) equine joints and joints affected by acute trauma (AT), chronic trauma (CT), osteochondritis dissecans (OCD), or sepsis (S)

Joint disease	Pro-MMP-2	Active MMP-2	Pro-MMP-9	Active MMP-9	Dimer MMP-9
N (15)	15	0	1	0	0
AT (24)	24	1	13	3	4
CT (22)	22	1	10	3	3
OCD (12)	12	0	6	4	4
S (3)	3	0	3	3	3
Total (76)	**76**	**2**	**33**	**13**	**14**

Values in parentheses indicate No. of synovial fluid samples tested.

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in only 3% (2/76) of the samples (Fig 1; Table 2). Samples from joints with all categories of joint disease had significantly (P = 0.001) greater concentrations of pro-MMP-2 than did samples from normal joints (**Fig 3**). There was a significant association between age and MMP-2 concentration; older horses with joints affected by AT had higher concentrations, and older horses with joints affected by CT had lower concentrations, compared with younger horses of each respective category.

Promatrix metalloproteinase-9 (92 kd) was detected in 33 of 76 (43%) samples, and active MMP-9 (86 kd) was detected in 13 of 76 (17%) synovial fluid samples (Fig 1; Table 2). All samples from horses with joints affected by S contained both forms of MMP-9, whereas only 1 sample from a normal joint had any MMP-9 activity. The dimer of MMP-9 (approx 225 kd) was detected in 14 of 76 (18%) synovial fluid samples. All samples for which dimer activity was detected had pro-MMP-9 activity as well. The dimer was also detected in 9 of 13 samples that had active MMP-9. Septic joints had significantly (P = 0.001) higher concentrations of pro- and active MMP-9, compared with all other categories of joint disease and normal joints (Fig 3). There was a significant association between age and MMP-9 concentrations that varied by joint disease category. A strong correlation (P = 0.001; R^2 = 0.88) between pro- and active MMP-9 was detected. Both pro-MMP-9 (R^2 = 0.61) and active MMP-9 (R^2 = 0.49) were also weakly correlated with WBC concentration in the synovial fluid (P = 0.001).

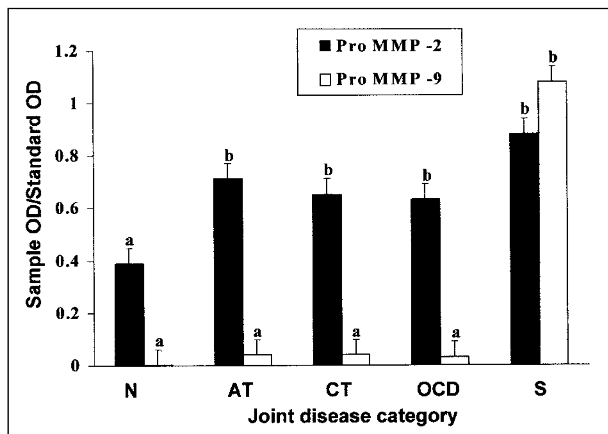

Figure 3—Mean ± SD pro-MMP-2 and -9 concentrations in synovial fluid samples obtained from horses with normal (N) joints or joints affected by AT, CT, OCD, or sepsis (S), expressed as a ratio of the optical density (OD) of samples to that of a standard. [a,b]Different superscripts indicate significant differences among groups for pro-MMP-2 (P < 0.001) or pro-MMP-9 (P < 0.05).

Western blot results—Western blots were performed on 6 representative synovial fluid samples (horses affected with AT [n =1], CT [2], OCD [1], S [1], and a clinically normal horse [1]) in which gelatin activity was detected by use of zymography. Cross-reactivity with the purified mouse monoclonal antibodies to the latent and active forms of MMP-2 and -9[b] was detected for all samples.

Results of qRT-PCR analysis—The mRNA from 104 tissue samples (45 articular cartilage and 59 synovial membrane) was analyzed by use of qRT-PCR. No tissue samples were obtained from septic joints. Collagenase-1 (MMP-1) mRNA expression was identified in only 3 of 59 (5%) synovial membrane samples and in no cartilage samples (**Table 3**). Because of this limited expression, no statistical evaluation could be performed on these data. Collagenase-3 (MMP-13) was expressed in 5 of 59 (8%) synovial membrane samples and 10 of 45 (22%) articular cartilage samples. No normal joints had MMP-13 mRNA expression. The MMP-13 was expressed most often in samples from joints affected by AT. The expression of MMP-13 mRNA was not associated with type of joint disease or age.

Stromelysin (MMP-3) was expressed in 51 of 59 (86%) synovial membrane samples and 36 of 45 (80%) articular cartilage samples (Table 3). All synovial membrane samples from joints affected by CT and OCD had MMP-3 mRNA expression, and MMP-3 was also identified in 25 of 30 (83%) articular samples from clinically normal horses (17 of 20 synovial membrane samples and 8 of 10 articular cartilage samples; Table 3). Stromelysin mRNA expression appeared to be influenced more by age than by joint disease category. Expression of MMP-3 was significantly higher in synovial membrane samples from older horses with OCD and CT, compared with younger horses in the same categories, and in articular cartilage samples of young horses with OCD, compared with older horses with OCD.

Interleukin-1α expression was detected in 37 of 59 (63%) synovial membrane samples and 20 of 45 (44%) articular cartilage samples (Table 3). Synovial membrane expression of IL-1α was detected in 16 of 20 samples from normal joints, but articular cartilage and

Table 3—Distribution (No. of samples with positive results) of various endopeptidases and cytokines in equine synovial membrane (SM) and articular cartilage (AC) samples obtained from normal (N) joints and joints affected by AT, CT, OCD, or S

Sample	Joint disease	MMP-1	MMP-13	MMP-3	IL-1α	IL-1β	TNF-α
SM							
	N (20)	1	0	17	16	12	8
	AT (18)	2	2	13	11	11	7
	CT (14)	0	1	14	10	13	8
	OCD (7)	0	2	7	0	7	2
	Total (59)	**3**	**5**	**51**	**37**	**43**	**25**
AC							
	N (10)	0	0	8	7	5	1
	AT (17)	0	6	14	9	5	3
	CT (12)	0	2	10	4	2	2
	OCD (6)	0	2	4	0	1	3
	Total (45)	**0**	**10**	**36**	**20**	**13**	**9**

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synovial membrane samples from joints affected by OCD did not have detectable IL-1α expression (**Fig 4**). There was no correlation between IL-1α expression in articular cartilage and synovial membrane, and the expression of IL-1α expression was not associated with type of joint disease or age.

Interleukin-1β was expressed in 43 of 59 (73%) synovial membrane samples and 13 of 45 (29%) articular cartilage samples (Table 3). Unlike IL-1α, IL-1β expression in synovial mebrane was detected in all joints affected by OCD and 13 of 14 samples from joints affected by CT. There were significantly ($P < 0.001$) higher levels of IL-1β expression in synovial membranes of joints affected by OCD versus all other categories of joint disease and normal joints (Fig 4). The 1 OCD sample with IL-1β mRNA expression in articular cartilage also had significantly ($P = 0.005$) higher levels of expression, compared with normal joints and those affected with AT, although a difference from values obtained from joints affected with CT was not detected (Fig 4). There was also a significant age effect ($P = 0.003$), with young horses having higher levels of IL-1β mRNA expression in synovial membrane samples, compared with older horses. In articular cartilage, IL-1β was expressed in 5 of 10 normal joints and rarely in joints affected by OCD (1/6) or CT (2/12). There was a correlation between IL-1β expression in synovial membrane and articular cartilage ($P = 0.041$; $R^2 = 0.69$). Expression of IL-1β was also weakly correlated with IL-1α expression in synovial membrane ($P < 0.001$; $R^2 = 0.42$). The levels of IL-1β expression in articular cartilage were also weakly correlated with MMP-3 concentrations ($P = 0.013$; $R^2 = 0.56$) and WBC concentration ($P = 0.009$; $R^2 = 0.55$).

Tumor necrosis factor-α was expressed in 25 of 59 (42%) synovial membrane samples and in 9 of 45 (20%) articular cartilage samples (Table 3). There were significantly ($P = 0.005$) higher levels of expression for TNF-α in articular cartilage samples from joints affected by AT and OCD, compared with normal joints and those affected by CT (Fig 4). No correlation was detected between TNF-α and IL-1α or β from either sample source.

There was expression of type-II collagen and aggrecan in all cartilage samples. Overall, expression of type-II collagen and aggrecan was not significantly different between samples from normal joints and those with any category of joint disease. Expression of aggrecan and type-II collagen was not associated with age.

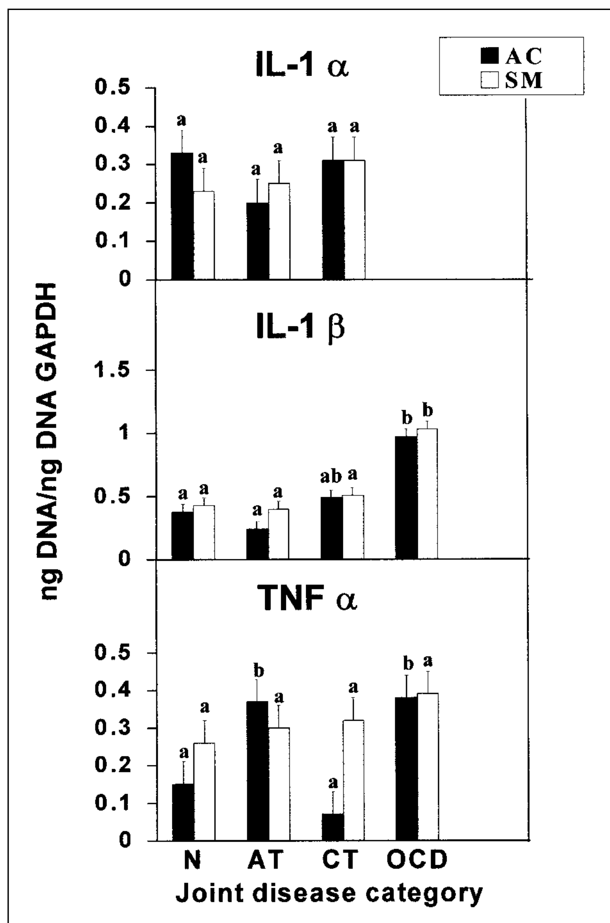

Figure 4—Expression of mRNA of interleukin (IL)1-α, IL-1β, and tumor necrosis factor (TNF)-α (mean ± SD values of the ratio of cDNA to glyceraldehyde-3-phosphate dehydrogenase [GADPH]) in articular cartilage (AC) and synovial membrane (SM) samples from horses with normal (N) joints or joints affected by AT, CT, or OCD. [a,b]Different superscripts indicate significant differences among groups for values obtained from AC ($P = 0.005$) or SM ($P < 0.001$) samples.

Discussion

The goal of this study was to identify specific MMP and cytokines in tissues and synovial fluid from horses with naturally occurring joint disease. One of the difficult aspects of using in vivo tissue samples is the inherent inaccuracy in quantifying disease duration at a given time during the development of the disease process.[25] Osteoarthritis is a cyclical disease with alternating periods of activity and remission, which can lead to overlapping of classification of cases in each category of joint disease. Arthroscopy allowed us to define multiple stages of clinically relevant equine joint disease as opposed to simply end-stage OA. In addition, arthroscopy allowed us to collect small specimens of articular cartilage and synovial membrane directly from local regions of joint injury, so that only unhealthy cartilage and synovium were removed, leaving healthy cartilage intact. Therefore, we believe our results provide important initial information in the examination of the interplay of many enzymes and inflammatory mediators in clinically relevant stages of natural joint disease in horses.

The WBC and TP concentrations were significantly higher in synovial fluid from septic joints, compared with normal joints and those affected with other diseases. Joints affected by AT also had significantly higher TP concentrations, compared with normal joints and those affected with CT and OCD. These results are consistent with inflammation[26] and suggest that synovitis is an important component of the acute phase of traumatic joint disease in horses.

Our results indicate that latent forms of the gelatinases MMP-2 and -9 are in greater concentrations than active forms in synovial fluid of horses with and without joint disease. Matrix metalloproteinase-2 is produced by

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resident cells within joint tissues,[18] and the concentrations of MMP-2 in synovial fluid have been measured in horses.[19] Latent MMP-2 was present in all synovial fluid samples in our study. The presence of MMP-2 in normal joints suggests a constitutive and homeostatic role for removing abnormal collagen from newly synthesized extracellular matrix.[27] Activation of MMP-2 was identified in only 2 horses with traumatic injuries in our study. This concurs with a report in which pro-MMP-2 was activated only in diseased joints and not in normal joints.[20] Unlike other MMP, pro-MMP-2 is not susceptible to activation via proteolytic enzymes but is instead activated at the cell surface by membrane-bound MMP.[20] Regardless of MMP-2 activation, the excess amount of latent MMP-2 in joints with all categories of disease in our study may indicate that MMP-2 plays a role in activation of other enzymes such as MMP-9[20,28]or -13.[10]

Reportedly, MMP-9 is produced and released into the synovial fluid by chondrocytes and synoviocytes in diseased joints in horses but not clinically normal joints.[19] In our study, latent and activated MMP-9 was present in synovial fluid from all septic joints. Similar findings have been reported in humans and horses, where an association has been identified between MMP-9 and inflammatory arthritides.[18,19,29,30] Matrix metalloproteinase-9 is mainly produced by neutrophilic granulocytes, which are believed to be the main source of synovial MMP-9.[31] A correlation between the WBC concentration and pro- and active MMP-9 was identified in our study and may suggest that increased concentrations of MMP-9 in septic joints are attributable to release from infiltrating neutrophils.[19] Matrix metalloproteinase-9 was present in low concentrations in all samples from horses with all categories of joint disease but was present in only 1 normal joint. These findings are supported by results of previous studies that revealed trace MMP mRNA in normal human cartilage,[32] and concentrations of MMP-9 in synovial fluid that increased as the grade of inflammation increased.[30,33] The presence of MMP-9 in noninflammatory arthritides in our study may be attributable to the fact that synovitis was a more prominent feature, compared with other studies. In other words, because of the cyclical nature of the early phases of OA, pro-MMP-9 concentrations may be low during periods of quiescence and increase during periods of inflammation.

The dimer for MMP-9 (approx 225 kd) was present in 18% of the samples in our study, which is similar to that reported elsewhere.[19] Dimerization of MMP-9 is thought to only occur when MMP-9 is present in excess of its tissue inhibitor,[18,19] and it is thought to be a critical step in the activation of MMP-9.[18] Results of our study support this, because 69% of samples with active MMP-9 also contained the dimer. Activation of MMP-9 has been ascribed to other MMP such as MMP-1, -2, and -3.[18,28,29] Once activated, MMP-9 can participate in cartilage destruction.[18] However, activation is inconsistent,[20] which may correspond to the degree of inflammation present during a given phase of joint disease. Although results of our study were inconclusive, we suggest that increased concentrations of pro-MMP-2 may play a role in activation of MMP-9 during periods of inflammation in horses with joint disease.

Among the 104 synovial membrane and articular cartilage samples in our study, only 3 synovial membrane samples expressed MMP-1 mRNA. One of the samples that expressed MMP-1 was from a normal joint, and 2 samples were from joints affected by AT. This suggests that MMP-1 may play a role in homeostasis and early joint disease in horses. Matrix metalloproteinase-1 has been reported to be synthesized primarily from the synovial lining cells of patients with OA[34] but has been identified in the superficial layers of articular cartilage at increased concentrations in humans with OA.[11,35] As our results suggest, MMP-1 has a low level of expression in articular cartilage, compared with other MMP.[10] The complete lack of expression of MMP-1 in articular cartilage samples in our study, however, is not consistent with current theories. Because MMP-1 was expressed only in synovial membrane samples of horses with AT and clinical signs of disease, MMP-1 may be involved in cartilage catabolism mostly during the inflammatory process. It has been stated that MMP-1 synthesized from the synovial lining cells is focal and related to the degree of local inflammation.[11] This enzyme could then diffuse into the synovial fluid and, eventually, into the superficial layer of the articular cartilage.[11] Once in the superficial layer, its catabolic effects could then predominate if preexisting mechanical trauma was present. Regardless, our results suggest that MMP-1 is only occasionally expressed by the synovial membrane during naturally occurring joint disease in horses.

Matrix metalloproteinase-13 was expressed more commonly in our study than MMP-1, with 10 of 45 articular cartilage samples and 5 of 59 synovial membrane samples expressing MMP-13. Matrix metalloproteinase-13 is expressed in normal articular cartilage and cartilage affected by OA as well as synovial membrane in humans with OA[17,22] but is expressed at increased concentrations mostly in articular cartilage that is affected by OA.[10,13] Interestingly, MMP-13 expression was not identified in any of the normal samples in our study, suggesting a more important role in joint disease than in homeostasis. This is similar to results of other studies[13,14] that suggest that expression of MMP-13 in normal tissues is limited because it is potentially damaging to normal tissues, and because it is a potent enzyme that cleaves the triple helix of type-II collagen 5 times faster than does MMP-1. Therefore, its catabolic effects may be better served when there is already an alteration in the articular cartilage. In support of this, it has been stated that MMP-13 may be related to the remodeling phase, including osteophyte formation.[10] Intuitively, one would consider the remodeling phase to occur during the chronic stage of joint disease. However, in our study, MMP-13 was only expressed in 3 of 15 samples (1 synovial membrane sample and 2 articular cartilage samples) from joints with CT. Expression actually occurred more often in joints affected by AT (8/15) and OCD (4/15). This could suggest that MMP-13 responds to the degree of inflammation and that the remodeling process actually begins in the acute phase of joint disease. Regarding MMP-13 expression in OCD, it is possible that cartilage fragments cause damage to the superficial layers of

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articular cartilage, similar to the damage caused by fragments associated with trauma; this could expose the intermediate and deep layers, which preferentially produce MMP-13.[10]

The low number of samples with expression of collagenase mRNA in our study was surprising. Although our results are contrary to reports in humans,[10,17] our findings may be an accurate indication of changes that occur during naturally occurring joint disease in horses. Areas of cartilage damage are hypocellular and may only contribute a small proportion of RNA to the total cartilage RNA content, which may not be detected by use of PCR.[36] Expression of the MMP-1 gene is regulated at multiple points, requiring transcriptional and post-translational mechanisms,[37] which can decrease the stability of MMP-1 mRNA and reduce the level of message obtained.[11] It is also possible that some of the horses in our population may have received intra-articular injections that were not reported to us prior to sample collection. Intra-articular anti-inflammatory agents such as corticosteroids reduce the mRNA expression of MMP-1[34] and -13.[12] However, one would suspect decreased concentrations of all MMP unless specific intra-articular corticosteroids target specific MMP.

Stromelysin (MMP-3) has been implicated as the key enzyme in the pathogenesis of osteoarthritis,[38] because it may play an important role in aggrecan and collagen degradation,[39] and it potentiates the activation of MMP-1, -8, -9 and -13.[3,18,40] Increased expression of MMP-3 mRNA has been identified in synovial membranes obtained during the final stages of rheumatoid arthritis and OA in humans[22,41] as well as in chondrocytes of the superficial and middle layers of cartilage affected by OA.[35,39,41] In our study, MMP-3 mRNA was expressed in 86% of synovial membrane and 80% of articular cartilage samples, confirming its importance in the pathogenesis of joint disease in horses. However, MMP-3 expression did not appear to be present at higher levels in diseased joints, compared with normal joints, which goes against conventional theories.[15,17] The abundance of stromelysin expression in normal and affected joints in our study suggests that MMP-3 may be secreted as the latent form and subsequently activated in articular tissue.[16,42] Because of the individual variation encountered in naturally occurring OA, our samples probably had wide variation in the extent of damage present, with MMP-3 production and activation occurring during different time periods. The presence of an enzyme does not necessarily imply activity.[43] Our results and those of others suggest that latent MMP-3 mRNA expression is abundant in normal joints and those affected by naturally occurring OA.[16,42] Activation by other mediators may then be important to disease progression.

Production of MMP-3 by many cell types is stimulated by IL-1 (α and β) and TNF-α.[3,5,44] In our study, expression of IL-1β in synovial membrane and articular cartilage samples correlated with expression of stromelysin in cartilage. In 1 study of partially meniscectomized rabbits, up-regulation of MMP-3 first occurred 4 weeks after injury.[45] This suggests that a certain length of time may be necessary for cytokine induction of MMP-3 and may correlate with the severity of the OA lesion and the availability of cytokine-specific cell surface receptors.[44] However, most MMP secreted in response to IL-1 may be secreted as zymogens and require further activation by such mediators as plasminogen activators.[3] Therefore, in our study, MMP-3 production may have been stimulated by IL-1β but mostly in its latent form.

Interleukin-1 (α and β) and TNF-α are produced by inflammatory cells, synoviocytes, and chondrocytes and have been identified at high concentrations in patients with OA.[6,8,11,46] Tumor necrosis factor-α is an important mediator of acute inflammation during joint disease.[26,44] However, as in our study, a lack of correlation has been reported between TNF-α and WBC concentrations in horses with joint disease.[26] The bioactivity of IL-1α and β has been identified in synovial fluid from normal and acutely diseased joints in horses,[3] and IL-1α has been used to stimulate equine cartilage explants to mimic changes seen during OA.[47] Interleukin-1β has recently been reported to be the most prominent cytokine in osteoarthritic joints[11,41] and plays an active role in synovitis.[48,49] Results of our study may be consistent with these findings, because expression of IL-1β mRNA in articular cartilage samples was correlated with the WBC concentration.

Overall expression of IL-1α and β was approximately equal in our study, with much lower expression of TNF-α (Table 3). Interleukin-1 (α and β) and TNF-α are produced primarily from synovial membrane and diffuse together into articular cartilage.[26,41] Presumably, affinity of superficial cartilage receptors for IL-1 and TNF-α increases in joints affected by OA, increasing chondrocyte production.[7,44] However, TNF-α expression in articular cartilage from joints affected by OA is lower than that of clinically normal joints.[26,46] Results of our study are consistent with this sequence of events, because IL-1β was expressed in 73%, IL-1α in 63%, and TNF-α in 42% of the synovial membrane samples. Similarly, articular cartilage expression was lower than synovial membrane expression for all the cytokines, with IL-1α (44%) expressed most commonly, followed by IL-1β (29%) and TNF-α (20%). Our results also support the contention that IL-1β is primarily produced by synovial membrane and that IL-1α is synthesized primarily from chondrocytes, which may be attributable to the fact that IL-1β is actually released into the extracellular environment, whereas IL-1α is primarily cell-associated.[46,49]

According to our results, IL-1 (α and β) and TNF-α are all expressed in normal tissues, with IL-1α being expressed most commonly. This expression suggests an important role for these cytokines in normal cartilage homeostasis. Interestingly, however, there was little difference between the number of samples and levels of expression of IL-1α and β in joints affected by AT and CT, compared with normal joints. This may be because there is little difference in cytokine amounts from normal in minimally affected cartilage and because cytokines increase with severity until the articular cartilage is completely destroyed.[50] This lack of difference between findings in joints affected by AT and CT may also be attributable to overlap in our categorization,

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but it more likely reveals the cyclical nature of inflammation during OA. Despite the low number of articular cartilage samples that expressed TNF-α, a significant difference in mRNA expression in the articular cartilage samples among joint disease categories was detected. Samples from joints affected by AT had significantly greater expression of TNF-α mRNA than did those from joints affected by CT and normal joints. This difference is most likely attributable to the low numbers of articular cartilage samples with expression of TNF-α, but it may also be influenced by the availability of chondrocyte receptors for TNF-α that are able to respond to inflammation. Our results were similar to those of another study that revealed high concentrations of TNF-α in the early stages of arthritis in racehorses,[26] supporting a pivotal role in the acute inflammatory process.[26,44]

The largest difference between IL-1α and IL-1β in our study was in expression in samples from horses with OCD; IL-1β was expressed at high levels in all synovial membrane samples and 1 articular cartilage sample, whereas IL-1α was not expressed in any of the samples. Tumor necrosis factor-α mRNA was also expressed at significantly higher levels in articular cartilage samples obtained from horses with OCD than from horses with CT or normal horses. This suggests that IL-1β and TNF-α may play an important role in OCD in horses. To the authors' knowledge, this is the first report of this finding. Age-related changes may offer a possible explanation for the high levels of IL-1β in samples from horses with OCD, because IL-1β concentration was significantly correlated with age and joint disease category; young horses with OCD had higher concentrations of IL-1β, compared with older horses or those with other joint diseases or normal joints. Because 67% of the horses ≤ 1 year of age in this study had OCD, it is suspected that younger horses have higher levels of expression of IL-1β. However, because we did not have age-matched controls, this is only speculative and does not explain the increased concentrations of TNF-α that were detected. Another possibility may be that small free-floating cartilage particles or instability of fragments associated with OCD (microtrauma) may induce synthesis of IL-1β by the synovial membrane and synthesis of TNF-α from the articular cartilage, causing secondary synovitis and joint effusion with continued exercise. The effusion that is often associated with OCD may also contribute to increased expression of these cytokines via mechanotransduction of the articular tissues.[51] The release of either of these cytokines into the synovial environment may also in turn stimulate increased expression of the other.

In our study, none of the MMP or cytokines were correlated with decreased concentrations of type-II collagen or aggrecan in samples from diseased joints, compared with normal joints. General consensus is that there is an increase in synthesis of aggrecan and type-II collagen early during the development of OA, leading to hypertrophy of the articular cartilage.[52] However, our study neither identified this nor the decrease in the matrix components reported during chronic disease.[52] A partial explanation is that even the most chronically affected joint in our study likely falls into the early OA category of studies using animal models and human patients. Complicating this is that the early phase of naturally occurring joint disease is difficult to study because of problems with accurate categorization. The chondrocytes in samples from horses with CT are obviously still metabolically active and capable of transcribing messages, but it is not known whether this is because of transition from quiescent to metabolically active cells or because of an increase in cell density, as has been described in early OA.[53]

Recent studies[15,54] that examined MMP activity in equine synovial fluid found higher activity in immature joints than mature joints. This was theorized to be attributable to increased physiologic tissue remodeling of the matrix associated with growth in young horses.[54] In our study, age was a strong confounding factor in interpretation of the results of the analyses of the gelatinases (MMP-2 and -9), stromelysin-1 (MMP-3), and IL-1β. Because of the prospective in vivo nature of this study, we were unable to get age- and sex-matched controls, which would have been preferable.[15,54] We did not detect any interactions between age and the matrix components. This may be attributable to the small number of synovial membrane and articular cartilage samples from horses that were < 1 year of age. Most young horses in this study were yearlings with OCD. At this age, there may be little difference from adults with regard to cartilage metabolism.

[a]NOVEX, Novel Experimental Technology, San Diego, Calif.
[b]Oncogene research products, Calbiochem-Novabiochem Corp, Cambridge, Mass.
[c]Sigma Chemical Co, St Louis, Mo.
[d]Personal densitometer SI, Molecular Dynamics, Sunnyvale, Calif.
[e]ImageQuant, Molecular Dynamics, Sunnyvale, Calif.
[f]Seikagaku Corp, Tokyo, Japan.
[g]TRIzol, Gibco BRL, Life Technologies Inc, Grand Island, NY.
[h]Brinkmann Instruments Inc, Westbury, NY.
[i]Promega, Madison, Wis.
[j]Fisher Laboratory Products, Pittsburgh, Pa.
[k]Courtesy of Dr. Dean Richardson, University of Pennsylvania, Pa.
[l]Courtesy of Dr. John Caron, Michigan State University, Mich.
[m]Genbank Nucleotide Databases, National Center for Biotechnology Information, Bethesda, Md.
[n]Macromolecular Resource Center, Colorado State University, Fort Collins, Colo.
[o]America Advanced Biotech, Proforma Biomed, Fullenton, Mass.
[p]The SAS system, SAS Institute Inc, Cary, NC.

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Correction: Direct and indirect contact rates among beef, dairy, goat, sheep, and swine herds in three California counties, with reference to control of potential foot-and-mouth disease transmission

In the report, "Direct and indirect contact rates among beef, dairy, goat, sheep, and swine herds in three California counties, with reference to control of potential foot-and-mouth disease transmission" (*AJVR*, Jul 2001, pp 1121–1129), the equations and figure on page 1123 should appear as the following:

$$\text{Mean center of livestock facilities} = (x, y) = \left(\frac{\Sigma x_i}{n}, \frac{\Sigma y_i}{n}\right)$$

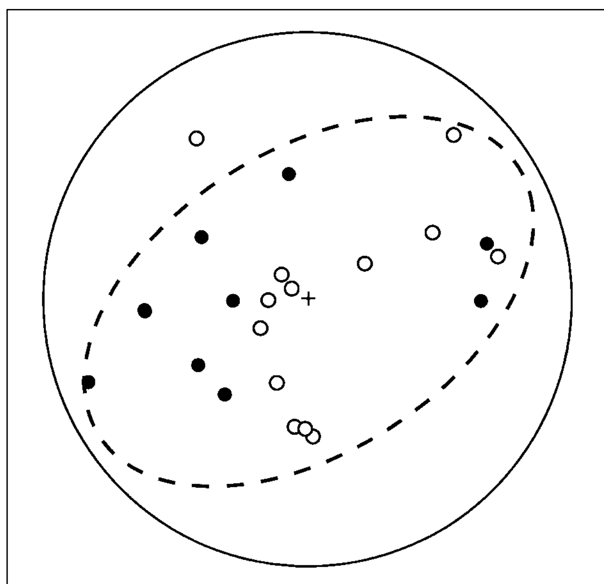
$$\text{Standardized coordinates } (x', y') = (x - x_i, y - y_i)$$

$$SD = \sqrt{\left(\frac{\Sigma x_i^2}{n} - \bar{x}^2\right) + \left(\frac{\Sigma y_i^2}{n} - \bar{y}^2\right)}$$

$$\sigma_x = \sqrt{\frac{\Sigma(x'^2)\cos^2\theta - 2(\Sigma x'y')\sin\theta\cos\theta + \Sigma(y'^2)\sin^2\theta}{n}}$$

$$\sigma_y = \sqrt{\frac{\Sigma(x'^2)\sin^2\theta + 2(\Sigma x'y')\sin\theta\cos\theta + \Sigma(y'^2)\cos^2\theta}{n}}$$

$$\tan\theta = \frac{(\Sigma x'^2 - \Sigma y'^2) + \sqrt{(\Sigma x'^2 - \Sigma y'^2)^2 + 4(\Sigma x'y')^2}}{2\Sigma x'y'}$$



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