

High-mobility group box chromosomal protein 1 as a potential inflammatory biomarker of joint injury in Thoroughbreds

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Objective—To investigate effects of osteochondral injury on high-mobility group box chromosomal protein 1 (HMGB-1) concentrations in synovial fluid (SF) from Thoroughbreds and to compare these results with radiographic and arthroscopic scores of severity of joint injury.

Animals—40 clinically normal rested Thoroughbreds (group 1) and 45 Thoroughbreds with osteochondral injury as a result of racing.

Procedures—SF was obtained from the metacarpophalangeal (MCP) joints, metatarsophalangeal (MTP) joints, middle carpal joints, and radiocarpal joints. For group 2, radiographic and arthroscopic scores were determined. Concentrations of SF HMGB-1 were determined by use of an ELISA.

Results—SF HMGB-1 concentrations in osteochondral-injured MCP-MTP joints were significantly higher than in normal MCP-MTP joints. Similarly, SF HMGB-1 concentrations in osteochondral-injured carpal joints were significantly higher than in normal carpal joints. Radiographic and arthroscopic scores were not correlated with SF HMGB-1 concentrations. Synovial fluid HMGB-1 concentrations ≥ 11 ng/mL for MCP-MTP joints and ≥ 9 ng/mL for carpal joints discriminated osteochondral-injured joints from normal joints. Horses with HMGB-1 concentrations ≥ 11 ng/mL for MCP-MTP joints were twice as likely to have an osteochondral injury, and horses with HMGB-1 concentrations ≥ 9 ng/mL for carpal joints were 4 times as likely to have an osteochondral injury.

Conclusions and Clinical Relevance—Osteochondral injury was associated with a significant increase in SF HMGB-1 concentrations in MCP-MTP and carpal joints, compared with results for clinically normal Thoroughbreds. Analysis of SF HMGB-1 concentrations may be useful for evaluation of joint injury in horses. (*Am J Vet Res* 2009;70:1230–1235)

High-mobility group box chromosomal protein 1 is an abundant and ubiquitous protein found in the nuclei and cytoplasm of nearly all cells. Its functions include maintenance of nucleosome structure and regulation of gene transcription.¹ More recently, it has been reported^{2,3} that HMGB-1 is secreted into extracellular fluid, where it acts as a proinflammatory cytokine. It mediates many inflammatory diseases, including many forms of experimentally induced and naturally developing arthritis.^{4–6} Extracellular HMGB-1 engages the receptor for advanced glycation end products (ie, RAGE)

ABBREVIATIONS

HMGB-1	High-mobility group box chromosomal protein 1
IL	Interleukin
MCJ	Middle carpal joint
MCP	Metacarpophalangeal
MTP	Metatarsophalangeal
RCJ	Radiocarpal joint
SF	Synovial fluid
TNF	Tumor necrosis factor

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to activate proinflammatory signaling pathways.⁷ In 1 study,⁸ intra-articular injections of recombinant HMGB-1 caused arthritis in mice, and it was concluded that HMGB-1 is not just an expression of inflammatory responses but initiates inflammation by activating macrophages and inducing IL-1 production via activation of nuclear factor κ B. In an immunohistochemical study⁸ of HMGB-1 in articular cartilage and synovial membrane from osteoarthritic patients, HMGB-1 was more prevalent in the cytoplasm than in the nuclei of the cells of these tissues, compared with results for normal samples. In that same study, the total amount of HMGB-1

was not significantly different between osteoarthritic and normal tissues; therefore, it was concluded that the location of this protein within cells influences its pro-inflammatory role.^a In other studies,^{9,10} concentrations of HMGB-1 were significantly higher in SF of patients with rheumatoid arthritis, compared with concentrations in those with osteoarthritis. Samples of SF from normal joints were not included for comparison. To our knowledge, there is no clear evidence that SF HMGB-1 concentrations are increased with osteoarthritis in any species. Although the authors of the aforementioned studies did not address the use of HMGB-1 as a possible biomarker of joint health, the potential for this application should be considered.

Early identification of joint injury and subsequent osteoarthritis could reduce the resulting loss of use and economic burden.¹¹ Because of the relative insensitivity of current diagnostic methods, such as radiography, diagnosis is often made when osteoarthritis is advanced. Difficulties in early diagnosis of joint injury and osteoarthritis have prompted considerable research for biomarkers, including biochemical markers that reflect quantitative and dynamic variations in joint tissue remodeling.¹² Synovial fluid biomarkers have been investigated in horses to identify injury-related changes in joints.^{13–16} Inflammatory biomarkers, such as prostaglandin E₂ and nitric oxide, have been used for equine SF,¹⁷ but they have the disadvantage that the assays require relatively large volumes of SF. By comparison, HMGB-1 requires a small sample volume (10 μ L), which makes it an attractive potential alternative inflammatory biomarker for use in equine SF.

Osteochondral fractures occur commonly in the carpal, MCP, and MTP joints of racehorses.^{18,19} Most horses with osteochondral injury develop osteoarthritis. Currently, no studies have been published on the association of osteochondral injury with SF HMGB-1 concentrations in horses. Therefore, the purpose of the study reported here was to investigate the association of osteochondral injury with HMGB-1 concentrations in SF obtained from the MCP, MTP, and carpal joints of racing Thoroughbreds. We hypothesized that SF HMGB-1 concentrations would be higher in joints with osteochondral injury than in normal joints.

Materials and Methods

Animals—Two groups of Thoroughbreds were included in the study. Group 1 consisted of 40 horses (age range, 14 to 20 months) purchased from July through September 2004 for resale at 2-year-old in training sales (February through April 2005). All horses in group 1 were unraced and confirmed healthy with no evidence of joint abnormalities on the basis of clinical and radiographic examinations conducted by an attending veterinarian prior to the study. Group 2 consisted of 45 racing Thoroughbreds (age range, 2 to 6 years) undergoing arthroscopy for removal of osteochondral fragments resulting from racing injury. These fragments were removed from the dorsal articular borders of the third, radiocarpal, and intermediate carpal bones; the distal aspect of the radius; and the proximal phalanx. The study protocol was approved by the University of Florida Institutional Animal Care and Use Committee.

Experimental design—Synovial fluid (1 to 4 mL) was collected from all horses. Horses of group 1 were sedated, and SF samples were collected without lavage from an MCP joint (n = 20 horses), MCJ (10), and RCJ (10). Horses of group 2 were anesthetized, and SF samples were collected without lavage before arthroscopy for removal of osteochondral fragments from an MCP joint (n = 14 horses), MTP joint (4), MCJ (17), or RCJ (10). Synovial fluid samples were aseptically collected via needle arthrocentesis, centrifuged, decanted, and stored at -80°C until assayed.

Procedure for the HMGB-1 immunoassay—Concentrations of HMGB-1 were measured in SF samples by use of a commercially available 2-step sandwich ELISA^b in accordance with the manufacturer's instructions. Briefly, a polyclonal antibody specific for human HMGB-1 was precoated onto the wells of the kit, and 10 μ L of SF was added. The HMGB-1 in SF bound specifically to the immobilized antibody. After addition of a peroxidase-linked anti-HMGB-1 and -2 monoclonal antibody to form an antigen-antibody complex, color development was initiated by the addition of tetramethyl benzidine. Color developed in proportion to the amount of HMGB-1 in the sample and was measured at 450 nm. Samples of SF were assayed without dilution or digestion.

Arthroscopic and radiographic scores—Available radiographs of osteochondral-injured horses were reviewed, and scores were assigned by use of a scoring system developed by the authors and reported elsewhere.¹⁶ Briefly, 10 categories of radiographic changes were graded from 0 to 3 to provide a total radiographic score of 0 to 30. Radiographic scores were determined for joint space, subchondral bone sclerosis and lucency, soft tissue swelling, and size and number of osteophytes, enthesophytes, and fragments. Similarly, available arthroscopic images, videos, and surgical reports for the osteochondral-injured horses were reviewed, and 11 categories were graded to provide a total arthroscopic score of 0 to 37. The total arthroscopic score accounted for 5 categories of inflammation (each graded from 0 to 3), fragment size and number (each graded from 0 to 3), and 4 categories of degenerative cartilage changes related to the fragments (each graded from 0 to 4).¹⁶

Statistical analysis—Statistical analysis was performed with personal computer-based statistical software.^c Normality plots of the data were assessed. Analysis of box plots identified possible outliers, and extreme Studentized deviate tests were then performed to determine whether the value was > 2 SDs or < 2 SDs from the mean. When the value was > 2 SDs from the mean, it was considered an outlier and eliminated from further analysis. To allow horses to be combined into MCP-MTP joint and carpal joint (MCJ and RCJ) groupings, a 1-way ANOVA was performed to determine whether there were significant differences between the MCP joints, MTP joints, MCJs, and RCJs within horses with normal joints and within osteochondral-injured horses. Differences between normal and osteochondral-injured joints were determined by use of an unpaired *t* test for the MCP-MTP and carpal joints. Sensitivity, specificity, positive predictive value, negative predictive value,

and likelihood ratios of SF HMGB-1 concentrations for identifying osteochondral injury were determined by use of the Fisher exact test. Correlations were assessed via the Spearman correlation.

Discriminant analysis was used to classify the carpal and MCP-MTP joints into the appropriate group (normal or osteochondral-injured) on the basis of SF HMGB-1 concentrations. A quadratic discriminant function was computed from a random sampling of the carpal and MCP-MTP joints by use of separate covariance matrices with the prior probabilities proportional to the population sizes. To determine how well the model could discriminate each joint for its respective group, a subset validation was performed. To eliminate bias, this subset consisted of a random sampling of respective carpal and MCP-MTP joints that were not used in creation of the original model. Values of $P < 0.05$ were considered significant.

Results

Seven SF HMGB-1 concentrations were considered outliers (2 normal MCP-MTP joints, 1 osteochondral-injured MCP-MTP joint, 2 normal carpal joints, and 2 osteochondral-injured carpal joints) and eliminated from further analysis. There were no MTP joints in the normal group. There was no significant ($P = 0.141$) difference between the mean \pm SD HMGB-1 concentrations for MCP joints (11.17 ± 6.10 ng/mL), MCJs (7.24 ± 5.95 ng/mL), and RCJs (10.45 ± 7.00 ng/mL) in the normal group. There was also no significant ($P = 0.493$) difference between the mean HMGB-1 concentrations for MCP joints (21.54 ± 15.47 ng/mL), MTP joints (23.00 ± 16.46 ng/mL), MCJs (16.61 ± 6.74 ng/mL), and RCJs (16.49 ± 9.07 ng/mL) in the osteochondral-injured group. Therefore, results of the normal and osteochondral-injured groups for the MCP and MTP joints and for the MCJs and RCJs were combined for further analyses.

Mean SF HMGB-1 concentrations in MCP-MTP joints of the osteochondral-injured group were significantly ($P = 0.01$) higher than in the MCP-MTP joints of the normal group. Similarly, mean SF HMGB-1 concentrations in osteochondral-injured carpal joints were significantly ($P < 0.001$) higher than in carpal joints of the normal group (Figure 1).

Analysis of the correlation between SF HMGB-1 concentrations in osteochondral-injured joints and radiographic scores or arthroscopic scores was conducted. For the carpal joints, SF HMGB-1 concentrations did not correlate with the radiographic ($r = 0.190$; $P = 0.386$) or arthroscopic ($r = 0.275$; $P = 0.240$) scores. Similarly for the MCP-MTP joints, SF HMGB-1 con-

centrations did not correlate with the radiographic ($r = -0.061$; $P = 0.809$) or arthroscopic ($r = -0.138$; $P = 0.573$) scores.

Synovial fluid HMGB-1 concentrations ≥ 11 ng/mL for MCP-MTP joints and ≥ 9 ng/mL for carpal joints were arbitrarily chosen for determining predictive values for discriminating osteochondral-injured joints from normal joints. This yielded a positive predictive value of 58% and a negative predictive value of 77% for MCP-MTP joints and a positive predictive value of 75% and negative predictive value of 88% for the carpal joints (Table 1). In other words, horses with SF HMGB-1 concentrations ≥ 11 ng/mL for MCP-MTP joints were twice as likely to have an osteochondral injury, and horses with SF HMGB-1 concentrations ≥ 9 ng/mL for carpal joints were 4 times as likely to have an osteochondral injury.

For horses with osteochondral-injured MCP-MTP joints, the SF HMGB-1 concentration made a significant contribution to the discriminant analysis model. The discriminant model was developed by use of 30 randomly selected horses (17 in the normal group and 13 in the osteochondral-injured group). This model was significantly able to separate the groups by overall correctly classifying 20 (66.7%) of the horses. In an attempt to verify the predictive nature of this model, an additional subset of 16 horses that were not used to

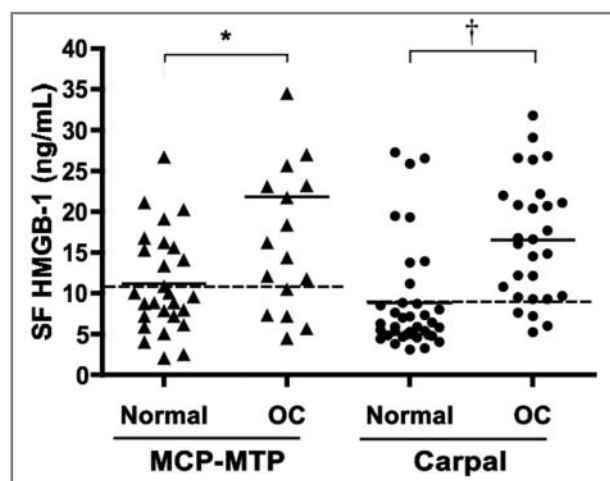


Figure 1—Scatterplot of SF HMGB-1 concentrations for MCP-MTP and carpal joints of Thoroughbreds in the normal ($n = 40$ horses) and osteochondral-injured (OC; 45) groups. The mean value for each group is indicated (solid horizontal lines). Notice the SF concentrations (≥ 11 ng/mL for MCP-MTP joints and ≥ 9 ng/mL for carpal joints; dashed horizontal lines) for which there were predictive values for use in discriminating horses in the osteochondral-injured group from horses in the normal group. *, † Mean values differ significantly (* $P = 0.01$; † $P < 0.001$) between groups.

Table 1—Sensitivity, specificity, positive predictive value, negative predictive value, and likelihood ratio for use of SF HMGB-1 concentrations to discriminate between joints of Thoroughbreds in the normal ($n = 40$ horses) and osteochondral-injured (45) groups.

Joint	Sensitivity (%)	Specificity (%)	Positive predictive value (%)	Negative predictive value (%)	Likelihood ratio
MCP-MTP joints*	74	63	58	77	2
Carpal joints†	86	78	75	88	4

*Cutoff value for the SF HMGB-1 concentration was ≥ 11 ng/mL. †Cutoff value for the SF HMGB-1 concentration was ≥ 9 ng/mL.

create this model was used, including 10 in the normal group and 6 in the osteochondral-injured group. This discriminant analysis model for the MCP-MTP joints correctly classified 13 of these 16 (81.3%) horses (10/10 in the normal group and 3/6 in the osteochondral-injured group), which suggested that this model can be used to correctly classify a horse into its appropriate group 8 out of 10 times on the basis of SF HMGB-1 concentrations. The model was more accurate for use in identifying horses in the normal group than in the osteochondral-injured group.

For horses with osteochondral-injured carpal joints, the SF HMGB-1 concentration made a significant ($P < 0.001$) contribution to the discriminant analysis model. The discriminant model was developed by use of 45 randomly selected horses (29 in the normal group and 16 in the osteochondral-injured group). This model was significantly ($P < 0.001$) able to separate the groups by overall correctly classifying 33 (73.3%) of the horses. In an attempt to verify the predictive nature of this model, an additional subset of 20 horses that were not used to create this model was used, including 8 in the normal group and 12 in the osteochondral-injured group. This discriminant analysis model for the carpal joints correctly classified 14 of these 20 (70%) horses (7/8 in the normal group and 7/12 in the osteochondral-injured group), which suggested that this model can be used to correctly classify a horse into its appropriate group 7 out of 10 times on the basis of SF HMGB-1 concentrations. The model was more accurate for use in identifying horses in the normal group than in the osteochondral-injured group.

Discussion

Osteochondral injury was associated with significantly higher SF HMGB-1 concentrations in MCP-MTP and carpal joints, compared with concentrations in normal joints. It has been suggested that HMGB-1 plays a role in both the inflammatory and destructive processes of rheumatoid arthritis and adjuvant-induced arthritis.⁴ To date, no association has been detected between osteoarthritis and increases in SF HMGB-1 concentrations. In addition, HMGB-1 has been identified in chondrocytes and synoviocytes.⁴ In 1 study,^a investigators determined that articular cartilage and synovial membrane from humans with osteoarthritis differed from normal tissues in the cellular location of HMGB-1 but not the total content. The increase in SF HMGB-1 concentrations in the horses reported here indicated that osteochondral injury was associated with release of HMGB-1 from these cells into the SF. This would require translocation of HMGB-1 from the cells into the extracellular fluid to cause an increase in SF concentrations of HMGB-1.

The release mechanism for HMGB-1 is not clearly understood.²⁰ Macrophages and monocytes in synovial membranes translocate HMGB-1 from the nucleus to the cytoplasm before it is released from cells.⁴ In a recent study,¹⁰ it was found that hypoxia causes substantial secretion or release of HMGB-1 from many kinds of cells. The investigators of that study suggested that the appearance of extracellular HMGB-1 might be associated with tissue hypoxia rather than with nuclear factor

κ B-mediated inflammatory pathways. They reported that HMGB-1 concentrations were significantly correlated with those of lactic acid, a marker of tissue hypoxia. Furthermore, those investigators proposed that the role of hypoxia in HMGB-1 release suggests a new concept for treatment. Treatments (such as hyperbaric oxygen) that reduce hypoxia may decrease extracellular HMGB-1 concentrations and thereby reduce inflammation and cartilage degradation during arthritis.¹⁰

A reciprocal relationship between the early (TNF and IL-1) and late (HMGB-1) cytokines has been proposed.²¹ Early proinflammatory cytokines have been defined as those released within the first few hours after onset of endotoxemia, whereas late cytokines are released later during endotoxemia.²¹ Although osteoarthritis does not involve endotoxemia, these terms serve a useful purpose in the description of the inflammatory response. Interferon- γ , TNF, and IL-1 cause HMGB-1 release.^{2,22} On the other hand, HMGB-1 stimulates monocytes to release TNF, IL-1 α , IL-1 β , IL-6, IL-8, macrophage inflammatory protein-1 α , and macrophage inflammatory protein-1 β .²¹ It has been suggested²³ that TNF and IL-1 play key roles in the pathogenesis of osteoarthritis. Both of these cytokines increase the production of matrix metalloproteinases (1, 3, 9, and 13) and a disintegrin and metalloprotease with thrombospondin motifs 4 (ie, ADAMTS4).²³ This strongly suggests that the interaction of HMGB-1 with other cytokines is of considerable importance in osteoarthritis. It would have been interesting to compare HMGB-1 concentrations with concentrations of other cytokines or markers of tissue hypoxia in the SF of these horses, but unfortunately, adequate volumes of SF were not available. Additional studies need to be conducted to determine this interaction.

We found it useful to use different cutoff values for SF HMGB-1 (≥ 11 ng/mL for MCP-MTP joints and ≥ 9 ng/mL for carpal joints) to calculate predictive values and likelihood ratios. The assay yielded good positive and negative predictive values, with higher values for the carpal joints (Table 1). Use of different cutoff values for these 2 joints for calculation of predictive values is consistent with the methods used in other studies^{16,24,d} conducted by our laboratory group. Thus, it may not be prudent to extrapolate biomarker values among joints, even though there were no significant differences in HMGB-1 concentrations among joints in this study.

The discriminant analysis model may prove to be useful in the diagnostic application for biomarkers of joint injury and disease. When separating normal and injured carpal or MCP-MTP joints, discriminant analysis yielded results similar to positive and negative predictive values. The model performed well at identifying carpal and MCP-MTP joints in the normal group.

In other studies conducted by our laboratory group, we have detected a correlation between radiographic and arthroscopic scores of joint injury and biomarker concentrations in SF or serum. These correlations were detected for serum concentrations and SF-to-serum concentration ratios of bone alkaline phosphatase,¹⁶ an enzyme believed to contribute to calcification of bone matrix²⁵ and thus assumed to be a biomarker of bone synthesis. We have also detected a correlation between

collagen degradation biomarkers in serum and SF with radiographic and arthroscopic scores.^{24,d} Therefore, we expected to detect a correlation of SF HMGB-1 concentrations, presumably a biomarker of joint inflammation, with radiographic and arthroscopic scores of joint injury. We have no definitive explanation for this lack of correlation. However, complete medical records were not available for some of the horses with osteochondral injury. Previous treatment and the duration of the osteochondral injury before collection of SF samples typically were unknown, which is often a limitation of clinical case material. Racehorses with musculoskeletal injury are commonly treated with anti-inflammatory drugs, such as intra-articularly administered corticosteroids, as well as phenylbutazone, an NSAID. It is possible that administration of these drugs may have indirectly affected SF HMGB-1 concentrations and thus may have accounted for the lack of correlation with radiographic and arthroscopic scores. In rodents with experimentally induced sepsis, extremely high doses of corticosteroids (such as dexamethasone and cortisone) and NSAIDs (such as aspirin, ibuprofen, and indomethacin) failed to reduce high serum concentrations of HMGB-1 caused by endotoxemia.²⁶ However, in a study²⁷ of 31 humans with chronic arthritides, evaluation of synovial membranes revealed that intra-articular administration of corticosteroids virtually eliminated extracellular HMGB-1 staining and markedly reduced cytoplasmic HMGB-1 staining in synoviocytes and macrophage-like cells. The potential effect of these drugs on SF HMGB-1 concentrations in the study reported here is unknown, but it may have been a factor. Thus, although there was no correlation between radiographic and arthroscopic scores and SF HMGB-1 concentrations, the assay was of use in discriminating between osteochondral-injured and normal joints. This lack of correlation would not preclude the use of HMGB-1 as a biomarker of joint injury. Additional studies in animals with experimentally induced osteoarthritis may allow further characterization of the effect of anti-inflammatory drugs on the HMGB-1 response.

The study reported here had other limitations. It was determined that 7 HMGB-1 concentrations were outliers; thus, they were eliminated from further analysis. Even though this was performed in accordance with accepted statistical analyses, it increased the possibility of a type I error. In other words, there was a slightly greater chance of stating that a difference existed between groups when there actually was no difference. We do not believe that elimination of these 7 concentrations dramatically affected our findings because there was no bias toward any one group (2 MCP-MTP joints in the normal group, 1 MCP-MTP joint in the osteochondral-injured group, 2 carpal joints in the normal group, and 2 carpal joints in the osteochondral-injured group were eliminated). Ages of horses in the normal and osteochondral-injured groups differed (horses in the normal group were younger). Although synthesis of HMGB-1 decreases in the liver with age,²⁸ the effect of age on SF concentrations is unknown. We evaluated only SF concentrations of HMGB-1. It is possible that comparison of these values with those in serum or urine may have provided useful information regarding

the relative concentrations of HMGB-1 between these compartments.

Another limitation of the study was that a full validation of the assay for application in equine SF was not performed. However, the equine HMGB-1 protein sequence has 99% homology with that of human HMGB-1^c; therefore, there is a high likelihood that the assay is valid in equine body fluids.

In the study reported here, we determined that osteochondral injury was associated with a significant increase in SF HMGB-1 concentration in the MCP-MTP and carpal joints of horses. Synovial fluid HMGB-1 concentrations were not correlated with radiographic or arthroscopic scores. Selection of arbitrary SF HMGB-1 concentrations for each joint yielded high negative predictive values and positive predictive values for the discrimination between normal and osteochondral-injured joints. This provides a rationale for using different SF HMGB-1 concentrations to establish criteria for the use of this biomarker as a diagnostic tool for various joints. On the basis of these findings, SF HMGB-1 analysis may be useful for evaluation of joint injury in horses.

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