Joint disease affects a substantial proportion of the equine population; this results in chronic pain, decreased mobility, and poor performance in horses; reduction in quality of life for both horses and owners; and, potentially, considerable expense for owners.  

Focal destruction of subchondral bone is a prominent feature of several types of joint disease. Bone erosion is an important component of osteoarthritis in high-load, low-motion joints and septic arthritis and in the development of subchondral cystic lesions. Although each of these disease processes has a unique inciting cause, the ultimate result can be loss of subchondral bone and the overlying cartilage, irreversible joint damage, and progressive pathological changes in affected joints.

Bone remodeling is controlled by a balance between the activities of bone-forming osteoblasts and bone-resorbing osteoclasts. Increasing the activity of osteoclasts relative to osteoblasts results in pathological bone resorption, such as that seen in progressive joint disease. Osteoclast development and activation depend on a system comprising RANK, RANKL, and OPG. A membrane-bound tumor necrosis factor superfamily ligand, RANKL, can be released from cells after cleavage by metalloproteinase enzymes. It is highly expressed by many cell types, including osteoclasts, osteoblasts, endothelial cells, bone marrow stromal cells, T lymphocytes, primitive mesenchymal cells within cartilage, synovial fibroblasts, and chondrocytes. Binding of RANKL to the RANK receptor leads to osteoclasto-

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In vitro expression of receptor activator of nuclear factor-κB ligand and osteoprotegerin in cultured equine articular cells

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**Objective**—To determine concentrations of receptor activator of nuclear factor-κB ligand (RANKL) and osteoprotegerin (OPG) in equine chondrocytes and synoviocytes and to quantify changes in the OPG:RANKL ratio in response to exogenous factors.

**Sample Population**—Samples of articular cartilage and synovium with grossly normal appearance obtained from metacarpophalangeal and metatarsophalangeal joints of 5 adult (1- to 8-year-old) horses.

**Procedures**—Cell cultures of chondrocytes and synoviocytes were incubated with human recombinant interleukin-1β (hrIL-1β; 10 ng/mL), lipopolysaccharide (LPS; 10 µg/mL), or dexamethasone (100 nM) for 48 hours. Negative control cultures received no treatment. Cells and spent media were assayed for RANKL and OPG concentrations by use of western blot and immunocytochemical analyses. Spent media were also assayed for OPG concentration by use of an ELISA.

**Results**—RANKL and OPG were expressed in equine chondrocytes and synoviocytes in vitro. Cell-associated RANKL and OPG concentrations were not impacted by exogenous factors. Soluble RANKL release into media was significantly increased by hrIL-1β in chondrocyte but not in synoviocyte cultures. Soluble OPG release into media was significantly increased by hrIL-1β and LPS in chondrocyte but not in synoviocyte cultures. The soluble OPG:RANKL ratio was significantly increased by LPS in chondrocyte cultures. Dexamethasone decreased OPG expression in synoviocytes.

**Conclusions and Clinical Relevance**—RANKL and OPG proteins were expressed in equine articular cells. Release of these proteins may affect osteoclastogenesis within adjacent subchondral bone. Thus, RANKL and OPG may have use as biomarkers and treatment targets in horses with joint disease. (Am J Vet Res 2010;71:615–622)
genesis from osteoclast precursors and stimulates bone resorption by mature osteoclasts. Osteoprotegerin is released by activated osteoblasts and acts as a soluble decoy receptor for RANKL, thus competing and interfering with RANK-RANKL interactions and thereby preventing osteoclast maturation and activation. Therefore, the balance between bone formation and pathological bone loss is dependent on the equilibrium between RANKL and OPG. Expression of RANKL and OPG is regulated in several cell types by many factors, including IL-1, tumor necrosis factor-α, prostaglandin E₂, glucocorticoids, and bacterial LPS. A decrease in the synovial OPG:RANKL ratio has been detected in humans with rheumatoid arthritis and osteoarthritis. This equilibrium shift from OPG excess toward RANKL excess favors osteoclast formation and activation, which leads to bone resorption. Thus, RANKL is a key agent that may be principally involved in bone and cartilage destruction in joint diseases, including rheumatoid arthritis and osteoarthritis. In support of this possibility, results of several experiments have revealed that this equilibrium can be manipulated to prevent bone loss. Blockade of the RANK-RANKL interaction through administration of OPG preserves bone and prevents cartilage destruction in rats with adjuvant-induced arthritis and mice undergoing meniscectomy. Importantly, OPG treatment prevented cartilage matrix proteoglycans in these animals as a result of subchondral bone protection, which illustrates the importance of healthy underlying bone to cartilage health.

Both RANKL and OPG may be useful as biomarkers of bone resorption in joint disease. Concentrations of RANKL and OPG have been measured in serum and synovial fluid obtained from humans with rheumatoid arthritis and osteoarthritis, and changes in the amounts of these markers have been correlated with clinical and radiologic signs of joint disease. To our knowledge, there have been no studies conducted on RANKL and OPG expression in equine articular tissues. The objective of the study reported here was to determine RANKL and OPG expression in vitro in equine chondrocytes and synoviocytes and to quantify changes in the OPG:RANKL ratio in response to exogenous factors. Our hypotheses were that RANKL and OPG are expressed in equine chondrocytes and synoviocytes and that the OPG:RANKL ratio is affected by exposure to hrIL-1β, bacterial LPS, and glucocorticoids.

Materials and Methods

Sample population—Samples of articular cartilage and synovium with a grossly normal appearance were obtained from metacarpophalangeal and metatarsophalangeal joints of 5 adult (1- to 8-year-old) horses that died or were euthanatized with an overdose of pentobarbital for conditions other than joint disease. Cartilage was dissected free of underlying subchondral bone, and synovium was dissected free from the subintima.

Sample preparation—Both tissues were incubated separately at 37°C for 1 hour in physiologic saline (0.9% NaCl) solution containing 1% penicillin and streptomycin. Chondrocytes and synoviocytes were isolated separately by digestion for 16 hours at 37°C with 0.15% collagenase in Dulbecco modified Eagle medium. Following digestion, cells were counted by use of a hemacytometer, and viability was determined via trypan blue stain exclusion. Chondrocytes and synoviocytes were isolated by centrifugation (300 g for 5 minutes), washed, and resuspended (5 x 10⁶ cells/mL) in Dulbecco modified Eagle medium supplemented with 1% penicillin and streptomycin, 4.5 g of glucose/L, 1% l-glutamine, 50 µg of ascorbic acid/mL, and 10% fetal bovine serum.

Cell cultures—Chondrocyte pellets (5 x 10⁶ cells) were formed via centrifugation (300 x g for 5 minutes) in 15-mL polypropylene centrifuge tubes and then incubated by use of standard cell culture conditions (37°C in an environment with 95% relative humidity and 5% carbon dioxide). Synoviocytes were seeded in culture flasks at a density of 5 x 10⁴ cells and grown to confluence as monolayers in 25-cm² flasks by use of identical conditions. Medium was renewed every 2 to 3 days. Cells were deprived of serum for 3 days prior to the start of an experiment. The first day of culture was designated as day 0. On day 7, treated cultures were supplemented with hrIL-1β (10 ng/mL), LPS (10 µg/mL), or dexamethasone (100nM) and incubated for an additional 48 hours in Dulbecco modified Eagle medium supplemented with 1% penicillin and streptomycin, 4.5 g of glucose/L, 1% l-glutamine, and 50 µg of ascorbic acid/mL. Negative control cultures were included that did not receive the additional treatments. Experiments were performed in duplicate. At the end of the 48-hour incubation period, cells and media were collected and stored at −80°C. Chondrocytes were collected and stored as pellets. Synoviocytes were collected via physical agitation in buffer solution.

Immunocytochemical analysis—Cyto centrifugation preparations of the equine synoviocyte and chondrocyte cell lines were prepared at concentrations of 250 cells/µL. Slides were incubated in acetone for 10 minutes, allowed to air dry, and loaded on an automated staining device. To minimize background staining of nonspecific peroxidase, all preparations were blocked with 10% hydrogen peroxide. Cyto centrifugation preparations were incubated for 30 minutes with polyclonal anti-human RANKL antibody (dilution, 1:20) or anti-human OPG antibody (dilution, 1:100). Then, samples were incubated for 30 minutes with the secondary goat anti-rabbit immunoglobulin (dilution, 1:200), followed by incubation for 7 minutes with 3,3′diaminobenzidine chromagen solution and counterstaining by incubation for 5 minutes with Mayer’s hematoxylin. The MG63 human osteosarcoma cell line was used as a positive control culture for RANKL and OPG. For each cell line, staining with only the secondary antibody was used as a negative control sample for the assay. Positive expression was characterized by staining of the membranes with or without staining of the cytoplasm. Antibodies were optimized with the MG63 human osteosarcoma cell line at dilutions of 1:20, 1:50, and 1:100.
Western blot analysis—Semiquantitative western blot analysis was performed on media supernatant and cell extracts. Briefly, media were precipitated with sodium acetate in ethanol and then resuspended. Cells were lysed by the addition of buffer solution. Protein content of each preparation was determined, and 1 to 3 μg of protein was separated on a 12.5% SDS-polyacrylamide gradient gel and then transferred to nitrocellulose membranes in a transfer cell. Membranes were blocked with 5% milk in TBS-T buffer solution and incubated with primary antibodies for 1 hour at 20°C. Primary antibodies for media samples included OPG (dilution, 1:1,000), RANKL (dilution, 1:750), and GAPDH (dilution, 1:2,000) as an internal standard. Primary antibodies for lysed cell samples included OPG (dilution, 1:1,000), RANKL (dilution, 1:1,250), and GAPDH (dilution, 1:8,000) as an internal standard. Membranes were washed 3 times with TBS-T buffer solution and incubated with secondary antibody for 1 hour at 20°C. After film was exposed for 5 minutes, target proteins were detected with a chemiluminescent system. Semi-quantitative densitometry was performed on protein bands by use of imaging software. Chemiluminescent bands from cell samples were standardized against GAPDH results, and protein expression was calculated as the ratio of the mean intensity of the RANKL or OPG band to the mean intensity of the GAPDH band.

OPG ELISA—Concentration of OPG in spent media from chondrocyte and synoviocyte cultures was determined by use of a commercial colorimetric ELISA performed in accordance with the manufacturer’s directions. Briefly, 96-well microplates were coated with rat anti-mouse capture antibody by overnight incubation. Plates were then washed, and wells were blocked by incubation with 1% bovine serum albumin in PBS solution for 1 hour at 20°C. Wells were washed, and 100 μL of sample was added to each well. Plates were incubated for 2 hours at 20°C and then washed. Biotinylated goat anti-mouse OPG detection antibody was added to each well, and plates were incubated for 2 hours at 20°C. Wells were washed, and streptavidin-horseradish peroxidase was added to each well. Plates were incubated in the dark for 20 minutes at 20°C. Plates were washed, and tetramethylbenzidine and hydrogen peroxide were added to each well. Plates were then incubated in the dark for 20 minutes at 20°C, followed by the addi-

Figure 1—Photomicrographs of representative cultured equine chondrocytes and synoviocytes immunocytochemically stained with primary antibodies against OPG or RANKL. Rows represent chondrocytes stained for OPG (OPG Chond), synoviocytes stained for OPG (OPG Syn), chondrocytes stained for RANKL (RANKL Chond), and synoviocytes stained for RANKL (RANKL Syn). Columns represent cells incubated without primary antibody (ie, with secondary antibody only) as an assay control sample (No antibody), untreated negative control cells (Neg), and cells treated with hrIL-1β (10 ng/mL), LPS (10 μg/mL), or dexamethasone (Dex; 100 nM). A positive result is indicated by uptake of brown stain at the periphery of cells. Bar = 30 μm.
tion of 2N H$_2$SO$_4$ to stop the reaction. Absorbance was measured at 450 nm and corrected for absorbance at 540 nm.$^4$ The OPG concentrations were determined by comparison to a standard curve.

**Statistical analysis**—Data (mean values of duplicate samples) were compared with a repeated-measures ANOVA by use of a statistical software program.$^2$ Significance was set at values of $P < 0.05$. Post hoc analysis was accomplished by use of the Student-Newman-Keuls method. Results were expressed as mean ± SE. Protein expressions of RANKL and OPG as well as the OPG:RANKL ratio were compared.

**Results**

**Immunocytochemical analysis**—Various amounts of staining for both RANKL and OPG were detected within all treatment groups (Figure 1).

**Western blot analysis**—Western blots had strong discrete staining for both RANKL and OPG proteins (Figure 2). A RANKL protein of approximately 40 kDa was detected in media supernatant from both chondrocytes and synoviocytes. Compared with results for untreated control cells, the RANKL concentration was significantly ($P = 0.032$) increased in spent media from chondrocytes stimulated with hrIL-1β (Figure 3). Treatment with hrIL-1β, LPS, or dexamethasone did not significantly impact the relative concentration of RANKL in media supernatant from synoviocytes. The RANKL proteins of approximately 40, 26, and 24 kDa were detected in both chondrocyte and synoviocyte cell cultures. Treatment with hrIL-1β, LPS, or dexamethasone did not impact relative amounts of RANKL associated with either cell type.

An OPG protein of approximately 55 kDa was detected in media supernatant from both chondrocytes and synoviocytes. Compared with results for negative control cells, OPG concentration was significantly increased in spent media from chondrocytes stimulated with hrIL-1β ($P = 0.034$) or LPS ($P = 0.018$; Figure 4). Treatment with hrIL-1β, LPS, or dexamethasone did not significantly impact relative

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**Figure 2**—Photograph of western blots for representative equine chondrocytes stained with primary antibody for RANKL (A) and OPG (B) and the GAPDH control sample (C). Lanes represent untreated negative control cells (Neg) and cells treated with hrIL-1β (10 ng/mL), LPS (10 µg/mL), or dexamethasone (Dex; 100nM). Numbers on the left side are number of kilodaltons.

**Figure 3**—Mean ± SE relative expression of RANKL protein released into media by cultured equine chondrocytes as determined by use of densitometric analysis of western blots. Treatment groups included untreated negative control cells (Neg) and cells treated with hrIL-1β (10 ng/mL), LPS (10 µg/mL), or dexamethasone (Dex; 100nM). Results represent the fold increase, relative to the results for cells incubated with antibody against GAPDH. *Value differs significantly ($P < 0.05$) from the value for the negative control group.

**Figure 4**—Mean ± SE relative expression of OPG protein released into media by cultured equine chondrocytes as determined by use of densitometric analysis of western blots. See Figure 3 for key.

**Figure 5**—Mean ± SE value for the OPG:RANKL ratio of proteins released into media by cultured equine chondrocytes as determined by use of densitometric analysis of western blots. See Figure 3 for key.
concentration of OPG in media supernatant from synoviocytes. The OPG proteins of approximately 60 and 55 kDa were detected in both chondrocyte and synoviocyte cell cultures. Treatment with hrIL-1β, LPS, or dexamethasone did not impact relative amounts of OPG associated with either cell type.

The OPG:RANKL ratio was significantly ($P = 0.019$) increased in media supernatant of LPS-treated chondrocytes (Figure 5). Treatment did not impact the OPG:RANKL ratio in media supernatant of synoviocytes. The OPG:RANKL ratio was not significantly affected by any treatment in chondrocyte or synoviocyte cell cultures.

ELISA—Concentration of OPG protein was significantly ($P < 0.001$) increased in media supernatant of chondrocytes treated with hrIL-1β or LPS (Figure 6). Concentration of OPG protein was significantly ($P = 0.01$) decreased in media supernatant of synoviocytes treated with dexamethasone (Figure 7).

Discussion

Analysis of the results of the study reported here indicated that RANKL and OPG were expressed in equine articular chondrocytes and synoviocytes in vitro, hrIL-1β increased RANKL and OPG concentrations in media supernatant of chondrocytes, LPS increased OPG concentrations in media supernatant of chondrocytes, and dexamethasone decreased OPG concentrations in media supernatant of synoviocytes. Cell-associated RANKL and OPG concentrations did not change significantly in response to treatment in vitro. In light of the fact RANKL and OPG were detected in articular cells, and both responded to exogenous factors, it is possible that they may play a role in periarticular bone metabolism in joint disease of horses.

Our results indicated an increase in RANKL and OPG concentration after stimulation with hrIL-1β, which is similar to results of other investigators. Expression of RANKL protein is upregulated by IL-1 in murine stromal cells. The RANKL protein is also upregulated in rat articular fibrochondrocytes exposed to IL-1. Expression of OPG protein is increased in osteoblasts and human chondrocytes in response to IL-1. It is possible that soluble OPG and RANKL produced by articular cells may impact osteoclastogenesis and osteoclast activity in adjacent subchondral bone in response to IL-1 in the joint environment. Specifically, binding of RANKL to the RANK receptor is expected to lead to osteoclastogenesis from osteoclast precursors and to stimulate bone resorption activity by mature osteoclasts, whereas OPG is expected to prevent osteoclast maturation and activation. However, it is the ratio of RANKL to OPG, rather than the absolute concentrations of each, that determines regulation of osteoclastogenesis.

To our knowledge, the effects of LPS on RANKL and OPG production in articular cells have not been investigated; however, LPS can increase OPG protein and mRNA production by periodontal ligament and gingival fibroblasts. Intraperitoneal injection of LPS in mice causes an increase in serum OPG concentrations. Equine chondrocytes in the present study had a dramatic increase in soluble OPG production in response to LPS, which is consistent with the aforementioned findings in other cell types and whole-body systems.

Our results indicated a decrease in OPG production by dexamethasone-treated synoviocytes, which is consistent with the findings of other investigators. To our knowledge, the effect of dexamethasone on OPG expression of articular cells has not been reported; however, other investigators have detected similar responses in other cell types. Glucocorticoids can downregulate OPG mRNA and protein in osteoblasts and marrow stromal cells and promote osteoclastogenesis. It should be mentioned that dexamethasone reduced OPG expression in the study reported here, as determined on the basis of results of the ELISA but not on the basis of results for western blot analysis. It is possible that the semiquantitative nature of western blot analysis was not as sensitive as the quantitative ELISA analysis for detecting differences. The larger SE observed in the dexamethasone-treated samples ana-
lyzed by use of western blot analysis, compared with the smaller SE in the same group evaluated by use of the ELISA, may have also reduced our ability to detect differences.

The soluble OPG:RANKL ratio was increased in media supernatant of chondrocytes treated with LPS, which may favor inhibition of osteoclastogenesis in adjacent bone. No other treatment induced a significant change in the ratio. Although response of the OPG: RANKL ratio to LPS treatment has not been examined in articular cells, this result is consistent with the findings of other investigators who detected an increase in serum OPG concentrations and a decrease in serum RANKL concentrations in mice injected intraperitoneally with LPS. Bone loss is a serious sequela to long-standing joint infection; therefore, an increase in the OPG:RANKL ratio in response to LPS stimulation is unexpected. However, some authors postulate that osteoclast differentiation may require a more specific sequence of events, rather than just exposure to LPS alone. Other investigators have determined that LPS alone fails to induce osteoclastogenesis, but LPS can induce osteoclastogenesis in bone marrow mononuclear cells after pretreatment of those cells with RANKL. Thus, LPS-induced osteoclastogenesis may be independent of the RANKL pathway and may instead depend on cyclooxygenase-2–mediated prostaglandin synthesis. Therefore, the cascade of events that lead to bone resorption may be dependent on factors other than exposure to LPS alone.

The RANKL and OPG responses to exogenous factors in the study reported here must be interpreted cautiously. In addition to evidence that suggests specific sequences of events are required for osteoclastogenesis, cellular responses may also change over time. Temporal regulation of RANKL and OPG expression has been reported in 1 study in which investigators determined that rat fibrochondrocytes increase RANKL expression but not OPG expression at 4 and 24 hours after stimulation with IL-1. These factors are upregulated in periodontal ligament fibroblasts 48 hours after LPS stimulation but not 24 hours after stimulation. In addition, LPS-stimulated osteoblasts cultured for up to 14 days had increased OPG expression early during culture, followed by a decrease in expression later during culture. Because the study reported here was based on a single time point (48 hours), further experiments are required to investigate the regulation of RANKL and OPG expression in equine articular cells over time. In the present study, we used hrIL-1β to treat cells, and a more vigorous response may have been detected had recombinant equine IL-1 been used. Furthermore, we used ex vivo tissues isolated from native extracellular matrix. Responses of cells within native matrix or for an in vivo system may differ from those reported here. Additionally, we were only able to obtain ELISA data for the OPG protein. We experienced difficulty obtaining ELISA data for the equine RANKL protein with both commercial and de novo protocols. We have also experienced difficulty with the RANKL ELISA in other veterinary species, and this aspect of RANKL detection requires further development.

Osteoprotegerin was identified as a soluble 55-kDa protein in media supernatant and as 55- and 60-kDa proteins associated with cells. There is good agreement between results reported here and reports of 55- and 60-kDa OPG proteins in mice, humans, 

ALTERNATIVELY, CROSS-REACTIVITY WITH RELATED PROTEINS CANNOT BE RULED OUT.

The study reported here indicates that RANKL and OPG are present in equine articular cells in vitro and that their expression changes in response to stimulation with exogenous factors. Liberation of these proteins may have an effect on osteoclastogenesis within adjacent subchondral bone, which may ultimately lead to changes in subchondral bone density seen with several types of joint disease. As has been reported in other species, RANKL and OPG may have use as biomarkers and treatment targets in joint diseases of horses. Additional studies are required to detail the RANK-RANKL-OPG axis in equine tissues, determine the specific cells that are targeted and influenced by RANKL in the joint environment, and determine concentrations of RANKL and OPG in naturally occurring disease.

2. Collagenase type 2, Worthington Biochemical Corp,Lakewood, NJ.
3. DMEM, Mediatech Inc, Herndon, Va.
4. Trypan blue stain 0.4%, Invitrogen, Carlsbad, Calif.
5. ω-glutamine 200mM, Invitrogen, Carlsbad, Calif.
6. WAKO, Richmond, Va.
8. R & D Systems, Minneapolis, Minn.
9. Sigma-Aldrich, St Louis, Mo.
10. Sigma-Aldrich, St Louis, Mo.
11. RIPA buffer, Sigma-Aldrich, St Louis, Mo.
12. RIPA buffer, Sigma-Aldrich, St Louis, Mo.
15. Biogenex Laboratories, San Ramon, Calif.
16. EZQ protein quantitation kit, Invitrogen, Carlsbad, Calif.
17. Rabbit polyclonal anti-human RANKL antibody, Axxora, San Diego, Calif.
20. DAKO autostainer, Dako, Carpinteria, Calif.
22. EZQ protein quantitation kit, Invitrogen, Carlsbad, Calif.
23. Mini-PROTEAN 3 cell, Bio-Rad Laboratories, Hercules, Calif.
24. Mini trans-blot electrophoretic transfer cell, Bio-Rad Laboratories, Hercules, Calif.
26. Donley anti-rabbit HRP Amersham Biosciences, Piscataway, NJ.
27. ECL, Amersham Biosciences, Piscataway, NJ.
29. Mouse OPG ELISA, R & D Systems, Minneapolis, Minn.
30. FluosStar Optima, BMG Laboratories, Durham, NC.
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