Immunohistochemical analysis of an equine model of synovitis-induced arthritis

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Objectives—To use lipopolysaccharide (LPS) to create synovitis in the midcarpal joint of ponies, and to assess the morphologic, histochemical, and immunohistochemical effects of synovitis on articular cartilage of the third carpal bone.

Animals—2- to 3-year-old ponies, 6 control (group 1) and 6 treated (group 2).

Procedure—Synovitis was induced in 1 midcarpal joint of group-2 ponies by intra-articular injections of LPS (0.02 μg/kg of body weight), morphine (0.1 mg/kg), and saline solution (group 2a) and morphine and saline solution alone in the contralateral midcarpal joint (group 2b). Articular cartilage sections and attached synovial membrane from the third carpal bones were examined by immunohistochemical distribution of interleukin 1β, tumor necrosis factor (TNF-α), TNF receptors (PSS, P75) and 3-B-3(−) epitopes, and by localization of proteoglycans (metachromatic staining). Proteoglycan extracts were assessed by metachromatic staining or western blotting and immunohistochemical staining, using anti-3-B-3 antibodies.

Results—Enhanced immunoreactivity for the cytokines and receptors was found in inflamed synovial membrane and noncalcified cartilage (group 2a more than 2b). Metachromasia of the noncalcified cartilage was greater in group-1 than in group-2a and group-2b specimens. In group 2a, chondrocyte hypertrophy and enhanced immunoreactivity for 3-B-3(−) epitope in areas of increased cytokine immunoreactivity suggested possible phenotypic change of the chondrocytes in response to synovitis. Immunohistochemical analysis by western blotting of proteoglycan extracts indicated strong 3-B-3(−) epitope immunolocalization in group-2a, weaker staining in group-2b, and barely detectable stain in group-1 specimens, which correlated with in situ immunolocalization.

Conclusions—Intra-articular administration of LPS may be used to induce a synovial environment conducive to increased immunoreactivity of interleukin 1β, TNF-α, and its receptors in equine synovial membrane and articular cartilage. These cytokines may be involved in the early phenotypic change of chondrocytes that is believed to occur in osteoarthritis and is characterized in this study by enhanced 3-B-3(−) epitope immunoreactivity and chondrocyte hypertrophy. (Am J Vet Res 1996;57:1080-1093)

L ameness that results from osteoarthritis (OA) is a major cause of poor performance and early retirement of equine athletes.1,2 Osteoarthritis is a disease of movable (synovial) joints and is characterized by variable degrees of pain, synovitis, and degeneration and erosion of articular cartilage.3-5 Normal articular cartilage is a dynamic tissue that undergoes continual remodeling by degradation and synthesis of its matrix.6 Chondrocytes produce the structural components of the extracellular matrix, principally collagens and hydrophilic proteoglycans, which are responsible for its biomechanical properties.6 Chondrocytes, synoviocytes, bone cells, and inflammatory cells produce cytokines, including interleukin (IL)-1 and tumor necrosis factor (TNF)-α, and degradative proteinases that effect matrix synthesis and degradation.6-10 The actions of IL-1 and TNF-α in suppressing proteoglycan synthesis and stimulating proteinase synthesis and release are mediated by binding to specific cell surface receptors.11-19,22,24-27 Homeostasis of the matrix is achieved through fine regulation of cytokines, growth factors, their respective receptors and antagonists, and degradative proteinases and their inhibitors, such as tissue inhibitor of metalloproteinase.6,7,9,12,14,21,27

Equine chondrocytes exposed to adverse conditions in vitro have been documented to produce IL-1,20 the metalloproteinase, stromelysin, and prostaglandin E2.21 Presumably, trauma, instability, acute inflammatory disease, or infection in joints causes release of pleiotropic cytokines in excess of their natural inhibitors, which in turn, activates proteinases in excess of their respective inhibitors. This imbalance produces a net loss of proteoglycans and collagen from the matrix and results in mechanical dysfunction that is characteristic of OA.4 Increased synovial fluid TNF-α, IL-1, and IL-6 activities have been measured in horses with naturally acquired and experimentally induced arthritides.29,30 Tumor necrosis factor-α and IL-1β and their specific receptors have been immunolocalized in porcine chondrocytes exposed to IL-129 and in cartilage and synovium of dogs with experimentally induced OA33 and people with naturally acquired arthritis,34 but have not been localized in horses to our knowledge.

A change in chondrocytic phenotype accentuated by hypertrophy and increased production of proteinases and matrical components appears to be a feature of OA.31,32 The latter includes the synthesis of glycosaminoglycans with abnormal sulfation patterns, such as 3-B-3(−) epitope.36,37 Recognition of glycosaminoglycans...
lycans in mature healthy cartilage by the 3-B-3 epitope antibody requires predigestion with chondroitinase or hyaluronidase, denoted as the expression of the 3-B-3(α) epitope. The oligosaccharide structure recognized by the antibody consists of a nonreducing, terminal, saturated (or Δ-4,5-unsaturated) glucuronic acid residue adjacent to N-acetyl-galactosamine-6-sulfate. Recognition of the 3-B-3 epitope without predigestion of samples, denoted as 3-B-3(−), reflects a natural, subtle change in the chondroitin sulfate of the glycosaminoglycans, indicating an atypical chondroitin sulfate chain termination. It has been recognized in the zone of hypertrophy in normal human and avian growth plates, in articular cartilage of human beings with OA and rheumatoid arthritis (RA), and in animals with naturally acquired and experimentally induced OA. It is present in low amounts in normal mature cartilage, but its expression is greatly upregulated in arthritis.

Traumatic arthritis, characterized by synovitis and effusion without gross articular damage, is common in the carpus and fetlock of young racehorses in training and may result in OA if untreated. Thus, models of synovitis may help us understand the early pathophysiologic processes of associated cartilage injury that result in OA. Intra-articular injections of lipopolysaccharides (LPS) in horses have been documented to cause synovitis, high synovial fluid TNF-α, IL-6, eosinomoids, and keratan sulfate values. Lipopolysaccharides are potent inducers of TNF-α and IL-1, which probably mediate their effects. Lipopolysaccharides have been documented to promote cartilage degradation in vivo in horses and rabbits and in vitro in equine, bovine, and canine cartilage.

Because of their potential role in the pathogenesis of equine synovitis-induced OA, IL-1β and TNF-α and its receptors were immunolocalized and compared in normal and inflamed synovial membrane. Expression of the cytokines, receptors, and degree of metachromasia in articular cartilage of normal and inflamed joints was used to identify degradative changes in the articular cartilage. Expression of 3-B-3(−) epitope was used as a sensitive marker of cartilage changes in response to that degradation.

Materials and Methods

Ponies—The experimental protocol was approved by the Auburn University Institutional Animal Care and Use Committee. Twelve healthy ponies between 2 and 3 years old were randomly assigned to 1 of 2 groups, each containing 6 ponies. Group-1 ponies were used for student surgical exercises at sites other than the carpus and received no medication. In group-2 ponies, on day 1, sterile synovitis was induced in 1 randomly chosen midcarpal joint by intra-articular injection of LPS (doseage, 0.02 µg/kg of body weight); morphine sulfate (0.1 mg/kg) was given for analgesia with saline solution to a 5-ml total volume (group 2a). As a treated control (group 2b), the contralateral midcarpal joint received the same dose of morphine sulfate with saline solution to a 5-ml total volume. Prior to the intra-articular injections, acepromazine maleate (0.05 mg/kg, IV) was given for tranquilization. At postinjection hour (PIH) 0, 8, and 24, synovial fluid samples were obtained. Total and differential systemic WBC counting was performed at PIH 0 and 8 because neutrophilia was evident at PIH 8 in previous studies of intra-articular injections of LPS. On postinjection days 6, 11, and 16, the injection procedure was repeated.

Medical status of each pony was assessed according to the clinic’s intensive care protocol, including recording of temperature and heart and respiratory rates every 3 hours for the first 24 hours, then twice daily until the next injection of LPS. After the initial 24 hours, attitude was assessed every 3 hours and physical examination was performed every 12 hours. Lameness and joint effusion (detected by palpation) were assessed hourly for the first 12 hours, every 3 hours for the next 24 hours, then twice daily. Additional analgesia (detomidine hydrochloride at a dosage of 0.05 mg/kg, butorphanol tartrate at a dosage of 0.1 mg/kg) was to be provided if the heart rate was > 64 beats/min, the pony was moderately lame at the walk for over 2 hours, and/or dysphagia was evident. Fever (> 40°C) was to be treated by administration of dipyrone (11 mg/kg, IV). The ponies were still rested and hand grazed twice daily. On postinjection day 18, the ponies were anesthetized, used for student surgical exercises performed at a site other than the carpus, then euthanatized. Right and left carpal joints were harvested for analysis immediately after euthanasia.

Necropsy—A sample of synovial fluid was collected from each joint at necropsy for total nucleated and differential cell counts and bacterial culture. The midcarpal joints were disarticulated and photographed.

Histologic examination of cartilage and synovial membrane—Slabs, 3 mm thick with attached synovial membrane (including both the transitional and villous areas), were removed by use of a band saw from the middle of each intermediate and radial facet of the third carpal bone (C3). The slabs were immersed in buffered 10% formalin for 72 hours. Each specimen was washed in running tap water, radiographed, and decalcified in a solution of 4% HCl and 4% formic acid. After decalcification, the specimens were rinsed in running tap water and stored in 70% ethyl alcohol. In preparation for embedment, the specimens were dehydrated in graded alcohols, cleared, and embedded in paraffin containing plastic polymers. Five-micron sections were cut by use of a rotary microtome and mounted on coated glass slides. Individual sections were stained with H&E (Harris hematoxylin) or toluidine blue. Each section was evaluated and subjectively assessed. To prevent interpretation bias, the treatment group identity was coded and not revealed to the evaluator.

Staining patterns of articular cartilage were described on the basis of topographic location along the radial or intermediate facet of C3 as region A (most dorsal 20%), B (next 20%), C (next 20%), and D (most palmar 40%). Depth into the noncalcified cartilage was described as superficial, middle, and deep zones. Location of staining, particularly for the immunolocalization procedures, was also described as cellular or matrical (pericellular, territorial, interterritorial). Staining patterns of synovial membrane were assessed by location in the synovial lining layer (including type-A/macrophage- and type-B/fibroblast-like cells) or subintimal layer (including fibroblasts, vascular smooth muscle and endothelial cells, and mononuclear and polymorphonuclear inflammatory cells). Hematoxylin and eosin-stained articular cartilage specimens were assessed for chondrocyte and nuclear shape (spindle, oval, or round), chondrocytic staining intensity, and presence or absence of matrical basophilia or acidophilia. Toluidine blue-stained articular cartilage specimens were graded for intensity of staining or metachromasia. Metachromasia was graded as negative, slight, mild, moderate, or severe.

Immunohistochemistry—Tumor necrosis factor-α was localized in articular cartilage and synovial membrane, using a rabbit anti-human TNF-α (IgG polyclonal) antibody at a dilution of 1:100. This antibody was found to be specific by the manufacturer for human TNF-α and had < 0.1% cross-reactivity for recombinant human TNF-β, IL-1β, IL-1α, IL-
3, IL-6, and mouse IL-6 by results of dot blot assay and radioimmunoassay.

Equine TNF-α has been characterized and cloned. A human monoclonal antibody to TNF-α was found to cross-react with TNF-α of several species, including the horse, and it was suggested that TNF-α was highly conserved between species. Tumor necrosis factor receptors P55 and P75 were immunolocalized in articular cartilage and synovial membrane, using rabbit anti-human P55 or P75 (IgG polyclonal) antibody at a dilution of 1:200. The antibodies were raised against the extracellular component of recombinant human P55 or P75 receptors.

Interleukin 1β was detected in articular cartilage and synovial membrane, using a rabbit anti-human IL-1β (IgG polyclonal) antibody at a dilution of 1:100. Specificity was determined by the manufacturer at 100% for human IL-1β and < 0.1% for human recombinant IL-1α, TNF-α, TNF-β, IL-3, IL-6, and mouse granulocyte-macrophage colony-stimulating factor by results of dot blot assay and radioimmunoassay. Interleukin 1β appears to be highly conserved between species because equine IL-1β has recently been purified and is active in assays used for detecting human IL-1β.

The 3-B-3(-) epitope of chondroitin sulfate was detected in articular cartilage, using mouse anti-human 3-B-3 (IgG monoclonal) antibody at a dilution of 1:100. Sections, except those intended for 3-B-3(-) epitope immunolocalization, were digested for 1 hour in bovine testicular hyaluronidase (1.0 mg/ml). All sections were methanol/hydrogen peroxide-blocked (1.5% hydrogen peroxide in methanol for 45 minutes), blocked with normal goat serum (10%) for 20 minutes, and incubated with the primary antibody overnight at 22 C. Commercial kits were used to label the primary antibodies with secondary antibodies. 3,3'-Diaminobenzidine tetrahydrochloride was used to give a colored reaction. Controls for the immunohistochemical procedures included rheumatoid synovium: with antibody, with and without digestion; and without antibody, with and without digestion. Intensity of immunolocalization was graded negative, slight, mild, moderate, or strong.

Biochemical analysis—Full-thickness specimens of the nonmineralized region of articular cartilage were excised, using a scalpel, from the dorsal aspect of the C3 between the radial and intermediate facets, placed in sealed vials, and extracted in 4M guanidine HCl, 0.05M sodium acetate, pH 6.8, containing protease inhibitors (100 mM 6-amino-hexanoic acid, 10mM EDTA, 5 mM benzamidine HCl, 0.1 mM phenylmethylsulfonyl fluoride) for 24 hours at 4 C. The solution containing the extracts was then exchanged with phosphate-buffered saline solution, using a concentrator. To allow equal loading of the extracts in the subsequent electrophoresis, a measurement of the amount of proteoglycan in aliquots of each extract was used, as determined by use of the safranin O dye-binding method. The solution containing the extracts was then exchanged with electrophoresis running buffer in the aforementioned concentrator and separated by composite agarose-polyacrylamide gel electrophoresis according to the method of Carney et al. The extracts were then stained either with toluidine blue to stain the B-3, either before or after washing briefly with TBST, then incubated with antibody 3-B-3(-) or after 3-B-3(-) treatment with 0.01 U of chondroitinase ABC/ml for 37°C. The antibody was diluted 1:1,000 in TBST containing 1% bovine serum albumin and incubated with the membrane for 1 hour. This and all subsequent steps were carried out at 20 to 22°C (room temperature). The blots were then washed for 3 x 10 minutes in TBST and incubated with anti-mouse immunglobulin conjugated to alkaline phosphatase at 1:7,500 dilution for 1 hour. After another rinse (3 x 10 minutes in TBST), bands were visualized by addition of nitroblue tetrazolium, 5-bromo-4-chloro-3-indolyl phosphate in 0.1M NaCl, 5 mM MgCl2, 0.1M Tris, pH 9.55, for color development. For toluidine blue staining and 3-B-3(-) blots, the extracts were loaded at 8 μg of chondroitin sulfate equivalents as determined by results of the safranin O assay. For the 3-B-3(+) blots, only 0.4 μg of the extract was loaded.
because the enzyme-generated epitope is so abundant on the proteoglycans that higher loading gave too high a signal, obscuring the resultant bands.

Statistical evaluation—Data for the synovial fluid results were analyzed, using the general linear models for ANOVA for PIH 0, 8, and 24. Level of significance was set at $P = 0.05$. The general linear model for ANOVA was used to determine within-group differences for each injection period. Data were blocked on individual ponies in each injection period to account for repeated measures. Scheffé's test for multiple comparisons was used to detect differences between each injection, using the mean values for PIH 0, 8, and 24.

Results
Clinical evaluation—Lameness of mild to moderate severity at the walk was evident in the LPS-treated limb between PIH 1 and 2. Between PIH 2 and 3, analgesia attributable to the morphine relieved the discomfort (negative to mild lameness at the walk). By PIH 24, lameness was not obvious at the walk and was mild to moderate at the trot in a straight line. Lameness worsened slightly after each subsequent injection so that the lameness was a grade more severe by the fourth injection (moderate lameness at the trot at PIH 24). Mild to moderate effusion was usually observed by PIH 2. At PIH 8, effusion was moderate to severe in group-2a joints and mild in group-2b joints. Effusion tended to become more obvious after each subsequent injection. Temperature became high (40 to 41°C) in 2 ponies between PIH 6 and 12, but responded to a single injection of dipyrene (11 mg/kg, IV). One of these ponies was also given butorphanol and xylazine at PIH 7 for analgesia (moderate lameness at the walk and dysphagia), to which it responded. Lameness improved in all ponies (none to mild at the walk) after removal of synovial fluid at PIH 8.

Hematologic variables—No alteration in total or differential systemic WBC counts was evident between PIH 0 and 8.

Synovial fluid—Marked infiltration with inflammatory cells, predominantly neutrophils, and an increase in total protein concentration were detected in group-2a joints after all injections, with a moderate...
response in group-2b joints by injection 4 (Fig 1). There was no significant difference between total WBC count and total protein concentration among ponies of the same group. A significant difference in total WBC count and total protein concentration was apparent between groups 2a and 2b after all injections, except injection 4. In group 2a, there was no trend for increased cell counts associated with subsequent injections. A significant (P < 0.05) decrease in mean WBC count (mean over PIH 0, 8, and 24) was apparent between injections 1 and 3 in group 2a. Mean total protein concentration in group-2a ponies increased with subsequent injections, but the increase was only significant between injections 2 and 3. In group-2b ponies, a significant (P < 0.05) increase in mean WBC count was observed after injection 4, relative to injections 1 and 3. Values in group-2b ponies indicated a trend toward high mean total protein concentration with progressive injections and a significant (P < 0.05) increase between injection 4 and the earlier injections. Culture of synovial fluid at the conclusion of the study yielded negative results.

Gross necropsy findings—No gross changes of the articular cartilage were evident at necropsy (Fig 2). The synovial membrane was thick, edematous, and hyperemic. The synovial membrane and overlying connective tissue were hemorrhagic at the sites of arthrocentesis. No synovial adhesions were evident.

Histopathologic examination of synovial membrane: H&E—The normal synovial lining layer of group-1 joints was 1 to 2 cells thick, with the subintima containing predominantly fibroblasts (Fig 3A). In group-2a joints, the synovial lining layer was 3 to 5 cells thick, and cellular infiltration into the subintimal layer of mononuclear and polymorphonuclear cells was apparent (Fig 4A). A proliferative, inflammatory response also was observed in group-2b joints, but the intensity was less than that in group-2a joints.

Immunohistochemistry—In group-1 specimens, IL-1β, TNF-α, and receptors were immunolocalized in a few fibroblasts and vascular smooth muscle or endothelial cells (Fig 3B–D). In group-2a (Fig 4B–D) and group-2b specimens, enhanced immunoreactivity was evident, with many positive-staining cells in the inflamed synovial membrane; greater intensity and number of cells staining were seen in the former. Moderate to strong immunoreactivity for IL-1β was apparent in approximately 75% of the synovial lining layer cells. The intensity of IL-1β immunoreactivity in vascular smooth muscle and endothelial cells, and in fibroblasts and infiltrating inflammatory cells, was uniform. Immunoreactivity of TNF-α was weak in the cells in the synovial lining layer of the transitional area. Approximately 50% of the villous synovial lining layer cells were immunoreactive. In the subintima, TNF-α was immunolocalized in more fibroblasts and inflammatory cells (particularly, mononuclear cells) and with greater intensity than was IL-1β. Immunoreactivity for the TNF receptors was similar to that for TNF-α, but of less intensity. Cytokines were immunolocalized with...
greater intensity in group-2a, compared with group-2b, joints.

Histopathologic examination of cartilage: H&E—Articular cartilage of group-1 ponies contained variable but strong matrical basophilia throughout the deeper zones of the noncalcified cartilage, particularly in the thicker cartilage of regions B and C (Fig 5A). Regions A and D were more acidophilic. In the superficial zone, flat chondrocytes with spindle-shaped nuclei were evident in regions A and D, whereas oval-shaped chondrocytes and associated nuclei were predominant in regions B and C. Isogenous groups of chondrocytes were apparent in the deep and middle zones. Some isogenous groups were observed in the superficial zone, but they were accompanied by increased isogenous groups in the deeper zones. Isogenous groups of chondrocytes in the deeper zones were arranged in columns perpendicular to the articular surface in regions A and D, whereas in regions B and C, they were arranged in round nests. In group-2a ponies, loss of basophilia and increased acidophilia were evident throughout the noncalcified cartilage, although variable degrees of basophilia remaining in the deep zone of regions B and C. Nuclei of chondrocytes in these regions were weakly basophilic. In the superficial zone of regions A and D, chondrocytes and their nuclei were predominantly oval-shaped, rather than spindle-shaped as in group-1 ponies. Most chondrocytes and associated nuclei in the superficial zone of regions B and C were round (Fig 6A) rather than oval. Isogenous groups of chondrocytes were similar to those observed in group-1 ponies; however, chondrocytic clones were present in the superficial zone of some specimens without concomitant cloning in the deeper zones. The appearance in group-2b ponies was similar; however, there was less chondrocytic hypertrophy and less loss of basophilia in the superficial zone in group-2b ponies.

Toluidine blue—The intensity of metachromatic staining was somewhat variable in group-1 ponies. Metachromasia was generally strongest in the deeper zones of the noncalcified cartilage, particularly in regions B and C (Fig 5B). The territorial matrix had more intense metachromatic staining than did the interterritorial matrix. Group-2a ponies had significant loss of metachromasia throughout the noncalcified cartilage (Fig 6B). The loss of metachromasia was greater in group-2a than in group-2b ponies. The loss of metachromatic staining was related to the increased immunolocalization of cytokines.

Interleukin 1β—Mild immunolocalization of IL-1β was observed in chondrocytes in the superficial and outer middle zones of the noncalcified cartilage in group-1 ponies (Fig 5C). Topographic heterogeneity among regions A, B, C, and D was evident with greater intensity of chondrocytic immunolocalization in regions B and C, followed by regions D, then A. Immunolocalization for IL-1β was found in chondrocytes, with greater intensity in regions B and C than in regions A and D. Immunolocalization for IL-1β of slight to mild intensity was observed in chondrocytes in the deep zone of regions B and C in 3 of 6 ponies. Immunolocalization in chondrocytes of the calcified layer was mild to moderate in intensity and was more consistent between regions than that in the noncalcified cartilage. Immunolocalization of IL-1β was slight to mild in intensity in the matrix of the noncalcified cartilage in most ponies. Matrical staining was confined to a narrow band through the middle zone and tended to be confined to regions B and C, although it was occasionally observed in regions A and D. Immunolocalization was not observed in the matrix of the deep or superficial zones. In the calcified cartilage, mild to moderate matrical immunolocalization was present in the territorial matrix around some chondrocytes, but this did not expand to form bands of immunoreactivity observed in the middle zone of the noncalcified cartilage.

Chondrocytes in group-2a ponies had heterogeneous patterns of intensity and distribution, compared with those in group-1 ponies. Heterogeneity of immunolocalization patterns between adjacent zones enabled differentiation of the zones. There were similarities between the radial and intermediate facets from the same C3. Heterogeneity was also evident among topographic regions A, B, C, and D. In the same specimen, interspersed in the same zone and region, or in a different zone but similar region, there was a tendency for contrasting intensities of cell staining (negative to strong). The intensity of immunoreactivity in chondrocytes was weaker (negative to mild) in areas of greater (mild to strong) matrical immunolocalization, whereas more immunoreactive chondrocytes (mild to moderate) were surrounded by weaker matrical localization (negative to slight). The intense immunoreactivity appeared in the territorial matrix and beyond, or alternatively, as a dark border to the chondrocyte, presumably in the pericellular matrix. The latter gave chondrocytes the appearance of nonimmunoreactive “ghosts,” halos by strong matrical immunolocalization. The calcified cartilage immunoreactivity was similar to that in group-1 control ponies, with no obvious differences.

Matrical immunolocalization of IL-1β was evident in all specimens from group-2a ponies. In region A, immunolocalization of slight to mild intensity was evident in the middle and superficial zones. In regions B and C, the intensity of immunolocalization in the matrix varied from mild to moderate and was concentrated principally in the middle zone. Immunolocalization in the matrix of the deep and superficial zones was obvious in the most immunoreactive specimens (3 of 6 ponies) in regions B and C, particularly in the pericellular and territorial matrix (Fig 6C). The pattern of chondrocytic and matrical immunolocalization in group-2b ponies was similar to that in group-2a ponies except that, conversely, the intensity of chondrocytic immunoreactivity was greater and matrical immunoreactivity was weaker (ie, greater chondrocyte-to-matrix ratio) and less widespread than that in group-2a ponies. Overall, groups 2a and 2b were distinguishable from each other and from group 1 on the basis of intensity of chondrocytic and matrical immunolocalization (compare Fig 5C controls and 6C LPS treated).

Tumor necrosis factor-α—In group-1 ponies, TNF-α had zonal and regional patterns of chondrocytic immunolocalization similar to those described for IL-1β (compare Fig 5C and D). Matrical immunolocalization of TNF-α was concentrated in the middle zone, but the intensity of TNF-α was slight to mild, whereas that
of IL-1β was mild to moderate. The chondrocyte-to-matrix immunoreactivity ratio was greater for TNF-α than for IL-1β.

Immunolocalization distribution of TNF-α in chondrocytes of group-2a ponies was similar to that of IL-1β; however, there was a tendency for TNF-α to be more intense (compare Fig 6C and D). Matrical immunolocalization of TNF-α was similar to that of IL-1β, although TNF-α was less widespread in the matrix. The intensity of TNF-α matrical stain tended to be lighter than that of IL-1β, except in the most immunoreactive specimens. The chondrocyte-to-matrix immunoreactivity ratio was less for TNF-α in group-2a, compared with group-1 ponies. However, relative to IL-1β, it had a greater chondrocyte-to-matrix ratio.

Chondrocytic and matrical patterns of immunolocalization in group-2b ponies were similar to those of group-2a ponies; however, similar to that for IL-1β, the chondrocytes stained more strongly whereas the matrix stained with less intensity and over less area in group-2b ponies.

Tumor necrosis factor (P55 and P75) receptors—The pattern of chondrocytic immunoreactivity in group-1 ponies was similar, but of lighter intensity, relative to TNF-α (compare Fig 5E and D). Matrical immunolocalization for TNF receptors was negative in group-1, but present in group-2, ponies. Receptor immunoreactivity in the matrix in group-2a ponies (slight to mild) was greater than that in group-2b ponies (negative to slight). Immunolocalization of both
Figure 6—Photomicrograph of a section of articular cartilage from region B of the intermediate facet of the third carpal bone from the lipopolysaccharide-treated midcarpal joint showing the superficial (a), middle (b), and deep (c) zones of the noncalcified cartilage. A—Chondrocyte hypertrophy and cloning in the superficial zone of the noncalcified cartilage. Compare with Figure 5A; H&E stain. B—Loss of metachromasia throughout the noncalcified cartilage with weakest stain closest to the chondrocyte. Compare with Figure 5B; toluidine blue stain. C—Enhanced immunoreactivity of IL-1β in the matrix of the noncalcified cartilage with diffuse reactivity in the middle zone and reactivity in the matrix of the deep and superficial zones close to the chondrocytes. Compare with Figure 5C; anti-IL-1β stain. D—Tumor necrosis factor-α showing a similar pattern of enhanced immunoreactivity in the matrix but with a stronger cell-to-matrix ratio than that of IL-1β. Compare with Figure 5D; anti-TNF-α stain. E—Tumor necrosis factor receptor, P55-R, showing enhanced matrical stain with greatest intensity adjacent to the chondrocytes in the deep zone. Compare with Figure 5E; anti-P55-R stain. F—Enhanced cellular and matrical expression of epitope 3-B-3(-) with the most diffuse matrical expression in the middle zone, similar to that of the cytokines. Compare with Figure 5F. Bar = 79 μm for all.

TNF receptors was more widespread in the matrix than was TNF-α in groups 2a and 2b (compare Fig 6D and E). The P55 receptor stained with greater immunoreactivity than the P75 receptor in the chondrocytes and matrix.

The 3-B-3(-) epitope—in group-1 ponies, chondrocytic immunolocalization of the 3-B-3(-) epitope was slight to mild in the superficial and middle zones in regions A and D, whereas in regions B and C, it was negative to slight (Fig 5F). Chondrocytic immunoreactivity was apparent in areas of weak metachromatic staining, but it was poor or absent in areas of strong metachromasia. Matrical immunolocalization of the 3-B-3(-) epitope was not seen in group-1 ponies.

Chondrocytic immunolocalization of 3-B-3(-) epitope in group-2a ponies (Fig 6F) was noticeably greater than that in group-1 ponies (Fig 5F) and had distribution which was similar to that of the cytokine immunostaining. Thus, less intense cytokine immunoreactivity paralleled less intense 3-B-3(-) epitope chondrocytic localization, and areas of strong cytokine immunoreactivity had correspondingly strong 3-B-3(-) epitope localization. In areas where cytokine immunolocalization was present in the matrix of the deep zone, immunoreactivity of 3-B-3(-) epitope in the...
chondrocytes was mild. Matrical immunolocalization of 3-B-3(−) epitope was evident in the middle zone in all group-2a (slight to mild) and most group-2b (negative to slight) specimens. For group-2a specimens with the greatest immunoreactivity for cytokines, matrical immunolocalization of 3-B-3(−) epitope paralleled metachromasia in the middle zone that was unusually strong, compared with that in the superficial and deep zones of these same specimens and with the less immunoreactive group-2a and group-2b specimens in this locale. Areas of chondrocyte hypertrophy in the superficial zone (Fig 6A) that coincided with the greatest cytokine immunoreactivity had the greatest immunolocalization for 3-B-3(−) epitope.

Biochemical analysis—Extracted proteoglycans were analyzed by separation of composite agarose-polyacrylamide gel electrophoresis followed by either toluidine blue staining or western blotting and immunohistochemical staining. Toluidine blue staining revealed migration of the major proteoglycans of the equine articular cartilage extracts (Fig 7A). The 3-B-3(+) staining correlated with that of toluidine blue for the proteoglycans (Fig 7B). The staining with toluidine blue and immunoblotting with 3-B-3(+) indicated that the proteoglycan extracts had been loaded equally, in correlation with the safranin O assay values used. These steps were performed to ensure that equal amounts of proteoglycan extracts were loaded from group-1 and group-2 specimens to ensure no bias in the 3-B-3(−) immunostaining results. In group-1 specimens, 3-B-3(−) was barely detectable (Fig 7C). In contrast, 3-B-3(−) staining was much stronger in the cartilage extracts from joints injected with LPS (group 2a) and weakly detectable in the group-2b specimens. These results correlated with the in situ immunolocalization of 3-B-3(−) epitope.

Discussion

Intra-articular injection of multiple doses of endotoxin into the midcarpal joint of ponies induced synovitis. Results of synovial fluid cytologic examination confirmed infiltration of inflammatory cells into both midcarpal joints of group-2 ponies, with LPS (group 2a) inducing a significantly greater inflammatory response than did morphine/saline solution alone (group 2b). Enhanced immunoreactivity of cytokines and receptors in the synovial lining and subintimal layers of inflamed synovial membrane (group 2a more than 2b), compared with normal synovial membrane (group 1), supports the potential role of cytokines in the pathogenesis of equine synovitis-induced cartilage changes. Cells in the synovial lining layer that were immunoreactive for IL-1β and TNF-α may be type-A/macrophase-like synoviocytes and/or type-B/fibroblastic synoviocytes. Further investigations are required to identify which synoviocytes are involved. The greater immunoreactivity of TNF-α in the subintimal cells and vascular tissue, compared with the synovial lining cells, may reflect its involvement in the subintimal inflammatory response, whereas IL-1β may be more involved in the response of synovial lining cells. Immunolocalization distribution of TNF receptors was similar to that of TNF-α, but was of lighter intensity. These findings agreed with immunohistochemical studies of synovial membrane from human beings with RA involvement where TNF-α and TNF receptors were principally immunolocalized in macrophages, and to a lesser extent, in fibroblasts. In people with RA, TNF-α and its receptors are concentrated at the cartilage-pannus junction, the major site of cartilage destruction. Up to 90% of endothelial cells in RA-affected synovial membrane and pannus tissue express TNF receptors.

Figure 7—Histochemical and immunohistochemical evaluation of major proteoglycans of equine articular cartilage extracted from the third carpal bone (C3) of normal (group 1) and inflamed (group 2a and 2b) midcarpal joints by use of composite agarose-polyacrylamide gel electrophoresis. Steps depicted in 7A and B were to confirm equal loading of proteoglycan extracts from groups 1 and 2 to avoid bias in the immunostaining of 3-B-3(−) epitope. 1 = both C3 from group-1 specimens; 2a = group-2a specimens; and 2b = group-2b specimens. A—Toluidine blue staining revealed the migration of the major proteoglycans and equal loading of extracts. B—Epitope 3-B-3(+) (ie, with predigestion with hyaluronidase) staining confirmed the specificity of toluidine blue for the proteoglycans and equal loading of extracts. C—In group-1 specimens, 3-B-3(−) epitope (ie, without predigestion) was barely detectable. Staining of the 3-B-3(−) epitope was slightly stronger in group-2b joints, but was more obvious in group-2a joints. These results correlate with the in situ immunolocalization of 3-B-3(−) epitope.
Increased matrixal and chondrocytic expression of IL-1β, TNF-α and its receptors P55 and P75, and 3-B-3(-) epitope, as well as chondrocyte hypertrophy (H&E stain) and loss of proteoglycans, as depicted by metachromatic staining, confirm that changes in the articular cartilage accompanied the synovitis. Collectively, these changes imply that synovial inflammation effected increased immunoreactivity of the cytokines and loss of metachromasia in the cartilage in this model. The exact mechanism(s) by which this happens remains to be determined.5,4,5,4,5,6 Equine neutrophils and synoviocytes have been found to produce collagenase and stromelysin when stimulated with equine IL-1 in vitro.33 Speirs et al24 found that activity of these proteolytic enzymes was high in synovial fluid of septic equine joints in conjunction with increased nucleated cell counts; however, in some horses, such correlation was not found because collagenase activity remained high despite cell counts returning to normal. In dogs with OA, no correlation of collagenolytic activity of the cartilage and that in the synovium was found; however, correlation between cartilage collagenolytic activity and the degree of synovial inflammation was observed.56 These studies support the hypothesis initially described by Fell and Jubb15 and Dingle et al16 that stimulatory factors, such as cytokines released from the synovium, may diffuse into the articular cartilage and mediate matrix degradation, rather than being a direct effect of synovial derived proteolytic enzyme activity.56

Synovitis, increased cytokine immunoreactivity, and loss of metachromasia in articular cartilage was evident in the morphine/saline-treated joints (group 2b). Proteoglycan loss in the contralateral saline-injected joint of rats26 and rabbits57 with LPS- and IL-1-induced unilateral synovitis has been reported.36,57 The proteoglycan loss was not considered to be a direct effect of the saline solutes alone, because intra-articular injections of saline solutions in control animals had no effect on proteoglycan content.26,56 Instead, a systemic action of LPS, TNF, or IL-1 was advocated. In this study, cells capable of producing cytokines may have been recruited and traveled via the circulation to the mildly inflamed saline/morphine-injected contralateral joint.5,37 An effect of morphine on cartilage metabolism is also possible. Hendrickson and Broadstone reported intra-articular administration of morphine in ponies had no effect on synovial fluid total protein concentration and WBC counts or on cartilage glycosaminoglycan or chondroitin sulfate contents. However, only a single injection and a much smaller dose were used in that study. Opioids may interact with cytokines and have been documented to regulate the production of IL-1 and IL-2.56 Endogenous opioids have been documented to augment IL-1 production by bone-marrow macrophages stimulated by LPS.59 Other factors that might have influenced the response of the articular cartilage include overse of the contralateral limb.56

The extracellular matrix of the middle zone, compared with other zones of the articular cartilage, particularly regions B and C, was most immunoreactive for IL-1β and TNF-α in normal and inflamed joints. This pattern of immunoreactivity may be a reflection of different metabolic and functional requirements of chondrocytes within different zones and topographic regions of the noncalcified cartilage.34,59,63 Chondrocytes of the middle and deep zones have more rough endoplasmic reticulum and better developed Golgi bodies than do superficial chondrocytes, and, as a result, are more active in protein synthesis.64 In weight-bearing areas, and particularly in the middle zone, chondrocytes may have a higher metabolic rate and greater surrounding concentration of proteoglycans (which contribute to matrix stiffness), compared with chondrocytes in less weight-bearing areas. This may be an adaptation to increased functional requirements.59,63 Such mechanism could explain the greater metachromasia, and therefore, proteoglycan content that was observed in regions B and C and with increasing depth in the noncalcified cartilage during this study.

Heterogeneity in immunolocalization of cytokines among neighboring chondrocytes in the treated specimens (group 2) was characterized by intensely immunoreactive chondrocytes adjacent to poorly immunoreactive chondrocytes. The ring of immunoreactivity in the territorial matrix around poorly immunoreactive chondrocytes gave the impression they had secreted their cytokines into the matrix. A similar pattern was reported in cartilage from dogs with OA. Also, after exposure of porcine articular chondrocytes to IL-1 in vitro, IL-1 was immunolocalized in chondrocytes of all zones, but only a fraction of chondrocytes were IL-1 positive at any time. This indicates either variable responsiveness to IL-1, or that responses were transient.32 Further, the metabolic responsiveness of cultured chondrocytes to IL-1 varies with depth in the cartilage.65,66 This may be attributable to receptor density differences, differences in capacity to synthesize IL-1, or variations in exposure to IL-1 owing to differences in the surrounding extracellular matrix.65 Variation in the density of the LPS CD14 receptors on chondrocytes may have contributed to the heterogeneity of immunostaining; however, analysis of these receptors was not done.

Immunolocalization of cytokines in chondrocytes and the matrix of normal articular cartilage may be related to their potential role in normal matrix turnover.1,5,10 Control of the catabolic effects of chondrocytes is likely balanced by numerous regulators, including growth factors (ie, insulin-like growth factor 1 and transforming growth factor B), and potential inhibitors of TNF and IL-1 (ie, α1 macroglobulin, IL-4, IL-6, and IL-10) and proteolytic enzyme inhibitors.5,7,13,14,67 In this study, the greater immunoreactivity of cytokines in the articular cartilage from inflamed joints and associated loss of metachromasia may reflect an imbalance between cytokines and their inhibitors. The resultant matrix degradation possibly reflects an early lesion of OA. This is supported by greater matrical immunoreactivity of cytokines in LPS-treated joints (group 2a), relative to joints receiving morphine/saline solution alone (group 2b), accompanying a greater loss of proteoglycans (metachromasia) in the LPS-treated ponies.

Tumor necrosis factor receptors had a similar distribution of immunoreactivity to TNF-α in chondrocytes of the noncalcified cartilage of controls (group 1), but matrical activity was not observed. However, in articular cartilage from inflamed joints (group 2), TNF receptors were expressed diffusely in the matrix of the superficial and middle zones, as well as the deep
zone in intensely immunoreactive specimens. Receptor expression followed the distribution of TNF-α in the LPS-treated cartilage, but spread further into the interterritorial matrix. This pattern of matrificial TNF receptor immunolocalization was similar to that detected in articular cartilage from dogs with experimentally induced OA. The matrical location possibly reflects shed (soluble) cell-surface receptors. Soluble TNF receptors have been purified from synovial fluid of RA patients with a correlation between the amount of soluble TNF receptor and severity of disease. Soluble TNF receptors bind directly to TNF-α forming soluble complexes. Thus, they may have a dual function where, at low amounts, they may potentiate TNF availability by stabilizing the TNF molecule, whereas at higher amounts, TNF-α may be neutralized.

Chondrocyte hypertrophy, which is an early change in OA, was evident in the treated specimens, especially in the LPS-treated joint (group 2a). Chondrocytes in the superficial zone have classically been described as spindle shaped, which was apparent in regions A and D in normal cartilage (group 1). However, more oval-shaped cells were apparent in regions B and C of normal cartilage in many specimens, which may be a function of weightbearing. Paler staining nuclei of chondrocytes in the LPS-treated specimens was suggestive of abundant euchromatin and reversion to an immature chondroblastic type of cell.

Chondrocyte hypertrophy in OA is not only associated with enlargement of the cell, but also with increased production of proteolytic enzymes, synthesis of unique gene products, including type-X collagen, and abnormal sulfation patterns of glycosaminoglycans, such as 3-B-3(-) epitope of chondroitin-6-sulfate. In this study, immunoreactivity for 3-B-3(-) epitope was strongest in group-2a ponies, less strong in group-2b ponies, and weakly reactive in group-1 ponies, in the in situ articular cartilage specimens and in proteoglycan extracts from C3. Although no immunoreactivity for 3-B-3(-) epitope was present in the matrix of normal specimens, mild immunolocalization was observed in chondrocytes in the superficial and outer middle zones of regions A and D of normal noncalcified cartilage of group-I ponies, in which metachromatic staining was weak. In contrast, immunoreactivity was absent or weak in the chondrocytes in regions B and C where metachromasia was strong. The relevance of this relation is unclear. Expression of 3-B-3(-) epitope in the normal growth plate in the zone of terminal hypertrophying chondrocytes occurs during normal growth and development. Immunolocalization of 3-B-3(-) epitope in normal human articular cartilage samples was weakly evident in adolescents (10 to 20 years old), making it indistinguishable from juvenile RA cartilage of adolescents. Presence of 3-B-3(-) epitope in normal cultured bovine explants was believed to be attributable to increased metabolic rate in the deep layers or degradation of proteoglycans during normal cartilage turnover. Thus, recognition of 3-B-3(-) epitope in chondrocytes from normal articular cartilage and in normal equine proteoglycan extracts may reflect immaturity of the individual, normal proteoglycan turnover, or some pathologic process. The latter seems unlikely because gross and microscopic examinations of articular cartilage from control ponies did not reveal any abnormalities.

Evidence of chondrocyte hypertrophy in this study (H&E staining) is supported by the belief that expression of the 3-B-3(-) epitope in cartilage from animals with OA reflects a change in chondrocyte phenotype, reverting to its embryonic origins. These hypertrophied chondrocytes produce glycosaminoglycans (found normally in the active growth plate) in an attempt at replacement of the proteoglycans in the matrix. Mankin and Lipiello first suggested that the increased C-4-S-to-C-6-S ratio observed in articular cartilage from human beings with OA was a reflection of altered chondrocyte phenotype to that of an immature chondroblast. In support of this, Platt and Bayliss observed that IL-1 in equine articular cartilage cultures induced greater suppression of sulfation of the N-acetylgalactosamine residue of the C-6-S loci relative to the C-4-S loci during posttranslational glycosylation, causing an alteration in the sulfation pattern of newly synthesized proteoglycans. Enhanced chondrocytic proteoglycan synthesis in areas of altered mechanical loading in cartilage from dogs with OA and in bovine cartilage undergoing mechanical stress appear to be part of the attempted repair of the extracellular matrix by the chondrocyte. Thus, expression of 3-B-3(-) epitope may reflect a specific anabolic state of activated chondrocytes and a repair process that, some believe, will probably fail.

In previous studies, the 3-B-3(-) epitope was expressed either before or with loss of metachromasia (as in most group-2 specimens). In contrast, in 3 of 6 ponies in group 2a, matrical expression of 3-B-3(-) epitope in the middle zone corresponded to intense metachromasia, compared with the superficial and deep zones in these same specimens and with the middle zone of other group-2 specimens. These specimens were also the most immunoreactive for IL-1β and TNF-α activities. This direct association of 3-B-3(-) epitope with increased proteoglycans in this study may represent a change in chondrocyte function to a heightened metabolic state during the early pathogenesis of OA.

To our knowledge, the relation of cytokines and 3-B-3(-) epitope, as depicted by immunolocalization, has not been reported in any species. In our study, cytokine and 3-B-3(-) epitope immunoreactivity followed similar patterns in the LPS-treated specimens. Although a cause-and-effect relation has not been established, cytokines may have a role in chondrocyte hypertrophy. Interleukin 1 has been immunolocalized in human hypertrophic chondrocytes in the cartilaginous epiphysis and active growth plate in the hypertrophic and calcified zones. Visco et al suggested a relation between chondrocyte hypertrophy and growth factors with similar patterns of immunoreactivity of 3-B-3(-) epitope in cartilage from dogs with OA, and type-X collagen and insulin-like growth factor-1 in articular cartilage from human beings with OA. This gives credence to the physiologic significance of the expression of 3-B-3(-) and type-X collagen as a reflection of a phenotypic change in chondrocytes to that of chondrocytes with an increased metabolic state.

In conclusion, intra-articular administration of LPS may be used to induce a synovial environment conducive to increased immunoreactivity of IL-1β.
INF-α, and its receptors in equine synovial membrane and articular cartilage. These cytokines may be involved in the early phenotypic change of chondrocytes that is believed to occur in OA and is characterized in this study by enhanced 3-β-3(-) epitope immunoreactivity and chondrocyte hypertrophy. Further chronicologic studies, using a similar model to the point of gross OA cartilage changes, may provide a better understanding of physiologic alterations in equine articular cartilage that are induced by synovitis and progress to OA. Such model may be feasible for controlled in vivo evaluation of antiarthritic agents, including those aimed at modulating the actions of cytokines and growth factors.

References


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