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ADVANCED TECHNIQUES IN THE DIAGNOSIS OF BONE DISEASE

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Introduction

It is now recognized that early disease in the subchondral bone (including microdamage, microcracks, apoptosis and inappropriate remodeling) all contribute to the development of osteochondral fractures. An ability to recognize this change early could potentially prevent many osteochondral fractures, as well as more severe fractures (including catastrophic ones). It is also recognized that stress fractures are commonly the result of bone remodeling. All of the initiators to bone remodeling are still not clarified, but it is recognized that stress fractures in the rehabilitation phase of horses coming back after surgery or lay-up can lead to catastrophic fractures requiring euthanasia. Early diagnosis of bone disease is key to the prevention of such injuries. It is also important to recognize that adaptation and pathologic change are very similar processes. Differentiation of these changes is also a key to early diagnosis.

The purpose of this paper is to outline some of these newer techniques, and compare what they can do to what is currently available.

Radiography and Computed Radiography

Radiography has been the standard clinical technique the veterinarian uses to diagnose fractures and obvious bone disease. The advent of computed radiography has improved the definition of the bone in many of these conditions. However, there are still major limitations to radiography, in that we need 30-50% loss in bone density for it to be apparent on a radiograph and do not get the best definition of early subchondral bone disease. We do not get definition of articular cartilage disease or definition of disease of other soft tissue such as ligaments and menisci.

Nuclear Imaging

There will be increased uptake of technetium in areas of early subchondral bone disease (Kawcak et al 2000). It is therefore useful as a sensitive diagnostic technique for early bone disease, but is relatively non-specific. It is also often unrewarding

for chronic problems. Nuclear scintigraphy has been extremely useful for the early recognition of stress fractures, and this has definitely decreased the number of catastrophic injuries resulting from stress fractures in athletes.

In southern California, the modality is available at the racetracks. If a horse has a hind-limb lameness that cannot be localized lower down, a nuclear scan is done, and typically will reveal a local area of uptake, representing a stress fracture. In many instances, this fracture cannot be defined with radiography. At the moment, nuclear scintigraphy is the gold standard for diagnosing stress fractures (it is now recognized that stress fractures lead to more severe fractures, so this serves as a huge prevention for catastrophic injury). The typical locations for these stress fractures are in the tibia, femur, pelvis, and humerus (in the forelimb).

Computed Tomography (CAT Scan/CT)

We have used this modality as a resource tool for a number of years, as has the Equine Research Group at Massey University (Prof. Elwyn Firth). In the work done by Dr. Chris Kawcak in our laboratory, we were able to show the development of subchondral bone sclerosis, as well as later development of subchondral bone necrosis using quantitated CT (osteoabsorptiometry). Prof. Firth's group similarly documented changes in the bone with exercise using a peripheral (portable) qCT unit and lowered bone mineral density (BMD) with OCD (Firth et al., 1999). A portable CT that can be used in the standing horse has recently been developed and is currently undergoing investigation. This could offer a practical means of monitoring bone density (or changes in bone density) with exercise in a clinical horse population. The use of CT, combined with modeling, is now a useful surgical tool for diagnosing fractures in more than one direction.

MRI

Most MRI usage, up until now, has been limited to the distal limb. However, the technology is becoming available to do limbs at least up to the carpus and tarsus. MRI will be extremely valuable for documentation of subtle changes in the bone, as well as other soft tissues of joints. There is a standing MRI (Hallmarq) that is being marketed from England. It will provide reasonable images of the distal phalanges and can image hind fetlocks (but not front fetlocks). At this stage, we are investing in an extremity MRI scanner (ONI) that will require anesthesia, but has the power to provide excellent images.

Optical Coherence Tomography

This is useful for evaluation of articular cartilage in vivo. It is currently being used as a research technique, but could be adapted in the future.

Fourier Transformed Infrared Imaging (FT-IR)

This is used on sections of bone. Infrared irradiation is directed toward a sample, and some passes through. The resulting spectrum represents the molecular absorption and transmission and an absorption spectrum is unique for each molecular structure. It has been used by the Utrecht group for examination of articular cartilage and evaluation of proteoglycan content.

Diagnostic Arthroscopy

The use of diagnostic arthroscopy has always been the gold standard technique for diagnosing and defining articular cartilage damage. However, it also needs to be recognized that it has been extremely useful in diagnosing and defining subchondral bone disease. Because of the focal nature of subchondral bone disease in some locations, specific localization of this problem is often only possible with the arthroscope. Scintigraphy can lead to the affected joint, but is not specific in location within that joint. CT can help define many of these when it is a practically available technique. Obviously, diagnostic arthroscopy requires anesthetic and is a minimally invasive procedure. At the present time, it is an important part of our armamentarium, however. The other advantage with arthroscopy is that, at the same time, we can commonly treat these subchondral lesions.

Synovial Fluid and Serum Biomarkers

The development of synovial fluid and serum biomarkers offers great potential for the early diagnosis of articular cartilage, bone, and tendon disease. Considerable work has been done with these biomarkers in various diseases and will be detailed below.

Principle of Biomarkers

The term "biomarker," "biochemical marker," and "molecular marker" have all been used to describe either direct or indirect indicators of abnormal skeletal tissue turnover. These markers are generally molecules that are the normal products and by-products of the metabolic processes occurring within the skeleton. Alterations occur in the balance between the anabolic and catabolic processes in disease, and, therefore, the concentrations of biomarkers may either increase or decrease. In joint disease, such molecules typically appear in the synovial fluid of affected joints. If the underlying subchondral bone is involved, the molecules of osseous origin will usually be cleared directly into the bloodstream.

Biomarkers can be used in one of the following ways:

1. As a diagnostic test to differentiate between affected and non-affected joints/animals.
2. As a prognostic test to identify joints/animals likely to show rapid progression or to predict response to therapy.
3. As an evaluative test to assess the severity, monitor change in disease status, or monitor response to therapy.

Cartilage Degradation Markers

AGGRECAN ASSAYS

Sulfated glycosaminoglycan (sGAG)—this is typically measured with a biochemical assay that uses the dimetylmethylene blue (DMMB). This assay identifies all sGAGs present in synovial fluid, regardless of their origin.

Chondroitin sulfate (CS)—by-products of aggrecan degradation have been measured, including proteoglycan fragments that contain "epitopes" that allow for their immunologic detection with antibodies. Antibodies have been developed to recognize both native epitopes in chondroitin sulfate and neoepitopes (new epitopes) created by the digestion of CS with enzymes. There has been little work done with these in horses.

Keratan sulfate (KS)—conflicting reports have been published concerning the relationship of KS in equine body fluids and joint disease. In a study in our laboratory, it was not useful.

Aggrecan core protein—antibodies have been developed against native epitopes in the protein core of aggrecan and against neoepitopes created by the digestion of the aggrecan core matrix, metalloproteinases (MMPs), and aggrecanases. Both of these enzymes are involved in the metabolic turnover of aggrecan in health and disease.

COLLAGEN ASSAYS

Cleaved Type II Collagen—initial degradation of fibrillar collagens occurs as a result of the action of collagenases. This digestion results in two collagen fragments of ¾ and ¼ length with newly created ends at the cleavage site. The COL2-¾Cshort antibody recognizes collagenase-cleaved fragments of both Type I and Type II collagen so is not specific to cartilage collagen. However, an antibody named 2¾CEQ has been developed in our laboratory that is specific for the collagenase-cleaved ¾ fragments of Type II collagen of the horse, and preliminary studies suggest that it may prove useful in assaying equine body fluids for abnormalities in Type II collagen turnover, as may occur in osteochondrosis.

Collagen cross-links—although mature collagen molecules possess cross-links that provide cohesiveness and stability to the collagenous framework, with collagen

degradation, these cross-links are released from the tissue. Although pyridinolone (PYD) cross-links predominate in cartilage, because they are major cross-links in all connective tissues, they do not provide specificity as a cartilage degradation marker. Another pyridinium crosslink called dioxypyridinolone (DPYD) is found in small amounts in mineralized tissue and will be described below as a marker of bone collagen degradation.

Cartilage Synthesis Markers

COLLAGEN ASSAYS

Type II procollagen peptide—Type II collagen is secreted by chondrocytes as individual procollagen chains that are further processed after triple helix formation by the enzymatic cleavage of the propeptides of both ends of the procollagen chain. It has been shown that the rate of release of the propeptide at the carboxi-terminus, the C-propeptide, is proportional to the rate of Type II collagen synthesis. We have seen increased levels of this C-propeptide in both synovial fluid and serum of horses with osteochondral fragmentation and also in the synovial fluids and sera of horses with osteochondrosis. We also showed a direct relationship between the levels of CPII and the severity of disease.

AGGRECAN ASSAYS

Chondroitin sulfate—large, newly synthesized aggrecan molecules have an epitope called 846 that can be measured to monitor aggrecan synthesis. This epitope progressively disappears from cartilage with aging, but reappears in joints with osteoarthritis. In horses with osteochondral fragmentation, significantly higher synovial fluid and serum levels of the 846 epitope were found compared with control horses.

Bone Degradation Markers

Bone markers have been found useful in diagnosis of osteochondrosis, as well as predicting progression and monitoring response to treatment. Bone markers may also be important in assessing bone remodeling in training, identifying abnormalities in the bones of exercising horses before they progress into potentially serious injuries, such as fractures.

COLLAGEN ASSAYS

Cleaved Type II Collagen—the COL2¾Cshort antibody recognizes collagenase-cleaved fragments of both Type I and Type II collagen, so it is not specific to bone collagen, but when this antibody is used in combination with the previously

described 2¾CEQ antibody that is specific for equine Type II collagen, a clear picture emerges in the relative breakdown of Type I collagen. Its use in equine studies has been restricted to measurement of serum levels in foals predisposed to osteochondrosis, where significantly increased levels of Type I collagen and less Type II collagen turnover during the first five months of life in those foals occurred when they had more severe or more numerous OCD lesions.

Collagen cross-links—the DPYD cross-links are almost exclusively found in bone, and may therefore be the best cross-link to monitor bone turnover. They have not been very rewarding in the horse. Another product of Type I collagen degradation are the cross-linked ends or "telopeptides" that can be measured in serum by using commercially available immunoassays. An assay for the carboxy-terminal link telopeptide of Type I collagen (ICTP) has been used in equine studies to show that serum ICTP levels decrease with age and differ between breeds. Increased levels have been reported in horses with DOD compared with age-matched controls.

Another cross-link assay that can be used in horses is the C-telopeptide cross-link (CTx) assay. Unlike most other bone markers, serum CTx levels appear to increase in foals with increasing age. More studies that assay CTx levels need to be performed in the horse to determine its value as a biomarker of joint disease.

Non-collagenous protein assays—potential non-collagenous protein markers of bone degradation exist, including bone sialoprotein (BSP) and tartrate-resistant acid phosophotase (TRAP), but no assays are currently available for their detection in the horse.

Bone Synthesis Markers

COLLAGEN ASSAYS

Type I procollagen propeptide—as with Type II collagen, Type I procollagen of bone also possesses propeptides that are cleaved and released from the collagen molecules upon fibril formation. Assays exist for both the N-terminal (PINP) and C-terminal (PICP) propeptides, but only the latter is currently applicable to horses. Serum PICP levels decrease with age and increase with exercise. Reduced serum levels were detected in horses with DOD compared with age-matched controls.

NON-COLLAGENOUS PROTEIN ASSAYS

Bone-specific alkaline phosphatase—as with PICP, an inverse relationship exists between age and the serum levels of bone-specific alkaline phosophotase (BALP) in the horse, and serum levels increase with exercise. Higher levels have been reported in clinically affected OA joints than the contralateral control joints with a strong positive correlation between BALP levels and the degree of articular cartilage damage.

Osteocalcin—osteocalcin is also produced by the osteoblasts and is an accepted marker of bone formation. Preliminary studies in osteochondrosis and osteochondral fragmentation suggest limited value in these conditions.

Use of Markers to Aid in Diagnosis of Early Joint Disease

CS 846 and CP-II were shown to be of use in diagnosing osteochondral damage in the horse (Frisbie et al., AJVR 1999). Synovial fluid and serum concentrations were evaluated in 38 horses with unilateral carpal osteochondral fragmentation, compared to 25 unaffected joints and also fluids from normal control horses. Synovial fluid marker changes with OCF included the total protein being significantly higher, the CS-846 being significantly higher, CP-II not being significantly higher (p=.06), keratan sulfate being not significantly higher (p=.28), and white blood cell count being not significantly higher. There was a significant linear increase with grade of fragmentation in both total protein and CS-846 and no significant increase in CP-II or KS. With serum markers, CS-846 and CP-II were both significantly higher, but KS was still not significantly higher. It was concluded that CS-846 and CP-II were useful markers in synovial fluid and serum, and that using these markers predicted the presence or absence of OCF in 80% of cases.

ALTERATIONS IN SERUM BIOMARKERS IN EQUINE OSTEOCHONDROSIS

Looking at osteochondrosis foals from zero to five months compared with foals with low scores, they have high levels of CP-II, high levels of COL2¾Cshort and lower levels of 2¾CEQ (Billinghurst et al., AJVR 2001).

Use of Markers in In-Vitro Studies

We have used markers to monitor degradation in IL-1/cartilage explant systems (Billinghurst et al., 1999). Articular cartilage degradation was inhibited by an MMP-inhibitor. A concentration of 100nM decreased collagenase-cleaved Type 2 collagen fragment generation and release, decreased proteoglycan release, and increased proteoglycan synthesis and DNA content.

Use of Markers to Distinguish Effects of Exercise versus Pathologic Change During Exercise

In this study, a number of synovial fluid and serum markers were assessed in a group of horses exercised on a treadmill, compared to a group of horses with osteoarthritis exercised on a treadmill. Levels of bone markers were higher in serum than synovial

fluid samples, whereas the level of articular cartilage markers was higher in synovial fluid than in serum. In osteoarthritis, cartilage metabolism preceded bone metabolism based on biomarker results. There was significant correlation between articular cartilage bone markers in the clinical examination, as well as gross and histologic changes of articular cartilage. The level of biomarkers was higher as a result of OA compared to exercise. This increase was seen in all bone markers except serum CTx-I and 2¾CEQ (Al-Soyabil F. Ph.D. Dissertation, Colorado State University, 2002).

Use of Markers in Detection of Osteomyelitis

Biomarkers seem to be of potential value in differentiating infected nonunion and aseptic nonunion fractures (Southwood et al., 2003).

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