Alterations in sclerostin protein in lesions of equine osteochondrosis

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ABSTRACT
Osteochondrosis (OC) is a common and clinically important joint disease that occurs in many species, including humans, pigs, chickens and horses. It has been described as a focal failure of endochondral ossification (EO), but no cellular/molecular mechanisms are fully described that explain the cause of this condition. Recently a Wnt signalling inhibitor, sclerostin, has been described in osteoarthritic cartilage, where it has been proposed to protect damaged cartilage from degradation. Cartilage degradation is a key event in EO, thus, abnormalities of sclerostin in growth cartilage could, potentially, lead to a failure of EO and, thus, OC. The aim of this study was to describe the distribution of sclerostin protein in normal and OC growth cartilage.

Immunohistochemistry (IHC) was used to localise sclerostin protein in normal and OC growth cartilage. Growth cartilage was harvested from the distal femur of horses aged between 6 and 18 months. Cartilage was classified as normal or having lesions consistent with a diagnosis of early OC. IHC was used to identify sclerostin protein in cartilage sections. Sclerostin protein distribution was semiquantified using a grading system and shown to be upregulated throughout all three zones of cartilage in lesions of OC (IHC score 8.1 compared to IHC score of 0.88). These results indicate that sclerostin may be contributing to the development of OC lesions by inhibiting extracellular matrix remodelling or may reflect the response of damaged cartilage. Clearly, further work is required to fully characterise this observation but, with antisclerostin antibodies used to treat human osteoporosis, the possibility of development of a systemic treatment of OC remains a potential goal.

INTRODUCTION
Osteochondrosis (OC) is a common and clinically important joint disease that occurs in many species, including humans, pigs, chickens and horses (Jeffcott 1991). Many different factors have been proposed to be involved in the aetiopathogenesis of OC, including altered biomechanics (van Weeren and Barneveld 1999, Ytrehus and others 2007), altered paracrine and growth factors (Semevolos and others 2002, Semevolos and others 2005, van Bezoijen and others 2005), and extracellular matrix (ECM) alterations (Laverty and others 2002, de Grauw and others 2011), with recent work also demonstrating that experimentally induced blood vessel ischaemia can cause OC lesions (Olstad and others 2011, Olstad and others 2013).

Equine OC is a disorder of articular/epiphysseal growth cartilage associated with abnormal endochondral ossification (Jeffcott and Henson 1998). The early lesions of OC occur during longitudinal bone growth (Ytrehus and others 2007), which occurs via endochondral ossification, the series of events that coordinate cartilage expansion, maturation and the formation of bone. At the current time, no cellular/molecular mechanisms are yet proven to cause changes in the chondrocyte maturation pathway during endochondral ossification in OC. In order to identify mechanisms involved in the aetiopathogenesis of OC, one strategy is to examine components of pathways known to be involved in the control of endochondral ossification. A number of major signalling pathways are important in this process, including the Wnt signalling pathway (Mak and others 2006). In OC cartilage, alterations have been identified in the Indian Hedgehog signalling pathway (Semevolos and others 2002, Semevolos and others 2005), which acts upstream of the Wnt pathway (Day and Yang 2008), and gene profiling of leukocytes from
horses affected by OC indicated that, amongst other changes, components of the Wnt signalling pathway are altered (Sertyn 2010). The canonical Wnt pathway involves binding of Wnt primarily to Frz and LP5/6 receptor complexes, leading to stabilisation of β-catenin, its accumulation in the cytoplasm and subsequent translocation to the nucleus where it affects gene transcription (Baron and Rawadi 2007). Two Wnt signalling pathways are described, the canonical and the non-canonical; sclerostin (along with Dkk-1) is a major inhibitor of the canonical pathway.

In the last decade, sclerostin has been demonstrated to be critically involved in bone biology (Baron and Rawadi 2007). In bone, sclerostin is primarily located in osteocytes (van Bezoijen and others 2005) and it is known to play a pivotal role in the control of bone mass. The importance of sclerostin is highlighted by the effects of deletion mutations of the sclerostin gene that causes osteosclerotic phenotypes including sclerosteosis and van Buchem syndrome (Balemans and Van Hul 2004). In addition to its well-described roles in bone, sclerostin has recently been identified in the chondrocytes of adult cartilage, and it has been demonstrated to be upregulated in osteoarthritic chondrocytes (Chan and others 2011). In OA, sclerostin is upregulated at sites of damaged cartilage, and it has been hypothesised that sclerostin protects damaged cartilage from degradation (Chan and others 2011). In addition to noting increased sclerostin in OA, recent studies have shown that sclerostin is increased in chondrocytes of a ‘transient’ phenotype compared to chondrocytes of a permanent phenotype, such as is found in mature hyaline cartilage (Gelse and others 2012). We therefore hypothesise that sclerostin will be increased in chondrocytes contained in lesions of OC.

The aim of this study was to investigate the distribution of sclerostin protein in normal and OC equine growth cartilage from the distal femur.

METHODS

Animals

The animals used formed part of a large archived study (Henson and others 1996, Shingleton and others 1997). Twenty-four animals were used in this study, aged 6–18 months (Table 1). All animals had been humanely euthanased with an overdose of intravenous barbiturates. Animals from which normal cartilage and bone were obtained were euthanased for non-orthopaedic reasons. Prior to euthanasia, all horses were examined to determine the presence of lameness and/or joint effusion. Additionally, radiographs of the femoropatellar joints were obtained in 10/12 OC cases (data for 4/12 affected animals described previously (Voute and others 2011)). Horses were considered to be ‘normal’ that is, free from OC if they were not lame, had no joint effusion and, at postmortem did not show any gross morphological abnormalities on the surface of the joints examined (proximal interphalangeal joints, fetlock joints, carpal and hock joints, elbow, shoulder and stifle joints). Additionally, samples harvested as ‘normal’ were examined histologically (see below). Horses with OC were age-matched to normal horse controls.

Sample collection

OC samples, including the full thickness of the cartilage, were collected from the lateral and medial trochlear ridges of the distal femur using sharp dissection. Samples were fixed in 4 per cent paraformaldehyde, routinely embedded in paraffin following decalcification (decalcification with a 10 per cent EDTA solution for times between 14 days and 4 weeks) and sectioned for histology and immunohistochemistry (IHC). Sections were stained with H&E and Toluidine blue. Samples of bone for western blotting were obtained from control animals from the distal femur and immediately snap-frozen in liquid nitrogen.

Sample classification

Osteochondral samples were classified as normal or early OC (van Weeren and Barneveld 1999, Riddick and others 2012) by two observers. All osteochondral samples were evaluated grossly at postmortem and histologically. Normal cartilage was defined as having no gross or histological abnormalities, early OC as having altered endochondral ossification (locally thickened cartilage only, loss of normal columnar arrangement of chondrocytes and chondrones, or separation along the osteochondral junction without concurrent superficial cartilage lesions.

Western blotting

The specificity of the antibody used in this study was demonstrated using western blotting techniques, as described by Towbin and others (1979). Two positive control samples were included in the western blotting: recombinant sclerostin protein (R&D systems, UK) and human bone tissue lysis samples (Abcam, UK). In the horse, protein was extracted from bone with cell lysis buffer (Invitrogen, UK) containing a protease inhibitor cocktail (Sigma, UK). Ten micrograms of protein was loaded in each lane onto 10 per cent polyacrylamide gels, and proteins separated by gel electrophoresis for two hours. Gels were transferred to Immobilon membranes (Millipore, UK), and antigens identified using the rabbit anti-human sclerostin antibody (Abcam, UK) at a concentration of 1 in 2000 and an HRP/ECL+ detection method (Invitrogen, UK). Gels were run under reducing conditions.

Immunohistochemistry

IHC was performed on 6 μm sections using a 1:100 dilution of rabbit anti-human sclerostin antibody (Abcam, UK). IHC was performed using a biotinylated amplification technique. In order to confirm specificity in equine tissue, a positive tissue control, equine cortical bone, was used. Negative controls included replacing non-immune rabbit serum in place of the primary antibody.
IHC scoring

IHC samples were evaluated as follows. Protein expression was scored from 0 to 3 by two blinded observers; 0 (no staining), 1 (mild staining), 2 (moderate staining) and 3 (strong staining) in each of the three zones of chondrocytes (superficial, middle and deep) (Hosgor and others 2004, Getgood and others 2012). A total cartilage score was then derived by adding all three zone scores together. In each animal a total of three adjacent sections were used, in order to increase the accuracy of the quantification. In each zone within each section, five high-power fields were scored. The total cartilage scores for normal horses and horses with OC were then compared using a Student’s t test to identify statistical significance (p<0.05).

RESULTS

Histology

Of the 24 samples studied, 12 were considered to be normal growth cartilage with 12 having histological changes consistent with a diagnosis of early OC. (Fig 1, Table 1).

Western blotting

Western blotting of equine bone samples confirmed that the antibody used in this study recognised sclerostin. Recombinant human sclerostin was seen to migrate with a strong band at 27 kD, the predicted weight of the protein. In both human and horse bone samples, sclerostin was detected with a molecular weight of approximately 27 KDa (Fig 2).

Sclerostin immunoreactivity in cortical bone

Sclerostin protein was detected in osteocytes within mature cortical bone (Fig 3). No staining was seen in osteoblasts or blood vessels. This staining confirmed the cross-reactivity of the antibody in equine tissue.

Sclerostin IHC in normal cartilage

Sclerostin was localised in all normal samples studied. Mild sclerostin immunoreactivity was detected in the hypertrophic chondrocytes within the mineralised cartilage adjacent to the subchondral bone (Fig 4a). No staining was seen in the superficial or mid-zones (Table 2).

| TABLE 1: Details of horses with early changes of osteochondrosis used in this study |
|---------------------------------|-----------------|----------------|-----------------|
| Case number | Age (months) | Sex | Femoropatellar joint effusion | Radiographic changes |
|-----------|-------------|-----|-------------------------------|---------------------|
| 1         | 14          | G   | Bilateral                     | Yes                |
| 2         | 6           | G   | Bilateral                     | Yes                |
| 3         | 12          | G   | None                          | No                 |
| 4         | 15          | G   | None                          | N/A                |
| 5         | 7           | G   | Bilateral                     | Yes                |
| 6         | 9           | F   | Bilateral                     | N/A                |
| 7         | 12          | F   | Bilateral                     | No                 |
| 8         | 18          | F   | Bilateral                     | Yes                |
| 9         | 14          | C   | Bilateral                     | Yes                |
| 10        | 7           | F   | Bilateral                     | Yes                |
| 11        | 8           | C   | Bilateral                     | No                 |
| 12        | 10          | C   | Bilateral                     | No                 |

FIG 1: Histological sections of growth cartilage from the lateral trochlear ridge of the distal femur of a six-month-old horse. Sections stained with H&E. (a) Cartilage taken from right hind limb and classified as osteochondrosis (OC). Marker – 200 μm. (b) Higher-power view of 1A. Cartilage taken from within a lesion of OC. In this section there is an increase in the thickness of the DZ cartilage, an increase in cell numbers and a loss of the organised morphology of the tissue. Marker – 400 μm. (c) Cartilage taken from left hind limb and classified as normal. There is normal organisation of the DZ. Marker – 400 μm. SZ, superficial zone; MZ, middle zone; DZ, deep zone.
The total sclerostin IHC score for normal cartilage was 0.88±0.33.

Sclerostin IHC in OC cartilage

Sclerostin was localised in all OC samples studied. Strong sclerostin staining was detected intracellularly in 26/27 zones (3 zones from 9 horses) of superficial, mid- and deep-zone chondrocytes within the osteochondrotic lesions (Fig 4b, Table 2). In the superficial zone, the sclerostin score was 2.1±1.033; in the mid-zone 3±0, and in the deep zone 3±0. The total sclerostin IHC score for early OC samples was 8.1, significantly different from the score for normal cartilage (P<0.05). Sclerostin was also present in chondrocyte clusters within and around lesions (Fig 4c).

DISCUSSION

The results presented in this study indicate that sclerostin, an inhibitor of the Wnt signalling pathway, is significantly increased in chondrocytes present within lesions of naturally occurring OC, in contrast with cartilage from normal horses where sclerostin was detected only in chondrocytes in mineralised cartilage and in the deep zone adjacent to bone. This observation suggests that cartilage sclerostin might be involved in the aetiology of OC.

In addition to its well-described roles in bone, sclerostin has recently been identified in the chondrocytes of adult cartilage, and it has been demonstrated that sclerostin is upregulated in osteoarthritic chondrocytes (Chan and others 2011). In OA, sclerostin is upregulated at sites of damaged cartilage, and it has been hypothesised that sclerostin protects damaged cartilage from degradation (Chan and others 2011). The findings reported in this study, namely that sclerostin is upregulated in areas of retained cartilage at sites of early OC, indicate that sclerostin may play a role in OC by inhibiting degradation of the cartilage ECM.

The mechanism by which sclerostin may protect cartilage from damage has been proposed to be via downregulation of proteases (Chan and others 2011). In normal chondrocytes, the addition of sclerostin protein led to downregulation of interleukin-1-induced proteases including matrix metalloproteinase 1, 13, ADAMTS4 and 5 (Chan and others 2011). Given that sclerostin protein is increased in lesions of OC, and that OC is associated with retained cartilage, it is possible that sclerostin may be having an effect on the cartilage via protease action, as proposed in OA, and further studies are required to test this hypothesis.

In addition to sclerostin playing a role in the pathogenesis of OC via a possible downregulation of proteases, sclerostin may be affecting or, indeed, reflecting, the maturation status of the chondrocytes directly. During endochondral ossification, chondrocytes are programmed to pass through a series of maturation processes before being replaced by mineralised tissue. Thus, these ‘temporary’ chondrocytes can be considered to have a transient chondrocytic phenotype. This is in direct contrast with the
chondrocytes of mature hyaline articular cartilage which retain their unique phenotype for many decades within the tissue and can be considered to have a ‘permanent’ phenotype (Gelse and others 2012). In OC, it has been hypothesised that there is an accumulation of ‘transient’ chondrocytes within the lesion, with molecular evidence indicating that pathways involving chondrocyte maturation are altered in the disease (Riddick and others 2012). Recent work has shown that osteophytic chondrocyte (considered as an example of a ‘transient’ phenotype) and mature hyaline articular chondrocytes (‘permanent’ phenotype) differ significantly in their gene expression pathways (Gelse and others 2012). In particular, an increase in sclerostin was noted in transient chondrocytes. This increase in sclerostin gene expression reflects the observed increase in sclerostin protein reported in this study on OC, with the accumulations of ‘transient’ chondrocytes expressing high levels of sclerostin protein. It has been suggested that the increase in sclerostin reported in the ‘transient’ chondrocyte of the osteophyte may be playing a modulatory role, decelerating the process of endochondral ossification (Gelse and others 2012) – the results in this study would support such a hypothesis and warrant further investigation.

The mechanisms by which sclerostin is increased in OC is unknown at present. Sclerostin can be modulated by a number of different factors, including load (Robling and others 2008, Moustafa and others 2012) and fracture healing (Sarahudi and others 2012). One well-described endocrine/paracrine control of sclerostin levels is via parathyroid hormone (PTH)/PTH-related peptide (PTHrp), with both PTH and PTHrp decreasing sclerostin levels (Aslam and others 2012). However, in OC, both PTH (Sloet van Oldruitenborgh-Ooste and others 1999) and PTHrp (Semevolos and others 2002) have been shown to be increased in association with the disease, which would lead to a potential decrease in sclerostin in lesions, rather than the increase in levels observed in this study, making this PTH/PTHrp route unlikely. Further work is clearly required to elucidate the mechanism of control of sclerostin biology in OC.

In conclusion, this study demonstrates that sclerostin protein is increased in naturally occurring lesions of equine OC, a disorder associated with a failure of ordered endochondral ossification. Further studies are clearly required to understand the mechanisms observed in this study.

|TABLE 2: Immunohistochemistry score for in superficial, mid and deep zones of normal and early osteochondrotic lesions |
|---|---|---|---|---|
|Histological sample | IHC score for superficial zone | IHC score for mid-zone | IHC score for deep zone | Total IHC score |
|Normal | 0 | 0 | 0.88±0.33 | 0.88 |
|Early OC | 2.1±0.33 | 3±0 | 3±0 | 7.1 |

IHC, Immunohistochemistry; OC, Osteochondrosis

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