Altered Gene Expression in Early Osteochondrosis Lesions

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ABSTRACT: Osteochondrosis is a condition involving defective endochondral ossification and retention of cartilage in subchondral bone. The pathophysiology of this condition is poorly characterized, but it has been proposed that the fundamental defect is failure of chondrocyte hypertrophy. The aim of the current study was to characterize phenotypic changes in chondrocytes associated with the initiation of osteochondrosis. Early lesions were induced in an equine model of osteochondrosis by feeding foals a high energy diet for 8 or 15 weeks. Lesions in articular-epiphyseal growth cartilage were examined histologically and by quantitative PCR analysis of expression of a number of genes representative of pathways that regulate chondrocyte behavior during endochondral ossification. There were more cells present in clusters in the lesions compared to normal articular cartilage. Expression of matrix metalloproteinase-13, type I collagen, type X collagen, and Runx2 mRNA was significantly greater in the lesions compared to normal cartilage from the same joint. Expression of vascular endothelial growth factor, type II collagen, connective tissue growth factor, aggrecan, Sox9, and fibroblast growth factor receptor 3 mRNA was not significantly different in lesions than in control cartilage. These observations suggest that osteochondrosis does not result from failure of chondrocytes to undergo hypertrophy. © 2008 Orthopaedic Research Society. Published by Wiley Periodicals, Inc. J Orthop Res 27:452–457, 2009

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During the process of endochondral ossification, chondrocytes in growth cartilage undergo proliferation, followed by hypertrophy and physiological death. This orderly series of events is regulated by a number of secreted proteins, the actions of which are mediated by specific receptors, signaling cascades, and transcription factors.[1] Several factors are of particular interest. The high-mobility-group transcription factor Sox9 is the earliest known determinant of chondrocyte differentiation and cartilage formation, also regulating expression of the chondrocyte-specific extracellular matrix proteins type II collagen and aggrecan.[2] Fibroblast growth factor receptor 3 (FGFR3) has been shown to inhibit chondrocyte proliferation, with FGF18 possibly the important ligand in growth cartilage.[3,4] Endochondral ossification is blocked in Runx2-deficient mice, indicating an essential role for this transcription factor in chondrocyte differentiation. It has been shown that expression of Runx2 induces hypertrophy, alkaline phosphatase activity, and expression of type X collagen and matrix metalloproteinase-13 (MMP-13)[5] in chondrocytes, as well as mineralization of the cartilage matrix. In the later stages of endochondral ossification, Runx2 is involved in the vascular invasion of the cartilage, inducing the expression of the angiogenic factor, vascular endothelial growth factor (VEGF), which is necessary for vascular invasion, as well as the expression of VEGF receptors.[6] Evidence from a mouse model has shown that the collagen-degrading enzyme MMP-13 plays a role in invasion of the ossification front, with delayed endochondral ossification and a markedly expanded hypertrophic zone in MMP-13 null mice.[7] Connective tissue growth factor (CTGF) is selectively expressed by hypertrophic chondrocytes, and regulates chondrocyte proliferation and differentiation in growth cartilage.[8]

Osteochondrosis (OC) is a developmental disorder of bone which has been described in a number of domestic animal species as well as in humans.[9,10] Failure of endochondral ossification in articular-epiphyseal growth cartilage (AEGC) results in the retention of cartilage foci in subchondral bone. The presence of these foci leads to clinically important manifestations, which include synovitis, subchondral fractures and cysts, and osteochondritis dissecans with flaps that may detach. It has recently been suggested that equine OC may serve as a model for human OC of the knee or talus.[11]

The pathophysiology of OC is poorly understood, although in horses it appears that diet plays an important role. Feeding horses a diet high in energy or phosphorus has been found to induce the formation of lesions.[12,13] Carlson et al. have provided evidence that OC is initiated at sites of ischemic necrosis of growth cartilage, resulting from necrosis of blood vessels within cartilage canals.[14,15] Other authors have speculated that the cellular defect is a failure of hypertrophic differentiation of chondrocytes.[10,11,16,17] We have provided evidence that a failure of hypertrophy occurs selectively at sites of patent cartilage canal blood vessels, and have suggested that this may be a mechanism by which systemic changes are translated into focal disease.[18]

The approach taken here was to feed young growing horses a high energy diet known to induce OC[12] to obtain early lesions. Most previous studies examining changes in morphology and gene expression in OC have used samples at a late stage of the disease. It is possible in such studies that the changes detected may be due to inflammation and other conditions subsequent to the formation of the initial lesion. The aim of the current

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study was to obtain a better understanding of the cellular changes occurring at the initiation of OC prior to clinical manifestations, by observing changes in morphology and gene expression in early focal lesions.

METHODS

Feeding Trial

The animal experiments were approved by the University of Melbourne Faculty of Veterinary Science Animal Experimentation Ethics Committee. Osteochondrosis lesions were generated by feeding growing horses a high energy diet.[12] The study group consisted of 14 foals ranging from 3 to 6 months at the time they were acquired. For the first 4 weeks, all foals were fed a control diet of chaff and Coprice G (pellets of mainly rice and rice bran), which contained the National Research Council (NRC) (1989) formulated nutrient requirement.[12] Following this period, the foals were divided into two groups of seven, one of which continued on the control diet, while the other was fed a high energy diet with approximately 129% of the dietary energy of the NRC diet through the addition of maize oil.

Postmortem Procedures

The foals were examined at two time points. Eight foals, four on the control diet and four on the high energy diet, were killed after 8 weeks on the different diets, and the remaining six foals, three on each diet, were killed after 15 weeks. Several joints from each animal were examined for signs of early OC lesions. Lesions were identified through pitted patches and/or discoloration on the articular surface, which on cutting revealed cartilage cores retained within the bone adjacent to the articular surface (Fig. 1). The sites examined, both on the left and right sides, were previously identified predilection sites for OC,[12] as follows: the glenoid of the scapula, head of the humerus, proximal and distal ends of the femur, including the lateral trochlear ridge, tibia, metacarpus, and metatarsus, including the distal metacarpal and metatarsal condyles and the proximal end of the first phalanx. In addition, the articular processes of the cervical vertebrae (C2–C7) were examined. None of the lesions showed signs associated with clinical OC, such as chondral or subchondral fractures or synovitis.

The four horses fed the control diet for 8 weeks had a total of four OC lesions, while the three horses fed the control diet for 15 weeks had seven lesions. The four horses fed a high energy diet for 8 weeks had a total of 11 OC lesions and the three horses fed the high energy diet for 15 weeks had a total of 14 lesions. The majority of the lesions were found in either the glenoid of the scapula or the cervical vertebrae, three were collected from the head of the humerus and one from a fetlock joint. The lesions chosen for histology and real-time qPCR analysis were from the glenoid of the scapula and cervical vertebrae of horses fed a high energy diet. Lesions from control horses were not used, since it was assumed that they may have been preexisting lesions, and therefore more advanced than the majority of lesions in horses fed the high energy diet.

Tissue Sample Collection

Lesions and normal articular cartilage tissue, from the articular surface to the bone–cartilage interface, were excised from horses killed for the study. Normal cartilage specimens were collected from each of the predilection sites examined. Tissue specimens were divided into two parts for RNA and histology. Samples for RNA extraction were snap-frozen in liquid nitrogen and stored at -80°C. Cartilage was dissected from bone for RNA extraction. Samples for histology were collected in a solution of 4% paraformaldehyde, 5% gluteraldehyde in PBS, and stored at 4°C. After 4–5 days, the samples were washed in PBS, demineralized in 0.33 M EDTA at 4°C, then embedded in Spurr's resin.

Histology

Semi-thin sections were stained with methylene blue. In order to count chondrocytes, digital images were acquired at a $200\times$ magnification of the cartilage immediately adjacent to the subchondral bone. Lesions and normal cartilage samples from three different horses were used from each time point. Cells from three fields of view were counted from the controls, and two from the lesions, due to the narrow width of the retained cartilage plugs. The images were printed, randomized, and the cells counted blind. The total number of cells per field was counted, as well as the percentage present as single cells, pairs, and clusters of more than two cells. Cell area was measured using Media Cybernetics' Image Pro Plus version 4.1 software by measuring the area of "dark objects" in the field of view. Images were examined to ensure cell debris and unrelated objects were not included in the count. The results are presented as mean $\pm$ SEM. Data were analyzed using Student's t-test; p values < 0.05 were considered significant.

Real-Time Quantitative RT-PCR (qPCR)

RNA was extracted from cartilage using TRI reagent (Sigma, St. Louis, MO) and further purified using the SV RNA Isolation Kit (Promega, Madison, WI). Reverse transcription was carried out using MMLV Reverse Transcriptase RNase H-minus (Promega). Primers for types I, II, and X collagen, MMP-13, Runx2, aggrecan, CTGF, VEGF, Sox9, FGFR3, and ribosomal protein S23 (RP-S23), and methods for qPCR have been previously described.[19,20] RP-S23 was used as a housekeeping gene, as its expression has been shown to be consistent

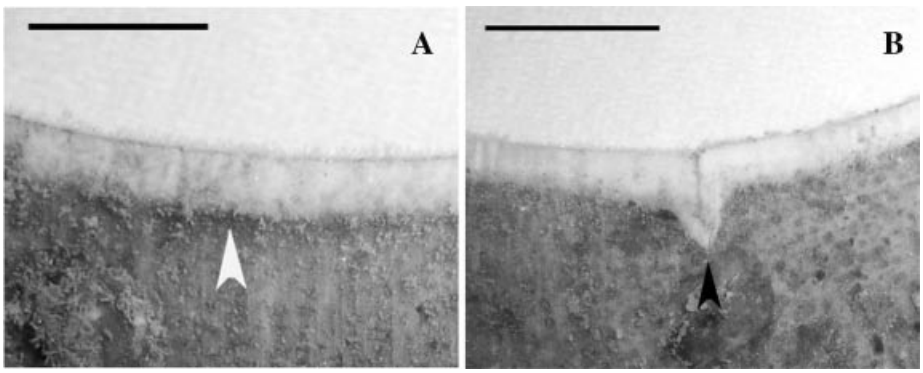

Figure 1. Macroscopic view of normal cartilage (white arrowhead; A) and subchondral bone, and osteochondrosis lesion (black arrowhead; B) from the glenoid of the scapula of a horse fed a high energy diet. Bar = 1 cm.

in both the proliferating and hypertrophic zones of the growth plate.[21] Automated sequencing was carried out on all PCR products to confirm their identity (AGRF, Melbourne, Australia).

For qPCR analysis, lesions from three horses from the 8-week high energy diet group were analyzed, comprising three lesions from the glenoid of the scapula and one lesion from the articular surface of a cervical vertebra. From the 15-week high energy diet group, lesions from three horses were analyzed comprising three lesions from the glenoid of the scapula and two lesions from cervical vertebrae. Results for qPCR were analyzed using the Pair-wise Fixed Reallocation Randomisation Test from the Relative Expression Software Tool—384, version 1.[22] The results for each gene of interest normalized to results for RP-S23 are presented as level of expression in each test sample relative to expression in the relevant control sample (mean ± SEM).

RESULTS

Morphology of Osteochondrosis Lesions

Histological studies were undertaken to investigate morphological changes in early OC lesions. Observation of the sections gave the impression that the cellular density was greater and that the individual chondrocytes were larger in the lesions than in controls. This led to cell counting and the measurement of cell area. Cell counts showed that the total number of chondrocytes per field was significantly higher in the lesions overall compared to controls (184 ± 17 cells per field for lesions, 142 ± 6 cells for the controls; $p < 0.05$) although there were no significant differences at the separate 8-week and 15-week time points. The area of each cell was significantly greater in the lesions compared to the controls at 15 weeks, but was not significantly different at 8 weeks (Fig. 2A). The cell area in control samples was lower at 15 weeks than at 8 weeks, but the difference was not significant (61 ± 13 μm^2 at 8 weeks, 44 ± 3 μm^2 at 15 weeks).

The distribution of the chondrocyte groupings also differed. The proportion of chondrocytes present in pairs was significantly higher in controls than in lesions, and the proportion of chondrocytes present in clusters of more than two cells was significantly higher in the lesions compared to the controls at both time points (Fig. 2B, C).

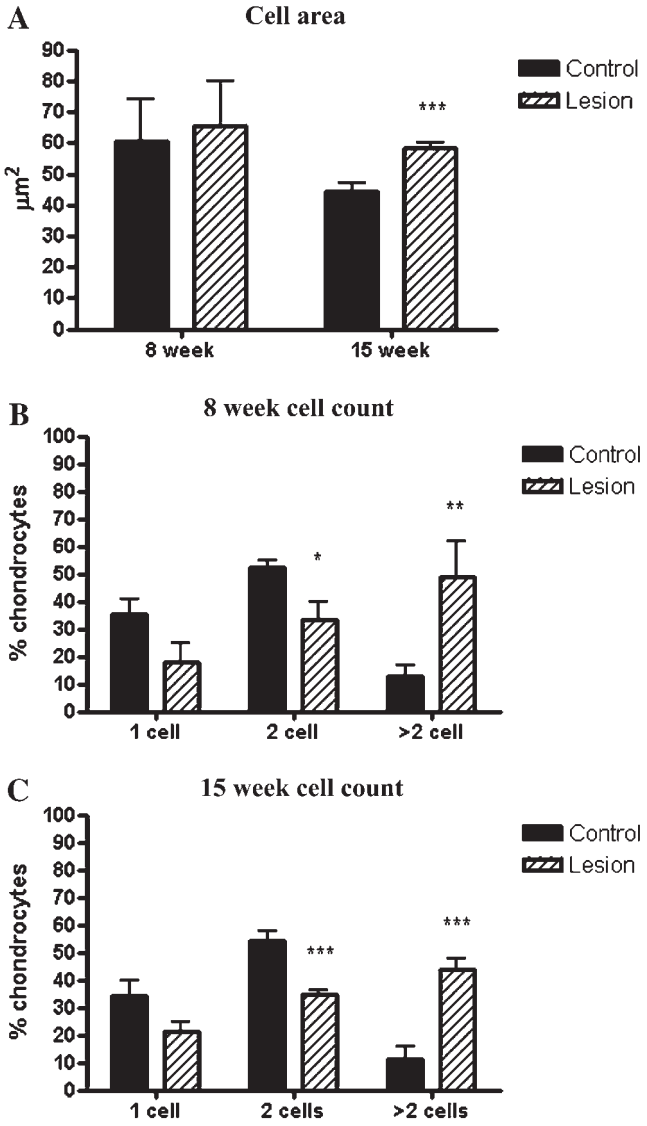

Figure 2. Histological analysis of lesions and control cartilage. (A) Average chondrocyte area in controls and lesions from horses fed a high energy diet for 8 or 15 weeks. (B, C) Distribution of cells in normal cartilage and lesions, expressed as percentage of cells in sections present as single cells, pairs, or clusters of more than two cells (mean ± SEM). Significant differences between lesions and controls: *$p < 0.05$; **$p < 0.01$; ***$p < 0.005$.

Gene Expression Studies

Initial studies investigated the variability in gene expression in normal cartilage samples between horses and between anatomical locations, in order to determine the optimal source of control cartilage for comparison with lesions. Expression of type II collagen and CTGF was assessed in normal cartilage samples from a number of joints in five randomly selected horses. Figure 3 shows the individual values for different joints grouped by horse (Fig. 3A, B) and the individual values for different horses grouped by joint (Fig. 3C, D). These data showed that there was considerable variation in gene expression between different joints within one animal, and between different animals within one joint. It was therefore decided that normal cartilage distant from the lesion but from the same articular surface would provide the best control.

Messenger RNA for a number of genes was found to be differentially expressed in lesions compared to normal cartilage from the same joint (Fig. 4). MMP-13 mRNA expression was increased significantly 36-fold in the 8-week lesions compared to normal cartilage, and 113-fold at 15 weeks. Both type I collagen and Runx 2 were significantly increased at both time points. Type X collagen was significantly increased at 15 weeks, while VEGF showed a nonsignificant trend to increased expression at both time points. No significant change was detected in the expression of type II collagen, aggrecan, Sox9 or FGFR3 mRNA.

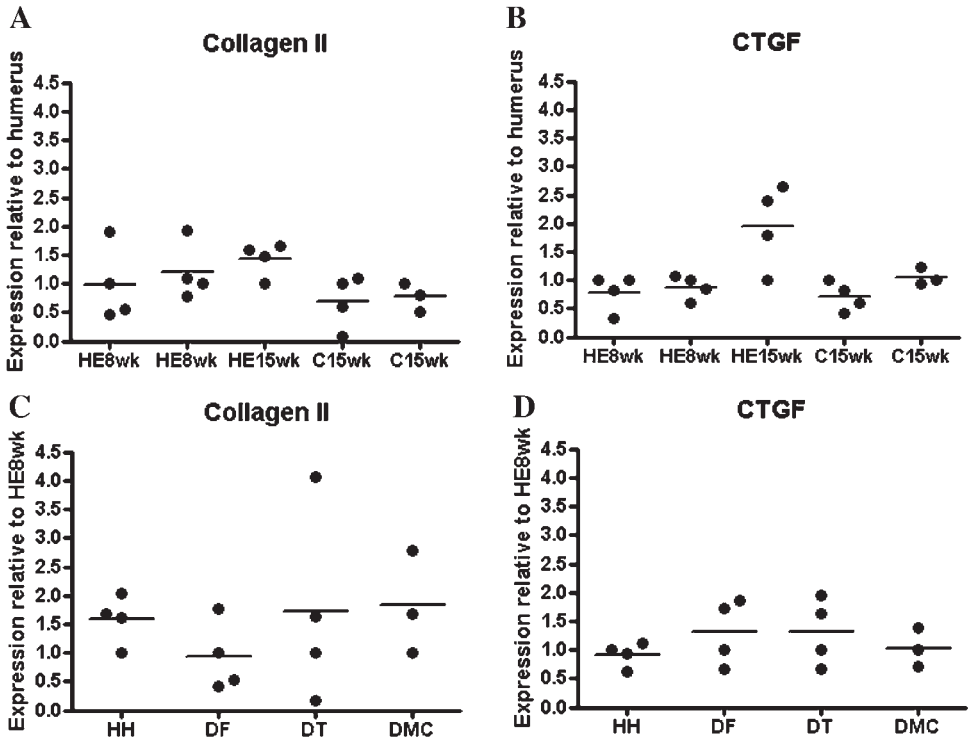

Figure 3. Expression of type II collagen (A, C) and CTGF (B, D) in normal cartilage from different joints of randomly selected horses fed a high energy (HE) or control (C) diet for 8 or 15 weeks (wk). (A, B) Individual values grouped by horse, expressed relative to expression in the sample from humerus. (C, D) Individual values grouped by joint, expressed relative to expression in one of the 8-week high energy diet (HE8wk) horses. HH, head of humerus; DF, distal end of femur; DT, distal end of tibia; DMC, distal end of metacarpus. Bars show the means.

DISCUSSION

The studies presented here were undertaken with the aim of identifying differences between normal cartilage and early OC lesions. The changes in morphology and gene expression described provide insights into the functional state of chondrocytes in early lesions.

Following formation of the secondary center of ossification in long bones, growth cartilage exists in two locations: the metaphyseal growth plate and the AEGC. Whereas chondrocyte proliferation in the metaphyseal growth plate drives longitudinal bone growth, AEGC contributes to expansion of the epiphysis, and thus occurs at a much lower rate. Thus, in young growing animals as used in the current study, the morphology of AEGC is substantially different from that of metaphyseal growth plate. In the normal AEGC specimens examined here, some chondrocytes were present in pairs or clusters but not the extended columns seen in metaphyseal growth plate. In lesions, there were significantly more cells present in multicellular clusters, suggestive of a higher rate of proliferation at the site of the lesions, although it is unknown whether this is a sign of persistent proliferation, or of the remnants of past proliferation. The cell area in control samples tended to decrease with time, which is consistent with a decrease in chondrocyte hypertrophy in AEGC with increasing skeletal maturity. The cell area of the lesions did not decrease between time points, and was significantly greater in lesions than in controls at 15 weeks, which suggests that there was persistence of a greater degree of hypertrophy in lesions than in controls.

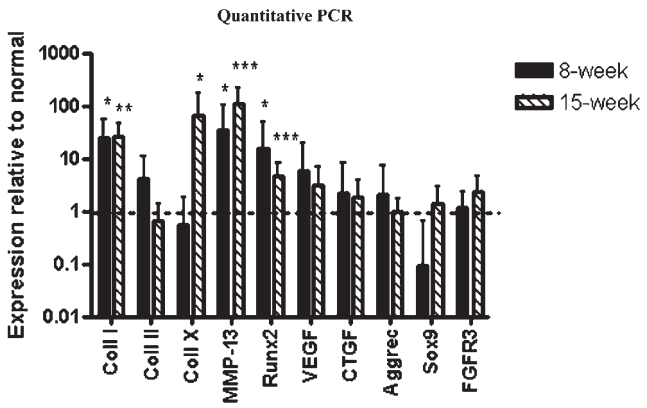

Figure 4. mRNA expression in osteochondrosis lesions from horses fed a high energy diet for 8 or 15 weeks. Gene expression in lesions was compared by quantitative PCR to normal cartilage adjacent to lesions. Results presented as mean ± SEM. Significant differences between lesions and controls: $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.005$. Dotted line marks expression level of controls.

It is worth noting that no cartilage canals were observed in any normal or lesion specimens, despite previous suggestions that they may play a role in the initiation of OC lesions.[15,18] Moreover, no cartilage necrosis was observed. These observations do not rule out a role for cartilage canals or cartilage necrosis in the pathogenesis of OC in some situations, but clearly indicate that they are not prerequisites for the initiation of an OC lesion.

Relative expression levels of a number of genes known to be important in the regulation of various stages of endochondral ossification were examined in lesions and normal cartilage. Of the genes examined, the one that showed the greatest relative difference between lesion and normal cartilage was MMP-13, which was significantly increased in the lesions compared to

controls at both 8 and 15 weeks. MMP-13 is expressed by hypertrophic chondrocytes, and appears to be critical for the normal removal of cartilage extracellular matrix during endochondral ossification in mice and humans.[7,23,24] The results of studies in MMP-13-null mice[7,24] had led us to predict that, if anything, MMP-13 would be down-regulated in lesions. The fact that the opposite is the case indicates that the retention of cartilage in subchondral bone in OC probably does not occur as a result of loss of the capacity to degrade cartilage matrix components. Indeed, the increased expression of MMP-13 is likely to be associated with increased protease activity leading to collagen and aggrecan degradation, and probably accounts for previous observations that collagen and aggrecan content are decreased in OC lesions.[25,26]

Type I collagen expression was significantly elevated at both 8 and 15 weeks, while type X collagen was only significantly increased at the later time point. Elevated type I collagen expression in clinical equine OC has previously been attributed to a healing response.[27] The fact that we have observed it in early lesions, however, suggests that it may be a primary alteration, reflecting an altered state of differentiation of the chondrocytes. Increased type I collagen expression in these lesions may be responsible for the elevated MMP-13 expression, since type I collagen has been shown to induce MMP-13 mRNA expression in chondrocytes.[28] The higher level of type X collagen expression suggests chondrocytes in the lesions may be undergoing hypertrophy, and are likely to be more hypertrophic than chondrocytes in normal articular cartilage. This observation is consistent with the finding that cell area was greater in lesions than in controls at 15 weeks.

There was a significant increase in Runx2 mRNA in lesions compared to control cartilage at both 8 and 15 weeks. Runx2 is normally expressed in proliferating and hypertrophic chondrocytes in the growth plate, with highest expression in terminal hypertrophic chondrocytes.[5] Runx2 induces genes typical of terminal chondrocyte differentiation, including type X collagen, MMP-13, and Ihh,[5,29,30] thus it is not surprising that these genes are also up-regulated in OC lesions (as demonstrated here for MMP-13, and by Semevolos et al., 2005[11] for Ihh).

During endochondral ossification, VEGF plays a vital role in invasion of the zone of hypertrophic chondrocytes by blood vessels.[31] It was included in the genes studied here in the expectation that there may be a decrease in VEGF expression in OC lesions, which could contribute to the retention of cartilage in subchondral bone; the fact that we observed no significant change in VEGF expression in lesions suggests that this is not the case. CTGF, Sox9, and FGFR3 do not appear to be involved in the establishment of OC, since their expression levels were not significantly altered in lesions.

We and others have previously hypothesized that OC results from a failure of chondrocytes to undergo hypertrophy, thus failing to prepare their surrounding matrix and provide appropriate signals for invasion by blood vessels and bone cells.[10,11,18] The morphological and gene expression studies presented here suggest that the fundamental defect is not failure to undergo hypertrophy per se. The chondrocytes in the lesions were larger than in control cartilage at 15 weeks; they expressed increased levels of MMP-13 and Runx2 at both time points, and increased collagen X at 15 weeks, factors which are associated with hypertrophy. The defect may be a continued stimulus to proliferate and undergo the early stages of hypertrophy in chondrocytes that would otherwise have almost stopped these activities.

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REFERENCES

1. Mackie EJ, Ahmed YA, Tatarczuch L, et al. 2008. Endochondral ossification: how cartilage is converted into bone in the developing skeleton. Int J Biochem Cell Biol 40:46–62.
2. Bi W, Deng JM, Zhang Z, et al. 1999. Sox9 is required for cartilage formation. Nat Genet 22:85–89.
3. Davidson D, Blanc A, Filion D, et al. 2005. Fibroblast growth factor (FGF) 18 signals through FGF receptor 3 to promote chondrogenesis. J Biol Chem 280:20509–20515.
4. Ohbayashi N, Shibayama M, Kurotaki Y, et al. 2002. FGF18 is required for normal cell proliferation and differentiation during osteogenesis and chondrogenesis. Genes Dev 16:870–879.
5. Enomoto H, Enomoto-Iwamoto M, Iwamoto M, et al. 2000. Cbfa1 is a positive regulatory factor in chondrocyte maturation. J Biol Chem 275:8695–8702.
6. Zelzer E, Glotzer DJ, Hartmann C, et al. 2001. Tissue specific regulation of VEGF expression during bone development requires Cbfa1/Runx2. Mech Dev 106:97–106.
7. Inada M, Wang Y, Byrne MH, et al. 2004. Critical roles for collagenase-3 (Mmp13) in development of growth plate cartilage and in endochondral ossification. Proc Natl Acad Sci USA 101:17192–17197.
8. Nakanishi T, Nishida T, Shimo T, et al. 2000. Effects of CTGF/Hcs24, a product of a hypertrophic chondrocyte-specific gene, on the proliferation and differentiation of chondrocytes in culture. Endocrinology 141:264–273.
9. Ytrehus B, Carlson CS, Ekman S. 2007. Etiology and pathogenesis of osteochondrosis. Vet Pathol 44:429–448.
10. Jeffcott LB, Henson FM. 1998. Studies on growth cartilage in the horse and their application to aetiopathogenesis of dyschondroplasia (osteochondrosis). Vet J 156:177–192.
11. Semevolos SA, Strassheim ML, Haupt JL, et al. 2005. Expression patterns of hedgehog signaling peptides in naturally acquired equine osteochondrosis. J Orthop Res 23: 1152–1159.
12. Savage CJ, McCarthy RN, Jeffcott LB. 1993a. Effects of dietary energy and protein on induction of dyschondroplasia in foals. Equine Vet J Suppl 16:74–79.
13. Savage CJ, McCarthy RN, Jeffcott LB. 1993b. Effects of dietary phosphorus and calcium on induction of dyschondroplasia in foals. Equine Vet J Suppl 16:80–83.
14. Carlson CS, Cullins LD, Meuten DJ. 1995. Osteochondrosis of the articular-epiphyseal cartilage complex in young horses: evidence for a defect in cartilage canal blood supply. Vet Pathol 32:641–647.

15. Carlson CS, Meuten DJ, Richardson DC. 1991. Ischemic necrosis of cartilage in spontaneous and experimental lesions of osteochondrosis. J Orthop Res 9:317–329.
16. Ekman S, Heinegard D. 1992. Immunohistochemical localization of matrix proteins in the femoral joint cartilage of growing commercial pigs. Vet Pathol 29:514–520.
17. Farnum CE, Wilsman NJ. 1986. Ultrastructural histochemical evaluation of growth plate cartilage matrix from healthy and osteochondritic swine. Am J Vet Res 47:1105–1115.
18. Shingleton WD, Mackie EJ, Cawston TE, et al. 1997. Cartilage canals in equine articular/epiphyseal growth cartilage and a possible association with dyschondroplasia. Equine Vet J 29:360–364.
19. Ahmed YA, Tatarczuch L, Pagel CN, et al. 2007. Physiological death of hypertrophic chondrocytes. Osteoarthritis Cartilage 15:575–586.
20. Ahmed YA, Tatarczuch L, Pagel CN, et al. 2007. Hypertrophy and physiological death of equine chondrocytes in vitro. Equine Vet J 39:546–552.
21. Wang Y, Middleton F, Horton JA, et al. 2004. Microarray analysis of proliferative and hypertrophic growth plate zones identifies differentiation markers and signal pathways. Bone 35:1273–1293.
22. Pfaffl MW, Horgan GW, Dempfle L. 2002. Relative expression software tool (REST) for group-wise comparison and statistical analysis of relative expression results in real-time PCR. Nucleic Acids Res 30:e36.
23. Kennedy AM, Inada M, Krane SM, et al. 2005. MMP13 mutation causes spondyloepimetaphyseal dysplasia, Missouri type (SEMD(MO)). J Clin Invest 115:2832–2842.
24. Stickens D, Behonick DJ, Ortega N, et al. 2004. Altered endochondral bone development in matrix metalloproteinase 13-deficient mice. Development 131:5883–5895.
25. Hernandez-Vidal G, Jeffcott LB, Davies ME. 1998. Immunolocalization of cathepsin B in equine dyschondroplastic articular cartilage. Vet J 156:193–201.
26. van de Lest CH, Brama PA, van El B, et al. 2004. Extracellular matrix changes in early osteochondrotic defects in foals: a key role for collagen? Biochim Biophys Acta 1690:54–62.
27. Semevolos SA, Nixon AJ, Brower-Toland BD. 2001. Changes in molecular expression of aggrecan and collagen types I, II, and X, insulin-like growth factor-I, and transforming growth factor-beta1 in articular cartilage obtained from horses with naturally acquired osteochondrosis. Am J Vet Res 62:1088–1094.
28. Ronziere MC, Aubert-Foucher E, Gouttenoire J, et al. 2005. Integrin alpha1beta1 mediates collagen induction of MMP-13 expression in MC615 chondrocytes. Biochim Biophys Acta 1746:55–64.
29. Takeda S, Bonnamy JP, Owen MJ, et al. 2001. Continuous expression of Cbfa1 in nonhypertrophic chondrocytes uncovers its ability to induce hypertrophic chondrocyte differentiation and partially rescues Cbfa1-deficient mice. Genes Dev 15:467–481.
30. Yoshida CA, Yamamoto H, Fujita T, et al. 2004. Runx2 and Runx3 are essential for chondrocyte maturation, and Runx2 regulates limb growth through induction of Indian hedgehog. Genes Dev 18:952–963.
31. Gerber HP, Vu TH, Ryan AM, et al. 1999. VEGF couples hypertrophic cartilage remodeling, ossification and angiogenesis during endochondral bone formation. Nat Med 5:623–628.