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Involvement of mitochondrial dysfunction and ER-stress in the physiopathology of equine osteochondritis dissecans (OCD)

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A B S T R A C T

Osteochondrosis (OC) is a developmental bone disorder affecting several mammalian species including the horse. Equine OC is described as a focal disruption of endochondral ossification, leading to osteochondral lesions (osteochondritis dissecans, OCD) that may release free bodies within the joint. OCD lesions trigger joint swelling, stiffness and lameness and affects about 30% of the equine population. OCD is considered as multifactorial but its physiopathology is still poorly understood and genes involved in genetic predisposition are still unknown. Our study compared two healthy and two OC-affected 18-month-old French Trotters diagnosed with OCD lesions at the intermediate ridge of the distal tibia. A comparative shot-gun proteomic analysis of non-wounded cartilage and sub-chondral bone from healthy (healthy samples) and OC-affected foals (predisposed samples) identified 83 and 53 modulated proteins, respectively. These proteins are involved in various biological pathways including matrix structure and maintenance, protein biosynthesis, folding and transport, mitochondrial activity, energy and calcium metabolism. Transmission electron microscopy revealed typical features of mitochondrial swelling and ER-stress, such as large, empty mitochondria, and hyper-dilated rough endoplasmic reticulum, in the deep zone of both OC lesions and predisposed cartilage. Abnormal fibril organization surrounding chondrocytes and abnormal features at the ossification front were also observed. Combining these findings with quantitative trait loci and whole genome sequencing results identified about 140 functional candidate genes carrying putative damaging mutations in 30 QTL regions. In summary, our study suggests that OCD lesions may result from defective hypertrophic terminal differentiation associated with mitochondrial dysfunction and ER-stress, leading to impaired cartilage and bone biomechanical properties, making them prone to fractures. In addition, 11 modulated proteins and several candidate mutations located in QTL regions were identified, bringing new insight into the molecular physiopathology and genetic basis of OCD.

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Introduction

Osteochondrosis (OC) is a developmental orthopedic disorder affecting joints of young individuals from different species including the horse. OC has been described as a focal failure of endochondral ossification (Ekman and Carlson, 1998). This condition often involves the subchondral bone, leading to dissecting lesions (osteochondritis dissecans, OCD) or subchondral bone cysts. OCD is a common developmental disease, affecting 10 to 30% of the equine population, depending on breed and joint. Clinical signs develop when the joint surface is breached by the dissecting lesion or when a fragment completely detaches and becomes a free body, leading to synovitis, varying degrees of lameness and development of arthritis. Thus, OCD has become a major concern in the horse industry (Jeffcott, 1996).

A multifactorial origin is commonly accepted, including environmental factors (dietary imbalance and biomechanical factors), physiological factors (growth, conformation and hormonal imbalance) and genetics (Jeffcott, 1991; Lepeule et al., 2009; McIlwraith, 2004; van Weeren, 2006; van Weeren and Barneveld, 1999; Ytrehus et al., 2007). Several gene mapping programs have been initiated worldwide in different breeds and several quantitative trait loci (QTL) regions have been identified (for review see Distl, 2013), but no gene or mutations have been identified to date. OCD pathogeny remains unclear, probably due to the confusion regarding disease definition and the lack of precise

Abbreviations: OC, Osteochondrosis; OCD, osteochondritis dissecans; ECM, extracellular matrix; TEM, transmission electron microscopy.

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data about mechanisms of primary lesion formation (Ytrehus et al., 2007). In this respect, two main hypotheses have been proposed: the vascular and dyschondroplasic hypotheses. In the first one, a focal interruption of canal blood supply leads to necrotic areas and abnormal ossification (Jeffcott and Henson, 1998). In the second hypothesis, primary lesions result from a local failure of endochondral ossification. Characteristic features of dyschondroplasic lesions include abnormal chondrocyte shape, presence of chondrocytes arrested in the pre-hypertrophic stage, abnormal matrix mineralization, necrosis, increased type VI collagen and cartilage cores retained in subchondral bone. Taken together, these features suggest a disruption of the endochondral ossification process at the chondro-osseous junction that impairs cartilage replacement by bone tissue as the ossification front advances with time (Muir, 1995). In both hypotheses, shearing biomechanical forces are thought to drive the development of dissecting lesions. Thus, the molecular mechanisms involved in primary lesions are still unknown.

The aim of this current study was to investigate the biological pathways involved in the development of OCD lesions from the intermediate ridge of the distal tibia in French Trotters by comparing non-wounded samples from non-affected joints (predisposed samples) harvested from OC-affected foals with samples from healthy foals. Consequently, results are thus not confounded by changes associated with the evolution of the lesion, but are focused on molecular mechanisms constitutively altered in predisposed samples. Firstly, a comparative shot-gun proteomic analysis was performed on normal cartilage and sub-chondral bone from affected and healthy horses. Based on the hypothesis drawn from this preliminary work, transmission electron microscopy (TEM) was then used to compare cartilage and bone structure and ultrastructure from OCD lesions as well as non-wounded tissues from healthy and predisposed samples. Clinical and genetic relevance of these findings are discussed.

Materials and methods

Sample collection

The study was based on four 18 month-old French trotters obtained from two different studs and intended for slaughter. Clinical and radiographic examinations were performed to evaluate their osteo-articular status. During necropsy, all joints were macroscopically examined to confirm radiologic diagnosis. Lesions and sections from talus and femoral trochlea were cut in the sagittal plane and included 5 mm of the sub-chondral bone. When possible, samples from the contra-lateral unaffected joint were collected. Samples were harvested at the same location in the joint (middle of talus and femoral trochlea) to avoid technical bias due to sample location in the joint. Some of these samples were fixed for 24 h in a solution of 4% paraformaldehyde for histological studies, whereas the others were flash frozen in liquid nitrogen for subsequent proteomics studies.

Histological analysis

After fixation, samples were decalcified in 10% EDTA pH 8.8 solution for one month before being embedded in paraffin and sectioned (5 μm). Sections were stained with Hematoxilin–Eosin–safranin-O (HES) and safranin-O and light green (LGS). Type-VI collagen immunohistochemistry was also performed.

Shotgun proteomics

Protein extraction was performed as previously described (Desjardin et al., 2012). Solubilization was achieved in ZALS–urea–thiourea (ZUT) buffer (urea 6 M, thiourea 2 M, DTT 10 mM, Tris pH 8.8 30 mM, 0.1% ZALS) and proteins were quantified using the PlusOne 2-D Quant kit (GE Healthcare). After reduction by dithiotreitol (DTT) and alkylation by iodoacetamine (IAA), proteins were digested by trypsin (2% w/w) overnight. Peptides were purified on solid phase extraction using polymeric C18 column (Phenomenex). After elution (40% acetonitrile) and speedvac drying, peptides were suspended in Peptide Suspension Buffer (2% acetonitrile, 0.05% formic acid, 0.05% TFA).

Peptides (1 μg) were analyzed by LC–MS/MS. A Nano2D Ultra system (Eksigent) was used to separate peptides on C18 column (15 cm, 75 μm i.d., Nanoseparation). Subsequent on-line analysis was performed with a Q Exactive mass spectrometer (Thermo Electron) using a nanoelectrospray interface (noncoated capillary probe, 10 μ i.d.; New Objective). Peptide ions were analyzed using Xcalibur 2.1 with the following data-dependent acquisition steps: (1) full MS scan on a 400 to 1200 range of mass-to-charge ratio (m/z) with a resolution of 70,000 and (2) MS/MS (normalized collision energy: 30%; resolution: 17,500). Step 2 was repeated for the 8 major ions detected in step 1.

Q Exactive raw files were converted with ProteoWizard 3.0.3706 (Kessner et al., 2008) and uploaded into PROTICdb (Langella et al., 2007). X!Tandem (2011.12.01.1, Craig and Beavis, 2004) was used to search three databases: (i) the NCBI Equus Caballus protein database (v20100519, 21,331 entries); (ii) the EBI Ensembl Equus Caballus protein database (v2010-05-19, 22,641 entries) and (iii) a contaminant database (trypsin, keratins). Only the proteins identified with at least two peptides were kept. Protein quantification was performed with MassChroQ software (Valot et al., 2011).

Statistics

Descriptive statistics were done using R scripts and the ade4 package (v1.5-1). Between-class correspondence analysis (dudi.bca) was performed to investigate differences between samples from healthy and predisposed animals. Proteins having inertia above the third quartile and showing at least a two-fold change in abundance were considered as associated with the pathology. Functional annotations were made using both Ingenuity Pathway Analysis (IPA) and Babelomics4 (http://babelomics.bioinfo.cipf.es).

Transmission electron microscopy (TEM)

After fixation, samples were decalcified with 2.5% glutaraldehyde, 2% paraformaldehyde, and 0.1 M EDTA, in 0.1 M Na pH 7.2 cacodylate buffer for five weeks at 4 °C. Samples were postfixed with 1% osmium tetroxide containing 1.5% potassium cyanoferrate, contrasted with uranyl acetate 2% in water, gradually dehydrated in ethanol (30%–100%) and embedded in Epon. For pre-visualization and selecting regions of interest, 1 μm sections were counterstained with methylene blue–Azur II and visualized using Nanozoomer (Hamamatsu). Thin sections (70 nm) of selected zones were collected onto 200 mesh copper grids, and counterstained with lead citrate before examination with Zeiss EM902 electron microscope operated at 80 kVe. Microphotographs were acquired using a charge-coupled device camera MegaView III CCD camera and analyzed with ITEM software (Eloïse, Roissy CDG, France).

QTL study, whole genome sequencing and variant calling

Genes located within QTL regions and their human orthologous genes, as well as their Gene Ontology (GO) annotations were retrieved from the Ensembl database (Version 74, http://www.ensembl.org/index.html). To identify functional candidates, GO terms were filtered based on enriched annotations, as calculated by Babelomics (Fatigo +, p-value < 0.05) using the proteomic datasets.

To search for candidate polymorphisms, 10 French Trotter horses from our previous QTL mapping project (Teyssedre et al., 2012) were sequenced on HiSeq2000 at the GET-PlaGe facility. These horses were chosen based on their genotype in order to be representative of allelic diversity at 10 QTL regions. One paired-end library with a 300-bp insert

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