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Disruption of biomineralization pathways in spinal tissues of a mouse model of diffuse idiopathic skeletal hyperostosis

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ABSTRACT

Equilibrative nucleoside transporter 1 (ENT1) mediates passage of adenosine across the plasma membrane. We reported previously that mice lacking ENT1 (*ENT1*$^{-/-}$) exhibit progressive ectopic mineralization of spinal tissues resembling diffuse idiopathic skeletal hyperostosis (DISH) in humans. Here, we investigated mechanisms underlying aberrant mineralization in *ENT1*$^{-/-}$ mice. Micro-CT revealed ectopic mineralization of spinal tissues in both male and female *ENT1*$^{-/-}$ mice, involving the annulus fibrosus of the intervertebral discs (IVDs) of older mice. IVDs were isolated from wild-type and *ENT1*$^{-/-}$ mice at 2 months of age (prior to disc mineralization), 4, and 6 months of age (disc mineralization present) and processed for real-time PCR, cell isolation, or histology. Relative to the expression of ENTs in other tissues, ENT1 was the primary nucleoside transporter expressed in wild-type IVDs and mediated the functional uptake of [^{3}H]2-chloroadenosine by annulus fibrosus cells. No differences in candidate gene expression were detected in IVDs from *ENT1*$^{-/-}$ and wild-type mice at 2 or 4 months of age. However, at 6 months of age, expression of genes that inhibit biomineralization *Mgp*, *Enpp1*, *Ank*, and *Spp1* were reduced in IVDs from *ENT1*$^{-/-}$ mice. To assess whether changes detected in *ENT1*$^{-/-}$ mice were cell autonomous, annulus fibrosus cell cultures were established. Compared to wild-type cells, cells isolated from *ENT1*$^{-/-}$ IVDs at 2 or 6 months of age demonstrated greater activity of alkaline phosphatase, a promoter of biomineralization. Cells from 2-month-old *ENT1*$^{-/-}$ mice also showed greater mineralization than wild-type. Interestingly, altered localization of alkaline phosphatase activity was detected in the inner annulus fibrosus of *ENT1*$^{-/-}$ mice in vivo. Alkaline phosphatase activity, together with the marked reduction in mineralization inhibitors, is consistent with the mineralization of IVDs seen in *ENT1*$^{-/-}$ mice at older ages. These findings establish that both cell-autonomous and systemic mechanisms contribute to ectopic mineralization in *ENT1*$^{-/-}$ mice.

1. Introduction

Aberrant calcification of soft connective tissues has been associated with a number of skeletal disorders including diffuse idiopathic skeletal hyperostosis (DISH) and related disorders such as ossification of the posterior longitudinal ligament of the spine (OPLL) [7,31]. DISH is diagnosed by radiographic detection of calcified outgrowths along the vertebrae of the spine [2,7]. This non-inflammatory spondyloarthropathy involves the pathological calcification of the anterolateral spinal ligaments, entheses, and fibrocartilaginous tissues present in the intervertebral disc (IVD) [27,31,44]. The early stages of DISH are largely asymptomatic. Therefore, individuals with DISH are often undiagnosed until the later stages of disease progression when DISH presents as back pain and stiffness and in severe cases, dysphagia [50] or compression of the spinal cord and nerve roots [23,50,58].

DISH is associated with a high rate of vertebral fractures in the elderly [12] and increasing evidence suggests that the presence of DISH is indicative of an underlying metabolic derangement [34]. The prevalence of DISH in North Americans over 50 years of age is 25% for males and 15% for females [55]. These numbers are expected to rise with the increase in life expectancy and the high prevalence of risk factors associated with the development of DISH, including obesity, diabetes and hypertension [32,33]. The aetiology of DISH is poorly understood and the local or systemic factors involved in initiation of ectopic mineralization are unknown.

Recent studies by our group characterized a novel mouse model of DISH [54]. These mice lack expression of the equilibrative nucleoside

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transporter 1 (ENT1, also known as solute carrier family 29 member 1, encoded by *Slc29a1*) [10]. ENT1 is a membrane protein involved in the bi-directional transport of nucleosides such as adenosine across the plasma membrane [3]. Calcified lesions in *ENT1*$^{-/-}$ mice were associated with paraspinal tissues in the cervical-thoracic region as early as 2 months of age, and extended to the lumbar and caudal regions at 6 and 12 months, respectively [54]. Histological examination revealed large, irregular accumulations of eosinophilic material in spinal fibrocartilaginous tissues including ligaments, entheses and the annulus fibrosus (AF) of the intervertebral disc. Interestingly, plasma levels of inorganic pyrophosphate were greater in *ENT1*$^{-/-}$ mice than in wild-type controls [54], consistent with disruption in the homeostasis of pyrophosphate — a regulator of biomineralization [59]. In this regard, a subsequent study reported that *ENT1*$^{-/-}$ mice have reduced bone mineral density in the femur, and the lower thoracic and lumbar spine compared to wild-type littermates [19]. Consistent with findings from the *ENT1*$^{-/-}$ mouse model, humans homozygous for a null mutation in *SLC29A1* (the first reported individuals lacking ENT1) demonstrate ectopic calcification in both periarticular and lumbar spinal tissues [11].

Equilibrative nucleoside transporters (ENTs) are Na^+-independent, facilitative diffusion carriers, which transport nucleosides bi-directionally down a concentration gradient. ENTs are widely distributed among cell types [4,25]. The ENT family consists of four members (ENT1-4), which share an ability to transport adenosine but differ in their capacities to transport other nucleosides, nucleobases and therapeutic analogues [3]. Considered as ubiquitously expressed, ENT1 is the most extensively characterized transporter and can be distinguished from ENT2 based on sensitivity to inhibition by nitrobenzylthioinosine (nitrobenzylmercaptopurine riboside; NBMPR) [60]. Unlike other ENTs, ENT3 is not found at the plasma membrane, but rather is located on intracellular membranes [5]. ENT4 is expressed primarily in the heart, liver and brain [60]. To date, the expression and function of nucleoside transporters in the IVD have not been investigated.

The current study investigated the mechanisms underlying ectopic mineralization in *ENT1*$^{-/-}$ mice. Our findings demonstrate that ENT1 is the primary nucleoside transporter in the murine intervertebral disc. We show that accumulation of ectopic calcification in the intervertebral discs of *ENT1*$^{-/-}$ mice is associated with aberrant alkaline phosphatase activity and a reduction in the expression of biomineralization inhibitors *Mgp*, *Enpp1*, *Ank* and *Spp1*. Based on the analysis of annulus fibrosus micromass cultures and intact IVD tissues, we suggest that both cell-autonomous and systemic mechanisms contribute to ectopic calcification of spinal tissues in *ENT1*$^{-/-}$ mice.

2. Materials and methods

2.1. Animals

ENT1$^{-/-}$ mice were generously provided by Dr. Doo-Sup Choi, Mayo Clinic College of Medicine, Rochester, MN, USA [10]. *ENT1*$^{-/-}$ mice were backcrossed with C57BL/6 mice and the mouse colony was maintained through the breeding of heterozygous animals (*ENT1*$^{+/-}$) to obtain wild-type (*ENT1*$^{+/+}$) and knockout (*ENT1*$^{-/-}$) littermates, as previously described [54]. Male and female mice were euthanized at 2, 4 or 6 months of age. All aspects of this study were conducted in accordance with the policies and guidelines set forth by the Canadian Council on Animal Care and were approved by the Animal Use Subcommittee of the University of Western Ontario.

2.2. Micro-computed tomography (micro-CT) imaging

At 2 or 6 months of age, male and female wild-type and *ENT1*$^{-/-}$ mice (n = 3–4 each group) were euthanized and formalin-fixed for imaging using a laboratory micro-CT scanner (eXplore speCZT, GE Healthcare Biosciences, London, ON, Canada). Data were acquired with an X-ray tube voltage of 90 kV and a current of 40 mA. In one continuous rotation, 900 views were obtained at an angular increment of 0.4° and an exposure interval of 16 ms per view. A calibrating phantom, consisting of a vial of water, air and a synthetic bone-mimicking epoxy (SB3, Gammex Inc. Middleton, WI, USA), was imaged together with the specimens. Images were acquired at isotropic voxel size of 50 or 100 μm and reconstructed into 3D images, using a modified conebeam algorithm [14]. High-resolution isosurfaced images of the thoracic spine were generated by outlining the region of hypermineralization (mineralization exceeding values of normal vertebral bone) in consecutive 50 μm-thick axial sections, spanning T1-T2, using a region-of-interest tool. The 3D region-of-interest corresponding to mineralized material within the IVD with density equivalent to or greater than that of normal cortical bone was pseudocoloured and displayed within the 3D co-registered isosurface of the thoracic spine.

2.3. Tissue isolation, RNA extraction and real-time PCR

Littermate-paired wild-type and *ENT1*$^{-/-}$ mice were dissected at specified time points to isolate intact intervertebral discs (IVDs), inclusive of annulus fibrosus, nucleus pulposus and cartilage endplates (n = 8 mice/genotype each timepoint; male and female mice). IVDs from each animal were separated according to anatomical location as body (cervical/thoracic, lumbar) or tail. Non-affected tissues (knee, heart, kidney, skeletal muscle, brain and liver) were harvested from wild-type animals at 6 months of age (n = 3). Tissues were placed directly in TRIzol reagent® (Life Technologies, Wilmington, DE, USA) and homogenized using a PRO250 Polytron homogenizer (PRO Scientific, Oxford, CT, USA). Total RNA was extracted according to the manufacturer's protocol and quantified using a NanoDrop 2000 spectrophotometer (Thermo Scientific, Mississauga, ON, Canada). Total RNA (500 ng/sample) was reverse transcribed using the iScript cDNA synthesis kit (Bio-Rad, Philadelphia, PA, USA).

Real-time PCR was conducted using the Bio-Rad CFX384 system. PCR reactions were run using 310 nM forward and reverse primers (sequences and annealing temperatures provided in Supplementary Table S1) with SsoFast™ EvaGreen® Supermix (Bio-Rad). Primers were designed with efficiency values between 90–110%, to amplify target genes with Cq values between 20–36. The PCR program consisted of the following: initial 3 min at 95 °C for denaturing; 95 °C for 10 s for denaturing; 30 s for annealing/elongation, for a total of 40 cycles. Gene transcript levels were determined relative to a six-point calibration curve made from cDNA generated from wild-type murine heart, brain, kidney, skeletal muscle, IVD, and calvarial RNA. The starting concentration of the cDNA standard (85–130 ng/μL) was inputted into the Bio-Rad CFX Manager 2.0 software to generate the standard curve (1/5 serial dilution). Transcript levels were expressed relative to the calibration curve in ng/μL.

2.4. Cell isolation and culture

IVDs were microdissected from wild-type and *ENT1*$^{-/-}$ mice at 2 or 6 months of age (n = 6 independent cell preparations/genotype at each timepoint, male and female mice used). The nucleus pulposus was removed from isolated IVDs, and AF tissues from the same animal were pooled for cell isolation, based on previously reported protocols [22,62]. Briefly, the AF tissue was minced and cells were isolated by enzymatic digestion in 0.2% type-2 collagenase (Worthington Biochemical Corp., Lakewood, NJ, USA) for 90 min at 37 °C. The cell suspension was filtered through a 100 μm sterile mesh and cells were seeded at 6×10^5 cells/cm^2 in DMEM/F12 supplemented with 10% fetal bovine serum and 1% Penicillin/Streptomycin (Invitrogen, Carlsbad, CA). Primary murine AF cells demonstrated a characteristic spindle-like morphology in monolayer culture. Once cultures reached ~80% confluence (5–6 days), cells were trypsinized and plated in either micromass cultures (5×10^4 cells in a 150 μL drop/well of a 12-well plate) or in monolayer (6×10^4 cells/cm^2). Maintenance of an AF cell phenotype (and lack