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Alteration of proteoglycan sulfation affects bone growth and remodeling

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ABSTRACT

Diastrophic dysplasia (DTD) is a chondrodysplasia caused by mutations in the *SLC26A2* gene, leading to reduced intracellular sulfate pool in chondrocytes, osteoblasts and fibroblasts. Hence, proteoglycans are undersulfated in the cartilage and bone of DTD patients. To characterize the bone phenotype of this skeletal dysplasia we used the *Slc26a2* knock-in mouse (dtd mouse), that was previously validated as an animal model of DTD in humans.
X-rays, bone densitometry, static and dynamic histomorphometry, and *in vitro* studies revealed a primary bone defect in the dtd mouse model.
We showed *in vivo* that this primary bone defect in dtd mice is due to decreased bone accrual associated with a decreased trabecular and periosteal appositional rate at the cell level in one month-old mice. Although the osteoclast number evaluated by histomorphometry was not different in dtd compared to wild-type mice, urine analysis of deoxypyridinoline cross-links and serum levels of type I collagen C-terminal telopeptides showed a higher resorption rate in dtd mice compared to wild-type littermates. Electron microscopy studies showed that collagen fibrils in bone were thinner and less organized in dtd compared to wild-type mice. These data suggest that the low bone mass observed in mutant mice could possibly be linked to the different bone matrix compositions/organizations in dtd mice triggering changes in osteoblast and osteoclast activities.
Overall, these results suggest that proteoglycan undersulfation not only affects the properties of hyaline cartilage, but can also lead to unbalanced bone modeling and remodeling activities, demonstrating the importance of proteoglycan sulfation in bone homeostasis.

Introduction

The diastrophic dysplasia sulfate transporter (DTDST, also known as SLC26A2) is a sulfate/chloride antiporter, widely expressed on the plasma membrane of many cell types, including fibroblasts, chondrocytes and osteoblasts [1].

Functional defects of the SLC26A2 can cause a reduction in the intracellular sulfate pool, leading to synthesis and secretion of undersulfated proteoglycans [2]. Proteoglycan undersulfation can result in altered architecture and mechanical properties of the extracellular matrix [3]. The consequences of these alterations are most evident at the cartilage level, since cartilage is a tissue very rich in proteoglycans that in normal conditions are massively sulfated. Thus, defects in the SLC26A2 can cause a chondrodysplastic phenotype. Mutations in the gene encoding for the SLC26A2 are indeed associated with a family of recessively inherited chondrodysplasias that include, in order of increasing severity, a recessive form of multiple epiphyseal dysplasia, diastrophic dysplasia (DTD), atelosteogenesis type 2, and achondrogenesis type 1B [4]. The different clinical phenotypes are related to the residual activity of the sulfate transporter and thus to the resulting degree of proteoglycan undersulfation [2].

We have previously generated a mouse model (dtd mouse) in which the murine homologue of the *SLC26A2* gene was knocked-in with a mutation previously identified in a DTD patient. Homozygous mutant mice were shown to reproduce some of the clinical, morphological and biochemical features of DTD in humans, being characterized by growth

Abbreviations: BER, bone elongation rate; BFR, bone formation rate; BMC, bone mineral content; BMD, bone mineral density; BrdU, 5-bromo-2′-deoxyuridine; CTX, C-terminal telopeptides of type I collagen; DEXA, dual energy X-ray absorptiometry; DLS/BS, double labeled surface per bone surface; DPD, deoxypyridinoline; DTD, diastrophic dysplasia; DTDST, diastrophic dysplasia sulfate transporter; FCS, fetal calf serum; MAR, mineral apposition rate; M-CSF, macrophage colony-stimulating factor; P, postnatal day; PBS, phosphate buffer saline; PTH, parathyroid hormone; RANK-L, receptor activator of nuclear factor kappa-B ligand; SLC26A2, solute carrier family 26 member 2; TRAP, tartrate resistant acid phosphatase.

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retardation, skeletal dysplasia, joint contractures and reduced viability. The skeletal phenotype included reduced toluidine blue staining of cartilage, chondrocytes of irregular size, proteoglycan undersulfation in articular cartilage and delayed secondary ossification center formation. Impaired sulfate uptake was observed in chondrocytes, osteoblasts and fibroblasts demonstrating the generalized nature of the sulfate uptake defect [5]. Consistent with the uptake defect, proteoglycan undersulfation was observed also in the growth plate of homozygous mutant mice, causing altered histomorphometric parameters, reduced chondrocyte proliferation, and altered Ihh signaling pathway [6]. However bone studies demonstrated that skeletal defects were not restricted to the articular cartilage or to the growth plate. The sulfate uptake defect was detected also in osteoblasts, and chondroitin sulfate proteoglycans from the femoral diaphysis of mutant mice were slightly but significantly undersulfated between postnatal days P7 and P60. Moreover, signs of early osteoporosis of long bones were detected in dtd mice at P60 [5]. These data demonstrated that a bone phenotype which has never been investigated in DTD patients was present in the dtd mouse.

When studying chondrodysplasias, attention is particularly focussed on the articular and growth plate cartilage, since common features of chondrodysplasias in humans and mice include retarded skeletal development, failure of growth plate chondrocytes to undergo the normal proliferation and maturation pathway, and osteoarthritis [7–15]. Usually patients do not appear to have bone problems such as bone fragility or osteoporosis and for these reasons bone studies are scarce. Nevertheless, the bone phenotype has been analyzed extensively in a few chondrodysplasia mouse models [16–21].

In order to better characterize the bone phenotype in dtd mice and to investigate whether it is a consequence of the cartilage defect or a primary bone defect, we performed radiographies, dual-energy X-ray absorptiometry, and static and dynamic histomorphometry on the long bones of dtd mice, as well as *in vitro* studies on cultured osteoblasts and osteoclasts, and analysis of markers of altered bone homeostasis in serum and urine.

Our results suggest that the bone phenotype in dtd mice is a primary bone defect, which is not due to defects in osteoblast mineralization, osteoclast differentiation or systemic alterations, but to increased degradation of the altered organic bone matrix. Ultimately, these results show an important role for proteoglycan sulfation in bone homeostasis.

Material and methods

Animals

The dtd mouse is a “knock-in” for a c1184t transition causing an A386V substitution in the eighth transmembrane domain of the SLC26A2, which strongly reduces the activity of the transporter. Homozygous mutant mice (dtd) show a chondrodysplastic phenotype that recapitulates essential aspects of human DTD [5]. In this study, 1 and 2 month-old male wild-type and dtd mice with a mixed C57Bl/6J × 129/SV background were used. Older animals couldn't be analyzed due to the reduced life span of dtd mice [5].

Genomic DNA was isolated from mouse tail clips and genotyping to distinguish homozygous mutant animals from heterozygous and wild-type littermates was then performed either by PCR or by Southern blotting.

Animals were bred with free access to water and standard pelleted food. Care and use of mice for this study were in compliance with relevant animal welfare guidelines approved by the Animal Care and Use Committee of the University of Pavia.

Only male animals were considered in this study, since age-related bone changes are more dramatic in C57BL/6J wild-type females than in male littermates [22].

Radiographies and dual-energy X-ray absorptiometry (DEXA)

Radiographies were performed on the tibiae of 1 (n = 12) and 2 (n = 6) month-old wild-type and dtd males mice using an X-ray cabinet (Faxitron).

DEXA was performed on the tibiae of 1 (n = 12) and 2 (n = 6) month-old wild-type and mutant animals using a PIXImus Mouse Densitometer (Lunar GE Medical Systems) in order to evaluate bone area, bone mineral density (BMD) and bone mineral content (BMC).

Bone histology and histomorphometry

Bone histomorphometry was performed as described elsewhere [23]. Briefly, mice were given two fluorochrome labels by intraperitoneal injection: 1 month-old mice (n = 12) were injected with 20 mg/kg tetracycline (Sigma) 3 days before sacrifice, and with 10 mg/kg calcein (Sigma) the day before sacrifice, while 2 month-old animals (n = 6) were injected with 20 mg/kg tetracycline 4 days before sacrifice, and with 10 mg/kg calcein the day before sacrifice.

Mice were sacrificed 24 h after the last injection. Femora were excised immediately after sacrifice and cleaned from the surrounding soft tissues. Bones were trimmed, and their distal halves were post-fixed in 70% ethanol, dehydrated in graded alcohols at 4 °C, defatted in xylene, and embedded without demineralization in methyl methacrylate.

Coronal sections (5 or 12 μm thick) of the central region of the distal femur were cut parallel to the long axis of the bone, using a SM2500S microtome (Leica) with a tungsten carbide knife. Consecutive sections were used for different purposes. Some of them (5 μm thick) were stained for tartrate resistant acid phosphatase (TRAP) detection using Naphthol AS-TR Phosphate (Sigma) as substrate, and counterstained with 0.5% toluidine blue (pH 4.3). Other 5 μm thick sections were stained with 1% toluidine blue (pH 4.3) for evaluation of bone formation parameters or with the Modified Masson's Trichrome staining for the evaluation of static parameters. Other sections (12 μm thick) were left unstained and analyzed under UV light for evaluation of the dynamic parameters. A mean of 6 sections per animal per staining was analyzed.

All measurements were performed in the secondary spongiosa, in an area of about 1.4 mm^2 located about 600 μm away from the growth plate (red squares in Fig. 2, panel A). The histomorphometric parameters were recorded in this standardized area in compliance with the recommendation of the American Society for Bone and Mineral Research Histomorphometry Nomenclature Committee [24].

Trabecular bone volume, trabecular thickness, trabecular number, trabecular separation, bone diameter, marrow diameter, cortical thickness, and osteoid thickness were measured using a semiautomatic image-analysis system (MicroVision) linked to a light microscope. The osteoclast, osteoblast and osteoid surfaces were measured using an objective eyepiece Leitz integrateplatte II. The dynamic parameters were measured in 12 μm thick unstained sections that were examined under UV light. The mineral apposition rate and the bone elongation rate were measured using the semiautomatic image-analysis system linked to a UV light microscope. The mineralizing surfaces were measured in the same area using the objective eyepiece Leitz integrateplatte II.

Scanning electron microscopy

Tibiae of 1 and 2 month-old wild-type and dtd mice (n = 4) were excised immediately after sacrifice, cleaned from the surrounding soft tissues and fixed for 24 h in 4% formaldehyde, 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.4. Tibiae were then isolated and fractured by a knife following a sagittal plane in the central region. After washing in 0.1 M sodium cacodylate buffer, pH 7.4, the samples were first incubated in 5% sodium hypochlorite to remove bone marrow for 10 min at room temperature, and then treated with a 35%