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Impaired osteoclast homeostasis in the cystatin B-deficient mouse model of progressive myoclonus epilepsy

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ABSTRACT

Progressive myoclonus epilepsy of Unverricht–Lundborg type (EPM1) is an autosomal recessively inherited disorder characterized by incapacitating stimulus-sensitive myoclonus and tonic-clonic epileptic seizures with onset at the age of 6 to 16 years. EPM1 patients also exhibit a range of skeletal changes, e.g., thickened frontal cranial bone, arachnodactyly and scoliosis. Mutations in the gene encoding cystatin B (CSTB) underlie EPM1. CSTB is an inhibitor of cysteine cathepsins, including cathepsin K, a key enzyme in bone resorption by osteoclasts. CSTB has previously been shown to protect osteoclasts from experimentally induced apoptosis and to modulate bone resorption in vitro. Nevertheless, its physiological function in bone and the cause of the bone changes in patients remain unknown. Here we used the CSTB-deficient mouse ($Cstb^{-/-}$) model of EPM1 to evaluate the contribution of defective CSTB protein function on bone pathology and osteoclast differentiation and function. Micro-computed tomography of hind limbs revealed thicker trabeculae and elevated bone mineral density in the trabecular bone of $Cstb^{-/-}$ mice. Histology from $Cstb^{-/-}$ mouse bones showed lower osteoclast count and thinner growth plates in long bones. Bone marrow-derived osteoclast cultures revealed lower osteoclast number and size in the $Cstb^{-/-}$ group. $Cstb^{-/-}$ osteoclasts formed less and smaller resorption pits in an in vitro assay. This impaired resorptive capacity was likely due to a decrease in osteoclast numbers and size. These data imply that the skeletal changes in $Cstb^{-/-}$ mice and in EPM1 patients are a result of CSTB deficiency leading to impaired osteoclast formation and consequently compromised resorptive capacity. These results suggest that the role of CSTB in osteoclast homeostasis and modulation of bone metabolism extends beyond cathepsin K regulation.

1. Introduction

Progressive myoclonus epilepsy of Unverricht–Lundborg type (EPM1, OMIM 254800, progressive myoclonus epilepsy type 1) is an autosomal recessively inherited neurodegenerative disorder with onset at the age of 6–16 years. The main symptoms include severely incapacitating action myoclonus, tonic-clonic epileptic seizures and ataxia (Koskiniemi et al., 1974, Kälviäinen et al., 2008). Other central findings include progressive gray and white matter degeneration and changes in cortical excitability (Koskenkorva et al., 2009, Koskenkorva et al., 2012, Danner et al., 2013, Manninen et al., 2013). In addition to the neurological features, EPM1 patients have heterogeneous bone findings (Korja et al., 2007, Suoranta et al., 2012, Danner et al., 2013). They exhibit diffuse thickening of cranial bones in head magnetic resonance imaging (Suoranta et al., 2012). Evaluation of digital head computed tomography (CT) scans have implied osteoporotic bone structure, but, bone mineral density has not been systematically determined (Suoranta et al., 2012). The majority of EPM1 patients also show other skeletal changes, most commonly arachnodactyly, scoliosis, enlarged sinuses, and accessory ossicles of the foot (Suoranta et al., 2012), the etiology of which is currently unknown.

EPM1 is caused by loss-of-function mutations in the *CSTB* gene encoding cystatin B (CSTB), an inhibitor of lysosomal cysteine cathepsins, including cathepsin K (Pennacchio et al., 1996, Lalioti et al., 1997). A mouse model ($Cstb^{-/-}$) of the EPM1 disease has been created with targeted disruption of the *Cstb* gene. The mouse model

Abbreviations: CSTB, cystatin B; EPM1, progressive myoclonus epilepsy of Unverricht–Lundborg type; μCT, micro-computed tomography.

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recapitulates the principal symptoms of EPM1, myoclonic seizures and progressive ataxia (Pennacchio et al., 1998). Pathological findings in *Cstb*$^{-/-}$ mice match those in the patients: the mice exhibit progressive loss in brain volume due to neuronal death and consequent white matter atrophy in the cerebrum and cerebellum (Tegelberg et al., 2012, Manninen et al., 2013, 2014). The earliest pathological finding in *Cstb*$^{-/-}$ mice is striking microglial activation present at postnatal day 14 and preceding the onset of myoclonus and progressive neuronal degeneration from 1 month of age onwards (Tegelberg et al., 2012).

Bone resorbing osteoclasts and bone forming osteoblasts continuously remodel the skeleton to maintain optimal bone structure (Kular et al., 2012). Cathepsin K is one of the fundamental enzymes in osteoclastic bone resorption (Krake et al., 1996; Rantakokko et al., 1996, Littlewood-Evans et al., 1997). CSTB is a known inhibitor of cathepsin K and an in vitro study has shown that it has a role in regulating bone resorption by inhibiting cathepsin K activity and osteoclast apoptosis (Laitala-Leinonen et al., 2006). Overexpression of cathepsin K is known to accelerate the resorption cycle (Morko et al., 2009) and to lead to thickening of cortex in long bone (Morko et al., 2005). The ossification of the calvaria and phalanges is different from rest of the skeleton and Suoranta et al. (2012) proposed that the bone changes in EPM1 patients, most importantly, the thickening of the skull and arachnodactyly, could be mediated through the lack of cathepsin K inhibition, caused by reduced CSTB in cells and this would lead to altered osteoclast function.

Here we utilized the *Cstb*$^{-/-}$ mouse model to evaluate the role of CSTB in bone metabolism and especially in osteoclastic bone resorption by combining micro-computed tomography (μCT) with histological and immunohistochemical analysis of bone tissue and by characterizing bone marrow-derived cultured osteoclasts. Our results show alterations in bone structure of *Cstb*$^{-/-}$ mice and imply impaired formation of mature osteoclasts with consequently compromised resorptive capacity as key phenomena contributing to the altered bone properties.

2. Materials & methods

2.1. Mouse model

Cstb$^{-/-}$ mice (Sv129-*Cstbtm1Rm/J*; stock 003486; Jackson Laboratories) between 1 of 4 months of age and wild-type animals of same gender and background as controls were studied. Animals were maintained under a 12 h light/12 h dark cycle, temperature 22 ± 1 °C, and with free access to food and water. The experiments were approved by the Animal Experiment Board of Finland (permits ESLH-2007-05788/Ym-23, ESAVI-2010-07744/Ym-23 and ESAVI/7039/04.10.03/2012) and conducted in agreement with the guidelines set by the European Community Council Directives 86/609/EEC.

2.2. Micro-computed tomography

Ex vivo micro-computed tomography (μCT) with SkyScan 1070 μm CT scanner (SkyScan, Kontich, Belgium) was performed on the right hind limb of 3-month-old male mice (*Cstb*$^{-/-}$, n = 5; controls, n = 4). Animals were perfusion fixed with 4% paraformaldehyde and hind limbs post fixed for 24 h, then stored immersed in 70% EtOH. The fixed hind limbs were inserted into a sealed tubular plastic imaging holder (manufactured in-house). The following parameters were used for the scanning: voxel resolution 20 μm; X-ray tube potential 70 kVp; current 200 μA; and integration time 3900 ms. The object was rotated in 0.45° steps (total 182.45°). Cross-sectional image reconstruction using Nrecon software version 1.4 (SkyScan, Kontich, Belgium) was performed with the following parameters: misalignment <3, ring artifact reduction 11, beam hardening correction 95% and intensity gap 0.005–0.150. Analysis and modeling were performed using CTan version 1.4.4 (SkyScan, Kontich, Belgium). Cross-sectional images were binarized using an attenuation coefficient range of 0.005 to 0.150. In trabecular bone, a volume of interest (VOI) excluding the cortical bone was defined at the metaphysis of the tibia, starting 20 layers (400 μm) below the lowest point of the growth plate and covering 50 layers (1000 μm). Cortical bone analysis was done from diaphysis of the femurs, with VOI starting 200 layers (4000 μm) above the growth plate and covering 100 layers (2000 μm). Finally, the 3D, 2D and bone mineral density results were calculated into numerical data, with low and high threshold values set to 35 and 250 respectively, after which the data were analyzed.

2.3. Bone histology

2.3.1. Samples

Bone histology was performed on the hind limbs of *Cstb*$^{-/-}$ and control mice, age 1 month (*Cstb*$^{-/-}$, n = 13; controls; n = 13) and 4 months (*Cstb*$^{-/-}$, n = 13; controls, n = 13) Animals were perfusion fixed with 4% paraformaldehyde, hind limbs removed and post fixed for 24 h, then stored immersed in 70% EtOH. Samples were prepared from distal femurs and proximal tibias, and the bones were decalcified in 5% formic acid, embedded in paraffin and cut into 5 μm-thick sections for mounting on microscopy slides. Femur length was also determined using a micrometer (*Cstb*$^{-/-}$, n = 5; controls, n = 3).

2.3.2. Quantification of bone morphology

Bone morphology was analyzed using hind limb samples from 1-month-old animals (*Cstb*$^{-/-}$, n = 8; controls, n = 8). Quantitative analysis of the thickness of the growth plate and its proliferative- and hypertrophic zones was performed as previously described (Nurmio et al., 2011). Briefly, bone sections were stained with hematoxylin and quantified using Leica QWin Pro analysis system (Leica Microsystems GmbH, Wetzlar, Germany). The number of mature tartrate-resistant acid phosphatase (TRACP, stained with the leukocyte acid phosphatase kit (Sigma Aldrich, St. Louis, Missouri, USA)) positive multinucleated osteoclasts was counted as described previously (Laitala-Leinonen et al., 2006) from samples taken from 1-month-old (*Cstb*$^{-/-}$, n = 13; controls, n = 13) and 4-month-old animals (*Cstb*$^{-/-}$, n = 13; controls; n = 13).

2.4. Characterization of cultured osteoclasts

2.4.1. Bone marrow-derived osteoclast cultures

Osteoclasts were cultured as described previously (Laitala-Leinonen et al., 2006). In brief, animals were euthanized with CO_2 and decapitated. Bone marrow was harvested from both femurs and tibias, and homogenized to a single cell suspension. The cell suspension was then allowed to adhere for 4 h, after which the non-adhering cells, including the macrophage precursors, were plated at 100,000 cells/well on 96-well plates in α-MEM. The culture medium was supplemented with macrophage colony stimulating factor (M-CSF) 20 ng/ml (RnD systems, Minneapolis, MN, USA), which induces the hematopoietic stem cells to differentiate into macrophages. Every 48 h half of the media was replaced and receptor activator of nuclear factor kappa-B ligand (RANKL, Peprotech, Offenbach, Germany) 100 ng/ml was added to facilitate osteoclast differentiation.

2.4.2. TRACP staining of cultured osteoclasts

First, osteoclast morphology and number was determined from cultures.

The osteoclasts were cultured for 6 days on RANKL, after which they were fixed with 4% PFA and stained with the leukocyte acid phosphatase kit (Sigma Aldrich, St. Louis, Missouri, USA). For determining the number and the area of mature osteoclasts, the samples were imaged using Olympus ScanR system (Olympus GmbH, Hamburg, Germany), a modular epifluorescence microscope designed for fully automated image acquisition. The number of mature osteoclasts, defined a TRACP-positive multinuclear (three or more nuclei) cells, was counted from 22 wells for both *Cstb*$^{-/-}$ and wild type controls (cells pooled from 3 mice per group in three independent experiments). The size of