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Bone matrix hypermineralization in prolyl-3 hydroxylase 1 deficient mice☆

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ABSTRACT

Lack of prolyl 3-hydroxylase 1 (P3H1) due to mutations in *P3H1* results in severe forms of recessive osteogenesis imperfecta. In the present study, we investigated the bone tissue characteristics of P3H1 null mice. Histomorphometric analyses of cancellous bone in the proximal tibia and lumbar vertebra in 1-month and 3-month old mice demonstrated that P3H1 deficient mice had low trabecular bone volume and low mineral apposition rate, but normal osteoid maturation time and normal osteoblast and osteoclast surfaces. Quantitative backscattered electron imaging revealed that the bone mineralization density distribution was shifted towards higher values, indicating hypermineralization of bone matrix. It thus appears that P3H1 deficiency leads to decreased deposition of extracellular matrix by osteoblasts and increased incorporation of mineral into the matrix.

1. Introduction

Osteogenesis imperfecta (OI) is a heritable connective tissue disorder that is mainly characterized by bone fragility. Extra-skeletal findings, such as tooth abnormalities (dentinogenesis imperfecta), blue or gray sclera and hearing impairment can be associated [1–3]. OI is usually transmitted in an autosomal dominant fashion and is mostly caused by mutations in *COL1A1* and *COL1A2*, the genes encoding the collagen type I alpha chains [3]. More recently, recessively inherited forms of OI have been discovered caused by genes whose molecules modify or interact with collagen post-translationally [3–7]. The first description of non-collagenous gene mutation leading to OI was a loss-of-function in cartilage-associated-protein (CRTAP) [8]. Deficiency in CRTAP causes OI type VII, a severe to lethal form of the disease [9–11]. CRTAP is a component of the 3-hydroxylation complex residing in the endoplasmic reticulum (ER), consisting of prolyl 3-hydroxylase 1 (P3H1) a collagen-modifying enzyme that is encoded by *P3H1*, CRTAP, the helper protein and Cyclophilin B (encoded by *PPIB*). The three proteins form a stable complex in a 1:1:1 ratio in the ER. The complex binds collagen post-translationally and hydroxylates a single residue, proline 986, on each α1(I) chain. Inactivating mutations in *CRTAP* result in a loss of function of the prolyl 3-hydroxylation complex and in an overmodification of the collagen helix by ER resident prolyl 4-hydroxylase and lysyl hydroxylases. The role of the highly conserved 3-hydroxylation during collagen biosynthesis remains largely unclear. The complex seems to be not only important for its enzymatic activity but also for its chaperone effects on the newly formed collagen chain [12–15]. Mice lacking any of the three components have a low bone mass phenotype, kyphosis, collagen fibrils with irregular fibril diameter, and skeletal abnormalities that resemble human osteogenesis imperfecta [8,16,17]. However, in contrast to the situation in affected humans who develop very severe forms of OI, these mice survive the perinatal period, indicating that the murine phenotype is less severe than in humans [8,16–19].

CRTAP knock-out animals lack 3-hydroxylation of proline 986 on both α1(I) and α1(II) collagen chains, have growth deficiency, osteopenia, rhizomelia and develop severe kyphosis [8]. Moreover, these mice have an increased bone matrix mineralization density, a characteristic also observed in other OI mouse models such as the oim mouse, and in children with autosomal dominant OI due to collagen gene mutations, as well as in patients with OI type VII having residual CRTAP activity [20–24].

P3H1 3-hydroxylates certain proline 986 residues in the triple helical domain of collagen type I and possibly other types of collagen [13]. Lack of P3H1 due to mutations in *P3H1* results in severe forms of recessive OI, which is very often lethal in the perinatal period and resembles

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OI types IIB and III [25,26]. Few long-term survivors with this type of mutations have been reported so far [27–31]. The general phenotype of the P3H1 deficient mouse model has previously been reported [16] and is characterized by abnormalities in skin, tendon and bone due to collagen fibril disturbances, as well as hearing loss [14]. P3H1 deficient mice were viable and fertile, but had shorter, less radiodense and weaker bones than wild type littermates. Collagen folding and secretion by skin fibroblasts was delayed, leading to overmodification of collagen molecules and disturbances in the assembly of higher-order collagen fibrils. However, the effects of P3H1 deficiency on bone at tissue and material levels have not been described in any detail. In the present study, we report on bone tissue characteristics of P3H1 null mice, a model of human OI.

2. Methods and materials

2.1. Animals

P3H1 null mice with deletions of exons 1–3 (nucleotides 15–817) of *P3h1* were used [16]. Animals were housed in the Animal Care Facility of the Shriners Hospital Portland and all procedures were performed in accordance with institutional and national guidelines and were approved by the Oregon Health & Science University Institutional Animal Care and Use Committee. Mice were bred multiple generations into a C57Bl6 background prior to analysis. Analyses were performed on homozygous P3h1$^{-/-}$ knockout mice, as well as their heterozygous P3h1$^{+/-}$ and homozygous P3h1$^{+/+}$ littermates. Mice were analyzed at either 1 month (growing period, 'pediatric model') or at 3 months of age (end of growth period, 'young adult model'). Male and female mice were analyzed separately. Eight mice of each sex per genotype were used. To enable the analysis of dynamic histomorphometric measures, each mouse received two intraperitoneal injections of calcein (30 mg per kg body weight) at 5 days and at 2 days before sacrifice.

2.2. Bone histomorphometry

Histomorphometric analyses of trabecular bone were performed at the left proximal tibia (50 μm distal to the growth plate for a distance of 1.4 mm) and at lumbar vertebra L4. Specimens were fixed in 10% phosphate-buffered paraformaldehyde, dehydrated in increasing concentrations of ethanol and embedded in methylmethacrylate. Undecalcified 6 μm thick sections were cut with a Polycut E microtome (Reichert-Jung, Heidelberg, Germany). The sections were deplastified with ethylene glycol monoethyl acetate to allow for optimal staining. In each sample, two consecutive sections were selected that were stained with Masson Goldner Trichrome for static parameters or mounted unstained for the measurement of dynamic parameters using fluorescence microscopy. Measurements were carried out using a digitizing table with Osteomeasure® software (Osteometrics Inc., Atlanta, GA, USA). Nomenclature and abbreviations follow the recommendations of the American Society for Bone and Mineral Research [32]. In the trabecular compartment of the proximal tibia, osteoblasts, osteoclasts and osteoid were not reliably identifiable. Therefore, parameters based on these tissue components were not measured at that location and results were limited to bone structural parameters and dynamic bone formation parameters. In contrast, osteoblasts, osteoclasts and osteoid were identifiable in the trabecular compartment of the L4 vertebra, and therefore parameters based on these tissue components were measured at the L4 vertebra. At both the proximal tibia and the L4 vertebra, we measured cartilage volume per bone volume, which represents the relative amount of growth plate material that persists within secondary trabeculae. Growth plate material within trabecular bone was identified by its light green color in Goldner stained sections (as opposed to the dark green color of mineralized bone).

2.3. Quantitative backscattered electron imaging (qBEI)

Backscattered electron imaging was performed exclusively at the tibia of male mice, in order to limit the number of samples. Sectioned bone surfaces of sample blocks were sequentially ground with sand paper with increasing grid number followed by polishing with diamond grains (size down to 1 μm) on hard polishing clothes by a precision polishing device (PM5 Logitech, Glasgow, Scotland). Finally, the sample surface was carbon coated by vacuum evaporation (Agar SEM Carbon Coater, Stansted, UK) for scanning electron microscopy.

Bone mineralization density distribution (BMDD) was determined in the metaphyseal spongiosa, the epiphyseal spongiosa and in the midshaft cortical bone by qBEI using a digital scanning electron microscope (DSM 962; Zeiss, Oberkochen, Germany) equipped with a four-quadrant semiconductor backscattered electron detector as described before [20,33]. The accelerating voltage of the electron beam was 20 kV, the current probe 110 pA, and the working distance 15 mm. We used carbon (Z number 6) and aluminum (Z number 13) as reference materials for the backscattered electron atomic contrast. Contrast and brightness control of the DSM were adjusted in such a way that the histogram peak of the carbon target was located at 25 ± 1 gray-level index and that of aluminum at 225 ± 1 gray-level index. Based on the fact that the bone tissue embedded in polymethylmethacrylate is composed of an organic phase (mainly collagen) with an average Z number ~ 6 and a mineral phase with an average Z number ~ 14 (hydroxyapatite), the gray-levels could be transferred to weight% mineral and/or weight% Ca values [34]. The cancellous and cortical bone areas were imaged at 200× nominal magnification (corresponding to a pixel resolution of 1 μm/pixel). From these digital images, gray level histograms were deduced, displaying the percentage of bone area occupied by pixels of a certain gray level. The transformation of these into calcium weight percent (wt% Ca) histograms led to a bin width of 0.17 wt% Ca. A technical precision of 0.3% was achieved. The derived BMDD parameters are: the mean calcium concentration (CaMean; weighted mean calcium content), the most frequently occurring calcium concentration (CaPeak; the peak position of the BMDD) in the sample, the width of the BMDD distribution (CaWidth; full width at half maximum) reflecting the heterogeneity in matrix mineralization; the percentage of lowly mineralized bone, CaLow, is defined as the area below 17.68 weight% calcium. This cut-off corresponds to the 5th percentile of the reference BMDD in human adults. We have previously shown that this value mirrors the transition from the phase of primary to secondary mineralization with a much lower speed of mineral accumulation. Thus, CaLow reflects the portion of the bone area undergoing primary mineralization [34]. The percentage of highly mineralized bone (CaHigh) is defined as the percentage of bone matrix, which is mineralized above the 95th percentile value of the corresponding control animals BMDD. This latter value was defined from the BMDDs of each age group of controls and each skeletal site (metaphyseal, epiphyseal and cortical midshaft).

2.4. Statistics

Eight mice per group were used to be able to identify group differences in mean values that corresponded to at least 1 standard deviation of the result in control animals. Normality of the data was tested by Kolmogorov–Smirnov test. Normally distributed data are given as mean and standard deviation (SD), non-normally distributed data by median [25th percentile; 75th percentile] in the tables. The significance of the differences in histomorphometric data between the three genotypes was assessed by analysis of variance (ANOVA) followed by Dunn's pairwise comparison if data were normally distributed, or by Kruskal Wallis tests if data were not normally distributed. The BMDD data were analyzed by two-factor analysis of variance (ANOVA) to test for the simultaneous effect of genotype and age followed by a pairwise comparison using Tukey's multiple comparison post-tests. Statistical analyses were performed using SPSS version 20 (SPSS Inc.) and Graph