Molecular Genetics and Metabolism xxx (xxxx) xxx–xxx

Contents lists available at ScienceDirect

Molecular Genetics and Metabolism

journal homepage: www.elsevier.com/locate/ymgme

Regular Article

Delayed development of ossification centers in the tibia of prenatal and early postnatal MPS VII mice

Zhirui Jiang[a,b], Ainslie L.K. Derrick-Roberts[a,b,c], Matilda R. Jackson[a,b], Charné Rossouw[b,c], Carmen E. Pyragius[b], Cory Xian[c,d], Janice Fletcher[b], Sharon Byers[a,b,c,*]

[a] Genetics & Evolution, University of Adelaide, Adelaide, SA, Australia
[b] Genetics and Molecular Pathology, SA Pathology, Adelaide, SA, Australia
[c] Paediatrics, Adelaide Medical School, University of Adelaide, Adelaide, SA, Australia
[d] School of Pharmacy and Medical Sciences, University of South Australia, Adelaide, SA, Australia

ARTICLE INFO

Keywords:
Mucopolysaccharidosis
Short stature
Murine models
Endochondral ossification
Primary ossification center
Secondary ossification center
Growth plate

ABSTRACT

Short stature is a characteristic feature of most of the mucopolysaccharidoses, a group of inherited lysosomal storage disorders caused by a single enzyme deficiency. MPS patients present with progressive skeletal defects from an early age, including short stature due to impaired cartilage-to-bone conversion (endochondral ossification). The aim of this study was to determine which murine MPS model best reproduces the bone length reduction phenotype of human MPS and use this model to determine the earliest developmental stage when disrupted endochondral ossification first appears. $Gus^{mps/mps}$ mice representing severe MPS VII displayed the greatest reduction in bone elongation and were chosen for histopathological analysis. Tibial development was assessed from E12.5 to 6 months of age. Chondrocytes in the region of the future primary ossification center became hypertrophic at a similar age to normal in the MPS VII mouse fetus, but a delay in bone deposition was observed with an approximate 1 day delay in the formation of the primary ossification centre. Likewise, chondrocytes in the region of the future secondary ossification center also became hypertrophic at the same age as normal in the MPS VII early postnatal mouse. Bone deposition in the secondary ossification centre was delayed by two days in the MPS VII proximal tibia (observed at postnatal day 14 (P14) compared to P12 in normal). The thickness of the tibial growth plate was larger in MPS VII mice from P9 onwards. Abnormal endochondral ossification starts *in utero* in MPS VII and worsens with age. It is characterized by a normal timeframe for chondrocyte hypertrophy but a delay in the subsequent deposition of bone in both the primary and secondary ossification centres, accompanied by an increase in growth plate thickness. This suggests that the signals for vascular invasion and bone deposition, some of which are derived from hypertrophic chondrocytes, are altered in MPS VII.

1. Introduction

Mucopolysaccharidoses (MPS) are a group of inherited lysosomal storage disorders resulting from the absence of or a deficiency in, enzymes that are involved in the step-wise degradation of glycosaminoglycans (GAGs) [1]. Undegraded GAGs accumulate in multiple tissues and organs, causing a range of symptoms including hepatosplenomegaly, corneal clouding, upper airway disease, cardiac defects, progressive central nervous system deterioration and a skeletal dysplasia characterized by distinctive radiographic changes, reduced joint mobility and short stature [1]. Skeletal disease is a common clinical manifestation in six types of MPS (MPS I, II, IVA, IVB, VI and VII). Bone growth velocity declines significantly after one year of age, with minimal long bone growth from two years of age, while other skeletal symptoms become progressively worse with age [2–7]. The final height z-score of affected children ranges from −3.0 to −6.0 standard deviations below normal [8,9]. Hematopoietic stem cell transplantation (HSCT) and enzyme replacement therapy (ERT) are clinically approved therapies for MPS patients. Although a small increase in growth velocity is observed in some MPS I, II and VI patients post HSCT or as a result of ERT [9–14], the long term benefit is limited [15–22].

The majority of bones in the human skeleton are formed by the process of endochondral ossification (EO) in which a cartilage template is replaced by bone. *In utero*, mesenchymal cells condense and differentiate into chondrocytes, forming a cartilaginous model of the future

* Corresponding author at: Genetics and Molecular Pathology, SA Pathology, 72 King William Rd, North Adelaide, SA 5006, Australia.
E-mail address: sharon.byers@adelaide.edu.au (S. Byers).

https://doi.org/10.1016/j.ymgme.2018.04.014
Received 7 March 2018; Received in revised form 30 April 2018; Accepted 30 April 2018

skeleton [23–25]. Chondrocytes in the centre of the model proliferate and increase in size (hypertrophy), while the surrounding matrix is calcified. Blood vessels originating in the periosteum invade the calcified region bringing osteogenic and endothelial precursor cells thus creating the primary ossification centre (POC) [26,27]. At either end of the POC chondrocytes assemble into the characteristic zonal morphology of the growth plate. After birth, the secondary ossification centers (SOC) develop in the epiphyseal regions in a similar manner as the POC *via* the invasion of cartilage canals from the perichondrium bringing osteogenic and endothelial precursors [28]. The growth plate occupies the region between the POC and SOC with chondrocytes arranged into three morphologically distinct zones: the resting zone (RZ), proliferative zone (PZ) and hypertrophic zone (HZ) [23,29]. Chondrocytes enter and progress through the cell cycle to undergo several rounds of proliferation, and then withdraw from the cell cycle, progressing to hypertrophic differentiation, in order to produce a mineralised cartilage matrix that becomes new bone [30,31].

In common with MPS patients, numerous animal models of MPS also display skeletal abnormalities [32–36]. Disorganized growth plate structures and decreased long bone lengths have been reported in MPS VII mice [32,37–40], suggesting impaired endochondral ossification. The mechanism linking progressive cellular GAG storage to skeletal pathology is not fully understood in MPS, limiting the development of effective therapies. Storage of undegraded GAGs occurs early in life and is observed in fetal tissues from MPS VII humans, MPS VII mice and MPS VI cats [41–44]. Although the pattern of chondrogenesis, osteogenesis and ossification during limb development have been well studied in normal mice [45,46], no comparative analyses have been reported in MPS mouse models.

In the present study, the greatest reduction in bone elongation was observed in the *GUS*$^{mps/mps}$ strain of murine MPS VII. This strain represents the more severe end of the MPS VII phenotype spectrum. Tibial development was therefore assessed from E12.5 to 6 months of age in normal and MPS VII mice (*GUS*$^{mps/mps}$). Chondrocytes underwent hypertrophic expansion as the primary and secondary ossification centres formed at the same age in both genotypes but bone deposition was delayed in both ossification centers in the MPS VII tibia. Abnormal development was observed in MPS VII tibiae from as early as E15.5 and was established by birth.

2. Materials and methods

2.1. Animal husbandry

All research procedures using mice were approved by the Womens and Children's Health Network and The University of Adelaide animal ethics committees. Mice were housed on a 14/10 light/dark cycle with food and water provided *ad libitum*. Founder animals for MPS I, MPS IIIA, severe MPS VII (Gus$^{mps/mps}$ strain) and attenuated MPS VII (Gus$^{tm((L175F)Sly}$ strain) were obtained from Jackson labs (Bar Harbor, Maine, USA), while MPS IX founder mice were obtained from MMRRC (NIH, USA). MPS affected and normal mice were bred from heterozygous parents. Heterozygous female and male mice were paired between 3.30 and 4.00 pm and the males removed the next morning (E0.5). Pregnant females were killed at E12.5 to E18.5 and embryos were dissected from the uterus and fixed in into 10% (*v*/v) neutral buffered formalin (NBF). A piece of tail was collected for genotyping as previously described [38,39,47,48]. Additional mice were killed between P1 and 6 months of age and the hindlimbs dissected out free of soft tissue fixed in 10% (v/v) NBF.

2.2. Radiography and bone length measurements

Femur, tibia and L4-L6 vertebrae from 6 month old normal and MPS mice were X-rayed on a detailed cassette at 100 cm at 56 kVp and 4 mAs using a Kodak CR system (Kodak, VIC, Australia) as previously described [38]. Additional radiographs of femurs from MPS I, severe MPS VII and attenuated MPS VII mice along with normal littermates were taken at 14 day, 1 month, 2 month and 3 months of age. X-ray film was scanned, converted to digital data and calibrated. Femur, tibia and L5 vertebrae lengths were measured using Olympus analySIS® LS Research Olympus Soft Imaging Solutions version 3.1 (Olympus Australia Pty. Ltd., Gulfview Heights, SA).

2.3. Proximal tibia morphology

Hindlimbs were fixed in 10% (v/v) NBF for 24 h and then decalcified in Immunocal™ decalcifier (StatLab, TX, USA) for one day (up to age E18.5) or for at least seven days (P5 and older) prior to routine processing and embedding in paraffin blocks in the sagittal plane. Three serial 5-micron sections were cut using a Leica RM2235 microtome at 50 µm intervals across the tibia (Leica Microsystems Pty Ltd., NSW, Australia). Sections corresponding to the mid-depth of the tibia were brought to water using Solv21C (United Bioscience, VIC, Australia), 100% ethanol, 70% (v/v) ethanol and dH_2O and then stained for five minutes in 0.07% (w/v) Fast-Green stain ((ProSciTech, QLD, Australia), dipped in 1% (v/v) acetic acid then stained with 0.07% (w/v) Safranin-O (ProSciTech, QLD, Australia) for five minutes. Sections were dehydrated in 70% (v/v) ethanol, 100% ethanol then xylene. Stained samples were mounted using Leica CV Mounting Media, viewed under an Olympus BX41 microscope (Olympus Australia Pty. Ltd., Gulfview Heights, SA, Australia) and analyzed using Olympus analySIS® LS Research Olympus Soft Imaging Solutions (version 3.1) software.

2.4. Quantification of the area occupied by the secondary ossification center

The area occupied by the SOC was measured on the proximal tibial epiphysis from normal and MPS VII mice aged from P14 days to 6 months. Serial sections were taken across the bone as described above. Three paraffin sections per animal corresponding to the mid-depth of the tibia were stained with Safranin-O/Fast Green. Using an Olympus BX41 microscope and Olympus analySIS® LS Research Olympus Soft Imaging Solutions (version 3.1) software a line was drawn around the SOC defined as containing bone or marrow but no cartilage and a line drawn around the epiphysis comprising the area bounded by the articular cartilage, perichondrium and the chondro-osseous junction. The area occupied by the SOC was calculated and expressed as a percentage of the total epiphyseal area. Measurements from the 3 sections were averaged to yield a single value for each animal.

2.5. Statistics

Statistical significance was determined by 2-way ANOVA plus Tukey's post-hoc test using Graph Pad Prism 7.0. Statistical significance was assumed when $p < 0.05$.

3. Results

3.1. Bone growth in mouse models of MPS

Radiographs were taken of MPS I, MPS IIIA, severe MPS VII (Gus$^{mps/mps}$), attenuated MPS VII (*Gus*$^{tm(L175F)Sly}$ strain) and MPS IX hindlimbs at 6 months of age with the most obvious visual difference being the shortened bone length of the severe MPS VII model (Fig. 1A). Femur length (Fig. 1B) was significantly decreased in murine MPS I and both the severe and attenuated MPS VII severe strains reaching 95.5 ± 0.7%, 66.8 ± 1.3%, and 92.6 ± 1.1% of normal length respectively. MPS IIIA and MPS IX femur length was not different to normal at 100.2 ± 0.7% and 99 ± 2.3% of normal respectively. Tibia length (Fig. 1C) was significantly reduced in murine severe and attenuated MPS VII strains (reaching 80.9 ± 3.3% and 96.3 ± 0.6% of