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Postnatal growth and bone mass in mice with IGF-I haploinsufficiency

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Abstract

We examined the influence of IGF-I haploinsufficiency on growth, bone mass and osteoblast differentiation in *Igf1* heterozygous knockout (HET) mice. Cohorts of male and female wild type (WT) and HET mice in the outbred CD-1 background were analyzed at 1, 2, 4, 8, 12, 15 and 18 months of age for body weight, serum IGF-I and bone morphometry. Compared to WT mice, HET mice had 20–30% lower serum IGF-I levels in both genders and in all age groups. Female HET mice showed significant reductions in body weight (10–20%), femur length (4–6%) and femoral bone mineral density (BMD) (7–12%) before 15 months of age. Male HET mice showed significant differences in all parameters at 2 months and thereafter. At 8 and 12 months, WT mice also showed a significant gender effect: despite their lower body weight, female mice had higher femoral BMD and femur length compared to males. Microcomputed tomography showed a significant reduction in cortical bone area (7–20%) and periosteal circumference (5–13%) with no consistent pattern of change in trabecular bone measurements in 2- and 8-month old HET mice in both genders. HET primary osteoblast cultures showed a 40% reduction in IGF-I protein expression and a 50% decrease in IGF-I mRNA expression. Cell growth and proliferation were decreased in HET cultures. Thus, IGF-I haploinsufficiency in outbred male and female mice resulted in reduced body weight, femur length and areal BMD at most ages. Serum IGF-I levels showed a high level of positive correlation with body weight and skeletal morphometry. These studies show that IGF-I is a determinant of bone size and mass in postnatal life. We speculate that impaired osteoblast proliferation may contribute to the skeletal phenotype of mice with IGF-I haploinsufficiency.

Keywords: Insulin-like growth factor I; Bone mass; Bone morphometry; Postnatal growth; Osteoblast

Introduction

The IGF regulatory system, comprised of multiple ligands, receptors, binding proteins and proteases, plays a key role in growth and development during both embryonic and postnatal life [1,2]. Although IGF-I and IGF-II are important growth factors in prenatal development, IGF-II expression diminishes after birth and IGF-I becomes the critical growth factor in postnatal life. The primary source of circulating IGF-I is liver; however, IGF-I is also expressed in almost all other tissues including bone. Both local and serum IGF-I levels are regulated by systemic hormones including growth hormone (GH), parathyroid hormone (PTH), gonadal steroids and local cytokines [3].

IGF-I mediates the stimulatory effect of growth hormone on longitudinal bone growth [1,2] and is required for the anabolic actions of PTH on bone [4]. IGF-I is the most abundant growth factor produced by osteoblasts. Once secreted, IGF-I is stored in bone matrix in association with IGF binding proteins and is released upon bone resorption. Many in vitro studies show that IGF-I increases osteoblast proliferation, matrix synthesis and mineralization and decreases apoptosis [3,5]. IGF-I also stimulates osteoclast formation and resorption [6,7], suggesting that IGF-I is an important coupling factor for bone remodeling.

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Studies in mice and humans have provided important information regarding the role of IGF-I in bone mass acquisition [8,9]. There is a positive correlation between serum IGF-I levels and bone mass in both inbred mouse strains and in humans [10–12]. Low circulating IGF-I is linked to low bone mineral density, a predisposition to the development of osteoporosis and a greater risk of hip and spine fractures [13,14]. By contrast, systemic administration of rhIGF-I or an IGF-I/IGFBP-3 complex increased bone formation parameters in patients [15,16]. However, high circulating IGF-I levels are associated with several neoplasias including prostate cancer and breast tumors [17–19].

Mice with a global knockout of the *Igf1* gene have greatly impaired prenatal development and a high degree of perinatal lethality [20]. Their extremely low survival rate makes it difficult to investigate the many functions of IGF-I in postnatal life. By contrast, *Igf1* heterozygous knockout mice (HET), which have one functional *Igf1* allele, are viable and breed normally. These mice have only moderately decreased serum IGF-I levels and body size [20,21]. Therefore, we reasoned that HET mice would provide a model for studying the age-related changes in bone that occur when IGF-I levels are only modestly decreased. Although outbred CD-1 mice may exhibit more phenotypic variability compared to genetically homogeneous inbred strains, they allowed us to take normal population variation into account while investigating the effects of IGF-I haploinsufficiency on postnatal growth and bone morphometry in WT and HET mice from 1 to 18 months of age.

Materials and methods

Experimental animals

Igf1 knockout mice were generated by Dr. Powell-Braxton and co-workers [20]. A neomycin cassette was inserted into exon 3 of the *Igf1* gene, which interrupts all reading frames of the B domain of IGF-I. Knockout mice were bred into the CD-1 background and housed in a pathogen-free barrier. Breeding pairs of male HET and female WT mice were established to obtain experimental mice. All animal work was performed using protocols approved by the institutional Animal Care Committee of the University of Connecticut Health Center, Farmington, CT.

PCR strategy for genotyping

Genotypes of the offspring were determined by PCR of tail DNA using a commercially available kit (Promega Corporation, Madison, WI). Two sets of primer pairs, one flanking exon 3 of *Igf1* (WT allele) and one for the *neo* cassette (disrupted allele), were used to genotype mice: primers 5′-GACCAGTAG-CAAAGGACTTACCAC-3′ and 5′-AAGTAAAAGCCCCTCGGTCCACAC-3′ generate a 366-bp product identifying the WT allele; primers 5′-TGACTGGGCACAA-CAGACAATCGG-3′ and 5′-GTAGCCAACGC-TATGTCCTG-ATAG-3′ generate a 608-bp product identifying the null allele [20]. PCR cycles (32) were 94°C, 30 s; 65°C, 30 s; and 72°C, 2 min. PCR products were fractionated by electrophoresis on a 1% agarose gel in 1× TBE buffer and visualized by ethidium bromide staining. DNA from HET mice produced both PCR products.

Body weight, femur length and bone mineral density (BMD)

Mice were sacrificed at 1, 2, 4, 8, 12, 15 and 18 months of age, and body weights were recorded. Femurs and L3 vertebrae were dissected from HET mice and WT littermates and stored in 70% ethanol. Femur length was measured from the distal end of the condyles to the proximal end of the femoral head using digital calipers (Fowler Eurocal III Newton, Massachusetts). Measurements were done for both right and left femurs, and the values were averaged for each mouse. Areal BMD and bone mineral content (BMC) were measured by dual energy X-ray absorptiometry (DEXA) with a Piximus Mouse Densitometer (Lunar corporation, WI, USA).

X-ray microcomputed tomography (microCT)

Trabecular morphometry within the metaphyseal region of distal femurs and centrum of the third lumbar vertebrae (L3) from 2- to 8-month-old mice was quantified using X-ray microcomputed tomography (μCT40, Scanco Medical AG, Bassersdorf, Switzerland). Specimens were scanned in 70% ETOH at 55 kV (145 μA), employing 1000 conebeam projections per revolution and an integration time of 300 ms within a 12.3 mm diameter field of view. Three-dimensional images were reconstructed at 12 μm resolution using standard convolution backprojection algorithms with Shepp and Logan filtering and rendered at a discrete voxel density of 578,704 voxels/mm^3 (isometric 12 μm voxels). Segmentation of bone from marrow and soft tissue was performed at a global threshold of 315 mg/cm^3 in conjunction with a constrained Gaussian filter to reduce noise. Volumetric analysis regions of trabecular bone were selected within the endosteal borders to include the central 80% of vertebral height and secondary spongiosa of femoral metaphyses located 960 μm (~5% of length) from the growth plate. Trabecular morphometric parameters were measured directly, without imposing a structural model (e.g., rod or plate) [22]. Cortical bone morphometry was averaged from fifty serial cross-sectional images (600 μm) extending distal from the diaphyseal mid-point between proximal and distal growth plates. The cross-sectional areas of segmented bone, periosteal perimeter and medullary space were measured directly. Cortical thickness and perimeter length of the periosteal and endosteal borders were derived from the areal measurements.

Primary osteoblast cultures

Hemicalvariae from 6- to 8-day-old neonatal mice were dissected free of sutures, placed in Dulbecco's modified eagle medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (HI-FBS), 100 U/ml penicillin and 50 μg/ml streptomycin and incubated overnight at 37°C in 5% CO_2 on a rocking platform. Hemicalvariae of the same genotype were grouped and subjected to four sequential 15-min digestions with an enzyme mixture of 1.5 U/ml collagenase P (Roche Applied Science, Indianapolis, IN) in phosphate-buffered saline (PBS) and 0.05% trypsin/1 mM EDTA at 37°C on a rocking platform. The first digest was discarded. The second to fourth digests were pooled and the cell pellet collected by centrifugation at 2000 rpm for 10 min. Cells were resuspended in DMEM and passed through a 40 μm cell strainer (Falcon, Becton Dickinson, Franklin Lakes, NJ). Cell number was determined using a Coulter counter (Coulter Corporation, Miami, USA). Cells were plated at a density of 15,000/cm^2 in 6-well culture dishes in DMEM containing 10% FBS and antibiotics. Medium was changed after 24 h and again on day 4. On day 7, 50 μg/ml ascorbic acid and 4 mM β-glycerophosphate (Sigma-Aldrich Chemical Company, St. Louis, MO) were added, and the medium was changed every other day for the duration of the experiment.

Bromodeoxyuridine (BrdU) incorporation

Cell proliferation was assessed using a BrdU Cell Proliferation Assay kit (Oncogene Research Products, San Diego, CA). Primary osteoblasts were plated at 10,000 cells/well in 96-well microtiter plates (Costar, Corning, NY) in DMEM supplemented with 10% FBS and antibiotics and allowed to attach overnight. After labeling with 20 μl/well of 1:2000 BrdU at 37°C for 6 h, 200 μl of the fixative/denaturing solution was added to each well and plates were incubated for 1 h at room temperature. Cells were washed once with PBS and stored at 4°C overnight. Then, 100 μl of 1× anti-BrdU antibody solution was added to each well, and plates were incubated for 1 h at room temperature. Wells were washed three times; a goat anti-mouse IgG horseradish peroxidase (HRP) conjugate (100 μl) was added to each well, and plates were incubated for 30 min at room temperature. After washing with deionized water, 100 μl of substrate