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LIM kinase 1 deficient mice have reduced bone mass☆

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ABSTRACT

The cytoskeleton determines cell shape and is involved in cell motility. It also plays a role in differentiation and in modulating specialized cellular functions. LIM kinase 1 (LIMK1) participates in cytoskeletal remodeling by phosphorylating and inactivating the actin-severing protein, cofilin. Severing F-actin to release G-actin monomers is required for actin cytoskeletal remodeling. Although less well established, LIMK1 may also influence the cell cycle and modulate metalloproteinase activity. Since the role of LIMK1 in bone cell biology has not been reported, the skeletal phenotype of LIMK1$^{-/-}$ mice was examined. LIMK1$^{-/-}$ mice had significantly reduced trabecular bone mass when analyzed by microCT ($p<0.01$). Histomorphometric analyses demonstrated a 31% reduction in the number of osteoblasts ($p=0.0003$) and a 23% reduction in osteoid surface ($p=0.0005$). The number of osteoclasts was no different in control and knock out animals. Consistent with the in vivo findings in osteoblasts, the number of osteoblast colony forming units in LIMK1$^{-/-}$ bone marrow was reduced by nearly 50%. Further, osteoblasts isolated from LIMK1$^{-/-}$ mice showed significantly reduced rates of mineralization in vitro. Osteoclasts from LIMK1$^{-/-}$ mice evidenced more rapid cytoskeletal remodeling in response to treatment with CSF1. In keeping with this latter finding, basal levels of phospho-cofilin were reduced in LIMK1$^{-/-}$ osteoclasts. LIMK1$^{-/-}$ osteoclasts also resorbed dentine slices to a greater extent in vitro and were more active in a pit assay. These data support the hypothesis that LIMK1 is required for normal osteoblast differentiation. In addition, its absence leads to increased cytoskeletal remodeling and bone resorption in osteoclasts.

Introduction

The cytoskeleton is highly dynamic and carefully regulated. It is modulated by the extracellular matrix, mechanical forces and intracellular signaling cascades activated by integrins, hormones and cytokines. In addition to its impact on cell size and shape, the cytoskeleton plays an important role in cell differentiation as well as in maintaining specialized cellular functions [1–3]. Despite this, the role of the cytoskeleton in regulating bone cell function, particularly in osteoblasts, has not been very well studied. None-the-less, available evidence underscores the importance of cytoskeletal architecture in regulating osteoblasts. Meyers et al. and Zayzafoon et al. have reported that osteoblastic differentiation of multipotent human mesenchymal stem cells (hMSCs) was suppressed when these cells were cultured in modeled microgravity that was associated with reduced integrin signaling. Interestingly, adipocyte differentiation was enhanced [4,5]. The same group of investigators also found that reduced gravitational force was associated with reduced RhoA activity, impaired phosphorylation of cofilin and reduced stress fiber formation in hMSCs, which demonstrated impaired differentiation to osteoblasts [6]. Forced expression of constitutively active RhoA restored stress fiber formation and enhanced osteoblast differentiation of these cells. In addition to F-actin, the microtubular component of the cytoskeleton is important for maintaining osteoblast function since cytochalasin D has been shown to reduce integrin signaling as well as osteopontin and alkaline phosphatase expression in osteoblast-like cells [7,8]. Furthermore, McBeath et al. reported that cell shape and cytoskeletal tension regulate commitment of hMSCs to different lineages [9].

Osteoclasts are highly motile cells that move along the bone surface while resorbing skeletal matrix. Since remodeling of the cytoskeleton is required for motility, regulation of the osteoclast cytoskeleton has been more extensively studied [10,11]. Although the physiologic regulators of cytoskeletal remodeling and motility in osteoclasts are not known, CSF1 is a likely candidate. Several groups, including our own, have shown that CSF1 is a potent chemoattractant for osteoclasts

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[12]. We have also shown that when CSF1 binds to its receptor, c-fms, it recruits PI3-kinase and c-Src. This activated complex results in increased activity of the guanine nucleotide exchange factor, Vav3. Vav3 in turn leads to activation of the small GTPase Rac, which induces actin cytoskeletal remodeling. Inhibiting Rac activity prevents CSF1-induced actin remodeling [12]. Wang et al. have reported that Rac plays an important role in regulating the actin cytoskeleton during osteoclast differentiation [13]. There are two major isoforms of Rac expressed in osteoclasts, Rac1 and Rac2, and we have reported that osteoclasts isolated from Rac2 knock out mice have impaired chemotaxis in a CSF1 gradient [14].

The small GTPases, Rac, Rho and Cdc42, control actin dynamics and focal adhesion assembly in response to extra- and intracellular stimuli [15]. Cofilin, one of the actin-severing proteins, is a key regulator of actin remodeling [16]. Cofilin severs F-actin to generate G-actin monomers making them available for cytoskeletal remodeling. The small GTPases, in particular Rac, are thought to regulate cofilin activity through a molecular pathway in which Rac activates the serine/threonine kinase Pak1 that in turn affects the activity of LIM kinase 1 (LIMK1) [17,18]. LIMK1 is a serine/threonine kinase that phosphorylates and inactivates cofilin [19]. The actin-binding activity of phospho-cofilin is reduced, resulting in actin polymerization and stabilization [20]. One might therefore predict that inactivation of LIMK1 would result in reduced cell motility. However, suppressing LIMK1 has been reported to either increase or reduce cytoskeletal remodeling and cell motility, depending on the cellular context [21,22]. In addition to its established role in regulating cofilin, emerging if still somewhat preliminary evidence suggests additional functions for LIMK1. Murine LIMK1 has been reported to bind to the cyclin inhibitor, $p57^{KIP2}$, raising the possibility that LIMK1 might influence cell cycle progression [23,24]. It has also recently been reported to regulate the activity of matrix metalloproteinases in a prostate cancer cell line [25].

Since the role of LIMK1 in regulating bone metabolism has not been explored, we examined the skeletal phenotype of 8–9 week-old LIMK1 knock out mice. Given the important role ascribed to LIMK1 in regulating actin remodeling we wondered whether bone cell function would be altered in LIMK1 knock out mice. We report that in the absence of LIMK1, the function of both osteoblast and osteoclast is altered and is associated with a low bone mass phenotype.

Materials and methods

LIMK1 knock out mice

LIMK1 knock out mice were kindly provided by Dr. Zhenping Jia, (The Hospital for Sick Children, Toronto CA) [26]. These animals are on a C56/Bl6 background. Heterozygous animals served as controls in this study. All animal studies were conducted with approval of the Yale Animal Care and Use Committee.

Materials

Recombinant human Colony Stimulating Factor 1 (CSF1) was a generous gift from Genetics Institute (Cambridge, MA). Recombinant mouse RANKL and TNF-α were from R & D Systems, Inc. (Minneapolis, MN). α-MEM cell culture medium was purchased from Sigma-Aldrich (St. Louis, MO) and fetal bovine serum from Atlanta Biologicals (Lawrenceville, GA). Ficoll-Paque® was from GE Healthcare (Kings Park, NY). Etched gridded coverslips were purchased from BELLCO Glass, Inc. (Vineland, NJ). Twenty four well Osteo Assay® plates were from Corning Inc. (Corning, NY). Type I collagen was purchased from Nitta Gelatin (Osaka, Japan). Collagenase A and Dispase II used in collagenase digestion protocols were purchased from Roche (Indianapolis, IN). Cofilin and phospho-cofilin antibodies were purchased from Cell Signaling (Danvers, MA). Phalloidin was from Molecular Probes (Eugene, OR). Vectashield mounting medium with DAP1 was from Vector Laboratories (Burlingame, CA).

Bone densitometry

Bone mineral density (BMD) determinations of spine, femur and total body were acquired using a PIXImus densitometer (GE-Lunar Corp., Madison, WI) running software version 1.45 as previously described [27]. BMD values are expressed in g/cm^2.

MicroCT analyses

Femurs were stripped of soft tissue and stored in 70% EtOH at 4 °C for subsequent microcomputed tomographic analyses (μCT). The bones were scanned using a Scanco μCT-35 instrument (Scanco, Bruttisellen, Switzerland) as previously described [14]. Volumetric regions for trabecular analyses, selected within the endosteal borders of the distal femoral metaphysis to include the secondary spongiosa located 1 mm from the growth plate and extending 1 mm proximally, were scanned at 12 μm resolution. Cortical morphometry was quantified and averaged volumetrically through 50 serial cross-sections (600 μm) extending distally from the diaphyseal mid-point between the proximal and distal growth plates. We used a customized thresholding technique (Scanco, Bruttisellen, Switzerland) that provided the best segmentation of the bone tissue. Both 2- and 3-D μCT data included bone volume to total volume fraction (BV/TV), and trabecular number (Tb.N), thickness (Tb.Th), space (Tb.Sp) and connectivity density (Conn.D). Cortical thickness averaged for both cortices (Cor.Th) was also quantified.

H&E staining

Femora were stripped of soft tissue and fixed in 4% paraformaldehyde for 24 h. They were then washed for 2–3 h in running water, decalcified in Deltaform (Delta Medical, Inc., Pewaukee, WI) for two weeks and paraffin sections prepared. After the slides were deparaffinized, they were stained with freshly filtered Harris's hematoxylin and eosin solutions.

Bone histomorphometry

Undecalcified tibiae embedded in methylmethacrylate utilizing the rapid embedding method, were cut into 4-μm longitudinal sections, mounted on chrome-alum gelatin-coated slides and stained with 2% toluidine blue (pH 3.7) in citric acid buffer for light microscopy as we have previously reported [27]. Sections were examined in a blinded fashion using the Osteomeasure software program (Osteometrics, Atlanta, GA) to quantify histomorphometric parameters. All indices were defined according to the American Society of Bone and Mineral Research histomorphometry nomenclature [28]. To assess mineral apposition and bone formation rates, animals received intraperitoneal injections of calcien (30 mg/kg) - seven days and again one day prior to sacrifice.

Quantification of marrow fat

Marrow fat was quantified in fixed bone slices from the tibiae of $LIMK1^{-/-}$ and control mice by measuring the area of adipocyte ghosts in the proximal tibiae using the Osteomeasure software. Four fields at 20× in the proximal tibiae just below the growth plate from 11 control and 11 knock out animals were evaluated. Percent fat for the total bone area (the latter also quantified using the Osteomeasure software) was determined.