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A potential role for the myeloid lineage in leptin-regulated bone metabolism

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Abstract

Leptin influences bone formation centrally through the hypothalamus and peripherally by acting on osteoblasts or their precursors. However, neither mechanism explains the divergent, gender-specific correlation between leptin and bone mineral density in humans. Although leptin is a potent regulator of pro-inflammatory immune responses, a potential role for leptin as an osteoimmunologic intermediate in bone metabolism has not been tested. Mice with myeloid-specific ablation of the long-form leptin receptor (ObRb) were generated using mice expressing cre-recombinase from the lysosyme M promoter. At 12 weeks of age the conditional knockout mice did not display any appreciable phenotype. However, at 52 weeks two changes were noted. First, there was a mild increase in liver inflammation. Second, a gender-specific, divergent bone phenotype was observed. Female mice displayed a consistent trend toward decreased trabecular bone parameters including reductions in bone volume fraction, trabecular number, and bone mineral content as well as a significant increase in marrow adipogenesis. Conversely, male mice lacked trabecular changes, but had statistically significant increases in cortical bone volume, thickness, and bone mineral density with equivalent total cortical volume. Since the year 2000, over twenty-five studies on more than 10,000 patients have sought to determine the correlation between leptin and bone mineral density. The results revealed a gender-specific correlation similar to that observed in our LysM transgenic animals. We hypothesize and show new evidence that regulation of myeloid lineage cells by leptin may facilitate their actions as an osteoimmunologic intermediate and contribute to leptin-regulated bone formation and metabolism in a gender-specific manner.

Keywords

osteoimmunology; leptin; bone mineral density; cytokines; macrophages; adipokines

INTRODUCTION

Leptin, a pro-inflammatory cytokine synthesized primarily by adipocytes, is a neuroendocrine regulator of appetite [1], energy metabolism [2], immune function [3], and bone mass [4,5]. Leptin is also a potent monocyte/macrophage chemoattractant [6] and plays a role in the regulation of pro-inflammatory immune responses [3,7]. Leptin binding to

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macrophages enhances secretion of pro-inflammatory cytokines TNF- α , IL-6, and IL-12 [8–10]. Macrophages are acutely sensitive to changes in their surrounding microenvironment and respond to leptin concentrations as low as 1ng/ml *in vitro* [11]. Though there are many studies that correlate obesity with increases in systemic inflammation and circulating leptin [12], the physiologic significance of leptin interactions with the myeloid lineage has not yet been determined.

Over twenty-five studies on more than 10,000 patients have sought to determine the relationship between circulating leptin and bone mineral density (BMD) in humans. The sum of the results, though varied, has revealed divergent patterns between males and females. While leptin often correlates positively with BMD in females, the opposite is noted in males [13–16]. Despite extensive analysis, the mechanism of peripheral leptin regulation of bone mass is not entirely understood. Though it is accepted that leptin may act through a central neuroendocrine relay to negatively modulate bone metabolism [5], the appearance of leptin resistance with age or obesity may impair these effects. Alternatively, leptin may act directly on osteoblasts. However, studies that examine this activity *in vitro* consistently use leptin concentrations far exceeding physiologic norms and mice conditionally lacking osteoblastic ObRb fail to demonstrate a bone phenotype [4,17–19]. Lastly, it has been shown that leptin may negatively regulate mesenchymal precursor cell differentiation to influence bone length and BMD [4]. However, this would likely contribute more to bone formation during growth and healing and not general bone turnover. We lack an experimental model that can mimic the modest, divergent, gender-specific changes in BMD that have been noted in adult humans. Because the macrophage can readily respond to subphysiologic leptin levels with changes in cytokine production and migration, we hypothesize that regulation of macrophage activity by leptin may facilitate its actions as an osteoimmunologic intermediate and contribute to bone formation and metabolism. To test this hypothesis, we generated a conditional myeloid lineage leptin-receptor (ObRb) deficient mouse and have evaluated parameters of bone mass with aging in both male and female mice.

MATERIALS AND METHODS

Conditionally Regulated ObRb Mice

All procedures were approved by the University Committee on the Use and Care of Animals. LysM-Cre mice were obtained from Jackson Laboratory (Bar Harbor, Maine, USA Cat:004781) [20]. Flox/Flox Jak2-ObRb mice were obtained from Dr. Martin Myers (University of Michigan) with permission of Dr. Streamson Chua (Columbia University) [21]. To generate LysM-Cre crossed with ObRb floxed/floxed (F/F) mice, one LysM^{Cre+F/-} male was bred with two LysM^{Cre-F/F} females and pups were genotyped via PCR analysis of tail biopsy DNA as reported previously [21]. Analysis was performed on LysM^{Cre-}, LysM^{Cre+F/-} and LysM^{Cre+F/F} littermates. Recombination and deletion of the loxP flanked allele was determined using the three-primer system designed by McMinn et al [21]. Serum markers of bone formation were analyzed as directed in kits for N-terminal propeptide of type I procollagen (P1NP, Immunodiagnostic Systems, AC-33F1) and tartrate-resistant acid phosphatase (Trap5b, Immunodiagnostic Systems, SB-TR103).

MicroCT

Femurs were scanned in a vivaCT 40 μ CT system (Scanco Medical, Switzerland) with an isotropic voxel size of 10.5 μ m (55 kVp, 145 μ A, 1000 projections per 180 degrees, 200 ms integration time) as described previously [4]. Briefly, 2D transverse slices were reconstructed into 2048 $\times$ 2048 pixel matrices. Cortical bone parameters were measured by analyzing 50 slices (0.525mm) of the mid-diaphysis. Trabecular bone parameters were

measured by analyzing 101 slices (1.06mm) of the distal metaphysis 0.105 mm proximal to the primary spongiosa in the marrow cavity to assure that only trabecular bone was analyzed. For both analyses, a Gaussian low-pass filter was used ($\sigma=0.8$, support=1). Analysis groups: 12 week female $\text{LysM}^{\text{Cre-}}$ (N=6), $\text{LysM}^{\text{Cre+F/F}}$ (N=6); 52 week female $\text{LysM}^{\text{Cre-}}$ (N=7), $\text{LysM}^{\text{Cre+F/F}}$ (N=4); 52 week male $\text{LysM}^{\text{Cre-}}$ (N=6), $\text{LysM}^{\text{Cre+F/F}}$ (N=6). One of the male 52 week old $\text{LysM}^{\text{Cre+F/F}}$ samples was eliminated as an outlier because values were greater than two standard deviations from the mean.

Histomorphometry

Tibia bones were fixed in 10% neutral buffered formalin, decalcified for 14 days in 14% EDTA pH 7.4, and embedded in paraffin. Paraffin sections were removed until the marrow cavity was exposed. At this point six sections of 5um thickness were made, one every 50um. Sections were stained for acid phosphatase (TRAP) activity (Sigma Diagnostics, 387-A) with Gil's haematoxylin counterstain. Briefly, sections were deparaffinized and rehydrated. Antigen retrieval was performed in 95°C sodium citrate buffer (10mM sodium citrate, 0.05% Tween-20, pH 6.0) for 40 minutes. After rinsing, slides were placed in TRAP stain for 45 minutes (1% Fast Garnet GBC Base, 1% sodium nitrite solution, 1% Naphthol AS-BI phosphate solution, 4% acetate solution, 2% tartrate solution) and counterstained with Gil's Haematoxylin. Sections were analyzed for osteoclast and adipocyte numbers at 10× and 40× magnification respectively using NIS-Elements software on a Nikon Eclipse E600 microscope.

Statistics

A two-tailed, homoscedastic t-test was used to calculate differences between control and experimental groups. Values are reported as the mean $\pm$ the standard deviation. $p<0.050$ was considered statistically significant. $p<0.150$ was considered to represent a non-significant trend.

RESULTS

Generation of myeloid lineage-specific leptin receptor knockout mice

Mice with loxP sites flanking the ObRb Jak2 signaling component [21] were bred with mice expressing Cre recombinase under the control of the Lysozyme M (LysM) promoter [20] to specifically eliminate ObRb function in myeloid lineage cells ($\text{LysM}^{\text{Cre+F/F}}$). DNA was isolated from tissues of 12 week old $\text{LysM}^{\text{Cre+F/F}}$ mice and used for PCR amplification to determine the extent of deletion of the floxed locus. DNA was also harvested from macrophages differentiated from primary bone marrow with 50ng/ml M-CSF for two days. Results revealed the highest level of deletion in cultured macrophages, whole bone marrow (WBM), tail, and spleen extracts (Fig.1A). Low-level recombination was observed in all other tissues except for the hypothalamus (Fig.1A). Mice were phenotypically similar with exception of slight obesity observed in both the male heterozygote $\text{LysM}^{\text{Cre+F/-}}$ (14% increase, $p=0.007$) and homozygote $\text{LysM}^{\text{Cre+F/F}}$ (11% increase, $p=0.062$) mice at 50 weeks (Data Not Shown). No differences were noted in the mass of females from 3 to 52 weeks of age (Fig.1B,C).

Because mice with complete ObRb knockout are known to have significant hepatomegaly [22], we quantified liver mass at necropsy. At 52 weeks, the $\text{LysM}^{\text{Cre+F/F}}$ female mice had an $11.3 \pm 0.9\%$ reduction in liver mass/body mass ratio ($P=0.016$) when compared to the $\text{LysM}^{\text{Cre-}}$ mice that was not present in the heterozygotes (Fig.1D). Histological analysis revealed moderate steatohepatitis with microscopic features including hepatocyte ballooning, finely divided fat droplets in the hepatocyte cytoplasm, intracytoplasmic accumulations of Mallory hyaline, and inflammatory infiltrates consisting predominately of

neutrophils with some lymphocytes (Fig.1E). Histological features of steatohepatitis were present in both the 52 week old $\text{LysM}^{\text{Cre-}}$ and $\text{LysM}^{\text{Cre+F/F}}$ mice, however, the presence of inflammatory islands of ten or more cells was significantly increased in the $\text{LysM}^{\text{Cre+F/F}}$ mice (Fig.1F). No changes in spleen size were noted (Data Not Shown).

Because complete ObRb KO mice have altered femoral bone parameters [23], femurs were harvested from mice at 12 and 52 weeks of age and cortical and trabecular bone parameters were analyzed with microCT. Statistically significant differences in bone parameters were not present at 12 weeks of age [4] (Table 1). At 52 weeks, females demonstrated a trend toward decreased trabecular bone volume fraction (-31% , $p=0.105$), trabecular number (-18% , $p=0.081$), and bone mineral content (BMC) (-31% , $p=0.136$) (Table 1). This corresponded to an increase in trabecular spacing ($+16\%$, $p=0.129$) (Table 1). These trends were not present in the males, however, increases in cortical parameters were observed including bone volume ($+9.3\%$, $p=0.036$), cortical bone volume fraction ($+9.0\%$, $p=0.089$), cortical thickness ($+10.5\%$, $p=0.038$), BMC ($+13.7\%$, $p=0.006$), and BMD ($+4.0\%$, $p<0.001$), but total tissue volume was unchanged (Table 1).

To determine a cellular explanation for the changes observed in bone geometry, serum N-terminal propeptide of type I procollagen (PINP) and tartrate-resistant acid phosphatase (Trap5b) were evaluated as measures of bone formation and resorption, respectively. These parameters were unchanged in serum from 52 week old males (Sup Table 1). In addition, osteoclast numbers were unchanged upon tibial histomorphometric analysis of males and females (Supplemental Table 1). However, there was a significant increase in the number of marrow adipocytes in female $\text{LysM}^{\text{Cre+F/F}}$ mice (Fig.1G).

DISCUSSION

Osteoimmunology is an emerging field of research that views regulation of bone metabolism within the context of immune function. While the macrophage has long been appreciated as a key component of the immune system, an essential role for macrophages in bone homeostasis was only recently confirmed when induced depletion of osteal tissue macrophages in a mouse model ablated the mature osteoblast bone-forming surface [24]. In addition to bone changes, we report the liver phenotype of the 52 week old $\text{LysM}^{\text{Cre+F/F}}$ animals as a new and interesting finding, though we do not yet understand its significance. The slight increase in liver inflammation observed in the conditionally regulated mice may indicate that leptin actions on myeloid lineage cells could regulate homing to the liver.

The trend toward decreased trabecular bone observed in the aged female but not the male $\text{LysM}^{\text{Cre+F/F}}$ mice and the increase in cortical bone mass in the male but not the female closely mimics the association between leptin and BMD observed in humans. For example, many studies cite a modest positive correlation between leptin and BMD in post-menopausal adult females [13,16]. Thus, inhibition of leptin receptor function should decrease bone parameters as was observed in our $\text{LysM}^{\text{Cre+F/F}}$ 52 week old females. Conversely, the negative association between circulating leptin and BMD in males would point toward increases in bone parameters after receptor ablation as was also noted in our animals. Though it is not yet possible to completely ascribe the effects of leptin on post-natal bone remodeling to the macrophage, this mouse model provides compelling evidence for the existence of a novel leptin-responsive myeloid osteoimmunologic intermediate. This is also the first leptin transgenic mouse model to mimic the human condition with a divergent gender-specific phenotype. In contrast, leptin signaling deficiency in the ob/ob (leptin deficient) or db/db (ObRb deficient) mouse results in decreased trabecular parameters at the distal femur that appear early in development and are not gender specific [23,25–27]. These changes may be due to altered actions of leptin in the periphery [4,25], brain [5], or an

unidentified systemic factor. Unlike these early changes during development, we hypothesize that with age, leptin receptors on the myeloid lineage may play a role in leptin-regulated maintenance of bone mass.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Generation of LysM^{Cre+/-F/F} Mice

LysM-Cre mice were mated with mice with loxP sites flanking exon 17 of the long-form leptin receptor (ObRb) to generate myeloid-specific ObRb knock-out mice. **(A)** Genomic DNA PCR of tissues from 12 week old animals or primary bone marrow macrophages cultured *in vitro* showing the intact (~646 base pairs) and deleted (~200 base pairs) bands, lanes cropped from the same gel. WBM = Whole Bone Marrow. **(B)** Representative photo, female, 3 months old. **(C)** Body mass of transgenic female mice from 3 to 52 weeks (N=6–8). **(D)** 52 week old female liver mass and liver mass/body mass ratio (N=6–8). **(E)** Liver histology. **(F)** Number of inflammatory islands per field (N=3 mice, 3 liver sections each, 10 fields/section). Inset contains genomic DNA PCR to check for loxP recombination. **(G)** Tibial bone marrow histology, 10× magnification. Numbers represent adipocyte number per marrow area (mm²) plus or minus the standard deviation (N=4–7). a: significant compared to Cre- female; b: significant over Cre- male; c: significant over Cre+ male.

Table 1

Femoral microCT analysis of LysM transgenic mice. No differences were noted in femoral cortical or trabecular parameters at 3 months of age. At 12 months, the LysM^{Cre+/F/F} females showed a trend toward decreased bone volume fraction and trabecular number. Conversely, the males had no noted changes in trabecular parameters, but had significant increases in cortical thickness, bone volume, bone mineral content (BMC) and bone mineral density (BMD).

LysMCre+F/F % Change vs Cre- Control									
	12 wk Female			52 wk Female			52 wk Male		
Trabecular	Control	LysM+	p value	Control	LysM+	p value	Control	LysM+	p value
Bone Volume Fraction (%)	24+/-3.9	25+/-5.0	0.581	6.9+/-1.9	4.8+/-1.9	0.105	7.4+/-1.3	7.7+/-3.1	0.842
Tb. Number (1/mm)	5.96+/-0.54	5.79+/-0.67	0.625	2.19+/-0.35	1.80+/-0.23	0.081	2.35+/-0.27	2.48+/-0.62	0.642
Tb. Thickness (mm)	0.061+/-0.002	0.060+/-0.005	0.759	0.063+/-0.007	0.063+/-0.006	0.982	0.058+/-0.007	0.058+/-0.007	0.879
Tb. Spacing (mm)	0.175+/-0.016	0.183+/-0.022	0.488	0.487+/-0.082	0.566+/-0.060	0.129	0.442+/-0.059	0.440+/-0.114	0.970
BMC (mg HA)	463+/-74	452+/-105	0.837	142+/-46	98+/-36	0.136	176+/-46	184+/-78	0.843

Cortical	Control	LysM+	p value	Control	LysM+	p value	Control	LysM+	p value
Total Volume (mm ³)	0.86+/-0.04	0.84+/-0.05	0.379	1.08+/-0.09	1.06+/-0.07	0.787	1.18+/-0.08	1.19+/-0.09	0.902
Bone Volume (mm ³)	0.408+/-0.010	0.398+/-0.036	0.512	0.509+/-0.047	0.512+/-0.021	0.900	0.538+/-0.046	0.588+/-0.020	0.036
Cortical Bone Fraction (%)	47.5+/-2.0	47.5+/-2.7	0.978	47.2+/-3.0	48.2+/-2.3	0.581	45.5+/-4.0%	49.6+/-3.4	0.089
Cortical Thickness (mm ³)	0.201+/-0.006	0.199+/-0.015	0.769	0.222+/-0.016	0.225+/-0.007	0.684	0.219+/-0.020	0.242+/-0.013	0.038
BMC (mg HA)	480+/-14	472+/-46	0.682	627+/-64	638+/-33	0.758	656+/-57	746+/-25	0.006
BMD (mg HA/ccm)	1176+/-18	1186+/-34	0.561	1232+/-27	1236+/-29	0.778	1219+/-6	1268+/-17	<0.001