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High-fat Diet Causes Bone Loss in Young Mice by Promoting Osteoclastogenesis through Alteration of the Bone Marrow Environment

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Abstract

Obesity is a severe health problem in children, afflicting several organ systems including bone. However, the role of obesity on bone homeostasis and bone cell function in children has not been studied in detail. Here we used young mice fed a high-fat diet (HFD) to model childhood obesity and investigate the effect of HFD on the phenotype of cells within the bone marrow environment. Five-week-old male mice were fed a HFD for 3, 6, and 12 weeks. Decreased bone volume was detected after 3 weeks of HFD treatment. After 6 and 12 weeks, HFD-exposed mice had less bone mass and increased osteoclast numbers. Bone marrow cells, but not spleen cells, from HFD-fed mice had increased osteoclast precursor frequency, elevated osteoclast formation and bone resorption activity, as well as increased expression of osteoclastogenic regulators including RANKL, TNF, and PPAR-gamma. Bone formation rate and osteoblast and adipocyte numbers were also increased in HFD-fed mice. Isolated bone marrow cells also had a corresponding elevation in the expression of positive regulators of osteoblast and adipocyte differentiation. Our findings indicate that in juvenile mice, HFD-induced bone loss is mainly due to increased osteoclast bone resorption by affecting the bone marrow microenvironment. Thus, targeting osteoclast formation may present a new therapeutic approach for bone complications in obese children.

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Disclosures

The authors Lei Shu, Eric Beier, Tzong Sheu, Hengwei Zhang, Michael Zuscik, J Edward Puzas, Brendan Boyce, Robert Mooney, and Lianping Xing declare that they have no conflict of interest.

Introduction

The prevalence of overweight or obese children and adolescents continues to be unacceptably high and of public health concern worldwide. With the rise of obesity, an important debate has developed as to whether excessive weight has a detrimental or protective effect on skeletal health in children and adults. The widely accepted opinion is that high body mass appears to be protective of bone in adults, as increased mechanical loading strengthens bone and minimizes bone loss in postmenopausal women [1–3] despite recent evidence suggesting that the effects of fat on bone mass and fracture risk may vary by skeletal site [4]. In contrast to adults, the incidence of bone fractures is found to increase in overweight and obese children and adolescents [5], suggestion that fat may be detrimental to bone accrual in children. This position is further examined by Dimitri and colleagues [1] in a review that highlights the potentially different mechanisms that are involved in mediating the effect of obesity on bone in children and adults.

Surprisingly, there are limited studies examining this issue in animal models. Animal studies using adult or aged mice or rats fed a diet containing high fat or high sugar [6, 7] show reduced bone mechanical properties (quality), but conflicting results on bone size and volume (quantity). In addition, studies where low bone mass in mice fed with high-fat diet (HFD) or sugar is reported, the changes in osteoclast and osteoblast function are not always consistent [4, 8, 9]. In general, a significant increase in osteoclast formation and bone resorption are accompanied by decreased bone mass. Proposed mechanisms that increase osteoclast formation under these conditions are complicated and likely include elevated circulating pro-inflammatory cytokines such as tumor necrosis factor alpha (TNF- α), interleukin-1 (IL-1), and receptor activator NF- κ B ligand (RANKL) [10–12].

Osteoclasts are derived from common myeloid progenitors in the bone marrow [13]. Osteoclast precursors (OCPs) have been characterized by their expression of c-kit and c-fms. In the spleen, CD11b⁺ cells are OCPs, but in bone marrow, both CD11b⁺ and CD11b⁻ cells can give rise to osteoclasts [14, 15]. These OCPs differentiate into normal resident osteoclasts that mediate focal erosions and maintain bone turnover. In the transgenic mouse expressing human TNF- α , chronic inflammatory signals produce an upregulation of c-fms and resultant increase in OCP frequency in peripheral tissues including blood, spleen, and bone marrow [14]. Increases in OCPs are also reported in patients with erosive bone diseases, such as psoriatic [16] and rheumatoid arthritis [17]. While obesity has been suggested to promote a chronic low level of inflammation and elevated TNF, its effects on OCP frequency and bone resorption remains unexplored.

Given the fact that obese children with prior fractures show a reduction in areal bone mineral density at multiple sites [18], it is possible that obesity also influences bone mass via various obesity-triggered cellular events. However, the influence of HFD on the cellular environment and differentiation potential of pluripotent bone marrow cells has not yet been adequately studied to address this question. The purpose of this study was to determine if HFD affects bone volume, bone cell function, and the bone marrow environment in young mice.

Materials and Methods

Animals

Male C57BL/6J mice were purchased from Jackson Laboratories (Bar Harbor, ME) and were housed on a 12-hour light/dark cycle. Mice were placed on a high-fat diet (HFD, 60% kcal, D12492) or low-fat diet (LFD, 10% kcal, D12450B) provided *ad lib* starting at 5 weeks of age (Open Source Diets, Research Diets Inc., New Brunswick, NJ). The HFD used is composed of animal fats that are high in saturated fat. This is comparable to the fat found in the red meat of a Western diet. The 10% kcal from fat provided by the LFD is very similar to the standard mouse chow used at the University of Rochester Medical Center (LabDiet 5010) that contains 12.7% kcal from fat. In contrast to the LabDiet 5010, however, the LFD diet from Research Diets, Inc., is matched to the HFD in that both are made from purified ingredients and have identical protein, vitamins, and minerals. In our recent publications we have confirmed that this HFD mouse model is associated with hyperglycemia and metabolic dysfunction using fasting blood glucose levels [19] and glucose tolerance testing (GTT) [20]. We demonstrated that the HFD-fed mice had 90% higher fasting blood glucose levels than LFD-fed mice (294 ± 33 mg/dl versus 155 ± 53 mg/dl; $p < .001$). GTT showed that HFD-fed mice were unable to restore basal blood glucose levels 120 minutes after glucose bolus while LFD-fed mice restored blood glucose levels after 90 minutes (380 ± 18 mg/dl versus 180 ± 11 mg/dl; $p < .001$). The Institutional Animal Care and Use Committee approved of all animal care and experimentation.

Serum biomarkers and glucose testing

All animals were fasted for 24 hours. Tail vein blood was collected and analyzed for whole blood glucose using the OneTouch Ultra glucose-testing meter (LifeScan, Milpitas, CA). Serum levels of leptin (ALPCO Diagnostics, Salem, NH) and total non-esterified fatty acids (NEFA) (Wako Diagnostics, Richmond, VA) were also measured using an ELISA method according to procedures provided from the manufacturer.

CD45⁻ and CD45⁺ cell isolation and real-time quantitative PCR

Primary bone marrow stromal cells and spleen cells were isolated according to our previously described methods [21, 22]. Cells were incubated with anti-CD45 antibody-conjugated microbeads (Miltenyi Biotec, Auburn, CA). The CD45⁻ and CD45⁺ populations were isolated according to the manufacturer's instructions. With this method, 98% enriched CD45⁻ cells were obtained [23, 24]. Total RNA was prepared using the RNeasy Mini Kit (Qiagen, Germantown, MD, USA) according to the protocol provided by the manufacturer. cDNAs were synthesized using the iSCRIPT cDNA Synthesis Kit (Bio-Rad, Hercules, CA, USA). Quantitative RT-PCR amplifications were performed in an iCycler using iQ SYBR Green supermix (Bio-Rad). The relative abundance of each gene was normalized to *Gapdh* expression. LFD group values were normalized to 1, and HFD gene expression reported as fold change vs. LFD group values. The sequences of primer sets are shown in a supplemental Table.

Osteoblast and adipocyte differentiation

Bone marrow cells were flushed from femoral and tibial cavities. For Colony-Forming Unit in Fibroblastic (CFU-F) assay, bone marrow cells were seeded at low density (1×10^6 cells/10 cm dish) and cultured in α -MEM plus 20% fetal bovine serum (FBS) for 7 days, and cells were stained with methyl blue. For alkaline phosphatase (ALP) colony assay, bone marrow cells were seeded at low density (1×10^6 cells/10 cm dish) to form CFU-F, and cells were then cultured in osteoblast differentiation medium (α -MEM containing 10% FBS with 50 μ g/ml ascorbic acid, 10 mM β -glycerophosphate) for 14 days, and stained for ALP activity using AS-MX phosphate and fast red staining. For bone mineralization assay, bone marrow cells were seeded at high density (5×10^6 cells/6-well dish) and cultured with α -MEM plus 20% FBS for 7 days to enrich mesenchymal stromal cells. Cells were then cultured in osteoblast differentiation medium for an additional 21 days. Bone mineralization was identified by Alizarin red staining. For adipogenesis, cells were allowed to become confluent and then cultured in adipocyte-inducing medium (AIM: α -MEM containing 10% FBS, 1 μ M dexamethasone, and 0.5 mM methylisobutylxanthine, 10 μ g/ml insulin, and 100 μ M indomethacin) for 3 days and then cultured in the adipocyte-maintaining medium (AMM, α -MEM containing 10% FBS and 10 μ g/ml insulin) for 2 days. After the first round, cells were subjected to a second round of treatment with AIM and AMM until adipocytes formed. Adipocytes were stained with oil red O as described previously [25]. For cell surface marker analysis, freshly isolated splenocytes and bone marrow cells were stained with APC-anti-CD11b, PE-Cy7-c-kit and PE-Cy5-c-fms (eBioscience, San Diego, CA, USA) and detected with Analytical Flow Cytometry (FACSCanto II). The results were analyzed using FlowJo 7.6 software.

Osteoclast formation and functional assays

Bone marrow cells (5×10^4 /well) and spleen cells (2×10^5 /well) from lean and HFD mice were seeded in 96-well-plates and cultured in 10% FBS α -MEM with conditioned medium from a macrophage-colony-stimulating factor (M-CSF) producing cell line (1:50) and RANKL (10 ng/ml, R&D Systems, Minneapolis, MN, USA) for 5–7 days to form osteoclasts as we described previously [26]. Cells were stained for tartrate-resistant acid phosphatase (TRAP) activity to identify osteoclasts and TRAP⁺ osteoclast area was assessed. For functional assays, cells were seeded in 96-well plates containing bovine cortical bone slices and cultured with RANKL and M-CSF for 10–12 days and resorption pit formation was assessed, as reported previously [27].

Microcomputed tomography (Micro-CT)

Distal ends of femora were scanned on a VivaCT40 micro-CT scanner (Scanco Medical, Basserdorf, Switzerland) using an integration time of 300 ms, energy of 55 kVp, and intensity of 145 μ A. Quantitative analysis of trabecular bone extended 1.06 mm, beginning 0.3 mm from the most proximal aspect of the growth plate. The three-dimensional images were generated using a constant threshold of 275 for all samples.

Histology and histomorphometry

Femoral bones were removed and fixed in 10% formalin for 24h, decalcified in EDTA-glycerol solution for 14 days, and embedded in paraffin. Specimens were cut at 5- μ m and stained with for TRAP activity. In brief, sections were preincubated in Naphthol AS-BI Phosphate Substrate (Sigma) solution for 45 minutes at 37C, and incubated in Sodium Nitrite (Sigma) and Pararosaniline Dye (Sigma) solution for 15 minutes at room temperature. Sections were then incubated in phosphomolybdic acid for 5 minutes and then counterstained with 1% fast green. Sections were converted to virtual slides using an Olympus VS120 whole slide imager and osteoclast numbers were assessed using an App developed using Visiopharm software. For calcein labeling, mice were injected intraperitoneally with 10 mg/kg calcein (Sigma) per gram body weight at 7 and 1 days prior to sacrifice, as reported previously [28]. Left femora were embedded in LR white acrylic resin (Sigma), and 5- μ m sections were imaged using fluorescence microscopy. The mineral apposition rates (MARs) and bone formation rates (BFRs) were calculated using a previously reported method [24].

Statistical analysis

Data are presented as mean $\pm$ SD. Statistical analyses were performed with StatView statistical software (SAS, Cary, NC, USA, www.sas.com). Differences between the two groups were compared using unpaired Student's t-test, while more than two groups were compared using one-way analysis of variance between groups (ANOVA), and p values less than 0.05 were considered to be statistically significant.

Results

Mice fed a high-fat diet for 12 weeks have systemic bone loss and increased osteoclast formation

To investigate the mechanism of obesity-induced bone loss in children, we fed 5-week-old young mice (equivalent to 10 year-old children [29]) a high-fat diet (HFD) or low-fat diet (LFD) for 12-weeks, a standard duration that we used for HFD studies [19, 20, 30] and examined bone volume and bone morphology. HFD-fed mice had significantly increased body weight, and associated increased levels of fasting blood glucose, serum fatty acids, and serum leptin, confirming diet-induced obese characteristics (Figure 1A). MicroCT of femoral bones from HFD-fed mice showed decreased trabecular bone volume, number and thickness and increased trabecular spacing, suggesting increased bone resorption-associated bone loss (Figure 1B). However, cortical bone changes were not significant (data not shown). Histomorphometric analyses indicated that HFD-fed mice had reduced trabecular volume in femoral metaphyseal sections and increased numbers of osteoclasts in the metaphyseal region. They also had more adipocytes in the diaphyseal area compared to LFD-fed mice (Figure 1C).

We then cultured spleen cells from HFD-fed mice or LFD-fed mice with RANKL and M-CSF to examine their osteoclast formation potential. The rationale of using osteoclast precursors (OCPs) from spleen cells is that they have similar osteoclastogenic potential as OCPs in peripheral blood, which is different from that of bone marrow cells [14]. We found

that osteoclast numbers and bone resorption capacity were comparable between spleen cells from HFD-fed and LFD-fed mice (Figure 1D). However, OCPs isolated from bone marrow of HFD-fed mice exhibited 2–3 fold increased osteoclast formation area and bone resorptive capacity compared to LFD-fed mice (Figure 1D).

Bone volume is maintained by a balance of osteoclast-mediated bone resorption and osteoblast-mediated bone formation. Adipocytes are derived from the same mesenchymal stem cell (MSC) pool as osteoblasts, and typically an increase in adipogenesis is associated with a decrease in osteoblastogenesis. To test if this is the case in our mouse model, we examined bone formation parameters in the long bones of HFD-fed mice using calcein labeling. Interestingly, HFD-fed mice had significantly increased mineral apposition rates and bone formation rates compared to LFD-fed controls. Increased bone formation rates were not observed in cortical bone (Figure 2A). We previously reported that CD45⁺ cells from mouse bone marrow represent a MSC-enriched population, which may have increased osteoblast formation potential [24]. To determine if HFD affects osteoblast or adipocyte differentiation of MSCs, we isolated CD45⁺ MSCs and examined the expression levels of osteoblast- and adipocyte-related genes. CD45⁺ MSCs from HFD-fed mice expressed increased levels of the osteoblast transcription factors *runx-related transcription factor 2* (*runx2*) and *osterix* (*sp7*), and the osteoblast marker *osteocalcin* (*ocn*), but not *alp* (Figure 2B, left panel). Bone marrow stromal cells from HFD-fed mice had no change in ALP⁺ colony numbers, but increased mineralized nodule formation, suggesting osteoblasts from HFD-fed mice may have higher capacity to produce bone matrix (Figure 2B, right panels). Cells from HFD-fed mice had no change in CFU-F colony formation compared to cells from LFD-fed mice (data not shown). As expected, CD45⁺ MSCs from HFD-fed mice expressed high levels of adipogenic factors including *ppar-γ* and *c/ebp* isoforms α , β , and δ (Figure 2C, left panel). Consistently, bone marrow stromal cells from HFD-fed mice also had significantly increased adipocyte differentiation (Figure 2C, right panels). To determine if HFD affects the chondrogenic potential of the MSCs, we examined the expression levels of *sox 9* and type 2 collagen (Col2) in CD45⁺ cells and found HFD slightly increased *sox 9* but not Col2 expression levels (HFD/LFD: 2.5±0.1 fold, $p<0.05$ for *sox 9* and 1.9±0.4 for Col2). Taken together, these findings indicate that although HFD causes both increased osteoclast and osteoblast parameters, osteoclast-mediated bone resorption clearly plays a dominant role over osteoblast-mediated bone formation, leading to a net bone loss.

Short-term HFD causes bone loss and increased osteoclastogenesis

Most reports studying the effect of HFD on skeletal homeostasis use a 12-week HFD-feeding protocol. Some studies feed mice with HFD for 16 weeks or even longer [31, 32]. To examine if short-term HFD could lead to bone loss and elevated osteoclastogenesis, we fed 5-week-old mice HFD or LFD for 6 weeks and examined their bone volume in vertebrae by microCT and in femoral bones by histology. Similar to mice fed a HFD for 12 weeks, mice fed HFD for 6 weeks had increased body weight and blood glucose levels (Figure 3A), decreased volume, number and thickness of trabecular bones, and increased trabecular spacing by microCT (Figure 3B), and increased osteoclast numbers and surface by histology (Figure 3C). These data suggest that increased bone resorption-associated bone loss also occurs in mice fed HFD for 6 weeks. The adipocyte numbers were comparative between

HFD-fed and LFD-fed mice. *In vitro* osteoclastogenesis assays indicated higher osteoclast formation potential of bone marrow mononuclear cells from 6 week HFD-fed mice (Figure 3D). Furthermore, we tested if mice fed a HFD for 3 weeks could develop bone loss and elevated osteoclastogenesis and found they also had higher body weight and blood glucose levels, lower bone volume, and more osteoclastogenic potential than mice fed LFD (Supplemental 2). Because mice fed HFD for 6 weeks had more obvious bone loss and osteoclastogenesis than those fed HFD for 3 weeks, we used 6-week-HFD fed mice in the subsequent experiments to study potential mechanisms for HFD-induced osteoclastogenesis in our model.

Increased osteoclast precursors are present in bone marrow, but not in the spleen of mice on high-fat diet

OCPs are defined as c-kit⁺ and c-fms⁺ cells. In the spleen and peripheral blood, CD11b⁺ cells are osteoclastogenic, but in bone marrow, both CD11b⁺ and CD11b⁻ cells can give rise to osteoclasts [14]. We next used FACS analysis to examine if HFD increases OCP frequency in bone marrow cells, but not in the spleen (Figure 4A). Bone marrow cells from mice fed HFD for 6 weeks had significantly increased percentage (Figure 4B) and numbers (Figure 4C) of c-kit⁺, c-fms⁺ or CD11b⁺ OCPs, but the OCP frequency in spleen cells was un-changed. If increased OCPs are the mechanism for increased osteoclast formation in HFD-fed mice, we should observe an elevated response to M-CSF and RANKL-induced osteoclast formation. To test this, we cultured bone marrow cells from LFD-fed and HFD-fed mice in the presence of different amounts of M-CSF and RANKL. In all situations, bone marrow cells from HFD-fed mice formed significantly more TRAP⁺ osteoclasts than lean mice (Figure 4D).

A high-fat diet supports a bone marrow microenvironment that favors osteoclastogenesis

To test if HFD affects the expression of genes involved in osteoclastogenesis, we isolated CD45⁻ MSCs (these give rise to RANKL-producing cells) and CD45⁺ hematopoietic lineage cells (these give rise to pro-inflammatory cytokine-producing cells) from bone marrow and spleen. In mice fed HFD for 6 weeks, we found an increase in the RANKL/OPG ratio and IL-1 levels in bone marrow CD45⁻ cells (Figure 5A, upper-left panel), and increased IL-1 and TNF in CD45⁺ cells (Figure 5A, upper-right panel). No changes in expression of these genes were detected in cells from spleens of the same mice (Figure 5A, lower panels).

Peroxisome proliferator-activated receptors (PPAR) and Ccaat-enhancer-binding proteins (C/EBP) are families of transcription factors that promote adipogenesis and are thus characterized as environmental obesogens [33]. PPAR- γ also positively regulates osteoclastogenesis [34]. We examined the expression of these obesogens in CD45⁺ cells and found that the expression of PPAR- γ was increased by 2–3 folds, whereas the expression of C/EBP members was no change ($-\beta$) or decreased ($-\alpha$ and $-\delta$) in cells from mice-fed HFD for 6 weeks compared to cells from LFD-fed mice (Figure 5B, upper panel). There was no change in the expression pattern of these genes in CD45⁺ cells from the spleens (Figure 5B, lower panel). These data suggest that HFD-induced osteoclastogenesis is likely mediated by

elevated osteoclastogenic factors (RANKL, IL-1 and TNF) indirectly and osteoclastogenic gene (PPAR- γ) directly.

Discussion

The effect of obesity on bone health has been difficult to assess, given the conflicting reports in the literature surrounding obesity and fracture risk [35, 36]. In this study, we used HFD-fed young mice to create a condition that mimics childhood diabetes and obesity to investigate its effects on bone volume and the cellular mechanisms that mediate bone cell dysfunction. Consumption of a HFD by mice resulted in bone loss within 3 weeks. Consistent with this, HFD promoted osteoclast formation and activity in the bone marrow cells and did not effect osteoclast formation in cells derived from spleen. HFD increased adipogenesis of MSCs and did not inhibit osteoblast differentiation, but rather stimulated osteoblast function. Furthermore, HFD up-regulated the expression of osteogenic, pro-inflammatory, and adipogenic genes in bone marrow cells, but not in spleen cells. Our findings suggest that in young mice, HFD-induced bone loss is mainly due to increased osteoclastic bone resorption by changing the bone marrow micro-environment in favor of osteoclastogenesis.

Several mechanisms have been proposed to explain HFD-induced osteoclastogenesis, including elevated levels of in the blood of the pro-inflammatory cytokines IL-1 and TNF derived from adipose tissue macrophages [37], increased RANKL expression from bones [7, 9], and decreased expression of the anti-osteoclastogenic cytokine IL-10 [38]. Interestingly, we found a considerable increase in osteoclast formation from M-CSF-dependent myeloid precursors from bone marrow, but not from spleen cells of HFD-fed mice. This result was surprising because if increased osteoclastogenesis is mediated by systemic inflammation, we should observe increased osteoclast formation in cells from both bone marrow and spleen, as in animals with TNF over-expression-induced inflammatory erosive arthritis induced by TNF over-expression [14, 39]. Our explanation for our prior results is that high TNF levels in the blood increases OCP frequency in peripheral tissues, blood, spleen, and in bone marrow [14]. Indeed, more OCPs are identified in blood of patients with psoriatic arthritis [16] and rheumatoid arthritis [17] associated with increased TNF levels in their serum. Currently, it is not known if diabetic patients or obese children have increased circulating OCPs. Further investigation into this is warranted.

Some studies have reported low osteoblast function and suppressed osteoblastic gene expression in HFD-fed mice [9], potentially as a consequence of a MSC differentiation switch favoring adipogenesis. However, we did not find decreased osteoblast activity in our model. Rather, osteoblast function was increased both *in vivo* and *in vitro*. The increase observed in osteoblast activity was apparent after 12 weeks of HFD, but the rise in osteoclast activity was recognized at 3 weeks of HFD treatment. The rise in osteoblast activity may be a compensatory effect of the elevated osteoclast activity at the time-points analyzed. Thus, according to our model and endpoints, the data suggests that osteoblast activity may not be uncoupled from osteoclast activity, as reported in other studies.

One difference between HFD-induced bone loss and fragility from the bone loss induced by other chronic inflammatory conditions is the massive increase in adipogenesis. Adipocytes produce and release various adipokines which regulate osteoclast and osteoblast function via paracrine mechanism [40]. Interestingly, we found increased PPAR- γ expression not only in CD45⁻ MSCs, but also in CD45⁺ hematopoietic cells of HFD-fed mice. PPAR- γ is a positive regulator of osteoclastogenesis and PPAR- γ ligands used clinically promote osteoclast differentiation and bone resorption [41]. Thus, PPAR- γ mediated molecular events in hematopoietic cells, from which osteoclasts are derived, likely contribute to osteoclastogenesis in HFD-fed mice. In our study, we did not detect increased PPAR- γ in CD45⁺ cells from spleen cells. One possibility is that HFD may not directly increase PPAR- γ expression in these cells. It may indirectly act on osteoblasts or osteocytes, cell types that are not present in the spleen. Further studies will be required to investigate which cell types within the CD45⁺ population express PPAR- γ and the cells that stimulate PPAR- γ expression in response to HFD.

HFD stimulates macrophages in visceral adipose tissue to produce various pro-inflammatory cytokines, including TNF, which is associated with the onset of insulin resistance [36, 42]. We found increased TNF expression in CD45⁺ cells from HFD mice, suggesting elevated TNF production in the bone marrow. At low doses, TNF acts synergistically with RANKL to stimulate osteoclast formation [43]. We reported that TNF increases the frequency of OCPs by promoting the differentiation of c-fms⁻ myeloid cells to c-fms⁺ cells [14]. Thus it is possible that TNF produced by CD45⁺ cells increases the number of OCPs by an autocrine mechanism within the bone marrow (Figure 6). TNF is a strong inhibitor of adipogenesis [44]. However, we observed increased adipogenesis in HFD mice with elevated TNF levels. We suspect that in the case of obesity, other adipogenic factors may override the inhibitory effect of TNF on adipogenesis or that CD45⁺ cells produce relatively low levels of TNF, which are unable to affect osteogenic cells in a paracrine fashion.

In this study, we demonstrated that mice fed HFD for 12 weeks clearly are obese and experience other metabolic changes, but 3-week and 6-week HFD-fed mice are not as obese although they do develop bone loss and elevated osteoclastogenesis. Thus, if bone loss in these short-term HFD-fed mice is mediated by obesity or other HFD-associated mechanisms need to be further elucidated. For instance it will be important to examine the time-course of bone loss and HFD-induced metabolic changes including body weight changes to determine if HFD-induced obesity affects bone volume, as well as if switching the HFD-fed mice to a LFD could rescue the bone and metabolic changes of these mice.

One of the important questions to address will be if bisphosphonates, or other clinically used osteoclast inhibitors, could prevent bone loss in HFD-fed mice. A recent study published by Cao and Picklo demonstrated that addition of the antioxidant N-acetylcysteine to HFD-fed mice prevented bone loss and suppressed the elevation in osteoclast differentiation. This study highlights the importance of the redox state of MSCs, which in the presence of HFD is increased and promotes osteoclast differentiation. Furthermore it demonstrates that intervention targeting the rise in osteoclastogenesis as a consequence of HFD has promise; we believe that targeting the bone marrow specifically may augment future strategies aimed at improving obese-related bone loss.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Decreased bone volume and increased osteoclasts in mice fed a HFD for 12 weeks
 Five-week-old male mice were fed a high-fat diet (HFD) or lean diet (LFD) for 12 weeks. (A) Body weight, blood glucose, serum leptin, and total non-esterified fatty acids (NEFA) were measured. (B) Representative μ CT scans (bar = 100 μ m) and morphometric data of BV/TV, trabecular number (Tb.N), trabecular separation (Tb.Sp) and trabecular thickness (Tb.Th) in the femoral bone. (C) TRAP-stained femoral sections (bar = 250 μ m) show decreased trabecular bone volume and increased osteoclasts in HFD-fed mice. Histomorphometric analyses including bone volume, adipocyte and osteoclast parameters were performed. (D) Spleen cells or bone marrow cells were cultured with M-CSF and RANKL for 5–7 days on plastic for osteoclast formation or for 10–12 days on bone slides for bone resorption. The area of TRAP⁺ osteoclasts and resorption pits were counted. Values are mean $\pm$ SD of 6 mice or 5 wells. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ versus lean group.

Figure 2. Increased osteoblast function in mice fed a HFD

Mice that were fed a HFD for 12 weeks were used. (A) Calcein labeled trabecular and cortical bones of femurs from mice fed with LFD and HFD. Mineral apposition rate (MAR), bone formation rate (BFR), and mineral surface per bone surface (MS/BS) were calculated. Values are mean $\pm$ SD of 4 mice. (B) Left panel: expression levels of osteoblast-related genes in CD45⁺ cells isolated from bone marrow cells. Right panels: osteoblast differentiation (Alkaline phosphatase staining) and mineralization (Alizarin Red staining) were determined in bone marrow stromal cells after they were cultured in the osteoblast inducing medium for 14 and 21 days, respectively. (C) Left panel: expression levels of adipogenic genes in CD45⁺ cells isolated from bone marrow cells. Right panels: adipocyte differentiation was assessed in cells that were cultured with the adipocyte-induced medium for 7 days. *, $p < 0.05$; **, $p < 0.01$, ***, $p < 0.001$, versus lean mice.

Figure 3. Decreased bone volume and increased osteoclasts in mice fed short-term HFD

Five-week-old male mice were fed a HFD or LFD for 6 weeks. (A) Body weight and blood glucose levels were measured. (B) Representative μ CT scans (bar = 100 μ m) and morphometric data of BV/TV, Tb.N, Tb.Sp and Tb.Th in the vertebral bone. (C) TRAP-stained femoral sections (bar = 250 μ m) show decreased trabecular bone volume and increased osteoclasts in HFD-fed mice. Histomorphometry including bone volume, adipocyte and osteoclast parameters were measured. (D) Bone marrow cells were cultured with M-CSF and RANKL for 5–7 days on plastic for osteoclast formation. The area of TRAP⁺ osteoclasts was counted. Values are mean $\pm$ SD of 6 mice or 5 wells. *, $p < 0.05$; **, $p < 0.01$, ***, $p < 0.001$ versus lean group.

Figure 4. Increased osteoclast precursors in cells from bone marrow, but not from the spleen, of mice fed a HFD

Mice that were fed a HFD for 6 weeks were used. (A) Osteoclast precursors defined as c-kit⁺, c-fms⁺ or CD11b⁺ cells were examined by FACS analysis. The percentage (B) and total number (C) of c-kit⁺, c-fms⁺ or CD11b⁺ cells vs total of bone marrow and spleen mononuclear cells were assessed. Values are mean $\pm$ SD of 3 mice per group. (D) Bone marrow cells were cultured with a series dilution of M-CSF and RANKL for 5–7 days to generate osteoclasts. The area of TRAP⁺ osteoclasts was counted. Values are mean $\pm$ SD of 5 wells. *, $p < 0.05$; **, $p < 0.01$, ***, $p < 0.001$, versus cells from lean group.

Figure 5. Alteration of osteoclastogenic genes in bone marrow cells of mice fed a HFD

Mice that were fed a HFD for 6 weeks were used. CD45⁺ and CD45⁺ cells were isolated from bone marrow or spleen using micro-beads conjugated with anti-mouse CD45 antibody. Relative expression levels of genes of interest were determined by qPCR. (A) The fold increases of osteoclastogenic gene expression in CD45⁺ and CD45⁺ cells that were isolated from bone marrow and spleen. (B) The fold changes of adipogenic gene expression in CD45⁺ cells that were isolated from bone marrow and spleen. Values are mean $\pm$ SD of 3 mice per group. *, $p < 0.05$; **, $p < 0.01$, versus lean mice.

Figure 6. A model of HFD induced bone loss in young mice

In the bone marrow cavity of young mice, HFD affects both CD45⁻ mesenchymal cells and CD45⁺ hematopoietic cells. HFD promotes osteoblastogenesis and adipogenesis by increasing the expression of positive regulators of osteoblasts and adipocytes in osteoprogenitors. At the same time, HFD stimulates osteoclast formation by up-regulating expression of osteoclastogenic factors, which increases the formation and differentiation of osteoclast precursors. Increased osteoclastic bone resorption overrides increased osteoblastic bone formation, resulting in net bone loss.