Post-menopausal women are at an increased risk for osteoporosis due to increased bone resorption associated with estrogen deficiency. Subcutaneous administration of leptin, a hormone produced primarily by adipocytes, attenuates bone loss in the ovariectomized (ovx) rat, an animal model for post-menopausal bone loss. Leptin has both direct peripheral and indirect central nervous system-mediated effects on bone. In the present study we evaluated the efficacy of increased central leptin, via hypothalamic leptin gene therapy, in preventing bone loss in ovx Sprague Dawley rats. Ovx rats were injected in the hypothalamus with either rAAV-lep (recombinant adeno-associated virus encoding leptin) or rAAV-GFP (control vector encoding green fluorescent protein) and maintained for ten weeks. As expected, bone volume/tissue volume in the lumbar vertebra and bone area/tissue area in the proximal tibia were lower in ovx compared to intact rats. Differences in osteoclast perimeter, an index of bone resorption, were not detected with treatment. Osteoblast perimeter, an index of bone formation, was higher in ovx rAAV-lep rats compared to intact rats. Differences between rAAV-lep and rAAV-GFP-treated rats were not detected for any endpoints evaluated. The results suggest that increasing central leptin has no positive or negative effect on bone mass or architecture in ovx rats.

Key Words: leptin, ovariectomy, hypothalamic gene therapy, bone, osteoporosis

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Hypothalamic Leptin Gene Therapy Does Not Prevent
Ovariectomy-Induced Bone Loss in Rats

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Melanie A. Jackson

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HYPOTHALAMIC LEPTIN GENE THERAPY DOES NOT PREVENT OVARIECTOMY-INDUCED BONE LOSS IN RATS

INTRODUCTION

The discovery of leptin began studies into a hormone essential to many pathways involving growth and energy metabolism [1-3]. Leptin-deficient animals have high body fat mass, diabetes, infertility, decreased immune function, and are osteopenic [1, 3]. Hypothalamic leptin administration, hypothalamic leptin gene therapy, and peripheral leptin administration [3-5] correct some or all of these abnormalities.

Estrogen shares hypothalamic pathways with leptin, and the estrogen receptors (ER) and leptin receptors (ObR) are expressed in many of the same brain regions [6]. Studies in growing estrogen-deficient rats show abnormalities in bone formation, affecting both the length and density of the bone, and indicating that estrogen is necessary for normal bone development. Ovariectomized (ovx) adult rats effectively model the estrogen deficiency seen in post-menopausal women and its skeletal effects. In both ovx rats and post-menopausal women, the decrease in estrogen leads to accelerated bone turnover with a net increase in bone resorption, resulting in net bone loss and in increased risk of osteoporotic fractures [7].

Leptin is an important factor in the normal development of bone, as leptin-deficient ob/ob mice have abnormal bone architecture, with shorter bones and lower total bone mass [5]. A correlation has been seen between bone mineral density (BMD) and fat mass in women, demonstrating that excess body fat may have a protective effect on bone [8]. The observed correlation cannot be explained by load-bearing effects alone, as many of the sites where greater bone mass is observed are not involved in carrying weight. In addition, a decrease in
bone mass is seen with weight loss, and bone fractures are much more common among people with a low body mass index (BMI) [9-11]. While leptin is likely not the sole factor in this relationship, circulating leptin levels have been found to correlate independently to BMD, fat mass, and reduced fracture risk [12-13].

Peripheral leptin administration corrects skeletal abnormalities in ob/ob mice [14], and leptin receptors are present in many peripheral tissues, including bone [4]. Studies to ascertain the effectiveness of these receptors show an increased differentiation of human mesenchymal stem cells to osteoblasts in the presence of leptin in vitro [15]. However, peripheral administration of leptin increases leptin levels in the central nervous system (CNS), Hypothalamic leptin gene therapy, using a recombinant adeno-associated viral vector encoding leptin (rAAV-lep), has been shown to correct the skeletal abnormalities in ob/ob mice, without a rise in circulating leptin [5]. This presents the possibility that multiple pathways are responsible for the normalization of bone seen with peripheral and central leptin replacement in ob/ob mice, with differing actions.

Explanations for these differences include absolute differences between the levels of peripheral and CNS leptin due to reduced blood-brain barrier transport of leptin at high levels, and differential target-tissue resistance to the hormone [16]. Conversely, many studies implicate leptin as a permissive factor, down-regulating growth processes in skeletal tissue during caloric sufficiency, but having little or no action once a threshold is reached [3].

A study investigating the effects of subcutaneous (sc) leptin injection following ovariectomy demonstrated an alleviation of both weight gain and bone loss typically seen with ovariectomy [17]. These authors suggested that leptin’s complex actions on bone could include both antiosteogenic central effects and osteogenic peripheral effects. We tested this
hypothesis by performing hypothalamic leptin gene therapy on ovariectomized adult rats, to examine the possibility of disparate pathways of leptin action.
METHODS

Animals:

Female Sprague Dawley rats weighing 230-250 g were obtained from Harlan (Indianapolis, IN). The rats were maintained in accordance with the NIH Guide for the Care and Use of Laboratory Animals, and the experimental protocols were approved by the Institutional Animal Care and Use Committee at the University of Florida (Gainesville, FL) where the study was conducted. The rats were housed individually in a temperature- (21-23°C) and light (lights on 6 am-6 pm)-controlled room at the McKnight Brain Institute under specific pathogen-free conditions. Food and water were provided *ad libitum* to all animals.

Construction and Packaging rAAV Vectors:

To enhance leptin transgene expression, a non-pathogenic and non-immunogenic recombinant adeno-associated virus encoding either leptin (rAAV-lep) or the control vector (green fluorescent protein, rAAV-GFP) was packaged purified, concentrated, and titered as previously described [18].

Surgery:

The rats were ovx and then were stereotaxically implanted with a permanent steel cannula in the third cerebroventricle under ketamine-xylazine (100 mg/kg and 15 mg/kg body weight) anesthesia as previously described [19]. After one week of recovery, the rats were weight-matched and injected intracerebroventricularly (icv) with either rAAV-lep (n=7) or
rAAV-GFP (n=6). Control groups included unoperated intact rats (n=6) and ovx rats pair-fed (n=6) to rAAV-lep rats. Animals were monitored for 10 weeks, then sacrificed by decapitation. Treatment effects on hypothalamic mRNA leptin expression, food intake, body weight, white adipose tissue mass, and serum leptin levels are reported in detail elsewhere [20].

μCT analysis:

μCT was used for nondestructive three-dimensional evaluation of cancellous bone mass and architecture. Second lumbar vertebrae were scanned using a Scanco μCT40 scanner (Scanco Medical AG, Basserdorf, Switzerland) at a voxel size of 16 x 16 x 16 μm. The threshold for analysis was determined empirically and set at 245 (scale 0–1000). Data were processed on an HP AlphaStation DS15 (Scanco Medical AG, Basserdorf, Switzerland). Analysis of the vertebral body included the entire region of secondary spongiosa between the cranial and caudal growth plates (Figure 1). Direct cancellous bone measurements included cancellous bone volume/tissue volume (volume of total tissue occupied by cancellous bone, %), trabecular thickness (μm), trabecular number (number of trabeculae within the tissue sample, mm⁻¹), and trabecular separation (distance between trabeculae, μm).
Histomorphometry:

For histomorphometric evaluation of cancellous bone, proximal tibiae were dehydrated in a graded series of ethanol and xylene, and embedded undecalcified in modified methyl methacrylate as described [21]. Longitudinal sections (4 μm thick) were cut with a vertical bed microtome (Leica 2065) and affixed to gelatin-coated slides. One section/animal was stained according to the Von Kossa method with a tetrachrome counter stain (Polysciences, Warrington, PA) and used for assessing bone area and cell-based measurements. Measurements were performed in a standard metaphyseal sampling site, 0.5 mm distal to the growth plate (Figure 2), using the OsteoMeasure System (OsteoMetrics, Inc., Atlanta, GA).
Measurements included cancellous bone area/tissue area (%) and the derived architectural indices of trabecular thickness (μm), trabecular number (mm\(^{-1}\)) and trabecular separation (μm). Osteoblast and osteoclast perimeters (Figure 3) were measured and expressed as % total bone perimeter. Adipocyte density (#/mm\(^3\)) and adipocyte size (μm\(^3\)) were determined as described [22]. Adipocytes were identified morphologically as large circular or oval shaped cells, bordered by a prominent cell membrane and deficient in cytoplasmic staining due to alcohol extraction of intracellular lipids during processing (Figure 3). All measured and derived data were generated according to standardized methods, derivations, and nomenclature.

Figure 3: Bone marrow magnified to 64x depicting osteoclasts (bone resorbing cells), osteoblasts (bone forming cells), and adipocytes (fat cells).

Figure 2: The sampling site evaluated in rat tibial metaphysis section stained with Von Kossa and a tetrachrome counterstain.
Statistical Analysis:

A one-way ANOVA followed by a Bonferroni post-hoc test was used to evaluate differences among treatment groups (SPSS 17.0, SPSS Inc., Chicago, IL). If ANOVA assumptions of homogeneity of variance were not met, a Kruskal-Wallis followed by a Tamhane post hoc test was used. Differences were considered significant at P<0.05. All data are expressed as mean ± SE.
RESULTS

The effects of ovx and central rAAV-lep gene therapy on body weight over the 10-week duration of treatment and on hypothalamic leptin mRNA, abdominal white adipose tissue (WAT) weight, and serum leptin at treatment termination are described in detail elsewhere [20]. Briefly, ovx-induced weight gain was attenuated by both leptin gene therapy and pair-feeding, with leptin-administered animals gaining significantly less than the rAAV-GFP group. Hypothalamic leptin mRNA expression in the rAAV-lep group was increased by 45% compared to rAAV-GFP. Serum leptin levels in rAAV-lep animals were 67% lower compared to rAAV-GFP animals. WAT in rAAV-lep animals was 64% lower compared to rAAV-GFP animals.

**Lumbar vertebrae (μCT):**

The effects of ovx and rAAV-lep gene therapy on cancellous bone in the second lumbar vertebra as measured by μCT are shown in Table 1. Cancellous bone volume/tissue volume was lower in all three ovx groups compared to intact controls, but did not differ among the ovx groups. In conjunction with the lower bone volume/tissue volume, lower trabecular number and higher trabecular spacing were observed in all three ovx groups compared to intact controls. Trabecular thickness was lower in both rAAV-treated ovx groups, but not in pair-fed ovx animals, compared to intact controls. No significant differences between rAAV-lep and rAAV-GFP ovx groups were observed for any endpoints evaluated.
The effects of ovx and rAAV-lep gene therapy on cancellous bone in the tibia as measured by histomorphometry are shown in Table 2. Cancellous bone area/tissue area was lower in ovx animals compared to intact controls. In conjunction, lower trabecular number and higher trabecular spacing was observed in ovx animals compared to intact control animals. Differences in trabecular thickness were not detected with treatment. Treatment differences in the proportion of trabecular bone perimeter covered by osteoclasts were not detected (Figure 4A). Osteoblast perimeter/bone perimeter was higher in rAAV-lep groups compared to the intact control animals (Figure 4B). Adipocyte density was higher in pair-fed and leptin-treated animals compared to intact control animals (Figure 4C). Changes in adipocyte size were not detected with treatment (Figure 4D). No significant differences were detected between rAAV-GFP and rAAV-lep ovx groups for any of the architectural or cellular endpoints measured.
Figure 4: Cellular indices of bone formation and resorption and marrow adiposity in the proximal tibial metaphysis. Effects of ovariectomy and rAAV-lep administration on (A) cancellous bone perimeter lined with osteoclasts, (B) cancellous bone perimeter lined with osteoblasts, (C) adipocyte density, and (D) adipocyte size. Data are mean ± SE.

* Significantly different from Intact Control, P<0.01
DISCUSSION

Post-menopausal women without estrogen replacement face the possibility of dysfunctional bone remodeling and severe bone loss that can lead to osteoporosis. As expected, this study showed an ovx-induced decrease in cancellous bone mass with a decrease in trabecular number in both the lumbar vertebra and tibial metaphysis. There were no additional effects of increased hypothalamic leptin, via leptin gene therapy, compared to rAAV-GFP animals.

Peripheral leptin administration attenuates both weight gain and bone loss in ovx animals [17]. Burguera et al. administered subcutaneous leptin to ovx rats and found that leptin reduced cancellous bone loss and changes in trabecular architecture associated with ovx. To determine their possible involvement in leptin-mediated actions, mRNA levels of OPG and RANK-L, an important negative and positive regulator of osteoclast differentiation, respectively, were assessed in cultured mesenchymal stem cells with or without leptin. Dose-dependent increases in OPG and decreases in RANK-L in the presence of leptin were observed, indicating a possible role for leptin in regulating bone resorption [17]. RANK-L and OPG are essential in regulating the coordinated activity of osteoblasts and osteoclasts. RANK-L bound to the RANK receptor is involved in activation and maturation of osteoclasts, while OPG is a decoy receptor for RANK-L [23-26].

Cell culture studies have shown increased proliferation of osteoblasts in the presence of leptin, indicating that leptin may increase bone formation [15, 27]. In the current study, leptin-treated animals showed a significant increase in osteoblast perimeter, an index of bone formation, compared to intact controls. However, since differences in osteoblast perimeter were not observed between leptin- and GFP-treated rats, we cannot conclude that central
leptin increases bone formation \textit{in vivo}. This is further supported by a lack of leptin-induced changes in cancellous bone volume.

Osteoporotic patients often have an increase in bone marrow adiposity accompanying a decrease in bone mass [28-29]. This finding was affirmed in the animal model, as ovx rats in this study had both a decrease in cancellous bone volume and an increase in marrow adiposity. It has been proposed that the direct actions of leptin on mesenchymal stem cells drive differentiation toward osteoblastic lineage at the expense of adipocytes [15]. Further studies examining the roles of leptin and other hormones in cell differentiation, however, have indicated that proliferation of either osteoblasts or adipocytes are not mutually exclusive [22, 30]. Indeed, the increase in osteoblasts in this study is not at the expense of adipocytes, as both were increased with leptin treatment. While adipocyte density in the bone marrow was increased in leptin-treated and pair-fed ovx rats compared to ovary-intact controls, no effects can be directly attributed to leptin’s effects on adipocyte differentiation or proliferation as differences were not detected between the leptin-treated and GFP-treated ovx animals.

Prior studies have shown rapid decreases in both peripheral and marrow adiposity following hypothalamic leptin administration [31]. While the peripheral fat mass losses observed in leptin-treated animals in the present study are in agreement with other studies, the bone marrow results are not. One possible explanation for the differences in marrow adiposity between studies lies in the length of the study duration; this and other long-term (10 weeks or longer) studies in rats conducted in our lab (data not shown) do not show a change in marrow adiposity with leptin administration, while short-term studies (<1 week) report reductions [31]. Given leptin’s association with increased levels of proinflammatory cytokines [23, 32], studies involving the hormone may be particularly susceptible to surgery effects, and thus show transient actions.
Effects of central leptin gene therapy on bone mass were not detected in the present study. However, effects on energy metabolism were quite pronounced, indicating that the gene therapy was successful. Hypothalamic leptin mRNA expression was significantly increased in rAAV-lep compared to rAAV-GFP animals. In addition to the decrease in serum leptin levels, the leptin-treated group showed a decrease in food intake, weight gain, and abdominal WAT [20]. These findings are consistent with numerous other studies documenting the efficacy of central leptin gene therapy in attenuating weight gain and increasing hypothalamic leptin levels with no rise in serum leptin levels [33-35].

Leptin's primary actions on energy metabolism are carried out in the arcuate nucleus (ARC) of the hypothalamus, where it binds to neurons expressing neuropeptide Y (NPY) and agouti-related peptide (AgRP). These peptides project to neurons in the paraventricular nucleus (PVN) expressing melanocortin receptor 4 (MC4R) and have orexigenic actions on food intake and energy metabolism [3]. In addition, leptin increases production of pro-opiomelanocortin (POMC), α-melanocyte-stimulating hormone (α-MSH), and cocaine-amphetamine–regulated transcript (CART), which decrease food consumption and increase energy expenditure [6, 26, 36-37]. Estrogen receptors and leptin receptors collocate to the ARC, and both hormones affect food intake and energy expenditure through central pathways [6]. Whether estrogen and leptin work in parallel to carry out similar actions or interact with each other is unclear. The situation is complicated by the presence of multiple estrogen metabolites, often exhibiting cell-specific locations and actions [38-41]. In contrast to the central acting pathways of leptin, the central energy-regulating actions of estrogen do not impact bone metabolism [6, 38, 42].
Leptin-deficient *ob/ob* mice have a mosaic skeletal phenotype characterized by shorter bones and reduced overall bone mass, with tissue-specific increases in cancellous bone [5, 8, 43]. The corrective effects of central leptin gene therapy on bone are rapid and sustained, with early correction of the abnormal bone, and no further changes occurring after longer-duration treatment [5]. In the current study performed in rats producing leptin, there was no specific effect of increased hypothalamic leptin levels on bone mass.

In agreement with the above, leptin has been shown to have no bone effects in normal animals [4]. However, sc leptin treatment reduced the bone loss associated with ovariectomy [17] and hind limb unloading [23]. In contrast, central leptin increased bone loss in hind limb unloaded animals [44]. Calorically-restricted and *ob/ob* mice experience improved longitudinal growth with central leptin administration, but other models of central administration show negative effects [5, 14, 45].

These conflicting results have led some researchers to postulate that central leptin has negative effects on bone, while peripheral leptin has positive effects. In this study, however, central leptin administration had no positive or negative effect on the skeleton of ovx rats. Weight maintenance following ovx was observed in both this and the Burguera study, while bone effects differed. Systemic leptin reduced bone loss following ovx, presumably by direct inhibition of bone resorption. In contrast, increasing hypothalamic leptin levels using leptin gene therapy in ovx animals was ineffective at attenuating bone loss. These findings suggest that peripheral, but not central leptin, is responsible for the protective effects on bone in the rat model of postmenopausal bone loss.
BIBLIOGRAPHY


