Osteoclastogenesis by Bone Marrow-Derived Macrophages Is Enhanced in Obese Mice\textsuperscript{1–3}

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Abstract

Obesity induces a low-grade systemic chronic inflammatory condition for which macrophages are responsible. We hypothesized that obesity affects osteoclastogenesis by acting on bone marrow-derived macrophages (BMM). Male mice were fed a high-fat diet (45\% of energy) or a standard diet (10\% of energy) for 13 wk. We found that the density of the femurs of obese mice was significantly lower than that of the femurs of lean mice. Osteoclastogenesis was enhanced in the BMM from obese mice. Lower levels of interleukin (IL)-10 were generated by the BMM from obese mice than by those from lean mice upon stimulation of receptor activator of nuclear factor-\(\kappa\)B ligand. Neutralization of IL-10 in the BMM from obese mice was not as effective in increasing osteoclast (OC) formation as that in those from lean mice. Exogenous IL-10 inhibited OC formation more strongly in the BMM from obese mice than those from lean mice. The elevated level of OC formation in the BMM from obese mice may thus be due to in part to the lower level of IL-10, a negative regulator of osteoclastogenesis. Our results suggest that obesity is associated with bone loss via enhanced osteoclastogenesis due to reduced IL-10 production by the BMM from obese mice. J. Nutr. 139: 502–506, 2009.

Introduction

There is growing evidence of a connection between fat and bone metabolism at both the clinical and molecular levels, although the systemic regulators of fat and bone metabolism have not been clearly identified. The relationship between obesity and bone loss, 2 metabolic derangements of fat and bone metabolism, is controversial, and the relationship between body weight and bone mineral density (BMD)\textsuperscript{6} is complex. Many clinical studies have demonstrated a positive association between body weight and bone mass (1,2). However, other results have pointed to an inverse relationship between percentage of body fat and BMD, with lean mass more strongly related to BMD (3). These conflicting clinical results suggest a complex interaction between fat and bone metabolism at the cellular and molecular levels. The results of gene targeting in animal models have provided greater insight into fat and bone metabolism, but the relationship between obesity and bone loss still remains controversial. Obese mice deficient in leptin, the fat-derived hormone, and its receptor have high trabecular bone volumes, suggesting a positive relationship between body weight and bone density (4). However, PPAR\(\gamma\) insufficiency leads to high trabecular bone volume and low body fat (5). Senescence-accelerated mice also have increased body fat and decreased bone mass (6,7).

Basic bone remodeling units, which consist of a bone-forming osteoblast and a bone-resorbing osteoclast (OC), are located close to stromal elements of the marrow and differentiation of the 2 cell types is closely coordinated during bone remodeling. OC is formed from hematopoietic stem cells and their precursors have properties in common with precursors of the monocyte and macrophage cell lineages. Cooperation between macrophage- colony stimulating factor (M-CSF) and receptor activator of nuclear factor-\(\kappa\)B ligand (RANKL) systems generates an essential signal for OC differentiation that results from an interaction between bone marrow stromal cells and cells of the OC lineage (8,9). The positive factors promoting osteoclastogenesis are mainly proinflammatory cytokines such as interleukin (IL)-1, IL-6, and tumor necrosis factor (TNF)-\(\alpha\), and the negative ones are IL-4, IL-10, and IL-12 (10). The antiinflammatory cytokine IL-10, which is generated by T cells and macrophages, acts as a negative regulator that suppresses the acute inflammatory response (11–14).

Obesity is considered to be a state of chronic inflammation. TNF\(\alpha\) was identified to be the first molecular link between inflammation and obesity. TNF\(\alpha\) is overexpressed not only in adipose tissue, although at extremely low circulating levels, but...
also in other tissues (15,16). A variety of approaches has identified many other inflammatory mediators to be overexpressed in adipose tissues during obesity, whereas adiponectin, which counteracts the proinflammatory effect of TNFα, is downregulated at both the transcriptional and plasma levels (17,18). Macrophages are thought to play a critical role in the course, although it is not clear which cell type is responsible for the inflammatory responses associated with obesity. Because environmental factors greatly influence the characteristics and state of activation of macrophages, they are functionally heterogeneous (19). Different stimuli induce macrophages to express different kinds and amounts of chemokines, surface markers, and signaling molecules, and this gives rise to the diversity of macrophages observed in physiological and pathological conditions. Macrophages not only play an important role in physiological bone remodeling but also participate in the bone destruction associated with chronic inflammatory disease (20).

In this study, we investigated whether obesity is associated with bone loss. We detected enhanced osteoclastogenesis by obese bone marrow-derived macrophages (BMM) and found that they generated reduced levels of IL-10 on stimulation by RANKL.

Materials and Methods

Reagents. Recombinant mouse M-CSF, RANKL, recombinant mouse IL-10, neutralizing anti-mouse IL-10 Ab, and biotin-labeled anti-mouse IL-10 Ab were obtained from R & D Systems. Antibodies against CD11b, F4/80, CD3, and CD45R were obtained from eBioscience, and α-modified minimal essential medium (α-MEM) and fetal bovine serum were purchased from Life Technologies.

Animals and osteoclastogenesis. Male C57BL/6J mice were rendered obese by ingesting a high-fat diet (45% of energy from fat) (D12451, Research Diets) for 13 wk starting at 9 wk of age (Supplemental Table 1). We chose to evaluate the effect of obesity on bone metabolism of male mice to minimize the complication of estrogen on our analysis. Food intake was monitored by assessing nonpurified diet weight every 7 d and prepared for tartrate-resistant acid phosphatase (TRACP) staining as described (14). Numbers of TRACP+ multinucleated cells (MNC) containing ≥3 nuclei were scored. OC was further characterized by assessing their ability to form pits on dentine slices, as described (22). Mature OC was generated by incubation with M-CSF and RANKL for 4 d, after which they were harvested with trypsin/EDTA. The cells obtained were seeded on dentine slices and samples of 2 × 106 cells were cleaned by ultrasonication in 1 mmol/L NH4OH to remove adherent cells and stained with Mayer’s hematoxylin (Sigma Chemical) according to the manufacturer’s directions to visualize resorption pits.

RNA isolation and RT-PCR. RANK, c-FMS, IL-10, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNAs were measured by RT-PCR analysis. Total RNA was isolated from M-CSF and RANKL-treated BMM with TRI reagent (Sigma Chemical) and cDNA was synthesized using the RT supplied with the cDNA synthesis kit (11745–250, Invitrogen). The cDNA was amplified by 30 cycles [RANK, M-CSF receptor(c-FMS), 32 cycles (IL-10), and 25 cycles (GAPDH) of PCR with the following specific PCR primers: mouse RANK, 5′-GGTTC-ACTGTCCTCAATCCGAC-3′ (forward) and 5′-GGTCTGCGTCAAC-TACACCACG-3′ (reverse); mouse c-FMS, 5′-GGCTGGCTGTTGCTTGG-GATGATCTT-3′ (forward) and 5′-GCTGTGTCAGTCTCTCGTCGTTG-3′ (reverse); mouse IL-10, 5′-CCCTGGTGAAGAGCTG-AAG-3′ (forward) and 5′-GGAAAGACCCCTCCCTCATC-3′ (reverse); mouse GAPDH, 5′-ACCAAGCTGCATGCTCACA-3′ (forward) and 5′-TCCACACCCTGTGGGTA-3′ (reverse). Each cycle consisted of 30 s of denaturation at 94°C, 30 s of annealing at 60°C, and 30 s of extension at 72°C. GAPDH was used as internal control. The sizes of the PCR products for mouse RANK, c-FMS, IL-10, and GAPDH were 563, 754, 526, and 452 bp, respectively.

Statistical analysis. All values were expressed as means ± SEM. Student’s t test was used to evaluate differences between samples of interest and the corresponding controls. Paired t test was performed to test the effect of obesity on bone parameters of <0.05 were considered significant. Some data were analyzed using Spearman correlation analysis, a rank-based method (Rs; Spearman correlation coefficient).

Results

Obese mice have reduced bone density, form more OC, and their BMM resorb more bone. At 22 wk of age, body weight was higher for the obese mice (42.65 ± 0.63 g) fed a high-fat diet than for the lean mice (29.31 ± 0.59 g) fed a standard diet (P < 0.001) and the visceral fat mass (intraorgan and periorigan fat) of the obese mice (4.56 ± 0.16 g) was greater than that of the lean mice (1.21 ± 0.06 g) (P < 0.001). The femoral bone density of obese mice was lower than that of lean mice, although the difference was small (Fig. 1; P < 0.01).

RANKL induced the formation of large numbers of mononuclear and MNC that were TRACP+ in cultures of BMM free of stromal cells and lymphocytes. The numbers of TRACP+ MNC formed by the BMM from obese mice were higher than those formed by the BMM from lean mice (Table 1; P < 0.01).
BMM from obese mice produce lower levels of IL-10, which results in enhanced osteoclastogenesis. Because it seemed possible that obese BMM generate increased amounts of some positively acting factor for OC formation or decreased amounts of some negatively acting factor, we first asked whether BMM from obese mice expressed higher levels of c-FMS and/or RANK to interact with M-CSF and/or RANKL, respectively. However, the 2 groups did not differ (data not shown).

We determined whether IL-10 levels were related to the enhanced osteoclastogenesis by the BMM of obese mice. The BMM from obese mice produced less IL-10 mRNA than those from lean mice (Supplemental Fig. 1; Supplemental Table 1; $P < 0.01$). Next, we compared the levels of secreted IL-10 after RANKL stimulation. The BMM from obese mice again generated lower levels of IL-10 than that from lean mice (Table 1; $P < 0.01$).

To determine whether the endogenous IL-10 level influenced osteoclastogenesis, we neutralized endogenously generated IL-10 with anti-IL-10 Ab and determined whether this affected OC formation. Neutralization of IL-10 in preparations of the BMM from lean mice accelerated the formation of TRACP$^+$ MNC (Table 1; $P < 0.01$) but did not change OC formation in preparations of the BMM from obese mice (Table 1; $P = 0.97$). These results suggested that the endogenously produced IL-10 inhibits osteoclastogenesis in the BMM from lean mice and that inhibition does not occur in the BMM from obese mice, because their level of IL-10 is lower.

To substantiate the role of IL-10, we determined the effect of exogenously added IL-10. Addition of exogenous IL-10 reduced the number of osteoclastic TRACP$^+$ MNC formed by the BMM from both sets of mice without reducing total cell numbers and the effect was greater in the obese BMM (Table 1). The decreases after IL-10 addition were 24% ($P < 0.05$) in the lean BMM and 48% ($P < 0.001$) in the obese BMM. Administration of IL-10 to BMM from obese mice reduced OC formation to the level observed in the BMM from lean mice. These results also confirm the presence of lower levels of IL-10 in obese BMM.

We have shown that obese mice have lower bone density and that RANKL-induced OC formation and bone resorption are enhanced in the BMM from obese mice. Furthermore, the level of IL-10, which is a negative regulator of OC formation, is greater in the BMM from lean mice than in those from obese mice in response to RANKL stimulation.

**Discussion**

There is disagreement in both human and animal studies about the relationship between body weight and bone mass (1–7). We have attempted to examine whether obesity affects bone density in mice. Our data show that the bone density of the femoral metaphysis is significantly reduced in obese mice, suggesting that obesity is associated with reduced bone density. It is possible that bone loss due to obesity would be more prominent at the lumbar spine, although we have not measured its bone density. More differences between sham-operated and OVX rats at the lumbar spine have been reported than at in the distal femur (23,24).

There is evidence supporting this conclusion, although it is controversial (25,26). Consistent with our results, alveolar bone loss after bacteria infection has been significant in obese mice compared with lean mice (25). However, uninfected obese mice do not exhibit any significant bone loss, even with an elevated level of TNF$\alpha$ in macrophages, which has been implicated in bone resorption (27). In addition, FFA and PPAR$\gamma$ activation, which are involved in obesity, stimulate bone resorption, and obesity-induced inflammatory cytokines, leptin, hyperglycemia, and insulinopenia impair bone formation (26). However, there is some evidence against our conclusion. Obesity could be protective for bone, because it increases the mechanical loading on skeleton and protection from fractures and leads to enhanced bone formation and reduced bone resorption via the actions of leptin and estrogen (26).

**TABLE 1** Enhanced OC formation, bone resorption, and reduced IL-10 by BMM from obese mice

<table>
<thead>
<tr>
<th></th>
<th>Lean</th>
<th>Obese</th>
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<tbody>
<tr>
<td>TRACP-positive MNC$^2$</td>
<td>119.80 ± 11.94</td>
<td>230.40 ± 23.46$^*$</td>
</tr>
<tr>
<td>Pit area, %$^3$</td>
<td>6.86 ± 0.44</td>
<td>13.81 ± 0.48$^{**}$</td>
</tr>
<tr>
<td>IL-10</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Transcript, $^4$ fold of control</td>
<td>1.01 ± 0.09</td>
<td>0.61 ± 0.06$^*$</td>
</tr>
<tr>
<td>Secreted protein, $^5$ μg/L</td>
<td>0.33 ± 0.01</td>
<td>0.25 ± 0.004$^*$</td>
</tr>
<tr>
<td>TRACP-positive MNC$^6$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control IgG</td>
<td>146.40 ± 8.69</td>
<td>406.20 ± 6.98</td>
</tr>
<tr>
<td>anti-IL-10 Ab</td>
<td>282.60 ± 17.55$^*$</td>
<td>407.00 ± 19.03</td>
</tr>
<tr>
<td>None</td>
<td>160.80 ± 10.52</td>
<td>283.90 ± 17.88</td>
</tr>
<tr>
<td>IL-10</td>
<td>129.10 ± 6.74$^*$</td>
<td>146.70 ± 11.99$^{**}$</td>
</tr>
</tbody>
</table>

$^1$ Values are means ± SEM, $n = 5$. $^2$ Asterisks indicate different from lean or obese. $^3$ Different from Ab or cytokine-treated ones: control IgG vs. anti-IL-10 Ab or none vs. IL-10; $^4$ $P < 0.01$. $^5$ $P < 0.001$. Similar results were obtained in 3 independent experiments. $^6$ BMM were incubated with M-CSF and RANKL for 3 d.

FIGURE 1 Bone density in lean and obese mice after 13 wk of consuming standard or high-fat diet. Radiograms of the femurs showed marked loss of mineralized cancellous bone in obese mice, especially in the distal metaphysis of the femur. Individual data points for 21 (lean) or 22 (obese) mice are shown; the bar represents the mean. **Means differ, $P < 0.01$.
Although the precise cell types involved in generating obesity-associated diseases have not been identified, macrophages may be directly or indirectly involved, because obesity induces the accumulation of macrophages in adipose tissues (28) and cross-talk between macrophages and adipocytes reinforces inflammatory conditions (29). Obesity also induces phenotypic changes in macrophages (30). In this study, we focused on the effect of obesity on OC formation by BMM, because macrophages provide the precursor cells for bone resorption leading to bone destruction. Our data have demonstrated that OC differentiation and bone resorption are stimulated in the BMM from obese mice. The degree of osteoclastic potential in the marrow including OC formation and bone resorption correlated significantly with the degree of obesity such as body weight and fat mass. This BMM generates less IL-10 in response to RANKL than that from lean mice, suggesting that obesity causes IL-10 depletion. Many supporting results are available to confirm our findings. Macrophages from the adipose tissues of obese mice have higher levels of TNFα and inducible nitric oxide synthase and lower levels of IL-10 (30). Obesity reduces an antiinflammatory process by reduced recruitment of NF-κB onto the promoter of IL-10 (25). Our data also implies an important role for IL-10 as a negative regulator of bone loss. Indeed, we found that neutralization of IL-10 elevated osteoclastogenesis in the BMM from lean mice but had almost no effect on the BMM from obese mice, indicating that the reduced level of IL-10 is involved in the enhanced osteoclastogenesis in the latter. In addition, exogenously added IL-10 inhibited osteoclastogenesis induced by RANKL in BMM from both lean and obese mice, but the effect was more pronounced in the obese mice. These results indicate that the enhanced osteoclastogenesis in obese mice is due to the lower level of IL-10, which is known to have a potent inhibitory effect on osteoclastogenesis by inhibiting the differentiation of progenitor cells (12). Enhanced osteoclastogenesis in the absence of 4-1BB, a T cell costimulator of the TNF receptor family, is also caused by a low level of IL-10 (14). IL-10 has been demonstrated to be a major regulator of bone homeostasis in vivo as well as in vitro. Increased alveolar bone loss is found in IL-10 knockout mice with significantly elevated bone resorption marker, type I collagen C-telopeptide (31), and IL-10 knockout mice also exhibit lower bone mineral content and BMD of femora and vertebrae (32). Viral delivery of IL-10 reduces RANKL-associated alveolar bone loss in pathogen-infected humanized mice (34).

Our data demonstrates that obesity is positively associated with bone loss, OC formation, and bone resorption in mice. Bone loss and obesity are affected by a common progenitor cell, because both involve the recruitment and differentiation of monocyctic cells that differentiate into more specific cell types at particular sites. In that sense, we have demonstrated the effect of obesity on BMM during osteoclastogenesis, although it is not yet clear what mediates obesity to lead to these changes of BMM. We are now investigating obesity-induced environmental changes in the bone marrow at the molecular level. These will hopefully provide an explanation of the epidemiological findings linking obesity and bone metabolism.

Literature Cited
18. Bastard JP, Maachi M, Lagathu C, Kim MJ, Caron M, Vidal H, Capeau J, Feve B. Recent advances in the relationship between obesity, inflammation, and insulin resistance. Eur Cytokine Netw. 2006;17:4–12.


