Correlation of obesity and osteoporosis: Effect of free fatty acids on bone marrow-derived mesenchymal stem cell differentiation

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Abstract. Studies on the relationship between obesity and bone have recently become widespread. The aim of this study was to investigate the effect of obesity on bone, utilizing a diet-induced obese mouse model, and to explore the role of free fatty acids (FFAs) in the osteogenesis/adipogenesis of mouse bone marrow-derived mesenchymal stem cells (BMSCs). An obese mouse model was established by a high-fat diet (HFD). Proximal femurs were collected at sacrifice, and bone mineral density (BMD) in the proximal femurs was measured by dual-energy X-ray absorptiometry. Bone histomorphometry was performed using undecalcified sections of the proximal femurs. The effect of obesity on the differentiation of mouse BMSCs was assessed by colony formation assays and gene expression analysis. In vitro, various osteogenic and adipogenic genes were determined by real-time quantitative PCR in mouse BMSCs after exposure to conditioned medium (CM) from FFA-treated 3T3-L1 adipocytes. Western blotting was further performed to analyze the representative protein expression of PPARγ and Runx2. BMD and trabecular thickness were significantly greater in the HFD mice than in the control mice. CFU-osteoid assay showed significantly increased osteogenesis of BMSCs. The mRNA level of Runx2 was significantly higher, while PPARγ and Pref-1 were significantly lower in BMSCs from the HFD mice compared to the control mice. In mouse BMSCs, the Sox9 and Runx2 genes were significantly up-regulated after exposure to CM from FFA-treated adipocytes, while PPARγ and CEBP-α were significantly down-regulated. Osteogenesis was significantly increased, while adipogenesis was significantly decreased. In conclusion, HFD-induced obesity may play a protective role in bone formation by concomitantly promoting osteogenic and suppressing adipogenic differentiation of BMSCs through factors secreted by FFA-treated adipocytes.

Introduction

Obesity and osteoporosis, two disorders of body composition, are growing in prevalence. Obesity is not only a state of increased adipose tissue, but also a state of chronic low-grade inflammation, characterized by macrophage infiltration (1). Osteoporosis is another highly prevalent disease characterized by accelerated bone resorption and attenuated bone formation, resulting in progressive loss of mineralized bone and a reduction in both bone quantity and quality (2,3). Recently, several studies have provided evidence suggesting their correlation. The traditional view is that obesity is detrimental to bone health, and a negative correlation between body weight or body mass index and bone mass has been reported (4,5). Challenging this widely held view, numerous studies have provided evidence to the contrary, indicating that adipose tissue protects the skeleton (6,7). Recently, the Gilsanz laboratory found that, in young adults, subcutaneous adipose tissue is directly related to bone size and density, whereas visceral adipose tissue is inversely correlated with bone mass (8). Given these discrepancies in results, further investigations aimed at elucidating the relationship between bone and adipose tissue are warranted.

High-fat diet (HFD)-induced obesity in animal models is believed to best mimic the physiological functions of an obese body. Animal experiments supporting the influence of obesity on bone formation in terms of bone marrow mesenchymal stem cell (BMSC) differentiation are limited. It is well-known that the developmental fate of BMSCs is largely determined by the expression of specific transcription factors that act as molecular switches to drive differentiation (9). However, it remains to be determined whether adipose tissue regulates specific transcription factors in BMSC differentiation.

In the present study, a HFD obese mice model was established, and BMSC differentiation between normal control (NC) and HFD mice was compared systematically. The aim was to determine whether adipocytes are an important source of factors that influence bone formation and, most importantly, whether dietary components, particularly free fatty acids, (FFAs) act as circulating regulators of BMSCs.
The marrow cavity was flushed with were obtained from the bone marrow of the mouse femurs. Mouse bone marrow mesenchymal stem cell cultures. lines of the aSBmr nomenclature committee (10). parameters were calculated and expressed according to guide measurements included total tissue area, trabecular area, osteolastic number and osteolastic surface. the studied region was cancellous bone between 0.25 and 2 mm distal to the growth plate-epiphyseal junction. Histomorphometry. The right proximal femur metaphysis (PFM) was opened to expose the marrow cavity using an isometric low speed saw (Buechler Ltd., USA), and was subsequently fixed in 10% phosphate-buffered formalin for 24 h. It was then dehydrated in ethanol, defatted in xylene and embedded undecalified in methylmethacrylate. The frontal sections were cut into 5-µm sections with a microtome (Leica RM2155, Germany), then stained with Goldner's trichrome staining and Masson-Goldner Trichrome staining for static histomorphometric measurement. A semi-automatic digitizing image analysis system (Osteometrics, Inc., Decatur, GA, USA) was used for quantitative bone histomorphometric measurements. The studied region was cancellous bone between 0.25 and 2 mm distal to the growth plate-epiphyseal junction. Regions 0.5 and 1 mm distal to the growth plate of PFM were omitted in order to exclude the primary spongiosa. Static measurements included total tissue area, trabecular area, trabecular perimeter, osteoclast number and osteoclast surface perimeter. These parameters were used to calculate trabecular number, trabecular thickness, trabecular separation, percent osteoclast perimeter and osteoclast number. Histomorphometric parameters were calculated and expressed according to guidelines of the ASBMR Nomenclature Committee (10). Mouse bone marrow mesenchymal stem cell cultures. BMSCs were obtained from the bone marrow of the mouse femurs. The marrow cavity was flushed with α-MEM (Invitrogen), and the marrow was centrifuged at 1,000 rpm for 10 min. The cell pellet was suspended in a basal culture medium containing 10% fetal bovine serum, 100 U/ml penicillin and 100 mg/ml streptomycin, and the cells were then seeded into 6-well plates at a concentration of 1-3×10^6 cells/well. Bone marrow cells were incubated at 37°C in a 5% CO₂ humidified atmosphere, and the medium was replaced every 3 days. Non-adherent cells were removed when the medium was replaced. Second passage cells were used for the experiment. Colony formation assay. For the CFU-osteoid assay, BMSCs were grown in basal culture medium for 6 days. The cells were then grown in an osteogenesis-inducing culture medium that comprised basal culture medium supplemented with 10⁻⁷ M dexamethasone, 0.2 mM ascorbic acid phosphate and 10 mM β-glycerophosphate (Sigma-Aldrich). After 21 days, the cells were fixed with 10% formalin and stained with 0.1% alizarin red, then the number of positive colonies was counted under a light microscope. Only colonies in which the majority (>50%) of cells were histologically stained were scored as positive.

Preparation of adipocyte-condition medium. Mouse fibroblast 3T3-L1 pre-adipocytes were purchased from the Institute of Biochemistry and Cell Biology (Shanghai, China). For adipogenesis, 3T3-L1 pre-adipocytes were grown to confluence in a 60-mm plate and incubated in an adipogenic cocktail (10⁻⁷ M dexamethasone, 0.5 mM isobutyl-methylxanthine and 3.3 µl/ml insulin). Oil Red O was then added to each well in order to stain the accumulated lipid vacuoles of the cells, and used as a marker of intracellular lipid accumulation in cells. The stained cells were counted under a light microscope.

RNA preparation and quantitative real-time PCR. Total RNA was extracted from the cultured cells using TRIzol and quantified using real-time PCR. Total RNA (2 µg) was reverse-transcribed with 200 units M-MLV (Promega, Madison, WI, USA) in the presence of 0.5 mmol/l deoxynucleotide triphosphate, 25 units RNase inhibitor and 0.5 µg N9 random primers, in a total volume of 25 µl. PCR primers were designed by Primer5 software (Table I). Each quantitative real-time PCR was carried out in triplicate in a 25 µl volume of SYBR Green.
treatment by intragastric administration. In particular, the level of glucose levels and at 15, 30, 60 and 120 min after glucose administration, obese mice exhibited sustained hyperglycemia at fasting blood glucose levels. In addition, an oral glucose tolerance test was performed (Fig. 1d).

The effect of the hFd on glucose homeostasis in adipose tissues, compared to the nc mice (p<0.01). To assess the difference between groups, the two-tailed t-test or rank-sum test according to the distribution of the data was used. Statistical analysis. Values are presented as the mean±SD. The difference between groups was tested by the two-tailed t-test or rank-sum test according to the distribution of the data. P<0.05 was regarded as a significant difference.

Results

In vivo study

Establishment of a high-fat diet-induced obese mice model. The physical and metabolic characteristics of the NC and HFD mice are presented in Fig. 1. As expected, body weight (Fig. 1B) and visceral adipose tissue weight (Fig. 1C) were significantly higher in the HFD mice, which had been maintained with hFd for 20 weeks, compared to the NC mice (p<0.01). To assess the effect of the HFD on glucose homeostasis in adipose tissues, an oral glucose tolerance test was performed (Fig. 1D). The obese mice exhibited sustained hyperglycaemia at fasting blood glucose levels and at 15, 30, 60 and 120 min after glucose treatment by intragastric administration. In particular, the level of sugar in blood was significantly elevated at 15 min (P<0.05). These results indicate that the HFD-induced obese mice model was successfully established.

BMD evaluations and static bone histomorphometric analysis. Significant differences in the weight of femurs from the HFD mice compared to the control mice were observed (P<0.05) (Fig. 2A). The BMD in the proximal femurs was examined by DEXA in the NC and HFD mice (Fig. 2B) and was significantly higher in the HFD mice compared to the NC group (P<0.01). More extensive histomorphometric studies were performed in both groups. Table II documents the static histomorphometric parameters of the femurs, which were fixed in sections and stained with toluidine blue (Fig. 2C) and Masson-Goldner Trichrome (Fig. 2D), both of which showed more bone trabecula in the HFD than in the NC mice. Trabecular thickness was significantly higher in the HFD mice (P<0.05). The trabecular number and trabecular surface were higher, while the trabecular separation was lower, in the HFD compared to the NC mice, although this was without significance. In addition, no significant differences were detected in any of the parameters related to the bone absorption rate, such as osteoclast number and osteoclast perimeter.

CFU assay and expression of differentiation genes in BMSCs. To investigate the cause of bone structure changes in the HFD mice, primary osteoblasts and osteoclasts were induced from BMSCs and bone marrow monocytes in both the HFD and NC mice. Tartrate-resistant acid phosphatase (TRAP) staining revealed no significant changes in the number of osteoclasts, and real-time PCR showed no significant changes in the mRNA levels of specific genes, including TRAP, integrin-α and cathepsin K expressed by osteoclasts, between NC and HFD mice (data not shown). These results indicate that obesity may have no impact on osteoclasts.

A BMSC differentiation experiment was performed to determine whether the effect of obesity occurred at the level of stem progenitor cells, which are able to differentiate into osteoblasts and adipocytes (11). As shown in Fig. 3A and B, there was a significantly increased CFU-osteoblast number (stained by alizarin red) in the HFD mice (P<0.01). As for CFU-adipo, no statistical difference was found between the HFD and NC groups (data not shown).

Table I. Primer sequences for real-time PCR.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer</th>
<th>Reverse primer</th>
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<tbody>
<tr>
<td>β-actin</td>
<td>TAAAGACCTCTATGCAACACAGT</td>
<td>CACGATGGAGGGCCGGGACTCATC</td>
</tr>
<tr>
<td>PPARγ</td>
<td>GACCACCTCGATTCCTTACGGTGTTT</td>
<td>CCACAGACTGCGGACTCA</td>
</tr>
<tr>
<td>C/EBPα</td>
<td>CAGTTGGAAGATTACGAGCA</td>
<td>GGTGAGTTGAGGAGGATA</td>
</tr>
<tr>
<td>Pref-1</td>
<td>GCCATTTTGTGGAGAGATTGG</td>
<td>GGTTCTTAGATAGCCGGATG</td>
</tr>
<tr>
<td>Runx2</td>
<td>GGTGAAACTTCTTCTCTCGTC</td>
<td>AGTCCTCACTCTGTGTC</td>
</tr>
<tr>
<td>Sox9</td>
<td>ACCATTTTGGAGGGAATTCC</td>
<td>GAAGGCTAAGGGACAC</td>
</tr>
<tr>
<td>Osterix</td>
<td>GCCAGGAGTAAAGATAATA</td>
<td>AGAGAGACACCCACAGA</td>
</tr>
<tr>
<td>Dlx5</td>
<td>TGAAAAACCGGGAGATGC</td>
<td>GCTGAGAGAAGCGGATG</td>
</tr>
</tbody>
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Forward (F) and reverse (R) primers are listed for each gene in a 5’ to 3’ direction, as specified.
In order to analyze the effect of obesity on the osteogenic and adipogenic potential of BMSCs at the molecular level, the expression levels of several osteogenic (Sox9, Runx2, Osterix and Dlx5) and adipogenic (PPARγ, Pref-1 and C/EBPα) genes were determined by real-time PCR. As shown in Fig. 3C and D, the expression level of Runx2 was significantly higher, while the mRNA transcripts of PPARγ and Pref-1 were significantly lower, in BMSCs from the HFD group compared to the NC group. As the prime regulator of BMSC differentiation, the protein expression of PPARγ in BMSCs from the NC mice was higher than that from the HFD mice (Fig. 4E), which coincided with PPARγ gene expression.

In addition, the activity of alkaline phosphatase (ALP) was assayed in isolated murine calvariae osteoblasts. Higher ALP activity in the HFD mice than in the NC mice was noted, although this was not significant (data not shown). The mRNA levels of BMP2 and COLIa1 in osteoblasts analyzed by real-time PCR were also higher in the HFD vs. the NC mice, although this was without significance (data not shown).

**In vitro study**

Direct and indirect effect of FFAs on mouse BMSCs. The diet administered to the HFD mice contained mainly FFAs, therefore BMSCs were directly stimulated by FFAs for 48 h (Fig. 4A and B). Compared to the control, direct treatment of FFAs significantly decreased only the expression level of PPARγ (P<0.05).

To obtain further insight into whether adipocytes are an important source of factors acting as circulating regulators of BMSCs, FCM was applied to the BMSCs in vitro for 24 and 48 h. As early as 24 h after the initiation of treatment, a significant decrease in specific adipogenic genes and an
Figure 2. Evaluation of bone mineral density (BMD) and static bone histomorphometric analysis. Femur weight (A) and BMD (B) measured in proximal femurs by DEXA were significantly higher in HFD vs. NC mice (n=6), *p<0.05; **p<0.01. Histological sections stained with toluidine blue (C) and Masson-Goldner Trichrome (D), which showed increased bone trabecula in HFD mice. Scale bar, 250 µm.

Figure 3. BMSC differentiation assays. BMSCs were obtained from the tibias and femurs of HFD as well as NC mice. (A and B) BMSCs were cultured in basal culture medium, which was then replaced with an osteogenic-inducing culture medium. On Day 21, the cultures were stained with alizarin red (A), and the number of positive colonies (CFU-oste) was counted (B). The number of CFU-oste colonies formed from the BMSCs of HFD mice was significantly higher than that formed from the control BMSCs (n=3, **p<0.01). (C and D) mRNA levels of adipogenic and osteogenic genes in BMSCs from NC and HFD mice. BMSCs were harvested after a 8-day culture, then RNA was extracted and gene expression was determined by real-time PCR. mRNA transcripts of PPARγ and Pref-1 (C) were significantly lower in BMSCs from HFD compared to NC mice. The expression level of Runx2 (D) was significantly higher. The relative expression level of each gene was normalized to β-actin. Data the means of three independent experiments conducted in triplicate (*p<0.05; **p<0.01). (E) Protein levels of PPARγ in BMSCs from NC and HFD mice. BMSCs were harvested after an 8-day culture. Total proteins were collected and PPARγ was detected by Western blotting. GAPDH was used as a loading control. PPARγ production in BMSCs from NC mice was higher than production in BMSCs from HFD mice.
increase in the early specific osteogenic gene Sox9 was observed (data not shown). After the BMSCs were exposed to FCM for 48 h, the expression levels of PPARγ, C/EBPα and Pref-1 were significantly decreased (Fig. 4C) (PPARγ, **p<0.01; C/EBPα, *p<0.05; Pref-1, *p<0.05 compared to control).
C/EBPα and Pref-1, P<0.05), while the expression levels of Sox9, Runx2 and Osterix were significantly increased (Fig. 4D) (Sox9, P<0.05; Runx2 and Osterix, P<0.01).

Effect of FCM on protein expression and mineralization in mouse BMSCs. As the prime adipogenic and osteogenic genes, the protein expression of PPARγ and Runx2 was analyzed by Western blotting. PPARγ expression was lower and Runx2 expression was higher in the FCM-treated BMSCs compared to the controls (Fig. 5A). BMSCs were also cultured for mineralization, during which FCM was added continuously. After induction for 21 days, the mineral nodus was stained with alizarin red, which was quantified by ethylpyridium chloride extraction by absorbance at 550 nm (Fig. 5B and C). As a result, FCM was found to significantly promote BMSC mineralization (P<0.01).

Discussion

Increased obesity is a major public health concern and a risk factor for many diseases, but determining whether it is beneficial or detrimental to bone health is difficult.

Our data showed that HFD-induced obesity exerts a protective effect in the development of osteoporosis, consistent with the findings of many clinical research studies (12). BMD, the parameter of femur trabecula and the number of CFU-oste were increased in obese mice, which may have been regulated through the enhancement of BMSC differentiation towards osteoblasts. However, there was no influence on the number and function of osteoclasts. These results indicate that HFD-induced obesity has an impact on osteoporosis at the level of osteoblasts, but not osteoclasts.

Several potential mechanisms have been proposed to explain the relationship between fat and bone mass. One explanation is that a greater fat mass imposes a greater mechanical stress on bone and, in response, bone mass increases to accommodate the greater load. However, only 27 and 38% of total body weight in white men and women are attributable to fat mass, respectively (13). Therefore, weight-associated gravitational forces associated with increased fat mass may be insufficient to explain the impact of fat mass on bone.

The dietary components fed to the HFD mice contained mainly FFAs, which serve as a major link between HFD and obesity (14). To date, the study of FFAs has focused largely on their roles in glucose metabolism and insulin resistance (15,16). However, in recent years, a novel viewpoint has emerged asserting that certain dietary components, specifically FFAs, may influence bone metabolism (17-19). In this study, FCM was found to significantly promote BMSC differentiation (Sox9, P<0.05; Runx2 and Osterix, P<0.01).

Indeed, adipose tissue is an endocrine organ that secretes multiple hormones, cytokines and inflammatory factors. It is likely that the important relationships between bone and obesity are mediated by adipocyte-derived factors that act on bone. However, different factors have various effects on bone. Inflammatory factors and their detrimental effects on vasculature as well as muscle have long been recognized; not surprisingly, they also inhibit bone formation (20). Several studies have shown that leptin directly promotes the differentiation of osteoblasts (21,22). As for adiponectin, it has a negative effect on bone formation due to an indirect induction of osteoclast formation and inhibition of osteoprotegerin production in osteoblasts (23,24).

It is well-known that active factors secreted by adipocytes can be altered when stimulated by FFAs. For example, acute elevation of plasma FFAs activated the pro-inflammatory nuclear factor NF-κB pathway, which resulted in increased gene expression of TNF-α and MCP-1 (14). Moreover, FFAs decreased the secretion of adiponectin and leptin into medium (25,26). Collectively, this research and our study indicate that adiponectin or other novel factors secreted by FFA-treated adipocytes may play a key role in BMSC differentiation.

In conclusion, our results provide evidence that HFD-induced obesity may play a protective role in bone formation through factors secreted by FFA-treated adipocytes. This supports the hypothesis that adiponectin or other factors, excepting leptin and inflammatory factors, may play a role in concomitantly promoting the osteogenic and suppressing the adipogenic differentiation of BMSCs. These possible factors may function as a suppressor of osteoporosis in future bio-therapy.

Acknowledgements

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References


