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Molecules and Cells

Increased Fat Due to Estrogen Deficiency Induces Bone Loss by Elevating Monocyte Chemoattractant Protein-1 (MCP-1) Production

Youn-Young Kim, Song-Hee Kim, Sora Oh, Ok-Joo Sul, Hye-Young Lee, Hyun-Ju Kim[1], Shin-Yoon Kim[1], and Hye-Seon Choi*

Ovariectomy (OVX)-induced estrogen withdrawal resulted in both bone loss and an increase in fat. We observed elevated osteoclast (OC) formation by bone marrow-derived macrophages treated with medium conditioned by fats from OVX mice, but not from sham-operated mice. Fats from OVX mice expressed and secreted higher levels of monocyte chemoattractant protein-1 (MCP-1) than those from sham-operated mice. Increased fat resulting from estrogen deficiency is thus responsible for bone loss due to enhanced OC formation, which is, at least partly, a consequence of elevated MCP-1 production.

INTRODUCTION

Growing evidence links fat and bone metabolism. Osteoporotic postmenopausal women have higher levels of lipid (Broulik and Kapitola, 1993), and a lipid-lowering drug, Statin, increases bone mineral density (BMD) in clinical studies (Uzzan et al., 2007). Animal studies have also shown that TLR4 deficiency results in not only increased BMD, but also decreased body fat (Johnson et al., 2004). However, a systemic modulator linking fat and bone metabolism has not been clearly identified yet. An increase in the mass of adipose tissue is associated with changes in its endocrine and metabolic functions. The altered production of adipokines by adipose tissue has been implicated in metabolic changes associated with obesity. Adipose tissue of obese mice expresses more monocyte chemoattractant protein-1 (MCP-1, CCL2), as compared with that of lean mice (Sartipy and Loskutoff, 2003). MCP-1 is secreted by a variety of cells upon stimulation by proinflammatory cytokines (Rollins, 1997) and is important for the recruitment of monocytes and T lymphocytes (Baggiolini, 1998; Lee et al., 2008). Recent findings suggest that MCP-1 also affects osteoclastogenesis. The receptor activator of nuclear factor-κB ligand (RANKL) induces MCP-1 and is then further increased by it during human osteoclastogenesis (Kim et al., 2005). These findings point to MCP-1 as a strong candidate for a systemic regulator of fat and bone metabolism.

Ovarian involution and estrogen deficiency induce postmenopausal bone loss by affecting bone marrow and bone cells. Steady-state bone mass depends upon a balance between rates of bone formation and bone resorption. However, markers of both bone resorption and formation are increased at menopause, suggesting that overall bone remodeling is accelerated, but that a net increase in bone resorption causes bone loss when estrogen is deficient (Ebeling et al., 1996; Parfitt et al., 1995). The most relevant consequence of estrogen deficiency due to ovariectomy (OVX) is elevated cytokine-induced osteoclast (OC) formation (Weizmann and Pacifici, 2006). Estrogen decreases osteoclastogenesis by acting on receptors in OCs (Srivastava et al., 2001). OCs are the cells responsible for bone resorption and are formed from hematopoietic stem cells. The mononuclear precursors of OCs fuse to form multinucleated OCs that differentiate into active OCs (Suda et al., 1999). Cooperation between the macrophage-colony stimulating factor (M-CSF) and RANKL systems generates an essential signal for OC differentiation. The factors that promote osteoclastogenesis are mainly proinflammatory cytokines, such as interleukin-1 and tumor necrosis factor (TNF). However, the concentrations of these factors in the bone marrow of OVX mice are insufficient to account for the increased bone resorption caused by estrogen withdrawal, suggesting that other bone-targeting cytokines are required for bone loss due to estrogen deficiency.

In this study, we investigated the relationship between increased fat and bone loss associated with OVX-induced estrogen withdrawal. We focused on the effect of fat on increased OC formation through the release of MCP-1 that acts as a link between bone and fat metabolism.

MATERIALS AND METHODS

Reagents

Recombinant proteins (mouse M-CSF, RANKL and MCP-1), neutralizing anti-mouse MCP-1 antibody (Ab), and biotin-labeled anti-mouse MCP-1 Ab were obtained from R&D Systems, Inc.

Department of Biological Sciences (Brain Korea 21 Program) and the Immunomodulation Research Center, University of Ulsan, Ulsan 680-749, Korea, [1]Skeletal Diseases Genome Research Center, School of Medicine, Kyungpook National University, Daegu 700-412, Korea
*Correspondence: hschoi@mail.ulsan.ac.kr

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(USA). Antibodies against CD11b, CD45R and CD3 were from eBioSciences (USA). The acid phosphatase kit was from Sigma Chemical Co. (USA).

Animals and OC formation

Six-week-old C57BL/6J (B/6) mice were subjected to OVX or sham operations. Eight or 13 weeks after surgery, animals were analyzed for body weight, femur phenotype, subcutaneous fat, and visceral fat, including intraperitoneal, extraperitoneal, and intrapelvic adipose tissue (Bays et al., 2008). All mice were housed in the specific pathogen-free animal facility of the Immunomodulation Research Center. Animal experimentation protocols were approved by the Institutional Animal Care and Use Committee (University of Ulsan, Immunomodulation Research Center).

Bone marrow cells were isolated from 4-5-week-old B/6 mice, as previously described (Lee et al., 2007). Femora and tibiae were aseptically removed and dissected free of adherent soft tissues. The bone ends were cut, and the marrow cavity was flushed out from one end of the bone with α-modified minimal essential medium (α-MEM), using a sterile 21-gauge needle. The bone marrow suspension was carefully agitated with a plastic Pasteur pipette to obtain single cells. These cells were washed twice and resuspended in α-MEM containing 10% fetal bovine serum (FBS). The suspension was incubated on plates with M-CSF (20 ng/ml). After 16 h, non-adherent cells were harvested and cultured for two more days at which time large populations of adherent monocyte/macrophage-like cells had formed on the bottom of the culture plates. The small number of non-adherent cells and was removed by washing the dishes with PBS, followed by incubation for 5 min in 0.25% trypsin/0.05% EDTA. The remaining adherent bone marrow-derived macrophages (BMM) were harvested, seeded at a density of 8×10^3 cells/well in 96-well plates, additional medium containing M-CSF and RANKL (40 ng/ml) was added, and the medium was replaced on Day 3. After incubation for the indicated times, the cells were fixed in 10% formalin for 10 min and prepared for tartrate-resistant acid phosphatase (TRAP) staining, as previously described (Kobayashi et al., 2000). The number of TRAP-positive multinucleated cells (MNC) containing three or more nuclei was scored.

Micro CT scanning was performed with the GE eXplore Locus SP system (Locus SP; GE Healthcare Company, USA), as previously described (Sheng et al., 2009). For three-dimensional histomorphometry and visualization of the long bone structure, femurs were scanned with a high-resolution micro CT imaging system, set to a 0.008-mm effective detector pixel size. The three-dimensional microstructure analysis software was provided by explore MicroView 2.2.

Real-time quantitative PCR

Total RNA was reverse-transcribed with oligo-dT and Superscript I enzyme (Invitrogen, USA), according to the manufacturer's instructions. Real-time quantitative RT-PCR was performed using SYBR Green 1 Taq polymerase (Qiagen, Germany) and appropriate primers in a DNA Engine Opticon Continuous Fluorescence detection system (MJ Research Inc., USA). The specificity of each primer pair was confirmed by melting curve analysis and agarose-gel electrophoresis. The housekeeping GAPDH gene was amplified in parallel with the gene of interest and the relative copy number calculated from the expression $2^{-\Delta\Delta Ct}$ (Livak and Schmittgen, 2001). The primer sequences used were as follows: 5′-tcatgcttctgggcctgctg-3′ and 5′-tctcatttggttccgatccaggtt-3′ (MCP-1); 5′-acccagaagactgtggatgg-3′ and 5′-cacattgggggtaggaacac-3′ (GAPDH).

ELISA

The concentration of MCP-1 in conditioned medium or serum was determined by a sandwich ELISA using a coating of anti-MCP-1 Ab and biotinylated anti-MCP-1 Ab (R&D Systems, Inc.).

Statistical analysis

All values are expressed as means ± standard error (SE). The Student's *t*-test was used to evaluate differences between samples of interest and the respective controls. *P* values of less than 0.05 were considered statistically significant.

RESULTS

Estrogen deficiency results in elevated levels of fat along with bone loss

We used a mouse model for estrogen deficiency-induced bone loss to examine the association of bone loss with increased adipose tissue. First, we determined the increase in subcutaneous and visceral area fat depots and body weight. Fat depots sampled from the visceral area weighed significantly more in OVX mice than in sham-operated mice at both 8 and 13 weeks after surgery (Fig. 1A). Although there was a higher trend in the weight of subcutaneous fat for OVX mice, as compared to sham-operated mice at 8 weeks post-surgery, this weight increase only became significant at 13 weeks, suggesting that estrogen deficiency affected visceral fat more than subcutaneous fat.

Next we examined femur bones from OVX and sham-operated mice by Micro CT. The trabecular bone volume fraction was 13% lower (Fig. 2A), and trabecular separation was higher (Fig. 2B), in OVX mice, as compared to sham-operated mice. Endpoint values of bone metabolism (trabecular BMD and bone mineral content (BMC)) were significantly lower in OVX mice than in sham-operated mice at four weeks after surgery (Figs. 2C and 2D). Three-dimensional CT analyses and X-ray of femora revealed that trabecular areas were less dense in OVX mice (Fig. 2E). Based on X-ray analysis, the femoral bone density of OVX mice was also significantly lower than that of sham-operated mice after 13 weeks of surgery (data not shown).

Enhanced osteoclast formation is due to MCP-1 released from mature adipocytes of OVX mice

Enhanced bone resorption plays a critical role in estrogen deficiency-induced bone loss (Ebeling et al., 1996) and is caused, in part, by elevated OC formation. To investigate whether increased fat contributes to bone loss, we first measured the effect of soluble factors released from adipose tissues on RANKL-induced OC formation by BMM. Since OVX resulted in a more prominent increase in visceral fat, mature adipocytes were isolated from visceral fat depots of OVX or control mice, and their conditioned media were collected. The medium from OVX mice had a significantly greater stimulatory effect on the number of TRAP-positive MNC (Fig. 3A), suggesting that visceral fat from OVX mice contained higher levels of osteogenic factors than that from sham-operated mice. The visceral fat depots from OVX mice also produced significantly higher levels of MCP-1 transcripts (Fig. 3B) and MCP-1 protein (Fig. 3C) than those from the sham-operated mice. A similar outcome was found for serum levels of MCP-1 (Fig. 3D). To confirm that the highly expressed MCP-1 in visceral fat of OVX mice contributes to the enhancement of OC formation, we neutralized MCP-1 with anti-MCP-1 Ab. The stimulatory effect of medium conditioned by mature adipocytes from the visceral fat of OVX mice on TRAP-positive MNC was abolished in the presence of anti-MCP-1 Ab but not in the presence of control IgG (Fig. 2E).

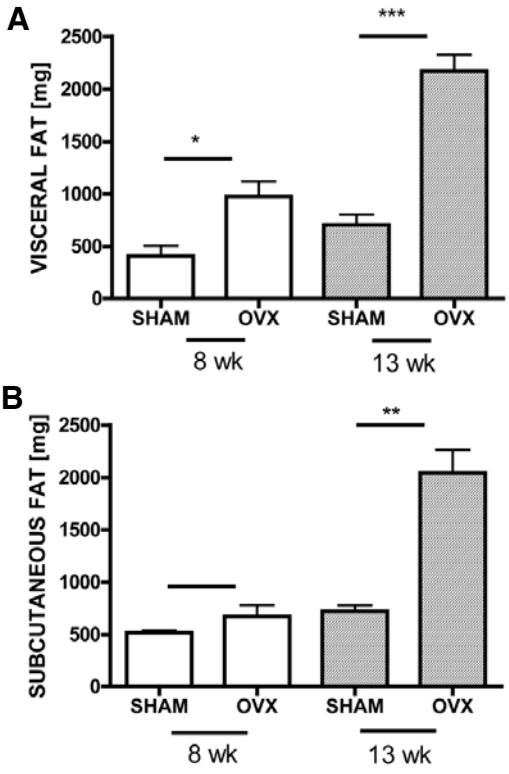

Fig. 1. OVX leads to increased visceral fat and subcutaneous fat. Visceral fat (A) and subcutaneous fat (B) were isolated from OVX and sham-operated mice after 8 and 13 weeks of surgery, and weighed.

We concluded that the elevated level of MCP-1 from visceral fat of OVX mice was responsible for increased OC formation by BMM.

DISCUSSION

We demonstrated that an elevated level of MCP-1 production in OVX-increased visceral fat was at least partly responsible for the simultaneous enhancement in OC formation, suggesting a strong connection between bone and fat metabolism.

In our experiments, OVX increased visceral fat at an early stage and subcutaneous fat at a later stage. Estrogen treatment is known to reverse increases in body weight and fat (Shinoda et al., 2002; Wade and Gray, 1985). White adipose tissue gradually increases in estrogen receptor (ER) α-knockout mice (Heine et al., 2000). Changes in adipose depots occur by adipocyte size changes. Estrogen deficiency is expected to result in an increase in both size and number of adipocytes, since these effects are observed in the absence of ER α in male and female mice (Heine et al., 2000). In addition, genistein, which is estrogenic in vivo and binds ER, decreases adipocyte size in a dose-dependent manner (Naaz et al., 2003). The location of fat stores influences the pathogenesis of metabolic disease. Obese individuals that are free of metabolic disease often have less visceral distribution of adipose tissue than those with metabolic disease (Karelis et al., 2004). Adipose tissue depots differ not only in their kind of metabolic activity, but also in the degree of metabolic activity. Visceral adipose tissues have an increased level of metabolic activity, as compared with subcutaneous adipose tissue (Tan et al., 2004). Adipose tissue is known as a reservoir for energy storage, as well as an important endocrine organ, expressing numerous receptors and secreting a variety of bioactive peptides acting at both local and systemic levels. Adipose tissue is, therefore, integrally involved in coordinating a variety of biological processes, such as neuroendocrine function and immunity. Although the relationship

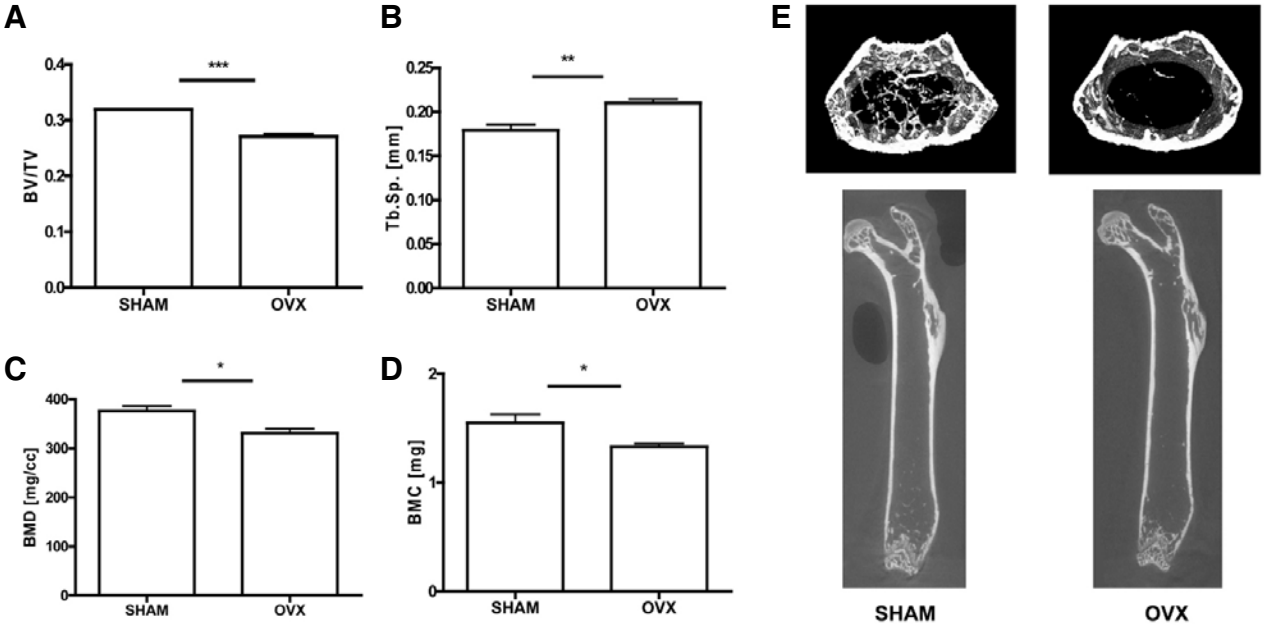

Fig. 2. Microstructural analysis of femora from OVX and sham-operated mice four weeks after surgery. Trabecular bone volume is expressed as a percentage of total tissue volume (BV/TV [%]) (A). Tb. Sp. (expressed in mm) refers to the distance between two trabeculae (B). BMD (C) and BMC (D) were measured at the right femora. Three-dimensional CT images of distal femora and plain X-ray images of femora from representative sham-operated and OVX mice (E). Data are expressed as means ± SE. The values of BV/TV, Tb. Sp., BMD, and BMC of OVX mice (n = 5) were significantly different from those of sham-operated mice (n = 5).

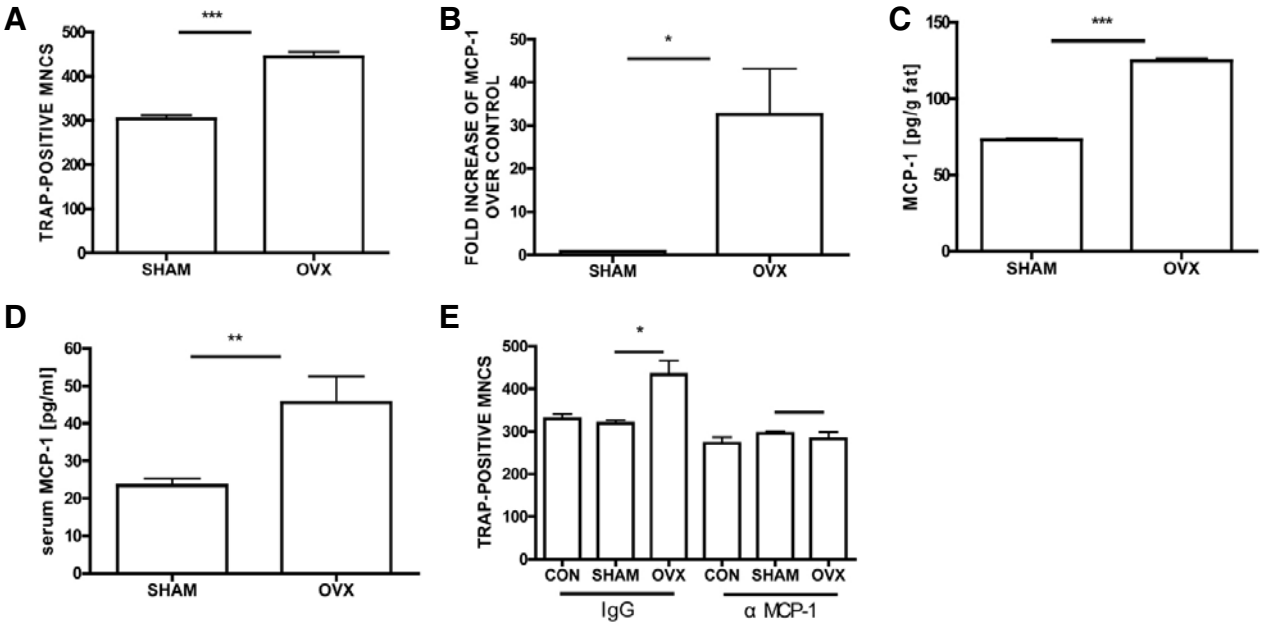

Fig. 3. Effect of visceral fat on OC formation. Visceral fat was isolated from OVX and sham-operated mice 13 weeks after surgery. Five hundred milligrams of visceral fat was incubated with α-MEM-containing collagenase II (0.7 mg/ml) and 5% BSA for 1 h at 37°C. Single cell suspensions were made by passage through a strainer, washed with 5% BSA twice, and incubated in α-MEM for 20 h to obtain conditioned medium. BMM were prepared as described in the "Materials and Methods" and incubated in 96-well plates (8×10^3 cells/well) in the presence of M-CSF (20 ng/ml) and RANKL (40 ng/ml) with medium conditioned by the visceral fat (A). After three days, cells were fixed and stained for TRAP, and the number of TRAP-positive MNC per well was scored (***, $P < 0.001$, n = 3). Total RNA was isolated from the visceral fat from OVX and sham-operated mice and subjected to quantitative PCR analysis of MCP-1 expression (B) (*, $P < 0.05$, n = 3). Conditioned medium (C) and serum (D) were used for determination of MCP-1 by ELISA (***, $P < 0.001$, n = 3; **, $P < 0.01$, n = 3). BMM were incubated with vehicle-only or the two types of conditioned medium from mature adipocytes of sham-operated and OVX mice in the presence of M-CSF and RANKL and exposed to control IgG (*, $P < 0.05$, n = 3) or anti-MCP-1 Ab (3 ug/ml) for three days, fixed, and stained for TRAP (E). There was no significant difference between the two types of conditioned media when treated with anti-MCP-1 Ab.

between excess fat mass and bone loss has not been clearly established, our previous results (Kyung et al., 2009) and those of others (Gao et al., 2009) suggested that excess fat induces bone loss. A lipid-lowering drug has been demonstrated to inhibit bone loss in a human study (Uzzan et al., 2007) and an experimental animal model (Gutierrez et al., 2008). Hyperlipidemia has been shown to decrease bone formation (Parhami et al., 2001) and to increase OC formation and bone resorption (Tintut et al., 2004). Free fatty acid is a strong candidate for the cause of bone loss by increasing osteoclast formation (Oh et al., 2009).

Adipose tissue secretes various adipokines, as well as free fatty acid, so it is plausible that adipokine production could mediate the association between fat gain and bone loss. We found an increase in MCP-1 in fat depots at both the mRNA and protein levels, as well as increased circulating MCP-1 levels during estrogen deficiency. However, whether visceral fat is directly responsible for elevated circulating MCP-1 still remains to be investigated. MCP-1 functions as both a local and an endocrine effector. An increased level of circulating MCP-1 in obesity results in increased circulating monocytes (Takahashi et al., 2003). Administration of MCP-1 promotes accumulation of monocytes in collateral arteries, increasing neointimal formation (van Royen et al., 2003). Exposure of cultured adipocytes to MCP-1 decreases insulin-stimulated glucose uptake (Sartipy and Loskutoff, 2003). These results suggest that MCP-1 acts as a mediator of metabolic abnormalities associated with excess fat. Expression of MCP-1 is elevated in human osteoporotic fractures (Hopwood et al., 2009), and elevated levels of MCP-1 produced by prostate cancer cells increases osteoclastogenesis and bone resorption (Lu et al., 2007).

The mechanism whereby estrogen deficiency induces bone loss is unclear. Although the key factors involved remain to be established, bone loss could be caused by the orchestrated actions of many different cells. Upregulation of OC formation is primarily responsible for bone loss induced by estrogen deficiency (Pacifici, 1996). Estrogen deficiency decreases the level of thiol antioxidants in OCs. This directly sensitizes OCs to osteoclastogenic signals and induces ROS-enhanced expression of cytokines that promote osteoclastic bone resorption (Lean et al., 2003). The absence of OVX-induced bone loss in T-cell deficient mice suggests that T cells are crucial for bone resorption (Cenci et al., 2000). The ability of estrogen to decrease M-CSF production by bone marrow stromal cells suggests that these cells contribute to the OVX-induced bone loss in vitro (Kimble et al., 1996). However, the net bone loss is limited, in part, by a compensatory increase in bone formation also mediated by estrogen deficiency. OVX significantly increases resistin expression at the transcript level and decreases leptin and adiponectin in perigonadal fat (Gui et al., 2004), although their contributions to bone loss require further investigation. Our findings suggest that estrogen affects adipose tissue by modulating the expression of adipokines. MCP-1 is overexpressed in white adipose tissue and elevated in the plasma of genetically obese mice (Sartipy and Loskutoff, 2003). The elevated expression of MCP-1 in adipose tissue may be responsible for the increased plasma level of MCP-1. Our finding of a relationship between estrogen deficiency and MCP-1 production is supported by other studies. Estrogen has been dem-

onstrated to decrease the expression of MCP-1 (Arci et al., 1999), and increased serum MCP-1 is observed in post-menopausal women (Koh et al., 2001). Furthermore, OVX results in elevated MCP-1 expression on T cells, without any change in its serum level at an early time point (Binder et al., 2009).

In conclusion, our results suggest that the bone loss observed as a result of estrogen deficiency is due, at least partly, to increased levels of fat depots and the consequent increase in MCP-1 production. This finding suggests that inhibition of MCP-1 may be effective in preventing bone loss in postmenopausal women.

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