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Effects of Estrogen Therapy on Bone Marrow Adipocytes in Postmenopausal Osteoporotic Women

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Abstract

Introduction—Aging is associated not only with bone loss but also with increases in bone marrow adipocytes. Since osteoblasts and adipocytes are derived from a common precursor, it is possible that with aging, there is a preferential “switch” in commitment of this precursor to the adipocyte over the osteoblast lineage. We tested the hypothesis that the apparent “age-related” increase in marrow adipocytes is due, at least in part, to estrogen (E) deficiency.

Methods—Reanalysis of bone biopsies from a randomized, placebo-controlled trial involving 56 postmenopausal osteoporotic women (mean age, 64 years) treated either with placebo (PL, n = 27) or transdermal estradiol (0.1 mg/d, n = 29) for 1 year.

Results—Adipocyte volume/tissue volume (AV/TV) and adipocyte number (Ad#) increased (by ~ 20%, $P < 0.05$) in the PL group, but were unchanged (Ad#) or decreased (AV/TV, by -24%, $P < 0.001$) in the E group. E treatment also prevented increases in mean adipocyte size over 1 year.

Conclusions—These findings represent the first *in vivo* demonstration in humans that not only ongoing bone loss, but also the increase in bone marrow adipocyte number and size in postmenopausal osteoporotic women may be due, at least in part, to E deficiency.

Keywords

Osteoporosis; adipocytes; bone

INTRODUCTION

Age-related bone loss in women and men and postmenopausal bone loss in women is due principally to an imbalance between bone resorption and bone formation (1). The impaired bone formation with aging was demonstrated in human iliac crest bone biopsies by Lips and colleagues (2) who measured mean wall thickness, an index of the work done by the team of osteoblasts in each basic multicellular unit (BMU), and found that this decreased by 43% over life. Dunnill and colleagues were the first to demonstrate an age-related increase in vertebral marrow fat and a decrease in bone volume as a function of age (3). Concomitant with an age-related decrease in bone formation at the BMU level, Meunier and colleagues (4) established over three decades ago that there was a parallel increase in bone marrow adipose volume with age. Using iliac crest bone biopsies, these investigators showed that, between the ages of 20 and 90 years, trabecular bone volume decreased by 40% and this was accompanied by a 300% increase in marrow adipose volume in both women and men.

Osteoblasts and adipocytes are believed to be derived from a common progenitor in the bone marrow mesenchymal stromal compartment (5). A number of *in vitro* studies using primary bone marrow stromal cells and stromal cell lines have established the existence of a bipotential osteoblast/adipocyte precursor (6,7). The commitment of this precursor cell to the osteoblast or adipocyte lineages is determined by the expression and/or activity of lineage-specific transcription factors. For example, *runx2* and *osterix* promote osteoblastic (8,9) and *C/EBP β* and *PPAR γ* promote adipocytic commitment and differentiation (10). That the balance between such lineage-specific nuclear transcription factors is important was highlighted by findings in mice with haplo-insufficiency of *PPAR γ* ; these mice have an increase in bone mass associated with increased osteoblastogenesis and decreased adipogenesis (11). On the other hand, marrow cultures derived from osteoporotic SAMP6 mice exhibited decreased osteoblast development and increased adipogenesis (12). Collectively, these findings have led to the concept of “reciprocity” between osteoblast and adipocyte cells and it is becoming increasingly clear that a change in bone marrow stromal cell dynamics can result in osteoporosis due to an increase in the number of marrow adipocytes at the expense of osteoblasts (13). Since the potential exists of the ability of single or a combination of agents to alter the commitment, or at least the differentiation pathway, of these bipotential osteoblast/adipocyte precursors (14), it has been proposed that either preventing further increases in marrow adipocytes, or better still, diverting marrow adipogenesis towards osteoblastogenesis would result in an increase in functional bone cells (15).

Estrogen (E) plays an important role in regulating both osteoblasts (1) and adipocytes (16) and is therefore a reasonable candidate for the modulation of the marrow stromal precursor population. Thus, ovariectomy in mice is associated not only with a decrease in bone mass, but also a significant increase in bone marrow adipocyte content (17). In *in vitro* studies, Okazaki et al. found that E dose-dependently promoted osteoblast development and inhibited adipogenesis of the murine bone marrow stromal cell line, ST2 (18). Similarly, Dang et al. (19) found that E upregulated osteoblast-related gene expression while reciprocally suppressing expression of adipocyte-related genes in both primary murine bone marrow stromal cells and in a fetal mouse calvarial cell line (KS483).

Since aging is also associated with significant decreases in circulating E levels following the menopause, in the present study we tested the hypothesis that the apparent “age related” increase in bone marrow adipocytes in postmenopausal women was due, at least in part, to E deficiency. Thus, we reanalyzed and compared paired iliac crest biopsy specimens obtained from an earlier study in postmenopausal osteoporotic women treated either with placebo or E therapy for 1 year who had displayed increases in bone mineral density (BMD) at various sites on E treatment for potential changes in adipocytic parameters (20).

MATERIALS AND METHODS

Study Population

We utilized archived transiliac bone biopsy slides from an earlier study by Lufkin and colleagues (20) examining effects of 1 year treatment either with placebo or transdermal estradiol (Estraderm, CIBA Pharmaceuticals, Edison, NJ, delivering 0.1 mg/d of estradiol) for days 1 to 21 and with medroxyprogesterone acetate (10 mg/d) for days 11 to 21 of a 28-day cycle, regardless of previous hysterectomy. The original study reported data on BMD, bone turnover, and selected bone histomorphometric variables in 39 women in the placebo group and 36 women in the E group (20). Inclusion/exclusion criteria are provided in detail in the previous publication (20). Briefly, all women were defined as having osteoporosis prior to entry based on the presence of one or more vertebral fractures and a BMD at the lumbar spine and proximal femur below the 10th percentile of normal premenopausal women. For the present study, we had access to the paired transiliac bone biopsy samples in 27 women in the placebo

group and 29 women in the E group; the remaining women in the original study either did not have baseline and 1 year biopsies or the biopsy samples were lost or not analyzable due to specimen artifact. All women provided informed consent for the original study and both that study and the subsequent reanalysis of the bone biopsy data were approved by the Mayo Institutional Review Board.

Bone histomorphometry and determination of adipocyte parameters

Partial results of changes in activation frequency and bone formation rate in these patients have previously been reported (20). The original data from bone histomorphometry regarding the bone parameters (bone volume/tissue volume, BV/TV; bone formation rate per bone volume, BFR/BV; percent surface covered by osteoblasts, %Ob surface; percent eroded surface, % eroded surface; and osteoclast number, OC#) were used. Those data were obtained using standard methods on a Bioquant IV computer system (R & M Biometrics, Nashville, TN), as previously described (20).

For the adipocyte parameters, we used sections stained with the Goldner's stain. Adipocyte number (AD#, 1/mm²), percent adipocyte volume per tissue volume (AV/TV), and total adipocyte perimeter (total AdPm, mm) were measured by tracing out individual adipocytes in all the fields analyzed; adipocytes appear as distinct, translucent, yellow ellipsoids in the marrow cavity. Since AdPm is a measure of total perimeter/total area, we also calculated the percent changes in adipocyte perimeter and diameter per adipocyte cell in order to assess the effect of treatments on the size of individual adipocytes. A uniform number of fields were read in all sections, starting three fields from the left end of the biopsy and three fields from the top endocortical surface. Four fields (2 consecutive fields on top followed by the immediate 2 consecutive fields below) were read in all paired samples from the placebo and E groups. When a field was observed to have a significant artifact due to disruption of adipocytes, that field was excluded from analysis and the adjacent field analyzed. Since all biopsies were read in a blinded manner, without knowledge of the treatment status, this approach should not lead to any bias in the analyses. All measurements were done at a 20× magnification using the Osteomeasure analysis system (OsteoMetrics, Atlanta, GA, USA). The photomicrographs shown in Figure 2 were captured at a 40× magnification. All biopsies were read in a blinded manner, without knowledge of the treatment status, in order to avoid any bias in the final analysis.

Serum lipids, leptin and estradiol measurements

Serum lipids were measured in stored and batched samples obtained at baseline and one year in the original study (20). Serum leptin was measured by radioimmunoassay (Millipore, Billerica, MA) with a lower limit of detection of 0.5 ng/mL and an inter-assay CV of <7%. High sensitivity estradiol measurements were carried out on serum samples using a double antibody radioimmunoassay (Diagnostic Products Corp., Los Angeles, CA) with a lower limit of detection of 5 pg/ml and an inter-assay CV of < 16%.

Statistical Methods

Paired t-tests were used to compare parameters at baseline versus 1 year of either placebo or E therapy. Changes in each of the parameters in the placebo versus E treatment group were compared using non-paired t-tests. All data are presented as mean ± SEM, and P < 0.05 was considered significant.

RESULTS

Table 1 shows the baseline characteristics of the subset of women in the placebo and E groups used in this study. As is evident, the subjects in the two groups were well matched for age,

anthropometric measures, years since menopause, BMD at various sites, baseline bone histology, as well as serum lipid, leptin, and estradiol levels. The subjects in the E group were slightly heavier than in the placebo group ($P=0.06$). Table 1 also shows the bone histomorphometric variables, and as is evident, these were also very similar between the two groups at baseline.

While the BMD and bone histomorphometric changes with placebo or E therapy have been previously reported for the entire study group (20), Table 2 shows the changes in these bone variables in the subset of patients specifically analyzed in this study. In this sub-group, as in the entire study group (20), BMD increased significantly at the lumbar spine in the E group, and these changes were significantly different in the E versus placebo groups. BMD remained stable in the E group at the mid-radius, whereas it declined in the placebo group. Although femur neck BMD increased significantly in the E group and remained unchanged in the placebo group, changes in femur neck BMD were not statistically different between groups. There were no discernible changes in BV/TV in either group over the limited period of 1 year but, as expected, E significantly reduced BFR at the tissue level, as well as the overall percent of bone surface covered by osteoblasts, consistent with the known effect of E in suppressing bone remodeling. Indices of bone resorption (% eroded surface and OC#) decreased in the E group but remained largely unchanged in the placebo group. Changes in blood lipids were not different between groups, whereas serum leptin levels increased significantly in the E group and were unchanged in the placebo group. Serum estradiol levels increased significantly in the E group and decreased slightly in the placebo group.

Figure 1(A-C) shows the changes in adipocyte parameters in the placebo and E groups over 1 year. As is evident, AV/TV, AD#, and AdPm (a measure of adipocyte size) all increased significantly in the placebo group (~20% for each of these parameters), but were either unchanged (AD#, AdPm) or decreased (AV/TV) following E therapy. The changes in AV/TV and AdPm in the placebo group were significantly different from the corresponding changes in the E group ($P < 0.001$ and < 0.005 , respectively); changes in AD# in the placebo versus E groups did not achieve statistical significance ($P = 0.075$). Figure 2 depicts photomicrographs obtained at a $40\times$ magnification from representative patients from either the placebo (Figure 2A) or E (Figure 2B) groups before start of treatment (baseline) and at the end of 1 year

In order to further assess whether the pattern of changes in adipocyte perimeter in the placebo and E groups were reflected at the level of an individual adipocytic cell, we calculated the changes in the mean adipocyte perimeter and diameter per adipocyte cell by dividing the adipocytic perimeter by total number of adipocytic cells counted and by deriving the diameter/cell. Figure 3 depicts the percent change from baseline in these parameters, both in the placebo and E groups. As evident, the mean adipocyte perimeter/cell and the adipocyte diameter/cell were significantly reduced in the E-treated group compared to baseline. In contrast to the increase in total adipocyte perimeter in the placebo group (Figure 1C), we did not observe significant increases adipocyte perimeter on a per cell basis in this group (Figure 3). Finally, changes in serum leptin or estradiol levels did not correlate with changes in any of the adipocyte parameters in either the placebo or E groups (data not shown).

DISCUSSION

We demonstrate in the present study that bone marrow adipocyte parameters (AV/TV, AD#, AdPm) increased significantly over one year (by about 20% each) in untreated postmenopausal osteoporotic women and that E therapy was able to either prevent (AD#, AdPm) or even reverse (AV/TV) these changes. The increases observed in the percent adipocyte volume (AV/TV) in untreated postmenopausal osteoporotic women were primarily due to an increase in adipocyte numbers. Although the total adipocyte perimeter was increased, we could not find an increase

in either the mean adipocyte perimeter or diameter per cell in this group. This can be explained by the fact that the total perimeter (which is a reflection of aggregate adipocyte surface per total area of the measured fields of the biopsy) would increase if the total number of cells per total area increased, regardless of the fact that the size of the individual adipocyte remained the same, and this appears to be the case in the placebo group. In the E-treated group, however, we did observe significant reductions in the size of the individual adipocyte perimeter and diameter, while adipocyte number did not increase. Thus, E appeared to both prevent increases in adipocyte number and to reduce the size of individual adipocytes, resulting in the observed net decrease in adipocyte volume in the bone marrow over one year in the E-treated women. We should also note that the fairly impressive (~20%) increases in AV/TV, Ad#, and AdPm observed in the placebo-treated women may not be representative of all postmenopausal women, since the study group consisted of women with significant osteoporosis, which may not be representative of changes occurring in non-osteoporotic postmenopausal women. Further, we did not observe any significant changes in the trabecular bone volume in either of the groups over the period of the study. This suggests that the increase in adipocyte volume in the absence of E in the control group is not merely a “passive” vacuum filling process to compensate for decreases in bone volume in these subjects.

While previous studies have shown increases in bone marrow adipocyte parameters with aging (4) or in patients with osteoporosis (21,22), our study is the first to demonstrate longitudinal increases in these adipocyte parameters in osteoporotic postmenopausal women and prevention or reversal of these changes with E therapy. These results are of interest in view of the well established fact that E is known to inhibit the lipogenic enzyme, lipoprotein lipase (23) and induce the lipolytic enzyme, hormone sensitive lipase (24). Thus, taken together, these actions of E are entirely consistent with its ability to decrease mean adipocyte size by preventing neutral lipid uptake into the adipocytic cell and at the same time inducing lipolysis of existing lipid stores within the adipocyte.

Even in animal models, there is a relative paucity of data directly demonstrating effects of E on bone marrow adipocytes, which may be regulated differently from peripheral adipocytes. In addition, peripheral adipocytes themselves are known to display distinct depot-specific responses to E (25). However, studies with female mice lacking either ER α or aromatase have found significant increases in total body or gonadal fat in these mice relative to wild type controls (26,27). For example, three month old female ER α knock out mice had increased fat pad weights as well as increased adipocyte size and numbers (28), and male ER α knock out mice have a near doubling of epididymal, perirenal, and inguinal white adipose tissue as compared to their wild type littermates by one year of age (28). These studies thus indicate that the absence of E signaling results in adipocytic hypertrophy and hyperplasia in mice, at least in peripheral fat depots. It would be important to ascertain the exact marrow adipocyte phenotype in the ER α knock out mice in order to understand the consequence of loss of E signaling on marrow adipogenesis.

It is of interest that serum leptin levels increased significantly following E treatment, whereas marrow adipocyte parameters showed a decrease; however, changes in serum leptin levels in either group did not correlate with changes in bone or adipocyte parameters. Consistent with our findings, E treatment of mice has been shown to increase leptin mRNA levels in peritoneal adipose tissues (29) and E also stimulates leptin secretion from human subcutaneous adipose tissue fractions *in vitro* (30). However, leptin has complex effects on bone metabolism, with evidence for both central (hypothalamic) negative effects on bone mass (31,32) as well as peripheral effects enhancing osteoblast differentiation and inhibiting adipocyte differentiation of bone marrow stromal cells (33), perhaps explaining the lack of any significant correlations between changes in serum leptin levels and any of the bone or adipocytic parameters in our study.

A recent review on the relationship between fat and bone discussed the implications of using therapeutic agents to alter the osteoblast/adipocyte balance in order to prevent or treat certain prevalent conditions such as osteoporosis and obesity (34). In support of this notion, it has recently been shown that the bisphosphonate, alendronate, can retard adipogenesis and enhance osteogenesis by inhibition of the adipogenic transcription factor, PPAR γ 2, in human mesenchymal stem cells (35). Intermittent teriparatide [PTH(1–34)] treatment of adult human bone marrow stromal cells also results in a decrease in adipogenesis and an increase in early osteogenesis (36). In addition, PTH (1–38) treatment of ovariectomized rats has been shown to increase osteogenic and decrease adipogenic markers in the distal femur metaphysis (37). It is also important to note that human mesenchymal stromal cells express FSH receptors (38), which could result in direct actions of FSH on marrow progenitors in humans; however, this needs to be tested.

To our knowledge, our findings represent the first *in vivo* demonstration, in humans, that the increase in marrow adipogenesis in postmenopausal osteoporotic women may be due, at least in part, to E deficiency and that E therapy, even for a relatively short time, can prevent or reverse these gains in marrow adipocyte parameters. Moreover, these data are consistent with earlier animal *in-vivo* studies (17,39) and in *in-vitro* studies (18) utilizing murine cell cultures that support the hypothesis that E deficiency leads to a shunting of mesenchymal precursor cells down the adipocyte and away from the osteoblast lineage, and that this mechanism may be a significant contributor to bone loss in elderly women. However, this is an area of active investigation, as not all studies point to this reciprocal osteoblast-adipocyte relationship. For example, in mice treated with the thiazolidinedione, troglitazone, there was an increase in bone marrow adipocyte content but no parallel reduction in trabecular bone volume (40). Mice knocked out for 11 β -hydroxysteroid dehydrogenase lack bone marrow adipocytes but maintain normal bone formation (41). In contrast, human studies utilizing iliac crest bone biopsies from aging and osteoporotic patients support the hypothesis there is reciprocity between adipocyte volume and bone volume (21). In addition, Martin and Zissimos found site-specific differences (between the epiphysis and metaphysis of the proximal tibia) in patterns of bone loss and also demonstrated a reciprocal relationship between marrow fat content and bone formation rate in these mice (42). Therefore, further studies aimed at identifying the specific pathways by which E inhibits adipogenesis and enhances osteoblastogenesis may lead both to a better understanding of the mechanisms of age-related bone loss and concomitant increases in marrow fat accumulation and also perhaps to the development of novel therapeutics aimed at directing mesenchymal precursor cells away from the adipocyte and towards the osteoblast lineage.

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Figure 1.

Changes in (A) adipocyte volume/tissue volume (AV/TV), (B) adipocyte number (AD#), and (C) adipocyte perimeter (AdPm) in the placebo (solid circles and lines) and E (open circles and dashed lines) groups over 1 year. * $P < 0.05$, *** $P < 0.001$, and † $P = 0.059$ for comparison with baseline. P-values for comparison of change in placebo versus change in E groups are as follows: AD#, 0.075; AV/TV, < 0.001 ; and AdPm, < 0.005 .

Figure 2.
Representative serial sections of Goldner's stained iliac crest biopsies from a patient either on placebo (Panel A) or on E (Panel B) at the start (baseline) and end of treatment (1 year). Photomicrographs taken at 40× magnification.

Figure 3. Percent changes from baseline in the mean adipocyte perimeter/cell (open bars) and mean adipocyte diameter/cell (filled bars) in the placebo and E groups. * $P < 0.05$, ** $P < 0.01$ vs. baseline.

Table 1

Baseline clinical characteristics, BMD, biochemical variables, and bone histomorphometric variables in subjects analyzed in the study. Data are mean $\pm$ SEM.

	Placebo (n = 27)	E (n = 29)	P-value
Age, years	63.9 $\pm$ 1.1	64.4 $\pm$ 1.1	0.759
Height, m	1.58 $\pm$ 0.01	1.58 $\pm$ 0.01	0.857
Weight, kg	62.7 $\pm$ 1.6	68.9 $\pm$ 2.8	0.060
Years since menopause	16.1 $\pm$ 1.3	16.1 $\pm$ 1.5	0.995
BMD, gm/cm ²			
Lumbar spine	0.78 $\pm$ 0.02	0.80 $\pm$ 0.02	0.581
Mid-radius	0.74 $\pm$ 0.02	0.74 $\pm$ 0.02	0.972
Femur neck	0.65 $\pm$ 0.02	0.65 $\pm$ 0.02	0.859
Serum lipids			
Total cholesterol, mg/dl	241.3 $\pm$ 8.9	238.7 $\pm$ 6.8	0.812
Triglycerides, mg/dl	108.7 $\pm$ 9.2	99.9 $\pm$ 7.4	0.460
HDL cholesterol, mg/dl	55.4 $\pm$ 2.2	56.7 $\pm$ 3.3	0.748
LDL cholesterol, mg/dl	169 $\pm$ 9.3	162.2 $\pm$ 7.0	0.555
Serum leptin, ng/ml	17.7 $\pm$ 2.0	20.9 $\pm$ 2.7	0.353
Serum estradiol, pg/ml	9.6 $\pm$ 1.5	12.6 $\pm$ 1.7	0.202
Bone histology			
BV/TV, %	17.3 $\pm$ 1.3	17.1 $\pm$ 1.0	0.877
BFR/BV, %/y	22.6 $\pm$ 2.6	24.0 $\pm$ 3.0	0.715
Ob surface/ BS, %	14.7 $\pm$ 2.2	16.1 $\pm$ 2.2	0.682
% eroded surface	10.02 $\pm$ 1.09	8.70 $\pm$ 0.73	0.318
OC#, 1/100mm B.Pm	21.0 $\pm$ 3.4	17.8 $\pm$ 3.5	0.508

Table 2

Changes over 1 year in BMD, bone histomorphometric variables, serum lipids, leptin, and estradiol levels in the subjects analyzed in this study.

	<u>Placebo</u>	<u>E</u>	<u>P-value^a</u>
BMD, gm/cm ²			
Lumbar spine	0.007 ± 0.008	0.052 ± 0.008 ***	0.0001
Mid-radius	-0.014 ± 0.004 **	0.003 ± 0.007	0.043
Femur neck	0.004 ± 0.009	0.028 ± 0.009 **	0.076
Bone histology			
BV/TV, %	1.645 ± 1.573	1.930 ± 1.241	0.887
BFR/BV, %/y	-0.082 ± 0.025 **	-0.176 ± 0.032 ***	0.023
Ob surface/ BS, %	3.098 ± 3.929	-7.447 ± 2.856	0.033
% eroded surface	1.90 ± 1.24	-1.65 ± 0.96	0.026
OC#, 1/100mm B.Pm	-2.7 ± 4.1	-6.8 ± 3.1 *	0.427
Serum lipids			
Total cholesterol, mg/dl	-15.3 ± 9.3	-15.1 ± 5.8 *	0.989
Triglycerides, mg/dl	-4.1 ± 9.0	-12.7 ± 6.8	0.446
HDL cholesterol, mg/dl	0.960 ± 2.1	-0.15 ± 2.5	0.739
LDL cholesterol, mg/dl	-16.6 ± 9.3	-6.7 ± 5.9	0.367
Serum leptin, ng/ml	-0.75 ± 0.89	3.1 ± 1.4 *	0.029
Serum estradiol, pg/ml	-2.5 ± 1.0 *	60.1 ± 9.0 ***	0.0001

^aP-value for comparison of change in the placebo versus E groups

* P < 0.05

** P < 0.01

*** P < 0.001 versus baseline