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Estrogen Action on Bone Marrow Osteoclast Lineage Cells of Postmenopausal Women In Vivo

J.A. Clowes^{‡,*}, G.Z. Eghbali-Fatourehchi^{*}, L. McCready^{*}, M.J. Oursler^{*}, S. Khosla^{*}, and B.L. Riggs^{*}

[‡] Division of Rheumatology and Internal Medicine, Mayo Clinic College of Medicine, 200 First Street S.W., Rochester, MN 55905

^{*} Endocrine Research Unit, Mayo Clinic College of Medicine, 200 First Street S.W., Rochester, MN 55905

Abstract

Introduction—Although mechanisms by which estrogen (E) decreases bone resorption have been extensively studied in rodents, little information is available in humans.

Methods—In bone marrow aspirates from 34 early postmenopausal women randomly assigned to receive 4-weeks of treatment (100µg/d of transdermal 17β-estradiol) or no treatment, we assessed osteoclast differentiation and surface receptors using flow cytometry with fluorescent-labelled specific antibodies.

Results—E-treatment decreased ($P<0.05$) the proportion of bone marrow mononuclear cells (BMMNCs) expressing the calcitonin receptor [CTR], a late osteoclast phenotype marker. There was an increase in c-Fms concentration in osteoclast lineage cells ($P<0.05$) and in the proportion of BMMNCs expressing TNFR2 ($P<0.05$), but there were no significant effects on other surface receptors for proresorptive factors (RANK, TNFR1, TREM2 or OSCAR). Changes in serum CTx and TRAP 5b, markers for bone resorption, correlated directly ($P<0.05$) with the proportion of BMMNCs expressing CTR and, for TRAP 5b only, TNFR2 and inversely with c-Fms concentration (all $P<0.05$).

Conclusion—E reduces bone resorption, in part, by decreasing differentiation of BMMNCs into mature osteoclasts. This action cannot be explained by decreased concentrations of surface receptors for proresorptive factors. The roles of increases in c-Fms concentration and the proportion of TNFR2⁽⁺⁾ cells are unclear.

Keywords

Estrogen; osteoclasts; bone resorption; cytokines; Osteoclast precursors; osteoclast differentiation; postmenopausal women; bone resorption markers

INTRODUCTION

Osteoporosis is a major public health problem resulting in a lifetime fracture risk of 40% in women and 15% in men (1). Postmenopausal estrogen (E) deficiency in women and age-related decreases in men are major causes of bone loss leading to osteoporosis and fractures (1). E-

Corresponding Author: B. Lawrence Riggs MD, Endocrine Research Unit, Guggenheim 7, Mayo Clinic College of Medicine, 200 First St. SW, Rochester, MN 55905, USA, Tel: (507) 255-6663, Fax: (507) 284-9111, riggs.lawrence@mayo.edu.

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deficiency increases bone resorption over bone formation resulting in net bone loss (2). The discovery of estrogen receptors (ER) in osteoblasts two decades ago (3,4) led to intensive research to discover the mechanisms for the post-receptor action of E. Based mainly on studies in ovariectomized rodents or in *in vitro* bone cell cultures, it has been variously reported that the effects of E on bone are mediated, at least in part, by modulation in the production of various cytokines, alone or in combination, including decreases in M-CSF, GM-CSF, TNF α , IL-1 β , IL-6, IL-7 or PGE $_2$; increases in TGF- β or osteoprotegerin; or by decreases in RANK ligand (RANKL) in the bone-bone marrow microenvironment [See reviews (2,5,6)].

However, osteoclast lineage cells also contain ER and respond directly to E-treatment, suggesting that they are target cells for E (7). Also, using RAW264.7 cells, an ER-positive murine monocytic cell preosteoclastic line, both Shevde et al. (8) and Srivastava et al. (9) found that E directly impaired intracellular signalling which inhibited the formation of multinucleated osteoclasts induced by M-CSF and RANK ligand (RANKL). Srivastava et al. (9) also found that effects were not mediated by altered concentrations of ER, RANK (the receptor for RANKL) or c-Fms (the receptor for M-CSF). Recently, Nakamura et al (10) have reported that mice with osteoclast-specific deletion of ER α lost trabecular bone to the same extent as ovariectomized wild-type mice. This strongly suggesting that direct E-action on osteoclast-lineage cells is of major importance in maintaining bone mass, but does not establish whether this action occurs at the level of differentiation of precursors into osteoclasts, the function of mature osteoclasts, or both.

With few exceptions, studies on the mechanism of E-action have been made exclusively in rodents or in cultured osteoclastic cells or cell lines *in vitro*, but these results may differ qualitatively or quantitatively from those in humans *in vivo*. Using flow cytometry of bone marrow aspirates, Eghbali-Fatourehchi et al (11) demonstrated that E-deficient postmenopausal women had increased surface concentration of RANKL on marrow stromal cells and T- and B-lymphocytes. Taxel et al. (12), using flow cytometry of bone marrow aspirates, reported that E-treatment of postmenopausal women decreased the proportion of cells in bone marrow aspirates expressing RANKL on their surfaces but did not find a decrease in concentration per cell. They also reported that E-treatment of cultured human bone marrow cells decreased RANKL expression in lymphocytes and inhibited the formation of osteoclasts *in vitro* in response to RANKL. Sorenson et al. (13) found that the addition of E to cultured CD14 $^{(+)}$ peripheral monocytes inhibited their differentiation into TRAP $^{(+)}$ cells.

Although there is strong evidence that E acts directly on osteoclast-lineage cells, the mechanism (s) of this effect is unclear, particularly in humans where few mechanistic studies have been made. Thus, we have employed 4-color quantitative flow cytometry with fluorescent-labelled specific antibodies in bone marrow aspirates from E-deficient and E-replete postmenopausal women to address two key mechanistic questions. First, does E decrease entry of precursor cells in bone marrow into the osteoclast differentiation pathway? Second, if this is so, is this action mediated by modulation of the surface concentration of key receptors for factors that modulate osteoclast differentiation?

MATERIALS AND METHODS

Study subjects

Thirty-four early postmenopausal women aged 40 to 65 years were randomised into an open label, controlled study to receive E-treatment or no treatment for 4 weeks. Menopausal status was defined by the absence of menses for >1 year in a woman over 50 years of age. Additionally, in women with previous hysterectomy or those under 50 years of age, an elevated value for serum FSH was required. All women were healthy and had no clinically significant abnormalities in laboratory values, no diseases known to affect bone metabolism and were not

taking any drug known to affect bone turnover. All subjects provided full informed consent and the study was approved by the Mayo Clinic Institutional Review Board.

Experimental Protocol

All subjects had a screening examination and blood drawn before enrollment into the study. Those with a value for serum 25-hydroxyvitamin D of <15 ng/ml on screening were treated with vitamin D, 1000 U/d for 6-weeks and a repeat serum determination was made prior to randomization to ensure that normal values were achieved. Those with values between 15 to 24 ng/ml on screening also received vitamin D treatment but serum values were not measured again prior to randomization. Subjects were randomly assigned to treatment groups by the pharmacy and study codes were stored in sealed envelopes. All investigators, technicians performing the assays and those performing statistical analyses were blinded to the randomization categories. Subjects randomised to E treatment (n = 17) received 17 β -estradiol (100 μ g/day) by cutaneous patches (Vivelle, Novartis, Inc.) that were changed every 3–4 days for 4 weeks. Serum was drawn at baseline and at 28 days for measurement of serum CTx and TRAP 5b levels. After 28 days of E-treatment, subjects were admitted to the Mayo Clinical Research Unit overnight and a bone marrow aspiration was performed under local anesthesia between 07:00 and 09:00 am. Bone marrow samples were processed immediately to examine the effects of E treatment on the surface expression of receptors.

Flow cytometry

Isolation and immunostaining of bone marrow mononuclear cells—We characterized surface markers by flow cytometry using 4-color direct and indirect immunofluorescence. Bone marrow mononuclear cells (BMMNCs) were concentrated using density gradient centrifugation (Ficoll-Paque), washed with ice-cold phosphate buffered saline (PBS; pH 7.2) containing 0.5% chicken albumin (Sigma) and counted. Viability was assessed using a hemocytometer and trypan blue exclusion. Non-specific binding sites were blocked by incubating MNCs with 5% normal donkey serum (Jackson ImmunoResearch) at 4°C for 20 minutes on an orbital shaker. Aliquots of 10⁶ cells were transferred into 5 ml polystyrene round-bottomed tubes and incubated at 4°C for 30 minutes with primary antibodies on an orbital shaker. Primary antibodies consisted of biotinylated goat anti-human RANK antibody (R&D Systems, BAF683), goat anti-human OSCAR antibody (R&D Systems, AF2004), goat biotinylated anti-human TREM2 IgG (R&D Systems; BAF1828) and polyclonal goat anti-human CTR (Santa Cruz Biotechnology; sc-8858). Isotype control antibodies were used at the same concentration as the primary antibodies to determine background staining.

Cells were washed and then incubated with corresponding secondary and conjugated antibodies at 4°C for 30 minutes on an orbital shaker. Conjugated antibodies included anti-human CD14 antibody (clone M5E2), anti-human c-Fms (CD115) antibody (clone 61708), anti-human TNFRI antibody (FAB 225) and anti-human TNFR2 antibody (FAB 226) from either R&D Systems or BD-Pharmingen. Secondary antibodies included FITC-conjugated AffinityPure IgG F(ab')₂ fragment donkey anti-goat (Jackson ImmunoResearch) and Streptavidin-Peridinin Chlorophyll-a Protein conjugate (Streptavidin-PerCP; BD-Pharmingen). Cells, while protected from the light, were washed twice with ice-cold buffer and transferred on ice to a flow cytometry core facility for immediate analysis.

Flow cytometry data acquisition—The details of the method of analysis of the flow cytometry data, including the setting of quadrants and gates were performed as previously described in detail (14). Cells in suspension were analyzed using a Becton Dickinson FACScan cytometer (Becton Dickinson Immunocytometry Systems) equipped with a 488 nm argon laser capable of detecting light scatter (forward and side) and a 340–360 nm UV excitation. Residual spectral overlap in the fluorescence channels was removed by electronic compensation. Data

were analyzed using Cellquest software. Gates were set around the lymphocyte-monocyte rich region R1 in forward and side scatter and 100,000 events counted for each sample. The threshold for the isotype control was set to include ~ 99% of background. The percentage of positive cells (as a fraction of BMMNCs) was calculated as the difference in the percentage of gated cells in each fluorescent channel between the sample and isotype control. The concentration per cell of surface receptors in selected gating regions during flow cytometry was determined using mean fluorescent intensity (MFI) per cell as a surrogate measurement. This was assessed automatically by an internal program of the flow cytometer instrument, as previously described (14). Data for each fluorochrome (FITC, PE, PerCP and APC) were normalized using Rainbow calibration beads (SPHERO™ Rainbow Calibration Particles, RCP-30-5 (6 peaks); Spherotech, Inc., Lake Forest, IL) from which a standard curve was constructed to quantify the MFI values. Figure 1 provides a composite of representative dot plot images that demonstrate key components of the flow cytometry analysis, using co-staining with CD14 and CTR as an example. Figure 2 gives composite dot plots of representative flow cytometry runs using fluorescent-labelled antibodies directed against epitopes for CD14 (y-axis) and RANK, TNFR1, TNFR2, TREM2, OSCAR, and c-Fms (x-axis).

Assessment of bone biochemical markers

All laboratory analyses were performed in-house, and all samples for each test were determined in a single assay. Serum cross-linked C-telopeptide of type I collagen (CTX) was measured using an ELISA (interassay CV < 10%; Nordic Biosciences, Herlev, Denmark). Serum tartrate resistant acid phosphatase isoform – type 5b (TRAP 5b) was measured by ELISA (inter-assay CV < 14%; Immunodiagnostic Systems (IDS) Ltd. Fountain Hills, AZ). CTx is believed to be a measure of osteoclast activity whereas TRAP 5b is believed to be a measure of osteoclast number (15). The percentage difference in bone resorption markers between baseline and the 4-week time point was used in all analyses.

Statistical analysis

The primary analysis involved a intergroup comparison of results from various bone marrow samples obtained from E-treated subjects and controls at 4 weeks. Because the majority of the distributions were non-Gaussian, the data are presented as medians and interquartile (25–75%) ranges (IQR). Intergroup differences were assessed using the Wilcoxon rank-sum test. The Spearman rank correlation test was used to examine the relationship of between variables obtained by flow cytometry microscopy with the biochemical markers for bone resorption. A P-value of less than 0.05 was considered significant.

RESULTS

Descriptive data

There were no significant differences at baseline for age [median (IQR), years; E-treatment, 55 (50, 57) vs. control, 53 (50, 59)], weight [kg; E treatment, 72 (67, 83) vs. control, 73 (63, 85)], postmenopausal interval [years; E treatment, 4 (1, 7) vs. control, 6 (3, 8)] or 25-hydroxyvitamin D [ng/mL; E-treatment, 32 (28, 38) vs. control, 30 (25, 40)] between the E treated subjects and controls. As assessed by peripheral leukocyte counting, there were no significant differences in numbers of monocytes [$\times 10^9/L$; E-treatment, 0.40 (0.39, 0.53) vs. control, 0.44 (0.36, 0.51)] or lymphocytes [$\times 10^9/L$; E-treatment, 1.6 (1.4, 2.1) vs. control, 1.7 (1.4, 2.0)].

Concentration of surface receptors per cell

Table 1 provides the medians and IQR for the concentrations per cell of epitopes for the surface receptors known to modulate osteoclast differentiation in controls and after E-treatment. There

was a 19.2% increase in surface concentration of c-Fms in the osteoclast lineage cells expressing the CTR. There was also an increase c-Fms surface concentration that was just below the level of significance for total BMMNCs, but not for CD14⁽⁺⁾ cells. No significant differences could be demonstrated for the effect of E-treatment on surface concentrations of RANK, TNFR1, TNFR2, TREM2 and OSCAR in BMMNCs or for TNFR1 and TNFR2 in CD14⁽⁺⁾ or CTR⁽⁺⁾ cells; no measurements were made for on RANK, TREM2 or OSCAR in the CD14⁽⁺⁾ or CTR⁽⁺⁾ cells

Proportion of BMMCs expressing various surface receptors

Table 2 provides the medians and IQR for the percentage of BMMNCs expressing one of the surface receptor epitope in the control and E-treatment groups. Values were calculated as the difference between sample and isotype control gated on the lymphocyte-monocyte rich region R1 and then compared between the E-treatment and control groups. The percentage of BMMCs that were CTR⁽⁺⁾ was decreased by about half in the E-treatment group ($P < 0.05$). The percentage of CD14⁽⁺⁾ cells that were CTR⁽⁺⁾ was decreased by E-treatment to a similar extent but, owing to a larger variability, fell just below the level of significance (control group, 2.20 [1.30, 4.57]; E-treated group, 1.28 [0.62, 3.41], $P = 0.07$).

Also, there was a large increase in the median percentage of cells expressing TNFR2 after E-treatment ($P < 0.05$). There was also a very large increase in the median value for c-Fms expressing cells that fell just below the level of statistical significance. Small decreases in the percentage of RANK⁽⁺⁾ and OSCAR⁽⁺⁾ cells in the E-treatment group also fell just below the level of significance. Values for TNFR1⁽⁺⁾ and TREM2⁽⁺⁾ cells were similar between groups.

Biochemical markers for bone resorption

Serum levels for CTx and TRAP 5b are given in Table 3. There were no significant differences at baseline between the control and E-treatment groups for either marker or between baseline and 28-day values in the control group. However, in the E-treatment group, differences between baseline and 28-day values were highly significant ($P < 0.01$ to $P < 0.001$).

Table 4 shows the relationship between the change in bone resorption markers from baseline and the concentration of surface receptors on BMMNCs for merged values for the combined (control plus treatment) groups. Changes in both serum CTx and TRAP 5b were inversely related to the concentrations of c-Fms on total BMMNCs ($P < 0.05$) and on osteoclast lineage cells [CTR⁽⁺⁾ cells] ($P < 0.01$). The changes in serum TRAP 5b were directly correlated with concentration of TNFR2 ($P < 0.05$) on CD14⁽⁺⁾ cells, whereas those for serum CTx was just below the level of significance.

Table 5 shows the relationship between the change in bone resorption markers from baseline and the proportion of total BMMCs expressing one of the surface receptors. Changes in both bone resorption markers correlated directly with the proportion of BMMNCs expressing CTR ($P < 0.05$). Note that this correlation is in the expected direction, since a lower percentage of CTR⁽⁺⁾ cells was associated with more negative changes in bone resorption markers. There were also direct correlations with the proportion of OSCAR⁽⁺⁾ cells (serum CTx falling just above and serum TRAP just below the level of statistical significance). Direct correlations with the proportion of RANK⁽⁺⁾ cells also fell just below the level of significance.

DISCUSSION

Our goal was to study the mechanisms of E-action on osteoclast lineage cells in bone marrow aspirates from postmenopausal women. After density gradient centrifugation, we employed quantitative flow cytometry using specific fluorescent-labelled antibodies to identify and

quantify cells expressing specific epitopes. Because the cells were processed immediately after they were obtained, the results are relevant to steady-state physiology *in vivo*. We designed our studies to focus on E-effects on osteoclast differentiation and on potential changes in surface receptors for pro-resorptive factors elaborated in the bone marrow microenvironment that might affect their differentiation.

We demonstrated that E-replete women had only half of the numbers of BMMNCs expressing CTR, an established marker of the committed osteoclast lineage cells, as compared to the E-deficient women. Moreover, the proportion of CTR⁽⁺⁾ BMMNCs correlated directly and significantly with changes in both serum CTx and TRAP 5b, markers for overall bone resorption. Additionally, the E-replete group also had lower proportions of BMMNCs expressing RANK or OSCAR than the E-deficient group although this difference was just below the level of significance. The proportion of BMMNCs expressing RANK and OSCAR correlated directly with changes in bone resorption markers and this was at or just below the level of significance. RANK and OSCAR are established markers for osteoclast differentiation (16). Thus, our collective findings strongly suggest that a reduction in the differentiation of mononuclear osteoclast precursors in bone marrow to mature osteoclasts is a major component of E action.

However, these findings do not establish whether E acts on osteoclast lineage cells directly or indirectly via elaboration of RANKL or other proresorptive cytokines by other cells in the bone marrow microenvironment, or both. Nor do they exclude a direct E-effect on mature osteoclasts. Indeed, based on studies using isolated avian (17) or human osteoclasts (18), such an effect seems likely. However, Sorensen et al. (13) found that the addition of E to cultured mature osteoclast-like cells that had been differentiated in the presence of M-CSF and RANKL did not impair their ability to produce pits on bone slices.

We also assessed the possibility that the anti-resorptive effect of E might be mediated by a reduction in one or more of the major surface receptors for factors that enhance osteoclast differentiation. As previously reviewed (5,19–22), osteoclast differentiation is regulated by multiple cytokines. It was originally believed that the combination of M-CSF and RANKL were both necessary and sufficient for osteoclast formation (22). M-CSF, acting through its receptor c-Fms, induces the proliferation of osteoclast precursors, supports their survival, and upregulates RANK expression. RANKL, acting through RANK, then completes the differentiation of osteoclast precursors and increases the function and lifespan of mature osteoclasts. More recently, however, Koga et al (23) demonstrated that co-stimulatory pathways involving the surface immunoglobulin-like receptors, OSCAR and TREM2, are also required for the completion of osteoclastogenesis. These receptors act through ITAM (the immunoreceptor tyrosine-based activation motif) containing adaptors DAP-12 and the Fc-receptor γ -chain (FcR γ), respectively, to enhance intracellular calcium signalling. Finally, TNF α can act synergistically with RANKL to maximize osteoclastogenesis, and some studies have suggested that it also can stimulate OC differentiation directly (24–26).

The only previous study of the effect of E-treatment on surface receptors of osteoclast-lineage cells was carried out by Shevde et al. (8) who failed to find changes in RANK or c-Fms after E-treatment of cultured RAW264.7 cells, a mouse osteoclast progenitor cell line. In the present study, using our highly sensitive and specific methods to study osteoclast-lineage cells *in vivo* from bone marrow, we were unable to demonstrate differences between E-deficient and E-replete postmenopausal women in the surface concentration per cell of RANK, OSCAR, TREM2, TNFR1 or TNFR2.

We found a small, but significant, increase in the concentration of c-Fms in CTR⁽⁺⁾ osteoclast lineage cells and similar increases in BMMCs, although this change was just below the level

of significance. This was in the opposite direction from predicted since c-Fms is the physiological receptor for M-CSF, a proresorptive cytokine required for osteoclast differentiation. However, we also demonstrated that the surface concentration of c-Fms was inversely and significantly correlated with the serum concentrations of CTx and TRAP 5b, suggesting a physiologically-relevant inhibitory effect. Recent studies have shown that there is a reciprocal relationship between M-CSF and c-Fms (27). Because M-CSF production has been shown to be necessary for ovariectomy-induced bone loss in rats (28), a reduction in M-CSF by E-treatment could lead to the increased expression of c-Fms that we observed in our study. Moreover, an increase in c-Fms on osteoclast precursor cells in bone marrow would not necessarily increase bone resorption if E leads to a “downstream” blocking of intracellular signalling in these cells, as has been found experimentally (8) (9). If the E-induced blocking of signalling is not present in other marrow cells expressing c-Fms, such as monocytes and macrophages, it could shunt precursor cells away from osteoclast differentiation into different pathways and thus decrease bone resorption. This possibility should be investigated in future studies. The failure to demonstrate an increase in c-Fms in CD14⁽⁺⁾ cells could be due to the inherent variability of the measurements or due to the fact that the CD14⁽⁺⁾ cells, which are still capable of differentiation to several different phenotypes, may be less responsive.

In transgenic mice expressing only TNFR2, TNF inhibits osteoclastogenesis whereas in transgenic mice expressing only TNFR1, it stimulates it (29). In the present study, we found that E-treatment of postmenopausal women was associated with increases in the proportion of BMMNCs expressing TNFR2 but not in the proportion of BMMNCs expressing TNFR1. Thus, our findings are consistent with the possibility that part of the E-effect on decreasing osteoclastogenesis is related to differential signalling of TNF through its two classes of receptors on osteoclast lineage cells. Further studies are needed to evaluate this possibility.

Our study has both strengths and limitations. Its major strength is that it was conducted in postmenopausal women under E-replete and E-deficient conditions rather than in rodents where mechanisms of E action may differ. Thus, our findings are directly relevant to bone loss in humans induced by menopause. Moreover, our methodology allowed us to assess changes in differentiation and receptor concentrations of osteoclast-lineage cells under virtually *in vivo* conditions. We also were able to validate our findings by demonstrating significant correlation of variables with biochemical markers of bone resorption. The major limitation is that studies of sorted human bone marrow cells have an inherently high variability. As a consequence, some of the differences that we observed were of only marginal significance. Also, because our studies were made *in vivo* rather than *in vitro*, we were unable to determine whether the observed differences were due to a direct effect of E on osteoclast-lineage cells or to E-induced indirect effects on “upstream” cytokines that then acted secondarily on osteoclast-lineage cells, or both. Further studies are needed to clarify this issue.

In conclusion, our data are consistent with the concept that a major action of E is to decrease the entry of bone marrow progenitor cells into the osteoclast pathway. Although this action has been previously demonstrated in rodents and in cultured bone marrow cells or cell lines stimulated by M-CSF and RANKL *in vitro*, we now show that it is also a major mechanism in postmenopausal women under physiological conditions *in vivo*. We further demonstrate that this effect is not mediated by changes in the surface concentration of receptors for the major proresorptive cytokines, except for the increase in c-Fms, or by the immunoglobulin-like receptors for the co-stimulatory pathway. The increase in c-Fms is presently unexplained as is the increase in the proportion of BMMCs expressing TNFR2. Thus, it is becoming increasingly clear that the action of E on bone cells is complex and involves multiple mechanisms (30). To define it completely, each potential mechanism should be examined individually and in combination. Our studies demonstrate that assessment of many of these mechanisms is now feasible in clinical investigative studies. A similar approach could be used in future clinical

investigation to assess the mechanisms of regulation of osteoclastogenesis by other hormones and cytokines.

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Figure 1.

Representative flow cytometry experiment images. (A) Side/forward light scatter profile of unstained cells following Ficoll-Paque density centrifugation in an untreated postmenopausal woman. The R1 gate represents the BMMCs. The R2 gate represents the excluded granulocyte precursor cells. (B) Dot plot profile of the fluorescence from the irrelevant isotype antibodies. (C) Dot plot profile for co-staining for CD14 and CTR expression. Numbers in each quadrant indicate the percent of gated cells present in that quadrant. Please see Methods for details regarding the flow cytometry analysis.

Figure 2.

Composite demonstrating representative examples of specific fluorescence for labelled-antibodies directed against epitopes for CD14 co-stained with (A) RANK, (B) TNFR1, (C) TNFR2, (D) TREM2, (E) OSCAR, and (F) c-Fms. Numbers in each quadrant indicate the percent of gated cells present in that quadrant.

Table 1

Median (25th and 75th percentile) concentration of surface receptors per cell on BMMNCs in untreated and E-treated postmenopausal subjects measured as assessed by mean fluorescent intensity (MFI) using flow cytometry.

Surface receptors	Concentrations on all BMMNCs		Concentrations on all CD14(+) cells		Concentrations on all CTR(+) cells	
	Control (n = 17)	E treatment (n = 17)	Control (n = 17)	E treatment (n = 17)	Control (n = 17)	E treatment (n = 17)
e-Fns ⁽⁺⁾	120 (110, 137)	135 (119, 170) [†]	59.4 (57.1, 64.9)	60.6 (57.2, 79.2)	68.2 (62.0, 78.1)	81.3(69.2, 101) *
RANK ⁽⁺⁾	317 (238, 358)	329 (280, 423)	NM	NM	NM	NM
TNFR1 ⁽⁺⁾	100 (93.5, 113)	104 (91.0, 115)	58.7(52.8, 66.2)	59.7 (50.7, 68.7)	66.4 (61.7, 80.3)	70.2 (64.1, 98.0)
TNFR2 ⁽⁺⁾	104 (98.4, 120)	102 (93.0, 113)	59.4(56.2, 65.6)	55.6 (53.7, 62.2) [†]	74.0 (62.0, 83.0)	67.8 (61.7, 75.8)
TREM2 ⁽⁺⁾	86.6 (77.4, 96.9)	82.0 (73.9, 90.7)	NM	NM	NM	NM
OSCAR ⁽⁺⁾	142 (103, 189)	142 (124, 197)	NM	NM	NM	NM

Wilcoxon rank sum test;
* P≤0.05,
[†] P > 0.05 and <0.10).
Not measured; NM.

Table 2

Percentages of total BMMNCs (median, 25th and 75th percentile) expressing specific surface receptors in postmenopausal subjects treated with E or controls assessed by flow cytometry.

Surface receptors	Percentage single positive BMMNCs	
	Controls (n = 17)	E treatment (n = 17)
Calcitonin R (CTR ⁽⁺⁾)	2.31(1.29, 3.76)	1.14(0.60, 2.72)*
RANK ⁽⁺⁾	2.65(1.61, 5.85)	1.83(1.04, 4.04) [†]
c-Fms ⁽⁺⁾	-0.30(-10.01, 6.85)	5.28(-2.28, 11.2) [†]
OSCAR ⁽⁺⁾	2.83(1.74, 6.29)	2.04(1.12, 4.43) [†]
TREM2 ⁽⁺⁾	1.46(0.83, 2.53)	0.79(0.60, 1.88)
TNFR1 ⁽⁺⁾	4.36(2.57, 7.58)	3.09(1.47, 11.6)
TNFR2 ⁽⁺⁾	-0.228(-12.2, 0.23)	2.73(-3.30, 6.49)*

Wilcoxon rank sum test;

* P ≤ 0.05,

[†] P > 0.05 and < 0.10.

Negative numbers result from instances where the staining with the particular antibody happened to be lower than the value for the isotype control (since the value for the isotype control was subtracted in each instance from the value for the specific antibody).

Table 3

Values for biochemical markers of bone resorption at baseline and 28 days and the significance of these changes between the two groups.

	Serum TRAP 5b, U/L		Serum CTx, ng/mL	
	Baseline	28 Days	Baseline	28 Days
Control	3.8 (3.4, 4.6)	3.8 (3.2, 4.4)	0.70 (0.55, 0.92)	0.64 (0.53, 0.77)
Treatment	4.1 (3.3, 5.3)	3.0 (2.6, 3.8)***	0.67 (0.51, 1.0)	0.40 (0.28, 0.63)**

Table 4

Spearman correlation coefficients for concentration of surface receptors on BMMNCs in merged postmenopausal subjects (n = 34) with changes in serum levels of biochemical markers between baseline and day 28 in both single positive and double positive cells.

Concentration of surface receptors	% Change in serum bone marker			
	Serum CTx		Serum TRAP	
	r	P	r	P
c-Fms on total BMMNCs	-0.389	0.023	-0.370	0.031
RANK on total BMMNCs	-0.018	NS	-0.04	NS
TNFR1 on total BMMNCs	-0.029	NS	-0.130	NS
TNFR2 on total BMMNCs	0.173	NS	0.203	NS
TREM2 on total BMMNCs	-0.050	NS	0.007	NS
OSCAR on total BMMNCs	0.012	NS	-0.045	NS
c-Fms on c-Fms ⁽⁺⁾ /CTR ⁽⁺⁾ cells	-0.453	0.007	-0.476	0.004
c-Fms on c-Fms ⁽⁺⁾ /RANK ⁽⁺⁾ cells	-0.004	NS	-0.084	NS
c-Fms on c-Fms ⁽⁺⁾ /CD14 ⁽⁺⁾ cells	-0.154	NS	-0.157	NS
RANK on RANK ⁽⁺⁾ /CD14 ⁽⁺⁾ cells	-0.147	NS	-0.223	NS
TNFR1 on TNFR1 ⁽⁺⁾ /CD14 ⁽⁺⁾ cells	-0.15	NS	-0.303	0.086
TNFR2 on TNFR2 ⁽⁺⁾ /CD14 ⁽⁺⁾ cells	0.302	0.088	0.391	0.024

Table 5

Spearman correlations of percentages of single positive BMMNCs expressing specific surface receptors in merged postmenopausal subjects (n = 34) with changes in serum levels of biochemical markers between baseline and day 28.

Surface receptor	% Change in serum bone marker			
	Serum CTx		Serum TRAP	
	r	P	r	P
CTR ⁽⁺⁾	0.437	0.011	0.387	0.026
RANK ⁽⁺⁾	0.341	0.052	0.328	0.062
c-Fms ⁽⁺⁾	-0.226	NS	-0.159	NS
OSCAR ⁽⁺⁾	0.349	0.047	0.334	0.058
TREM2 ⁽⁺⁾	0.262	NS	0.242	NS
TNFR1 ⁽⁺⁾	-0.001	NS	0.029	NS
TNFR2 ⁽⁺⁾	-0.131	NS	-0.213	NS

Appendix 1

Combinations of antibodies and fluorochemicals used in flow cytometry analysis

Antibody 1	Antibody 2
CD14-APC	CTR-PERCP
CD14-APC	RANK-PERCP
CD14-APC	TNFR1-FITC
CD14-APC	TNFR2-FITC
CD14-APC	TREM2-PERCP
CD14-APC	OSCAR-PE
CFMS-FITC	BRANK-PERCP
CFMS-FITC	CTR-PERCP
CFMS-FITC	OSCAR-PE
RANK-PERCP	OSCAR-PE
TNFR1-FITC	CTR-PERCP
TNFR1-FITC	RANK-PERCP
TNFR2-FITC	CTR-PERCP
TNFR2-FITC	RANK-PERCP