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Bone Loss in Surgically Ovariectomized Pre-Menopausal Women is Associated with T Lymphocyte Activation and Thymic Hypertrophy

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Abstract

Post-menopausal osteoporosis is associated with estrogen deficiency and rapid bone loss. The mechanism by which estrogen deficiency results in bone loss has not been fully explained. Studies in mice rendered acutely estrogen deficient by ovariectomy have suggested that estrogen deficiency results in an activated T-lymphocyte phenotype and increased production of pro-osteoclastic cytokines. The aim of this study was to translate these findings from mouse models that suggest that the T-lymphocyte plays an important role in the etiology of post-menopausal osteoporosis. We recruited pre-menopausal women who underwent ovariectomy (OVX) for benign gynecologic conditions or for prophylaxis against ovarian cancer and a group of matched control women without OVX. Subjects provided blood samples to characterize T-lymphocyte phenotype by Fluorescence-activated cell sorting (FACS) and for T-lymphocyte culture and collection of conditioned media. Bone mineral density at the lumbar spine and left femoral neck was performed annually for two years and volumetric measurements by computed tomography (CT) of the thymus were obtained during the first 6 months. We enrolled 6 OVX and 13 control women. The OVX subjects had a significant loss of bone mineral density at the lumbar spine and left femoral neck. The volumetric thymus measurements suggested an increase in thymus size in the OVX subjects but did not reach statistical significance due to the small sample size. The T-lymphocyte phenotype in the OVX subjects demonstrated increased T-lymphocyte activation by FACS compared to the control subjects. Our preliminary findings support the hypothesis that estrogen deficiency leads to an activated T-lymphocyte phenotype which may contribute to the bone loss seen in estrogen deficiency. Larger clinical studies are necessary to confirm these findings.

Introduction

Post-menopausal osteoporosis is a common and debilitating disease of the aging population. Based on National Health and Nutrition Examination Survey (NHANES III), an estimated 13-18% (4 – 6 million) of post-menopausal Caucasian women in the United States has

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osteoporosis (1). The National Osteoporosis Foundation (NOF) has estimated that more than 10 million Americans 50 years old already have osteoporosis with women comprising almost 80% of these individuals (2). An additional 33.6 million have low bone density at the hip with increased risk for an osteoporotic fracture (2). Approximately one out of two Caucasian women will sustain an osteoporosis-related fracture at some point in her lifetime (3). In 2005, the annual cost for osteoporosis-related fractures was estimated at \$ 16.9 billion and is expected to rise to \$ 25.3 billion in 2025 (4). According to a recent systematic review, health care costs associated with osteoporotic fractures were 1.6-6.2 fold higher compared to costs for patients with no fractures (5).

Estrogen deficiency occurring after menopause is a major factor that accelerates bone loss in middle aged women. However, the mechanism of estrogen deficiency related bone loss has remained unclear. Pre-clinical studies conducted in mice suggest that estrogen deficiency results in increased thymic output of T-lymphocytes (8), leading to increased production of pro-osteoclastic cytokines TNF α and RANKL with resultant bone loss (6,7,8). Very few clinical studies have been conducted to establish a central role for T lymphocytes in post-menopausal osteoporosis (22,23,24,25). D'Amelio et al found that estrogen deficiency in post-menopausal women was associated with an increased production of RANKL and TNF α by T lymphocytes and an increase in the number of osteoclast precursors (24). Luo et al isolated peripheral blood mononuclear cells from healthy adults and examined the dose response of 17 β -estradiol on various cytokines in purified T regulatory (Treg) lymphocytes (25). This study demonstrated that estradiol enhanced Treg production of pro-osteoclastic cytokines IL-10 and TGF- β 1 thereby suppressing osteoclast differentiation and bone resorption. These findings again support a role for estrogen in regulating pro-osteoclastic cytokines.

While studies conducted in animals suggest a role of T cells in the etiology of post-menopausal women, there have not been any prospective studies conducted in women to examine the T-cell phenotype and early immunologic events leading to bone loss immediately following estrogen deficiency. As the menopause in humans occurs over a period of time it is more difficult to investigate acute changes in immune function associated with bone turnover in postmenopausal women. To overcome this confounder we examined changes in T cell production of the pro-inflammatory/osteoclastogenic cytokines TNF α and RANKL in women rendered acutely estrogen deficient by surgical ovariectomy. The hypothesis of our study was that T- cells derived from such women would show increased T-cell activation and proliferation, enhanced production of the pro-osteoclastogenic cytokine TNF α , demonstrate increased thymic T-cell output and thymic hypertrophy, leading to accelerated bone loss.

Material and Methods

Study Design and Participants

The study was IRB approved by the Emory University Human Subjects Committee (IRB). Subjects were enrolled for the study between October 2006 and August 2010. We identified subjects by posted advertisements, screening of the electronic medical record or by referral by physicians. We recruited a surgical menopause group of women rendered acutely estrogen deficient consisting of pre-menopausal women undergoing hysterectomy with ovariectomy (OVX) for benign gynecologic disease (fibroid uterus, endometriosis, dysfunctional uterine bleeding, chronic pelvic pain) or for prophylaxis against ovarian cancer (subjects with mutations in BRCA) and a control group of women who were estrogen sufficient consisting of pre-menopausal women either undergoing abdominal surgery without OVX or not undergoing any surgery. Women were confirmed to be pre-menopausal by history defined as regular spontaneous menstrual bleeding every 21 – 35 days or

documented FSH value less than 10 IU/L. Inclusion criteria for all groups included: age between 18 and 55 and no current estrogen therapy. Exclusion criteria included: history of active cancer including breast and uterine cancer, treatment with chemotherapy or glucocorticoids, history of an immune deficiency syndrome including HIV infection, history of severe anemia with hematocrit < 25. All subjects provided written informed consent prior to participation. All of the subjects were seen in the Emory University Clinical Interactions Network site (formally Emory General Clinical Research Center) at Emory University Hospital. The trial was registered at clinicaltrials.gov under trial registration number NCT00787904.

Bone Mineral Density Determinations

Subjects underwent measurement of bone mineral density of the lumbar spine and left femoral neck with a GE Prodigy Densitometer at baseline, 1 year and 2 years upon enrollment into the study. For the estrogen deficient group, the baseline bone mineral density measurement was performed either prior to the surgery or within 1 month after surgery.

Thymus measurements

Overlapping 1.25 mm images were reconstructed through the chest following a single breath-hold, multi-channel helical acquisition. Images were obtained from the apices of the lungs to at least the aortic arch; some patients were imaged to the lower chest. No intravenous contrast material was utilized. Multi-planar reformatted images were performed in real-time at the GE Advantage work station to better characterize and analyze the anatomy and any findings in the thorax. Thymic measurements were obtained after scanning was completed on a work station utilizing images in the axial and coronal planes, as indicated.

T-Cell collection, isolation and activation

Subjects provided whole blood for flow cytometry at baseline (prior to surgery in the OVX and non-OVX surgical groups) and at 3 months. Whole blood was collected in two 8 ml BD Cell Preparation Tubes (CPT™). Peripheral blood mononuclear cells were transferred to a sterile 50 ml conical tube and washed twice with PBS. Cell suspension was re-suspended in MACS® buffer and incubated for 10 minutes at 4°C with Fc blocking reagent followed by a 20 minutes incubation at 4°C with anti-CD3 coated microbeads (Miltenyie Biotec, Cambridge, MA). T-cells were positively selected using MACS® Cell Separation Columns and suspended at a concentration of 10⁶ cells per ml of DMEM, 2mM L-glutamine, 0.1 mg/ml ampicillin. Isolated T cells were kept as such or stimulated for 4h with 1 µM of ionomycin and 50 ng/ml of PMA in the presence of BD Golgi Plug™ (BD Biosciences, San Jose, CA). Cells were washed and stained for cell surface markers and for intracellular TNFα as described below.

Flow Cytometry

Purified un-stimulated or stimulated T cells were stained for various cell surface markers using multiple antibody panels. Antibodies used included: Anti-human CD3, CD4, CD8, CD45RA, CD45RO and CD69 (all from Biolegend, San Diego, CA). Following surface staining cells were washed twice with FACS buffer (PBS+ 2% fetal bovine serum). Cells were fixed with 2% paraformaldehyde (PFA) and divided into two parts. One part was kept at 4°C while other was permeabilized using BD Perm/Wash™ (BD Biosciences, San Jose, CA). Permeabilized cells were then stained for intracellular TNFα by using anti-human-TNFα antibody (Biolegend, San Diego, CA) or an isotype control. Cells were washed and

data acquired on a BD LSR II flowcytometer (BD Biosciences) and analyzed using Flowjo software (Tree Star Inc., Ashland, OR).

Cytokine measurements of T-cell conditioned media

Additional purified T-cells were cultured at a concentration of 250,000 cells/mL in T-cell specific media for 48 hours under ionomycin and PMA stimulation. The T-cell conditioned media was collected and stored at -80°C . Commercially available ELISA kits were used to determine the concentrations of TNF α (R&D Systems, Minneapolis, MN) and total sRANKL (Alpco, Salem, NH).

Results

Subject Demographics

We enrolled 24 subjects for this prospective observational study. One subject discontinued participation in the study. One subject did not follow-up after the initial visit and three subjects attended only two visits. Two subjects in the OVX group initiated estrogen therapy at 1 month and 3 months respectively after the baseline visit. One subject in the control group initiated estrogen at 18 months after the baseline visit.

For the final analysis, we included 6 estrogen deficient subjects who underwent OVX and 13 control subjects who remained estrogen sufficient. Both groups were equally matched in terms of age, race, BMI, prior estrogen use (both none), smoking history, age at menarche, family history of osteoporosis, gravidity and parity (Table 1). The bone mineral density at baseline for both groups at the lumbar spine and femoral neck were equally matched as well (Table 1).

Bone Density Measurements

As expected, subjects in the estrogen deficient group had significant declines in bone mineral density at their lumbar spine (Figure 1) and left femoral neck (Figure 2). Exclusion of the three subjects who initiated estrogen therapy had no effect on the results of femoral neck BMD but made L1-L4 BMD changes non-significant. Two years after ovariectomy, the estrogen deficient group lost 6% and 3% of bone mineral density at the spine and left femoral neck respectively. Two years after enrollment, the control group had a non-significant change in bone mineral density at the spine (-2%) and a significant increase in bone mineral density at the left femoral neck ($+4\%$) ($p=0.049$). The control group and ovariectomy group had significant differences in the change of bone mineral density at the lumbar spine ($p=0.047$) and left femoral neck ($p=0.004$) over a 2 year period.

Thymus Size

The estrogen deficient subjects had a trend towards increase in volumetric thymus size 3 months after surgery ($p=0.13$) which persisted to up to 6 months following surgery (Figure 3). The control group had an expected age related decrease (26) in thymus size with decreases in size of up to 13% and 7% at 3 and 6 months following enrollment. Exclusion of the three subjects who received estrogen therapy during the trial did not change these results significantly.

T-cell Phenotype before and after ovariectomy

Phenotypic analysis of various cell surface markers on isolated CD3 $^{+}$ T cells before and after ovariectomy showed no significant differences in the frequencies of CD4 and CD8 T cells between controls and OVX patients over a three-month follow up period (data not shown). Both CD4 and CD8 frequencies were stable in control as well as OVX group over

this period. OVX patients had significantly higher numbers of activated circulating CD3+CD69+ T cells ($p < 0.05$) and CD3+TNF+ cells ($p < 0.05$) than the controls at one month and three months post ovariectomy (**Figure 4**).

T cell pool of OVX patients, displayed a trend towards a memory phenotype. Compared to the control group, subjects undergoing OVX had significantly higher frequencies of CD3+CD45RO+ memory T cells ($p = 0.06$) and lower frequencies of CD3+CD45RA+ naïve T cells ($p = 0.051$) at day 0 (**Figure 5**). There was no significant change in the frequency of CD45RA+ or CD45RO+ T cells over the course of the three month follow up period both in control and OVX group (Figure 5) and exclusion of the OVX subject on estrogen replacement at month 1 did not change these findings.

RANKL and TNF α from T-cell conditioned media

The presence of sRANKL could not be detected in any of the T-cell conditioned media from both groups. There were no significant differences in the initial and 3 month follow-up TNF α concentration in the T-cell conditioned media in the OVX group (61.9 ± 34 pg/mL and 44.9 ± 10 pg/mL, respectively) and in the control group (31.4 ± 18 pg/mL and 50.6 ± 29 pg/mL, respectively).

Discussion

This observational study demonstrated significant declines in bone mineral density in women rendered acutely estrogen deficient following ovariectomy which corresponded with a T-cell phenotype that was more activated compared to a control group of pre-menopausal women. The women rendered acutely estrogen deficient by OVX experienced a short term increase in volumetric thymus size. In contrast, there was a decrease in thymus size in the control women. We found no significant changes in the frequencies of CD4 and CD8 T cells between the OVX and control women. However, compared to the control group, the T cell pool of OVX women demonstrated a shift towards a memory phenotype. The OVX subjects also had a higher proportion of activated circulating CD69+ T cells ($p < 0.05$) and TNF secreting T cells ($p < 0.05$) than the controls at one and three months post ovariectomy.

Both T cells and B cells cooperate for maintenance of peak bone mass via production of OPG by B cells, and augmentation by T cells, via CD40/CD40L co-stimulation (8). T lymphocytes appear to play a key role in estrogen deficiency induced bone loss (10,11,12). Animal models have shown that estrogen deficiency stimulates T cell activation and production of osteoclastogenic cytokines (particularly TNF α) with resultant augmented RANKL-induced osteoclastogenesis leading to bone loss (6,13-18). Ryan et al demonstrated that mice undergoing OVX resulted in 1.5 times increased cellularity of thymus tissue compared to sham operated mice (8). In addition, OVX resulted in increased T-lymphocyte activation assessed by increased expression of the cellular marker CD69 (8). Recently, Li and colleagues determined that T lymphocytes require the co-stimulatory molecule CD40 ligand (CD40L) for OVX induced bone loss by expanding stromal cells, promoting osteoblast proliferation and differentiation and regulating osteoclastic cytokines m-CSF, RANKL and OPG (19). However, a recent study by Onal et al demonstrated that RANKL deletion from T lymphocytes had no impact on OVX induced bone loss whereas deletion of RANKL from B lymphocytes partially protected mice from bone loss (20). In our study, we found an increased proportion of T lymphocytes producing TNF α from women undergoing OVX as compared to control women.

There has been interest in developing therapies that target the T lymphocyte induced bone loss in post-menopausal women. Tyagi et al found that the Isoflavonoid daidzein reduced the production of TNF α from CD4 T lymphocytes. They also found that co-culture of T

lymphocytes with bone marrow cells enhanced osteoclastogenesis. In contrast, treatment with daidzein reduced osteoclastogenesis in the T lymphocyte/bone marrow co-culture (21)

Similar to our study, D'Amelio et al (24) observed higher T cell production of TNF α in post-menopausal compared to pre-menopausal women. A limitation of their study was that they did not longitudinally examine the T lymphocyte phenotype and function after acute estrogen deficiency. Our study found that women rendered estrogen deficient by OVX had increased activation and proliferation of T lymphocytes which was associated with the expected immediate loss of bone density.

The strengths of our study include collection of T cells before and after OVX in women with examination of the T-cell phenotype by flow cytometry. In parallel, we also measured thymus size by volumetric CT and bone mineral density. Another strength of the study was recruitment of a matched control group. The limitations of our study include the relatively small number of OVX subjects due to difficulty with recruitment because of timing of the study in relationship to surgery. Since this was an observational study, we did not evaluate other factors important for bone including dietary calcium and vitamin D intake.

In conclusion, our study demonstrates that T lymphocytes demonstrate a pro-osteoclastic phenotype by increased expression of TNF by FACS analysis which was associated with acute bone loss and increased thymus size. Larger studies need to be conducted using human models of estrogen deficiency to examine the function of these lymphocytes on pre-osteoclastic cells.

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Figure 1. Bone Mineral Density of Lumbar Spine

Compared to the control group, the OVX group had significant declines in lumbar spine BMD at year 1 (-4%) and year 2 (-6%).

Figure 2. Bone Mineral Density of Left Femoral Neck

The OVX group showed a significant decline in left femoral neck BMD at year 1 (–6%) and year 2 (–3%). A significant increase in left femoral neck BMD was seen at year 2 (+4%) in the control group.

Figure 3. Thymus Size

The OVX group had a trend towards increase in volumetric thymus size 3 months after surgery which persisted up to 6 months following ovariectomy. Volumetric thymus size decreased in the control group.

Figure 4. Markers of T-cell activation and differentiation in subjects undergoing ovariectomy compared to control subjects

Peripheral blood mononuclear cells were collected and T-cells were purified from women before and after ovariectomy (squares) and pre menopausal control subjects (diamonds). Flow cytometry was performed using the indicated cell surface and intracellular markers. Frequencies of both CD69 (A) and TNF α (B) positive cells increased significantly in OVX T-cells at 1 month compared to baseline and remained significantly elevated at 3 months ($p < 0.05$).

Figure 5. Naïve and memory T-cell frequencies in subjects undergoing ovariectomy compared to control subjects

Peripheral blood mononuclear cells were collected and T-cells were purified from women before and after ovariectomy (squares) and pre-menopausal control subjects (diamonds). Flow cytometry was performed using the indicated cell surface markers. A. Compared to the control subjects, there were higher frequencies of CD45RO+ memory T-cells (A) in the ovariectomy group at baseline ($p=0.05$). B. There were no significant changes in the frequencies of CD45RO+ or CD45RA+ T-cells in control and OVX groups over 3 months follow up period.

Table 1

Patient Demographics and Baseline Characteristics

	Control Group (n=13)	Surgical Menopause Group (n=6)	p-value
Age (years)	41.4 ± 7.11	46.3 ± 5.1	0.1
Race n (%)	8 (61.5%)	2 (33.3%)	
BMI (kg/m²)	32.3 ± 11.3	30.8 ± 8.8	0.8
OCP use (%)	0%	0%	
Smokers (%)	7.7%	0%	
Age at Menarche (years)	13.2 ± 1.63	12.8 ± 1.7	0.7
Family history of osteoporosis (%)	23.0%	0%	
Gravidity n	1.6 ± 1.7	2.0 ± 1.3	0.6
Parity n	1.2 ± 1.2	1.7 ± 0.8	0.4
Type of Surgery			
Hysterectomy only	2	0	
Oophorectomy only	0	2	
Hysterectomy + Oophorectomy	0	4	
Myomectomy	1	0	
Colpopexy+ Urethral Suspension	2	0	
No surgery	8	0	
Baseline Bone Mineral Density			
Spine (L1-L4) (g/cm ²)	1.257 ± 0.1	1.319 ± 0.2	0.4
Left Femoral Neck (g/cm ²)	1.040 ± 0.1	1.047 ± 0.1	0.9
Baseline 25(OH)D Level (nmol/L)	51.1 ± 17.3	59.1 ± 22.5	0.5
Vitamin D insufficient (%)	92.3%	66.7%	
Baseline PTH Level (pg/mL)	27.8 ± 17.1	27.2 ± 15.7	0.9