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Cytokine Production and Bone Mineral Density at the Lumbar Spine and Femoral Neck in Premenopausal Women

L. M. Salamone,[1] T. Whiteside,[2] D. Friberg,[3] R. S. Epstein,[4] L. H. Kuller,[1] J. A. Cauley[1]

[1]University of Pittsburgh, Graduate School of Public Health, Department of Epidemiology, 130 DeSoto Street, Pittsburgh, Pennsylvania 15261, USA
[2]University of Pittsburgh, Department of Pathology and University of Pittsburgh Cancer Institute, W1041 BST 200 Lothrop Street, Pittsburgh, Pennsylvania 15213, USA
[3]University of Pittsburgh, Cancer Institute Immunologic Monitoring Laboratory, W1041 BST 200 Lothrop Street, Pittsburgh, Pennsylvania 15213, USA
[4]Merck-Medco Managed Care, LLC., 100 Summit Avenue, Montvale, New Jersey 07645, USA

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Abstract. Cytokines such as interleukin-1 (IL-1β), interleukin-6 (IL-6) and tumor necrosis factor (TNF-α) can influence both bone resorption and bone formation. The objective of this cross-sectional study was to examine the relationship between cytokine production by peripheral blood mononuclear cells (PBMC) and bone mineral density (BMD); the annual rate of change in BMD was examined. Subjects participating in a randomized clinical trial entitled the Women's Healthy Lifestyle Project in Allegheny County, Pennsylvania were used. They included 50 healthy premenopausal women, aged 45–52 years, who had regular menses within the past 3 months and were not on replacement estrogens. Dual-energy X-ray absorptiometry measurements at the AP lumbar spine and femoral neck were made at baseline and at the first annual exam using a Hologic QDR 2000 densitometer. Cytokine production of IL-1β, IL-6, and TNF-α by PBMC was measured at the annual exam. The median values for stimulated cytokine production by PBMC were 3.92 ng/ml, 31.3 ng/ml, and 1.05 ng/ml, for IL-1β, IL-6, and TNF-α, respectively. There were modest correlations between cytokine production and cross-sectional BMD, ranging from r = −0.30 to r = −0.13. Trends of greater spinal bone loss were observed in women with ''high'' (⩾75th percentile) cytokine production of stimulated IL-1β and IL-6 (IL-1β: ''high'' = −1.56% ± 0.70 versus ''low'' (<75th percentile) = −0.56% ± 0.35, P = 0.21). In contrast, greater annual gains in femoral neck BMD were observed in those with high cytokine production of IL-1β and IL-6 (IL-1β: high = 3.39% ± 1.16 versus low = −0.85 ± 0.58, P = 0.002). There was no association between stimulated TNF production and annual change in BMD. In this population of healthy premenopausal women, the relationship between cytokine production by PBMC and the rate of change in BMD was significantly different for the lumbar spine and femoral neck, possibly reflecting differences in the proportion of trabecular and cortical bone at these sites.

Key words: Cytokines — Bone mineral density — Premenopausal — Interleukin-1 — Interleukin-6 — Tumor necrosis factor.

Correspondence to: L. M. Salamone

The abatement in ovarian hormones associated with the menopause appears to accelerate the risk of certain diseases. Such hormonal declines are particularly detrimental for skeletal homeostasis. With the menopause, an uncoupling of bone remodeling occurs, in which bone resportion exceeds bone formation, resulting in progressive bone loss and postmenopausal osteoporosis. Accordingly, bone loss in women is the highest in the years immediately following the menopause [1]. Estrogen replacement therapy can be most effective in attenuating this postmenopausal bone loss [2], although the mechanism by which estrogens exert this bone-conserving effect remains largely unresolved. Since estrogen receptors have been found on both osteoclasts and osteoblasts [3, 4], it is likely that a direct skeletal effect is involved.

Most recently, it has been suggested that estrogen mediates the production of cytokines involved in bone remodeling such as interleukin-1 beta (IL-β), interleukin-6 (IL-6) or tumor necrosis factor alpha (TNF-α). Cytokines are local intracellular mediators that are produced by many cells, particularly bone and hematopoietic cells and thereby provide a potential functional link between bone and bone marrow [5]. Cytokines produced in the bone microenvironment influence bone remodeling by regulating the differentiation as well as activity of osteoclasts and osteoblasts [6–9].

Several studies have suggested that differences in cytokine production by various tissues or hematopoietic cells, including peripheral blood mononuclear cells (PBMC) might be associated with changes in BMD and perhaps, in the development of osteoporosis. For example, Pacifici et al. [10] demonstrated that PBMC produced significantly higher levels of IL-1β in patients with higher rates of bone remodeling than in those with low turnover osteoporosis and nonosteoporotic healthy subjects. Some [11, 12], but not all investigators [13], have reported similar findings. Bismar et al. [14] demonstrated that IL-6 secretion by bone marrow cells was elevated at menopause or after discontinuation of estrogen therapy, thereby supporting the idea that changes in cytokine concentrations may be partially responsible for enhanced bone resorption in estrogen-

depleted women. Indeed, when postmenopausal osteoporotic women were treated for 1 month with a combined estrogen and progesterone therapy, IL-1β secretion was significantly reduced [15]; this finding, however, is not universally accepted [16].

Since a gradual decline in ovarian function precedes the menopause, we hypothesized that premenopausal women may show differences in cytokine production levels that may correlate with pre- and perimenopausal rate of bone change. The objectives of this study were first, to determine whether cytokine production by PBMC was associated with cross-sectional DXA measures at the lumbar spine and femoral neck and more importantly, to evaluate whether cross-sectional cytokine production rates can predict the rate of bone change in premenopausal women.

Materials and Methods

The baseline and annual clinic examination included measurements of weight and height. Weight was measured using a standardized balance beam and height was measured using a stadiometer (Perspective Enterprises Inc., Kalamazoo, MI). BMI was calculated as weight in kilograms divided by height in meters squared. Blood was drawn following a 10-hour fast. Standard procedures for blood handling and processing were followed.

Experimental Subjects

The 535 women enrolled in this study entitled the Women's Healthy Lifestyle Project (WHLP) were recruited from voter registration lists from selected zip codes in Allegheny County, Pennsylvania. The protocol was approved by the Human Investigation Review Committee at the University of Pittsburgh and written informed consent was obtained from each subject. The criteria for entry into this study were good general health, aged 44–50 years, menses within the past 3 months, body mass index (BMI) between 20 and 33.9 kg/m^2, alcohol intakes <5 drinks per day, diastolic blood pressure <95 mmHg (on three occasions), total cholesterol between 160 and 260 mg/dL, and blood glucose <140 mg/dL. Women were excluded if they had a history of cancer within 5 years, psychiatric hospitalization within 1 year, drugs for hypertension and lowering cholesterol, hormone replacement therapy (including thyroid medications and estrogens), psychiatric disorders, hysterectomy, and bilateral oophorectomy.

This cross-sectional study was part of a clinical trial designed to determine whether the increase in LDL-c around the time of menopause can be prevented by dietary intervention aimed at reduction in saturated fat and cholesterol, weight loss, or the preventio of weight gain. Women were randomly assigned to either the Assessment-only or Assessment-plus dietary intervention group. Women assigned to the intervention group participated in a dietary intervention aimed at reducing fat intake to no more than 20% of calories and reducing saturated fat and cholesterol in the diet. The subjects in the Assessment group were given general nutrition and low fat guidelines.

Cytokine Study

Because of the potential confounding effect of weight change on cytokine production, only those women in the Assessment-only (control group) were eligible for this study. The first 52 consecutive Caucasian women who attended their annual clinic exam, having complete DXA measurements at baseline and annual exam, regular menses within the past 3 months, and were not on replacement estrogens participated in this study.

Of the 52 women completing this study, two women were excluded. The first had significantly higher cytokine secretion compared with the study population and was discovered to have a history of endometriosis and polycystic ovaries. Another woman was excluded who had unusually low levels of stimulated cytokine levels with an IL-6 of 0.12 ng/ml as compared with the range of values for the study population of 10.0–85.0 ng/ml. Thus, the total number for this study was 50. Only two of these women were past smokers.

Dual-Energy X-ray Absorptiometry Measurements

Dual-energy X-ray absorptiometry measurements at the AP lumbar spine, proximal femur (femoral neck, trochanter, and intertrochanter), whole-body and whole-body soft-tissue composition (total lean and total fat mass) were made with a Model QDR 2000 densitometer (Hologic Inc., Waltham, MA). DXA measurements were performed at baseline within the first 12 months of randomization and again at the annual clinic exam. A standard protocol for patient positioning and utilization of software was used. The coefficients of variation, as based on *in vitro* phantoms scanned each month were 0.6% and 1.3% for the lumbar spine and femoral neck, respectively (unpublished).

Cytokine Production Measurements

Spontaneous and stimulated production of cytokines by PBMC was measured as previously described [17]. Upon *in vitro* stimulation with phytohemagglutinin or lipopolysaccharide, PBMC secrete cytokines; the levels of these proteins in the cellular supernatant, as measured by immunoassays (ELISA), are a reliable indicator of immunocompetence [17]. Twenty milliters of venous blood was collected in heparin-coated vacutainer tubes, and within 2 hours of blood draw, PBMC were separated on Ficoll-Hypaque density gradients, washed, counted, and resuspended in complete tissue culture medium. Cells were then cryopreserved and stored in vapor phase liquid nitrogen freezers. On the day of the assay, PBMC were thawed, washed, and adjusted to the cell concentration of 1×10^6 ml in complete RPMI medium containing 20 mM L-glutamine, 100 U/ml of penicillin, 100 μg/ml of streptomycin, and 5% (v/v) of fetal calf serum. All reagents were purchased from GIBCO Life Technologies (Gaithersburg, MD). Aliquots of cell suspension (100 μl per well) were added to wells of a 96-well round bottom plate containing 100 μl of either medium alone (for measurement of spontaneous release), lipopolysaccharide (LPS; Escherichia coli 026:B6) (Sigma, St. Louis, MO) at 5 μg/ml for stimulation of TNF-α and IL-1β production, or phytohemagglutinin (PHA-P; Sigma) at 10 μg/ml for IL-6 production. Plates were incubated for 24 or 48 hours at 37°C in 5% CO_2 in air, and supernatants were collected and stored at −70°C for testing in cytokine immunoassays. Commercially available ELISA kits were used to measure IL-1β (Cistron Biotechnology, Pine Brook, NJ) and TNF-α (Biosource, Camarillo, CA) and IL-6 (R&D Systems, Minneapolis, MN). Reliability and precision for cytokine production assays, as performed in our laboratory, have been recently described [17, 18].

Quality Control for Cytokine Assays

All of the immunoassays were standardized against reference standards for cytokines available from the WHO through the National Institute for Biological Standards and Control in England or from the Biological Response Modifier Program (Frederick, MD) [3]. An internal laboratory control consisting of a supernatant with the known levels of each cytokine was divided into aliquots in small vials, stored at −80°C and tested every time the assay was performed to determine interassay variability as well as intraassay variability. The coefficients of variation (CV) for cytokine assays in our laboratory did not exceed 10–15%, as previously reported [17]. Normal values, expressed as the middle 80% range, from normal female blood donors (ages 20–48 years) are 0.53–3.95 ng/ml for TNF-α production (n = 44), 2.19–7.47 ng/ml for IL-1β

Table 1. Clinical characteristics of the 50 premenopausal women

Characteristic	Mean (SD)	Range
Age (yrs)	48.6 (1.9)	45.5–52.3
Height (cms)[a]	64.9 (2.2)	60.5–69.5
Weight (kg)		
Baseline	65.1 (7.4)	52.0–88.6
Annual	63.4 (7.7)	53.9–90.5
% Change[b]	−2.73 (6.17)	−20.7–10.9
Total lean mass (kg)		
Baseline	40.2 (4.3)	33.4–52.3
Annual	41.1 (4.2)	34.4–57.1
% Change	2.6 (4.2)	−5.5–15.5
Total fat mass (kg)		
Baseline	21.1 (5.7)	10.6–34.1
Annual	20.8 (5.3)	12.3–33.8
% Change	−0.67 (12.4)	−32.0–24.8
Lumbar spine BMD (g/cm^2)		
Baseline	1.081 (0.12)	0.777–1.386
Annual	1.073 (0.12)	0.776–1.323
% Change[b]	−0.76 (2.2)	−6.43–4.22
Femoral neck BMD (g/cm^2)		
Baseline	0.819 (0.12)	0.597–1.263
Annual	0.817 (0.11)	0.624–1.155
% Change[b]	−0.003 (4.00)	−8.49–12.99

[a] Unless specified, these measurements are at the annual examination
[b] % Change calculated as (annual value − baseline value)/(baseline value × years) × 100

production (n = 38), and 2.7–12.0 ng/ml for IL-6 production (n = 8).

Statistical Analyses

Statistical analyses were performed using the SAS Statistical package (SAS Institute, Cary, NC) [19]. Means ± standard deviations (SD) are presented. Spearman rank-order correlations were calculated to examine the association between non-normally distributed variables. Since cytokine production values for IL-1β, IL-6, and TNF-α were not normally distributed, the variables were dichotomized based on frequency distributions into quartiles, with the middle groups combined. The 75th percentile cutpoints for IL-1β, IL-6, and TNF-α were IL-1β, 5.18 ng/ml, IL-6, 42.5 ng/ml, and TNF-α, 1.44 ng/ml. A general linear model for least-squared means was conducted to test for significant differences in the cross-sectional BMD and the annual rate of bone change by categories of cytokine production. Adjustments for body weight, annualized change in body weight, and baseline BMD were made. Multiple regression analyses were conducted with both cross-sectional BMD and the rates of change in BMD as the dependent variables and each stimulated cytokine (designated as ''high'' levels (⩾75th percentile) or ''low'' levels (<75th percentile) of cytokine production) as well as all potential two-way interactions between cytokines, i.e., IL-1β × IL-6; IL-1β × TNF; and IL-6 × TNF, as independent variables. All *P*-values less than 0.05 were considered to indicate statistical significance.

Results

Cross-sectional Studies

The clinical characteristics of the 50 premenopausal women are given in Table 1. The median values for stimulated production by PBMC of IL-1β, IL-6, and TNF-α were 3.92 ng/ml, 31.3 ng/ml, and 1.05 ng/ml, respectively; only stimulated cytokine levels are reported since spontaneous cytokine levels were all approximately zero. There were modest Spearman correlations between cross-sectional BMD measurements and cytokine production, ranging from r = −0.30 to r = −0.13 (Table 2). Cytokine production by PBMC was weakly correlated with body weight (r = 0.04 to r = 0.15, $P = 0.800$ to $P = 0.287$, respectively). Lean mass at baseline and follow-up was negatively related to IL-1β and IL-6 production and fat mass was positively related to production of all three cytokines (Table 2). When we examined potential interactions between stimulated cytokine production as determinants of BMD, the interaction between categories of IL-6 and TNF-α approached significance in determining lumbar spine BMD at the annual exam ($P = 0.0624$). Women having a ''high'' production (⩾75th percentile) of both IL-6 and TNF-α appeared to have significantly lower spinal BMD [0.963 ± 0.18 g/cm^2 (SD)] as compared with women with ''low'' levels (<75th percentile) of both cytokines (1.08 ± 0.07 g/cm^2, $P = 0.0495$). A similar interaction was observed for IL-1β and TNF-α in determining annual spinal BMD (interaction term; $P = 0.0825$).

Table 2. Spearman correlation matrix for the associations between cytokine production by peripheral blood monocytes and bone mineral density and body composition

Characteristic	Stimulated IL-1β	Stimulated IL-6	Stimulated TNF-α
Lumbar spine BMD (g/cm^2)			
Baseline	−0.26	−0.21	−0.19
Annual	−0.25	−0.19	−0.15
Femoral neck BMD (g/cm^2)			
Baseline	−0.23	−0.20	−0.30[a]
Annual	−0.14	−0.13	−0.27[b]
Weight (kg)			
Baseline	−0.10	−0.12	0.09
Annual	0.04	0.06	0.16
Total lean mass (kg)			
Baseline	−0.35[a]	−0.28[a]	−0.06
Annual	−0.30[a]	−0.14	0.04
Total fat mass (kg)			
Baseline	0.32[a]	0.23	0.20
Annual	0.28[a]	0.19	0.20

[a] Significant correlation at $P < 0.05$
[b] Significant correlation at $P = 0.057$

Longitudinal Studies

When we compared the annual rate of change in spinal BMD by each cytokine production category, there tended to be a greater rate of bone loss at the lumbar spine in the ''high'' category (⩾75th percentile) of cytokine production for IL-1β and IL-6 as compared to the ''low'' category (<75th percentile), even after adjusting for baseline BMD, baseline body weight, and the annualized percent change in body weight (Table 3). In contrast, the annual rate of femoral neck bone gain appeared to be the highest for those women in the ''high'' category of cytokine production. The annual rate of change in BMD at the spine and femoral neck were not consistent for categories of TNF-α. From univariate regression analyses, categories of IL-1β ($P = 0.002$) and IL-6 ($P = 0.037$) appeared to be significant determi-

Table 3. Annual percent rates of change in BMD by categories of stimulated cytokine production

% Change/ Year in BMD	<75th Percentile (n = 40)	⩾75th Percentile (n = 10)	*P*-value[b]
IL-1β			
Lumbar spine			
Unadjusted	−0.56 (0.35)	−1.56 (0.70)	0.208
Adjusted[a]	−0.47 (0.35)	−1.67 (0.69)	0.130
Femoral neck			
Unadjusted	−0.85 (0.58)	3.39 (1.16)	0.002
Adjusted	−0.67 (0.56)	2.59 (1.13)	0.021
IL-6			
Lumbar spine			
Unadjusted	−0.55 (0.36)	−1.42 (0.64)	0.247
Adjusted	−0.52 (0.36)	−1.37 (0.67)	0.272
Femoral neck			
Unadjusted	−0.66 (0.63)	2.09 (1.11)	0.037
Adjusted	−0.44 (0.59)	1.50 (1.12)	0.136
TNF-α			
Lumbar spine			
Unadjusted	−0.96 (0.37)	−0.18 (0.62)	0.281
Adjusted	−0.86 (0.37)	−0.29 (0.65)	0.464
Femoral neck			
Unadjusted	−0.22 (0.66)	0.62 (1.12)	0.521
Adjusted	−0.01 (0.63)	0.003 (1.06)	0.997

[a] Adjusted for baseline BMD, baseline body weight, and change in body weight from baseline to annual visit
[b] *P*-values represent differences between mean rates of change

nants of the annualized change in femoral neck BMD, explaining about 18% and 9% of the total variance in BMD, respectively. From multiple regression analyses, there were no significant interactions between cytokine categories in determining the annual rates of change in either femoral neck or spinal BMD.

Discussion

Changes in cytokine levels (or cytokines in general) have been involved in and may be responsible for bone resorption in premenopausal women [20–22]. In this study of healthy premenopausal women, significant cross-sectional correlations were observed between cytokine production by PBMC and BMD at the lumbar spine and femoral neck, providing additional support for the role of cytokines in BMD. However, in contrast to our findings, Kania et al. [23] observed no correlations between plasma levels of IL-6 and BMD, at either the spine or femoral neck, that was independent of age. The negative findings of Kania et al. may relate to the fact that plasma levels, not cellular production of IL-6, were measured. As previously reviewed, plasma levels of cytokines often do not reflect the status of the locoregional cytokine production [18]. Several limitations in measuring plasma IL-6 have been identified [24, 25] and it appears that systemic cytokine levels are unlikely to correlate with those in the microenvironment of bone. Cytokines have short half-lives in serum (minutes) and the presence of soluble cytokine receptors or other inhibitors in serum may sequester cytokine proteins. In addition, the presence of natural antagonists may interfere with assay interpretation. The ability of cytokines to bind proteins may interfere with their detection, and membrane-associated or cell-bound cytokines are not detectable in serum. Finally, in some cases, antibodies to cytokines may be present and may interfere with cytokine determination in serum [18]. For these reasons, measurements of cytokine production by PBMC rather than plasma cytokine levels are preferable and might better reflect the capability of an individual's cells to release cytokines upon stimulation.

In this study, cytokine production by PBMC was measured in lieu of that by bone-associated cells. We used PBMC as surrogate cells for the obvious reason that they are easily accessible for studies. Cytokine production by stimulated PBMC may not reflect local effects in bone, but it seems to serve as an index of the overall capability of circulating immune cells to secrete a particular mix of cytokines. It is possible that circulating immune cells are influenced by local events in bone through complex cytokine networks active *in vivo*. In fact, our studies indicate that such is the case, because cytokines production by PBMC was found to correlate with BMD.

Although we observed a correlation between cytokine production and BMD, we were surprised at our finding of greater spinal bone loss in the groups of women with high (⩾75th percentile) levels of IL-1β and IL-6. The women who showed low (<75th percentile) levels of IL-1β and IL-6 experienced loss in the femoral neck. The observation that levels of cytokine production influence changes in the lumbar spine differently than in the femoral neck is not entirely clear. It may reflect differences in the proportion of trabecular and cortical bone between the lumbar spine (largely trabecular) and femoral neck (largely cortical). Because approximately 25% of trabecular bone is resorbed and replaced every year, as compared with only 3% of cortical bone [26], it is likely that the rate of bone remodeling is largely controlled by local rather than systemic factors. Additionally, trabecular bone has a high suface-to-volume ratio with 70–85% of the bone's surface in contact with the bone marrow as compared with cortical bone [21]. These differences may partly explain the more dominant effect of local factors residing in the bone's microenvironment on trabecular, not cortical bone remodeling. Cytokines are known to modulate the microenvironment either positively or negatively, depending on local requirements.

Postmenopausal bone loss is associated with excessive osteoclast activity, whereas aging-associated bone loss is related to a decline in osteoblast supply [27, 28]; thus, postmenopausal bone loss primarily occurs in highly trabecular sites whereas bone loss with aging is mainly in cortical sites. These basic etiologic differences between menopausal bone loss and aging bone loss offer some explanation for the perhaps more dominant effect of cytokines in mediating a more site-specific bone loss (trabecular versus cortical sites) rather than modulating more general skeletal effects. This explanation is consistent with the general view that under normal conditions cytokines act as local rather than systemic mediators.

The observation that greater cytokine production by PBMC is associated with elevated spinal or trabecular bone loss could have a variety of explanations. As estrogen withdrawal increases in women who become perimenopausal, estrogen can no longer modulate the local production of cytokines which are released and which ultimately stimulate osteoclastogenesis. IL-1β, for example, is a potent stimulator of osteoclastogenesis activity with a primary effect on osteoblasts [29] and is involved in inducing the recruitment and differentiation of osteoclastogenesis precursors [6]. Moreover, the time course of events for estrogen depletion,

with increased cytokine production and accelerated bone loss, is consistent with a causal role for cytokines in postmenopausal osteoporosis, as suggested by others [30, 31]. Elevated IL-1β activity in osteoporotic women is supported by many [13, 32], but not all studies [33].

In summary, cytokines are associated with cross-sectional measurements of BMD at both the lumbar spine and femoral neck. Cytokines appear to have different site-specific effects on bone, depending on whether trabecular or cortical bone is examined. This observation is important in supporting etiological differences between postmenopausal and aging bone loss. The ability to use cytokine secretion as markers of accelerated postmenopausal bone loss remains largely unexplored. Monitoring cytokine production serially during the menopausal transition may provide an additional means of assessing the risk of developing osteoporosis or perhaps, for identifying the best candidates for homone replacement therapy.

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