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Ovarian steroid treatment blocks a postmenopausal increase in blood monocyte interleukin 1 release

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ABSTRACT **In previous studies, we showed that blood monocyte elaboration of interleukin 1 (IL-1), a known stimulator of bone resorption, was higher in osteoporotic patients with rapid bone turnover than in those with slow turnover and in nonosteoporotic subjects. Since an acceleration of bone loss following menopause contributes to the risk of osteoporosis in women, we have studied the effects of menopause and ovarian steroid treatment on IL-1 release by monocytes obtained from nonosteoporotic and osteoporotic women. IL-1 activity in the monocyte culture medium derived from untreated postmenopausal women (nonosteoporotic and osteoporotic) was higher than in the medium derived from either untreated premenopausal or estrogen/progesterone-treated postmenopausal women. A significant negative correlation was found between IL-1 and years since menopause in both the healthy ($r = -0.75$; $P < 0.005$) and the osteoporotic ($r = -0.61$; $P < 0.01$) untreated postmenopausal women. The difference between the two slopes was significant at $P < 0.05$. Premenopausal IL-1 levels were achieved within 8 years of menopause in the nonosteoporotic, but not in the osteoporotic, subjects in whom increases were evident as long as 15 years after menopause. IL-1 also correlated inversely with vertebral mineral density ($r = -0.37$; $P < 0.05$), as measured by quantitative computed tomography. In prospective studies, treatment with estrogen/progesterone for 1 month caused a substantial highly significant decrease in IL-1 activity in each of three nonosteoporotic and five osteoporotic women, confirming the apparent effect of hormone therapy observed in the cross-sectional analysis. Although a cause–effect relationship has not been established, it is our hypothesis, based on these data, that alterations in IL-1 production may underlie the postmenopausal acceleration in bone loss and its inhibition by ovarian steroids. Persistent elevation of IL-1 secretion appears to be a feature of postmenopausal osteoporosis.**

Postmenopausal osteoporosis is an extremely common disabling condition characterized by a reduced bone mass and a heightened risk of fracture (1). It stems in part from a dramatic acceleration of bone loss that begins in the perimenopausal period and lasts for 5–10 years thereafter (2–4). The bone loss is attributable to a defect in bone remodeling in which bone resorption is excessive (5).

Although estrogen deficiency underlies (2–4) and estrogen therapy mitigates this defect (6–8), the nature of the estrogen-responsive resorption stimulus is unknown. There are no consistent changes in the levels of endocrine resorption stimulators, parathyroid hormone and 1,25-dihydroxyvitamin D_3 (9, 10), and plasma calcitonin, an inhibitor of resorption, while lower in women than in men (11), is not remarkably diminished in postmenopause (12). These findings have suggested that estrogen may act by modifying the production of one or more of the local factors now known to influence remodeling events. Among the most potent of these is interleukin 1 (IL-1), one of several closely related proteins produced by many types of mammalian cells, including skeletal cells (13–16). Initially discovered because of its participation in lymphocyte mitogenesis (17, 18), IL-1 is a powerful stimulator of bone resorption *in vitro*, where it is active at a concentration as low as 1 pM (19–21).

Recently, we reported that blood monocytes from patients with osteoporosis in whom bone remodeling rates were high elaborated significantly more IL-1 than patients with "low turnover" osteoporosis and nonosteoporotic healthy subjects (22). We now report that the appearance of the menopause, a state accompanied by rapid bone turnover, heralds a marked increase in blood monocyte IL-1 production that is suppressed by ovarian steroid therapy. We also show that osteoporotic women, in contrast to their nonosteoporotic counterparts, fail to achieve premenopausal levels of monocyte IL-1 release within 8 years after menopause.

MATERIALS AND METHODS

Normal Subjects and Patients. The study population consisted of healthy Caucasian subjects, 7 premenopausal (age range, 26–53 years), 8 hormone-treated postmenopausal (age range, 46–60 years), and 14 non-hormone-treated postmenopausal (age range, 45–59 years) women. They had normal vertebral body mineral density (VMD) as assessed by single energy quantitative computed tomography (23) and had no history of fractures, back pain, or loss of height. In addition, we studied 16 untreated (age range, 54–75 years) and 12 hormone-treated (age range, 53–64 years) postmenopausal women with osteoporosis. They all had suffered at least one spontaneous fracture and had evidence of spinal demineralization by quantitative computed tomography. Hormone treatment consisted of conjugated estrogen (0.625 mg/day for days 1–25 of the month) and medroxyprogesterone acetate (10 mg/day for days 15–25 of the month). All gave their informed consent and were ambulatory and in good health. Patients with osteoporosis attributable to secondary causes (e.g., corticosteroid excess, hyperthyroidism, hyperparathyroidism, lymphoma, myeloma, or other neoplasia) were excluded. Additional criteria for exclusion were documented in high alcohol intake, autoimmune disease or diseases that could potentiate changes in bone remodeling or monocyte function, as well as therapeutic regimens (other than estrogen or progesterone) for treatment of osteoporosis. As shown in Table 1, within each of the two postmenopausal groups (nonosteoporotic treated and untreated; osteoporotic treated and untreated), subjects were similar in age, years since menopause, and VMD.

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Abbreviations: IL-1, interleukin 1; VMD, vertebral body mineral density.

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Table 1. Mean (±SD) age, years since menopause (YSM), and VMD in nonosteoporotic and osteoporotic subjects

	n	Age, years	YSM	VMD, mg of Ca per ml
	Nonosteoporotic women			
Untreated premenopausal	7	39.4 ± 10.8	—	163.5 ± 20.6
Untreated postmenopausal	14	51.2 ± 4.9	5.4 ± 4.8	109.6 ± 17.1
Hormone-treated postmenopausal	8	53.7 ± 4.0	5.7 ± 4.7	111.8 ± 18.3
	Osteoporotic women			
Untreated premenopausal	16	62.8 ± 6.6	13.7 ± 6.0	55.3 ± 25.6
Hormone-treated postmenopausal	12	57.5 ± 3.0	11.6 ± 6.9	74.0 ± 20.2

Age, years since menopause, and VMD were similar in the untreated and the hormone-treated nonosteoporotic postmenopausal women and in the untreated and hormone-treated osteoporotic women.

Monocyte Cultures. To assay IL-1, blood was drawn into EDTA-containing tubes and monocyte cultures were prepared as described (22). Briefly, freshly drawn blood was fractionated on Ficoll/Hypaque, and the mononuclear cells were removed from the interface and washed twice with RPMI 1640 medium. The medium was assayed for endotoxin by the limulus amoebocyte lysate assay (Microbiological Associates). Endotoxin was not detected at the level of sensitivity of the assay (<1 ng/ml). The cells were resuspended in medium at a concentration of 1×10^6 cells per ml. One-milliliter aliquots were allowed to adhere to the plastic surface of 16-mm cells in a 24-well tissue culture plate for 1 hr at 37°C, in a humidified atmosphere of 5% CO_2/95% air. After incubation, the nonadherent cells were removed from the wells, and the adherent population was washed twice to remove any remaining nonadherent cells. The adherent cell population was then incubated in 1 ml of complete medium [RPMI 1640 medium supplemented with 5% (vol/vol) heat-inactivated fetal bovine serum (defined, Sterile Systems, Logan, UT; endotoxin, 0.038 ng/ml)]. The supernatant fractions were collected 48 hr later, then passed through 0.22-μm filters and stored at −20°C until assayed. The adherent population was subsequently stained for the monocyte–macrophage-specific enzyme α-naphthyl acetate esterase (Sigma) and was found to be >95% monocytes. Monocytes composed 17.0% ± 6.9% of the entire original mononuclear population isolated by Ficoll density-gradient centrifugation. Monocytes so processed spontaneously release IL-1 in a reproducible fashion (22), although a contribution from other cellular blood elements cannot be entirely excluded.

IL-1 Assay. The monocyte-conditioned media were assayed for total IL-1 activity (IL-1α and -β) by assessing the increment in mitogen-induced proliferation of the helper T cell D10.G4.1 (D10 cells) (24) as described by Kurt-Jones *et al.* (25, 26). The IL-1 standard used in the assays was ultrapure IL-1 (Genzyme), except for the IL-1 used in the neutralization assay, which was recombinant IL-1α or IL-1β (Genzyme). The IL-1 activity was measured by quantitating the thymidine incorporation in the D10 cells and is expressed as units/ml by performing a log-logit transformation of the serial dilution curves and determining the dilution of the test sample that yielded a value corresponding to 50% of the standard IL-1 maximum activity. The standard IL-1 activity was arbitrarily set at 100 units/ml. The test sample units were then determined as follows:

$$\text{Activity}_{(\text{sample})} = 2^{(a-b)} \times 100 \text{ units/ml}_{(\text{standard IL-1})},$$

where a is the dilution ($\log_2$) of the test sample yielding 50% of the standard IL-1 activity and b is the dilution ($\log_2$) of the standard IL-1 yielding 50% maximal activity.

Since agents in addition to IL-1 are comitogenic in the T-cell assay (24, 27, 28), we verified our findings as indicative of the presence of IL-1 in two ways: first, by demonstrating inhibition of the conditioned medium effect in the presence of polyclonal anti-IL-1 antibody, which preferentially recognizes IL-1β (data not shown); and second, by a specific ELISA for IL-1β on replicate samples, generously performed by John Kenney and Anthony Allison (Syntex, Palo Alto, CA). When the two assays were compared by linear regression, a positive correlation was found between the results of the T-cell assay and ELISA for IL-1β ($r = 0.98$; $P < 0.0001$).

Statistical Analysis. Group mean values were compared by two-tailed Student's t test or analysis of variance, as appropriate. Subsequent mean comparison tests were performed by Tukey's honestly significant difference test. Comparisons for differences in IL-1 activity were made by using either a ln transformation of the data or the Wilcoxon rank sum test since the values obtained were not normally distributed as shown by a Filliben's test. Simple linear regression was used to determine the relationship between IL-1 activity and years since menopause. Differences between slopes were tested by covariance analysis. Stepwise regression, multiple regression, and partial correlation analysis were used to assess the relationship between VMD, age, and IL-1. The distribution of IL-1 values among nonosteoporotic and osteoporotic women 8–15 years after menopause was compared by the Fisher's exact test. Discriminant function analysis was used to classify patients in groups according to years since menopause.

RESULTS

Blood monocytes from both nonosteoporotic and osteoporotic women spontaneously released IL-1 into the culture medium (Fig. 1). However, IL-1 activity (mean ± SEM) in the conditioned medium from cultured monocytes derived from the nonosteoporotic untreated postmenopausal women (101.2 ± 42.1 units/ml) was significantly higher ($P < 0.01$) than in the medium derived from either the nonosteoporotic premenopausal (1.5 ± 1.0 units/ml) or the estrogen/progesterone-treated postmenopausal women (1.2 ± 0.5 units/ml). Similarly, IL-1 activity in the untreated osteoporotic women (41.0 ± 17.3 units/ml) was significantly ($P < 0.001$) higher than in either the nonosteoporotic untreated premenopausal women or the estrogen/progesterone-treated osteoporotic women (0.6 ± 0.2 unit/ml). Mean IL-1 monocyte secretion from untreated nonosteoporotic and osteoporotic women was not significantly different because of the large scatter of individual values. Moreover, the hormone-treated subjects all exhibited monocyte IL-1 production <7 units/ml, the IL-1 upper normal limit defined as the mean IL-1 activity for the premenopausal group plus 2 SD. The differences for IL-1 observed between the groups were not affected by normalizing IL-1 activity for the number of adherent monocytes (data not shown). Increased IL-1 production was most marked in nonosteoporotic women during the first 7 years after menopause. There was considerable overlap between

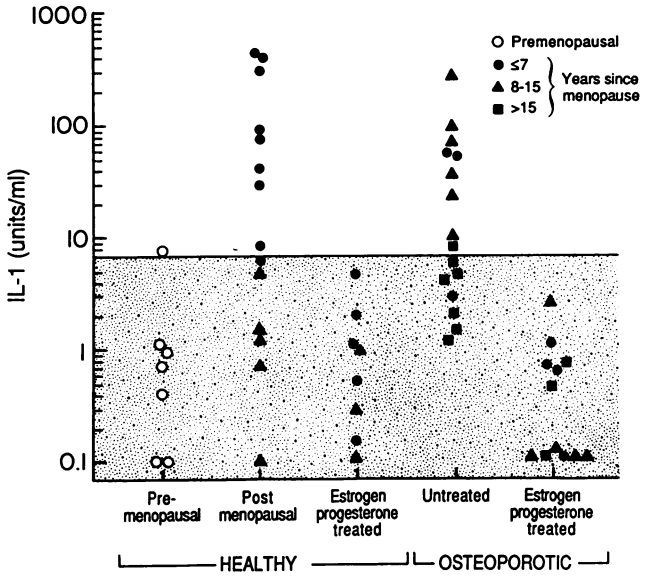

FIG. 1. IL-1 activity in the peripheral blood monocyte culture medium from nonosteoporotic and osteoporotic women. Untreated nonosteoporotic postmenopausal women had higher IL-1 activity ($P < 0.01$) than either premenopausal or estrogen/progesterone-treated subjects. Similarly, mean IL-1 levels were higher ($P < 0.001$) in the untreated osteoporotic subjects than in either the nonosteoporotic premenopausal or the estrogen/progesterone-treated osteoporotic women.

premenopausal and 8- to 15-year postmenopausal subjects (Fig. 1). By contrast, IL-1 production exceeded that of premenopausal subjects among osteoporotic patients who were up to 15 years postmenopausal and, in most cases, even in those beyond 15 years.

Fig. 2 shows the temporal relationship between IL-1 activity and menopausal status in osteoporotic and nonosteoporotic untreated subjects. In both groups, there was a significant negative correlation between IL-1 production and years since menopause (nonosteoporotic women: $r = -0.75$; $P < 0.005$; osteoporotic women: $r = -0.61$; $P < 0.01$) but the regressions in the two groups differed significantly from each other ($P < 0.05$). No relationship was found between IL-1 and age. When differences in the distribution of IL-1 levels among the untreated nonosteoporotic and osteoporotic women in the group between 8 and 15 years since menopause were analyzed with a Fisher's exact test (Table 2), all of the subjects >7 years postmenopausal who still had elevated monocyte IL-1 production were osteoporotic, whereas all subjects who had normal IL-1 secretion were nonosteoporotic ($P < 0.005$), suggesting a relationship between IL-1 and excessive bone loss.

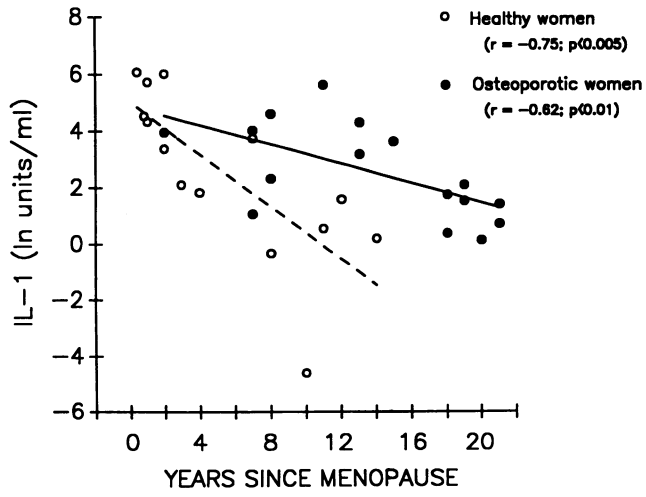

FIG. 2. Linear regression of IL-1 and years since menopause in nonosteoporotic and osteoporotic untreated postmenopausal women. When analyzed with analysis of covariance, the difference between the slope of the two curves was significant at $P < 0.05$. A significant correlation between IL-1 and years since menopause was also found in the entire untreated postmenopausal population ($r = -0.50$; $P < 0.005$).

Table 2. Fisher's exact test to evaluate differences in IL-1 activity between groups of untreated women 8–15 years after menopause

IL-1	Nonosteoporotic, *n*	Osteoporotic, *n*
Normal	5	0
High	0	6

The untreated women 8–15 years after menopause were divided into two groups, those whose IL-1 activity was above the upper normal limit (defined as the mean IL-1 activity of the premenopausal women plus 2 SD), and those with normal levels of IL-1. Based on this criterion, a significant difference ($P = 0.002$) was found between the two groups in the distribution of the nonosteoporotic and the osteoporotic subjects.

When the role of IL-1 as a predictor of bone density was further analyzed by linear regression analysis, both age and IL-1 were found to account for the variance of VMD among the untreated women. However, when the impact of age on VMD was held constant with stepwise and multiple regression analysis (Table 3), the negative partial correlation between VMD and IL-1 persisted, in keeping with a significant and independent contribution of IL-1 on the VMD variance.

The apparent effect of hormone therapy observed in the foregoing cross-sectional analysis was demonstrable in prospective studies. IL-1 activity declined significantly (Table 4) in monocytes from each of three nonosteoporotic and five osteoporotic women upon initiation of hormone treatment, and it did so within 1 month.

DISCUSSION

These results disclose a close association between menopause and an increased capacity of blood monocytes to release IL-1 *in vitro*. This association, and the decline in monocyte IL-1 release upon ovarian steroid replacement, indicates that it is the change in ovarian hormone status that underlies the postmenopausal increment. Since all of our treated patients received both estrogen and progesterone, either steroid (or both) could have produced this effect.

In this study, IL-1 activity was higher, although not significantly, in the nonosteoporotic than in the osteoporotic untreated women. This does not contradict our previous study, in which we reported that monocytes from osteopo-

Table 3. Stepwise and multiple regression analysis between VMD, age, and IL-1 activity in the untreated study population

Independent variable	*t* value	Partial correlation coefficient	% variance	*P*
Age	6.87	−0.78	60.0	<0.0001
IL-1*	−2.32	−0.39	6.0	<0.05

In the untreated population, a significant correlation was found between VMD and both age ($r = -0.77$; $P < 0.0001$) and ln IL-1 ($r = -0.37$; $P < 0.05$) by simple linear regression. When the influence of age on the variance of VMD was taken into account by stepwise and multiple regression analysis, a portion of the VMD variance was independent of age and was found to be significantly related to IL-1.
*Since the IL-1 values were not normally distributed, the ln of the values was used to compute the correlation coefficients.

Table 4. IL-1 activity in the culture medium of peripheral blood monocytes from three nonosteoporotic and five osteoporotic postmenopausal women before and after 1 month of treatment with estrogen and progesterone

	IL-1, units/ml	
	Before	After
Nonosteoporotic women		
T.R.	406.0	3.9
S.W.	29.2	0.0
N.K.	6.2	2.5
Osteoporotic women		
E.W.	72.9	0.0
D.A.	54.6	2.0
A.B.	52.4	8.0
C.P.	10.1	0.1
Z.O.	1.5	0.1

Treatment with estrogen and progesterone for 1 month resulted in a significant ($P < 0.01$) decrease in IL-1 activity, from 79.1 ± 47.5 to 2.1 ± 1.0 units/ml.

rotic subjects secrete higher IL-1 levels than those from nonosteoporotic subjects (22). In that study, in fact, the nonosteoporotic and the osteoporotic groups included women a comparable number of years past menopause as well as eugonadic men of similar age. In the present investigation, the nonosteoporotic subjects were closer to menopause than the osteoporotic patients. Thus, as one would have predicted from the regression of IL-1 and years since menopause, they exhibited higher IL-1 levels.

There is some reason to believe that monocyte IL-1 production is related to the skeletal impact of the menopause. First, bone loss accelerates after menopause (2, 3) and decreases upon ovarian steroid replacement (6–8). Second, increased IL-1 elaboration is characteristic of patients with osteoporosis in whom bone remodeling is particularly vigorous (22). Third, increased monocyte IL-1 elaboration has also been reported in several medical conditions associated with a high incidence of osteoporosis, including rheumatoid arthritis (29–31) and, more recently, endometriosis (32, 33). Fourth, in the present studies, the degree of IL-1 elaboration was found to predict part of the bone density variance, and the transmenopausal increase in IL-1 seemed to diminish more slowly with the passage of time in osteoporotic than in nonosteoporotic subjects.

Although the significance of ovarian steroid-dependent IL-1 production by blood monocytes is uncertain, its possible association with bone turnover deserves serious consideration. Three relationships can be visualized. First, IL-1 mediates the enhanced bone turnover caused by ovarian steroid deficiency. In this model, IL-1 would be derived from intraskeletal monocytes or their progeny, or from bone cells (13–16) that might mirror the behavior of blood monocytes, since there is no evidence for systemic IL-1 release in postmenopausal women. The lack of published evidence for estrogen receptors in monocytes indicates that another cell type must be the primary target cell for ovarian steroids if, as seems most likely, it is estrogen and not progesterone that is the influential steroid. Lymphoid cells and bone cells, recently shown to possess estrogen receptor activity (34–36), are candidates. A second possible relationship is that a substance or substances released from bone during the postmenopausal acceleration of bone turnover determines the capacity of blood monocytes to release IL-1, either by direct action on the monocytes themselves or on their bone marrow precursors. Certain bone peptides are chemotactic for monocytes (37–39). Among these factors is transforming growth factor type β, highly enriched in bone tissue, which stimulates the expression of IL-1 messenger RNA in monocytes (39) and osteocalcin, the most abundant noncollagen bone protein (38). Third, we cannot exclude the possibility that factors unrelated to bone metabolism mediate the relationship of monocyte IL-1 release to ovarian steroid status.

Postmenopausal enhancement of IL-1 elaboration from various other types of cells could underlie additional phenomena associated with menopause. The recent demonstration of IL-1 in hypothalamic neurons (40) and the proposed association of gender with the effects of IL-1 on the hypothalamic pituitary axis (41) suggest that IL-1 secretion might mediate hot flashes and other autonomic responses accompanying estrogen withdrawal.

Although a cause–effect relationship has not been established, our results indicate that IL-1 secretion relates closely to and may explain the postmenopausal acceleration in bone loss and its inhibition by ovarian hormones. Monitoring of IL-1 activity, as the time progresses from menopause, may represent another method for assessing the risk of developing osteoporosis and for determining the best candidates for ovarian steroid therapy.

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