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Original Article

Bone-Resorbing Cytokines from Peripheral Blood Mononuclear Cells after Hormone Replacement Therapy: A Longitudinal Study

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Abstract. Conflicting results have been reported in several cross-sectional studies measuring cytokine production from adherent monocytes in pre- and postmenopausal women. Furthermore, the target cells for the action of estrogen are still debated. We therefore assessed in a longitudinal manner the cytokine production from different fractions of peripheral blood mononuclear cells (PBMC) cultured for 48 h. PBMC were obtained from 30 postmenopausal women before and after 6 months of hormone replacement therapy (HRT). Women were randomly allocated to two groups: an adherent PBMC group ($n = 20$) and a total PBMC group ($n = 9$). After 6 months of treatment, urinary pyridinoline levels were markedly decreased in both groups (353 ± 24 vs 114 ± 13 μg/mmol creatinine and 325 ± 35 vs 164 ± 31 μg/mmol creatinine respectively, $p<0.01$). Culture supernatants were assayed for interleukin 1β (IL-1β), interleukin 6 (IL-6), soluble IL-6 receptor (IL-6rs) and tumor necrosis factor alpha (TNF-α). In the adherent PBMC group, HRT induced a nonsignificant trend toward decreased levels of IL-1β (35 ± 10 vs 13 ± 5 pg/ml), TNF-α (333 ± 58 vs 222 ± 30 pg/ml) and IL-6 (115 ± 70 vs 17 ± 10 pg/ml). In contrast, in the total PBMC group, HRT induced a consistent and dramatic decrease in levels of IL-1β (104 ± 22 vs 25 ± 8 pg/ml), IL-6 (5950 ± 1041 vs 1011 ± 361 pg/ml), IL-6rs (148 ± 33 vs 35 ± 12 pg/ml) ($p<0.01$) and TNF-α (1468 ± 315 vs 585 ± 207 pg/ml, $p = 0.05$). We then evaluated whether HRT had the same effect in vitro. Adherent or total PBMC of 8 postmenopausal women were cultured with or without 10^{-8}M 17β-estradiol or tibolone for 48 h. Production of IL-1β, TNF-α, IL-6 and IL-6rs was not affected by the presence of 17β-estradiol or tibolone in cultures of these cell fractions. In conclusion, our data indicate that non-adherent PBMC could mediate the response to HRT. HRT may exert its action indirectly via noncirculating cells, as suggested by the absence of an in vitro effect.

Keywords: Cytokines; Hormone replacement therapy; Peripheral mononuclear cells

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Introduction

Many studies have shown the involvement of cytokines both in the regulation of bone remodeling and in the physiopathology of bone loss [1–3]. These factors produced locally induce osteoclastic bone resorption. Animal models have shown the importance of bone-resorbing cytokines in ovariectomy-related bone loss [4–6]. In women, much evidence is now available confirming the mediating role of cytokines in postmenopausal bone loss [7]. However, conflicting results have been reported in several cross-sectional studies measuring cytokine production from adherent monocytes in pre- and postmenopausal women with or without estrogen treatment. The discrepancies are related to the assay used and to the large inter-patient variability observed in all studies. These cytokines are regulated in a complex network involving many types of cells present in the microenvironment. Recently, the role of lymphocytes has been emphasized, especially as a possible target cell for estrogen, as adherent monocytes,

lymphocytes and macrophages express estrogen receptors [8], and ovariectomy induces an enhanced lymphopoiesis, which can be reversed by estrogen treatment [9]. We therefore assessed cytokine production from peripheral blood mononuclear cells (PBMC) in order to: (1) evaluate the in vivo effect of hormone replacement therapy on the production of bone-resorbing cytokines in a longitudinal manner, (2) assess the role of estrogen on non-adherent cells (monocytes or lymphocytes), and (3) compare the in vivo and in vitro effect of hormonal treatment on the different fractions of PBMC. The results suggest that estrogen action involves many cell types which communicate through several cytokines.

Patients

In Vivo Study

The longitudinal study was performed on 29 early postmenopausal women (mean age 56 $\pm$ 5 years) who were involved in a double-masked clinical trial designed for the prevention of postmenopausal bone loss. These women had either not received hormone replacement therapy (HRT) or previous HRT was stopped for at least 6 months. Exclusion criteria were also any treatment interfering with bone metabolism or any acute inflammatory condition influencing cytokine production at the time of blood withdrawal. The women were randomized to receive either an estroprogestative compound (Kliogest, Novo-Nordisk Pharmaceuticals) containing 17β-estradiol 2 mg/day and norethisterone acetate 1 mg/day, or a synthetic steroid compound (Livial, Organon) at two dosages: tibolone 1.25 or 2.5 mg per day. Recent data suggest that tibolone acts as an estrogen and progesterone agonist on bone [10]. Tibolone has been shown to prevent bone loss in early [11] and late postmenopausal women [12].

Blood samples were taken at baseline during the inclusion visit prior to any treatment and at the 6 month visit when subjects were taking HRT. Cell culture experiments were performed the day of blood withdrawal. Supernatants and RNA were stored at -80 °C until the end of the inclusions. Cytokine assays and reverse transcription polymerase chain reaction (RT-PCR) were performed within a year in masked fashion. Final analysis of the data was performed at the end after the opening of the randomization treatment code.

In Vitro Study

Effects of both compounds on in vitro cytokine production was assessed in 8 postmenopausal women (mean age 66 $\pm$ 7 years). Estradiol was purchased from Sigma (France) and tibolone was kindly provided by Organon Laboratory (Dr de Maricourt, France). No women were receiving any other treatment that interferes with bone metabolism.

Methods

Bone Density Measurements and Pyridinoline Excretion

Bone mineral density (BMD) was measured at the lumbar spine and at the femoral neck using dual-energy X-ray absorptiometry (QDR 2000, Hologic, Waltham, MA). Results at baseline and after 6 months of treatment are expressed as grams per square centimeter.

Pyridinoline excretion was measured at baseline and after 6 months of HRT on morning spot urine samples. The method used was ELISA (Crosslaps; Laboratoire Cis-Bio, France), and results are expressed as the pyridinoline/creatinine ratio (μg/mmol creatinine).

Cell Cultures

In vivo Study. Isolation of peripheral mononuclear cells was performed in the same conditions for the in vivo and in vitro evaluation. Thirty milliliters of heparinized blood was centrifuged in a Ficoll-Hypaque gradient, the fraction of mononuclear cells was washed twice with DMEM (Dulbecco's Modified Eagle's Medium), and resuspended at a final concentration of 10^6 cells/ml of medium (DMEM, 2 mM L-glutamine, 0.1 mg/ml ampicillin). Cells were cultured in the absence of serum and lipopolysaccharide in order to avoid activation. Cells were plated in 24-well dishes at a final volume of 2 ml/well.

For each patient, blood was drawn at baseline and after 6 months of treatment. Without knowledge of hormone treatment, 30 postmenopausal women were randomly assigned to one of two groups: group A, where only adherent PBMC were cultured and group T where total PBMC were cultured. Randomization was performed as follow: the first 10 patients were allocated to group A, the following 10 to group T, and again the following 10 to group A. We did plan to allocate the last 10 samples to group T, but the clinical trial was unfortunately stopped because enough patients had been included. Therefore we were not able to include the last 10 patients. When the randomization was opened at the end of the study, one patient in group T was found not to have been compliant with treatment, and was removed from the analysis. The distribution in groups A and T is not equilibrated, but the results of this human study were interesting enough to be reported.

Twenty women were studied in group A (mean age 55 $\pm$ 2 years). Cells were allowed to adhere for 1 h at 37 °C in a humidified atmosphere (95% air, 5% CO_2), then washed to remove the non-adherent cells. Only adherent cells were cultured in the presence of medium for 48 h. Nine women were studied in group T (mean age 57 $\pm$ 2 years). Total mononuclear cells (adherent and non-adherent) were cultured in the same conditions. At the end of culture, supernatant was collected and stored until assayed for bone-resorbing cytokines.

In Vitro study. To compare the in vivo and in vitro effect of HRT, peripheral blood cells were treated in vitro with both compounds. Blood was drawn and assessed as for the longitudinal study. The following culture protocol was applied to the cells after Ficoll separation: (1) total cell fraction (both adherent and non-adherent cells), (2) adherent mononuclear cells only, and (3) non-adherent mononuclear cells. Each of these fractions was cultured in the presence or absence of 17β-estradiol or tibolone at a final concentration of 10^{-8} and 10^{-9} M. For each group, cultures were made in triplicate. After 48 h, supernatants were harvested and stored at -80 °C until assayed.

Cytokine Measurements in Cell Culture Supernatants

Measurements of the cytokines IL-1β, TNF-α and IL-6, and the IL-6 soluble receptor (IL-6rs) which potentiated the effects of IL-6, were performed in supernatant using the same assay method. Cytokine concentrations were measured by a immunoenzymatic method using specific antibodies: IL-1β (Immunotech, detection 5 pg/ml), IL-6 (high-sensitivity hIL6 ELISA system, Amersham Life Science, detection threshold 0.1 pg/ml), IL-6rs (Quantikine hIL6Sr Immunoassay, RD Systems, detection threshold 3.5 pg/ml) and TNF-α (IRMA Biosource, Europe SA, detection threshold 5 pg/ml). Inter- and intra-assay reproducibility was below 10% for each kit. There was no cross-reactivity of the cytokines to other than their specific assay.

RNA Expression

RNA was extracted from cells after 48 h of culture. Cells were treated with RNAnow (Ozyme, France), and extraction was performed according to the method of Chomczynski and Sacchi. RNA extracts were stored for reverse transcription polymerase chain reaction (RT-PCR) evaluation. Because of the small amount of total mRNA products, we chose to evaluate only the expression of IL-1β and IL-6. Semiquantitative PCR was performed using GAPDH as an external standard. Quantification of density of the band was expressed as the ratio between IL-1β or IL-6 and GAPDH. Preliminary tests of control mRNA showed that a better signal was obtained with 1 μg of total RNA. Thus, reverse transcription of mRNA was performed from 1 μg total RNA according to a method previously described [13]: 1 h at 37 °C for the reverse transcription, then 95 °C for 7 min. Amplification of the RT products was performed with the following primers:

Amplification of IL-1β mRNA:
sense primer: 5′ GGATATGGAGCAACAAGTGG 3′
antisense primer: 5′ ATGTACCAGTTGGGGAACTG 3′
Amplification of IL-6 mRNA:
sense primer: 5′ TCAATGAGGAGACTTGCCTG 3′
antisense primer: 5′ GATGAGTTGTCATGTCCTGC 3′
Amplification of GAPDH mRNA:
sense primer: 5′ AACAGCCTCAAGATCATCAGC 3′
antisense primer: 5′ GGATGATGTTCTGGAGAGCC 3′

Reverse transcription products were separated into three aliquots of 9.5 μl, and then 4 μl PCR buffer, 25 pmol of each of the corresponding sense and antisense primers, and 0.1 μl *Taq* polymerase 5 U/ml (Appligene, France) were added. In order to determine the number of cycles, a cycle-effect was performed in control RNA. We chose to amplify with 27 cycles, which corresponds to a point on the ascending part of the slope (data not shown). Twenty-seven cycles (95 °C, 55 °C and 72 °C) were performed for each amplification. Amplification products were allowed to migrate in 2% agarose gel containing TEB. DNA fragments were visualized under UV light. The DNA contained in the gel was transferred onto nylon membrane (Genescreen) for 18 h. Membranes were hybridized with specific probes as follows:

Probe IL-1β: 5′ GTCCTGCGTGTTGAAAGATGAT AAGCCCACTCTACAGCTC 3′
Probe IL-6: 5′ GTAGTGAGGAACAAGCCAGAG CTGTGCAGATGAGTACAAAAG 3′
Probe GAPDH: 5′ ATGACAACTTTGGTATCGTGGA AGGACTCATGACCACAGTCCATG CCATC 3′

Signals were analyzed with a scanning densitometer (Transydine General Corporation) and results are expressed in arbitrary units (AU), using the GAPDH as reference.

Statistical Analysis

Results are expressed as means $\pm$ SEM. Comparison tests were analyzed by ANOVA, and paired *t*-tests or signed rank tests (Statview Abascus Software, Microsoft).

Results

Results were analyzed after the opening of the randomization code for treatment. In the adherent PBMC group, the allocation of treatment was: 7 women, Kliogest; 6 women, tibolone 1.25 mg; 7 women, tibolone 2.5 mg. In the total PBMC group, only 1 woman was not compliant with treatment and was excluded from the analysis. The allocation was: 2 women, Kliogest; 5 women, tibolone 1.25 mg; 2 women, tibolone 2.5 mg. We found no difference in the effect of the three treatments on pyridinoline levels and bone density of the patients.

Bone Density Measurements

BMD was assessed at both the lumbar spine and femoral neck. In the adherent PBMC group, after 6 months BMD had increased significantly at the lumbar spine (0.877 ± 0.027 vs 0.858 ± 0.027 g/cm^2, $p = 0.002$) and femoral

neck (0.712 ± 0.026 vs 0.690 ± 0.023 g/cm^2, $p = 0.001$) compared with baseline. In the total PBMC group, BMD increased significantly at the lumbar spine (0.852 ± 0.049 vs 0.836 ± 0.050 g/cm^2, $p = 0.044$) but not at the femoral neck (0.716 ± 0.044 vs 0.719 ± 0.043, p = NS).

In Vivo Bone Resorption

Bone resorption was assessed using pyridinoline excretion at baseline and after 6 months of hormone treatment. Compared with baseline levels, pyridinoline levels decreased in patients in the adherent PBMC group (353 ± 24 vs 114 ± 13 μg/mmol creatinine, $p<0.01$) and in the total PBMC group (325 ± 35 vs 164 ± 31 μg/mmol creatinine, $p<0.01$). Pyridinoline levels decreased more than 50% in each patient (Fig. 1).

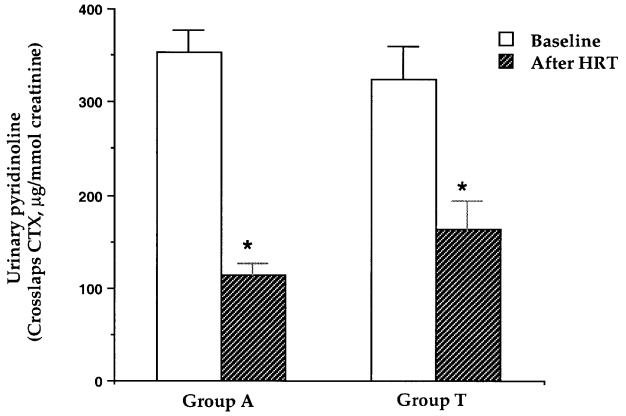

Fig. 1. Urinary pyridinoline excretion at baseline and after 6 months of hormone replacement therapy in patients of group A and group T. Pyridinoline was measured in a morning spot by ELISA (Crosslaps). Results are expressed as the pyridinoline/creatinine ratio (μg/mmol creatinine). There was a significant decrease in pyridinoline levels in group A and in group T (*$p<0.01$).

In Vivo Cytokine Production and Expression

Cytokine Release. In the adherent PBMC group as well as in the total PBMC group, cytokine levels and the changes in level did not differ according to the treatment group, as shown in the individual data in Figs. 2 and 3. Therefore, data were analyzed together regardeless of treatment.

In the adherent PBMC group, hormonal treatment induced a trend towards decreased levels of IL-1β (35 ± 10 vs 13 ± 5 pg/ml), IL-6 (115 ± 70 vs 17 ± 10 pg/ml) and TNF-α (333 ± 58 vs 222 ± 30 pg/ml) (Fig. 2). Although close to significance, the decreases did not reach significance ($p = 0.06$, $p = 0.18$, $p = 0.14$, respectively). Cytokine levels decreased in only half the patients, and remained unchanged in the others. IL-6 levels were zero at baseline and after HRT in 9 patients, as shown in the overlap in Fig. 2. IL-6rs was undetectable in the supernatants of adherent mononuclear cell cultures.

In the total PBMC group, HRT induced a significant decrease in the levels of IL-1β (104 ± 22 vs 25 ± 8 pg/ml, $p<0.006$), IL-6 (5950 ± 1041 vs 1011 ± 361 pg/ml, $p<0.001$) and IL-6rs (148 ± 33 vs 35 ± 12 pg/ml, $p<0.005$). The decrease was smaller for TNF-α (1468 ± 315 vs 585 ± 207 pg/ml, $p = 0.05$) (Figs. 3, 4). Interestingly, the decrease occurred in each individual patient, except for one whose levels of TNF-α and IL-1β increased after treatment with tibolone 2.5 mg daily.

mRNA Expression of IL-1β and Il-6. Amplification products of mRNA for IL-1β and IL-6 are expressed as the ratio of GAPDH products. In the adherent PBMC group (adherent cells), the RNA of only 15 patients was available for correct analysis. Results are presented in Fig. 4. After in vivo treatment, we observed a trend towards a decrease in transcript expression from adherent cells (IL-1β: 0.63 ± 0.20 vs 0.40 ± 0.16 AU (−37%); IL-6: 0.29 ± 0.10 vs 0.20 ± 0.08 AU (−32%)). Of the 15 patients, expression decreased in 7 for IL-1β, and in 6 for IL-6. However, the decrease did not reach statistical significance.

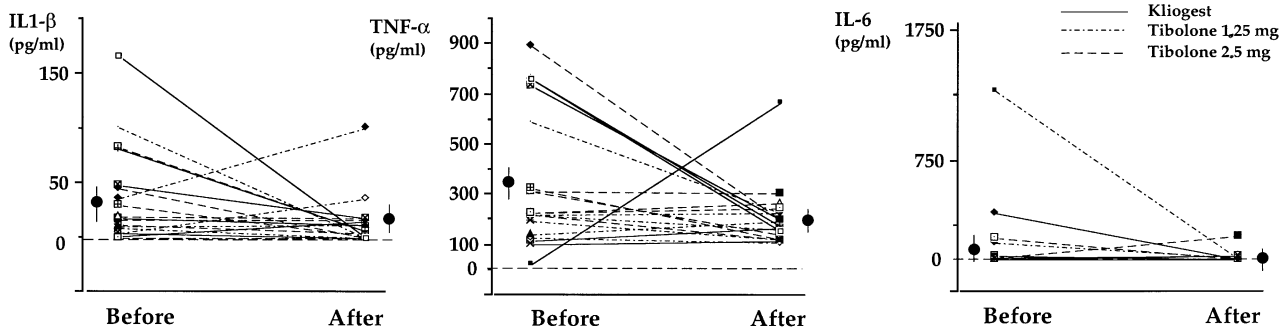

Fig. 2. Changes in cytokine levels in adherent mononuclear cell cultures before and after treatment with HRT. Adherent mononuclear cells were cultured for 48 h in red phenol-free DMEM. Cytokine levels were measured at the end of the cultures in the supernatants. Graphs show individual data of patients ($n = 20$) according to the treatment received. There are fewer points an lines in the IL-6 graphs because of overlaps. *Filled circles* to the left and right of the panels represent the mean, and the *vertical lines* the SEM. Statistical analysis was performed using paired *t*-test. The changes before and after treatment did not reach statistical significance: IL1-β, p=0.06; TNF-α: p=0.14, IL-6: p=0.18.

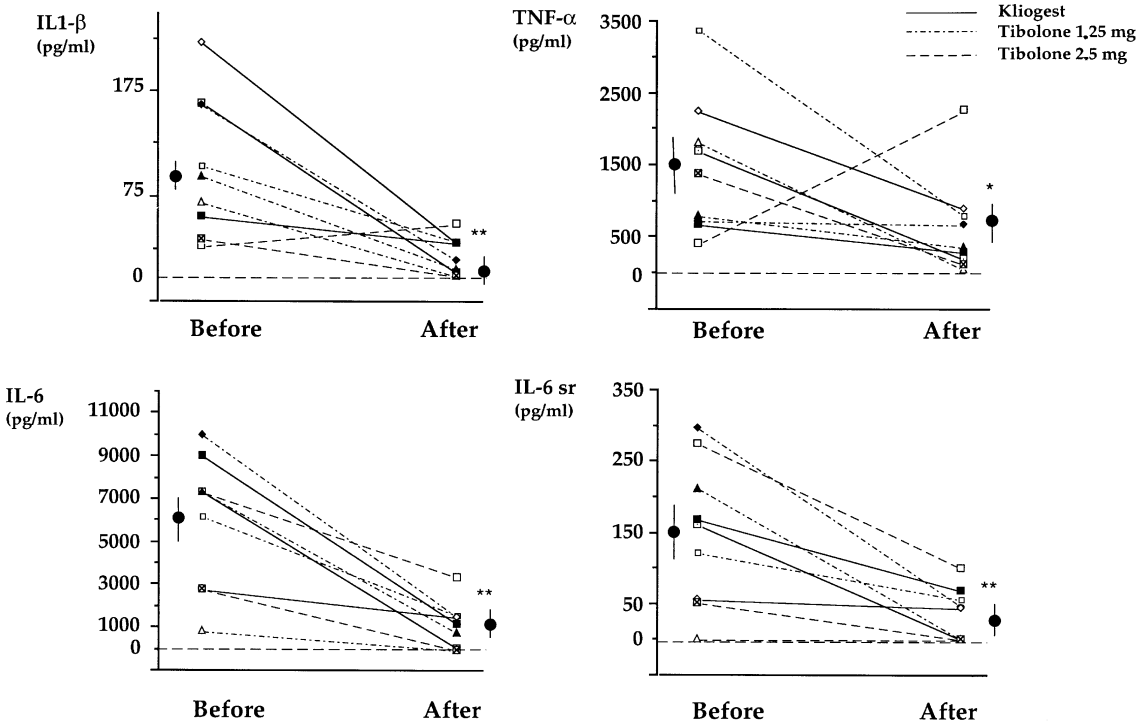

Fig. 3. Changes in cytokine levels in total mononuclear cell cultures before and after treatment with HRT. Total mononuclear cells (both adherent and non-adherent cells) were cultured for 48 h in red phenol-free DMEM. Cytokine levels were measured at the end of the cultures in the supernatants. Graphs show individual data of patients according to the treatment received ($n = 9$). *Filled circles* to the left and right of the panels represent the mean, and *vertical lines* the SEM. Statistical analysis was performed using paired *t*-test. There was a statistically significant decrease in three cytokines: IL1-β, IL-6 and IL-6sr (**$p<0.01$ for all) and TNF-α: *$p=0.05$.

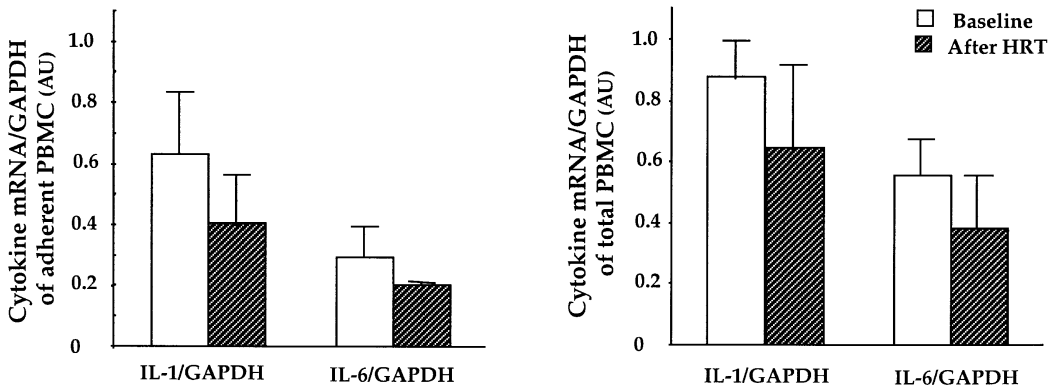

Fig. 4. Expression of mRNA for Il-1β and IL-6/GAPDH for adherent mononuclear cells and for total mononuclear cells before and after HRT. Cell cultures and RT-PCR were performed as described in Methods. Results are expressed as the means $\pm$ SEM. *Left panel* represents the results of group A ($n = 15$) and *right panel* the results of group B ($n = 9$).

In the total PBMC group (total cells), RNA of 9 patients was analyzed. A similar trend to a 30% decreased density of transcripts was observed (IL-1β: 0.87 ± 0.12 vs 0.64 ± 0.27 AU (-27%); IL-6: 0.55 ± 0.12 vs 0.38 ± 0.17 AU (-31%)), but was not significant. In the total PBMC group, inhibition of IL-1β expression was observed in 5 of 9 patients, and for IL-6 in 4 of 9 patients. This inhibition of expression is parallel to a marked decrease in IL-1β and Il-6 protein levels.

In Vitro Cytokine Production

In this study, the adherent, non-adherent and total cells were cultured in the presence or absence of 17β-estradiol or tibolone in vitro. Results are reported in Tables 1 and 2. We observed no change in the production of any cytokines after addition of 17β-estradiol or tibolone regardless of the cell fraction, suggesting that neither compound has any direct action on these cells in culture.

Table 1. In vitro effect of 17β-estradiol and tibolone on IL-1β and TNF-α production

	IL-1β (pg/ml)			TNF-α (pg/ml)		
	Total cells	Adherent cells	Non-adherent cells	Total cells	Adherent cells	Non-adherent cells
Controls	76.8 ± 46	74.5 ± 58	29.3 ± 23	503 ± 248	312 ± 170	352 ± 165
17β-estradiol 10^{-8} M	75.6 ± 50	64.8 ± 49	34.6 ± 28	483 ± 243	305 ± 153	317 ± 128
17β-estradiol 10^{-9} M	82.4 ± 52	64.1 ± 49	35.4 ± 23	448 ± 215	258 ± 103	322 ± 133
Tibolone 10^{-8} M	81.9 ± 54	60.4 ± 48	40.6 ± 25	452 ± 228	278 ± 134	322 ± 150
Tibolone 10^{-9} M	90.4 ± 57	52.8 ± 38	37.3 ± 26	479 ± 233	198 ± 39	327 ± 137

Peripheral blood mononuclear cells were cultured in the presence of 10^{-8} and 10^{-9} M 17β-estradiol and tibolone for 48 h. IL-1β and TNF-α levels in supernatants were measured at the end of the culture. Data are means ± SEM (n = 8). No differences in IL-1β and TNF-α levels were observed after 17β-estradiol or tibolone in vitro treatment at any dose.

Table 2. In vitro effect of 17β-estradiol and tibolone on IL-6 and Il-6sr production

	IL-6 (pg/ml)			IL-6sr (pg/ml)		
	Total cells	Adherent cells	Non-adherent cells	Total cells	Adherent cells	Non-adherent cells
Controls	6.06 ± 4.94	0.68 ± 5.85	1.43 ± 1.22	45 ± 23	4.37 ± 4.37	18.62 ± 13.18
17β-estradiol 10^{-8} M	6.65 ± 5.39	0.77 ± 0.65	0.28 ± 0.18	37 ± 22	0	7.57 ± 7.57
17β-estradiol 10^{-9} M	5.86 ± 4.61	5.86 ± 4.61	1.93 ± 1.59	31 ± 21	0	16.62 ± 11.27
Tibolone 10^{-8} M	6.91 ± 5.33	0.79 ± 0.67	1.50 ± 1.21	31 ± 21	4.25 ± 4.25	15.25 ± 10.88
Tibolone 10^{-9} M	6.39 ± 4.81	0.58 ± 0.39	2.38 ± 1.90	42 ± 21	0	21.5 ± 14.20

Peripheral blood mononuclear cells were cultured in the presence of 10^{-8} and 10^{-9} M 17β-estradiol and tibolone for 48 h. IL-6 and IL-6sr levels in supernatants were measured at the end of the culture. Data are means ± SEM (n = 8) and are expressed as pg/ml. No differences in IL-6 and IL-6sr levels were observed after 17β-estradiol or tibolone in vitro treatment at any dose.

Discussion

Postmenopausal osteoporosis is characterized by bone loss related to a negative bone balance, where bone resorption is higher than bone formation. High bone resorption is regulated by hormonal factors that are mediated through local factors, acting on bone cells and marrow cells in the bone microenvironment. In recent years much evidence has suggested a role for local factors in animal models [1,6]. In humans, estradiol decreases cytokine production in in vivo-treated women [7]. We have shown that levels of bone cytokines produced by adherent monocytes from estrogen-treated women were decreased compared with those of untreated women [14]. In vivo, estrogen and progesterone decrease the IL-1 bioactivity of PBMC [15]. In vitro, estrogen inhibits the release of TNF-α from adherent PBMC only in osteoporotic women and not in premenopausal women [16]. Most of the studies on PBMC have been peformed on adherent monocytes, some showing decreased levels after in vivo treatment and others showing no changes [2]. The discrepancies observed in the literature could be due to differences in the assays used, but are also influenced by the delay of menopause and the high inter-patient variation in cytokine release. The lack of significant changes might be due to inter-patient variability. Because of this variation, we tried to reduce this bias through a longitudinal study, each patient being her own control at baseline and for the effect of treatment.

For each patient, treatment led to a 50% decrease in pyridinoline excretion, confirming the compliance with treatment and the efficacy of inhibition of bone resorption in the three treatment groups. When adherent mononuclear cells only were cultured in our subset of patients, we observed no difference in mean levels of the three cytokines at baseline and after 6 months of treatment. However, IL-1β, IL-6 and TNF-α levels decreased in half the patients. This inhibition was inconsistent in our study, in agreement with the literature. There was no difference in the effect of the three treatments on pyridinoline excretion or cytokine levels, justifying the pooling of the data. It is unlikely that the data might have been influenced by a decrease in monocyte number or adherence. Only one previous study has reported an increased number of blood monocytes after menopause [17], but there are no data on the adherence of such PBMC in vitro. Indeed, we have investigated the effect of estradiol on PBMC and we did not observe any effect on the prolfieration of these cells (unpublished data).

In the present study, in patients treated with HRT, total mononuclear cells release significantly and constantly decreased levels of IL-1β, TNF-α, IL-6 and Il-6sr. This suggests that the effect of estrogen may be

mediated through non-adherent monocytes or lymphocytes. Several arguments suggest the involvement of lymphocytes in the effect of estradiol on bone marrow cells. Like monocytes [18], lymphocytes express estrogen receptors [8], and estrogen is a negative regulator of lymphopoiesis. In ovariectomized normal mice, estrogen deficiency leads to enhanced lymphopoiesis in vivo [9], in parallel with enhanced bone resorption, suggesting local interactions between cells in the bone microenvironment [19]. Normal mice treated with estrogen have decreased thymic T cells due to decreased precursor levels [20]. In normal rats, ovariectomy induced increased B lymphopoiesis [21]. There is less evidence of involvement of lymphocytes after menopause in humans. Immune cell subsets were modulated by estrogen in vivo [22]. Moreover, bone loss was negatively correlated with T cells, but estrogen treatment does not affect lymphocyte subsets in healthy postmenopausal women [23].

In order to test a direct effect on PBMC, we treated the cells in vitro using the same cell culture protocol. For each patient, cells were fractionated and cultured separately: total cells, adherent cells and non-adherent cells, both in the presence and in the absence of estradiol and tibolone. We observed no change in any cytokines produced in the presence of either compound. These results show that the target cells are not circulating PBMC, and raise again the hypothesis of the role of cells present in the bone microenvironment. Many cells are candidates: hematopoietic cells, osteoclast precursors or stromal cells. As already shown in mice, estrogen suppresses lymphopoiesis through an effect on stromal cells, which involves several types of receptor [24,25]. Moreover, in mice they are necessary for the differentiation of osteoclast precursors in marrow cultures [21]. In our study, steroids may modulate cytokine production from stromal cells and lymphocytes, which in turn regulate local bone resorption by generating monocyte-macrophage precursors in bone marrow. Another hypothesis is that decreased cytokine release may be secondary to decreased bone resorption, since both estrogen and tibolone induced a similar decrease in cytokine release. This could be in agreement with previous data showing that monocytes cultured on bisphosphonate-treated bone showed reduced IL-2 release [26].

The design of the study has limitations which weaken the conclusions. The first is the lack of a placebo-treated group. A trial including a placebo group would have been more convincing in order to relate the effects of the two treatments, but such a clinical trial could not be performed for ethical reasons as estrogens are known to prevent bone loss in this population. However, the constant decrease observed in the total PBMC group is quite striking and parallels the decrease in pyridinoline levels, suggesting a link with the reduction in bone turnover. Secondly, cytokine production in an individual may vary according to unknown conditions, and a better estimate would have been provided with two basal measurements. In this study, only one cell culture was performed at baseline and after 6 months, but more blood could not be taken at a close visit for ethical reasons. Finally, total and adherent cells were cultured from different patients in vivo. Again, it would have been better to culture both adherent and total cells from the same individuals, but this required more blood than we could obtain.

Gene expression of bone-resorbing cytokines has been poorly assessed. Transcriptional gene expression has been studied for IL-6. In murine stromal cells and in human osteoblasts, transcripts of IL-6 decreased significantly after in vitro 17β-estradiol addition [27,28]. The effect of 17β-estradiol is not direct since there is no estrogen response element on the IL-6 gene promotor, but it may be mediated through transcripton factors such as NFk-B or C/EBPb [29]. We evaluated protein production as well as the gene transcription before and after HRT. Although not significant, we found a marked trend towards decreased gene expression for IL-1β and IL-6 after HRT, for both the adherent and total PBMC groups. The reduction in IL-1β and IL-6 was 32–37% in adherent cells and 31% and 27% in total cells, which contrasts with the amplitude of the decrease in cytokine release. The absence of significance may be due to the low number of subjects together with the high variability observed. However, compared with baseline, expression of transcripts was decreased in 50% of patients after HRT, suggesting transcriptional mechanisms. In 2 patients for IL-1β and 3 patients for IL-6, there was a discrepancy between expression and protein production. A decrease in half-life or an increase in degradation of RNA could be involved. Post-transcriptional regulation of RNA may also account for the diminished protein production.

In conclusion, we have shown that hormone treatment negatively regulates the production of bone-resorbing cytokines from mononuclear cells in vivo. The data also stress the action of estrogen on lymphocytes, which may amplify the cytokine response through stromal cells. Therefore, estrogen may act directly on several different cell types in the bone microenvironment.

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