Estrogen deficiency increases osteoclastogenesis up-regulating T cells activity: A key mechanism in osteoporosis

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A B S T R A C T
Compelling evidences suggest that increased production of osteoclastogenic cytokines by activated T cells plays a relevant role in the bone loss induced by estrogen deficiency in the mouse. However, little information is available on the role of T cells in post-menopausal bone loss in humans. To investigate this issue we have assessed the production of cytokines involved in osteoclastogenesis (RANKL, TNFα and OPG), in vitro osteoclast (OC) formation in pre and post-menopausal women, the latter with or without osteoporosis. We evaluated also OC precursors in peripheral blood and the ability of peripheral blood mononuclear cells to produce TNFα in both basal and stimulated condition by flow cytometry in these subjects.

Our data demonstrate that estrogen deficiency enhances the production of the pro-osteoclastogenic cytokines TNFα and RANKL and increases the number of circulating OC precursors. Furthermore, we show that T cells and monocytes from women with osteoporosis exhibit a higher production of TNFα than those from the other two groups.

Our findings suggest that estrogen deficiency stimulates OC formation both by increasing the production of TNFα and RANKL and increasing the number of OC precursors. Women with post-menopausal osteoporosis have a higher T cell activity than healthy post-menopausal subjects; T cells thus contribute to the bone loss induced by estrogen deficiency in humans as they do in the mouse.

Introduction

Post-menopausal osteoporosis is a systemic skeletal disorder characterised by reduced bone mineral density (BMD), micro architectural deterioration of bone tissue resulting in fragility and susceptibility to fractures [1] and uncoupling of osteoblast-mediated bone formation and osteoclast (OC)-mediated bone resorption.

Post-menopausal osteoporosis stems from the cessation of ovarian function at menopause and from genetic and non genetic factors which heighten and prolong the rapid phase of bone loss characteristic of the early post-menopausal period. The anti resorptive activity of estrogen is a result of multiple genomic and non genomic effects on bone marrow and bone cells, which leads to decreased OC formation, increased OC apoptosis and decreased capacity of mature OCs to re-absorb bone. Although it is now recognized that stimulation of bone resorption in response to estrogen deficiency is mainly due to cytokine-driven increases in OC formation, the responsible factors are not completely understood. OC formation occurs when monocytes are co-stimulated by the essential osteoclastogenic factors RANKL and M-CSF [2–4], but additional inflammatory cytokines are responsible for the up-regulation of OC formation observed in estrogen deficiency. One of the cytokines responsible for the augmented osteoclastogenesis in states of estrogen deficiency is TNFα, a factor which enhances OC formation by up-regulating stromal cell production of RANKL and M-CSF, and by augmenting the responsiveness of OC precursors to RANKL [5,6]. Furthermore, TNF directly induces narrow precursor differentiation into OCs in the absence of RANKL, although according to some studies, TNF is not osteoclastogenic in cells not previously primed by RANKL [7]. However, the source of this cytokine, which is relevant to post-menopausal bone loss in humans, remains to be determined. Studies in mice suggest that activated T cells are the most relevant source of TNF in conditions of estrogen deficiency [8,9]. In support of this hypothesis there are reports demonstrating that T cell deficient nude mice are protected against post-ovariectomy bone loss [10,11]. Furthermore, data show that adoptive transfer of wild type T cells restores the capacity of ovariectomy to induce bone loss, while transfer of T cells from TNF null mice does not.

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Other studies argued against a pivotal role of T cells in bone loss induced by ovariectomy in mice models [13–15]. In particular, Lee et al [13] suggested that nude mice lose trabecular bone as well as wild type after ovariectomy and that T cells may have important effects on the cortical rather than on the trabecular compartment.

Little information is available regarding the role of T cells in human bone loss. Studies show a key role of T cell produced TNF in rheumatoid arthritis [16], multiple myeloma [17,18] and bone metastasis [5,19]. Other reports show that estrogen deficiency increases the production of TNF and RANKL by bone marrow cells, including T cells, and that their increase correlate with indices of bone resorption [20–22].

This cross-sectional investigation was designed to determine how estrogen deficiency affects the production of osteoclastogenic cytokines by peripheral blood mononuclear cells (PBMC), OC formation and bone resorption.

We report that estrogen deficiency increases the ability of PBMC to form OCs in vitro and that this ability is due both to increased output of OC precursors from bone marrow and to an increased production of TNFα. Furthermore, we demonstrate that T cells are more active in women affected by post-menopausal osteoporosis as respect to post-menopausal healthy women and to pre-menopausal healthy controls. We suggest T cell cytokine production as the primary driver of osteoclastogenesis in post-menopausal osteoporosis.

Materials and methods

Experimental subjects and markers of bone turnover

The study was approved by the human study review board of the Azienda Sanitaria Ospedaliera San Giovanni Battista in Torino. The study population included twenty-five post-menopausal women with osteoporosis, 23 healthy post-menopausal and 10 healthy pre-menopausal women. All study subjects had levels of 25-OH vitamin D, bone alkaline phosphatase (BAP) activity and routine blood tests within normal limits. Subjects with secondary forms of osteoporosis or taking drugs active on bone turnover such as calcium and vitamin D, thyroid hormones, corticosteroids, estrogen, bisphosphonates and raloxifene were excluded. The patients and the post-menopausal controls were matched for age, years since menopause and body mass index. Patients had a bone mineral density (BMD) T score of −2.5 S.D. or less according to the WHO criteria as measured by double-emission X-ray absorptiometry with a Hologic QDR 4500. BMD was measured at both lumbar spine and femoral neck; none of the patients had sustained recent fragility fractures. Post-menopausal controls had a T score > −1 S.D. in both the lumbar spine and the femoral neck. BMD was not measured in pre-menopausal controls. Serum osteocalcin and urinary CTX were measured by RIA using commercially available kits (DiaSorin for osteocalcin and α-Cross Laps® RIA from Osteometer Biotech A/S CTX, respectively) in patients and post-menopausal controls. All biochemical measurements were performed on a single blood sample at a single time point per subject.
PBMCs were obtained in all the subjects with the Ficoll–Paque method from 40 ml peripheral blood in lithium heparin as previously described [22]. All cultures were performed in triplicate in 16-well plates BD BioCoat™ Osteologic™ Bone Cell Culture System (Becton Dickinson & Co. 4×10⁵ cells/well) [17,23] using alpha minimal essential medium (α-MEM; Gibco) supplemented with 10% fetal bovine serum (FBS), benzyl penicillin (100 IU/ml) and streptomycin (100 μg/ml) (called “complete medium”), or complete medium plus M-CSF [25 ng/ml] and RANKL [30 ng/ml]. RPMI (Gibco) was used for cell isolation. All cultures were maintained at 37 °C in a humidified 5% CO₂ atmosphere. To validate resorption of the hydroxyapatite matrix, cells from 5 patients, 5 post-menopausal and 5 pre-menopausal controls randomly selected were also double-plated on dentin slices [2×10⁶ cells/slice]. The capacity of OCs to resorb bone in vitro was equally detected by measuring resorption pits on dentin slices and the resorption of hydroxyapatite-coated wells (data not shown) as also demonstrated by other studies [17,18,24]. In this study bone resorption was quantified by measuring the resorption of hydroxyapatite in all the subjects.

**Osteoclast formation and activity**

PBMCs were fed every 3 days. On the 21st day, they were fixed and stained for Tartrate Resistant Acid Phosphatase (TRAP) (Acid Phosphatase, Leukocyte staining kit, Sigma Diagnostics) and stained with an immuno technique to express the vitronectin receptor (VNR) (Becton Dickinson & Co). The supernatants of cultures without M-CSF and CD11b+ cells gated on CD14+ cell. The graphs show MFI and percentages of positive cells, the bars represent the mean and SD obtained for all the experiments performed in 25 osteoporotic, 23 post-menopausal and 10 pre-menopausal controls. The quadrant gates were set against isotype control. P values were calculated with ANOVA: °p=0.02, †p=0.04, **p=0.04.

**Cell isolation and cultures**

PBMCs were obtained in all the subjects with the Ficoll–Paque method from 40 ml peripheral blood in lithium heparin as previously described [22]. All cultures were performed in triplicate in 16-well plates BD BioCoat™ Osteologic™ Bone Cell Culture System (Becton Dickinson & Co. 4×10⁵ cells/well) [17,23] using alpha minimal essential medium (α-MEM; Gibco) supplemented with 10% fetal bovine serum (FBS), benzyl penicillin (100 IU/ml) and streptomycin (100 μg/ml) (called “complete medium”), or complete medium plus M-CSF [25 ng/ml] and RANKL [30 ng/ml]. RPMI (Gibco) was used for cell isolation. All cultures were maintained at 37 °C in a humidified 5% CO₂ atmosphere. To validate resorption of the hydroxyapatite matrix, cells from 5 patients, 5 post-menopausal and 5 pre-menopausal controls randomly selected were also double-plated on dentin slices [2×10⁶ cells/slice]. The capacity of OCs to resorb bone in vitro was equally detected by measuring resorption pits on dentin slices and the resorption of hydroxyapatite-coated wells (data not shown) as also demonstrated by other studies [17,18,24]. In this study bone resorption was quantified by measuring the resorption of hydroxyapatite in all the subjects.

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**Cytokine measurement**

ELISA kits were used to measure TNFα (Quantikine; R&D System), OPG (BioMedica; Biomedica Medizinprodukte GmbH & Co KGA) and free s-RANKL (BioMedica; Biomedica Medizinprodukte GmbH & Co KGA) levels in 3 and 6 day unstimulated culture supernatants from all the subjects. To evaluate the in vivo cytokines production we measured the same cytokines also in the serum. We measured the levels of total s-RANKL (Apoptech; Apoptech Corporation & Immunodiagnostik) in the serum to exclude possible variation in free s-RANKL due to OPG level as previously suggested [26]. Each measurement was performed in duplicate for each patient.

**Table 1**

<table>
<thead>
<tr>
<th>Patients</th>
<th>Controls</th>
<th>Osteoporotic (25)</th>
<th>Post-menopausal (23)</th>
<th>Pre-menopausal (10)</th>
<th>P values</th>
<th>Across groups</th>
<th>Patients vs. post-menopausal</th>
<th>Patients vs. pre-menopausal</th>
<th>Post-menopausal vs. pre-menopausal</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNFα day 3</td>
<td>40±3.8</td>
<td>30.4±2.7</td>
<td>0.4±0.05</td>
<td>0.001</td>
<td>0.04</td>
<td>0.001</td>
<td>0.037</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TNFα day 6</td>
<td>178.2±2.5</td>
<td>53.7±7.2</td>
<td>38.2±7.7</td>
<td>0.029</td>
<td>0.24</td>
<td>0.03</td>
<td>0.029</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RANKL day 3</td>
<td>0.29±0.09</td>
<td>0.05±0.03</td>
<td>0</td>
<td>0.024</td>
<td>0.24</td>
<td>0.000</td>
<td>0.04</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RANKL day 6</td>
<td>0.32±0.08</td>
<td>0.03±0.01</td>
<td>0.0</td>
<td>0.002</td>
<td>0.003</td>
<td>0.002</td>
<td>0.04</td>
<td></td>
<td></td>
</tr>
<tr>
<td>OPG day 3</td>
<td>11.39±23.66</td>
<td>10.57±2.88</td>
<td>12.38±16.62</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td></td>
<td></td>
</tr>
<tr>
<td>OPG day 6</td>
<td>14.45±24.61</td>
<td>29.31±26.63</td>
<td>19.31±2.39</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Supernatant TNFα, OPG and free s-RANKL values (pg/ml: means±SE) at 3 and 6 days of culture of PBMC cultures without addition of M-CSF and RANKL from the patients and the two control groups. The P values were obtained with the one way ANOVA corrected with the Bonferroni post hoc test. The values in parenthesis correspond to the number of analysed subjects.
appears as two bands at 49 and 29 kDa. Western blot analysis was performed on protein extracted from PBMC cultures supernatants from three osteoporotic women whose RANKL levels were determined by real-time quantitative RT-PCR. The results were normalized for the expression of S14. All samples were analysed in triplicate. The specificity of the PCR results was confirmed by melt curve analysis.

**Flow cytometry**

OC precursors were detected in all the subjects by staining the fresh blood samples with FITC-conjugated anti-CD11b, PE-conjugated anti CD14 and APC-conjugated anti-CD11b mAb, or with the corresponding isotype control followed by incubation at 4 °C for 30 min. We treated the triple-positive (CD14+/CD11b+/VNR+) cells as OC precursors –...
[10^6 PBMC/well] were analysed for the basal production of TNFα or plated in triplicate in 96-well plates using RPMI (supplied by GIBCO) supplemented with 10% fetal bovine serum (FBS), benzyl penicillin (100 IU/ml) and streptomycin (100 μg/ml), plus PHA [10 μg/ml] and analysed after 24 h of culture for the production of TNF after stimulus in all the subjects. For intra-cytoplasmic detection of TNFα in these subsets, 1 x 10^6 PBMC were stained with PerCP-conjugated anti-CD3 and APC-conjugated anti-CD14 or with the corresponding isotype control and then incubated for 30 min at 4 °C, fixed with 1 ml cold 0.4% paraformaldehyde in PBS and incubated for 15 min at 4 °C. Fixed cells were washed twice with PBS supplemented with 0.5% BSA, 0.1% sodium azide and 500 μl of FACS permeabilizing solution (Becton Dickinson & Co.) was added to the pellet. After 10 min incubation at room temperature, cells were washed with PBS supplemented with 0.5% BSA, 0.1% sodium azide and incubated with PE-conjugated anti-TNF alpha mAb (according to the manufacturer’s protocols) or with the corresponding isotype control for 30 min at 4 °C, as previously described [33]. Flow cytometry was performed on a FACSCalibur flow cytometer (Becton Dickinson & Co.), membrane antigens expression was analysed through CellQuest (Becton Dickinson & Co.) software and displayed as bivariate dot plots or histograms. Each plot represents the results from 10,000 events of viable cells gated by size and granularity. All the antibodies were purchased from Becton Dickinson & Co.

**Cell co-cultures**

To clarify the role of lymphocytes in OC differentiation and activation in vitro, we cultured monocytes alone and together with either T or B cells obtained by negative separation with monocyte, Pan T cell and Pan B cell isolation kits (Miltenyi Biotech). CD14+ cells were cultured alone (200,000/well), with CD3+ cells (420,000/well) and with CD19+ cells (360,000/well) on biocoated plates with and without growth factors.

**Table 2**

<table>
<thead>
<tr>
<th>Patients</th>
<th>Controls</th>
<th>P values</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Osteoporotic (25)</td>
<td>Post-menopausal (23)</td>
</tr>
<tr>
<td>TNFα</td>
<td>163.04 ± 107</td>
<td>25.14 ± 2.65</td>
</tr>
<tr>
<td>RANKL</td>
<td>3107 ± 28.96</td>
<td>0.1 ± 0.05</td>
</tr>
<tr>
<td>OPG</td>
<td>1863 ± 373.3</td>
<td>1191 ± 141.65</td>
</tr>
</tbody>
</table>

Serum TNFα, OPG and total s-RANKL values (pg/ml; means ± SE) in patients and the two control groups. The P values were obtained with the Kruskal-Wallis test. The values in parenthesis correspond to the number of analysed subjects.

![Fig. 5](image-url)
Cytokine inhibition experiment

To evaluate the role of endogenous TNFα and RANKL production in supporting osteoclastogenesis, we carried out a double cytokine inhibition experiment on three osteoporotic patients whose RANKL and TNF levels correspond to the mean of the analysed patients. Increasing doses of anti-TNF mAb (Peprotek) and anti-RANKL mAb (RANKL-Fc, R&D System) or a neutral isotype were added during a 21-day culture, after which the cells were fixed, stained and counted under a light microscope as described above.

The FACS analyses, the cultures and the cytokine measurement experiments were performed for all the enrolled subjects.

Statistics

The data were processed with the SPSS 14.0 for Windows software package, with \( p < 0.05 \) as the significance cut-off. One-way ANOVA was used to compare patients and controls for menopausal period, BMI, bone metabolism markers, TNFα, RANKL and OPG levels in the supernatants, number of OCs per well, percentage of resorbed hydroxyapatite and TNFα production measured with FACS. The post hoc Bonferroni test was used to point out differences between groups. To analyse the differences among patients and controls for serum levels of TNF, RANKL and OPG we used a Kruskal–Wallis test as these variables were not distributed according to a Gaussian curve. To determine which of the significantly different variables were predictors of the number of OCs, we used a linear regression model with stepwise analysis.

**Results**

Osteoclasts formation is higher in women affected by post-menopausal osteoporosis

Unstimulated PBMC cultures from osteoporotic women produced more OCs than those from controls, whereas this difference disappeared when the cultures were stimulated with M-CSF and RANKL. Furthermore, the OCs formed in patients are more active than those formed in controls in unstimulated condition as demonstrated by the higher percentage of hydroxyapatite resorbed (Fig. 1). PBMC from pre-menopausal controls do not form OCs nor reabsorb hydroxyapatite in unstimulated condition by contrast with post-menopausal controls. This finding confirms that estrogen deficiency induces osteoclastogenesis. The addition of osteoclastogenic cytokines in culture annuls the difference between the three groups, suggesting that an adequate stimulation is able to induce the formation of mature and active OCs even in PBMC from subjects in pre-menopausal period. The markers of bone metabolism confirm the experimental data on the higher OC activity: in fact the bone resorption marker (CTX) is higher in patients, as respect to post-menopausal controls (8.02±1.88 versus 4.17±2.56 μg/L, \( p = 0.04 \)) and correlated to the number of OCs formed in culture (\( R=0.7, p=0.03 \)).

To assess the effects of menopause and osteoporosis on the process of OC differentiation we next measured the number of OC precursors in...
PBMC harvested from the 3 study groups. The percentage of OC precursors (CD14+/CD11b+/VNR+) was found higher in the patients than in the two control groups. The mean fluorescence intensity (MFI) of CD11b and VNR was higher in patients than in samples from the control groups, while the MFI of CD14 was higher in the pre-menopausal controls and inversely correlated with age ($R = -0.5, p = 0.002$) (Fig. 2). This finding suggests that OC precursors in patients were more committed toward osteoclastic lineage as compared to controls.

Patients produce more TNF and RANKL as respect to both post-menopausal and pre-menopausal controls

The production of RANKL and TNF by unstimulated PBMC was assessed by measuring the levels of these two cytokines in 3 and 6 day culture media by ELISA. TNFα levels were significantly higher in the patients than in both control groups at each point studied. Furthermore, post-menopausal had higher levels of TNF than pre-menopausal controls. These findings suggest that estrogen loss induce a greater production of TNFα by PBMC and that osteoporosis further increases this production. At day 3 and 6 RANKL levels were significantly higher in the patients than in controls (Table 1). The Western blot technique used to verify the specificity of the antibody at low RANKL concentration confirms the reliability of our results: serial dilutions of PBMC supernatant protein revealed dominant bands of RANKL at 49 and 29 kDa with intensities that were linear over the range from 100–600 µg protein. Thus, the antibody recognized RANKL in a linear fashion over the protein range studied. The lower RANKL dose (0.25 pg) was also recognized (Fig. 3). The real-time RT-PCR data confirm that PBMC from patients produce more RANKL than those from controls (Fig. 4).

In contrast, OPG levels were similar in all three groups at each point studied (Table 1). The ratio between RANKL and OPG measured at day 6 was significantly higher in patients ($0.7 ± 0.2$) as respect to post-menopausal and pre-menopausal controls ($0.5 ± 0.4$ and 0, respectively).

In order to evaluate the in vivo cytokines production we measured the serum levels of TNFα, OPG and total RANKL (free RANKL+OPG-bound RANKL). We choose to evaluate total RANKL in serum to avoid possible interference due to changes in OPG levels in osteoporotic subjects [26,34]. Our data show that both TNFα and OPG are higher in patients as compared to the controls, while RANKL is not statistically different even if higher in patients (Table 2). The ratio between serum RANKL and OPG was not significantly different between patients and controls.

T cells are the major source of TNF and RANKL in osteoporosis

To determine whether the increased levels of TNFα in vitro were due to a greater production of the cytokine per cell, or to an increase in their number, we measured the number of TNF producing cell by FACS in PBMC and in PBMC after stimulus with PHA for 24 h. We found that TNF was produced by monocytes and T cells both with and without PHA. As expected, PHA increased TNF production by T cells, but not by monocytes. T cells from pre-menopausal and post-menopausal controls also displayed a greater response to PHA than those from patients (80, 30 and 1 fold respectively) (Fig. 5). These data suggest that T cells from post-menopausal women are less prone to immune

<table>
<thead>
<tr>
<th>OCs without factors</th>
<th>Standardized B</th>
<th>T</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>MIF VNR</td>
<td>0.95</td>
<td>5.5</td>
<td>0.012</td>
</tr>
<tr>
<td>OC precursors %</td>
<td>0.98</td>
<td>227.57</td>
<td>0.000</td>
</tr>
<tr>
<td>6th culture day RANKL level</td>
<td>0.022</td>
<td>70.21</td>
<td>0.000</td>
</tr>
<tr>
<td>BMD</td>
<td>B</td>
<td>T</td>
<td>P</td>
</tr>
<tr>
<td>8th culture day RANKL level</td>
<td>-0.461</td>
<td>-6.9</td>
<td>0.006</td>
</tr>
</tbody>
</table>

Stepwise linear regression models for osteoclast (OC) formation without added M-CSF and RANKL and bone mineral density (BMD) as dependent variables.
stimulation as respect to pre-menopausal healthy women. However in patients TNF production was significantly higher at baseline and after PHA stimulation as respect to controls. Monocytes from pre-menopausal produced more TNFα than those from post-menopausal controls also (Fig. 5), and this points to a greater reactivity of the immune system in youth.

To investigate the role of T cells in the RANKL production, we performed a real-time RT-PCR experiment on T cells and monocytes separated from 3 osteoporotic and 3 post-menopausal healthy controls to check for RANKL production; our data demonstrated that T cells produced a greater amount of RANKL as respect to monocytes and that T cells from osteoporotic subjects produced more RANKL than healthy controls (Fig. 4). Furthermore stimulus with PHA increases the amount of RANKL in T cells both in patients and in controls, as expected ($p=0.016$). The percentage of T cells in PBMC range between 58 and 63% without significant differences between patients and controls.

**T cells are pivotal for OC formation**

To further investigate the effects of T cells on OC formation in vitro, we examined the capacity of cultures of monocytes to form OCs in the presence and in the absence of T cells. The number of OCs formed in the monocyte plus T cell co-cultures was the same as in the un-fractioned PBMCs, while the absence of T cells suppressed OC formation in cultures without the addition of exogenous growth factors. The addition of a permissive amount of M-CSF and RANKL in the cultures of monocytes and of monocytes plus B cells restored osteoclastogenesis (Fig. 6). These findings suggest that T cells are pivotal in OC formation through the production of osteoclastogenic cytokines. This datum is also supported by dose response studies conducted by adding increasing amounts of neutralizing antibodies directed against TNFα and RANKL. These experiments revealed that neutralization of both TNFα and RANKL reduces OC formation in a dose dependent fashion (Fig. 7).

Analysis by linear regression showed that the number of OCs formed in the absence of exogenous growth factors was 100% ($R^2=1$) predicted by the circulating precursor percentage, the precursors’ degree of maturation and the RANKL level. BMD levels were 94% ($R^2=0.94$) predicted by the RANKL production (Table 3).

**Discussion**

Although estrogen deficiency is known to induce bone loss through various mechanisms, to our knowledge, this report is the first to demonstrate the contribution of T cells to cytokine-driven osteoclastogenesis in post-menopausal osteoporosis. We found menopause to increase the number of OC precursors in the PBMC and the ability of T cells to produce pro-osteoclastogenic cytokines. Estrogen deficiency leads to an increase in OC formation more relevant in patients affected by osteoporosis. Menopause stimulates osteoclastogenesis through increased production of RANKL and TNF by monocytes and T cells and this phenomenon is more relevant in osteoporotic women. Our findings confirm the result of an earlier report from our laboratory [22] and demonstrate that T cells play a pivotal role in the stimulation of osteoclastogenesis induced by post-menopausal osteoporosis in humans. Thus, this study validates in humans earlier findings in ovariectomy mice.

A relevant finding of our study is that PBMC from healthy post-menopausal women produce fewer OCs than those from osteoporotic subjects, but only in the absence of exogenous cytokines. In fact, the addition of permissive doses of M-CSF and RANKL induces the formation of a similar number of OCs in cultures from all groups, suggesting that estrogen deficiency does not increase the capacity of OC precursors to differentiate into mature OCs, but rather increases the levels of osteoclastogenic cytokines in the bone microenvironment. Here we show that mature and active osteoclasts can be differentiated from PBMC cultures and that their activity is significantly greater in patients with post-menopausal osteoporosis compared with post-menopausal and pre-menopausal healthy controls, and that enhanced osteoclast formation is largely dependent on the greater ability of PBMC from such patients to produce pro-osteoclastogenic cytokines (TNFα and RANKL). It has been demonstrated that circulating osteoclast precursors exist primarily within the monocytic fraction of peripheral blood, [19,28–32] and their presence in the circulation serves both as a reservoir for replenishing pre-osteoclast populations in the bone marrow as needed and as a potentially abundant source of pre-osteoclasts that can be recruited into bone or joint tissue in response to reparative or pathological signals. In contrast to the beneficial nature of pre-osteoclast recruitment during normal bone remodelling or fracture repair, excessive pre-osteoclast recruitment in pathological conditions causes significant bone loss in many skeletal disorders characterized by increased osteoclast formation and activity [35–37]. Several studies have identified the surface markers that define the phenotype of monocytes with osteoclastogenic potentials [19,28,29,31,38]. Analysis of the MFI of these markers by flow cytometry can be used as an indirect indicator of greater precursor differentiation in the osteoporotic patients. Here we demonstrate that there is an increased number of mature circulating OC precursors in osteoporotic patients as respect to controls.

Furthermore, we found that pre-menopausal controls had a larger expression of CD14 on cellular surface, as compared to menopausal controls and to patients, and that CD14 MFI was inversely correlated with age. This points to greater reactivity of the immune system in youth, as suggested by the greater TNFα expression induced by PHA stimulation of young women CD3+ cells, whereas it had little effect in healthy post-menopausal women.

Our findings suggest the hypothesis that estrogen deficiency reduces the ability of T cells to answer to immune stimulation both in healthy and in an osteoporotic subject, whilst in patients T cells are already activated to a greater degree at baseline. Greater spontaneous and PHA stimulated TNFα and RANKL production by monocytes, and even more so by T cells in samples from patients suggest that estrogen deficiency stimulates OC formation through TNF and RANKL. T cells are a key source of these cytokines not only because a significant fraction of TNF and RANKL producing cells are T cells, but also because in vitro OC formation was impeded by the absence of T cells and restored by the addition of exogenous cytokines. It has been suggested that T cells survive in PBMC cultures and are able to prolong osteoclast viability in cultures until 35 days in myeloma patients [17].

Our data on the role of T cells and of the production of TNFα in culture confirm recent data obtained both in humans, in vivo and in vitro [5,16–19], and in the animal model [12]. The present study demonstrates an increase in the production of two potent pro-osteoclastogenic cytokines, TNFα and RANKL, by PBMC cultures from patients as compared to controls. These findings may provide an explanation of the increased osteoclastogenesis characteristic of women with osteoporosis. In post-menopausal women we observed a greater production of pro-osteoclastogenic cytokines as compared to pre-menopausal subjects, in confirmation of the previous data in the literature which established a relationship between estrogen deficiency and the greater production of pro-inflammatory cytokines [39–41]. These findings are consistent with the result of the antibody neutralization experiments which show that silencing of TNF and RANKL blocks OC formation and with the linear regression model that suggests the MFI of the VNR, the percentage of osteoclast precursors and the RANKL levels in the supernatant are able to predict the formation of osteoclast in vitro.

The level of TNF is greater in patients than in controls also in the serum, this datum confirms that there is a higher production of this cytokine also in vivo. Total s-RANKL was not significantly different in patients and controls even if higher in patients. The lack of statistical
significance could be due to the small cohort studied and to the high individual variability. Serum OPG were higher in patients than in controls: this datum confirms previous studies by Yano et al [26] in post-menopausal osteoporosis and by Oh et al in males [34]. In conclusion, the present study suggests that estrogen deficiency up-regulates osteoclastogenesis through the production of the pro-osteoclastogenic cytokine TNF alpha and RANKL. It confirms previous reports demonstrating that osteoclastogenesis is greater after the menopause in women suffering from post-menopausal osteoporosis compared with controls matched for menopausal period. It also suggests that the factors predictive of osteoclastogenesis are to be sought in both the greater marrow output of precursors committed in the osteoclast direction and in the cytokine production, and in particular of RANKL and TNF. Furthermore, it suggests a fundamental role for T lymphocytes in post-menopausal bone loss and in the induction of osteoclastogenesis in humans.

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