

The FASEB Journal express article 10.1096/fj.04-2214fje. Published online December 20, 2004.

Spontaneous osteoclast formation from peripheral blood mononuclear cells in postmenopausal osteoporosis

Patrizia D'Amelio,* Anastasia Grimaldi,* Gian Piero Pescarmona,† Cristina Tamone,*
Ilaria Roato,‡ and Giancarlo Isaia*

*Department of Internal Medicine, †Department of Genetics, Biology and Biochemistry
Research, and ‡Department of Medical Oncology Center on Experimental Medicine (CeRMS),
University of Torino, Italy

Corresponding author: Dr. Patrizia D'Amelio, Dipartimento di Medicina Interna, Corso Dogliotti
14, 10126 Torino, Italy. E-mail: patriziadamelio@netscape.net

ABSTRACT

Osteoclasts are cells involved in bone reabsorbing and hence in postmenopausal bone loss. There is no evidence of increased in vitro spontaneous osteoclast formation in postmenopausal osteoporosis. The aim of our study was to evaluate spontaneous osteoclastogenesis in osteoporosis. Bone mineral density, markers of bone turnover, and cultures of peripheral blood mononuclear cells (PBMC) on dentine slices with or without the addition of 1,25-OH vitamin D₃ ($[10^{-8}$ M]) were obtained from 18 osteoporotic women and 15 controls. To verify cytokine production by PBMC cultures, supernatants were collected on days 3 and 6 and tested for TNF- α and RANKL. The data obtained were compared between patients and controls by one-way ANOVA and correlated by Pearson's coefficient. We found a significant increase in osteoclast formation and bone reabsorbing activity in patients with respect to controls; in addition, the production of TNF- α and RANKL is significantly higher in patients. Furthermore, osteoclast number is inversely correlated with bone mineral density and directly with RANKL in culture supernatants. Our data demonstrated an increased spontaneous osteoclastogenesis in women affected by postmenopausal osteoporosis: this increase may be explained by the higher production of TNF- α and RANKL by PBMC cultures of osteoporotic patients.

Key words: TNF- α • RANKL • cytokines

Postmenopausal osteoporosis is a common disorder characterized by decreased bone density and increased fracture risk (1); an imbalance between bone formation and bone resorption is thought to underlie the pathogenesis of reduced bone mass in osteoporosis. Bone resorption is carried out by osteoclasts, which are multinucleated cells formed by the fusion of marrow-derived cells. Some studies demonstrate the presence of an osteoclast precursor in peripheral blood in the mononuclear cell fraction (2–5) and show that it is possible to obtain in vitro mature osteoclasts from the culture of peripheral blood mononuclear cells (PBMC; refs 4–5); in particular, it has been demonstrated that osteoclasts derive from the CD 14+ fraction (4, 6). Osteoclast-stimulating factors such as M-CSF, RANKL, and/or TNF- α are usually added to the medium in previous studies.

In recent years, many studies have been carried out to elucidate the role of cytokines in the pathogenesis of postmenopausal osteoporosis and, hence, their role in stimulating or inhibiting osteoclasts formation. Plenty of data show the important role played in bone demineralization after menopause by cytokines, which are also active in the inflammation and regulation of the immune system; the most important of these factors in bone demineralization seems to be IL-1 β , IL-6, RANKL, and TNF- α (7–10). In our previous study on animal models, we demonstrated that after ovariectomy the production of IL-1 β and TNF- α significantly increases (7). An increased cytokine production in supernatants of PBMC cultures after menopause has also been demonstrated (11–13), with an increase in bone resorbing activity in these cultures (13); furthermore, a recent study of ex vivo cultures of bone marrow demonstrates an increase in RANKL expression in mononuclear cells of bone marrow in postmenopausal women as compared with premenopausal women and with postmenopausal women receiving hormone replacement therapy and show that RANKL expression is directly correlated with markers of bone resorption (14). However, there are no data on the direct effect of osteoporosis on the production of RANKL in PBMC cultures and on the direct effect observed on osteoclast formation in human PBMC cultures.

The aim of the present study was to evaluate a possible increase in spontaneous (i.e., without the addition of M-CSF, TNF- α , or RANKL in the medium) in vitro osteoclast formation in postmenopausal osteoporotic women with respect to healthy subjects (age, sex, and body mass index (BMI) match) and to assess the sensitivity of the osteoclast precursors to 1,25-OH vitamin D₃ in patients with respect to controls. Furthermore, we investigated the relationship between osteoclastogenesis and bone mineral density (BMD) and the common markers of bone turnover used in clinical practice. We also analyzed the role of RANKL and TNF- α in in vitro osteoclast formation.

MATERIALS AND METHODS

Patients and markers of bone turnover

Eighteen patients and 15 controls after at least 1 yr of spontaneous menopause were enrolled in the study. The subjects had not assumed drugs active on bone metabolism for at least 6 months; patients assuming calcium and vitamin D, thyroid hormones, corticosteroids, estrogen, bisphosphonates, and raloxifene were excluded.

We considered osteoporotic those patients with a BMD T-score value of -2.5 SD or less, according to the World Health Organization (15). BMD was measured by double emission X-ray absorptiometry (DXA) by means of a Hologic QDR 4500. The presence of secondary osteoporosis was excluded by anamnesis, physical examination, and common exams such as calcemia, phosphoremia, bone alkaline phosphatase (BAP), and 25-OH vitamin D. In all the subjects, we measured serum osteocalcin (BGP, using an RIA technique with DiaSorin, Saluggia, Italy) and urinary cross laps (using α -Cross LapsTM RIA by Osteometer Biotech A/S Copenhagen, Denmark) as markers of bone metabolism.

The controls (densitometric T-score >-1 SD at lumbar and femoral neck) were recruited among healthy women in menopause for at least 1 yr and age and BMI matched to the patients; in addition, in the controls the same blood and urinary exams were performed.

Cells isolation and cultures

In all the subjects, we obtained circulating mononuclear cells from peripheral blood (PBMC). The PBMC were obtained with the Ficoll-Paque method, as described previously in the literature (5) from 20 ml of peripheral blood in lithium heparin (LH).

All cell incubations were performed in triplicate in 96-well/plates (2×10^5 cells/well) using α -minimal essential medium (α -MEM, supplied by GIBCO) supplemented with 10% fetal bovine serum (FBS), benzyl penicillin (100 IU/ml), and streptomycin (100 μ g/ml) or in the above-mentioned medium plus 1,25-OH vitamin D₃ ($[10^{-8}$ M] dissolved in ethanol and used <0.1% in the medium). RPMI (supplied by GIBCO) was used for cell isolation. All cell cultures were maintained at 37°C in a humidified atmosphere 5% CO₂. To evaluate functional activity of osteoclasts, the same cultures were plated in triplicate on dentin slices (supplied by Pantec; 1×10^6 cells/well) with or without the addition of 1,25-OH vitamin D₃ ($[10^{-8}$ M]).

To evaluate possible differences in osteoclast formation with M-CSF and RANKL in our cultures from five patients and five controls, cultures with the above-mentioned medium and the addition of M-CSF (25 ng/ml) and RANKL (30 ng/ml) were also obtained.

Cell viability assay

To exclude possible bias in the number of osteoclasts due to different cell viability between patients and controls, cell viability was measured by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. PBMC were cultured in 96-well/plates in the presence or absence of M-CSF (25 ng/ml) and RANKL (30 ng/ml) and with or without the addition of 1,25-OH vitamin D₃ ($[10^{-8}$ M]). At days 10 and 21, 200 μ l/well of MTT 0.5 mg/ml were added, followed by 4 h incubation at 37°C in humidified 5% CO₂ atmosphere. The reaction was stopped by the addition of 150 μ l of 0.04 N HCl in absolute isopropanol. The optical density was read at 570 nm using an automatic plate reader (Automatic Microtiter Reader, Bio-Rad).

Osteoclast formation and activity

The cells were fed every 3 days, and supernatants were collected after 3 and 6 days of culture. On the 10th day, the cells were fixed and stained for Tartrate-resistant acid phosphatase (TRAP; acid phosphatase, leukocyte staining kit, Sigma Diagnostics, St. Louis, MO) and stained immunohistochemically by an indirect immunoperoxidase technique for the expression of vitronectin receptor (VNR; antibody for VNR was 12030 by Serotech).

To quantify the formation of TRAP⁺ and VNR⁺ multinucleated (>3 nuclei) cells, the number of stained cells in each well was counted and identified as osteoclast by the same operator for all the plates (5, 7). The count was in blind with respect to health status of the patients.

On the 21st day of culture, dentine slices were removed from the wells, rinsed in PBS, and placed in 0.25% trypsin for 15 min; they were then washed in distilled water and left overnight in 0.25 M ammonium hydroxide. The slices were then washed in distilled water and stained with 0.5% (w/v) toluidine blue. To avoid aspecific staining, the dentine slices were left in 2 N NaOH for a few seconds and examined by a light microscope. The total surface of each dentine slice was

inspected, and the resorption areas were photographed with a digital camera (Nikon Coolpix). The extent of lacunar resorption was determined by analyzing each micrograph with a computer image analysis system (Image J, 1.3, Wayne Rasband National Institutes of Health, available at free domain <http://rsb.info.nih.gov/ij/>) and was expressed as the total percentage of surface area reabsorbed.

Cytokine measurement

In all the subjects, the supernatant level of TNF- α (Quantikine; R&D Systems, Minneapolis, MN) and RANKL (Biomedica; Biomedica Medizinprodukte GmbH and Co. KGA) was measured with the ELISA method.

Statistical analyses

The statistics were performed using SPSS 8.0 for Windows, and in particular patients and controls were compared for age, postmenopausal period, BMI, markers of bone metabolism, levels of TNF- α and RANKL in supernatants, number of osteoclasts for 10^5 cells, and percentage of lacunar resorption using one-way ANOVA.

The cell viability, the number of osteoclasts, the percentage of bone resorption, and the level of cytokines in culture supernatants with or without addition of 1,25-OH vitamin D₃ ($[10^{-8}$ M]) and of M-CSF and RANKL were compared by means of paired Student's *t* test.

The correlations between age, postmenopausal period, BMD, markers of bone metabolism, levels of measured cytokines, and the number of osteoclasts for 10^5 cells were carried out by Pearson's coefficient correlation.

To evaluate the possible predictors of the number of osteoclasts, we applied a linear regression model with stepwise analyses for variables significantly different among patients and controls.

In all the statistical analyses performed, the result was considered significant if the *P* value was <0.05.

RESULTS

Patients and markers of bone turnover

The comparison between patients and controls for age, postmenopausal period, BMI, and markers of bone metabolism, demonstrated a significant increase in the level of urinary cross laps in patients with respect to controls ([Table 1](#)).

Cell viability assay

Statistical analyses demonstrate no differences between patients and controls in cell viability assay when patients and controls are compared in the presence or absence of M-CSF (25 ng/ml) and RANKL (30 ng/ml) and with or without the addition of 1,25-OH vitamin D₃ ($[10^{-8}$ M]) at days 10 and 21 ([Fig. 1](#)).

Osteoclast formation and activity

By the TRAP and VNR staining on the 10th day of culture, it was possible to detect the formation of large multinucleated TRAP and VNR positive cells, which are defined as osteoclasts according to previous literature (3-6, 15; [Fig. 2](#)).

In the patients, the average number of osteoclasts was 48.23 ± 25 (without 1,25-OH vitamin D₃) and 28.5 ± 24.6 (with 1,25-OH vitamin D₃) per 10⁵ cells ($P=0.000$). In the controls, the number of osteoclasts was 10.41 ± 13 (without 1,25-OH vitamin D₃) and 4.4 ± 6.6 (with 1,25-OH vitamin D₃) per 10⁵ cells ($P=NS$). The reduction in osteoclast number, considering patients and controls together, in wells treated with 1,25-OH vitamin D₃ is significant as shown by the paired Student's *t* test ($P=0.01$). The percentage of lacunar resorption area ([Fig. 3](#)) calculated on dentine slices was significantly higher in patients with respect to controls ([Table 1](#)). The percentage of lacunar resorption area is significantly higher in the wells treated with 1,25-OH vitamin D₃ with respect to wells without the addition of 1,25-OH vitamin D₃ ($P=0.001$). Regarding the number of osteoclasts obtained in the cultures with the addition of M-CSF and RANKL, we observed a significant increase in osteoclast number and in percentage of lacunar resorption in controls, although we did not observe significant differences after the addition of these factors in patients ([Table 2](#)); furthermore, there were no differences between patients and controls and between wells treated or not treated with 1,25-OH vitamin D₃ in both the number of osteoclast and the percentage of lacunar resorption in presence of M-CSF and RANKL.

Cytokines

Levels of TNF- α and RANKL are significantly higher in patients with respect to controls in both samples with and those without the addition of 1,25-OH vitamin D₃ ([Table 1](#)).

Our data showed significant inverse correlations between BMD measured at lumbar spine and femoral neck with number of osteoclasts, level of RANKL, and TNF- α ; regarding the markers of bone metabolism there was a significant inverse correlation between the level of urinary cross laps and lumbar BMD, while the correlation between level of urinary cross laps, RANKL, and TNF- α is direct ([Table 3](#)). The stepwise regression demonstrated that lumbar BMD is a predictor of the number of osteoclasts formed without addition of 1,25-OH vitamin D₃ ($r^2=0.56$), while RANKL alone is a predictor of the number of osteoclasts formed with the addition of 1,25-OH vitamin D₃ ($r^2=0.63$; [Table 4](#)).

DISCUSSION

To our knowledge, there are no data in the literature that correlate clinical features with in vitro osteoclast formation and with the level of cytokines in PBMC cultures in postmenopausal osteoporosis. In the present study, we analyzed the relationship between osteoclast formation and clinical parameters such as BMD and common markers of bone metabolism: BGP, BAP, and cross laps and their relationship with the cytokines most involved in osteoclast formation, i.e., TNF- α and RANKL.

From a methodological point of view, we decided to culture PBMC without the addition of stimulating cytokines such as M-CSF and without using stromal cells to test the spontaneous

ability of PBMC of osteoporotic patients to generate osteoclasts. Considering that osteoclasts derived from CD14⁺ cells (2, 4, 6), the use of PBMC allows coculture of lymphocytes and monocytes and a previous study (8) demonstrated the possibility of obtaining osteoclasts generation in cocultures of lymphocytes and monocytes. It has recently been demonstrated that TNF- α and IL-1- α are able to induce osteoclasts formation from PBMC (9), and these cytokines predominantly derive from monocytes and macrophages (10); in addition, T lymphocytes produce several pleiotropic cytokines (17), many of which are capable of regulating osteoclasts differentiation and function (7, 8, 18).

Our data, in contrast with a recent study (19), demonstrate an increase in osteoclast formation in postmenopausal osteoporotic women as compared with healthy controls, age, sex, and BMI matched. The above-mentioned study by Jevon et al. (19) demonstrates an increased osteoclast activity in osteoporotic patients with respect to controls, without any increase in the number of osteoclasts formed. It is important to point out that the data by Jevon and et al. are obtained in cultures of PBMC with addition of M-CSF and RANKL; it is our opinion that the addition of exogenous cytokines could alter the endogenous differences in production of these cytokines in patients with respect to controls and consequently a possible increase in spontaneous osteoclastogenesis in osteoporotic patients. To reinforce this hypothesis, it is interesting to note that our experiments in analogous condition (i.e., in the presence of M-CSF and RANKL) confirm the data of the study by Jevon et al. Furthermore, the addition of M-CSF and RANKL to the PBMC cultures from osteoporotic patients did not significantly change the number of osteoclasts, compared with the parallel unstimulated cultures. However, the above cytokines were essential in triggering osteoclastogenesis of PBMC from patients without osteoporosis. These results are consistent with the findings of increased TNF- α and RANKL endogenous production in PBMC cultures of osteoporotic women with respect to controls. Similar data were recently found in multiple myeloma patients with osteolysis (20).

Our data on cytokine production could explain the increase in osteoclast formation and function in PBMC cultures. An increase in TNF- α and M-CSF production in PBMC cultures of women after oophorectomy has previously been demonstrated, with a consequent increase in markers of bone turnover (11, 12). Also, an increase in levels of TNF- α and in bone resorbing activity in cultures of PBMC of postmenopausal women as compared with premenopausal has been previously demonstrated (13).

We evaluated osteoclast formation in in vitro culture of PBMC adding only 1,25-OH vitamin D₃ ($[10^{-8}$ M]) to determine a possible difference in the sensitivity of osteoclast precursors to this metabolite in patients and controls, and we found a significant reduction in osteoclast formation considering patients and controls adding 1,25-OH vitamin D₃, while there is a significant increase in osteoclast reabsorbing activity. There is plenty of literature on the use of 1,25-OH vitamin D₃ in culture of PBMC with different results: some authors (21) showed an increased osteoclast formation in PBMC treated with 1,25-OH vitamin D₃, while other authors (22) showed a reduction in osteoclast activity adding only 1,25-OH vitamin D₃; this contrast may be due to different methods of culture, as far as some authors use coculture between PBMC and an osteoblast line or addition of cytokines such as M-CSF or RANKL.

Regarding the possible relationship between clinical features and osteoclast formation and activity, it is interesting to note the significant inverse correlations between these parameters and

BMD; these findings confirm our hypothesis that there is an increased spontaneous osteoclastogenesis in osteoporotic women with respect to controls; also, the level of TNF- α and RANKL is inversely related to BMD at lumbar spine and femoral neck, while the correlation between these cytokines and cross laps is direct. These data confirm the role of the production of TNF- α and RANKL in PBMC cultures in determining postmenopausal bone loss by increasing osteoclast number and activity. The linear regression models reinforce this hypothesis, lumbar BMD being a predictor of the number of osteoclasts; the lumbar BMD explained ~60% of spontaneous osteoclastogenesis in wells not treated with vitamin D, while in wells treated with vitamin D, the level of RANKL on day 6 explains ~60% of osteoclastogenesis; these data could suggest a mechanism of action of vitamin D RANKL dependent.

In conclusion, our data demonstrate that 1) spontaneous osteoclast formation and activity are increased in osteoporotic patients with respect to healthy controls. 2) There is no difference in PBMC sensitivity to 1,25-OH vitamin D₃ in osteoporotic patients compared with controls and vitamin D in vitro acted as a promoter of osteoclast activity. 3) 60% of the osteoclast formation in the presence of 1,25-OH vitamin D₃ is explained by the level of RANKL; these data could suggest a vitamin D RANKL-dependent mechanism of action. 4) The number of osteoclasts is inversely correlated with BMD, and lumbar BMD is a predictor of osteoclast formation. 5) Levels of TNF- α and RANKL are significantly higher in patients than in controls and are inversely correlated with BMD but directly correlated with levels of cross laps.

ACKNOWLEDGMENTS

This work was supported by a grant from Ministry for Universities and Research (MIUR) and a grant support from the Fondazione Internazionale Ricerche Medicina Sperimentale (FIRMS) Compagnia San Paolo. The authors are grateful to T. R. Arnett and C. Costamagna for scientific support.

REFERENCES

1. National Institutes of Health. (1984) Consensus conference: Osteoporosis. *JAMA* **252**, 799-802
2. Shalhoub, V., Elliott, G., Chiu, L., Manoukian, R., Kelley, M., Hawkins, N., Davy, E., Shimamoto, G., Beck, J., Kaufman, S. A., et al. (2000) Characterization of osteoclast precursors in human blood. *Br. J. Haematol.* **2**, 501–512
3. Faust, J., Lacey, D. L., Hunt, P., Burgess, T. L., Scully, S., Van, G., Eli, A., Qian, Y., and Shalhoub, V. (1999) Osteoclast markers accumulate on cells developing from human peripheral blood mononuclear precursors. *J. Cell. Biochem.* **72**, 67–80
4. Massey, H. M., and Flanagan, A. M. (1999) Human osteoclasts derive from CD14-positive monocytes. *Br. J. Haematol.* **106**, 167–170
5. Shih, C., and Bernard, G. W. (1996) Peripheral blood mononuclear cells develop into multinucleated osteoclasts in tissue culture. *Anat. Rec.* **245**, 41–45

6. Nicholson, G. C., Malakellis, M., Collier, F. M., Cameron, P. U., Holloway, W. R., Gough, T. J., Grgorio-King, C., Kirkiland, M. A., and Myers, D. E. (2000) Induction of human osteoclasts from CD14-positive human peripheral blood mononuclear cells by receptor activator nuclear factor κ B ligand (RANKL). *Clin. Sci.* **99**, 133–140
7. Roggia, C., Gao, Y., Cenci, S., Weitzmann, M. N., Toraldo, G., Isaia, G., and Pacifici, R. (2001) Up-regulation of TNF-producing T cells in the bone marrow: a key mechanism by which estrogen deficiency induces bone loss in vivo. *Proc. Natl. Acad. Sci. USA* **98**, 13960–13965
8. Kotake, S., Udagawa, N., Hakoda, M., Mogi, M., Yano, K., Tsuda, E., Takahashi, K., Furuya, I., Ishiyama, S., Kim, K. J., et al. (2001) Activated human T cells directly induce osteoclastogenesis from human monocytes: possible role of T cells in bone destruction rheumatoid arthritis patients *Arthritis Rheum.*, **44**, 1003-1012
9. Kudo, O., Fujikawa, Y., Itonaga, I., Sabokbar, A., Torisu, T., and Athanasou, N. A. (2002) Proinflammatory cytokines (TNF α /IL1 α) induction of human osteoclasts formation *J. Pathol.* **198**, 220-227
10. Mundy, G. R. (1993) Cytokines of bone. In: *Physiology and Pharmacology of Bone* (Mundy, G. R., and Martin, T. J. eds) pp. 185-214, New York, Springer Berlin
11. Pacifici, R., Brown, C., Puscheck, E., Friedrich, E., Slatopolsky, E., Maggio, D., McCracken, R., and Avioli, L. V. (1991) Effect of surgical menopause and estrogen replacement on cytokine release from human blood mononuclear cells. *Proc. Natl. Acad. Sci. USA* **88**, 5134–5138
12. Bernard-Poenaru, O., Roux, C., Blanque, R., Gardner, C., de Vernejoul, M. C., and Cohen-Solal, M. E. (2001) Bone-resorbing cytokines from peripheral blood mononuclear cells after hormone replacement therapy: a longitudinal study. *Osteopor Int* **12**, 769–776
13. Cohen-Solal, M. E., Boitte, F., Bernard-Poenaru, O., Denne, M. A., Graulet, A. M., Brazier, M., and de Vernejoul, M. C. (1998) Increasing bone resorbing activity of peripheral monocyte culture supernatants in elderly women. *J. Clin. Endocrinol. Metab.* **83**, 1687–1690
14. Eghbali-Fatourehchi, G., Khosla, S., Sanyal, A., Boyle, W., Lacey, D. L., and Riggs, B. L. (2003) Role of RANK ligand in mediating increased bone resorption in early postmenopausal women. *J. Clin. Invest.* **111**, 1221–1230
15. World Health Organization. (1994) Assessment of fracture risk and the application to screening for postmenopausal osteoporosis. In *WHO Technical Report Series No. 843*, WHO, Geneva
16. Horton, M. A., Lewis, D., McNulty, K., Pringle, J. A., and Chambers, T. J. (1985) Monoclonal antibodies to osteoclastomas (giant cell bone tumors): definition of osteoclasts-specific cellular antigens. *Cancer Res.* **45**, 5663–5669

17. Salgame, P., Abrams, J. S., Clayberger, C., Convit, J., Modlin, R. L., and Bloom, B. R. (1991) Differing lymphokine profiles of functional subsets of human CD4 and CD8 T cell clones. *Science* **254**, 279–282
18. Romas, E., and Martin, T. J. (1997) Cytokines in the pathogenesis of osteoporosis *Osteopor. Int.*, **7**, S47-S53
19. Jevon, M., Hirayama, T., Brown, M. A., Wass, J. A. H., Sabokar, A., Ostelere, S., and Athanasou, N. A. (2003) Osteoclast formation from circulating precursors in osteoporosis. *Scand. J. Rheumatol.* **32**, 95–100
20. Colucci, S., Brunetti, G., Rizzi, R., Zonno, A., Mori, G., Colaianni, G., Del Prete, D., Faccio, R., Liso, A., Capalbo, S., et al. (2004) T cells support osteoclastogenesis in an in vitro model derived from human multiple myeloma bone disease: the role of the OPG/TRAIL interaction *Blood* **104**, 3722-3730
21. Itonaga, I., Sabokbar, A., Neale, S. D., and Athanasou, N. A. (1999) 1,25-Dihydroxyvitamin D(3) and prostaglandin E(2) act directly on circulating human osteoclast precursors. *Biochem. Biophys. Res. Commun.* **264**, 590–595
22. Li, B., and Yu, S. (2003) Genistein prevents bone resorption diseases by inhibiting bone resorption and stimulating bone formation. *Biol. Pharm. Bull.* **26**, 780–786

Received June 11, 2004; accepted November 8, 2004.

Table 1

Results of ANOVA with means \pm SD for age markers of bone metabolism, levels of TNF- α , RANKL, number of OC for 10^5 cells, and percentage of bone resorption either treated with 1,25-OH vitamin D₃ or not

	Patients	Controls	P
Number of OC without vitD₃	48.23 \pm 25	10.41 \pm 13	0.000
Number of OC with vitD₃	28.5 \pm 24.6	4.4 \pm 6.6	0.001
Percentage of bone resorption without vitD₃	9.5 \pm 8.5	0.85 \pm 5.6	0.049
Percentage of bone resorption with vitD₃	11.2 \pm 8.22	0.93 \pm 0.52	0.024
Cross laps (μg/l)	752 \pm 311.3	311 \pm 247.7	0.016
TNF α (pg/ml) without vitamin D day 3	53 \pm 16.3	32.9 \pm 6.6	0.000
TNF α (pg/ml) without vitamin D day 6	31.9 \pm 8.2	33.9 \pm 9.5	NS
TNF α (pg/ml) with vitamin D day 3	53 \pm 16.3	38.1 \pm 8.2	0.003
TNF α (pg/ml) with vitamin D day 6	36.12 \pm 13.6	25.1 \pm 7.2	0.008
RANKL (pg/ml) without vitamin D day 3	2 \pm 1.4	0.14 \pm 0.2	0.000
RANKL (pg/ml) without vitamin D day 6	2.4 \pm 1.2	0.4 \pm 0.6	0.000
RANKL (pg/ml) with vitamin D day 3	2 \pm 0.12	0.012 \pm 0.4	0.000
RANKL (pg/ml) with vitamin D day 6	2.2 \pm 0.4	0.16 \pm 0.4	0.000

BMI, body mass index; OC, osteoclasts.

Table 2

Results of ANOVA with means±SD for number of OC for 10^5 cells and percentage of lacunar resorption in presence or absence of M-CSF (25 ng/ml) and RANKL (30 ng/ml) and of 1,25-OH vitamin D₃ [10^{-8} M] or not.

	Ustimulated	Stimulated	P
Number of OC without vitD₃ in patients	45.5±13.4	50.20±20	NS
Number of OC with vitD₃ in patients	35.5±24.6	40.4±20	NS
Number of OC without vitD₃ in controls	15.1±10.5	40.8±18	0.001
Number of OC with vitD₃ in controls	8.4±5.4	45.6±20.6	0.003
Percentage of bone resorption without vitD₃ in patients	8.5±7.5	10.5±8.3	NS
Percentage of bone resorption with vitD₃ in patients	10.3±8.5	10.7±7.6	NS
Percentage of bone resorption without vitD₃ in controls	0.78±4.5	3.82±1.9	0.016
Percentage of bone resorption with vitD₃ in controls	1.2±0.48	5.44±3.2	0.026

OC, osteoclasts.

Table 3

Pearson's coefficient correlation between number of OC treated with vitamin D₃ or not (expressed as osteoclasts for 10⁵ cells) age, lumbar and femoral neck BMD, BGP, cross laps, TNF- α , and RANKL

	Number of OC without vitD ₃		Number of OC with vitD ₃		BMD L (g/cm ²)		BMD FN (g/cm ²)		Cross laps	
	<i>r</i>	<i>P</i>	<i>r</i>	<i>P</i>	<i>r</i>	<i>P</i>	<i>r</i>	<i>P</i>	<i>r</i>	<i>P</i>
Number of OC without vitD ₃	-	-	0.5	0.005	-0.6	0.0001	-0.5	0.01	0.2	NS
BMD L (g/cm ²)	-0.6	0.001	-0.6	0.000	-	-	0.73	0.000	-0.5	0.01
BMD FN (g/cm ²)	-0.5	0.01	-0.37	0.048	0.73	0.000	-	-	0.02	NS
TNF- α without vitamin D day 3	0.3	NS	0.3	NS	-0.6	0.000	-0.2	NS	0.48	0.006
TNF- α (pg/ml) without vitamin D day 6	0.02	NS	0.06	NS	0.14	NS	0.3	NS	0.03	NS
TNF- α (pg/ml) with vitamin D day 3	0.4	0.026	0.4	0.048	-0.65	0.000	-0.3	NS	0.55	0.002
TNF- α (pg/ml) with vitamin D day 6	0.4	0.034	0.4	0.016	-0.6	0.000	-0.2	NS	0.34	0.063NS
RANKL (pg/ml) without vitamin D day 3	0.3	NS	0.3	NS	-0.6	0.001	-0.5	0.005	0.42	0.019
RANKL (pg/ml) without vitamin D day 6	0.4	NS	0.3	NS	-0.77	0.000	-0.65	0.000	0.21	NS
RANKL (pg/ml) with vitamin D day 3	0.36	0.048	0.44	0.014	-0.63	0.000	-0.47	0.007	0.43	0.019
RANKL (pg/ml) with vitamin D day 6	0.4	0.025	0.45	0.011	-0.81	0.000	-0.72	0.000	0.3	NS

OC, osteoclasts.

Table 4

Stepwise linear regression models for OC with and without vitamin D

OC without vitamin D	Beta	Standard Error	<i>t</i>	<i>P</i>
BMD lumbar (g/cm²)	-90.7	27.1	-0.564	0.003
OC with vitamin D	Beta	Standard Error	t	<i>P</i>
RANKL (pg/ml) with vitamin D day 6	332.5	86	0.63	0.001

OC, osteoclasts.

Fig. 1

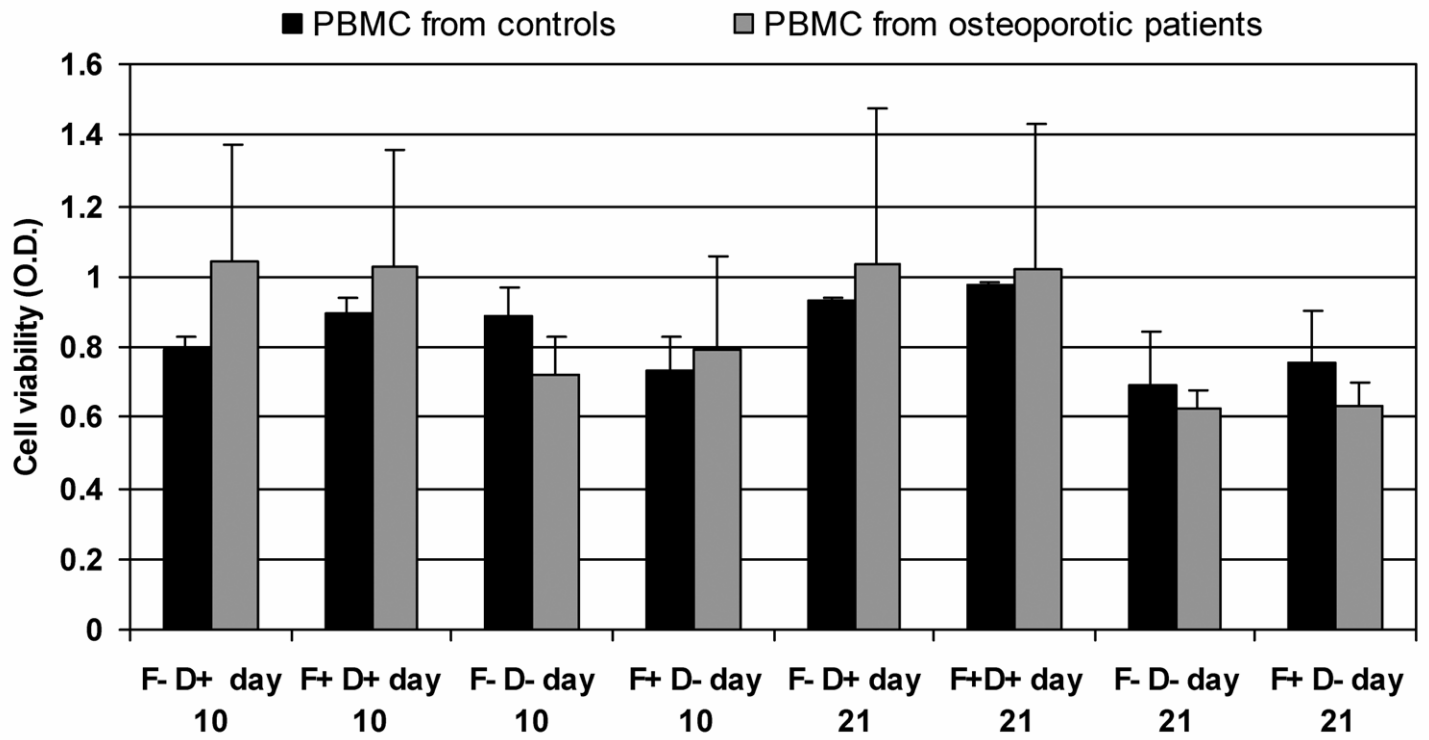


Figure 1. Graph shows cell viability assay (optical density: means \pm SD). The comparison between patients and controls in presence (F+) or absence (F-) of M-CSF and RANKL, and in presence (D+) or absence (D-) of vitamin D is not significant ($P > 0.05$).

Fig. 2

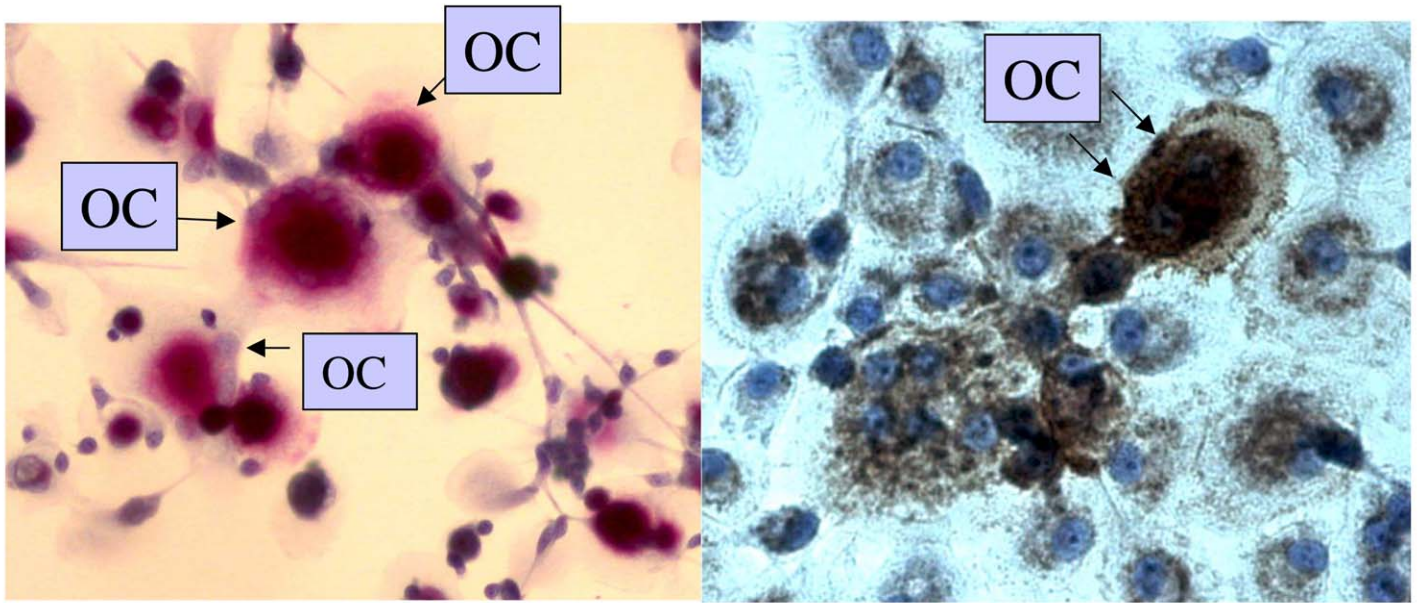


Figure 2. Photographs show osteoclasts (indicated by arrows) VNR staining ($\times 40$) on the right and TRAP staining on left ($\times 40$).

Fig. 3

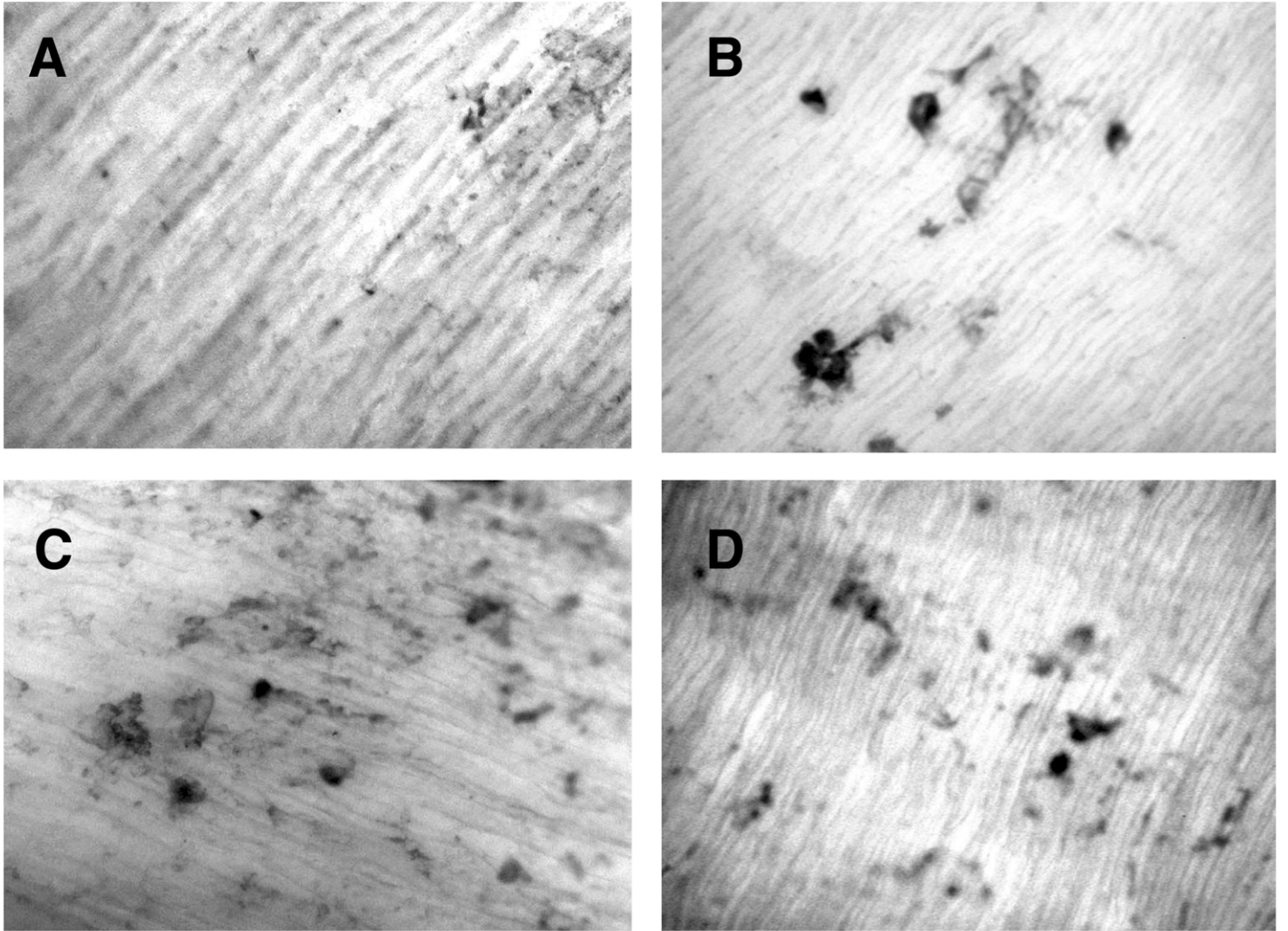


Figure 3. Micrographs showing dentine slices of control in absence (*A*) or presence (*B*) of M-CSF and RANKL and osteoporotic patient in absence (*C*) or presence (*D*) of M-CSF and RANKL.