Journal of Steroid Biochemistry & Molecular Biology 113 (2009) 46–51

Contents lists available at ScienceDirect

Journal of Steroid Biochemistry and Molecular Biology

journal homepage: www.elsevier.com/locate/jsbmb

RANKL/OPG in primary cultures of osteoblasts from post-menopausal women. Differences between osteoporotic hip fractures and osteoarthritis

Mercè Giner[a,*], Mª José Rios[b], Mª José Montoya[b], Mª Angeles Vázquez[b], Latifa Naji[a], Ramón Pérez-Cano[a,b]

[a] Bone Metabolism Unit, Department of Internal Medicine, University Hospital "Virgen Macarena", Av. Dr. Fedriani s/n, 41018 Seville, Spain
[b] Department of Medicine, University of Seville, Seville, Spain

ARTICLE INFO

Article history:
Received 11 March 2008
Received in revised form 28 October 2008
Accepted 18 November 2008

Keywords:
Human osteoblast culture
Osteoporosis
OPG/RANKL
17-Beta-estradiol
1,25-Dihydroxyvitamin D

ABSTRACT

The OPG/RANKL/RANK system is important in the balance between bone formation and resorption.

We used primary human osteoblasts (hOBs) cells to examine the impact of 17-β-estradiol (E2) or/and 1,25-dihydroxyvitamin D (1,25D) in OPG/RANKL system in 28 post-menopausal (PM) women; (a) with hip fracture (OP) or (b) with osteoarthritis (OA). The hOB from OP patients proliferated slower during the first stage, than the OA women (31.5 ± 2.6 and 21.4 ± 1.3 days, respectively, $p < 0.05$). The OP group secreted significantly higher OPG protein levels than the OA women (10.1 ± 2.6 and 4.4 ± 0.8 pmol/L, respectively, $p < 0.05$). The 1,25D and 1,25D+E2 induce an increase in RANKL and RANKL/OPG mRNA expression in OP patients above 200% ($p < 0.05$).

HOBs from the osteoporotic hip initially proliferate slower but after reaching the first cellular confluence, the proliferation rate is equal in both groups. Furthermore, hOBs from hips with OP present a higher protein secretion of OPG, and higher RANKL and RANKL/OPG expression ratio in response to 1,25D and 1,25D+E2, than hOBs from OA women. All this could suggest that the greater bone loss that characterizes OP patients can be mediated due to differences in the secretion and expression of the RANKL/OPG system in response to different stimuli.

1. Introduction

Osteoporosis and osteoarthritis (OA) are two chronic age-related disorders of the skeleton and articular joints, but they are rarely present together in the same patients [1]. Inversely related alterations have been described in both of them. In OA, bone mineral density (BMD) is increased, there is a reduced bone loss and a tendency towards a reduced risk of femoral neck hip fractures, comparing healthy elderly women. In OP patients, however, there is low BMD, a higher bone loss and increased risk of bone fractures. These bone and fragility differences are caused by an imbalance between bone resorption and formation in both pathologies [2–5].

Bone remodelling is carried out by osteoblast and osteoclast cells. The first one, in addition to being directly responsible for bone formation, also controls bone resorption by regulating the proliferation, differentiation and recruitment of osteoclastic progenitor. The bone remodelling is regulated at the systemic level by sex and calciotropic hormones and, at local level, by a range of growth factors and cytokines. Amongst these, RANKL (receptor activator of NF-κB) is a particularly central and potent physiological inducer of osteoclastogenesis [6]. Interaction through the RANK receptor induces preosteoclast differentiation, increases osteoclast activity, and prolongs osteoclast lifespan [7]. Osteoprotegerin (OPG) is the corresponding decoy receptor, which acts as a competitive inhibitor of RANKL action [8]. Both OPG and RANKL are expressed by osteoblastic cells and bone marrow stromal cells [9] and it is now thought that the final step in the osteoclast regulatory pathway may be determined by the relative ratio of the RANKL/OPG system. Accordingly, an increase in OPG serum levels with age has been described in women with post-menopausal osteoporosis [10]. Most studies that analyze RANKL and OPG expression in osteoblasts are performed in animal cultures, tumor cells or cellular lines; those that analyze these molecules in hOB primary cultures are very scarce and no studies have been carried out that compare results in vitro of hOB patients with OP as opposed to those with OA. Recently, two different groups have studied the gene expression of these molecules from macerate of bone biopsies to compare OP and OA patients, which have reported that mRNA levels of OPG were lower, and RANKL and RANKL/OPG ratio levels were higher

* Corresponding author. Fax: +34 954556617.
E-mail addresses: merce_giner@yahoo.es (M. Giner), mayorios@hotmail.com (M.J. Rios), pmontoyag@inicia.es (M.J. Montoya), AVAZQUEZC@terra.es (M.A. Vázquez), najilatifa@hotmail.com (L. Naji), rpcano@us.es (R. Pérez-Cano).

0960-0760/$ – see front matter © 2008 Elsevier Ltd. All rights reserved.
doi:10.1016/j.jsbmb.2008.11.005

in patients with fractures in respect to those with osteoarthrosis [5,11].

Oestrogen has been reported to stimulate, to inhibit and to have no effect on OPG protein secretion [12,13] and expression mRNA OPG [12–15].

About 1,25D, some authors have published a rise in mRNA RANKL expression and a decrease in mRNA OPG [16–18], while others find an increase in the levels of mRNA OPG and protein secretion in response to 1,25-dihydroxyvitamin D [19].

The aim of the present study was to investigate whether primary human osteoblastic cell cultures derived from patients with osteoporotic hip fractures and those with OA showed differences in the RANKL/OPG system as well as evaluating whether the hormones that modulate the expression of this system, such as oestrogens and 1,25D, induce a different response on the osteoblasts derived from both pathologies.

2. Materials and methods

2.1. Patients and cell culture

The study population consisted of two groups of women. The first was formed by 14 post-menopausal (PM) women with osteoporotic fractures (OP), between 73 and 89 years old and the second by 14 women with OA, between 65 and 88 years old. The criteria for inclusion in both groups were to be aged 60 or over, not to lead a sedentary life, to walk for 1–2 h everyday, to take more than 700 mg/day of calcium (calculated by dietetic questionnaire), not to receive treatment with a negative influence on the bone metabolism. The OP group was formed by PM women with an ostoporotic hip fracture that required arthrosplasty (seven of whom had antecedents of non-traumatic fractures at least one, at ages above 45 years old) and the OA group was formed by PM women with OA, admitted for elective hip arthroplasty and not to have presented an osteoporotic fracture during their life. Exclusion criteria were malignant diseases (unless cured with no signs of relapse over the past 5 years), hyperthyroidism, hyperparathyroidism, osteomalacia, any previous bisphosphonate treatment or systemic steroid treatment or use of vitamin D supplements or current use of hormone replacement therapy, nor any other drugs with negative effects on bone metabolism.

Patients were included in the study in a consecutive manner over a period of 2 years as they were directed to arthroplasty by the Trauma & Orthopedics Department in the University Hospital "Virgen Macarena" of Seville, Spain. The study was approved by the ethical committee and an informed consent was obtained from all the participants in the study.

During the surgical procedure, a femoral osteotomy was performed and the femoral head and neck were removed. Briefly, trabecular bone was cut into pieces (1–2 mm) and thoroughly rinsed with phosphate-buffered saline (PBS) six times. The cells from each donor were examined separately in different experiments, they were seeded into 10-cm^2 plates and cultured in Dubelcco Modified Eagle (DMEM; Gibco Grand Island, NY, USA) containing 4.5 g/L glucose, supplemented with 100 UI/ml of penicillin, and 100 μg/ml of streptomycin and 10% foetal bovine serum (FBS; Gibco), previously inactivated during 1 h at 37 °C. The cultures were incubated at 37 °C in a humidified atmosphere with 5% CO_2. The medium was changed twice a week until confluence was achieved (first stage) (4–6 weeks). Confluent cells were detached by tripsinization, and then 300.000 cells in were sub-cultured in the same medium in six-well plates and tested in the second passage. The number of cells (index of proliferation) was counted with an hematocytometer. Tripan blue was used to screen out dead cells after harvesting. We confirmed osteoblastic phenotype by using alkaline phosphatase staining. In all the cultures, ≥85% of the cells showed intense staining for alkaline phosphatase activity.

2.2. Histochemical detection of ALP

After rinsing monolayer cells with PBS, the cells were fixed in 3.7% formaldehyde and 90% ethanol solution for 2 min and washed in TBS for 10 min. Then, the cells were stained with fast 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium (BCIP/NBT) alkaline phophatase substrate (Amresco, OH, USA) for 10 min at room temperature. The reaction was stopped by removing the substrate solution and washing it out with distilled water.

2.3. Cell treatment with E2 and 1,25D

To assess OPG secretion and expression and RANKL expression, confluent hOB cells were incubated for 24 h in serum free DMEM and then we added different agents during 24 h. The concentration used for each agent is listed as follows: 10-7M 17-β-estradiol (E2; Sigma, St. Louis, MO, USA) and/or 10-8M 1,25-dihydroxyvitamin D (1,25D; Sigma). The conditioned medium was collected and the supernatant stored at −80 °C until analysis. All experiments were performed in duplicate.

2.4. Measurement of OPG secretion

The levels of OPG were analyzed using an ELISA (Immundiagnostik, Bensheim and Biomedica, Wien). Briefly, the samples and the biotinylated antibody against OPG react simultaneously with the pre-coated antibody on the microtiterplate, in a second step, streptevidin-peroxidase is added which reacts with the detection antibody, and the solid phase is incubated with the substrate, TMB. An acidic stopping solution is subsequently added. A dose–response curve of the absorbance units (at 450 nm) vs. concentration is generated. The OPG present in the samples is determined directly from the calibration curve. Detection limit was 0.14 pmol/L, intra-assay and inter-assay variation were <10%. Protein concentrations were normalized to the number of cells and expressed as pg/ml per million cells.

2.5. Semi-quantitative RT-PCR for the mRNAs of OPG and RANKL

The cells were lysed and the RNA was isolated using High Pure RNA Isolation Kit according to the manufacturer's instruction (Roche, Germany). Total RNA was quantified by measuring the OD_{260}. 0.2 μg was amplified by RT-PCR using Titan One Tube Kit (Roche, Germany), primers and conditions were presented in Table 1. Amplified products were analyzed by 2% agarose gel electrophoresis and visualized with ethidium bromide. Band densities were measured using Scion computer software program. Relative expression of the different transcripts was calculated as a ratio to the β-actin signal.

2.6. Statistical analysis

Statistical analysis was carried out by ANOVA and Student's test using SPSS 15.0 software. The correlations between variables were carried out by Pearson and Rho de Spearmen coefficient. The critical value for significance was $p < 0.05$.

3. Results

The osteoporotic PM women were older (83 ± 1.5 years) than those with OA (74.1 ± 1.7) and confluence time, at the first stage, was positively related to age ($r = 0.38$, $p = 0.042$). The proliferation of hOBs in cultures coming from OP patients was more slowly than in

Table 1
Human oligonucleotide primers used for PCR. Primers are presented in a 5′–3′ orientation, with that for coding strand (+) and the noncoding strand (−). Tm and cycle number correspond to the linear part of the amplification curve.

Molecule	Primers	Strand	Size (bp)	Conditions (Tm; cycle no.)
OPG	GAACCCCAGAGCGAAATACA CGCTGTTTTCACAGAGGTCA	+ −	441	54 °C, x25
RANKL	ATCCCATCTGGTTCCCATAA CCCTGACCAATACTTGGTGC	+ −	276	53.5 °C, x30
β-Actin	TTGTAACCAACTGGGACGATATGT GATCTTGATCTTCATGGTGCTAGG	+ −	746	54 °C, x25

Table 2
Study population and effect of 17-β-estradiol 10-7M (E2), or/and 1,25-dihydroxyvitamin D 10-8M (1,25D) in patients with osteoporosis (hip fracture) or osteoarthritis on OPG secretion.

	Hip fracture ($n = 14$)	Osteoarthritis ($n = 14$)
Age (year)	83.8 ± 1.5 (73–89)	74.1 ± 1.7* (65–88)
BMI (body mass index)	27.84 ± 2.4	34.97 ± 1.2*
Confluence time (day) (first stage)	31.5 ± 2.6	21.4 ± 1.3*
OPG secretion basal (pmol/L)	10.1 ± 2.6	4.4 ± 0.8*
OPG secretion after E2 10-7M (pmol/L)	11.2 ± 3.3	3.9 ± 0.7*
OPG secretion after 1,25D 10-8M (pmol/L)	10.3 ± 2.3	4.3 ± 0.8*
OPG secretion after E2+1,25D (pmol/L)	9.2 ± 2.5	4.1 ± 0.8*

Comparison was assessed by Student's *t*-test. Values are mean ± standard error.
* $p \leq 0.05$ inter-group.

cultures from the OA group, with significant different time to confluence (31.5 ± 2.6 days OP; 21.4 ± 1.3 days OA, $p < 0.05$) (Table 2). Although OP and OA patients were not perfectly age-matched, the slower growth in OP cells was maintained even after adjusting for age (OP: 32.5 (CI 95%: 27.45–37.56) days; OA: 20.4 (CI 95%: 15.4–25.5) days, $p = 0.005$). Afterwards, in the second stage, the growth was similar in both groups (6.9 ± 2.5 days OP; 5.6 ± 3.9 days OA).

Body mass index (BMI) was lower in OP patients (27.84 ± 2.4) than in OA group (34.97 ± 1.2) ($p < 0.05$) (Table 2). However, there was not any correlation between BMI and confluence time.

3.1. OPG secretion

The OPG secretion from the cell cultures was important in all of the cultures. The basal secretion of OPG obtained from the hOB cultures in the OP group (10.1 ± 2.6 pmol/L) were double of those presented in the OA group (4.4 ± 0.8 pmol/L), and remained significantly higher in all conditions of the study ($p < 0.05$). The treatment with E2, 1,25D or E2+1,25D did not change the secretion levels of OPG in either of the two studied groups (Table 2).

Nor age nor confluence time was correlated with OPG protein secretion in any of the groups under study and nor do they influence the response to the stimulations analyzed.

3.2. OPG and RANKL expression

All mRNA levels were normalized to β-actin gene mRNA level. The expression of genes encoding OPG and RANKL were compared between the hip fracture and OA groups.

The OPG expression from hOB cultures was comparable in PM women with OP and OA (0.97 ± 0.1 relative units (r.u.) OP; 0.96 ± 0.08 r.u. OA). The cell stimulation, with oestrogen and/or 1,25D, did not induce any apparent change (Fig. 1A).

The RANKL expression in basal conditions was similar in both groups (0.26 ± 0.07 r.u. OP; 0.32 ± 0.09 r.u. OA). However, there was a significant increment, above 200%, in the RANKL expression within the hOB cultures of the OP group in response to 1,25D ($p = 0.027$) and E2+1,25D ($p = 0.025$). Nevertheless, this increase was less pronounced in the OA group and only became significant, as regards to the basal value, after the E2+1,25D stimuli. E2 did not produce any modification in the conditions used (Fig. 1B).

There was no relationship between the OPG protein secretion levels, mRNA OPG and the mRNA RANKL expression, neither between these genes and the cellular confluence time, or the patients' age in any of the studied groups.

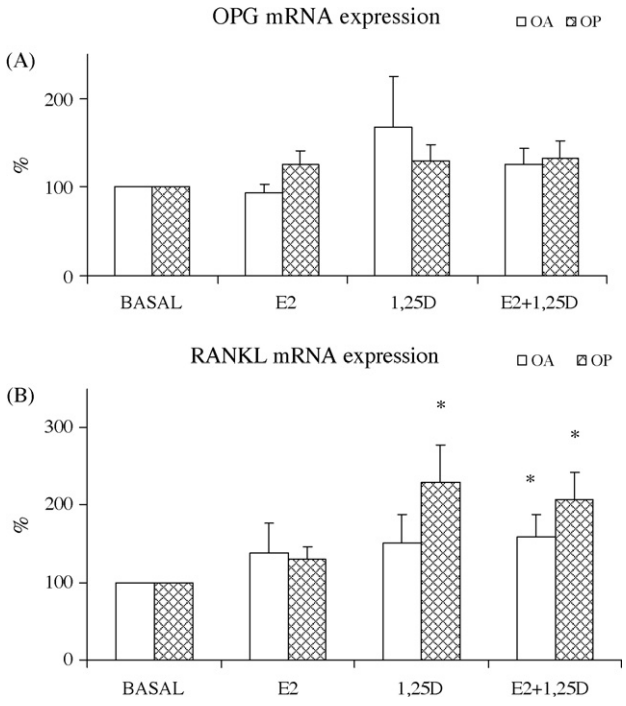

Fig. 1. Expression of OPG (A) and RANKL (B) mRNA by human osteoblasts, in basal condition, and in the presence of 17-β-estradiol (E2) 10-7M or/and 1,25-dihydroxyvitamin D (1,25D) 10-8M during 24 h. Results were normalized to β-actin levels. Data are expressed as mean ± S.E. (%) vs. untreated control, *$p < 0.05$.

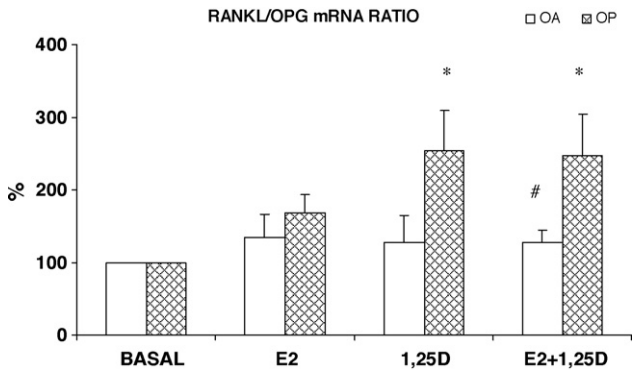

Fig. 2. RANKL/OPG mRNA ratio in basal condition, and in the presence of 17-β-estradiol (E2) 10-7M or/and 1,25-dihydroxyvitamin D (1,25D) 10-8M during 24 h. Data are expressed as mean ± S.E. (%) vs. untreated control, $^{*}p < 0.025$; #OA vs. OP, $p = 0.006$.

3.3. RANKL/OPG mRNA ratio

The RANKL/OPG mRNA expression ratios in basal condition were similar in both groups (0.31 ± 0.07 r.u. OP; 0.20 ± 0.04 r.u. OA). After stimulating with 1,25D and E2+1,25D there was an increase of 250% in the OP group ($p = 0.024$ and $p = 0.001$, respectively). As in the RANKL expression, the presence of E2 on the culture medium did not change the RANKL/OPG ratio. In the OA group, there was no change in the RANKL/OPG ratio in any of the studied conditions (Fig. 2). When both groups were compared, the increase obtained in the ratio after E2+1,25D was double in the OP group as regards to the OA group ($p = 0.006$). The RANKL/OPG mRNA expression ratio was calculated by dividing the absolute values of each patient. The average ratio of all the patients and the standard error were then calculated. This average ratio was considered as 100% when in basal conditions and the variation of this ratio with the different stimulus was expressed as a percentage change.

4. Discussion

In this study we have found that primary hOB cultures from PM women with an osteoporotic hip fractures initially have a slower proliferation than cultures from OA groups. However, the proliferation was similar after reaching cellular confluence and in successive stages. Also the OP group secreted higher OPG protein levels and expressed higher RANKL mRNA levels, making RANKL/OPG mRNA ratio higher in response to 1,25D and E2+1,25D than hOB cultures from PM women with hip OA.

Culture time, until first stage cellular confluence, was homogeneous in patients from either group, but it was significantly slower in hOB cultures of OP than in OA group and this difference remained after the adjustment for age. Other authors [20], have found that osteoblastic cell from OP patients show significantly less attachment to serum-coated tissue culture plastic. These results are in agreement with our finds. Although the traumatic injury of the fracture could play a role in the slower proliferation in OP cultures, we believe this is unlikely, because during the second stage, growth time and cellular proliferation of hOBs were similar in both groups, while, OPG secretion and RANKL and OPG expression were different in responding to stimuli. So the differences we found are mainly due to true differences between both pathologies and not to traumatic injury.

This study demonstrates, for the first time, that OPG secretion in hOB from patients with hip fractures is elevated with respect to OPG secretion from OA cells. No study has been carried out with hOBs in which OPG secretion was appraised in the culture medium in OA. OPG levels in peripheral blood have been described which show higher levels in elderly people than in young people, in post-menopausal than premenopausal women, in osteoporotic women than healthy controls [10,21] underling that high levels of OPG serum are associated with an increased risk of hip fractures in women [21].

There are some previous papers where the expression of OPG and RANKL genes has been studied, basically in cell pools but not in isolated OBs, and under different experimental conditions, with inconsistent results [5,11,22,23]. The strongest aspect of this study is the current measurement of the OPG and RANKL mRNA expression in osteoblasts cultures of bone from patients with osteoporotic fractures and patients with OA. As we have studied isolated OBs, we have eliminated the effect of different growth factors and cytokines coming from other cells that may influence the system. We have found that the absolute levels of OPG and RANKL mRNA, as well as the RANKL/OPG mRNA ratio, are comparable in both groups of patients studied in basal conditions. However, other authors, based on biopsies of the iliac crest and not on isolated hOb cultures, find an increase in RANKL expression and the RANKL/OPG ratio in OP patients in respect to OA.

However 1,25-dihydroxyvitamin D alone and E2 plus 1,25D increased the mRNA expression of RANKL and the RANKL/OPG ratio becoming significant in the osteoporotic hip fracture group. We chose concentrations of 10-7M E2 and 10-8M 1,25D after performing previous studies of dose–response curves and observed that they were the concentrations that showed higher activity, without provoking a toxic effect on cellular viability and they were also similar to those used by other authors [9,13–15,18,24,25]. The mechanism by which oestrogen affects bone metabolism is complex and has not been fully elucidated, but its main effect is to prevent bone loss and the consequence of the estrogenic deficit is the stimulation of the proliferation of osteoclast precursors [23,26]. In different experimental conditions (hOB, human cellular lines or untransfected ST-2 cells, a mouse bone marrow stromal cell line) it has been studied the effect of E2 on the RANKL/OPG system with inconclusive results. Oestrogens have been indicated as being able to produce a decrease or an increase of OPG protein secretion; they produce or not changes in OPG mRNA expression; they modify or not in RANKL mRNA expression and, finally, neither an increase nor change in the RANKL/OPG mRNA ratio [9,12–15,27,28]. The variability of results on the E2 effect can be attributed to the heterogeneity of the osteoblastic cell systems, to differences in culture conditions, the differences in the people's age from whom bone samples were taken for the culture (young and elderly people), the differences in the state of health of the bone metabolism (healthy, osteoporotic or osteoarthritic people) and differences in ER expression and the proportion between ERα and ERβ receptors. Some authors describe a maximum OPG stimulation in cells that over express ERα [28] and others have pointed out a negative effect regulated by ERβ [9]. Our study is performed on an important number of patients with osteoporotic hip fracture as well as on patients with OA, in cultures of samples extracted from the same location of the femoral neck. We did not find a homogenous effect on the OPG/RANKL system induced by oestrogen stimulus. While an increase in response was observed in some women, the opposite happened in others. We have not found any association between the difference in response shown by each patient and factors such as culture confluence time, age, and years of menopause or physical activity. This cannot be due to the absence of estrogen receptors as previous studies carried out by our group, using the same dose as we have actually employed, showed the presence of estrogen receptors α and β in primary hOB cultures in a very variable range between patients (data not published). Further studies will be necessary to clarify these results.

1,25-Dihydroxyvitamin D affects many aspects of bone cell biology. Numerous "in vitro" studies have implicated 1,25D in the regulation of both osteoblastic and osteoclastic activity. In

osteoblasts, 1,25D regulates gene transcription, proliferation, differentiation and mineralization. In our study 1,25D does not modify OPG protein secretion or expression; nevertheless, it increases the RANKL mRNA expression and the RANKL/OPG mRNA ratio in both groups, obtaining statistical significance in patients with OP hip fractures. The up-regulation of RANKL and the increased RANKL/OPG mRNA ratio by 1,25D treatment agreed with a number of reports in cell line and human primary cells [16–18,24,25,29,30]. Other authors have found different results such as, an increase in the levels of mRNA and protein secretion of OPG [28], a down-regulation of OPG expression [16,31], and even a decrease of the RANKL/OPG mRNA ratio [24]. These differences in the results may be due to the fact that the studies were carried out at different moments in osteoblastic maturation with changes in the VDR expression [24]. As we analyzed cultures when they had reached cellular confluence, in both groups, the differences we found must be due to true differences between both diseases [22,23,32].

The joint effect of both hormones, oestrogen and 1,25-dihydroxyvitamin D was able to verify that it induced an increase of more potent osteoblastic activity than each one did separately [33], which could be determined by an up-regulation of the estrogenic receptors by 1,25D when they are associated [34,35]. This led us to use the combination of both substances as a stimulus in this study and we have found an even more potent effect than isolated 1,25D on RANKL expression and RANKL/OPG mRNA ratio, that was more marked in osteoporotic patients.

We have verified that the 1,25D and E2+1,25D effect on the RANKL/OPG system is basically performed by modifying RANKL expression in hOBs, this could be influenced by the doses used in the study.

We believe that the differences that we found between the OP and OA groups are not due to the damage produced by the fracture but to differences in the bone metabolism inherent to the pathology. Although a slower proliferation of hOB cultures in patients with fractures would be expected, the traumatic damage cannot explain the greater metabolic activity in respect to the OPG and RANKL system in basal conditions and after different stimuli that we observe in OP patients in respect to the OA group but completely the opposite would be expected.

This study presents as the main limitation not being able to compare the results with healthy people's bone given the difficulty in obtaining samples.

In summary, we have demonstrated that the proliferation of the osteoblastic cells in culture is significantly slower in patients with osteoporotic fractures compared to cultures from OA patients during the first stage; we have also demonstrated, for the first time, that OPG protein secretion is higher in OP than in OA, in all studied conditions. And finally, RANKL mRNA expression and RANKL/OPG mRNA ratio are higher in OP than in OA group in response to 1,25D and E2+1,25D. The main modification on the RANKL/OPG system induced by 1,25D and E2+1,25D is exercised by an increase the RANKL mRNA expression. All of which indicates that the hOB in PM women with OP hip fracture are more sensitive to these hormonal stimulations, which could contribute to a greater osteoclastic activity in remodelled bones.

Conflict of interest

We here declare that there is no conflict of interest of any of the participant institutions.

Acknowledgments

This work was supported in part by the "Fondo de Investigación Sanitaria (FIS PI031164-2003)" of Spain. The authors thank the staff in the Biochemistry and Trauma & Orthopaedic Department, Hospital Virgen Macarena.

References

[1] H. Pogrund, M. Rutenberg, M. Makin, G. Robin, J. Menczel, R. Steinberg, Osteoarthritis of the hip joint and osteoporosis: a radiological study in a random population sample in Jerusalem, Clin. Orthop. Relat. Res. 164 (1982) 130–135.
[2] N.K. Arden, M.C. Nevitt, N.E. Lane, L.R. Gore, M.C. Hochberg, J.C. Scott, A.R. Pressman, S.R. Cummings, Osteoarthritis and risk of falls, rates of bone loss, and osteoporotic fractures, Study of Osteoporotic Fractures Research Group, Arthritis Rheum. 42 (1999) 1378–1385.
[3] G.R. Jordan, N. Loveridge, J. Power, M.T. Clarke, J. Reeve, Increased cancellous bone in the femoral neck of patients with coxarthrosis (hip osteoarthritis): a positive remodeling imbalance favoring bone formation, Osteoporosis Int. 14 (2003) 160–165.
[4] G.R. Jordan, N. Loveridge, K.L. Bell, J. Power, G.R. Dickson, S. Vedi, N. Rushton, M.T. Clarke, J. Reeve, Increased femoral neck cancellous bone and connectivity in coxarthrosis (hip osteoarthritis), Bone 32 (2003) 86–95.
[5] D.B. Logar, R. Komadina, J. Prezelj, B. Ostanek, Z. Trost, J. Marc, Expression of bone resorption genes in osteoarthritis and in osteoporosis, J. Bone Miner. Metab. 25 (2007) 219–225.
[6] K. Fuller, B. Wong, S. Fox, Y. Choi, T.J. Chambers, TRANCE is necessary and sufficient for osteoblast-mediated activation of bone resorption in osteoclasts, J. Exp. Med. 188 (1998) 997–1001.
[7] D.L. Lacey, E. Timms, H.L. Tan, M.J. Kelley, C.R. Dunstan, T. Burgess, R. Elliott, A. Colombero, G. Elliott, S. Scully, H. Hsu, J. Sullivan, N. Hawkins, E. Davy, C. Capparelli, A. Eli, Y.X. Qian, S. Kaufman, I. Sarosi, V. Shalhoub, G. Senaldi, J. Guo, J. Delaney, W.J. Boyle, Osteoprotegerin ligand is a cytokine that regulates osteoclast differentiation and activation, Cell 93 (1998) 165–176.
[8] W.S. Simonet, D.L. Lacey, C.R. Dunstan, M. Kelley, M.S. Chang, R. Luthy, H.Q. Nguyen, S. Wooden, L. Bennett, T. Boone, G. Shimamoto, M. DeRose, R. Elliott, A. Colombero, H.L. Tan, G. Trail, J. Sullivan, E. Davy, N. Bucay, L. Renshaw-Gegg, T.M. Hughes, D. Hill, W. Pattison, P. Campbell, S. Sander, G. Van, J. Tarpley, P. Derby, R. Lee, W.J. Boyle, Osteoprotegerin: a novel secreted protein involved in the regulation of bone density, Cell 89 (1997) 309–319.
[9] L.C. Hofbauer, S. Khosla, C.R. Dunstan, D.L. Lacey, T.C. Spelsberg, B.L. Riggs, Estrogen stimulates gene expression and protein production of osteoprotegerin in human osteoblastic cells, Endocrinology 140 (1999) 4367–4370.
[10] M.A. Vázquez, M.J. Montoya, M.J. Gómez de Tejada, R. Moruno, A. Carrillo, J.M. Guerrero, R. Pérez-Cano, Niveles séricos de osteoprotegerina en mujeres sanas y osteoporóticas, REEMO 12 (2003) 63–67.
[11] B.M. Abdallah, L.S. Stilgren, N. Nissen, M. Kassem, H.R. Jorgensen, B. Abrahamsen, Increased RANKL/OPG mRNA ratio in iliac bone biopsies from women with hip fractures, Calcified Tissue Int. 76 (2005) 90–97.
[12] V. Viereck, C. Grundker, S. Blaschke, H. Siggelkow, G. Emons, L.C. Hofbauer, Phytoestrogen genistein stimulates the production of osteoprotegerin by human trabecular osteoblasts, J. Cell Biochem. 84 (2002) 725–735.
[13] J. Cheung, Y.T. Mak, S. Papaioannou, B.A. Evans, I. Fogelman, G. Hampson, Interleukin-6 (IL-6), IL-1, receptor activator of nuclear factor kappaB ligand (RANKL) and osteoprotegerin production by human osteoblastic cells: comparison of the effects of 17-beta oestradiol and raloxifene, J. Endocrinol. 177 (2003) 423–433.
[14] M. Saika, D. Inoue, S. Kido, T. Matsumoto, 17beta-estradiol stimulates expression of osteoprotegerin by a mouse stromal cell line, ST-2, via estrogen receptor-alpha, Endocrinology 142 (2001) 2205–2212.
[15] A. Taranta, M. Brama, A. Teti, V. De luca, R. Scandurra, G. Spera, D. Agnusdei, J.D. Termine, S. Migliaccio, The selective estrogen receptor modulator raloxifene regulates osteoclast and osteoblast activity in vitro, Bone 30 (2002) 368–376.
[16] T. Suda, N. Takahashi, N. Udagawa, E. Jimi, M.T. Gillespie, T.J. Martin, Modulation of osteoclast differentiation and function by the new members of the tumor necrosis factor receptor and ligand families, Endocr. Rev. 20 (1999) 345–357.
[17] N.J. Horwood, J. Elliott, T.J. Martin, M.T. Gillespie, Osteotropic agents regulate the expression of osteoclast differentiation factor and osteoprotegerin in osteoblastic stromal cells, Endocrinology 139 (1998) 4743–4746.
[18] D. Zhang, Y.Q. Yang, X.T. Li, M.K. Fu, The expression of osteoprotegerin and the receptor activator of nuclear factor kappa B ligand in human periodontal ligament cells cultured with and without 1alpha,25-dihydroxyvitamin D3, Arch. Oral Biol. 49 (2004) 71–76.
[19] L.C. Hofbauer, C.R. Dunstan, T.C. Spelsberg, B.L. Riggs, S. Khosla, Osteoprotegerin production by human osteoblast lineage cells is stimulated by vitamin D, bone morphogenetic protein-2, and cytokines, Biochem. Biophys. Res. Commun. 250 (1998) 776–781.
[20] H. Perinpanayagam, R. Zaharias, C. Stanford, R. Brand, J. Keller, G. Schneider, Early cell adhesion events differ between osteoporotic and non-osteoporotic osteoblasts, J. Orthop. Res. 19 (2001) 993–1000.
[21] W.S. Browner, L.Y. Lui, S.R. Cummings, Associations of serum osteoprotegerin levels with diabetes, stroke, bone density, fractures, and mortality in elderly women, J. Clin. Endocrinol. Metab. 86 (2001) 631–637.
[22] H. Tsangari, D.M. Findlay, J.S. Kuliwaba, G.J. Atkins, N.L. Fazzalari, Increased expression of IL-6 and RANK mRNA in human trabecular bone from fragility fracture of the femoral neck, Bone 35 (2004) 334–342.
[23] N.L. Fazzalari, J.S. Kuliwaba, G.J. Atkins, M.R. Forwood, D.M. Findlay, The ratio of messenger RNA levels of receptor activator of nuclear factor kappaB ligand to

osteoprotegerin correlates with bone remodeling indices in normal human cancellous bone but not in osteoarthritis, J. Bone Miner. Res. 16 (2001) 1015–1027.
[24] P.A. Baldock, G.P. Thomas, J.M. Hodge, S.U. Baker, U. Dressel, P.D. O'Loughlin, G.C. Nicholson, K.H. Briffa, J.A. Eisman, E.M. Gardiner, Vitamin D action and regulation of bone remodeling: suppression of osteoclastogenesis by the mature osteoblast, J. Bone Miner. Res. 21 (2006) 1618–1626.
[25] G.J. Atkins, P.H. Anderson, D.M. Findlay, K.J. Welldon, C. Vincent, A.C. Zannettino, P.D. O'Loughlin, H.A. Morris, Metabolism of vitamin D3 in human osteoblasts: evidence for autocrine and paracrine activities of 1 alpha,25-dihydroxyvitamin D3, Bone 40 (2007) 1517–1528.
[26] T.C. Spelsberg, M. Subramaniam, B.L. Riggs, S. Khosla, The actions and interactions of sex steroids and growth factors/cytokines on the skeleton, Mol. Endocrinol. 13 (1999) 819–828.
[27] S. Bord, D.C. Ireland, S.R. Beavan, J.E. Compston, The effects of estrogen on osteoprotegerin, RANKL, and estrogen receptor expression in human osteoblasts, Bone 32 (2003) 136–141.
[28] M.K. Lindberg, S.L. Alatalo, J.M. Halleen, S. Mohan, J.A. Gustafsson, C. Ohlsson, Estrogen receptor specificity in the regulation of the skeleton in female mice, J. Endocrinol. 171 (2001) 229–236.
[29] M. Nagai, N. Sato, Reciprocal gene expression of osteoclastogenesis inhibitory factor and osteoclast differentiation factor regulates osteoclast formation, Biochem. Biophys. Res. Commun. 257 (1999) 719–723.
[30] G.P. Thomas, S.U. Baker, J.A. Eisman, E.M. Gardiner, Changing RANKL/OPG mRNA expression in differentiating murine primary osteoblasts, J. Endocrinol. 170 (2001) 451–460.
[31] T. Murakami, M. Yamamoto, K. Ono, M. Nishikawa, N. Nagata, K. Motoyoshi, T. Akatsu, Transforming growth factor-beta1 increases mRNA levels of osteoclastogenesis inhibitory factor in osteoblastic/stromal cells and inhibits the survival of murine osteoclast-like cells, Biochem. Biophys. Res. Commun. 252 (1998) 747–752.
[32] B. Hopwood, S. Gronthos, J.S. Kuliwaba, P.G. Robey, D.M. Findlay, N.L. Fazzalari, Identification of differentially expressed genes between osteoarthritic and normal trabecular bone from the intertrochanteric region of the proximal femur using cDNA microarray analysis, Bone 36 (2005) 635–644.
[33] F.P. Chen, N. Lee, K.C. Wang, Y.K. Soong, K.E. Huang, Effect of estrogen and 1alpha,25(OH)2-vitamin D3 on the activity and growth of human primary osteoblast-like cells in vitro, Fertil. Steril. 77 (2002) 1038–1043.
[34] T. Bellido, G. Girasole, G. Passeri, X.P. Yu, H. Mocharla, R.L. Jilka, A. Notides, S.C. Manolagas, Demonstration of estrogen and vitamin D receptors in bone marrow-derived stromal cells: up-regulation of the estrogen receptor by 1,25-dihydroxyvitamin-D3, Endocrinology 133 (1993) 553–562.
[35] A.M. Vertino, C.M. Bula, J.R. Chen, M. Almeida, L. Han, T. Bellido, S. Kousteni, A.W. Norman, S.C. Manolagas, Nongenotropic, anti-apoptotic signaling of 1alpha,25(OH)2-vitamin D3 and analogs through the ligand binding domain of the vitamin D receptor in osteoblasts and osteocytes. Mediation by Src, phosphatidylinositol 3-, and JNK kinases, J. Biol. Chem. 280 (2005) 14130–14137.