Modifying RANKL/OPG mRNA Expression in Differentiating and Growing Human Primary Osteoblasts

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- 17-β-estradiol
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- osteoporosis
- osteoarthritis

Abstract

The OPG/RANKL system in primary cultures of human osteoblasts has been studied by different authors. However, very few studies have been performed on gene expression of RANKL and OPG at different stages of maturation on human osteoblast cultures. The effect of 17-β-estradiol and 1,25dihydroxyvitamin D3 on the OPG/RANKL system is not known during the different states of cellular maturation. In this work we quantified OPG and RANKL protein levels (ELISA) and the mRNA of OPG, RANKL, collagen type I, alkaline phosphatase, and osteocalcin (semi-quantitative RT-PCR) in human osteoblasts. We analyzed these in basal conditions and after incubation with 17-β-estradiol and 1,25dihydroxyvitamin D3 in the first and second phases. We found that OPG secretion and expression levels increased throughout cellular growth. RANKL proteins were detected only in the first stage, and the expression increased throughout the first phase. Thus, the RANKL/OPG ratio was higher in immature osteoblasts than in mature osteoblasts. The evolution of RANKL gene expression was related to collagen I and alkaline phosphatase, while OPG was related to osteocalcin. We observed no modifications after estradiol and 1,25dihydroxyvitamin D3 treatment. Our results suggest that the OB is a positive stimulator at precocious stages of differentiation on osteoclastogenic modulates.

Introduction

Bone turnover is a continuous physiological process depending on the balance between bone formation and resorption. This balance involves the coordinated regulation and interaction of two cell types: the osteoclast (OC) and the osteoblast (OB) [1,2]. The OPG/RANKL/RANK system is fundamental for regulation between the OBs and OCs [3,4]

Previous in vitro studies of OPG/RANK/RANKL regulation have used stromal cells, immature primary osteoblasts, and osteoblast cell lines. Recent studies using cell lines induced to differentiate into a more mature osteoblastic phenotype have indicated that RANKL and OPG expression levels can be altered [5,6]. However, expression models of RANKL and OPG have not been established during primary human osteoblastic differentiation. Only Thomas et al. [7] have demonstrated that RANKL and OPG expression is different during the growth of murine osteoblastic cells.

An OB cell has different levels or stages of maturity during its growth. At each stage the phenotype, including the morphologic appearance and biosynthetic activity, is different. OB differentiation includes three distinct periods: 1) Growth (proliferation) and extracellular matrix (ECM) biosynthesis, 2) ECM development and maturation, and 3) ECM mineralization. During the period of active proliferation, many genes are expressed, such as cell cycle genes (c-fos, c-myc, histone) and those of extracellular matrix proteins (procollagen I, fibronectin). This is followed by a stage of matrix maturation characterized by a high expression of bone alkaline phosphatase. When mineralization begins, genes for proteins such as osteocalcin, bone sialoprotein and osteopontin are expressed [8,9].

The effects of 1,25dihydroxyvitamin D3 on OB have been well characterized in rats and human osteoblast cultures; in both cases, stimulation and inhibition have been described in genes related to the differentiation of osteoblastic phenotypes [10,11]. It is well established that estrogens influence osteoblastic growth and differentiation [12,13]. Nevertheless, the simultaneous action of both stimuli has not been described.

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Therefore the main aim of this study was to examine OPG and RANKL expression and secretion in primary cultures of human OBs throughout their developmental sequences. The second point was to analyze the response of the RANKL/OPG system to 17-β-estradiol and 1,25dihydroxyvitamin D3 throughout the different maturation stages. The final goal was to relate the OPG and RANKL gene expression with the expression of other genes implicated in bone structural proteins such as osteocalcin (BGP), collagen type 1 (COL 1), and alkaline phosphatase (ALP) during the proliferation and maturation of osteoblasts.

Materials and Methods

Cell culture

Primary human OB (hOB) cells were isolated from bone fragments taken from four patients (3 women and 1 man, aged 58–72 years), without bone metabolic disease, who had undergone knee or hip arthroplasty due to the diagnosis of osteoarthritis. Ethical approval had been obtained from the local Research Ethics Committee. Briefly, trabecular bone was cut into pieces (1–2 mm) and thoroughly rinsed with PBS six times. The cells from each donor were examined individually, they were incubated in a humidified CO_2 incubator at 37 °C, and the medium was changed twice a week until confluence was achieved. This was defined as first passage cells. hOB was cultured in α-DMEM supplemented with 10% FCS and antibiotics. In all the cultures, >85% of the cells showed intense staining for alkaline phosphatase activity. Trypan blue was used to screen out dead cells after harvesting. We tested cells at 14 and 28 d and at confluence, and then 300 000 cells/plate were sub-cultured and tested again after 14 and 28 d in the second passage. We confirmed the osteoblastic phenotype by using alkaline phosphatase staining.

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 nitroblue tetrazolium (BCIP/NBT) alkaline phosphatase substrate (Amresco, Ohio, USA) for 10 min at room temperature. The reaction was stopped by removing the substrate solution and washing with distilled water.

Cell treatment with E2 and vitD

Confluent hOB cultures were then washed and incubated in a serum-free medium for 24 h. The concentration used for each agent was as follows: 10^{-6} M 17-β-estradiol and 10^{-8} M 1,25dihydroxyvitamin D3.

Measurement of OPG secretion

OPG levels were analyzed using an ELISA (Immundiagnostik, Bensheim and Biomedica, Vienna). Briefly, the samples and biotinylated antibody against OPG reacted simultaneously with the pre-coated antibody on the microtiter plate; in a second step, streptavidin-peroxidase, which reacts with the detection antibody, was added, and the solid phase was incubated with the substrate, TMB. An acidic stopping solution was subsequently added. A dose–response curve of the absorbance units (at 450 nm) versus concentration was generated. The OPG present in samples was determined directly from the calibration curve. The detection limit was 0.14 pmol/1, the intra-assay variation was 4%, and the inter-assay variation was < 10%. Protein concentrations were normalized to the number of cells.

Measurement of sRANKL secretion

sRANKL levels were analyzed using an ELISA (Immundiagnostik, Bensheim and Biomedica, Vienna). In a first step, the sample and biotinylated anti-sRANKL detection antibodies were pipetted into the wells. Human sRANKL, if present in the sample, binds to the pre-coated recombinant osteoprotegerin (OPG) and forms a sandwich within the detection antibody. Streptavidin–HRP conjugate was added to the wells, and then TMB was added as a substrate. A dose–response curve of absorbance units (at 450 nm against 690 nm or 620 nm as a reference) versus concentration was generated. The sRANKL present in the samples was determined directly from the calibration curve. The detection limit was 0.08 pmol/l, the intra-assay variation was 4%, and the inter-assay variation was 9%. Protein concentrations were normalized to the number of cells.

Semi-quantitative RT-PCR for the mRNAs of OPG, RANKL, ALP, COLI, and BGP

The cells were lysed and the RNA was isolated using a High Pure RNA Isolation Kit (Roche, Germany) according to the manufacturer's instructions. Total RNA was quantified by measuring the OD at 260 nm. An amount of 0.2 μg was amplified by RT-PCR using a Titan One Tube Kit (Roche, Germany); primers and conditions are presented in **Table 1**. Amplified products were analyzed by 2% agarose gel electrophoresis and visualized with ethidium bromide. Band densities were measured using a Scion Image 4.0.2. (Maryland, USA) computer software program. The relative expression of the different transcripts was calculated as a ratio to the β-actin signal.

Statistical analysis

Statistical comparisons were carried out by ANOVA using the SPSS 14.0. software (Illinois, USA). The correlations between variables were carried out by Pearson coefficient. The critical value for significance was $p < 0.05$.

Results

Primary human osteoblast cultures progressed throughout the osteoblastic development sequence. All the cultures behaved homogenously, and cellular confluence was reached between 33 and 35 d after starting the culture.

Osteoclastogenesis regulatory proteins

OPG protein secretion was detected at all the time points analyzed, although the higher levels were reached at 14 d of the second passage, with a significant increase regarding the first study point ($p = 0.003$). (**Fig. 1A**).

sRANKL protein secretion was detected only in the initial phase of osteoblastic proliferation, with a decrease of 96% at day 28 of the first passage with respect to the initial value, while it was not detectable in the rest of the determinations (**Fig. 1B**).

The treatment with estrogens and 1,25D for 24 h apparently did not exert any effect on the OPG secretion when we analyzed the joint data from the four patients. There was a decrease of between 14 and 58% in two patients and an increase of between 6 and 162% in the other two patients. Secondly, on analyzing

Molecule	Primers	Strand	Size (bp)	Conditions (Temp; no. cycles)
OPG	GAACCCCAGAGCGAAATACA	+	441	54°C; ×25
	CGCTGTTTTCACAGAGGTCA	−		
RANKL	ATCCCATCTGGTTCCCATAA	+	276	53.5°C; ×30
	CCCTGACCAATACTTGGTGC	−		
ALP	ACGTGGCTAAGAATGTCATC	+	475	52°C; ×30
	CTGGTAGGCGATGTCCTTA	−		
BGP	CATGAGAGCCCTCACA	+	310	52°C; ×30
	AGAGCGACACCCTAGAC	−		
COL1	TGACGAGACCAAGAACTG	+	599	52°C; ×30
	CCATCCAAACCACTGAAACC	−		
β-actin	TTGTAACCAACTGGGACGATATGT	+	746	54°C; ×25
	GATCTTGATCTTCATGGTGCTAGG	−		

Note: Temperature and cycle numbers correspond to the linear part of the amplification curve

Table 1 Human oligonucleotide primers used for PCR. Primers are presented in a 5′ to 3′ orientation for coding (+) and noncoding (−) strands.

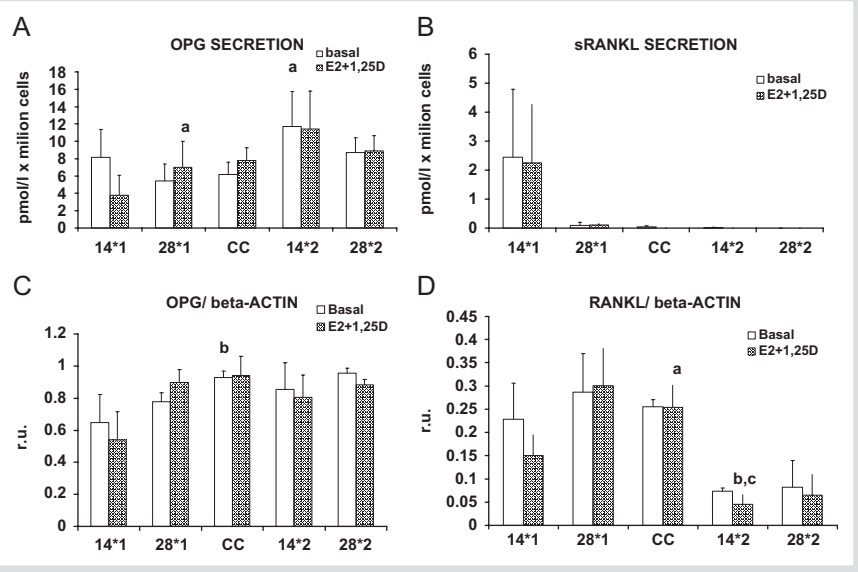

Fig. 1 Protein secretion and gene expression during in vitro maturation and differentiation of human primary osteoblasts (hOBs) in cultures under the influence of 10^{-6}M 17-β-estradiol (E2) and 10^{-8}M vitamin D3 (1,25D) compared with control. Results are plotted in percentage of maximum secretion. (**A**) OPG protein secretion, (**B**) RANKL protein secretion. Gene expression was normalized with the expression of β-actin. (**C**) OPG gene expression, (**D**) RANKL gene expression. Mean values ± SE of four experiments with two or three replicates; statistical significance: [a]p < 0.05 vs. day 14 of first passage, [b]p < 0.05 vs. day 28 of first passage, [c]p < 0.05 vs. confluence. 14*1 = day 14 of first passage; 28*1 = day 28 of first passage; CC = cellular confluence; 14*2 = day 14 of second passage; 28*2 = day 28 of second passage.

sRANKL, while the levels were undetectable on the 28th day of the second passage in three out of four patients, after stimulating with E2 + 1,25D, the patients presented sRANKL secretion in the culture medium, thus indicating a positive net effect on this protein.

There was a negative correlation between the values of OPG and sRANKL secretion throughout cellular growth, with r = −0.332, p = 0.036.

Osteoblast phenotype

The mRNA levels were measured by semi-quantitative RT-PCR. The expression pattern of key genes such as COL1, ALP, and BGP was evident throughout the cellular culture, with higher concentrations for COL1 and ALP on the 28th day of the first passage and for BGP at the moment of cellular confluence in the first phase. Although no significant differences were found for any of the genes among the points studied, we were able to verify that the maximum increase in ALP values was 5 times the initial value, while it was 3.5 times for BGP expression. (**Fig. 2A–C**).

mRNA expression of OPG was nearly unchanged in two out of four patients, and the other two underwent a decrease in the range of 2.2–20%. We observed no apparent change in RANKL expression.

Osteoclastogenesis regulatory genes

The OPG mRNA levels were measured by semi-quantitative RT-PCR. These levels progressively increased throughout culture development until the 28th day of the second passage, when values were highest. The overall increase was 32%, with significant differences between day 28 of the first passage and its cellular confluence in the same passage (p = 0.031) (**Fig. 1C**, **Table 2**).

Cellular stimulation with estrogens and 1,25dihydroxyvitamin D3 did not induce any apparent change in OPG expression during culture with regard to the results of cellular cultures in basal conditions.

We were able to observe a positive correlation between OPG mRNA and BGP mRNA values (r = 0.716, p = 0.001).

RANKL expression kept within the same range of values in the first passage; however, it decreased significantly in the second passage, where values fell up to 3.5 times with respect to the maximum values obtained on day 28 of the first passage (p = 0.024) (**Fig. 1D**, **Table 2**). Once again, the E2 and 1,25D stimuli did not modify the RANKL expression throughout the study with regard to the values obtained in nontreated cells.

RANKL mRNA levels were correlated with those found for ALP (r = 0.479, p = 0.012) and for COL1 (r = 0.405, p = 0.029).

The modulation of osteoclastogenic activity is related to the RANKL/OPG ratio, and we observed that this ratio decreased during culture time until reaching the lowest values at the last point studied. This means that in the initial studies of cell growth,

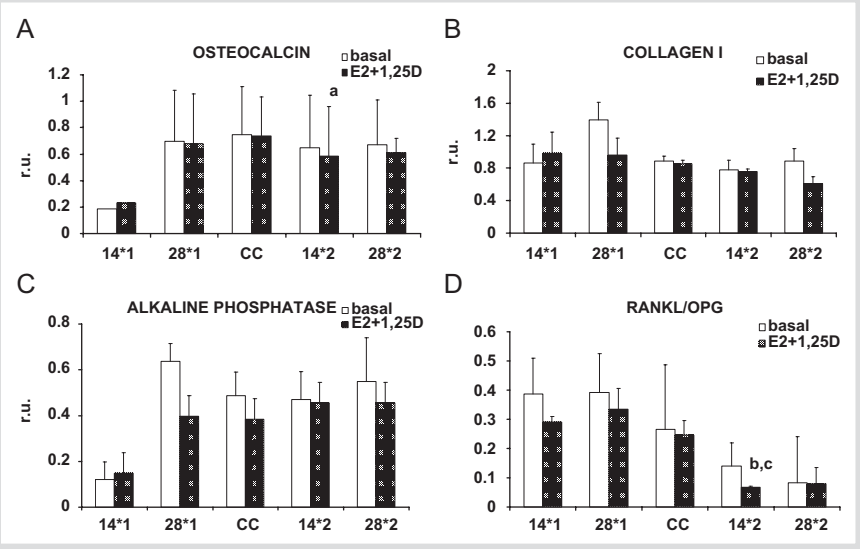

Fig. 2 Time course of relative changes in hOB markers. Semi-quantitative RT-PCR of (**A**) BGP, (**B**) COL1, and (**C**) ALP. Comparison between basal culture and treated cultures (10^{-6} M E2 and 10^{-8} M 1,25D for 24 h). Results are expressed as ratio to β-actin. (**D**) RANKL mRNA and OPG mRNA ratios. Mean values ± SE of four experiments with two or three replicates; statistical significance: $^{a}p<0.05$ vs. day 28 of first passage, $^{b}p<0.05$ vs. day 14 of first passage, $^{c}p<0.05$ vs. confluence. 14*1 = day 14 of first passage; 28*1 = day 28 of first passage; CC = cellular confluence; 14*2 = day 14 of second passage; 28*2 = day 28 of second passage.

Table 2 Effect of β-estradiol and 1,25$(OH)_2D_3$ (E2 + VD) on OPG, RANKL expression (respect β-actin) and RANKL/OPG ratio. 14*1 = 14 days of 1st passage; 28*1 = 28 days of 1st passage; CC = cellular confluence 1st passage; 14*2 = 14 days 2nd passage; 28*2 = 28 days 2nd passage (r.u. relative unit).

	OPG (r.u.)		RANKL (r.u.)		RANKL/OPG	
	Basal	After E2 + VD	Basal	After E2 + VD	Basal	After E2 + VD
14*1	0.65 ± 0.2	0.54 ± 0.17	0.23 ± 0.08	0.15 ± 0.04	0.39 ± 0.1	0.29 ± 0.01
28*1	0.78 ± 0.05	0.89 ± 0.08	0.29 ± 0.08	0.31 ± 0.08	0.39 ± 0.1	0.34 ± 0.07
CC	0.93 ± 0.04*	0.94 ± 0.12	0.26 ± 0.01*	0.25 ± 0.05	0.27 ± 0.2	0.25 ± 0.05
14*2	0.85 ± 0.1	0.81 ± 0.14	0.07 ± 0.01*	0.05 ± 0.02	0.14 ± 0.1*	0.07 ± 0.003
28*2	0.96 ± 0.03	0.88 ± 0.03	0.08 ± 0.05	0.06 ± 0.04	0.08 ± 0.1	0.08 ± 0.06

Mean values ± SE ; *p < 0.05

OBs modulate osteoclastogenic activity positively in the system (▶ **Fig. 2D**, **Table 2**).

Discussion

In the present study we have shown that the patterns of different genes change throughout the time of hOBs cultures. The cultures were obtained from bone biopsies of adult patients who had undergone knee or hip arthrodesis. All of them similarly reached cellular confluence at 33–35 d. During their growth, the percentage of cells obtained at each time of analysis was very similar. Confluence time was very similar to that obtained by other authors for primary osteoblastic cultures from hip bone biopsies [14–16], between 3 and 6 weeks, although it was more prolonged than that of other species such as rat primary OBs [17] or chicken embryo calvarial cells [18], which took 6–8 d, or hOBs from other sources such as iliac crest biopsies [9], which reached cellular confluence after 21 d of culture.

There are three osteoblastic developmental phases [17]: an initial proliferative phase characterized by higher COL1 expression, followed by a matrix production and maturation phase in which ALP is increased, and, finally, a third phase of initiation of mineralization marked by higher levels of BGP mRNA. We detected COL1, ALP, and BGP levels throughout the culture growth up to cellular confluence. We were able to verify that the highest levels corresponded with day 28 of the first passage for the gene expression of COL1 and ALP. At the moment of cellular confluence for BGP mRNA in the second passage, the levels were maintained, and we did not detect significant changes in the levels of mRNA of these genes. There are very few studies that have evaluated the expression of these genes in the first phases of cellular growth hOBs, and our results coincide with these studies [11, 14].

Expression of OPG and RANKL, key osteoblast regulatory genes, has hardly been studied throughout the development of the osteoblastic growth phases, although there are multiple authors who have studied OPG and RANKL expression at the moment of cellular confluence. Some have also studied OPG and RANKL protein secretion but found problems in detecting the latter [12, 19]. Although the soluble form of sRANKL in the medium is well known [20, 21], its detection has been difficult. We have detected soluble RANKL secretion in culture medium in the most precocious phases of cellular growth; in fact, only the determination carried out on the 14th day of the first culture passage showed sRANKL levels above the minimum threshold of sensibility of the test. Other authors have not found soluble levels of RANKL in the hOB culture medium, mainly because the sRANKL determinations were performed at later stages of cellular growth [22]. We did not evaluate the RANKL form anchored to a membrane in this study and, therefore, do not know whether this protein is modified throughout the differentiation phases and cellular maturation. Other studies will be necessary to determine this. In the present study we wanted to analyze protein secretion as well as OPG and RANKL expression during the growth of hOB cultures, first by evaluating the most incipient growth phases until cellular confluence was reached and then by relating the levels of these genes at the different time points

with COL1, BGP, and ALP genes. Protein secretion as well as OPG expression were higher in studies of more immature cultures, coinciding with the BGP maximum peak (beginning of cellular maturation phase). These results have also been described in murine OBs in post-confluent growth [7] and in hOB cellular lines [23]. The behavior of sRANKL was different, with the highest secretion at the beginning of cellular culture and a sharp decrease from then onwards, reaching undetectable levels during the follow-up. This could explain the fact that some authors do not find a soluble form of the protein in the culture medium when they refer to the moment of cellular confluence [19,22]. RANKL gene expression was also greater at the beginning of cellular culture and coincided with ALP and COL1 expression, indicative of immature cells in the proliferative phase. These results are similar to the data shown by Gori et al. [23].

The RANKL/OPG mRNA ratio, indicative of positive modulation osteoclastogenic activity, showed higher values in the initial phases of the study, coinciding with more immature OBs, and decreased progressively in the latter phases of cellular maturation [23]. This makes us believe that the stimulation of osteoclastogenesis is a major role of immature osteoblasts. It would be necessary to perform, in the future, in vitro studies with both cellular types, osteoblasts and osteoclasts, and to observe this phenomenon throughout the different phases of cellular growth and development. Although the in vitro observations could not necessarily be extrapolated to in vivo physiologic mechanisms, we believe that the regulation of the RANKL/OPG ratio observed in vitro during cellular growth could contribute, in vivo, to the coordination of osteoblastic differentiation and osteoclastic activity.

E2 and 1,25D are hormones that influence bone turnover activity. Several in vitro studies have evaluated the effect each one has on the OPG/RANKL system, showing contradictory results [19,24,25]. In our case the results were not consistent, showing a stimulating effect on some cultures and inhibitory effects on others. We chose concentrations of 10^{-6} M E2 and 10^{-8} M 1,25D after performing previous studies of dose–response curves and observing that these concentrations showed higher activity. We used a combination of E2 and 1,25D because we were able to verify that the joint effect of both hormones induced more potent osteoblastic activity than each one did separately, coinciding with data from other authors [26,27]. This greater effect could be due to the upregulation of the estrogenic receptors by 1,25D when both substances are present, as other groups have proposed [28,29]. This led us to use a combination of both substances as a stimulus in the development of primary human cultures; however, we saw no relevant effect on the genes studied that could be related to the dose and time of incubation employed.

Overall, it appears that the differentiation over time is much stronger in regulating RANKL and OPG than E2 and 1,25D are. Thus, when assays to evaluate the effect of different pharmaceutical treatments on osteoblasts are designed, we have to bear in mind at what stage of proliferation and/or maturation the cell cultures are found [14].

In this work we did not measure the α or β estrogen receptor (ER) concentrations in hOB cultures, which could limit our understanding of the results. Nevertheless, the existence of ER in OBs has been repeatedly observed, though at very low concentrations, which could justify the scarce response found [30–32].

In conclusion, in this study we have shown that human OB differentiation in vitro is associated with a high RANKL/OPG production ratio in early cellular growth stages [23,33]. We speculate that human OBs have a positive effect on modulating the osteoclastogenic activity at precocious stages of its cellular growth which is lost on reaching a higher degree of cellular maturation and that may contribute to the coordinated sequence of bone turnover cycles.

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