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Journal of Cellular Biochemistry 110:304–310 (2010)

Journal of Cellular Biochemistry

Effect of IL-1β, PGE_2, and TGF-β1 on the Expression of OPG and RANKL in Normal and Osteoporotic Primary Human Osteoblasts

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ABSTRACT

The *RANKL*/*RANK*/*OPG* pathway is essential for bone remodeling regulation. Many hormones and cytokines are involved in regulating gene expression in most of the pathway components. Moreover, any deregulation of this pathway can alter bone metabolism, resulting in loss or gain of bone mass. Whether osteoblasts from osteoporotic and nonosteoporotic patients respond differently to cytokines is unknown. The aim of this study was to compare the effect of interleukin (IL)-1β, proftaglandin E_2 (PGE_2), and transforming growth factor-β1 (TGF-β1) treatments on *OPG* and *RANKL* gene expression in normal (n = 11) and osteoporotic (n = 8) primary osteoblasts. *OPG* and *RANKL* mRNA levels of primary human osteoblastic (hOB) cell cultures were assessed by real-time PCR. In all cultures, *OPG* mRNA increased significantly in response to IL-1β treatment and decreased in response to TGF-β1 whereas PGE_2 treatment had no effect. *RANKL* mRNA levels were significantly increased by all treatments. Differences in *OPG* and *RANKL* responses were observed between osteoporotic and nonosteoporotic hOB: in osteoporotic hOB, the *OPG* response to IL-1β treatment was up to three times lower ($P = 0.009$), whereas that of *RANKL* response to TGF-β1 was five times higher ($P = 0.002$) after 8 h of treatment, as compared with those in nonosteoporotic hOBs. In conclusion, osteoporotic hOB cells showed an anomalous response under cytokine stimulation, consistent with an enhanced osteoclastogenesis resulting in high levels of bone resorption. J. Cell. Biochem. 110: 304–310, 2010.

KEY WORDS: CYTOKINES; EXPRESSION; OPG; OSTEOBLASTS; PROTEIN; RANKL

Osteoporosis is a complex disease that affects mainly postmenopausal women and has a dramatic impact on health. The cellular interactions between osteoblasts and osteoclasts, mediated in part by the receptor activator of the NF-κB (*RANK*)/*RANK* ligand (*RANKL*)/osteoprotegerin (*OPG*) pathway, are essential for the regulation of bone remodeling. The interaction between *RANKL*, either at the osteoblast cell surface or soluble [Lacey et al., 1998; Yasuda et al., 1998], and its receptor *RANK* [Anderson et al., 1997] on the membrane of osteoclast precursors initiates a cascade of signaling events [Wong et al., 1997; Darnay et al., 1998], resulting

Grant sponsor: Carlos III Health Institute (ISCIII), Spanish Ministry of Science and Innovation; Grant number: PI030708; Grant sponsor: Spanish Ministry of Education and Science; Grant number: SAF2007-64654; Grant sponsor: Catalan Government; Grant numbers: 2005SGR 00848, 2009SGR 971.

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Received 19 October 2009; Accepted 15 January 2010 • DOI 10.1002/jcb.22538 • © 2010 Wiley-Liss, Inc.
Published online 11 March 2010 in Wiley InterScience (www.interscience.wiley.com).

in the differentiation of these precursors to form mature osteoclasts. *OPG*, an osteoblast-secreted glycoprotein of the tumor necrosis factor receptor superfamily [Simonet et al., 1997], acts as a decoy receptor and blocks the interaction between *RANKL* and *RANK*, thus inhibiting osteoclastogenesis [Hofbauer and Heufelder, 2001]. Imbalances between *OPG* and *RANKL* mRNA levels alter the equilibrium between bone formation and bone resorption. Regulation of *OPG* and *RANKL* gene expression by osteoblasts has been extensively studied. Many known cytokines and hormones have been found to regulate either *OPG* or *RANKL*, or both, and in comparable or opposite directions [Hofbauer and Heufelder, 2001]. Moreover, the effects of these cytokines and hormones differ by cell source [Hofbauer et al., 1998].

Interleukin-1β (IL-1β) is mainly produced by activated monocytes and macrophages. It stimulates bone resorption and enhances prostaglandin E_2 (PGE_2) production in several cell types, including calvarial osteoblasts [Dinarello, 1988]. PGE_2 plays an important role in inflammatory processes, including osteoporosis and periodontitis. It has been shown to stimulate bone resorption in vivo and osteoclastogenesis in cell culture [Raisz, 1999]. Unexpectedly, though, IL-1β was found to stimulate *OPG* expression [Hofbauer et al., 1998, 1999; Sakata et al., 2002]. Transforming growth factor-β1 (TGF-β1) is a cytokine that plays a major role in the regulation of bone formation and resorption [Centrella et al., 1994]. TGF-β1 induces *OPG* expression in osteoblastic cells, inhibiting osteoclast differentiation and survival [Murakami et al., 1998; Takai et al., 1998] and bone resorption in fetal rat bone cultures [Hofbauer et al., 1999]. In this study, we hypothesized that these cytokines, IL-1β, PGE_2, and TGF-β1, could act differently to modulate the *OPG*/*RANKL* ratio in an osteoporotic environment. Thus, we compared their effects on the expression of *OPG* and *RANKL* in primary cultures of human osteoblast (hOB) cells from osteoporotic and nonosteoporotic subjects.

MATERIALS AND METHODS

SUBJECTS

Unselected postmenopausal women scheduled for knee replacement surgery at the orthopedic outpatient clinic were asked to participate in the protocol. After informed consent was obtained, a bone densitometry was performed, and women were classified as osteoporotic or normal according to the WHO criteria (osteoporosis T-score $= < -2.5$; normal T-score > -1.0). All the postmenopausal women had no history of bone, metabolic, or endocrine diseases. They were neither treated with any drug or hormone-replacement therapy that could affect bone mass. Eight osteoporotic and 11 normal (nonosteoporotic) subjects were finally selected. This study was approved by the Local Ethics Committee of our institution.

CELL CULTURE

hOB cells were obtained from bone specimens extracted from knee samples otherwise discarded at the time of orthopaedic surgery in postmenopausal women, as described previously [Garcia-Moreno et al., 2002; Velasco et al., 2009]. Briefly, the trabecular bone was separated and cut into small fragments, washed in phosphate-buffered solution (PBS) to remove nonadherent cells, and placed on a petri dish. Samples were incubated in Dulbecco's Modified Eagle medium (DMEM; Gibco®; Invitrogen, Paisley, Scotland, UK) supplemented with sodium pyruvate (1 mM), L-glutamine (1 mM), 1% penicillin/streptomycin, 10% fetal calf serum (FCS), 0.33% fungizone, and 1.66% ascorbic acid. At confluence, the cells were subcultured twice to obtain larger numbers of cells. If not used immediately in experiments, the cells were frozen in liquid nitrogen with dimethyl sulfoxide (DMSO) until use. For the experiments, the cells were plated in the aforementioned medium on six-well dishes. At confluence, the medium was replaced by the same medium without FCS and supplemented with 2% bovine serum albumin (BSA) to synchronize the cells. After 2 days, the cells were refed with 1 ml of this medium containing the agonists, IL-1β (10^{-9} and 10^{-8} M), PGE_2 (10^{-8} and 10^{-7} M), and TGF-β1 (10^{-10} and 10^{-9} M), and incubated for 8 and 24 h. These concentrations were within the range of those previously used to test the effects of several cytokines and growth factors in various human and nonhuman bone cell preparations [Brandstrom et al., 1998; Hofbauer et al., 1998; Fuller et al., 2000]. For each treatment, concentration, and time, three replicate plates were used.

EXTRACTION OF TOTAL CELL RNA AND REAL-TIME PCR

Total cell RNA was prepared from cultured hOB cells by the Tri Reagent method, according to the manufacturer's protocol (Molecular Research Center, Inc., Cincinnati, OH). Reverse transcription reaction for cDNA synthesis was performed using 14 μl of total RNA, 2 μl of 10× RT buffer, 2 μl of 10× random primers, 1 μl of 100 mM dNTPs mix, 0.4 μl of RNAse inhibitors, and 1 μl of multiscribe reverse transcriptase (Applied Biosystems, Foster City, CA). The total volume of the reaction was 20 μl. The extension reaction was performed at 25°C for 10 min and then at 37°C for 120 min. To check for the existence of cDNA we performed β-actin PCR as a constitutive control; cDNA was kept at −20°C.

Real-time PCR amplification was performed with the ABI PRISM 7900 HT Sequence Detector (Applied Biosystems). Taqman assays were used to quantify *OPG* and *RANKL* expression (Hs00171068 and Hs00243522, respectively; Assays-on-Demand Gene Expression, Applied Biosystems). The general housekeeping gene *ACTB* (Hs99999903) was analyzed in every sample; for further statistical analysis, the *ACTB* level was used as an endogenous control for data normalization. Samples were assayed in triplicate in an optical plate in a final volume of 20 μl. All reactions consisted of 2 μl of cDNA, 10 μl of × Taqman Universal PCR Master Mix, 900 nM concentrations of each of the primers, and 250 nM concentration of the fluorescent probe. Samples of cDNA for *OPG* and *ACTB* measurements were diluted 10-fold because levels were highly concentrated. The PCR mixture was incubated for 2 min at 50°C for AmpErase™ uracil-*N*-glycosylase-mediated decontamination, followed by 10 min at 95°C to activate AmpliTaq Gold DNA polymerase. Subsequently, 40 cycles of a 15-s denaturation step at 95°C and a combined annealing–extension step of 1 min at 60°C were performed. Relative quantification studies of collected data (threshold cycle, C_t, numbers) were performed with Sequence Detector software (SDS 2.1; Applied Biosystems). As a result of the large number of parameters analyzed (three cytokines, at two concentrations and two time points), each sample was processed separately

and the data from nontreated cultures at 8 h were used as control to normalize the results for relative quantification. Final results are thus expressed as percentages of the corresponding nontreated cell culture. This allows us to compare among the different samples tested, treated for different days. One additional real-time PCR plate with only cDNA from nontreated osteoblasts from all subjects was prepared to analyze the differences in *RANKL* and *OPG* expression between osteoporotic and nonosteoporotic hOB cells. Values from osteoporotic hOB cell cultures were pooled and the corresponding mean was used to normalize those from each nonosteoporotic hOB cell culture. A mean was then calculated for all the normalized nonosteoporotic cell cultures.

ENZYME-LINKED IMMUNOSORBENT ASSAY

This method was used to quantify the amounts of *OPG* protein in hOB cell culture supernatants. Enzyme-linked immunosorbent assay (ELISA) plates were from Biomedica (Biomedica Gruppe, Wien, Austria), and the procedure was according to the manufacturer's protocol. Quantification was performed by measuring absorbances at 450 nm and 620 nm (as a reference). Duplicates of each sample were analyzed.

STATISTICAL ANALYSIS

Statistical analysis was performed using SPSS for Windows, release 12.0 (SPSS, Chicago, IL). Comparisons between two groups (osteoporotic and nonosteoporotic cell cultures) were made by using nonparametric exact Mann–Whitney U-test; the nonparametric exact Wilcoxon test was used for comparisons between total (normal plus osteoporotic) nontreated and treated cell cultures. For all analyses, a two-tailed nominal *P*-value less than 0.05 was considered significant.

RESULTS

GENE EXPRESSION

Basal expression of *OPG* and *RANKL* in hOB cells from osteoporotic (n = 8) and nonosteoporotic (n = 11) bone samples is shown in Figure 1. Osteoporotic hOB cells had a lower mean value for *OPG* mRNA levels than the nonosteoporotic hOB cells, but the difference was not statistically significant ($P = 0.397$); meanwhile, *RANKL* mRNA levels were similar in both hOB cell groups ($P = 0.232$). Figures 2 and 3 show the response capacity of osteoporotic and nonosteoporotic hOB cells to IL-1β, PGE_2, and TGF-β1. All the data were normalized to those in nontreated cultures at 8 h post-treatment; thus, gene expression levels are relative to those in nontreated cultures and expressed as fold induction. These results show a different response dependent on the hOB cell group tested. Hence, a significant increase in *OPG* gene expression was induced by IL-1β in all conditions used (cytokine concentration and time of treatment) in both osteoporotic and nonosteoporotic hOB cells (Fig. 2A). However, nonosteoporotic hOB cells showed a significantly higher response to 10^{-9} M IL-1β treatment than those from osteoporotic subjects at both 8 h ($P = 0.033$) and 24 h ($P = 0.009$). With 10^{-8} M of IL-1β, a similar trend was observed, although there were no significant differences among the hOB cell types (Fig. 2A).

In normal hOB cells, PGE_2 treatment showed a tendency to increase *OPG* mRNA levels at 8 h and to decrease them at 24 h, at both doses tested; although these effects did not reach statistical significance. Meanwhile, in osteoporotic hOB cells, PGE_2 failed to affect *OPG* mRNA levels.

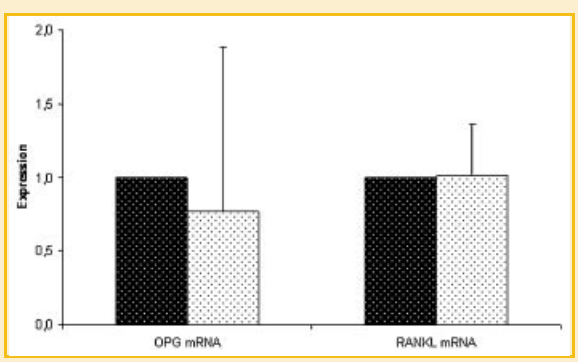

Fig. 1. *OPG* and *RANKL* gene expression in hOB cell cultures from nonosteoporotic (n = 11; black bars) and osteoporotic (n = 8; white bars) subjects. Corresponding levels in the osteoporotic group were normalized against those in the nonosteoporotic group. Results are means ± SD.

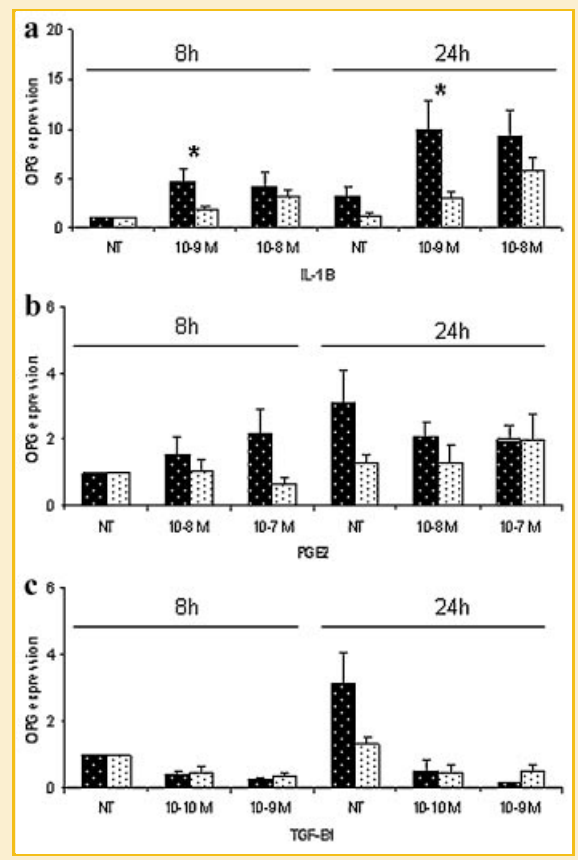

Fig. 2. *OPG* gene expression in response to IL-1β (a), PGE_2 (b), and TGF-β1 (c) treatment in nonosteoporotic (n = 11; black bars) and osteoporotic (n = 8; white bars) hOB cells, at 8 and 24 h post-treatment. Data are expressed relative to those in corresponding untreated cultures at 8 h (represented as NT). * $P < 0.05$ versus osteoporotic cell cultures.

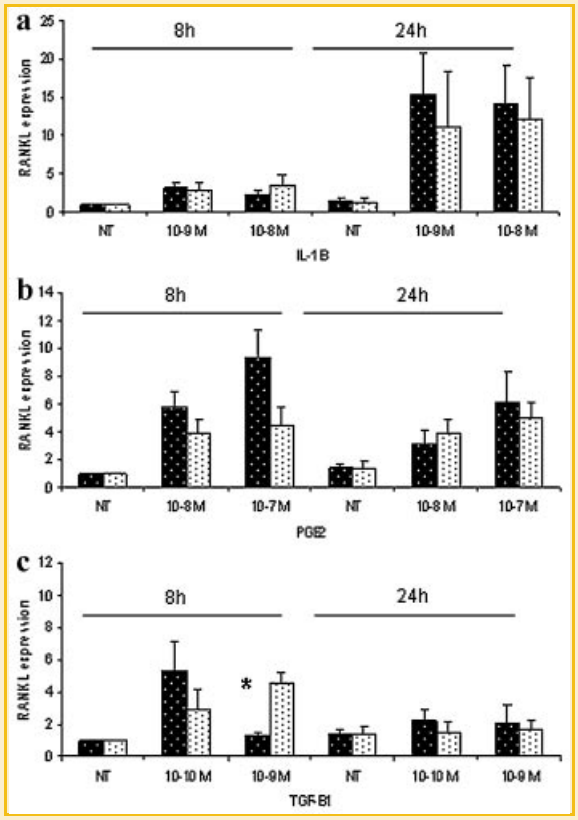

Fig. 3. *RANKL* gene expression in response to IL-1β (a), PGE_2 (b), and TGF-β1 (c) treatment, in nonosteoporotic (n = 11; black bars) and osteoporotic (n = 8; white bars) hOB cells and osteoporotic (n = 8) cell lines, at 8 and 24 h post-treatment. Data are expressed relative to those in corresponding untreated cultures at 8 h (represented as NT). $^*P < 0.05$ versus osteoporotic cell cultures.

When both types of hOB cells were treated with TGF-β1, a significant decrease ($P < 0.001$) in *OPG* expression was observed, corresponding to a two- and threefold reduction at 10^{-10} and 10^{-9} M doses, respectively, at 8 h; after 24 h, this decrease induced was even higher (Fig. 2C). No differences in this response were found between osteoporotic and nonosteoporotic hOB cell cultures (Fig. 2C).

Regarding *RANKL* expression, an increase in its mRNA levels was observed for all cytokine treatments examined (Fig. 3). In the case of IL-1β, this response was time-dependent and up to 10-fold after 24 h ($P < 0.0001$; Fig. 3A). When we compared *RANKL* gene expression in osteoporotic and nonosteoporotic hOB cells, a significant difference was found after 8 h of exposure to 10^{-9} M of TGF-β1; so that osteoporotic hOB cells expressed fivefold higher *RANKL* mRNA levels than these nonosteoporotic cells ($P = 0.002$; Fig. 3C). After 24 h, this effect was lost. For the other conditions tested, no differences were found between osteoporotic and nonosteoporotic hOB cell cultures (Fig. 3A,B).

The *OPG*/*RANKL* ratio was calculated from relative quantification data for each sample. The corresponding mean values for nonosteoporotic and osteoporotic hOB cells in untreated cultures were 23.30 ± 5.62 and 15.42 ± 2.21, respectively. Therefore, nonosteoporotic hOB cells had a higher ratio of *OPG* to *RANKL* expression than osteoporotic hOB cells, although the difference was not significant. We next analyzed this *OPG*/*RANKL* ratio for each cytokine treatment tested (Fig. 4). All treatments significantly affected the *OPG*/*RANKL* mRNA ratio. IL-1β increased this ratio, whereas PGE_2 and TGF-β1 caused a significant decrease, with respect to that of the untreated cells (Fig. 4). When nonosteoporotic and osteoporotic hOB cells were compared, the former cells showed a significantly higher ratio than the latter with 10^{-9} M of TGF-β1 treatment for 8 h ($P = 0.035$; Fig. 4C). The effects of IL-1β and PGE_2 did not differ for these two cell sources (Fig. 4A,B).

PROTEIN SYNTHESIS

The changes in OPG protein levels after the different treatments were consistent with those observed in mRNA levels (Table I). However, we could not detect any significant differences in these levels between nonosteoporotic and osteoporotic hOB cells.

DISCUSSION

The *RANK*/*RANKL*/*OPG* system, in conjunction with various cytokines, growth factors, and calciotropic hormones, plays a pivotal role in bone remodeling. Deregulation of this system has been implicated in the pathophysiology of multiple bone remodeling disorders [Vega et al., 2007]. In this study, we examined the

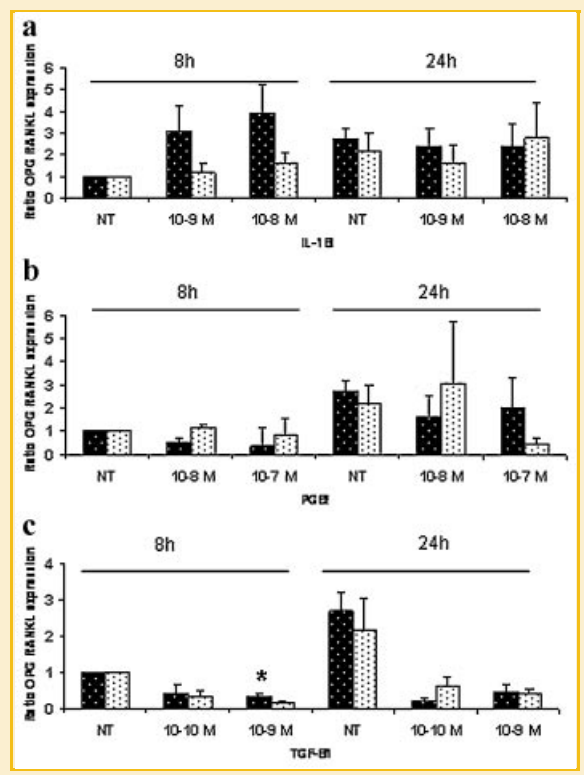

Fig. 4. *OPG*/*RANKL* mRNA ratio in response to IL-1β (a), PGE_2 (b), and TGF-β1 (c) in nonosteoporotic (n = 11; black bars) and osteoporotic (n = 8; white bars) hOB cells. Data are expressed relative to those in corresponding untreated cultures at 8 h (represented as NT). $^*P < 0.05$ versus osteoporotic cell cultures.

TABLE I. *OPG* Protein Quantification by ELISA in Response to IL-1β, PGE_2, and TGF-β1 Treatment, in Nonosteoporotic (n = 11) and Osteoporotic (n = 8) Cell Lines, at 8 and 24 h Post-Treatment

Treatment	Nonosteoporotic	Osteoporotic	$P < 0.05$ (vs. NT)
8 h			
NT	22.93 ± 9.52	24.70 ± 13.53	
IL-1β (10^{-8})	30.05 ± 9.87	29.16 ± 10.85	Yes
IL-1β (10^{-9})	29.11 ± 9.55	26.64 ± 11.13	Yes
PGE_2 (10^{-7})	23.88 ± 6.75	18.54 ± 7.25	No
PGE_2 (10^{-8})	22.84 ± 8.78	18.01 ± 9.36	No
TGF-β1 (10^{-9})	19.66 ± 9.80	21.62 ± 13.22	Yes
TGF-β1 (10^{-10})	19.56 ± 7.97	20.46 ± 10.98	Yes
24 h			
NT	25.58 ± 8.87	30.70 ± 13.12	
IL-1β (10^{-8})	35.52 ± 8.07	37.60 ± 15.23	Yes
IL-1β (10^{-9})	35.39 ± 10.61	34.72 ± 16.70	Yes
PGE2 (10^{-7})	32.35 ± 6.94	20.23 ± 10.69	No
PGE2 (10^{-8})	28.41 ± 7.85	26.22 ± 15.47	No
TGF-β1 (10^{-9})	20.13 ± 10.03	26.11 ± 12.75	Yes
TGF-β1 (10^{-10})	21.11 ± 9.15	25.63 ± 11.78	Yes

*P-values below 0.05 when nontreated (NT) cell cultures were compared with treated cell cultures.
Data are expressed as means (pmol/L) ± standard deviations.

putative differences in the response of this system to IL-1β, PGE_2, and TGF-β1, all of which are important local regulators of bone remodeling, in osteoporotic and nonosteoporotic hOB cells.

Previous studies have assessed potential gene expression differences between osteoporotic and nonosteoporotic cells. Balla et al. [2008] searched for different gene expression patterns in bone tissue from osteoporotic and nonosteoporotic women, with a sample size similar to that in our study, and found no differences in the basal amount of *OPG* and *RANKL* mRNA between both types of subjects. Although our results here using primary hOB cells are consistent with these previous findings, we observed a trend toward less *OPG* mRNA in osteoporotic hOB cells than in the nonosteoporotic cells. Still, osteoporotic hOB cells might behave in an altered way in response to an inflammatory challenge.

In this study, we demonstrate that osteoporotic and nonosteoporotic hOB cells display different gene expression levels of *OPG* and *RANKL* after treatment with IL-1β and TGF-β1, respectively. PGE_2 affects bone turnover stimulating bone resorption and promoting osteoclast formation. This effect seems to be osteoblast-dependent and the results in our experiments confirm that hOB from osteoporotic patients express less OPG after response to PGE_2 than nonosteoporotic patients [Brandstrom et al., 1998]. However, this increase did not reach statistical significance perhaps because of a lack of power. A submaximal dose of IL-1β induces a greater increase of *OPG* expression in nonosteoporotic hOB cells than in these osteoporotic cells, suggesting that the former cells might be better protected, against a proresorptive stimulus, than the latter. However, at a higher IL-1β dose (10^{-8} M) the differences in OPG mRNA levels between normal and osteoporotic hOB cells were not significant, further confirming that the latter are less efficient in this regard. However, PGE_2 failed to significantly affect OPG expression in these cells, suggesting that IL-1β did not act through PGE_2 to increase OPG in hOB cells. In this regard, a previous study showed that IL-1β increased OPG expression at a transcriptional level in a PGE_2-independent manner in human periodontal ligament cells [Sakata et al., 2002]. In addition, in this study, osteoporotic hOB cells showed a significantly higher stimulation of RANKL expression by TGF-β1 than the nonosteoporotic cells within 8 h. Thus, whereas the latter expression was not affected by a submaximal dose (10^{-9} M) of TGF-β1 in nonosteoporotic hOB cells, it was increased in those from osteoporotic subjects. *RANKL* expression was also increased by IL-1β and PGE_2 treatments, although no differences were found between osteoporotic and nonosteoporotic cells. Moreover, the highest stimulation by IL-1β was at 24 h, whereas that of PGE_2 occurred earlier (at 8 h). Thus, it is likely that IL-1β might induce PGE_2 synthesis, which would subsequently result in *RANKL* transcription.

Our *OPG* protein results were consistent with those of mRNA expression, although no differences were observed between both types of hOB cells evaluated. This might have been owing to several reasons. The effects of treatments on protein synthesis, however, are usually slower than those on gene expression, and thus putative differences in the former may have needed longer times than those used here to be disclosed. Moreover, real-time PCR is able to detect very small differences in gene expression whereas ELISA is not such a sensitive technique to detect small differences in protein expression.

A limitation of this study is the sample size. hOB cell cultures were obtained from 8 osteoporotic and 11 nonosteoporotic patients. We found a great deal of variability among individual cell cultures in each group, and this might be the reason why some of our differences between both groups studied were trends but failed to reach significance. However, an important strength of this work is the use of ex vivo primary cultures of hOB cells which are a good model to approximate the pathophysiologic conditions in humans, as compared with the use of established osteoblastic cell lines or primary osteoblasts from other species. In this regard, various studies have examined the effects of the bone cytokines examined herein on *OPG* and *RANKL* in different osteoblastic cell preparations, including hOB cells [Hofbauer et al., 1998, 1999; Li et al., 2002; Liu et al., 2006; Sakata et al., 2002]. Our results are consistent with the majority of these studies, showing that IL-1β increases *OPG* and *RANKL* gene expression whereas PGE_2 increases *RANKL* gene expression in these cells. However, some of these previous studies do not support our results regarding the effect of TGF-β1 on *OPG* and *RANKL* gene expression and that of PGE_2 on *OPG*. Thus, TGF-β1 was shown to exert an opposite action on *OPG* and *RANKL* than that reported previously in hOB cells [Takai et al., 1998]. Moreover, in our study, PGE_2 failed to significantly affect *OPG* gene expression whereas other studies found an inhibitory effect [Brandstrom et al., 1998; Sakata et al., 2002; Liu et al., 2006]. Different cell sources and/or different cell culture environments might explain these apparently opposite results [Torricelli et al., 2003]. In this regard, Hofbauer et al. [1998] have suggested that the osteoblastic cell type seems to determine the response capacity to a hormone or cytokine. Moreover, it is well known that the differentiation status of osteoblastic cells determines the gene expression levels of specific osteoblastic genes; consequently, it would affect the response to external stimuli. In fact, another report using primary mouse OB cell cultures [Li et al., 2002] also showed that PGE_2 did not affect *OPG* transcription.

A theoretical issue might be if some of the donors had been treated with NSAID before the surgery, hypothesis that seems plausible given the underlying process (OA) as this might interfere with our experiments. However, the potential effect of such a treatment on the *OPG* expression in our cell culture system is negligible because the required concentrations are much higher [Kolar et al., 2009]. Therefore we do not consider that NSAID, which indeed play a role in the PG-mediated phenomena in clinics, may have jeopardized our results.

In this study, we also examined the cytokine-induced changes in the *OPG*/*RANKL* mRNA ratio, as an unbalanced ratio is more important than changes of each individual component to predict osteoclast activation. All treatments used in this study were found to significantly alter this ratio. In this manner, our results further confirm that these cytokines, namely IL-1β, PGE_2, and TGF-β1, can affect bone resorption through the modulation of the *OPG*/*RANKL* ratio. In addition, only TGF-β1 was shown to alter osteoporotic and nonosteoporotic hOB cells differentially, suggesting that the presence of TGF-β1 is more detrimental in an osteoporotic tissue environment than in healthy tissue.

In summary, this study shows, for the first time to our knowledge, the comparative effects of three important bone cytokines on *OPG* and *RANKL* expression in primary hOB cells isolated from postmenopausal women. Our original findings demonstrate that osteoporotic hOB cells respond differently to these cytokines than these nonosteoporotic cells. These results add new data to previous studies performed with different osteoblastic cell sources and help to clarify the interaction between several pro-inflammatory cytokines and the *RANK*/*RANKL*/*OPG* pathway.

ACKNOWLEDGMENTS

This study was supported by grants from Carlos III Health Institute (ISCIII), Spanish Ministry of Science and Innovation (PI030708), the Spanish Ministry of Education and Science (SAF2007-64654), and the Catalan Government (2005SGR 00848 and 2009SGR 971). The authors also thank the support from CIBER de Enfermedades Raras (CIBERER) and the Red Temática de Investigación Cooperativa en Envejecimiento y Fragilidad (RETICEF), which are initiatives of the ISCIII.

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