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Osteogenic Growth Peptide Effects on Primary Human Osteoblast Cultures: Potential Relevance for the Treatment of Glucocorticoid-Induced Osteoporosis

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Abstract The osteogenic growth peptide (OGP) is a naturally occurring tetradecapeptide that has attracted considerable clinical interest as a bone anabolic agent and hematopoietic stimulator. In vivo studies on animals have demonstrated that the synthetic peptide OGP (10–14), reproducing the OGP C-terminal active portion [H-Tyr-Gly-Phe-Gly-Gly-OH] increases bone formation, trabecular bone density and fracture healing. In vitro studies performed on cellular systems based on osteoblastic-like cell lines or mouse stromal cells, have demonstrated that OGP (10–14) increases osteoblast proliferation, alkaline phosphatase (ALKP) activity and matrix synthesis and mineralization. In view of a potential application of OGP (10–14) in clinical therapy, we have tested different concentrations of OGP (10–14) on primary human osteoblast (hOB) cultures. We have observed significant increases of hOB proliferation (+35%), ALKP activity (+60%), osteocalcin secretion (+50%), and mineralized nodules formation (+49%). Our experimental model based on mature hOBs was used to investigate if OGP (10–14) could prevent the effects on bone loss induced by sustained glucocorticoid (GC) treatments. A strong decrease in bone formation has been attributed to the effects of GCs on osteoblastogenesis and osteocyte apoptosis, while an increase in bone resorption was due to a transient osteoblastic stimulation, mediated by the OPG/RANKL/RANK system, of osteoclasts recruitment and activation. Moreover, GCs act on hOBs decreasing the release of osteoprotegerin (OPG) a regulator of the RANKL/RANK interaction. Here, we provide evidences that OGP (10–14) inhibits hOB apoptosis induced by an excess of dexamethasone (−48% of apoptotic cells). Furthermore, we show that OGP (10–14) can increase OPG secretion (+20%) and can restore the altered expression of OPG induced by GCs to physiological levels. Our results support the employment of OGP (10–14) in clinical trials addressed to the treatment of different bone remodeling alterations including the GC-induced osteoporosis. J. Cell. Biochem. 98: 1007–1020, 2006.

Key words: osteogenic growth peptide; osteoblasts; glucocorticoids; osteoporosis; bone formation; osteoprotegerin; osteoblast apoptosis

The osteogenic growth peptide (OGP), initially isolated from the osteogenic phase of postablation regenerating bone marrow, is a naturally occurring tetradecapeptide that has attracted considerable clinical interest as a bone anabolic agent and hematopoietic stimulator [Bab and Chorev, 2002].

OGP sequence is identical to the C-terminal amino acid sequence 89–102 (H-Ala-Leu-Lys-Arg-Gln-Gly-Arg-Thr-Leu-Tyr-Gly-Phe-Gly-Gly-OH) of histone H4 (H4) [Bab et al., 1992]. Endogenous OGP is a proteolytic cleavage product of PreOGP translated from H4 mRNA via alternative translational initiation at a downstream initiation codon [Bab et al., 1999]. OGP in high abundance occurs physiologically in human and rodent serum and in serum-free

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medium of osteoblastic and fibroblastic cells. It is mainly found as an inactive complex with α2-macroglobulin (α2 M) [Gavish et al., 1997]. Following its dissociation from the complex with α2 M, the peptide is proteolytically cleaved, thus generating the C-terminal pentapeptide H-Tyr-Gly-Phe-Gly-Gly-OH [OGP (10–14)], which activates an intracellular Gi-protein-MAP kinase signaling pathway [Gabarin et al., 2001].

It has been recently demonstrated that the systemical administration to rats and mice of synthetic OGP (10–14), or some of its analogs, increases bone formation and trabecular bone density and stimulates fracture healing [Gabet et al., 2004]. In vivo it also regulates the expression of type I collagen, transforming growth factors beta1, beta2, and beta3, fibroblast growth factor-2, insulin-like growth factor-1 and aggrecan [Brager et al., 2000]. In addition, OGP and OGP (10–14) enhance hematopoiesis, including the stimulation of bone marrow transplant engraftment and hematopoietic regeneration after ablative chemotherapy [Gurevitch et al., 1996; Fazzi et al., 2002].

In vitro studies regarding the osteogenic properties of OGP (10–14) have been mostly performed on cellular systems based on osteoblastic-like cell lines of murine or human origins or by human and mouse marrow stromal cells [Robinson et al., 1995; Greenberg et al., 1997]. In these experiments OGP (10–14) seems to regulate cell proliferation, alkaline phosphatase activity, and matrix mineralization via an autoregulated feedback mechanism. In view of the potential important application of OGP (10–14) in the clinical treatment of human skeletal diseases, we have assumed that a cell system composed by human bone cells in an advanced stage of differentiation could be helpful for the evaluation of particular aspects of the OGP activity.

With this aim, we have evaluated the biological effects of different concentrations of OGP (10–14) on different primary human osteoblast (hOB) cultures at their first passage, obtained from up to 10 different human bone specimens. The treated hOB cultures were analyzed measuring in different periods of time, the cellular proliferation and differentiation, in terms of alkaline phosphatase (ALKP) activity, osteocalcin (OCN) production, and bone-nodules formation and mineralization.

In order to demonstrate if OGP (10–14) could be effective as a regulator of altered mechanisms of bone homeostasis that were evidenced in particular bone disorders, we have assayed if OGP (10–14) could restore some pathogenic mechanisms retained to be involved in osteoporosis induced by glucocorticoids (GCs). It is generally accepted that GCs can cause a rapid bone loss decreasing bone formation and increasing bone resorption in vitro as well as in vivo [reviews: Delany et al., 1994; Weinstein and Manolagas, 2000, Weinstein, 2001; Zalavras et al., 2003; Xing and Boyce, 2005]. The decrease in bone formation has been mainly attributed to GC effects on osteoblastogenesis and osteocyte apoptosis, while the increase in bone resorption has been referred to an increase of osteoclast activity and proliferation. In the last years, it has been demonstrated that osteoclastogenesis and osteoclast activation can be stimulated through the interaction of [receptor activator of NF-KB]-ligand (RANKL), a member of the TNF ligand family expressed on the surface of mesenchimal cells (MSC) of the osteoblast lineage, with its physiological receptor [receptor activator of NF-KB] (RANK), expressed on the surface of osteoclast lineage cells [Li et al., 2000; Khosla, 2001; Hofbauer et al., 2004]. This binding is regulated by osteoprotegerin (OPG), a neutralizing soluble decoy receptor, produced by MSCs and osteoblasts [Simonet et al., 1997]. The relative ratio of RANKL to OPG is considered the critical determinant and final step in the regulation of osteoclast biology and bone resorption, and its ratio is modulated by various osteotropic hormones, cytokines and drugs. Of note, some studies regarding the GCs effects on OPG and RANKL expression in various human osteoblast lineage cells have shown that GCs such as dexamethasone, can strongly inhibit OPG mRNA expression and protein secretion and concurrently stimulate RANKL mRNA expression [Hofbauer et al., 1999].

In this work, we present evidence that OGP (10–14) administration to hOB cultures could prevent apoptosis of mature osteoblasts induced by a high dose of dexamethasone (5×10^{-5} M). Furthermore, we show that OGP (10–14) can increase OPG synthesis and can restore the alteration of the expression of OPG and RANKL induced by GCs.

MATERIALS AND METHODS

Bone Samples

Bone samples were obtained from 10 women and men (aged 56–78 years, with a mean age of 66 years) who underwent total hip replacement surgery for degenerative joint disease. The patients were selected excluding those who had sex steroid deficiency or had received a previous therapy with bisphosphonates, hormone replacement, or GC treatment during the previous 2 years. The experiments were conducted using cells from individual patients, and the specimens were not pooled. First-passage human osteoblastic cells (hOB) from primary cultures of trabecular bone explants were used.

OGP (10–14) Synthetic Peptide

The synthetic linear pentapeptide OGP (10–14), (Tyr-Gly-Phe-Gly-Gly; MW: 499.7), was kindly provided by ABIOGEN Pharma Research Laboratories (Pisa, Italy). The pentapeptide was prepared according to standard solid-phase peptide synthesis methodology [Chen et al., 2000].

Cell Cultures

Fragments of trabecular bone were washed extensively in PBS to remove blood and bone marrow, and then explanted into culture in Dulbecco's modified Eagle's Medium (DMEM) (Gibco, Life Technologies, UK) supplemented with 10% FCS (Sigma, USA), 2 mM L-glutamine (Gibco, Life Technologies). The cultures were incubated at 37°C in a humidified atmosphere of 7% CO_2/93% air [Gallagher et al., 1996]. Cells were maintained by removing the conditioned medium and replacing it with fresh DMEM supplemented with 10% FCS every 7 days. After 3–6 weeks in culture, a cellular confluent monolayer had grown out from the bone fragments. The primary cell layers (E1) were washed in PBS and then treated for 5 min with a 0.05% trypsin/0.02% EDTA solution (Gibco, Life Technologies). The detached cells were passaged at a density of 60,000 cell/well into a 12-well multiplate (Corning Costar Corp.) and cultured in DMEM with 10% FCS until confluence (E1P1). The medium was changed twice a week, and after this period, the cells were washed in PBS and incubated for further 48 h with serum-free medium supplemented with 0.1% BSA (Sigma). During drug treatment, each culture medium was changed twice a week, adding 1 ml of DMEM with 10% FCS containing different concentrations of OGP (10–14). The effect of each OGP (10–14) concentration was tested in triplicate for each different cell culture.

Proliferation Assay

The proliferative response of osteoblast cells to different OGP concentrations (10^{-13}–10^{-8} M) was examined by a colorimetric assay system based on tetrazolium salts (MTT, Boehringer Mannheim, Germany) reduction [Testi et al., 1995]. Briefly, each hOB culture was incubated with 0.5 mg/ml 3-(4,5-dimethylthiazol-2-yl)-2,5-dyphenyl-tetrazolium bromide labeling reagent for 4 h in a humidified atmosphere. The resultant purple formazan salt crystals were solubilized with sodium dodecyl sulfate (SDS) in 10 mM HCl. After 1 h of incubation, the solubilized formazan product was spectrophotometrically quantified. An increase in the number of living cells resulted in the increased total metabolic activity in the sample. This increase was directly correlated to the amount of purple formazan crystals formed, as monitored by the absorbance.

Biochemical ALKP Assay

The ALKP activity was measured directly on monolayer cultures. The medium was removed, and the cells were washed three times with PBS and shaken for 30 min at 37°C in 1 ml of 10 mM *p*-nitrophenilphosphate (Sigma). The *p*-nitrophenylphosphate solution was removed and the reaction was stopped by adding 1 ml 1N NaOH. The optical density was measured at 405 nm [Rattner et al., 1997]. The ALKP activity values were normalized to the relative number of viable cells as determined in 12-well plates using the above-mentioned proliferation assay.

Measurement of Osteocalcin Production

Osteocalcin production was measured in supernatants of hOB cultures treated with different concentrations of OGP (10–14). After the attainment of semi-confluence, hOB cultures were washed and incubated in serum free medium containing 10 mg/ml BSA with 5 ng/ml $1,25(OH)_2D_3$. After 72 h, the medium of each culture was removed and assayed using a commercial ELISA kit (h-Ost EASIA Kit, BIOSOURCE Europe, Belgium) and following the manufacturer's instructions. The OCN values were normalized to the relative number

of viable cells as determined in parallel experiments using the above-mentioned proliferation assay.

Nodules Formation and Mineralization

The mineralized nodule formation and the degree of mineralization were determined for osteoblast cell cultures grown in 6-well plates for different periods of time (7, 12, 15, and 20 days) using alizarin red S staining. Briefly, different hOB cultures were grown in culture medium for with 10% FCS and different concentrations of OGP (10–14), at the day fixed for the assay, the medium was aspirated from the wells, and the cells were rinsed twice with PBS. The cells were fixed with ice-cold 70% (v/v) ethanol for 1 h; the ethanol was removed, and the cells were rinsed twice with deionized water. The cells were then stained with 40 mm alizarin red S (Sigma, USA) in deionized water (adjusted to pH 4.2) for 10 min at room temperature. The alizarin red S solution was removed by aspiration, and the cells were rinsed five times with deionized water. The water was removed by aspiration, and the cells were incubated in PBS for 15 min at room temperature on an orbital rotator. The PBS was removed, and the cells were rinsed once with fresh PBS. After counting of the alizarin red-positive nodules present in each well using light microscopy, the cells were destained for 15 min with 10% (w/v) cetylpyrinium chloride in 10 mM sodium phosphate (pH 7.0). The extracted stain was then transferred to a 96-well plate, and the absorbance at 562 nm was measured using a plate/reader spectrophotometer. For each culture assayed, the mineralization degrees were normalized to the number of viable cells calculated in parallel experiments.

FACS Analyses of hOB Apoptosis Induced by Dexamethasone

To quantify the percentage of apoptotic cells by analysis of DNA content, we used the flow cytometric method described by Nicoletti et al. [1991]. Different hOB cultures, obtained in 6-well plates, were pretreated or not with various concentrations of OGP (10–14) (10^{-12}, 10^{-9}, and 10^{-7} M). After 12 h, dexamethasone was added to each culture to obtain a final concentration of 5×10^{-5} M and cells were incubated for additional 72 h. About 1×10^{6} cells for each treated culture were washed 3 times in PBS and fixed overnight in ice-cold 70% ethanol at −20°C. Then, the cell suspension was centrifuged, washed twice with 1 ml of PBS and resuspended in 1 ml of a PBS solution containing ribonuclease (Type-1A, Sigma, 1 mg/ml final concentration) and propidium iodide (PI) (Sigma, 50 μg/ml final concentration). The tubes were placed on ice in the dark until the cellular orange fluorescence of PI was collected in a linear scale using a FACS-calibur flow cytometer (Becton Dickinson, USA) equipped with an excitation laser line at 488 nm and a 575 ± 15 nm band pass filter. At least 20,000 events were collected for each sample using the Cell Quest software (Becton Dickinson) and the pulse processing module for doublet discrimination; debris was excluded from the analysis by an appropriate morphological gate of forward scatter versus side scatter.

Detection of Apoptotic Cells Using the TUNEL Reaction

The TUNEL method was used to detect apoptotic cells [Gavrieli et al., 1992] Primary hOB cultures were grown in monolayer using sterile shell vials (Sarstedt, Germany) containing a circular glass slide (diameter: 1 cm) in the bottom. One milliliter of cell suspension (50,000 cells/ml in DMEM with 10% FCS) was seeded in each vial and incubated for 24 h at 37°C. The mediums of separate cultures were then replaced with others containing different OPG (10–14) concentrations (10^{-13}, 10^{-11}, 10^{-9}, and 10^{-7} M) in DMEM with 10% FCS. After 12 h, dexamethasone was added to each culture to obtain a final concentration of 5×10^{-5} M and cells were incubated for additional 72 h. The apoptotic cells of each culture were then evidenced using the in situ cell death detection kit, POD (Roche Applied Science, Germany) following the manufacturer's instructions. The kit was used for immunohistochemical detection and quantification of apoptosis at single cell level, based on staining of DNA strand breaks with the TUNEL reaction mixture. A second DNA labeling reaction with a specific converter-POD and DAB substrate was performed in order to observe the apoptotic cells under a light microscope.

Osteoprotegerin and RANKL Protein Measurements

Different hOB cultures, grown in 12-well plates in DMEM with 10% FCS, were treated

with different concentrations of OGP (10–14) (from 10^{-15} to 10^{-7} M) in the presence or the absence of 5×10^{-5} M dexamethasone. After 48 h of incubation, conditioned mediums were harvested from cultured cells and centrifuged to remove debris. Samples were stored at -80°C until use. OPG and total soluble RANKL protein secretion were determined in triplicate measurements using two specific ELISA immunoassay kits from Biomedica (Germany). According to manufacturer's instructions, the lower limits for protein detection in cellular supernatants were 0.5 pmol/L for OPG and 0.08 pmol/L for RANKL. The OPG and RANKL values obtained were normalized to the relative number of viable cells.

Statistical Analyses

Data were expressed as mean $\pm$ SD of all cultures that received the same treatment. Differences between the values were tested for statistical significance by analysis of variance (ANOVA) using the Tukey test and the Dunnett test. The Tukey test allows comparison of more than two means at once since this reduces the error associated with multiple t-tests. The Dunnett test allows comparison between the control mean and every other mean in the group. The values of $P < 0.05$ were considered to be statistically significant.

RESULTS

Primary Human Osteoblast Cultures

In this study we analyzed the effects of OGP (10–14) on up to 10 different primary human osteoblast cultures obtained from healthy trabecular bones of patients undergoing total hip replacement surgery. All experiments were performed by adding different concentrations of OGP (10–14) to confluent human osteoblast cultures at their first passage. At this cultural stage, cells have been shown to further differentiate and, according to the time course of osteoblastic differentiation, represent the phenotype within the matrix maturation phase or intermediate phenotype [Siggelkow et al., 1999]. The expression of specific markers for this stage of the osteoblastic differentiation sequence (Cbfa1, alkaline phosphatase, and osteocalcin) has been however checked by RT-PCR (data not shown) as described in a previous paper [Frediani et al., 2004].

Effect of OGP on Primary Human Cell Culture Proliferation

Dose-response and time-course experiments were performed to determine the effects of OGP (10–14) on normal human osteoblast cells proliferation.

In Figure 1 is reported the proliferative response of different human osteoblast cultures grown in culture medium containing 10% FCS and treated with different OGP (10–14) concentrations (10^{-13}–10^{-8} M) for 24 and 72 h. As reported in literature for other cellular experimental models, low doses of exogenous OGP (10–14) seems to enhance cell proliferation tracking a bell-shaped dose-response curve. In comparison with hOB cells grown without the addition of OGP (10–14), the proliferation rate of hOB cells stimulated with OGP (10–14) 10^{-12} M increase of the 20% ($P < 0.05$) after 24 h and 35% ($P < 0.05$) after 72 h. According to some authors [Greenberg et al., 1997; Bab and Chorev, 2002], high levels of OGP accumulate in tissue culture medium of cells, which respond to a stimulation with exogenous OGP or OGP (10–14) at very low doses. It was calculated in vitro that after 4 h from the exogenous OGP or OGP (10–14) stimulation, the amount of endogenous OGP increases from two- to five-fold. The bell-shaped proliferation curve is probably due to an autocrine/paracrine mode of regulation consisting of a positive feedback loop at suboptimal to optimal extracellular OGP concentrations followed by negative regulation at supraoptimal concentrations. The endogenous OGP is a precursor of the bioactive OGP (10–14). In our experiments, OGP released in the culture medium probably binds to α2 M or other OGP-binding proteins (OGPB) present in FCS and forms a complex that should present OGP to its target cells. Upon dissociation of the OGP-OGPB complexes, the inactive OGP undergoes proteolytic activation generating the bioactive OGP (10–14), which is able to interact with a yet unidentified receptor and activates the Gi protein MAP kinase cascade.

Biochemical Assay for ALKP

Dose response experiments were performed to examine the effects of OGP on alkaline phosphatase activity in hOB cultures. Different concentrations of OGP (10–14) (from 10^{-13} to 10^{-4} M) were added to cell cultures grown in normal medium supplemented with 10% FCS.

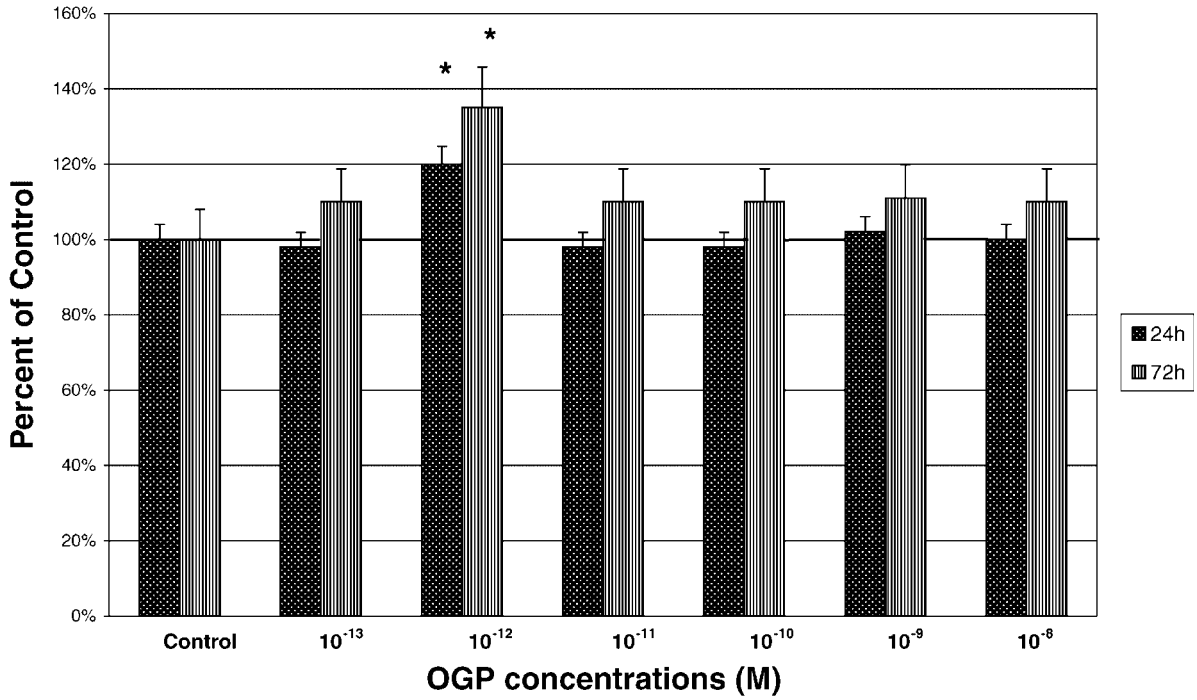

Fig. 1. Effects of different OGP (10–14) concentrations on human osteoblasts proliferation. Cells were seeded in 12-well plates and cultured in normal growth medium with 10% FCS in absence or presence of OGP (10–14). The graph shows mean values obtained from eight different cell strains after 24 and 72 h of culture. The data are expressed as percentage considering the control as 100%. Error bars, SDs from the mean values. The significant statistical differences, compared with the control, are marked with $*P < 0.05$.

The mean values obtained from distinct osteoblast cultures derived from five different specimens were expressed as percentages, considering the enzymatic activity value in the absence of OGP (10–14) as 100%. A significant variation for ALKP activity, in comparison with the control, was mainly observed after 7 days of culture. At this stage, the increase in ALKP activity was maximum (+60%, $P < 0.01$) for an OGP (10–14) concentration 10^{-9} M (Fig. 2).

Osteocalcin Synthesis

To assess if OGP stimulation is able to induce the expression of different markers of osteoblast maturation and differentiation, we analyzed the OCN release in the culture medium of hOB cells treated with different OGP (10–14) concentrations (from 10^{-13} to 10^{-5} M). In order to obtain detectable OCN values all the cultures were co-treated with vitamin D. A significant variation for OCN levels, in comparison with the control, was observed after 72 h of culture (Fig. 3). At this time, the increase of OCN was maximum (+50%, $P < 0.01$) for an OGP (10–14) concentration 10^{-9} M.

Nodules Formation and Mineralization

To examine the effects of OGP to osteoblast activity, we have used the alizarin red S staining method to count the total mineralized nodules present in each treated culture. This staining method also allowed to obtain a measure of the calcium incorporation in relation to the number of viable cells of each culture. As shown in Figure 4, a significant increase of the mineralized nodule formation in respect of the control was observed in cultures stimulated with OGP (10–14) at concentrations 10^{-10} and 10^{-9} M (max +49%, $P < 0.05$ for 10^{-9} M OGP (10–14) at day 20). A similar course for calcium incorporation was observed analyzing at day 7 the total alizarin red S staining of each treated culture (data not shown).

OGP (10–14) Inhibits Apoptosis of hOB Induced by a High Dose of Dexamethasone

As described in other reports [Gohel et al., 1999; Plotkin et al., 1999; Eberhardt et al., 2000], exposure of hOB cultures to GC dexamethasone at concentrations above 10^{-7} M

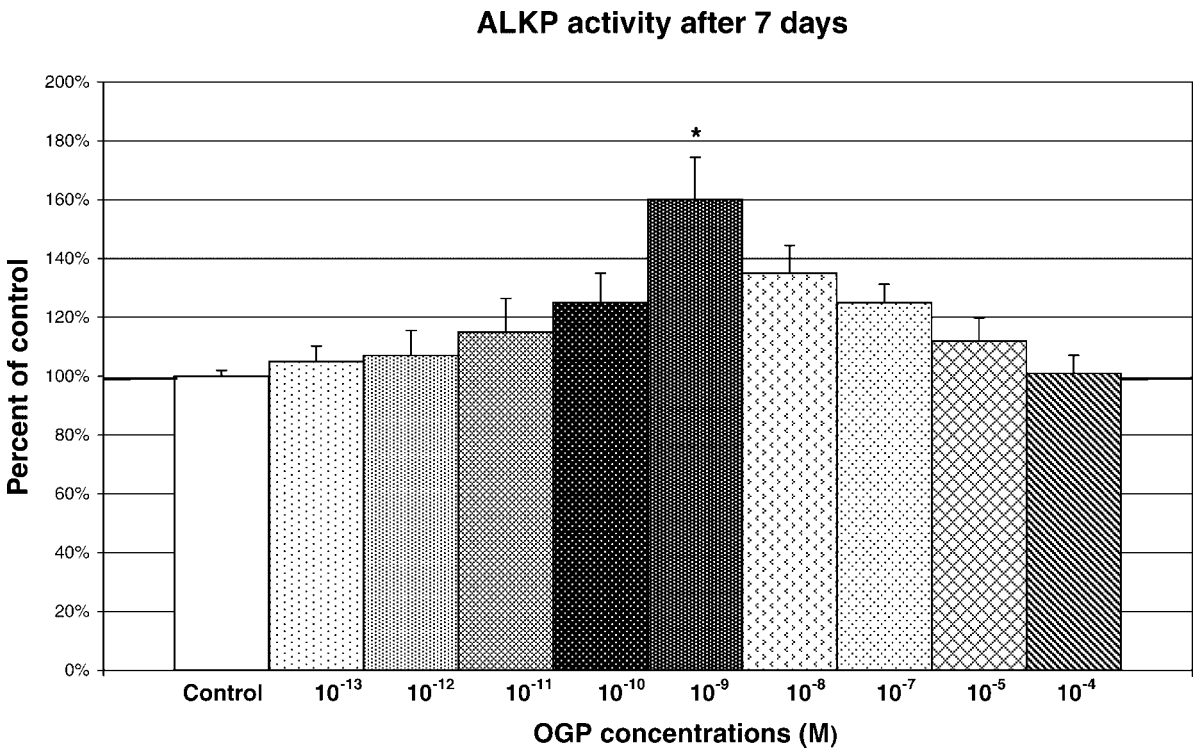

Fig. 2. Effect of OGP (10–14) on alkaline phosphatase activity in human osteoblast cells cultured in normal growth medium with 10% FCS. Alkaline phosphatase activity was determined in 12-well plates after 7 days of culture and normalized to the relative number of viable cells. The data, expressed as percentage considering the control as 100%, represents the average of the mean values obtained for five different osteoblast specimens. Error bars, SDs from the mean values. Statistical significance compared with the control: $^{*}P < 0.01$.

increases the percentage of apoptotic cells. In preliminary experiments by FACS analyses, we have defined the experimental conditions to obtain a sensible apoptotic effect in hOB cells, cultured in DMEM with 10% FCS, and treated with different concentrations of dexamethasone (from 1×10^{-9} to 5×10^{-5} M). As reported in Table I, we have observed a significant proapoptotic effect after 72 h of treatment with dexamethasone 5×10^{-5} M. In order to maximize the potential antiapoptotic effects of OGP (10–14) we have chosen to adopt a protocol similar to that proposed by Plotkin et al. [1999].

Pretreatment of the hOB cultures with OGP (10–14) for 12 h before addition of dexamethasone 5×10^{-5} M inhibited apoptosis, with a maximal inhibitory effect for 10^{-9} M OGP (10–14) (−48% of apoptotic cells in comparison with the control) (Fig. 5).

Comparable results were obtained by means of a qualitative TUNEL assay on hOB cultures treated in the same experimental conditions (Fig. 6). As shown in Figure 6b, a prevalence of apoptotic osteoblasts was evident after a 72 h treatment with 5×10^{-5} M dexamethasone. The number of apoptotic nuclei substantially decreased when the cultures were pretreated with different concentrations of OGP (10–14). Amongst the tested OGP (10–14) concentrations, the more effective seems to be the 10^{-9} M. At concentrations lower than 10^{-13} M, or higher than 10^{-7} M, the antiapoptotic effect is strongly decreased or lost.

OGP Effects on OPG and RANKL Secretion in hOB Cultures Treated With Dexamethasone

Some studies have shown that GC treatment of human osteoblastic lineage cells inhibits the production of OPG and can increase the mRNA levels of RANKL [Vidal et al., 1998; Hofbauer et al., 1999]. In our experiments, we have initially tested if OGP (10–14) could influence the steady state levels of OPG and RANKL production (Fig. 7). In our cellular system, and for the OGP (10–14) concentrations that we have tested, we observed after 48 h a significant

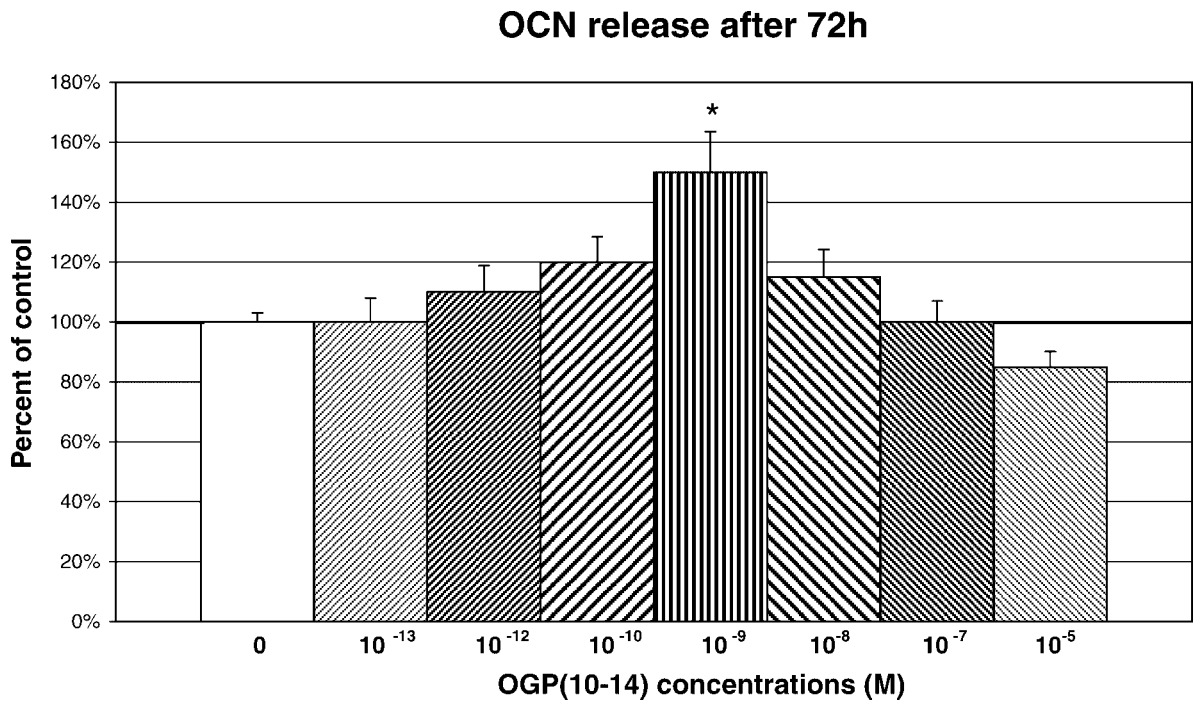

Fig. 3. Effect of different OGP (10–14) concentrations on osteocalcin production of hOB cultures seeded in 12-well plates and grown in DMEM medium with 0.1% (w/v) BSA and 5 ng/ml $1,25(OH)_2D_3$. The amount of OCN was measured in the conditioned culture supernatants harvested after 72 h from the beginning of the OGP (10–14) administration. The OCN values were normalized to the relative number of viable cells as determined in parallel experiments using the above mentioned proliferation assay. The data, expressed as percentage considering the control as 100%, represents the average values of the means obtained from three different osteoblast specimens. Error bars, SDs from the mean values. Statistical significance compared with the control: $^*P < 0.01$.

increase in OPG production that reached a maximum for 10^{-12} M OGP (10–14) stimulation (+20%, $P < 0.05$). Thereafter, we have observed that the decrease of OPG secretion induced by 5×10^{-5} M dexamethasone (−52%, $P < 0.05$) can be reduced by the addition of OGP (10–14). Amongst the different concentrations tested, we have observed a maximal inhibitory effect of OPG reduction for the 10^{-12} M OGP (10–14) concentration (+72% of inhibition, $P < 0.01$).

A variation of soluble RANKL secretion was not detected in our experiments. Even if we have used a high sensitivity test, the level of soluble RANKL molecules released in the culture medium, and not bound by OPG, is very low, at the limit of the assay reliability. A low expression of RANKL mRNA by hOB grown in our experimental conditions treated or not with dexamethasone, was confirmed by means of real-time PCR analyses (data not shown).

DISCUSSION

Glucocorticoid-induced osteoporosis is a common and serious complication of systemic GC use. Like other types of osteoporosis, this disease is the result of too little bone formation in relation to the amount of bone that has been resorbed in each remodeling cycle. This could be due either: (1) to a numeric lack of osteoblasts and an insufficient capacity of osteoblasts to synthesize and subsequently mineralize bone matrix; (2) to an increase of osteoclast proliferation and activation.

It is well known that the cells responsible for bone matrix synthesis are derived from a mesenchimal stem cell with regenerative capacity. These cells proliferate and subsequently differentiate into mature osteoblasts and osteocytes. Any disturbance in this process will ultimately lead to loss of bone and fragility of the skeleton. Even if the magnitude of hOB response to GC treatment can be extremely variable, several in vitro studies on hOB cultures have demonstrated that GC administration at supraphysiological concentrations can generally cause a substantial decrease of osteoblastic proliferation, concomitant with variations of osteoblastic differentiation and activity [Walsh et al., 2001; Leclerc et al., 2005].

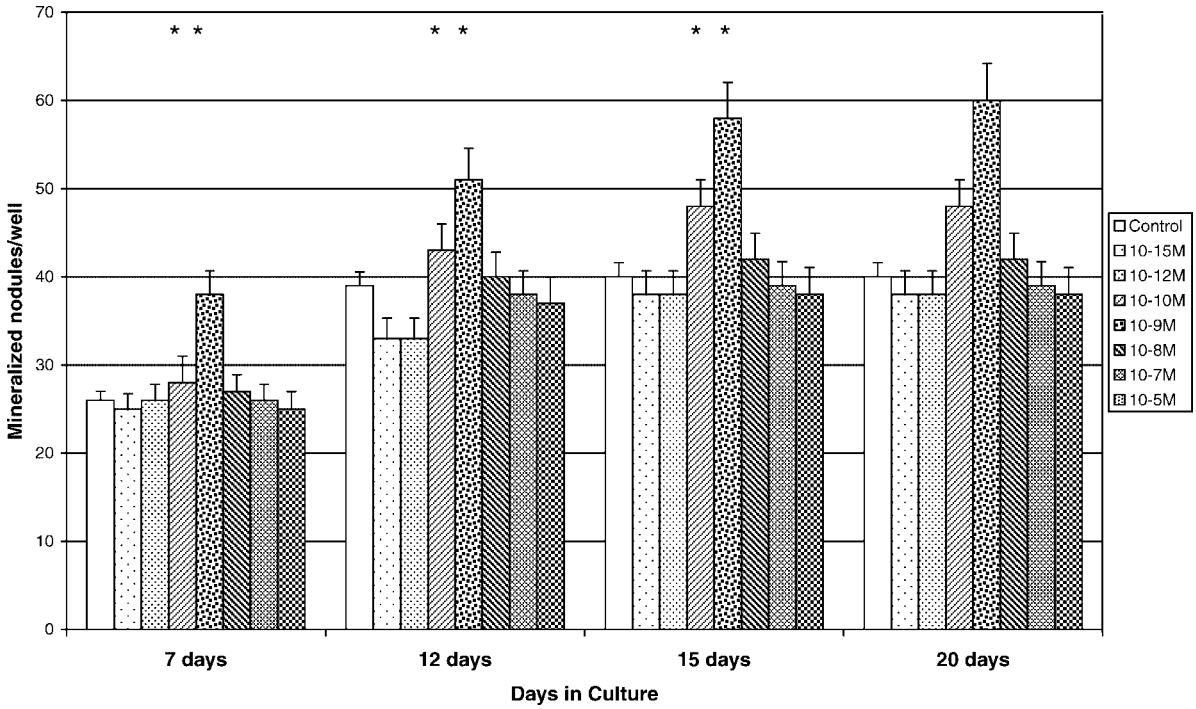

Fig. 4. Effect of OGP (10–14) on mineralized nodules formation in human osteoblastic cells cultured in normal growth medium with 10% FCS. Cells were cultured in 6-well plates in the absence or presence of various concentrations (from 10^{-15} to 10^{-5} M) of OGP (10–14). The graph shows the counting of mineralized nodules, positive for alizarin red S staining, performed after 7, 12, 15, and 20 days. The data represents the average of the mean values obtained for 5 different osteoblast specimens. Error bars, SDs from the mean values. Statistical significance compared with the control: $^*P < 0.05$.

To understand the complexity of GC effects on hOB, it should be considered, for example, that in short term cultures (2–3 days) dexamethasone seems to stimulate the proliferation in a dose-dependent way; conversely, in more prolonged cultures, dexamethasone exerts a biphasic effect, with stimulation at 1–10 nM and a marked inhibition of cell proliferation at and above 100 nM. The decrease in osteoblast and osteocyte numbers caused by GC excess may be explained in part by the increased prevalence of cell apoptosis [Weinstein et al., 1998; O'Brien et al., 2004]. The GC proapoptotic effect has been well demonstrated to occur in vitro and in vivo and its molecular mechanism has been related to suppression of survival factors such as IGF, IL-6 type cytokines, integrins, MMPs, bone morphogenetic proteins, and Cbfa1.

A further mechanism by which GCs seem to exert a negative effect on bone homeostasis, may be the modulation that they induce on the production of essential pro- or anti-resorptive regulators, such as RANKL and OPG, by cells of the osteoblastic lineage. A number of studies performed in vivo, have demonstrated that GC administration causes a rapid initial bone loss due to an increased osteoclast activity and/or life span. At a molecular level, as observed in several experimental models based on murine or human osteoblastic lineage cells treated with GCs [Vidal et al., 1998; Hofbauer et al., 1999], this biological effect should be caused by the suppression of OPG synthesis and increase in

TABLE I. Percentage of Apoptotic Cells in hOB Cultures Grown in Culture Medium With 10% FCS and Treated With Increasing Concentrations of Dexamethasone for 72 h

	Control	D 10^{-9} M	D 10^{-8} M	D 10^{-7} M	D 10^{-6} M	D 10^{-5} M	D 5×10^{-5} M
Apoptotic cells (%)	$1.56 \pm 22\%$	$1.55 \pm 33\%$	$1.67 \pm 31\%$	$1.97 \pm 33\%$	$2.06 \pm 25\%$	$2.46 \pm 25\%$	$6.58 \pm 15\%$; ($P < 0.001$)

Data were obtained from FACS analyses.

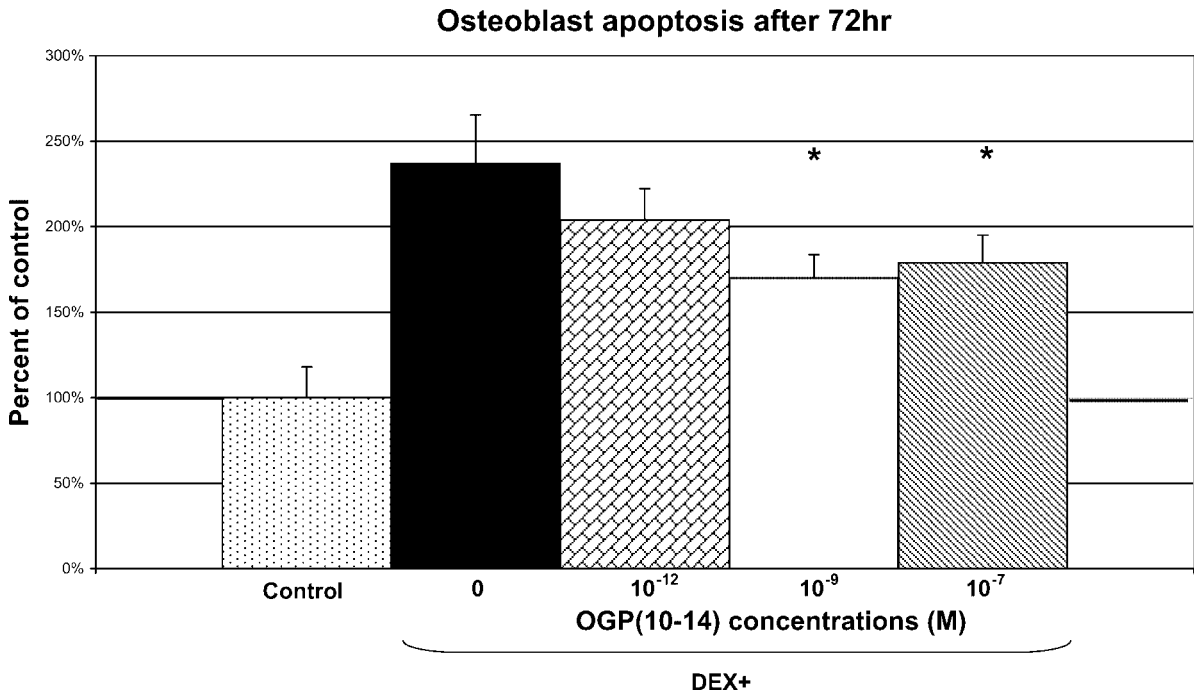

Fig. 5. Antiapoptotic effect of OPG (10–14) on hOB cultures incubated with an excess of dexamethasone. Cells, cultured in DMEM with 10% FCS, were pretreated with different OGP (10–14) concentrations (from 10^{-12} to 10^{-7} M). After 12 h, cells were coincubated for 72 h with 5×10^{-5} M dexamethasone. FACS analyses were then performed on each treated culture to quantify the relative subpopulation of apoptotic cells. Data are expressed as mean percent of four independent experiments with different osteoblast specimens. Error bars, SDs from the mean values. Statistical significance compared with the control: $^{*}P < 0.05$.

RANKL expression. Decreased levels of OPG allow the RANKL, expressed on committed preosteoblastic cells to increase osteoclastogenesis by unopposed binding to its specific receptor, RANK, on the surface of hematopoietic osteoclast progenitor cells. The rapid and consistent bone loss observed in patients during the first year of GC therapy (about 12%) has been generally observed to become slower with time [Lo Cascio et al., 1990]. This reduction of bone resorption has been supposed to be caused by the GC-induced decrease in osteoblastogenesis and resulting decline in osteoclast-supporting preosteoblasts.

Presently, the availability of new agents or drugs that can compensate the negative skeletal effects of GCs in comparison with their desirable immunosuppressive effects could be very appreciable. In this direction, the search for a suitable medication that can be taken concomitantly with the steroid drug is still active. Today's treatment for osteoporosis is primarily focused on antiresorptive agents, for example, estrogen, bisphosphonates, and calcitonin. However, in view of the belief that a dysfunctional osteoblast is the major cause of cortisone-induced osteoporosis, it should be important to develop an anabolic treatment for this type of osteopenia. In recent years new therapies have been proposed based on drugs that could decrease the prevalence of apoptosis on osteoblasts possibly increasing their proliferation and/or functional activity. Amongst them, treatment with low doses of biphosphonates or therapies with calcitonin or PTH seem to be quite effective and well tolerated.

In the present report, we provide in vitro evidence of new important functional properties of OGP (10–14) that suggest its potential application in treatment of GC-induced osteoporosis as an osteogenic agent and a bone-resorbing inhibitor.

In the first phase of our work we evaluated the anabolic effect of OGP on primary hOB. The obtained results are similar with those reported in literature using other cell systems [rewiewed by Bab and Chorev, 2002]. Moreover, we have produced new results regarding the OGP (10–14) ability to stimulate hOB differentiation in terms of OCN secretion and mineralized nodules formation. Our data confirm the role of OGP (10–14) in the physiologic regulation of

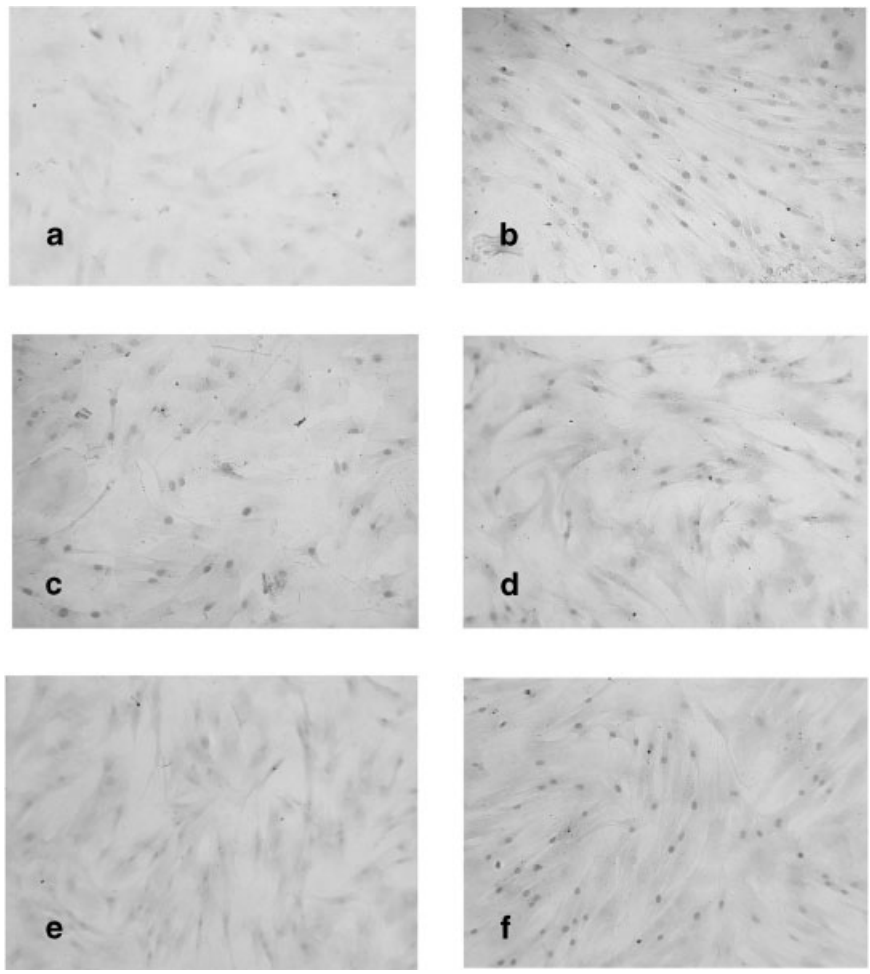

Fig. 6. TUNEL staining for the detection of apoptotic cells in hOB cultures grown in culture medium with 10% FCS. **a**: Control culture without OGP (10–14) and dexamethasone. **b**: Cells treated with 5×10^{-5} M dexamethasone alone for 72 h. **c–f**: Cells pretreated for 12 h with the following OGP (10–14) concentrations: 10^{-13} M (c), 10^{-11} M (d), 10^{-9} M (e), 10^{-7} M (f) and incubated for 72 h with 5×10^{-5} M dexamethasone. Original magnification 20×.

osteoblastic activity and its therapeutic potential as a bone anabolic agent. The second phase of our work has been addressed towards the demonstration of antiapoptotic effects of OGP (10–14) for hOBs treated with an excess of GCs. In order to best resemble the real in vivo conditions, we have tested the OGP (10–14) antiapoptotic activity on hOBs fed with 10% FCS rather than 1%BSA as often suggested in analogous experimental protocols. The FCS contains many OGP binding proteins (OGPBs) that are retained to interact with OGP biological functions. Indeed, the OGPBP-OGP complex formation seems to regulate the availability of OGP to its target cells and its biological contribute should be, even roughly, considered in vitro. On one side, OGPBPs may increase the availability of OGP by rendering protection against proteolytic cleavage, thus maintaining an extracellular reservoir of the OGP from which it can be controllably released over time. Such mechanism may be associated with the regulation of free OGP levels and/or the peptide transport and presentation to its specific tissues or acceptors. On the other side, it has been observed that some OGPBPs, like the α2 M, if activated by particular cell-derived factors, can convert their function sequestering the OGP and promoting the peptide clearance from the body.

The supplementation of FCS in culture during the GC administration, has also allowed to treat, even for a sustained period of time, hOB cells grown in an optimal condition. This

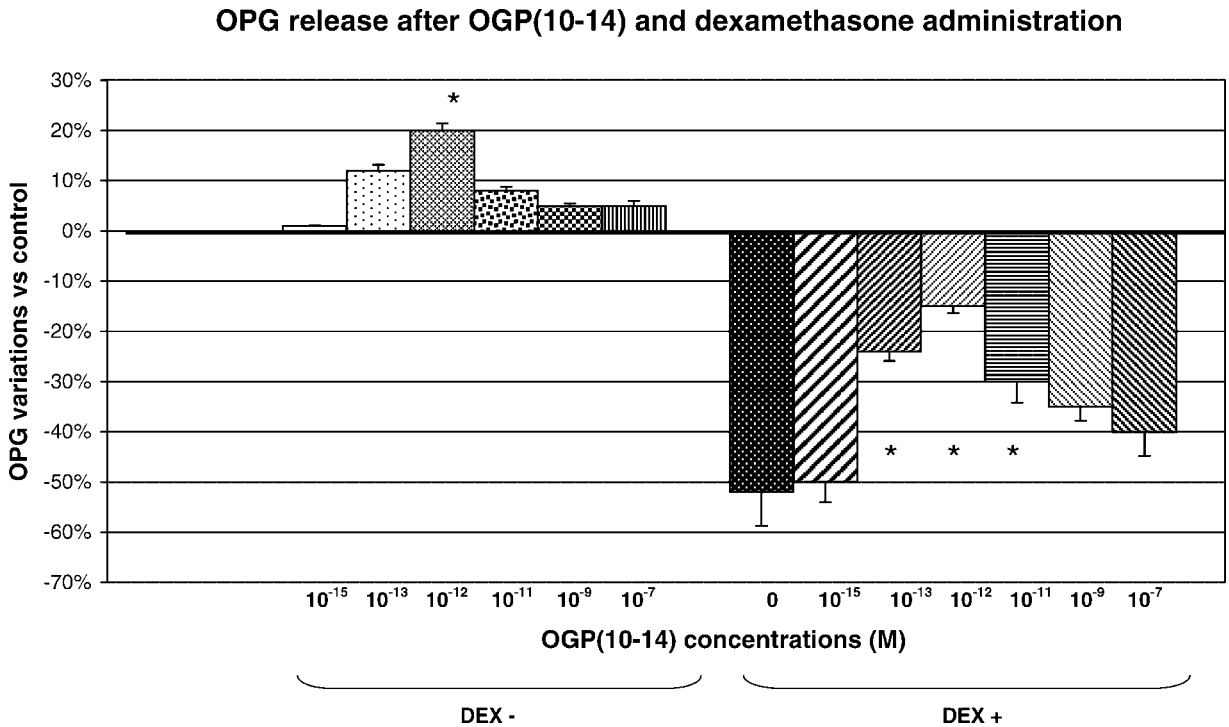

Fig. 7. Effect of OGP (10–14) on OPG secretion by human osteoblasts. hOB cultures were grown in culture medium containing 10% FCS with different concentrations of OGP (10–14) in absence (**left panel**) or presence (**right panel**) of 5×10^{-5} M dexamethasone. The OPG values were normalized to the relative number of viable cells as determined in parallel experiments using the above mentioned proliferation assay. The data, expressed as percentage considering the control as 100%, represents the average values of the means obtained from four different osteoblast specimens. Error bars, SDs from the mean values. Statistical significance compared with the control: $^{*}P < 0.01$.

experimental protocol make us more confident in our results since the observed biological effects (hOB apoptosis and OPG decrease) should be induced by GC itself and not by other causes, like cell starvation.

A relevant in vitro antiapoptotic effect (about −48% of apoptotic dead cells) was observed for an OGP stimulus 10^3 folds higher than that necessary for cell proliferation. The relationship between OGP (10–14) doses and hOB cellular activity in terms of proliferation and differentiation is complex and it is still not completely elucidated. As described by some authors, the osteoblast sensitivity for OGP (10–14) changes in relation to the OGP that is endogenously secreted in the extracellular medium. Even if low doses of exogenous OGP (10–14) are enough to alter the OGP steady-state level and to stimulate cells to start a mitogenic activity, this effect on cell proliferation decreases with time in consequence of a negative feedback loop induced by higher OGP concentrations. However, our results suggest that for higher OGP concentrations other molecular mechanisms related to cell maturation and apoptotic resistance seem to be activated. These observations could roughly indicate that, in consequence of an initial stimulation with a low dose of OGP or OGP (10–14), the hOB response occurs temporally with an initial mitogenic activity followed by an increase in cell differentiation and osteoblast life-span when the endogenous OGP concentration becomes higher. At the molecular level, the antiapoptotic effect of OGP is probably triggered by the stimulation of ERK phosphorylation through the Gi protein-MAP kinase signaling cascade described by Gabarin et al. [2001]. It is now well demonstrated that phosphorylation of ERKs enhances cell survival and that these families of protein kinases are activated by several other anti-apoptotic agents like PTH and bisphosphonates [Swarthout et al., 2001; Plotkin et al., 2005].

Another important aspect of OGP activity that we have investigated was its ability to modulate the osteoblastic OPG and RANKL production. When added alone to hOB cultures, OGP (10–14) induced, after 48 h, a fair increase of OPG production and did not seem to alter

RANKL expression. However, when administered to hOBs treated with an excess of dexamethasone, a potent depressor of OPG synthesis, OGP (10–14) restores the OPG levels to that of cells cultured in normal conditions. That OPG production could be stimulated by OGP is not surprising since it is now accepted that OPG synthesis is positively correlated with osteoblastic differentiation [Gori et al., 2000; Viereck et al., 2003]. It is very probable that OGP promotes osteoblastic differentiation through activation of the transcription factor Cbfa-1 and the up-regulation of TGF-β release [Robinson et al., 1995]. Since the OPG gene contains binding sites for Cbfa1 and TGF-β, it is possible that OGP could enhance OPG gene expression through a direct genomic mechanism. In our experimental model, RANKL gene expression was very low and was not found to be significantly modulated by OGP, suggesting that OGP regulates the OPG/RANKL system mainly by altering the OPG component. The observed OGP prevention of the OPG/RANKL ratio decrease induced by excess of GCs, suggests that OGP treatment can indirectly act on osteoclasts inhibiting their proliferation and activity. In this way, OGP systemic treatment could prevent the typical and substantial bone loss observed after the start of a GC therapy.

The increase of OPG production consequent to the addition of OGP (10–14) alone, suggests that therapeutic applications of the peptide could be extended to other bone diseases characterized by an excess of osteoclast activation, such as post-menopausal osteoporosis and articular and periarticular osteoporosis developed in arthritis patients.

In summary, our data indicate that OGP (10–14) exerts in vitro positive effects on bone formation. Moreover, when OGP (10–14) has been tested on hOB cells challenged with an excess of GCs, it exerts a significant antiapoptotic effect concomitant with a positive regulation of OPG production.

The whole of our results further supports the employment of OGP (10–14) in clinical trials addressed to the treatment of different human bone remodeling alterations.

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