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Stimulation of Osteoprotegerin Ligand and Inhibition of Osteoprotegerin Production by Glucocorticoids in Human Osteoblastic Lineage Cells: Potential Paracrine Mechanisms of Glucocorticoid-Induced Osteoporosis*

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ABSTRACT

Osteoporosis is a serious complication of systemic glucocorticoid use. However, while glucocorticoids increase bone resorption *in vitro* and *in vivo*, the mechanism(s) of this effect are at present unclear. Recent studies have identified the osteoprotegerin (OPG) ligand (OPG-L) as the final effector of osteoclastogenesis, an action that is opposed by the soluble neutralizing receptor, OPG. Thus, we assessed glucocorticoid regulation of OPG and OPG-L in various human osteoblastic lineage cells using Northern analysis, RT-PCR, and ELISA. Dexamethasone inhibited constitutive OPG messenger RNA (mRNA) steady-state levels by 70–90% in primary (MS) and immortalized stromal cells (hMS), primary trabecular osteoblasts (hOB), immortalized fetal osteoblasts (hFOB), and osteosarcoma cells (MG-63). In hFOB cells, dexamethasone inhibited constitutive OPG mRNA steady-state levels in a dose- and time-dependent fashion by 90%, and also suppressed cytokine-stimulated OPG mRNA steady-state levels. Dexamethasone-induced inhibition of OPG mRNA levels was not affected by the protein synthesis inhibitor, cycloheximide, and was shown to be due to inhibition of OPG gene transcription using a nuclear run-on assay. Moreover, dexamethasone also dose dependently (10^{-10} M–10^{-7} M) inhibited constitutive OPG protein concentrations in the conditioned medium of hFOB cells from 2.59 ± 0.02 ng/ml (control) to 0.30 ± 0.01 ng/ml (88% inhibition; $P < 0.001$ by ANOVA). Concurrently, dexamethasone stimulated OPG-L mRNA steady-state levels in MS and hFOB cells by 2- and 4-fold, respectively. Treatment of murine marrow cultures with conditioned medium harvested from dexamethasone-treated MG-63 cells increased tartrate-resistant acid phosphatase (TRAP) activity by 54% ($P < 0.005$) compared with medium harvested from control-treated cells (in the presence of OPG-L and macrophage colony-stimulating factor). Moreover, dexamethasone (10^{-8} M) promoted osteoclast formation *in vitro*, as assessed by a 2.5-fold increase of TRAP activity in cell lysates ($P < 0.001$) and the appearance of TRAP-positive multinucleated cells. Our data are thus consistent with the hypothesis that glucocorticoids promote osteoclastogenesis by inhibiting OPG and concurrently stimulating OPG-L production by osteoblastic lineage cells, thereby enhancing bone resorption. (*Endocrinology* **140:** 4382–4389, 1999)

GLUCOCORTICOID-INDUCED osteoporosis is a common and serious complication of systemic glucocorticoid use (1, 2). However, the precise mechanism(s) underlying its pathogenesis have not been defined. It is generally accepted that glucocorticoids decrease bone formation (as reviewed by Refs. 1–3) and increase bone resorption *in vitro* (4–6) as well as *in vivo* (7–9). The combination of decreased bone formation and increased bone resorption then leads to extremely rapid bone loss (1). While the decrease in bone formation has been attributed to glucocorticoid effects on osteoblastogenesis (10), osteocyte apoptosis (10), and alteration of skeletal growth factors such as insulin-like growth factor-1 and transforming growth factor-β (11), the mechanism(s) of the glucocorticoid-induced increase in bone resorption are unclear.

Osteoprotegerin (OPG) has recently been identified by several groups (12–16) as a novel, secreted cytokine receptor that is a member of the tumor necrosis factor (TNF) receptor (TNF-R) superfamily. Overexpression of *OPG* in transgenic mice results in osteopetrosis (generalized increased bone mass) and the administration of OPG to normal animals prevents ovariectomy-induced bone loss (12). By contrast, targeted ablation of the *OPG* gene in knock-out mice leads to early-onset, severe osteoporosis (17, 18). More recently, two groups have independently identified the cognate ligand for OPG (OPG-L; osteoclast differentiation factor, ODF) (19, 20). OPG-L is identical to a previously described novel member of the TNF ligand superfamily named TRANCE [TNF-related activation-induced cytokine (21)] and RANKL [receptor activator of NF-κB ligand (22)], and has been implicated in T cell and dendritic cell maturation and activation (21–23).

OPG-L exists in a cell membrane-associated and a soluble form, both of which stimulate osteoclastogenesis and osteoclast action after binding to and activating a high-affinity receptor located on osteoclast precursors (19, 20). Recent studies have shown that OPG-L, in the presence of macrophage colony-stimulating factor (M-CSF) is both sufficient

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and necessary for osteoclast development *in vitro* (19, 20, 24), and when administered to normal mice results in enhanced osteoclastogenesis, severe osteoporosis, and malignant hypercalcemia (19). The soluble cytokine receptor OPG counteracts the biological effects of OPG-L by competing for both forms of OPG-L and preventing them from binding to the OPG-L receptor on osteoclast precursors (19). Of note, OPG-L knock-out mice have recently been shown to develop severe osteopetrosis and completely lack mature osteoclasts (23).

We and others have previously demonstrated that OPG gene expression and protein production in osteoblastic cells is regulated by various calcitropic hormones and cytokines (25–29). OPG production is stimulated by 1,25-dihydroxyvitamin D_3 (25), bone morphogenetic protein-2 (25), TNF-α and -β (25, 27), interleukin (IL)-1α and -β (25, 28) as well as by estrogen (26) and decreased by prostaglandin E_2 (29). Here we report that glucocorticoids concurrently decrease OPG and increase OPG-L production by human osteoblastic lineage cells, and increase osteoclastogenesis *in vitro*. These findings thus provide a potential paracrine mechanism for glucocorticoid effects on bone resorption.

Materials and Methods

Materials

Culture flasks and dishes were obtained from Corning, Inc. (Corning, NY). The random primer labeling kit (Decaprime II) was from Ambion, Inc. (Austin, TX) and [α-^{32}P]-dCTP was from NEN Life Science Products (Boston, MA). The human *β-actin* complementary DNA (cDNA) insert and ExpressHyb solution were obtained from CLONTECH Laboratories, Inc. (Palo Alto, CA). Recombinant human TNF-α was from R & D Systems (Minneapolis, MN). All other reagents were purchased from Sigma (St. Louis, MO).

Cell cultures

The following human osteoblastic cells were used: 1) a conditionally immortalized bipotential marrow stromal cell line (hMS) (30); 2) a conditionally immortalized fetal osteoblastic cell line (hFOB) that displays the complete characteristics of the mature osteoblastic phenotype (31); 3) primary osteoblasts (hOB) obtained from cultures of trabecular bone explants from corrective orthopedic procedures (32); 4) primary marrow stromal cells (MS) from healthy subjects (33); and 5) the human osteosarcoma cell line, MG-63, obtained from American Tissue Culture Collection. The hOB and MS cells were obtained following approval by the Institutional Review Board. Both conditionally immortalized cell lines, hMS and hFOB, proliferate at 33.5 C (the permissive temperature, when the temperature-sensitive mutant SV 40 large T antigen is active) and differentiate at 39.5 C (the restrictive temperature, when the SV 40 large T antigen is inactive) (30, 31). At the restrictive temperature, these cells are essentially a clonal population of normal preosteoblastic (hMS) and osteoblastic (hFOB) cells. All other cells were grown at 37 C. All cells were maintained in phenol-free medium supplemented with 10% double charcoal-stripped FCS and were grown in serum-free medium supplemented with 0.125% (wt/vol) BSA for 4 days before RNA isolation.

Northern blot analysis

Total RNA was isolated from cell cultures using the QIAGEN RNeasy kit in combination with the QiaShredder from QIAGEN (Hilden, Germany). Poly-A RNA was isolated using the PolyATract messenger RNA (mRNA) kit from Promega Corp. (Madison, WI). Ten micrograms of total RNA or 1 μg of poly-A+ RNA were separated on a 1.5% (wt/vol) agarose/formaldehyde gel using continous buffer circulation (34) and then transferred to a nylon membrane (Hybond N+, Amersham Pharmacia Biotech, Arlington Heights, IL) by capillary blotting (35). The human cDNA inserts, a *β-actin* cDNA that hybridized to a 2.0 kb mRNA, a full-length *OPG* cDNA that hybridized to three mRNA species of 2.9 kb, 4.4 kb, and 6.6 kb (25), and an *OPG-L* cDNA that hybridized to a mRNA species of 2.4 kb (19) were radiolabeled by random primer labeling (36). Hybridization and stringent washing were carried out as reported elsewhere (25). Band intensity was quantified by densitometry. Control hybridization with human *β-actin* cDNA verified that equal amounts of RNA were loaded. All experiments were carried out at least three times, and representative blots are shown.

Semiquantitative RT-PCR

RT was performed with 2 μg of total RNA as previously described (36). PCR reactions were carried out in 25 μl reactions at a cycle number ensuring a linear amplification profile (OPG-L, 2 min at 94 C, 35 cyles [of 30 sec at 94 C, 30 sec at 58 C, 1 min at 72 C], 7 min at 72 C; GAPDH, 2 min at 94 C, 24 cyles [of 30 sec at 94 C, 30 sec at 55 C, 30 min at 72 C], 7 min at 72 C). The oligonucleotides for OPG-L (sense: 5′ TCAGAAGATGGCACTCACTG 3′; antisense: 5′ AACATCTCCCACTGGCTGTA 3′) were synthesized at the Mayo Oligonucleotide Core Facility. For radiolabeled PCR reactions, [^{32}P]-dCTP (0.5 μl/reaction) was used. PCR products were analyzed by electrophoresis on a 1.5 (wt/vol)% agarose gel and visualized under UV light. For quantitative analysis of radiolabeled PCR products gel slices were prepared from the gel and the radioactivity was determined by a liquid scintillation counter (37).

Nuclear run-on assay

Two micrograms of full-length OPG cDNA were denatured and fractionated in an agarose/formaldehyde gel under denaturing conditions and transferred to a nylon membrane analogous to the Northern blot procedure. MG-63 osteosarcoma cells (2×10^8 cells) were treated either with vehicle or dexamethasone (10^{-8} M) for 24 h. Then cellular nuclei were prepared according the method of Dignam *et al.* (38). Nuclear RNA was radiolabeled using [^{32}P]-dCTP (15 μl/reaction), and extracted using the QIAGEN RNeasy kit from QIAGEN (Hilden, Germany). The two lanes on the nylon membrane were then cut and separately hybridized each with radiolabeled RNA (10^7 cpm/μg) as described in (25). The membrane strips were then exposed to an autoradiography film.

OPG protein measurement

Conditioned media from cultured cells was centrifuged to remove cell debris. OPG protein concentration was determined in triplicate measurements with a sandwich ELISA (CV: $< 3\%$; lower limit of detection: 0.1 ng/ml) as described previously (12, 25).

In vitro osteoclastogenesis assay

Bone marrow cells from 4- to- 6-week-old male CSH/HeN mice (Charles River Laboratories, Inc. Wilmington, MA) were prepared as previously described (19) and cultured for 7 days in α-MEM containing 10% of FCS. To assess the activity of conditioned medium on osteoclastogenesis, the murine marrow cells (2×10^5 per well in 96-well-plates) were cultured for 7 days in an 1:1 mixture of fresh α-MEM/sterile-filtered conditioned medium (harvested from MG-63 cells treated for 48 h either with vehicle or dexamethasone at a concentration of 10^{-8} M) supplemented with FCS (10%), recombinant human OPG-L (20 ng/ml), and recombinant human M-CSF (60 ng/ml). To assess the direct effects of dexamethasone, the cells were treated with either recombinant human OPG-L (10 ng/ml), recombinant human M-CSF (30 ng/ml, R & D Systems, Minneapolis, MN), recombinant human OPG (10 ng/ml), or dexamethasone (10^{-8} M). Tartrate-resistant acid phosphatase (TRAP) activity of cell lysates (n = 4) was assessed by a solution assay using the Acid Phosphatase Activity Assay from Sigma. TRAP cytochemistry (n = 3) was performed in the plates following formaldehyde fixation by using a leukocyte acid phosphatase assay from Sigma.

Statistical analysis

Unless otherwise stated, all values are expressed as mean ± SEM. Student's paired *t* test was used to evaluate differences between the sample of interest and its respective control. For analysis of time course

and dose response, multiple measurement ANOVA was used. A P value of < 0.05 was considered significant.

Results

Regulation of osteoblastic OPG mRNA steady-state levels and protein production by dexamethasone

To assess the regulation of constitutive OPG mRNA levels by dexamethasone in human osteoblastic lineage cells, the conditionally immortalized cell lines hMS and hFOB were grown at 39.5 C, the temperature at which these cells differentiate, and the osteoblastic cells hOB and MG-63 were cultured at 37 C in serum-free medium + 0.125% (wt/vol) BSA. A single OPG mRNA species of 2.9 kb was detected in all osteoblastic lineage cells (Fig. 1). Of note, constitutive OPG mRNA steady-state levels were low in the bipotential, uncommitted marrow stromal cell line (hMS) and high in differentiated osteoblastic cell systems that have a mature osteoblastic phenotype (hFOB, hOB) as well as in the osteosarcoma cell line, MG-63 (Fig. 1). Regardless of the baseline OPG mRNA levels, treatment with dexamethasone at a concentration of 10^{-8} M for 24 h inhibited constitutive OPG mRNA levels by 70–90% (Fig. 1).

Because the inhibition of constitutive OPG mRNA levels occurred in all osteoblastic cell systems examined, the hFOB cell line was used for further analysis. We next assessed the inhibitory effect of dexamethasone on constitutive OPG mRNA levels of the hFOB cell line using a dose response and a time course. Dexamethasone (after 24 h of treatment) dose dependently inhibited constitutive OPG mRNA levels by 80% with a maximum effect at 10^{-8} M (Fig. 2A). The chronological pattern indicated that dexamethasone (10^{-8} M) inhibited constitutive OPG mRNA levels after 6 h (by 70%) with a maximum effect of 90% after 12 to 24 h of treatment (Fig. 2B). A similar dexamethasone-induced dose-dependent and chronological reduction of constitutive OPG mRNA steady-state levels was also detected in primary marrow stromal cells (by 60% and 90%, respectively) and MG-63 cells (by 90% and 70%, respectively) (data not shown). As shown in Fig. 3, constitutive OPG mRNA levels in hFOB cells were 10-fold higher at 39.5 C (when proliferation ceases, and the cells differentiate and display a mature osteoblast phenotype) compared with 33.5 C (when the cells proliferate, but do not differentiate). However, dexamethasone dose dependently inhibited constitutive OPG mRNA levels of hFOB cells by 90% at both temperatures (Fig. 3). Thus, the action of dexamethasone was independent of the activity of the SV40 large T antigen, which is active at 33.5 C but inactive at 39.5 C.

To assess whether dexamethasone also regulated OPG

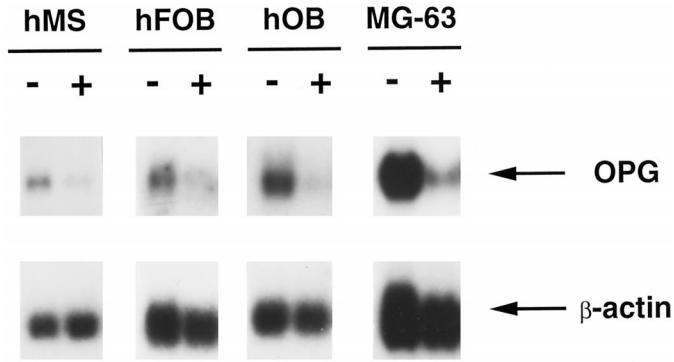

FIG. 1. Inhibition of constitutive OPG mRNA levels by dexamethasone in various human osteoblastic lineage cells as assessed by Northern analysis. The cells were grown at either 39.5 C (hMS; hFOB) or 37 C (hOB; MG-63) for 2 days in serum-free medium + 0.125% (wt/vol) BSA, and then treated with either vehicle (-) or dexamethasone (10^{-8} M; +) for 24 h. Ten micrograms of total RNA were analyzed by Northern blot. Expression of OPG mRNA (2.9 kb) (*upper panel*) and β-actin mRNA (2.0 kb) (*lower panel*) [hMS, human marrow stromal cell line; hFOB, human fetal osteoblastic cell line; hOB, primary adult trabecular osteoblastic cells; MG-63, osteosarcoma cell line MG-63].

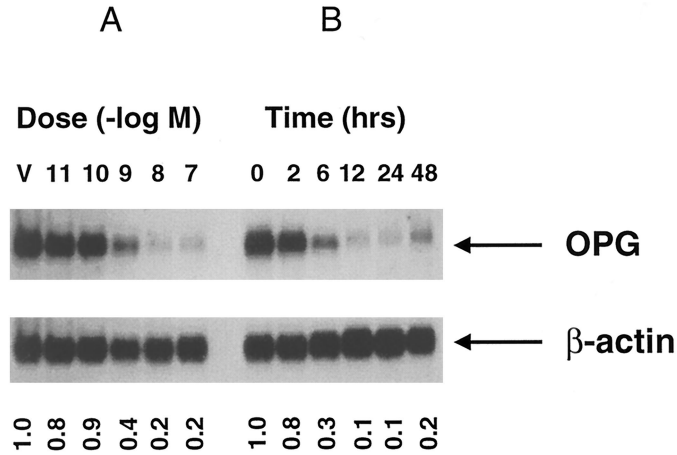

FIG. 2. Dose response (A) and time course (B) of constitutive OPG mRNA expression following dexamethasone treatment of hFOB cells as assessed by Northern analysis. Ten micrograms of total RNA were isolated from cells grown at 39.5 C for 2 days in serum-free medium + 0.125% (wt/vol) BSA. A, Dose response: The cells were then treated with either vehicle (ethanol) or dexamethasone (10^{-11} M–10^{-7} M) for the last 24 h (the numbers indicate the dose in − log M). B, Time course: The cells were treated with either vehicle (ethanol) or dexamethasone (10^{-8} M) for the time (in hours) indicated. Northern analysis demonstrates the expression of OPG mRNA (2.9 kb) (*upper panel*) and β-actin mRNA (2.0 kb) (*lower panel*). The numbers underneath the β-actin bands indicate the OPG/β-actin ratio, normalized to the vehicle control (A) or the control at 0 h (B).

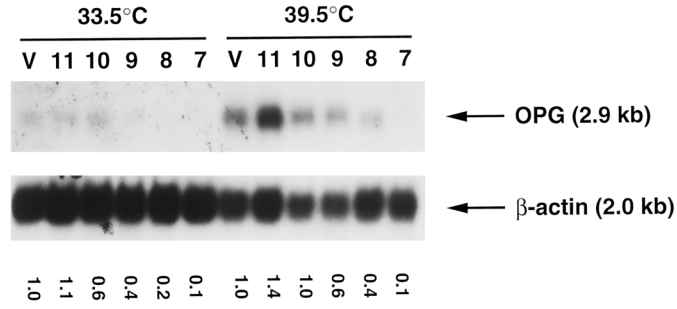

FIG. 3. Dose response of constitutive OPG mRNA expression following dexamethasone treatment of hFOB cells at either the restrictive (33.5 C) or the permissive (39.5 C) temperature as assessed by Northern analysis. Ten micrograms of total RNA were isolated from cells grown at either 33.5 C (*left*) or 39.5 C (*right*) for 2 days in serum-free medium + 0.125% (wt/vol) BSA. The cells were then treated with either vehicle (ethanol) or dexamethasone (10^{-11} M–10^{-7} M) for the last 24 h (the numbers indicate the dose in − log M). Northern analysis demonstrates the expression of OPG mRNA (2.9 kb) (*upper panel*) and β-actin mRNA (2.0 kb) (*lower panel*). The numbers underneath the β-actin bands indicate the OPG/β-actin ratio, normalized to the vehicle control.

mRNA levels stimulated by proinflammatory cytokines, the hFOB cells were pretreated with either vehicle (ethanol) or dexamethasone at concentrations of 10^{-11} M, 10^{-9} M, or 10^{-7} M and then treated with either vehicle or TNF-α (9 nM). The latter has recently been demonstrated to stimulate OPG mRNA and protein levels (25, 27). TNF-α stimulated OPG mRNA levels by 3-fold, whereas cotreatment with dexamethasone (at 10^{-7} M) completely abrogated the stimulatory effects of TNF-α on OPG mRNA levels (Fig. 4).

To confirm the inhibitory effects of dexamethasone on constitutive OPG mRNA steady-state levels at the protein level, OPG protein concentrations were measured by ELISA in the conditioned medium harvested from hFOB cells grown at 39.5 C. Consistent with the Northern analyses, dexamethasone dose dependently decreased OPG protein production from 2.59 ± 0.02 ng/ml (control) to 0.30 ± 0.01 ng/ml (88% inhibition) at 10^{-7} M ($P < 0.001$ by ANOVA) (Fig. 5).

Nuclear run-on studies and effects of protein synthesis inhibition on OPG mRNA regulation by dexamethasone

To assess whether dexamethasone-induced inhibition of OPG mRNA required *de novo* protein synthesis, we treated MG-63 osteosarcoma cells either with vehicle, dexamethasone (10^{-8} M), the protein synthesis inhibitor, cycloheximide (10 μg/ml), or dexamethasone (10^{-8} M) and cycloheximide (10 μg/ml). As shown in Fig. 6A, cycloheximide failed to abrogate the inhibitory effect of dexamethasone on OPG mRNA levels. This suggests that no newly synthesized protein is required for or involved in the inhibition of OPG mRNA expression by dexamethasone. Next, we assessed the effects of dexamethasone treatment on OPG gene transcription by MG-63 cells directly by using a nuclear run-on assay. Compared with vehicle treatment, dexamethasone markedly decreased OPG mRNA expression (Fig. 6B). Collectively, these results indicate that the inhibition of osteoblastic OPG production occurs mainly at the transcriptional level.

Regulation of osteoblastic OPG-L mRNA steady-state levels by dexamethasone

The regulation of constitutive OPG ligand (OPG-L) mRNA levels by dexamethasone was assessed in hFOB cells using semiquantitative RT-PCR, because Northern analysis failed to detect OPG-L mRNA levels in hFOB cells despite the use of 1.0 μg of poly-A+ RNA. Dexamethasone stimulated the

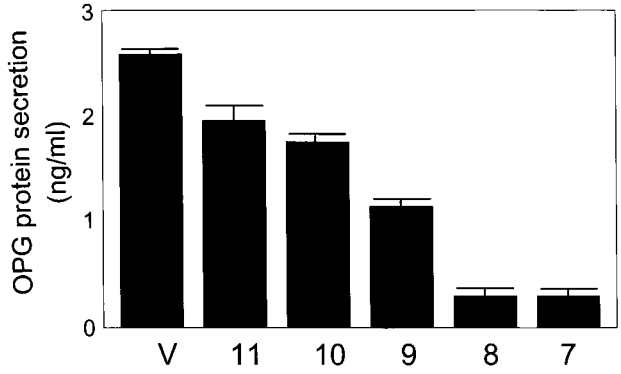

FIG. 5. Dose response of OPG protein production following dexamethasone treatment of hFOB cells as assessed by ELISA. Conditioned medium was harvested from hFOB cells grown at 39.5 C for 2 days in serum-free medium + 0.125% (wt/vol) BSA. The cells were then treated with either vehicle (ethanol) or dexamethasone (10^{-11} M–10^{-7} M for the last 24 h. The numbers indicate the dose in − log M. Values given are the mean ± SEM of triplicates ($P < 0.001$ by ANOVA).

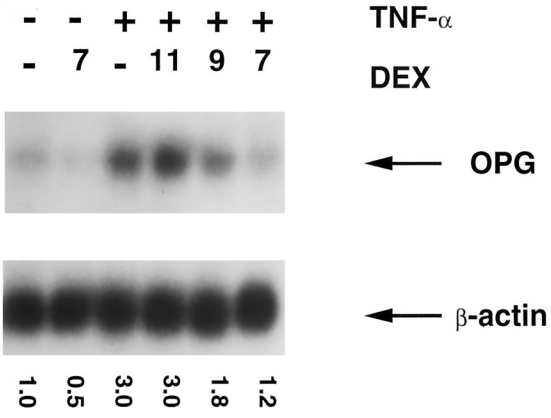

FIG. 4. Inhibition of TNF-α-stimulated OPG mRNA expression by dexamethasone in hFOB cells as assessed by Northern analysis. The cells were grown at 39.5 C for 2 days in serum-free medium + 0.125% (wt/vol) BSA. The cells were then treated with either vehicle (−) or dexamethasone (DEX; dose in − log M) for 48 h and in the last 24 h either vehicle (−) or tumor necrosis factor-α (TNF-α, +) at a concentration of 9 nM was added. Seven micrograms of total RNA was fractionated by electrophoresis and transferred to a filter membrane. Northern analysis demonstrates the expression of OPG mRNA (2.9 kb) (*upper panel*) and β-actin mRNA (2.0 kb) (*lower panel*). The numbers underneath the β-actin bands indicate the OPG/β-actin ratio, normalized to the vehicle control without dexamethasone and TNF-α.

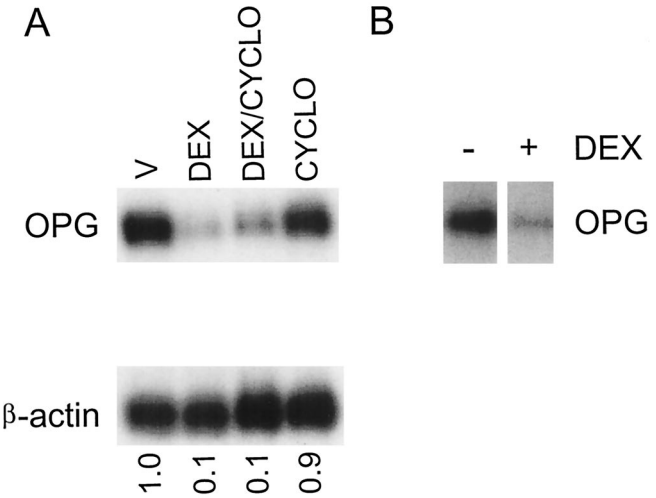

FIG. 6. Molecular mechanisms of dexamethasone-induced osteoblastic OPG mRNA inhibition. A, Effect of inhibition of *de novo* protein synthesis on OPG mRNA levels. MG-63 cells were treated for 24 h with vehicle, dexamethasone (10^{-8} M), the protein synthesis inhibitor, cycloheximide (10 μg/ml), or dexamethasone (10^{-8} M) and cycloheximide (10 μg/ml). 10 μg of total RNA were then assessed for OPG mRNA (2.9 kb) and β-actin mRNA (2.0 kb) levels by Northern hybridization. B, Effects of dexamethasone on OPG gene transcription. 2 μg of OPG cDNA were assessed by Southern blot analysis using radiolabeled OPG mRNA (200,000 cpm) from vehicle-treated (−) or dexamethasone-treated (+) MG-63 cells as a probe.

OPG-L/GAPDH ratio in a dose- and time-dependent fashion (Fig. 7A). Quantitative analysis using liquid scintillation counting of gel slices from radioactive PCR reactions demonstrated a 2-fold increase of OPG-L mRNA steady-state levels following treatment with dexamethasone (for 24 h) in a glucocorticoid dose-dependent fashion ($P < 0.001$ by ANOVA). Similarly, a 4-fold increase was observed after treatment with dexamethasone (10^{-8} M) for 6 h ($P < 0.001$ by ANOVA) (Fig. 7B).

To confirm these results by Northern analysis, up to 20 μg of total RNA harvested from hFOB cells were analyzed using a human *OPG-L cDNA* probe but did not reveal a detectable signal (data not shown). Thus, we analyzed OPG-L mRNA levels using 1.0 μg of poly-A+ RNA isolated from hFOB cells and primary marrow stromal cells treated for 24 h with either vehicle or dexamethasone (10^{-8} M). As shown in Fig. 8, while OPG-L mRNA levels in hFOB cells were barely detectable, marrow stromal cells expressed high constitutive OPG-L mRNA levels that increased by 2-fold following treatment with dexamethasone.

Effects of dexamethasone on osteoclast formation

Because the observed effects of dexamethasone on OPG and OPG-L would be expected to favor the formation of osteoclasts, we next directly assessed the ability of dexamethasone to promote osteoclast formation in murine marrow cells (12, 19). As shown in Fig. 9A, conditioned medium

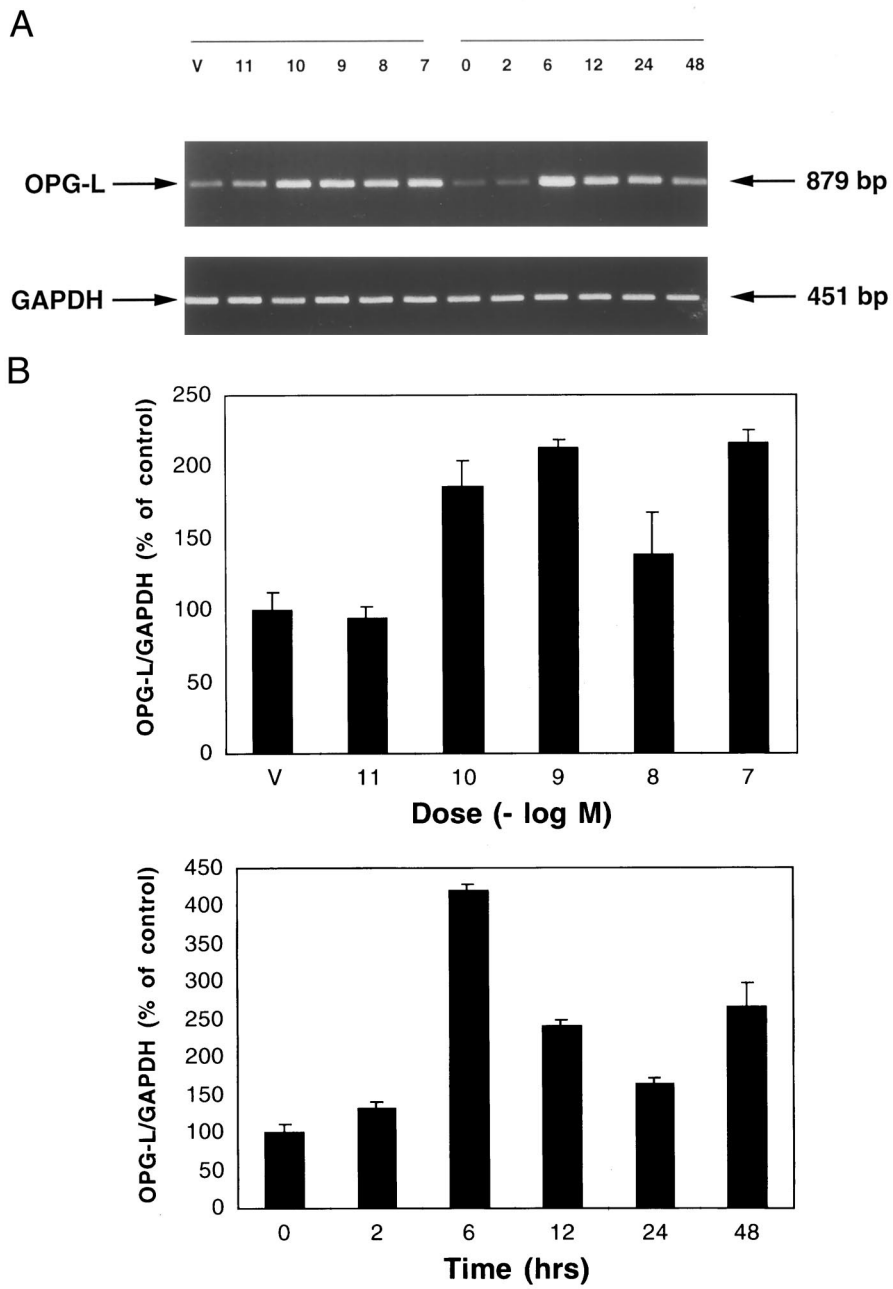

FIG. 7. Dose response and time course of constitutive OPG ligand (OPG-L) mRNA expression following dexamethasone treatment of hFOB cells as assessed by RT-PCR. A, Agarose gel electrophoresis demonstrating OPG-L (879 bp) and GAPDH (451 bp) PCR products. For the dose response (*left*), the cells were treated with either vehicle (ethanol) or dexamethasone (10^{-11} M–10^{-7} M) for 24 h (the numbers indicate the dose in $-$ log M). For the time course (*right*), the cells were treated with either vehicle (ethanol) or dexamethasone (10^{-8} M) for the time (in hours) indicated. B, Quantitation of radiolabeled PCR products using [^{32}P]-dCTP. The numbers are given as the mean $\pm$ SEM in triplicate measurement of OPG-L/GAPDH ratios normalized to the vehicle control (*left*) or the control at 0 h (*right*), $P < 0.001$ by ANOVA.

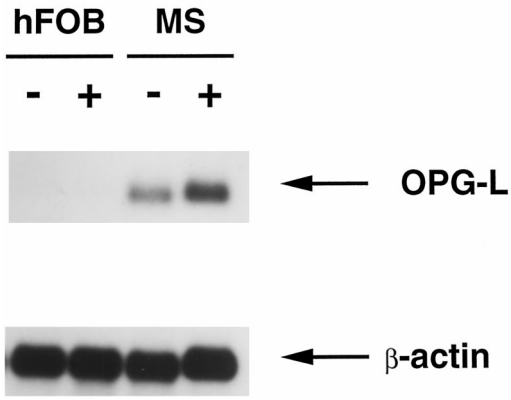

FIG. 8. Stimulation of OPG-L mRNA levels by dexamethasone in primary marrow stromal (MS) cells as assessed by Northern analysis. The cells (hFOB, MS) were grown for 2 days in serum-free medium + 0.125% (wt/vol) BSA, and then treated with either vehicle (−) or dexamethasone (10^{-8} M; +) for 24 h. Poly (A)+ RNA (1.0 μg) was fractionated by gel electrophoresis. Expression of OPG-L mRNA (2.4 kb) (*upper panel*) and β-actin mRNA (2.0 kb) (*lower panel*).

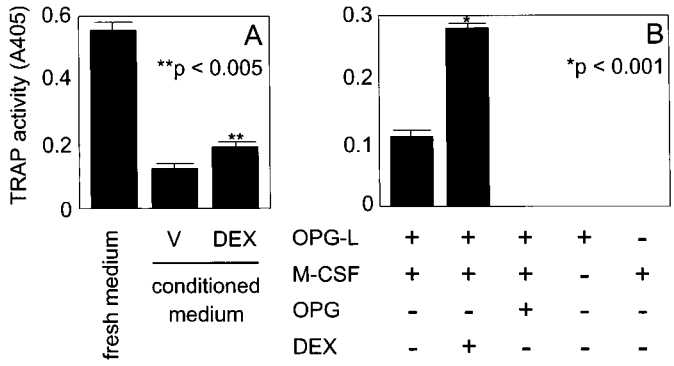

FIG. 9. Effects of OPG-L, M-CSF, OPG and dexamethasone on osteoclast formation. A, Murine marrow cells were cultured for 7 days at 37 C in the presence of OPG-L (20 ng/ml) and M-CSF (60 ng/ml) and either fresh medium, or a 1:1 mix of fresh medium with conditioned medium from vehicle-treated or dexamethasone-treated (10^{-8} M) MG-63 cells. Tartrate-resistant acid phosphatase (TRAP) activity (n = 4) was assessed by a TRAP solution assay using absorption at 405 nm. $P < 0.005$ by Student's paired t test. B, Murine marrow cells were treated either with (+) or without (−) recombinant OPG-L (10 ng/ml), M-CSF (30 ng/ml), OPG (10 ng/ml) or dexamethasone (10^{-8} M) for 7 d at 37 C. TRAP activity of cell lysates (n = 4) was assessed as in (A). $P < 0.001$ by Student's paired t test.

harvested from MG-63 cells following treatment with dexamethasone (10^{-8} M) increased TRAP activity by 54% compared with conditioned medium from control-treated cells (n = 4, $P < 0.005$). Of note, the finding that TRAP activity in cell lysates treated with vehicle-treated conditioned medium was inhibited by 77% compared with TRAP activity in cell lysates cultured in fresh medium may be due to the inhibitory effect of OPG, which is abundantly expressed by MG-63 cells under basal conditions (see Fig. 1). In addition, cotreatment of cells treated with OPG-L and M-CSF at concentrations of 10 ng/ml and 30 ng/ml, respectively, with dexamethasone (10^{-8} M) increased TRAP activity of cell lysates by 2.5-fold (n = 4, $P < 0.001$), compared with cells in the absence of dexamethasone (Fig. 9B). By contrast, TRAP activity of OPG-L- and M-CSF-treated cells was inhibited by cotreatment with OPG (10 ng/ml), and was not detected in the absence of either M-CSF or OPG-L (Fig. 9B). Consistent with the data in Fig. 9B, TRAP cytochemistry demonstrated the presence of numerous, large, multinucleated TRAP-positive osteoclasts following treatment with dexamethasone compared with treatment with vehicle (data not shown).

Discussion

Although glucocorticoid-induced osteoporosis represents the most prevalent form of secondary osteoporosis (1), the mechanisms of glucocorticoid effects on enhancing bone resorption remain unclear. While the glucocorticoid-induced increase in bone resorption has been attributed, at least in part, to increased PTH secretion (39), glucocorticoids also directly increase bone resorption *in vitro* (4–6), indicating direct skeletal effects of glucocorticoids on bone resorption. The potential mediator(s) of these direct effects, however, have not been previously defined. In the present study, we report that glucocorticoid treatment (at concentrations ranging from 10^{-11} M–10^{-7} M) of human osteoblastic lineage cells concurrently inhibited the production of the recently identified soluble antiresorptive receptor, OPG, and increased the mRNA levels of the proresorptive cytokine, OPG-L. The inhibition of OPG concentrations by dexamethasone (from 2.59 ng/ml to 0.30 ng/ml) corresponds to the steep part of the OPG dose response for its inhibitory effects on osteoclastogenesis (12, 13). Thus, the OPG protein concentrations measured in the conditioned medium of hFOB cells are within the biologically relevant dose range. We then hypothesized that differential regulation of OPG-L and OPG by dexamethasone would favor the formation of osteoclasts, and we tested this by using a murine osteoclastogenesis assay. Using this assay, we found that conditioned medium from MG-63 cells treated with dexamethasone stimulated OPG-L/M-CSF-induced TRAP activity by 54% (compared with conditioned medium from vehicle-treated cells), and that dexamethasone increased OPG-L/M-CSF-induced osteoclast formation by 2.5-fold, as assessed by TRAP activity in cell lysates. Enhanced osteoclast formation after treatment with the conditioned medium from cells treated with dexamethasone most likely resulted from decreased OPG secretion by these cells. Taken together, our data thus suggest that the OPG/OPG-L system may play a key role in mediating glucocorticoid effects on osteoclastogenesis. We recognize, however, that our assay assessed osteoclast formation and not activity. Given the evidence that OPG-L and OPG can affect both processes (19, 20), clearly further studies are needed to test glucocorticoid effects on bone resorption.

OPG and OPG-L are potent regulators of bone homeostasis because they are expressed by cells of the osteoblastic lineage (12, 13, 15, 16, 25) and act in opposite directions on the differentiation and activity of osteoclasts (12–14, 16, 19, 20, 24). OPG-L has been shown to be a prerequiste for osteoclastogenesis *in vitro* (19, 20, 24). Furthermore, OPG and OPG-L production is regulated by major calcitropic hormones and cytokines known to regulate bone resorption (20, 25–29). The importance of the OPG-L/OPG system for bone metabolism is further supported by the phenotypic extremes of osteopetrosis (when the *OPG* gene is overexpressed in

transgenic mice, and thus the effects of OPG-L are completely blocked) (12) and severe osteoporosis (when the *OPG* gene is deleted in knock-out mice and the effects of OPG-L are unopposed) (17, 18). The latter phenotype can also be generated by exogenous administration of recombinant OPG-L to normal mice (19). These data thus suggest that the OPG-L/OPG system may be the final and common pathway for mediating the effects of other candidate cytokines on osteoclastogenesis and bone resorption.

The inhibition of OPG mRNA levels by glucocorticoids was detected in all human osteoblastic cell systems, including the immortalized fetal osteoblastic cell line (hFOB), the immortalized adult marrow stromal cell line (hMS), primary trabecular osteoblasts (hOB), primary marrow stromal cells (MS), and the osteosarcoma cells line, MG-63. Thus, glucocorticoids inhibit OPG mRNA levels in osteoblastic lineage cells regardless of their stage of differentiation, phenotype, or absolute constitutive OPG mRNA levels. The inhibition by glucocorticoids was demonstrated in hFOB cells for both constitutive and TNF-α-stimulated OPG expression and was present both at the mRNA and the protein levels. The inhibition was substantial in magnitude (~90%) and was glucocorticoid dose and time dependent. Moreover, the inhibition of OPG in hFOB cells by glucocorticoids was detected at both the restrictive temperature and the permissive temperature, indicating that the inhibition of OPG by glucocorticoids was independent of the activity of the SV 40 large T antigen. Thus, the inhibition of OPG production following glucocorticoid treatment meets the criteria for a physiologic response. During the review process of this manuscript, the inhibitory effects of glucocorticoids on OPG mRNA levels were also reported by Vidal *et al*. (40), although this study did not assess glucocorticoid effects on OPG-L mRNA expression or the biologic consequences of these changes. In the present studies, we also demonstrate direct inhibition of OPG gene transcription by dexamethasone (by a nuclear run-on assay) and failure of the protein synthesis inhibitor, cycloheximide, to prevent dexamethasone-induced suppression of OPG mRNA steady-state levels, indicating that glucocorticoids inhibit OPG production mainly at the transcriptional level and that this does not require *de novo* protein synthesis.

In addition to effects on OPG production, glucocorticoids concurrently increased OPG-L mRNA levels in hFOB and MS cells, as assessed by RT-PCR and Northern analysis, by up to 2- to 4-fold in a time- and dose-dependent fashion. In this study, we did not assess OPG-L regulation at the protein level because no antibodies are as yet available for an ELISA or Western analysis. Thus, glucocorticoids increased the OPG-L/OPG ratio in these osteoblastic cells by 20- to 40-fold. Obviously, OPG-L/OPG mediation of the stimulatory effects of glucocorticoids on bone resorption does not exclude the contribution of other proinflammatory and bone-resorbing cytokines and cytokine receptors such as TNF-α, IL-1, and IL-6 (41–43). However, while OPG-L is induced by glucocorticoids, the synthesis of TNF-α, IL-1, and IL-6 is suppressed by glucocorticoids (44, 45). In contrast to the marked and consistent inhibition of OPG by glucocorticoids, soluble or cell-associated cytokine receptors and endogenous antagonists for other bone-resorbing cytokines (IL-1 receptors, IL-1 receptors, and TNF-R-1 and -2) appear not to be significantly regulated by glucocorticoids (44, 46). Moreover, glucocorticoids did not affect the production of M-CSF by various osteoblastic cell systems studied (Hofbauer, L. C., and S. Khosla, unpublished data).

In conclusion, we find that glucocorticoids concurrently inhibit production of the antiresorptive cytokine receptor, OPG, while stimulating the mRNA levels of the bone-resorbing cytokine, OPG-L in various human osteoblastic lineage cells. We also demonstrate stimulatory effects of conditioned medium from osteoblastic cells treated with glucocorticoids and of glucocorticoids on osteoclastogenesis *in vitro*. These findings thus provide a potential paracrine mechanism for glucocorticoid effects on bone resorption. Strategies aimed at reducing the OPG-L/OPG ratio during the systemic use of glucocorticoids may therefore be useful in preventing glucocorticoid-induced osteoporosis.

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