Osteoprotegerin mRNA is expressed in primary human osteoblast-like cells: down-regulation by glucocorticoids

N O A Vidal, H Brändström[1], K B Jonsson[1] and **C Ohlsson**

Research Centre for Endocrinology and Metabolism, Department of Internal Medicine, Sahlgrenska University Hospital, Gothenburg, Sweden and [1]Department of Internal Medicine, University of Uppsala, Uppsala, Sweden

(Requests for offprints should be addressed to C Ohlsson, Department of Internal Medicine, Division of Endocrinology, RCEM, Sahlgrenska Hospital, S-41345 Göteborg, Sweden)

Abstract

Osteoprotegerin (OPG) is a recently cloned member of the tumour necrosis factor receptor family. It has been suggested that this secreted glycoprotein acts as an inhibitor of osteoclastic differentiation. Expression of OPG has previously been demonstrated in a number of tissues. However, it is still unclear whether or not OPG is expressed by human osteoblasts. We have used the RNase protection assay to demonstrate the OPG transcript in primary cultured human osteoblast-like cells, human marrow stroma cells and osteosarcoma cell lines. Furthermore, we have studied the effect of glucocorticoids on OPG mRNA levels in these cells. We demonstrate that glucocorticoids decrease the OPG transcript in a dose- and time-dependent manner. The time-course study reveals that hydrocortisone (10^{-6} M) decreases OPG mRNA levels within 2 h. This decrease is transient, reaching control levels again after 24 h.

Our findings demonstrate that human osteoblasts express the mRNA corresponding to OPG, an inhibitor of osteoclast differentiation. The finding that OPG mRNA levels are decreased by glucocorticoids indicates that a reduced production of OPG from osteoblasts and/or marrow stroma cells could, in part, explain glucocorticoid-induced bone resorption.

Journal of Endocrinology (1998) **159,** 191–195

Introduction

The regulation of bone metabolism results from a balance between bone resorption caused by osteoclasts and bone formation caused by osteoblasts. Imbalance in the remodelling process results in metabolic bone diseases such as osteoporosis. It appears that osteoblasts play a permissive role during the resorptive phase as it has been demonstrated that osteoclastic differentiation and activity requires the presence of osteoblastic cells (Takahashi *et al.* 1988, Martin & Ng 1994, Jimi *et al.* 1996). Several studies have demonstrated that many factors which increase bone resorption, such as interleukin-6, parathyroid hormone and prostaglandin E_2, regulate osteoclast differentiation indirectly via receptors on osteoblasts (Jilka 1986, Udagawa *et al.* 1995, Kaji *et al.* 1996). These observations indicate that osteoblasts, in addition to being directly responsible for bone formation, also control bone resorption by regulating the development of osteoclastic progenitor cells as well as the activity of mature osteoclasts.

Osteoporosis is a well known side-effect of long-term treatment with glucocorticoids (Canalis 1996). The mechanism responsible for glucocorticoid-induced osteoporosis is not fully understood but it has been demonstrated that high levels of glucocorticoids inhibit bone formation (Canalis & Avioli 1992, Delaney *et al.* 1994, Advani *et al.* 1997). The effect on bone resorption is unclear. Stimulatory (Tobias & Chambers 1989, Dempster *et al.* 1997) as well as inhibitory (Conaway *et al.* 1996, Kaji *et al.* 1997) effects on osteoclast development and activity have been demonstrated *in vitro*, whereas *in vivo* more conclusive evidence for glucocorticoid-induced bone resorption exists (Bockman & Weinerman 1990, Lowe *et al.* 1992).

Whereas the effect of glucocorticoids on bone formation is believed to be a direct effect on the osteoblast lineage, the effects of glucocorticoids on bone resorption are unclear and may involve direct effects on osteoclasts as well as secondary osteoblast-mediated effects.

Osteoprotegerin (OPG), also known as osteoclastogenesis-inhibitory factor, is a recently cloned member of the tumour necrosis factor (TNF) receptor family (Simonet *et al.* 1997, Tsuda *et al.* 1997). OPG is a soluble cytokine receptor which binds to the recently cloned OPG ligand (OPGL) (Lacey *et al.* 1998), also called osteoclast-differentiation factor (ODF) (Yasuda *et al.* 1998). Recombinant OPG inhibits osteoclast differentiation by binding to OPGL on the cell surface of marrow stroma cells and osteoblasts, thereby blocking the binding of OPGL to a putative membrane-bound TNF receptor member on osteoclasts (Yasuda *et al.* 1998). Recombinant

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OPG increases bone volume in bones from normal and ovariectomised rats. Furthermore, transgenic mice over-expressing OPG have increased bone density compared with normal litter mates (Simonet *et al.* 1997).

Because OPG could be a candidate factor in the local paracrine signalling between osteoblasts and osteoclasts in bone, we decided to study the expression of OPG mRNA in cultured human osteoblast-like (hOB) cells. We here demonstrate the novel findings that the OPG transcript is expressed by primary cultured hOB cells and that OPG mRNA levels are down-regulated by glucocorticoids.

Materials and Methods

Cell culture

Cultured hOB cells were isolated from cancellous bone from orthopaedic surgery as previously described (Nilsson *et al.* 1995). The bone explants were minced into small bone chips, extensively washed in DMEM/F12 1:1 (Gibco, Paisley, UK) and thereafter transferred into 162 cm^2 culture flasks (Costar, Cambridge, MA, USA). The hOB cells and osteosarcoma cell lines MG-63 and SaOS-2 were cultured in DMEM/F12, 1:1 with the addition of 10% fetal calf serum (FCS; Gibco), Fungizone (500 µg/l; Gibco), gentamycin sulphate (50 mg/l; Sigma, St Louis, MO, USA), L-glutamine (2 mmol/l; Gibco) and L-ascorbic acid (100 mg/l; Merck, KGaA, Darmstadt, Germany), in a humidified 5% CO_2 atmosphere at 37 °C. Human marrow stroma cells (hMS) were isolated as follows. Bone fragments were rinsed with PBS and bone marrow cells were collected and centrifuged through a column of histopaque 1077. Cells from the interface were pelleted, counted and seeded into 75 cm^2 culture flasks. The cells were incubated in a humidified CO_2 incubator at 37 °C and the medium was changed weekly. At confluence, cells were detached with trypsin–EDTA (0·05/0·02% w/v) and subcultured in 75 cm^2 flasks containing α-MEM supplemented with 10% FCS and antibiotics (100 U/ml of penicillin, 100 mg/ml streptomycin, amphotericin-B, L-glutamine (2 mM)) and FCS (10%). Only first passage cells were used in the experiments.

When the effect of glucocorticoids, testosterone or oestrogen was tested, hOB cells and MG-63 cells were starved in DMEM without phenol red (Gibco) (in order to avoid oestrogen-like effects of phenol red) with the addition of 0·5% dextran-coated charcoal-stripped FCS (DCC-FCS; kindly provided by Dr M Slootweg, The Netherlands) for a period of 48 h during which hydrocortisone (H-0135, Lot: 44H9402, 115H4629, Sigma) was added at intervals ranging from 24 to 47·5 h. All cultures were harvested after 48 h. Dose dependence was studied in a similar manner, including starvation for 24 h, followed by the addition of glucocorticoids at final concentrations ranging from 10^{-9} to 10^{-6} M (hOB and MG-63 hydrocortisone, hMS, dexamethasone, Sigma). The cultures were harvested after 8 h. Hydrocortisone was dissolved in absolute ethanol and diluted 1:1000 in the culture medium. Testosterone (Sigma, T-1500) and oestrogen (β-oestradiol, Sigma, E-2758, Lot: 38F-07655) were dissolved and diluted as described for hydrocortisone above.

The study was approved by the ethical committee at Sahlgrenska University Hospital in Gothenburg.

Probes

A 219 bp (nucleotides 496–714) cDNA fragment, part of the full-length human OPG cDNA (genebank U94332), was generated with reverse transcriptase PCR and total RNA from hOB cells, using the following primer pairs: 5′-AACCCCAGAGCGAAATAC-3′ and 5′-AAGAAT GCCTCCTCACAC-3′. The cDNA fragment was inserted into a PCR-II vector (Invitrogen, Leek, The Netherlands) by T/A-cloning and the sequence was verified by sequencing. The vector was linearised with HindIII prior to *in vitro* transcription with SP6-polymerase in the presence of [^{33}P]αUTP. The 85 bp human ribosomal 18S internal standard (Ambion, Austin, TX, USA) was linearised with HindIII and T7-polymerase was used for *in vitro* transcription and incorporation of [^{33}P]αUTP (hOB and MG–63). Alternatively, the 154 bp glyceraldehyde phosphate dehydrogenase internal standard (Ambion), linearised by XbaI and transcribed with T3 polymerase, was used (hMS).

RNase protection assay

RNA was prepared as described elsewhere (Chomczynski & Sacchi 1987). The OPG transcripts were quantified with an RNase protection assay (RPA II kit, Ambion). Total cellular RNA (10 µg) was hybridised at 45 °C overnight with [^{33}P]αUTP-labelled human OPG riboprobe and 18S internal standard, then digested with RNase. The RNA/RNA hybrids were precipitated, resuspended and separated on a 6% TBE (tris-borate (90 mM)–EDTA (2 mM))–urea gel. The protected fragments were visualised and quantified using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA, USA).

Results

First we wanted to investigate if the OPG transcript is expressed in primary cultured hOB cells. The RNase protection assay demonstrated that the OPG transcript was expressed in hOB cells and in the mineralising human osteosarcoma cell line SaOS-2 (Fig. 1). Furthermore, the OPG transcript was highly expressed in the non-mineralising osteosarcoma cell line MG-63 (Fig. 1). Levels in MG-63 cells were 198 ± 5% over SaOS-2 levels (n=3).

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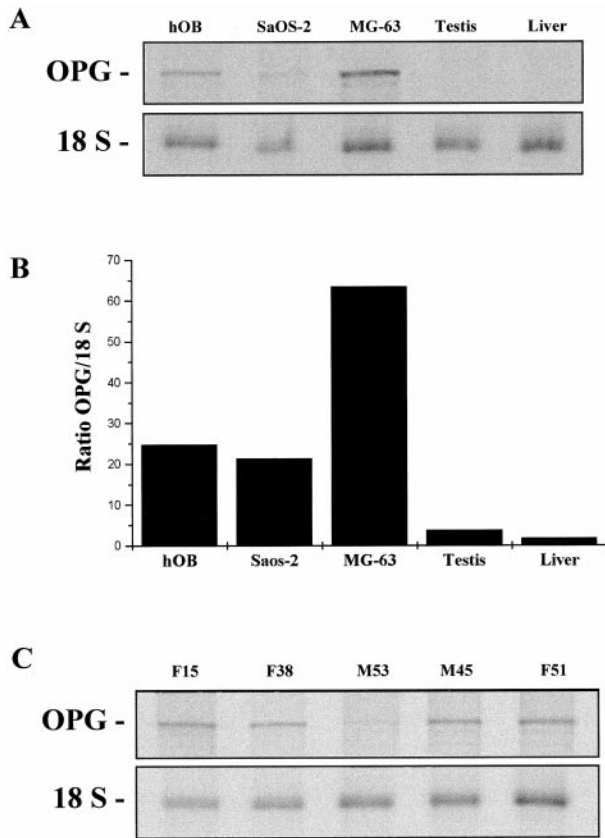

Figure 1 Expression of OPG mRNA levels in human osteoblasts (hOB), two osteosarcoma cell lines (MG-63 and SaOS-2) and in human liver and testis. (A) RNase protection assay and (B) the mean OPG/18S ratios of two separate RNA-samples from one patient (testis and liver) or from two separate cell cultures (hOB, MG-63 and SaOS-2). (C) RNase protection assay of OPG expression in hOB preparations from five different patients (F15=female, 15 years old; F38=female, 38 years old; M53=male, 53 years old; M45=male, 45 years old, F51=female, 51 years old).

We studied the OPG mRNA levels in hOB cell preparations from eight different cell donors (six females and two males, 15–58 years old) and detected OPG mRNA in all but one of these cell preparations. We could not find any correlation between OPG mRNA levels and cell density (data not shown).

The next question was to study if glucocorticoids regulate OPG mRNA levels. Hydrocortisone decreased the levels of OPG mRNA in hOB cells in a dose-dependent manner (Fig. 2). Treatment of MG-63 cells with hydrocortisone (10^{-6} M) and hMS cells with dexamethasone (10^{-6} M) for 8 h resulted in a similar decrease in OPG mRNA levels (Fig. 2A and B). Time-course studies (Fig. 3) revealed that hydrocortisone (10^{-6} M) decreased OPG mRNA levels in hOB cells within 2 h (72 ± 4% of control culture) and that this decrease was present 8 h (58 ± 8% of control culture) after hydrocortisone stimulation. Control levels were reached again after 24 h (109 ± 13% of control culture).

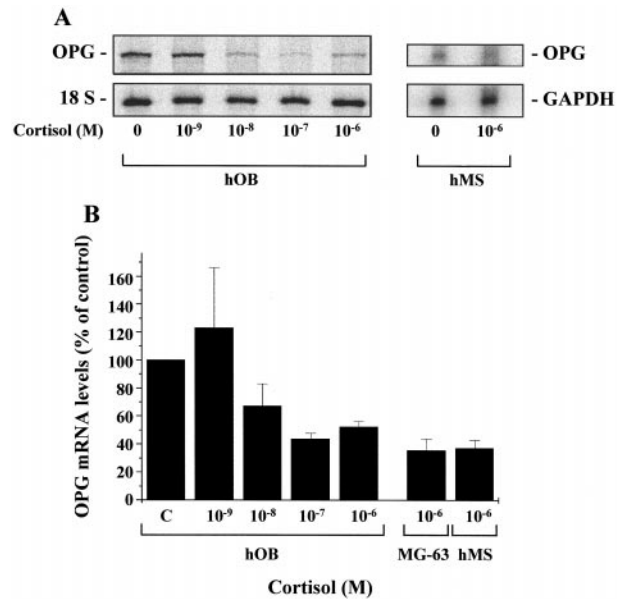

Figure 2 Dose–response effect of glucocorticoids on OPG mRNA levels in cultured human osteoblasts (hOB, hydrocortisone), human marrow stroma cells (hMS, dexamethasone) and the osteosarcoma cell line MG-63 (hydrocortisone). Cells were cultured in 10% FCS, starved in DMEM without phenol red with addition of 0·5% DCC-FCS for 24 h and then stimulated with different doses of glucocorticoids 8 h before harvest. Each experiment was performed using cells from one patient.
(A) Representative RNase protection assay gel picture.
(B) Densitometric quantification of the results from hOB-cells from three different patients, three cultures of MG-63 cells and two separate hMS cell cultures. Values are expressed as OPG mRNA as per cent of control ± S.E.M.

The effects of testosterone (10^{-12} to 10^{-8} M) and oestrogen (10^{-15} to 10^{-8} M) were also studied under similar culture conditions, but no statistically significant effects were found (data not shown).

Discussion

Osteoclast differentiation is regulated by many hormones and growth factors (Suda *et al.* 1992, 1995, Lerner & Ohlin 1993, Hofbauer & Heufelder 1996, Lerner 1996). It is generally believed that the presence of osteoblastic cells is required for normal osteoclast development (Takahashi *et al.* 1988, Martin & Ng 1994, Jimi *et al.* 1996). We here report the novel finding that the OPG transcript is expressed in primary cultured hOB cells. This finding strongly suggests that local production of OPG may be of importance for the coupling between osteoblasts and osteoclast development in human bone tissue.

Previously presented *in vitro* and *in vivo* results, on the effects of OPG in rodents, indicate that the main effect of

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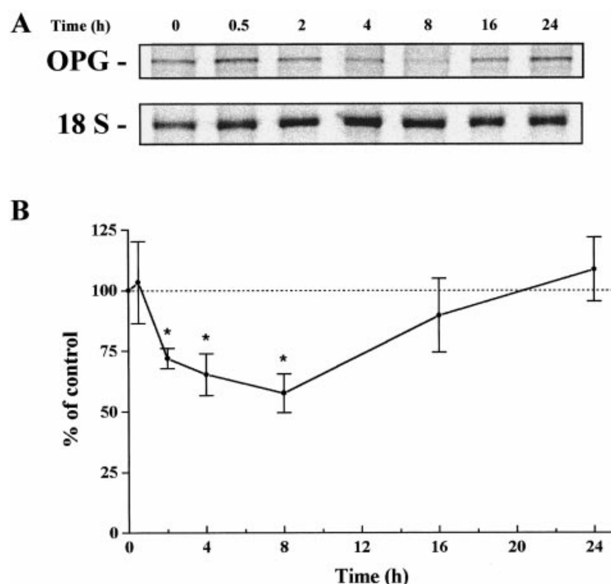

Figure 3 Time-course effect of hydrocortisone on OPG mRNA levels in hOB cells. Cells were cultured in 10% FCS, then starved in DMEM without phenol red with addition of 0·5% DCC-FCS for a total length of 48 h and stimulated with hydrocortisone (10^{-6} M) at different times before harvest. (A) RNase protection assay of hOB from a 53-year-old male. (B) Densitometric quantification of the pooled results from three experiments. Each experiment was performed using cells from one patient. The results from three different patients were then pooled and are shown in (B). Values are expressed as OPG/18S ratio as per cent of control ± S.E.M. *P<0·05 compared with untreated cells.

OPG is to inhibit the differentiation of osteoclasts. Glucocorticoid-induced bone resorption could therefore, in part, result from a local down-regulation of OPG expression in bone. This notion is supported by recent findings that high levels of glucocorticoids increase osteoclast recruitment rather than activity in mouse bone and spleen cultures (Kaji *et al.* 1997) and by the finding that the effect of glucocorticoids on resorption is dependent on cellular replication in neonatal mouse calvariae (Conaway *et al.* 1996). We therefore chose to investigate the effects of glucocorticoids on osteoblastic expression of OPG mRNA. Our results clearly demonstrate that glucocorticoids decrease the OPG transcript in hOB cells and in human marrow stroma cells. Other steroids with well-documented effects on bone metabolism, such as testosterone and oestrogen, did not cause a change in OPG mRNA levels under similar culture conditions, demonstrating that the effect of glucocorticoids is specific. Our finding that glucocorticoids decrease the OPG transcript is intriguing, not only because it provides a possible explanation of how glucocorticoids may influence bone resorption, but also because it indicates that the local cross-talk taking place between osteoblasts and osteoclasts in bone may be of either a stimulatory or inhibitory nature. This notion is clearly supported by recent reports that OPGL or ODF, the membrane-bound ligand for OPG (Lacey *et al.* 1998, Yasuda *et al.* 1998), is present on marrow stroma cells and osteoblasts (Yasuda *et al.* 1998). The finding that osteoblasts produce OPG as well as ODF is evidence for a negative feed-back loop in which the osteoblast itself may modulate the recruitment of osteoclast precursors by presenting ODF molecules on its surface, which in turn can be made unavailable by systemic or locally produced OPG.

It is interesting to note that the mineralising osteosarcoma cell line SaOS-2 together with hOB cells expressed significantly lower amounts of OPG transcript when compared with the non-mineralising osteosarcoma cell line MG-63. This finding indicates that the OPG transcript could be differentially regulated during osteoblast differentiation. However, in the present study we failed to detect any significant alterations of OPG mRNA levels in hOB cells at different stages of confluence.

In conclusion we have, for the first time, demonstrated that the OPG transcript is expressed in primary cultured hOB cells and in hMS cells. Furthermore, OPG mRNA levels were shown to be decreased by glucocorticoids in these cells. Our findings indicate that glucocorticoid-induced bone resorption in part may result from a decrease in the local secretion of OPG from osteoblasts/marrow stroma cells. The biological significance of this finding remains to be demonstrated and will remain unresolved until commercial antibodies for OPG are made available.

Acknowledgements

This work was supported by grants K95–19P-11328–01A and K96–19P11837–02B from the Swedish Medical Research Council, grants from Pharmacia & Upjohn (Stockholm, Sweden), the Novo Nordisk Foundation (Bagsvaerd, Denmark), the Lundberg Foundation, and the Göteborg Medical Society. We thank A Hansevi for her excellent assistance. We would also like to thank the Department of Orthopaedics, Sahlgrenska Hospital, and Dr Anders Nilsson for providing us with bone samples.

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Received 2 March 1998
Revised manuscript received 5 May 1998
Accepted 29 May 1998

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