

Changing *RANKL/OPG* mRNA expression in differentiating murine primary osteoblasts

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Abstract

Osteoblast–osteoclast coordination is critical in the maintenance of skeletal integrity. The modulation of osteoclastogenesis by immature cells of the osteoblastic lineage is mediated through receptor activator of NF κ B (RANK), its ligand RANKL, and osteoprotegerin (OPG), a natural decoy receptor for RANKL. Here, the expression of *OPG* and *RANKL* in primary mouse osteoblastic cultures was investigated to determine whether the osteoclastogenic stimulus depended on the stage of osteoblastic differentiation and the presence of the calcitrophic hormone 1,25-dihydroxyvitamin D₃ (1,25-(OH)₂D₃).

OPG mRNA expression was increased in osteoblastic cultures after the onset of mineralisation relative to less mature cultures, but did not alter in response to 1,25-(OH)₂D₃ treatment. In contrast, basal *RANKL* mRNA expression did not change during differentiation but was significantly enhanced by 1,25-(OH)₂D₃ treatment at all times. The stimulatory effects of 1,25-(OH)₂D₃ on

RANKL were lessened in more mature cultures, however. The *RANKL/OPG* ratio, an index of osteoclastogenic stimulus, was therefore increased by 1,25-(OH)₂D₃ treatment at all stages of osteoblastic differentiation, but to a lesser degree in cultures after the onset of mineralisation. Thus the 1,25-(OH)₂D₃-driven increase in osteoclastogenic potential of immature osteoblasts appears to be mediated by increased *RANKL* mRNA expression, with mature osteoblasts having relatively decreased osteoclastogenic activity due to increased *OPG* mRNA expression. These findings suggest a possible mechanism for the recently proposed negative regulatory role of mature osteoblasts on osteoclastogenesis and indicate that the relative proportions of immature and mature osteoblasts in the local microenvironment may control the degree of resorption at each specific bone site.

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Introduction

Skeletal structure is continually adapting to metabolic and mechanical demands. Osteoblastic (bone forming) and osteoclastic (bone resorbing) cells maintain the integrity of bone during this remodelling process, with tight regulation and coordination of their activities. *In vivo* histological and *in vitro* culture studies suggested a role for osteoblastic cells in the direct regulation of osteoclast activity (Rodan & Martin 1981, Martin & Ng 1994).

Resorption is stimulated by a number of factors, including the hormones 1,25-dihydroxyvitamin D₃ (1,25-(OH)₂D₃) and parathyroid hormone (Teti *et al.* 1988, Flanagan *et al.* 1995, Suda *et al.* 1997). Enhancement of osteoclast differentiation and activity by 1,25-(OH)₂D₃ is largely mediated via the immature cells of the osteoblastic lineage (Martin & Ng 1994). Regulatory factors involved in this interaction have been identified. Stromal and osteoblast precursor cells express a member of the tumour necrosis factor ligand family, receptor activator of NF κ B ligand (RANKL), also identified as osteoclast differentia-

tion factor (ODF), osteoprotegerin ligand (OPG-L) and tumour necrosis factor-related, activation-induced cytokine (TRANCE) (Anderson *et al.* 1997, Wong *et al.* 1997, Lacey *et al.* 1998, Yasuda *et al.* 1998b). This cell-surface ligand stimulates osteoclastogenesis and osteoclast activity by binding to its cognate receptor, RANK, also termed ODF receptor (ODFR), on the surface of osteoclast precursors (Hsu *et al.* 1999). Addition of the cleaved and thus soluble form of RANKL can increase osteoclast formation and activity *in vitro* (Lacey *et al.* 1998, Matsuzaki *et al.* 1998, Yasuda *et al.* 1998b, Burgess *et al.* 1999). A further level of regulation is provided by the production of a RANKL 'decoy receptor', osteoprotegerin (OPG) or osteoclastogenesis inhibitory factor (OCIF), by osteoblasts and other cell types (Simonet *et al.* 1998, Yasuda *et al.* 1998a). OPG binds to RANKL, blocking its interaction with RANK on osteoclast precursors and thus inhibiting osteoclast activity (Lacey *et al.* 1998, Yasuda *et al.* 1998b, Hsu *et al.* 1999). The importance of this pathway has been demonstrated in *OPG* knockout mice, which exhibit severe osteoporosis (Bucay *et al.* 1998,

Mizuno *et al.* 1998), and transgenic mice overexpressing OPG, which have osteopetrosis (Simonet *et al.* 1997). On the basis of these and other studies, it has been suggested that the ratio between *RANKL* and *OPG* expression levels in osteoblastic cells is a key factor in osteoclast regulation (Horwood *et al.* 1998, Nagai & Sato 1999).

Previous *in vitro* studies of OPG/RANK/RANKL regulation have utilised stromal cells, immature primary osteoblasts and osteoblast cell lines (Horwood *et al.* 1998, Hofbauer *et al.* 1999, Lee & Lorenzo 1999, Nagai & Sato 1999, Shirai *et al.* 1999, Suda *et al.* 1999, Yasuda *et al.* 1999, Deyama *et al.* 2000). Immature osteoblasts and stromal cells enhance osteoclast recruitment and differentiation (Martin & Ng 1994). Recent studies using cell lines induced to differentiate into a more mature osteoblastic phenotype have indicated that levels of expression of *RANKL* and *OPG* may be altered (Shirai *et al.* 1999, Deyama *et al.* 2000). However, the patterns of expression of *RANKL* and *OPG* have not been established during primary osteoblastic differentiation.

Primary osteoblasts cultured *in vitro* progress through a developmental sequence considered to mirror the sequence *in vivo*, with three overlapping stages of osteoblast differentiation and characteristic patterns of gene expression (Robey & Tremine 1985, Marie *et al.* 1989, Owen *et al.* 1990, Lian *et al.* 1990). Collagen type 1 α 1 (*COL1*; the main structural component of the bone extracellular matrix), is expressed from an early stage during osteoblast differentiation (Owen *et al.* 1990). Expression of non-collagenous matrix proteins such as osteopontin (OPN) and alkaline phosphatase (ALP), which are believed to stabilise the matrix, is associated with the matrix formation/maturation phase of differentiation (Owen *et al.* 1990, Stein & Lian 1993). Osteocalcin, the non-collagenous matrix protein expressed almost exclusively in bone, is up-regulated at the late stage of differentiation coincident with the onset of mineralisation, and thus is believed to have a role in the regulation of this process (Owen *et al.* 1990, Ducy *et al.* 1996). The effects of 1,25-dihydroxyvitamin D₃ (1,25-(OH)₂D₃) on osteoblasts have been well characterised in rat and human osteoblastic cultures, with both stimulation and inhibition of genes associated with the differentiated osteoblastic phenotype, but an overall inhibitory effect on osteoblast differentiation, particularly by longer-term treatment (Aufmkolk *et al.* 1985, Owen *et al.* 1991, Ishida *et al.* 1993, Siggelkow *et al.* 1999).

The present study examined primary mouse osteoblasts throughout their developmental sequence and their response to 1,25-(OH)₂D₃ treatment. Basal and 1,25-(OH)₂D₃-stimulated expression patterns of mouse genes encoding bone structural proteins and osteoclastogenic regulators were examined. *RANKL* mRNA expression was stimulated by 1,25-(OH)₂D₃ at all times, but was down-regulated in untreated late cultures. In contrast, *OPG* mRNA expression was increased at the onset of

mineralisation and remained high throughout, thereby resulting in a lower *RANKL/OPG* ratio in mature osteoblasts. These changes in gene expression correlate with the previously proposed lower osteoclastogenic potential of mature osteoblasts in mice and suggest that immature and mature osteoblasts may have different roles in the maintenance of the bone remodelling balance.

Materials and Methods

Cell culture

Primary calvarial osteoblast cultures were established from 2-day-old FVB/N mice by enzymatic digestion (Wong & Cohn 1975), as described previously (Thomas *et al.* 2000). Briefly, frontal and parietal bones were removed, minced, transferred to digest mix (1 mg/ml collagenase (Boehringer-Mannheim, Mannheim, Germany), 0.05% trypsin (CSL, Sydney, Australia), 0.02% EDTA (ICN, Costa Mesa, CA, USA) in PBS; 1 ml/5 calvaria) and stirred for 30 min at 37 °C. Cells released from the first digest were discarded and the cells released from the second digest were seeded at a density of 10⁴ cells per cm² into six-well plates and cultured in α -MEM (Trace, Sydney, Australia) containing 10% fetal calf serum (Gibco, Grand Island, NY, USA), 100 U/ml penicillin, 100 mg/ml streptomycin, 40 mg/ml gentamycin, 20 mM HEPES, 2 mM glutamine (all Gibco), 10 mM β -glycerophosphate, 50 mg/ml ascorbic acid (Sigma, St Louis, MO, USA). Culture medium was changed after 3 days and every 2–3 days thereafter.

Cells were treated with 10⁻⁸ M 1,25-(OH)₂D₃ or 0.1% isopropanol vehicle at the time-point indicated and collected for analysis 48 h later.

Biochemical determinations

Cells were lysed in 0.5 ml/well of 0.1% Triton-X100, 0.5 mM MgCl₂ in 20 mM Tris base, pH 10 and stored at -20 °C until required for assay.

DNA was assayed using a fluorimetric assay (Downs & Wilfinger 1983, Rao & Otto 1992). Briefly, 0.05 ml lysate was mixed with 0.2 ml of 1 mg/ml bisbenzimidazole in water in clear polystyrene 96-well plates in duplicate, allowed to stand for 5 min, then read on a Fluoroskan II (Labsystems, Helsinki, Finland) with excitation at 355 nm and emission at 460 nm. DNA values were calculated using the Delta-Soft analytical software (Biometallics, Princeton, NJ, USA) and expressed as μ g DNA.

ALP concentrations in the cell lysates were assayed by colourimetry (Lowry *et al.* 1954). Lysate (0.25 ml) was incubated at 37 °C for 15 min with 0.075 ml AMP buffer (0.075 M AMP 221 buffer (Sigma), 10 mM MgCl₂, 2 mg/ml *p*-nitrophenol phosphate) in clear polystyrene 96-well plates. NaOH (0.1 ml of 0.5 M) was added to stop

the reaction and the plates were read at 405 nm. ALP concentrations were calculated against a *p*-nitrophenol standard curve. Values are expressed as mol *p*-nitrophenol produced per μg DNA per h.

Osteocalcin concentrations in the culture medium were determined by radioimmunoassay (Gundberg *et al.* 1992) in 50 μl samples and expressed as ng osteocalcin per μg DNA. Primary antibody and osteocalcin standards were kindly provided by C Gundberg (New Haven, CT, USA). Iodinated osteocalcin was purchased from Biomedical Technologies Inc., Stoughton, MA, USA. Donkey anti-goat IgG secondary antibody was purchased from Sigma.

RNA isolation and analysis

RNA was isolated from osteoblastic cell cultures in six-well plates using 1 ml/well of Trizol reagent (Gibco) with 5 μg glycogen as carrier. RNA (15 μg) was analysed by northern blot analysis on a nylon filter (HybondN+, Amersham, Buckinghamshire, UK), and probed with the random primed α - ^{32}P -dCTP labelled *osteocalcin* probe followed, after stripping, by the mouse *osteopontin*, *COL1*, *OPG*, *RANKL* and *GAPDH* probes. Radioactivity bound to the filter was measured on a phosphorimager (Molecular Dynamics, Sunnyvale, CA, USA) and normalised to the *GAPDH* signal.

Semi-quantitative RT-PCR

Total RNA was isolated as for the northern blot analysis. cDNAs were generated by reverse transcriptase (Superscript II, Gibco) with random hexamer primers. PCR amplification was carried out with gene-specific primers (Sigma) using Taq polymerase (Amplitaq, Perkin Elmer, Norwalk, CT, USA). Reactions were carried out in foil-sealed 96-well plates (Thermofast 96, ABGene, Epsom, Surrey, UK) on a GeneAmp 9700 machine (Perkin Elmer). Both *OPG* and *RANKL* were duplexed with *GAPDH* after conditions were optimised to produce linear amplification for both genes. For both *OPG*–*GAPDH* and *RANKL*–*GAPDH*, annealing temperatures were 65 °C with the reactions run for 29 cycles. Specific primers were as follows: *OPG* (product 578 bp) forward 5'-TCCTG GCACCTACCTAAAACAGCA-3', reverse 5'-CTAC ACTCTCGGCATTCACTTTGG-3'; *RANKL* (product 394 bp) forward 5'-CACACCTCACCATCAATGCT GC-3', reverse 5'-GAAGGGTTGGACACCTGAA TGC-3'; *GAPDH* (product 983 bp) forward 5'-GGTCCGGTGTGAACGGATTTGG-3', reverse 5'-ATGTAGGCCATGAGGTCCACC-3'. PCR products were run on 1.5% agarose gels, stained with ethidium bromide, the image captured using GelDoc (BioRad, Hercules, CA, USA) and the bands quantified using MolecularAnalyst software (BioRad). Data are represented as the ratio to *GAPDH* PCR product.

Statistical analysis

Data shown are from at least two separate culture experiments and are the means of four to six replicates in each experiment for biochemical determinations. For RNA analysis, three experiments were conducted with two to six replicates in each experiment. Statistical comparisons were carried out by ANOVA using SuperAnova software (Abacus Concepts, Berkeley, CA, USA).

Results

Osteoblast phenotype

FVB/N primary mouse osteoblasts progressed through the osteoblastic developmental sequence, reaching confluence after approximately 10 days of culture, followed by matrix formation and maturation. At approximately 10 days post-confluence, mineralisation was detectable and continued for a further 20 days (as determined by visual inspection). These phases were reflected in the patterns of expression of key genes such as *COL1*, *ALP*, *osteopontin* and *osteocalcin*, and in mineralisation (Fig. 1). *Osteopontin* mRNA expression was evident at confluence and did not change significantly throughout the culture period. *COL1* mRNA expression was high at confluence and decreased markedly by 10 days post-confluence before increasing at 20 and 30 days post-confluence. Although osteocalcin protein was measurable at confluence, its mRNA was negligible by northern blot analysis at this time-point. The increase in *osteocalcin* mRNA at later times (20 and 30 days post-confluence) was not as marked as the increase in osteocalcin protein. The ALP protein profile was similar to that of osteocalcin protein. Mineralisation, as measured by uptake of calcium-45, was low during the matrix maturation phase (8 days post-confluence) but increased through days 17 to 25 days post confluence (Fig. 1).

The effects of short-term 1,25-(OH) $_2$ D $_3$ treatment on expression of individual genes varied (Fig. 1). *Osteocalcin* RNA was reduced at all times post-confluence and osteocalcin protein was decreased by 20 days post-confluence. *Osteopontin* mRNA was strongly up-regulated by 1,25-(OH) $_2$ D $_3$ throughout the culture period. In contrast, there was no consistent effects of 1,25-(OH) $_2$ D $_3$ on *COL1* mRNA expression. ALP protein was decreased after 1,25-(OH) $_2$ D $_3$ treatment at both time-points post-confluence. Short-term (2 day) treatment with 1,25-(OH) $_2$ D $_3$ inhibited mineralisation at 8 and 17 days post-confluence.

Osteoclastogenesis regulatory genes

RANKL RNA levels, measured by semi-quantitative RT-PCR, were evident at confluence, increased at 10 days post-confluence, then returned to confluence values at 20 days and remained low at 30 days post-confluence

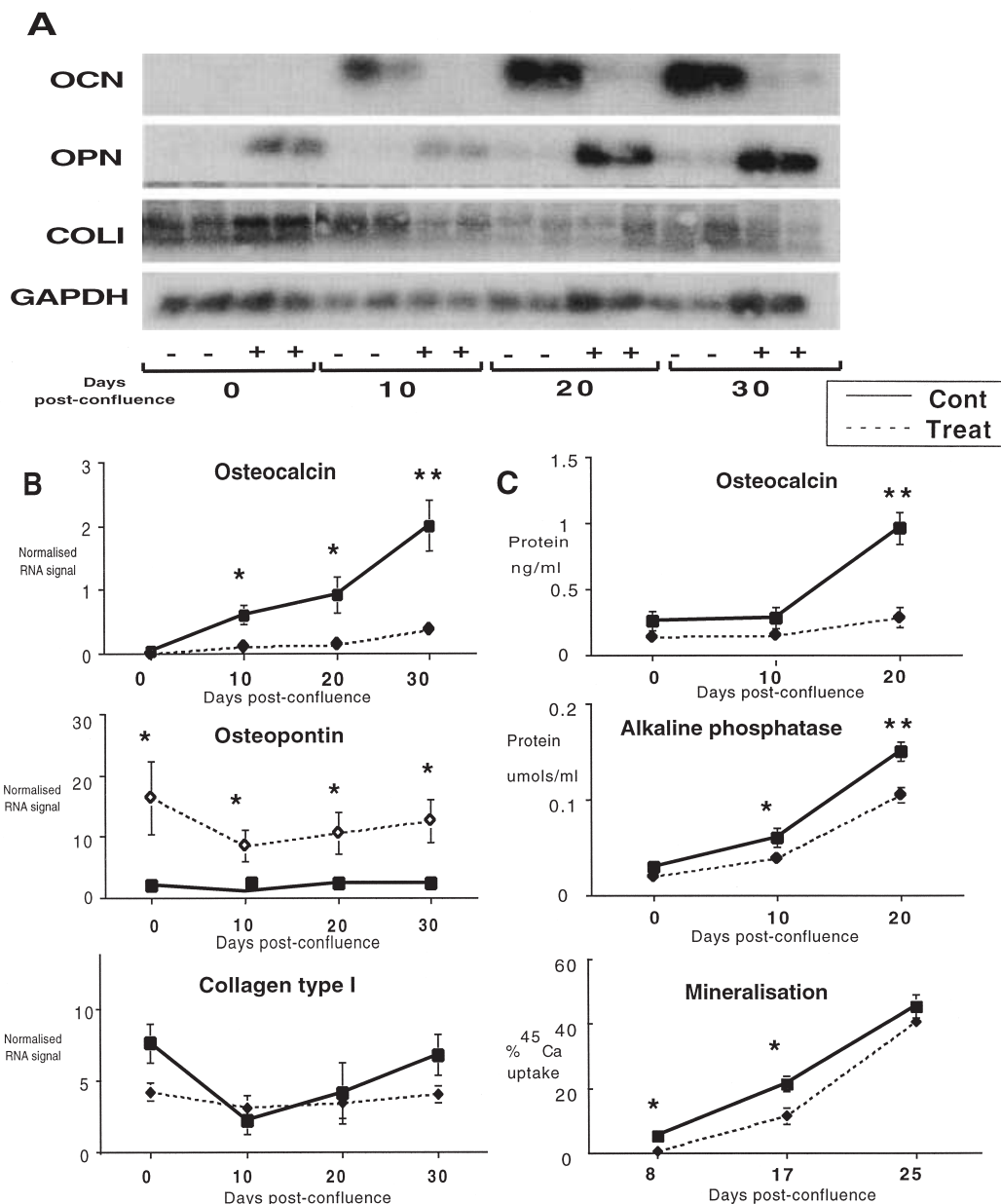


Figure 1 Osteoblast gene expression in primary osteoblast cultures. (A) Northern blot showing expression of genes in basal conditions and in $1,25\text{-(OH)}_2\text{D}_3$ -treated osteoblasts, at various stages during the culture period. Large changes in *osteocalcin* (OCN) and *osteopontin* (OPN) in response to $1,25\text{-(OH)}_2\text{D}_3$ treatment are evident. Data are from duplicate wells. On longer exposures (not shown), signal was evident in all lanes of vehicle-treated cultures. (B) Expression of the *osteocalcin* gene was low at confluence and increased as differentiation progressed and was decreased significantly by $1,25\text{-(OH)}_2\text{D}_3$ treatment at all time-points post-confluence. *Osteopontin* RNA levels were relatively stable throughout the time course and were significantly increased by $1,25\text{-(OH)}_2\text{D}_3$ treatment at all time-points. *COL1* (Collagen type 1) RNA expression was high at confluence, decreased by 10 days post-confluence then increased again by 30 days post-confluence, but showed no response to $1,25\text{-(OH)}_2\text{D}_3$ treatment. (C) Osteocalcin and ALP (Alkaline phosphatase) protein levels both increased markedly after the onset of mineralisation (beyond 10 days post-confluence). Treatment with $1,25\text{-(OH)}_2\text{D}_3$ decreased osteocalcin protein at 20 days post-confluence and ALP at 10 and 20 days post-confluence. Mineralisation increased with the progression of differentiation in the cultures and was significantly inhibited by $1,25\text{-(OH)}_2\text{D}_3$ treatment at 8 and 17 days, but not 25 days post-confluence. Note the different time-points of the experiments. Cont (—), vehicle-treated; Treat (---), $1,25\text{-(OH)}_2\text{D}_3$ -treated. Values are means \pm S.E.M. of two or three experiments with two to four replicates. * $P < 0.05$, ** $P < 0.01$, $1,25\text{-(OH)}_2\text{D}_3$ -treated compared with vehicle-treated cultures.

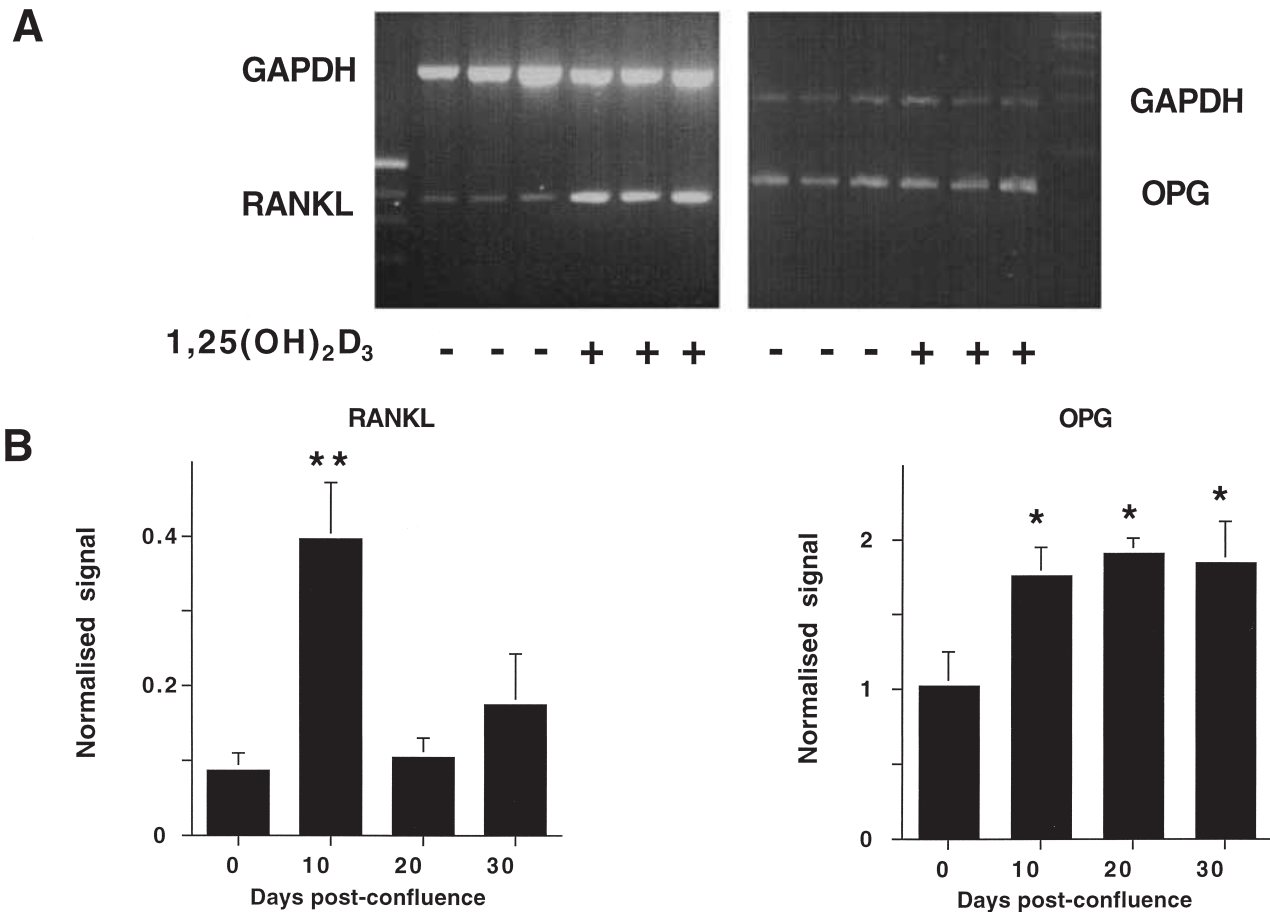


Figure 2 Levels of expression of osteoclastic regulatory molecules (RANKL and OPG) during osteoblast development in primary cultures. (A) Representative semi-quantitative RT-PCR for RANKL and OPG at 10 days post-confluence with and without 1,25-(OH)₂D₃ treatment. The upper band represents GAPDH (983 bp) and the lower band RANKL (394 bp) or OPG (578 bp). (B) RANKL and OPG RNA levels in untreated cultures, detected by semi-quantitative RT-PCR. RANKL expression increased between confluence and 10 days later, then returned to basal values at 20 and 30 days post-confluence. OPG expression increased by 10 days post-confluence relative to levels at confluence and remained increased at 20 and 30 days post-confluence. Values are means \pm S.E.M. of three experiments with two to six replicates. * $P < 0.05$, ** $P < 0.01$, time-point compared with confluent cultures.

(Fig. 2). OPG RNA was evident at confluence, increased by 10 days post-confluence and remained at a similar level thereafter (Fig. 2).

The effects of 1,25-(OH)₂D₃ treatment on expression of RANKL and OPG mRNAs were examined at various times up to 30 days post-confluence (Fig. 3). RANKL mRNA expression was increased by 1,25-(OH)₂D₃ treatment at all time-points. This was in contrast to OPG mRNA expression, which did not change in response to 1,25-(OH)₂D₃ treatment. As a consequence, the RANKL/OPG ratio was increased significantly by 1,25-(OH)₂D₃ treatment at all time-points, but to a lesser extent in the more mature primary osteoblastic cells.

Discussion

Expression of RANKL and OPG, key osteoclast regulatory genes, has previously been characterised predominantly in

immature non-mineralising osteoblasts. In the present study, osteoblastic development through to mature mineralising cells was examined in primary cultures, as were the effects of 1,25-(OH)₂D₃ treatment on gene expression during this differentiation sequence. The developmental profile of mouse primary osteoblasts has been described previously (Lian *et al.* 1997, Thomas *et al.* 2000), although RANKL and OPG gene expression in fully mature mineralising primary osteoblastic cultures have not been studied.

The three osteoblastic developmental phases (Owen *et al.* 1991) can be clearly delineated, with the initial proliferative phase during which COL1 and osteopontin mRNA expression were evident, followed by the matrix production and maturation phase in which expression of the non-collagenous proteins osteopontin, ALP and osteocalcin were increased. The final mineralisation phase was marked by the increase in ⁴⁵Ca uptake concomitant with

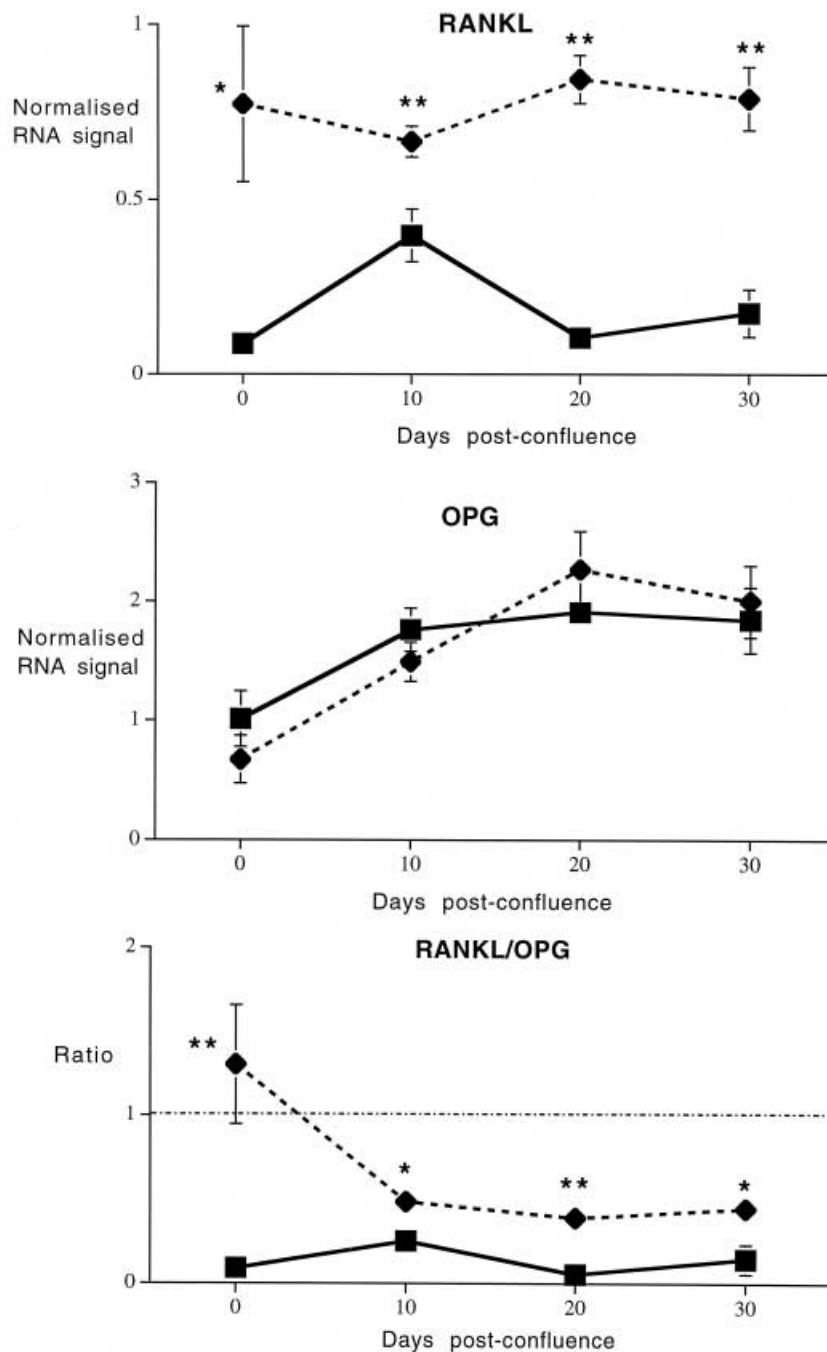


Figure 3 Effects of short-term $1,25(\text{OH})_2\text{D}_3$ treatment on *RANKL* and *OPG* expression levels in osteoblastic cultures, measured by semi-quantitative RT-PCR. *RANKL* expression was up-regulated at all time-points by $1,25(\text{OH})_2\text{D}_3$ treatment. *OPG* expression was unaffected by $1,25(\text{OH})_2\text{D}_3$ treatment. *RANKL/OPG* ratios were increased at confluence by 48 h of treatment with 10^{-8}M $1,25(\text{OH})_2\text{D}_3$. At later time-points the increase was less marked. —, Vehicle-treated cultures; ---, $1,25(\text{OH})_2\text{D}_3$ -treated cultures. Values are means \pm S.E.M. of three experiments with two to six replicates. * $P < 0.05$, ** $P < 0.01$, $1,25(\text{OH})_2\text{D}_3$ -treated compared with vehicle-treated cultures.

further up-regulation of osteocalcin production. This differentiation profile is similar to that of other species (Robey & Termine 1985, Marie *et al.* 1989, Owen *et al.* 1990). The increase in *COL1* mRNA expression observed during the later stages of osteoblast differentiation is consistent with a number of other studies in differentiated mouse, but not rat, osteoblasts (Owen *et al.* 1991, Yamashita *et al.* 1996, Lian *et al.* 1997, Qu *et al.* 1998).

As for other species, 1,25-(OH)₂D₃ treatment suppressed mineralisation of these primary mouse osteoblasts. Changes in gene expression were also consistent with earlier studies, including the inhibition of *osteocalcin* expression (Clemens *et al.* 1997, Lian *et al.* 1997, Sims *et al.* 1997, Zhang *et al.* 1997, Thomas *et al.* 2000) and up-regulation of *osteopontin* expression (Beresford *et al.* 1994, Owen *et al.* 1991, Lian *et al.* 1997). Short-term 1,25-(OH)₂D₃ treatment had no significant effect on *COL1* mRNA expression, consistent with previous studies in mice and other species reporting only minimal differing responses to 1,25-(OH)₂D₃ (Kurihara *et al.* 1984, Lian *et al.* 1997, Siggelkow *et al.* 1999). ALP protein was down-regulated by 1,25-(OH)₂D₃ at all time-points measured, consistent with reports in rat primary osteoblasts (Owen *et al.* 1991), but contrasting with its up-regulation by 1,25-(OH)₂D₃ in the mouse osteoblastic MC3T3-E1 cell line (Kurihara *et al.* 1984, Matsumoto *et al.* 1991). Studies in human primary osteoblasts and cell lines have documented both up-regulation (Murray *et al.* 1987, Beresford *et al.* 1994, Harris *et al.* 1995) and minimal effects (Marie *et al.* 1989) on ALP by 1,25-(OH)₂D₃. Although mouse bone cells exhibit osteoblastic morphology and general response to 1,25-(OH)₂D₃ treatment that are similar to those in other species, differences in patterns of expression of specific gene and responses to 1,25-(OH)₂D₃ do occur. The significance of these species differences remains to be elucidated.

RANKL, a stimulator of osteoclast differentiation and activity (Yasuda *et al.* 1998b, Burgess *et al.* 1999), and *OPG*, the inhibitor of osteoclastogenesis (Simonet *et al.* 1997, Yasuda *et al.* 1998a), were differentially expressed during osteoblast development. *RANKL* mRNA expression was detectable at confluence, increased transiently during matrix formation and initiation of mineralisation, then decreased in cells during the mineralisation phase. *OPG* mRNA expression was evident from confluence, increased during matrix production and maturation, and remained high in the mineralisation phase. The *RANKL/OPG* ratio, an index of osteoclast activation, did not alter significantly over the time-course of the study under basal conditions, with only a slight increase at the onset of mineralisation followed by a return to baseline as mineralisation progressed. Importantly, however, the *RANKL/OPG* ratio in response to 1,25-(OH)₂D₃ treatment decreased during maturation of these primary osteoblastic cultures. In this present study, protein levels of *RANKL* and *OPG* were not measured. However, recent studies

have demonstrated that protein levels of *RANKL* and *OPG* closely correlate with mRNA levels *in vitro* (Hofbauer *et al.* 1999, Deyama *et al.* 2000). Furthermore, *RANKL* and *OPG* mRNA levels have been shown to correlate with altered resorption in response to physiological stimuli such as calcium concentration (Yasuda *et al.* 1998b, Takeyama *et al.* 2000) and hormonal treatment (Lee & Lorenzo 1999), in pathologic states such as Paget's disease of bone (Menaar *et al.* 2000) and in a breast cancer bone metastasis model (Thomas *et al.* 1999).

Unlike the present study of mature mineralising osteoblastic cultures, previous *in vitro* characterisation of *RANKL* and *OPG* expression has focused on immature primary osteoblasts and osteoblastic cell lines. On the basis of these studies, it was previously suggested that regulation of *RANKL* and *OPG* by osteotropic agents such as 1,25-(OH)₂D₃ did not occur or was significantly reduced in more mature osteoblastic cell lines (Horwood *et al.* 1998, Nagai & Sato 1999). In the present study, however, a significant increase in the *RANKL/OPG* ratio was elicited by 1,25-(OH)₂D₃ treatment at all time-points, including the most mature cultures. The up-regulation of *RANKL* by 1,25-(OH)₂D₃ treatment was consistent with the known osteoclastogenic action of 1,25-(OH)₂D₃ and agrees with a number of recent reports in cell line and immature primary cells (Horwood *et al.* 1998, Yasuda *et al.* 1998b, Nagai & Sato 1999). *OPG* was not significantly altered by 1,25-(OH)₂D₃, contrasting with its down-regulation reported in previous studies in immature osteoblastic cells (Horwood *et al.* 1998, Murakami *et al.* 1998). The greater *RANKL/OPG* ratio in less mature 1,25-(OH)₂D₃-treated confluent cultures, driven by the lower *OPG* level at this time-point, is consistent with the osteoclastogenic activity of precursor stromal cells and early osteoblasts (Martin & Ng 1994). The decrease in the *RANKL/OPG* ratio in more differentiated cultures, whether under basal or 1,25-(OH)₂D₃-treated conditions, is driven primarily by the relative increase in *OPG* mRNA expression in mature osteoblasts, as there was little change in *RANKL* response to 1,25-(OH)₂D₃ during osteoblast development. The fact that *OPG* is a secreted polypeptide that may act locally or systemically, while *RANKL* is a cell-surface molecule and therefore can only act locally on neighbouring cells, may also influence this putative regulatory pathway. A recent study reported only partial site-specific rescue of osteopetrosis in *RANKL*-deficient mice by a lymphocyte-expressed *RANKL* transgene (Kim *et al.* 2001).

Recently, a decreased *RANKL/OPG* ratio in differentiated MC3T3.E1 osteoblasts was reported (Deyama *et al.* 2000). In that study, however, reduction of the *RANKL/OPG* ratio was driven by decreased expression of *RANKL* rather than by the increase in *OPG* expression seen here, possibly reflecting a difference between established cell lines and primary cultures. The differentiated MC3T3.E1 osteoblasts also exhibited decreased osteoclastogenic

potential (Deyama *et al.* 2000), consistent with a previously proposed negative regulatory effect of mature osteoblasts on osteoclast activity (Gardiner *et al.* 2000). Taken together, these data therefore suggest that altering the *RANKL/OPG* ratio may provide a simple reciprocal mechanism by which 1,25-(OH)₂D₃ could mediate both stimulation of osteoclastogenesis through immature stromal cells/osteoblasts, and the proposed inhibition of osteoclast activity by mature osteoblasts.

The mechanisms that define the locations at which bone resorption occurs throughout the skeleton have been unexplained. The present data suggest that physical proximity of osteoclastic precursors with both immature and mature osteoblasts may be an important aspect of local regulation of bone resorption during bone turnover.

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