Interleukin-6 (IL-6), IL-1, receptor activator of nuclear factor κB ligand (RANKL) and osteoprotegerin production by human osteoblastic cells: comparison of the effects of 17-β oestradiol and raloxifene

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Abstract

Oestrogen inhibits bone resorption, at least in part, by regulating the production of several cytokines, including interleukin-6 (IL-6), IL-1, receptor activator of nuclear factor κB ligand (RANKL) and osteoprotegerin (OPG) by cells of the osteoblastic lineage. The selective oestrogen receptor modulator raloxifene (RAL) acts on bone in a similar manner to oestrogen, although the mechanisms of action of RAL on osteoblasts still remain unclear. We investigated and compared the effects of 17-β oestradiol (E_2) and RAL on the regulation of IL-6, IL-1, RANKL and OPG *in vitro* in primary human osteoblastic (HOB) cells and in an immortalised clonal human bone marrow stromal cell line (HCC1) with osteoblastic characteristics. We tested E_2 and RAL at concentrations ranging from 10^{-12} to 10^{-6} M. IL-6, IL-1α and IL-1β, OPG and RANKL were measured by ELISA. RANKL and OPG mRNA steady state level was assessed by quantitative PCR analysis. Both E_2 and RAL led to a significant reduction in IL-6 production in the HOB cells, although the effect was more marked with E_2 ($P<0·05$). IL-1α and IL-1β also decreased significantly following treatment with E_2 and RAL in the HCC1 cells (E_2 (10^{-8}, 10^{-7} and 10^{-6} M), % reduction (means ± S.E.M.) compared with vehicle-treated cells – IL-1α: 84 ± 7·4, 70·8 ± 2·9*, 78·2 ± 4·8*; IL-1β: 79 ± 10, 72·8 ± 8·2*, 66·6 ± 2·8*; RAL (10^{-8}, 10^{-7} and 10^{-6} M) – IL-1α: 72·4 ± 5*, 79 ± 5·2*, 102 ± 7·7; IL-1β: 67·9 ± 3·2*, 69 ± 2·5*, 73·8 ± 6·2*; *$P<0·05$). OPG protein concentration decreased significantly in a dose-dependent manner following treatment with E_2 and RAL (% reduction E_2 (10^{-8}, 10^{-7} and 10^{-6} M) – HOB: 72·5 ± 8·4*, 80 ± 6·7*, 62·8 ± 8·9*; HCC1: 109 ± 4, 98·8 ± 6, 54·5 ± 3·4*; RAL (10^{-8}, 10^{-7} and 10^{-6} M) – HOB: 81·5 ± 5·5*, 62·7 ± 7·4*, 55·2 ± 10·9*; HCC1: 92·7 ± 7·4, 67 ± 12·2*, 39 ± 4·5*; *$P<0·05$). In the HCC1 cells, RANKL protein did not change significantly following E_2. In contrast, a significant reduction in RANKL was seen with RAL at 10^{-7} and 10^{-6} M (66 ± 6·4% and 74 ± 3% respectively). There was no change in OPG mRNA expression following E_2 or RAL in the HCC1 cells, although in the HOB cells we observed a significant reduction in OPG mRNA. RANKL mRNA decreased significantly in the HCC1 cells following RAL (10^{-8}, 10^{-7} and 10^{-6} M) treatment (% change from controls: 52 ± 2*, 62 ± 1*, 53 ± 5·8*; *$P<0·05$). Similar results were seen in the HOB cells with RAL at 10^{-6} M (RANKL mRNA: 72 ± 5·5, $P<0·05$). In addition, there was a significant decrease in the RANKL/OPG ratio after RAL at 10^{-6} M (HOB: 65·6 ± 5*, HCC1: 56·9 ± 20*; *$P<0·05$). RANKL/OPG ratio did not change significantly in the HCC1 cells following E_2. However, in contrast to RAL, we observed an increase in the RANKL/OPG ratio in the HOB cells following treatment with E_2. In conclusion, the study shows that RAL and E_2 have divergent cell-specific effects on the regulation of cytokines. The data also suggest that, in contrast to E_2, RAL may exert its anti-resorptive actions, at least in part, via the RANKL/OPG pathway. Further *in vivo* studies are required to confirm this.

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Introduction

Oestrogen prevents bone loss by inhibiting bone resorption (Compston 2001). Although some of the anti-resorptive effects of oestrogen are via direct actions on osteoclasts and haematopoietic cells, the steroid hormone has also been shown to have indirect effects by regulating osteoblasts and bone marrow stromal cells (Oursler 1998).

Oestrogen has thus been shown to modulate osteoclastic bone resorption, at least in part, by regulating the production of several pro-resorptive paracrine factors such as interleukin-6 (IL-6), IL-1β and tumour necrosis factor-α (TNF-α) by cells of the osteoblastic lineage (Spelsberg *et al.* 1999). More recently, a cell-surface member of the TNF ligand family termed receptor activator of nuclear factor κB ligand (RANKL) (Yasuda *et al.* 1998), its receptor (RANK) (Hsu *et al.* 1999) and osteoprotegerin (OPG) (Simonet *et al.* 1997) have been shown to be central in both osteoclast development and activity. OPG acts as a decoy receptor for RANKL, thus preventing it from binding to and activating RANK on the osteoclast surface. Both OPG and RANKL are expressed by osteoblastic cells and bone marrow stromal cells (Hofbauer *et al.* 2000). It is now thought that the final step in the osteoclast regulatory pathway may be determined by the relative ratio of RANKL to OPG (Hofbauer *et al.* 1999). The RANKL/OPG system may thus be an important paracrine mediator of the anti-resorptive effects of oestrogen. Indeed, recent *in vitro* studies in human osteoblastic (HOB) and murine stromal cells have shown that oestrogen stimulates OPG production (Saika *et al.* 2001).

Selective oestrogen receptor modulators (SERMs) such as raloxifene (RAL) are now being used as anti-resorptive agents for the prevention and treatment of osteoporosis (Etinger *et al.* 1999). Although an oestrogen agonist on bone, the mechanism of action of RAL on osteoblasts at the tissue level is still unclear. SERMs interact with the oestrogen receptor and, in those tissues where they have agonistic actions, it is thought that they modulate gene transcription in a manner similar to oestrogen, albeit at a different site to the classic oestrogen response element (Compston 2001). The effects of RAL on the regulation of the cytokines detailed above by HOB cells remain to be established. In order to explore further the mechanisms of action of RAL on bone, we examined and compared the effects of RAL and 17-β oestradiol (E_2) on the regulation of the bone-resorbing cytokines such as IL-6, IL-1 and RANKL and the anti-resorptive factor OPG in primary HOB cells as well as in an immortalised human bone marrow stromal cell line (HCC1) representative of an early osteoblastic cell line (Harrison 1996, Davies *et al.* 2002, Ogston *et al.* 2002).

Materials and Methods

Cell culture

We studied the osteosarcoma-derived human cell line, SaOS-2, an immortalised human bone marrow stromal cell line with osteoblastic characteristics (HCC1) (Harrison 1996, Davies *et al.* 2002, Ogston *et al.* 2002) and primary HOB cells.

SaOS-2 cells The SaOS-2 cells were grown in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, Paisley, Scotland, UK), supplemented with 5% foetal calf serum (FCS, Invitrogen), penicillin (100 IU/ml), streptomycin (100 μg/ml) and amphotericin B (2·5 μg/ml) in 24-well plates (Gibco BRL) at 37 °C in a humidified 5% CO_2 incubator. The cells were seeded at a density of 5×10^4 per cm^2 and grown to confluence.

HCC1 cells The HCC1 cells were grown to confluence in minimum essential medium (MEM, Gibco BRL) containing 10% FCS, penicillin (100 IU/ml), streptomycin (100 μg/ml) and amphotericin B (2·5 μg/ml) in 24-well plates as described above. They were not grown in differentiating culture conditions. The HCC1 cell line was derived from a bone marrow aspirate from a 45-year-old male donor undergoing spinal fusion. The immortalised cell line was established following insertion of the SV40 large T and has been shown to express several osteoblastic markers such as alkaline phosphatase, osteocalcin and the PTH/PTH-rp receptor (Harrison 1996, Davies *et al.* 2002, Ogston *et al.* 2002).

Primary HOB cells The HOB cells were obtained from trabecular bone obtained at operation from four different subjects (two males, two females). Ethical approval had been obtained from the local Research Ethics Committee. The cells were grown from the bone explants in α-MEM and 10% FCS as previously described (Cheng *et al.* 1994). At confluence, they were sub-cultured in 24-well plates. Only cells of the first four passages were used in the experiments. The HOB cells were shown to express several osteoblastic markers including alkaline phosphatase, type 1 collagen, CBFA1 and osteocalcin mRNA. The HOB and HCC1 cells were also shown to express the oestrogen receptor (ERα and ERβ) by RT-PCR and confirmed by sequencing, as shown in Fig. 1a and b. Primer sequences for ERα were as previously described (Morishita *et al.* 1999). RT-PCR was also carried out to assess the expression of the bone-specific ERβ isoform (Poola *et al.* 2002) using the following primer sequences: Forward primer: 5′-ACCTTACCTGTAAACAGGACA-3′ and reverse primer 5′-CTGTGACCAGAGGGTACAT-3′. The expected PCR product size was 459 bp. ERβ mRNA expression could be detected in both the HCC1 and HOB cells, although the expression was lower and more variable than ERα.

Cell treatment with E_2 and RAL

At confluence, the cells were washed with PBS and changed to serum-free DMEM.

For the experiments designed to study the effects of E_2 and RAL at supra-physiological concentrations of 10^{-8}, 10^{-7} and 10^{-6} M on IL-6 production, the Sa-OS-2, HCC1 and HOB cells were incubated in serum-free DMEM

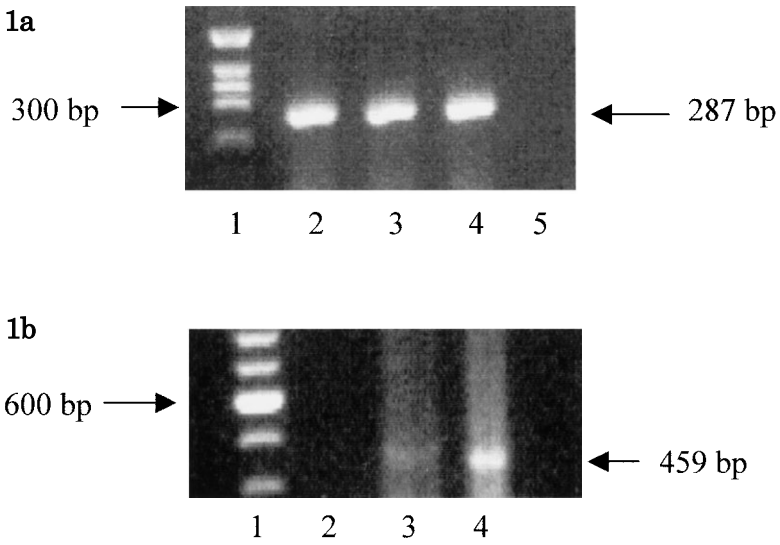

Figure 1 (a) Human oestrogen receptor gene (ERα) expression by RT-PCR in the HCC1 and HOB cells. PCR product size is 287 bp. Lane 1: molecular weight markers; lane 2: basal ERα mRNA expression in the HCC1 cells; lane 3: ERα mRNA expression in the HCC1 following dexamethasone (10^{-7} M); lane 4: basal ERα mRNA expression in the HOB cells; lane 5: blank. (b) ERβ expression by RT-PCR in the HOB and HCC1 cells. PCR product size is 459 bp. Lane 1: molecular weight markers; lane 2: blank; lane 3: basal ERβ mRNA expression in the HOB cells; lane 4: basal ERβ mRNA expression in the HCC1 cells.

with either vehicle or E_2 or RAL for 72 h. Lower concentrations of E_2 and RAL ranging from 10^{-12} to 10^{-9} M were also tested. The conditioned medium was then collected and the supernatant stored at –20 °C until analysis. For the studies on OPG secretion, the HCC1 and HOB cells were grown in serum-free DMEM for 24 h. The medium was then changed and the cells were treated with either vehicle or E_2 or RAL for 24 h in fresh serum-free DMEM. The culture medium was then harvested and stored at –20 °C until analysis. For the measurement of RANKL, IL-1α and IL-1β, the HCC1 cells were grown in serum-free DMEM with either vehicle, E_2 or RAL for 72 h. The cells were then trypsinised and suspended in 0·5 ml PBS. The cell suspension was sonicated for 120 s and centrifuged at 4000 ***g*** for 3 min. RANKL, IL-1α and IL-1β were measured in the supernatant. All experiments were carried out at least two or three times in triplicates.

Measurement of IL-6, IL-1α, IL-1β, OPG and RANKL

IL-6, IL-1α and IL-1β were measured by an ELISA using kits from R & D Systems (Abington, UK). OPG was also determined by ELISA. The assay consists of a monoclonal mouse anti-human OPG antibody as a capture antibody in combination with a biotinylated goat anti-human OPG affinity-purified polyclonal detection antibody (R & D Systems). Recombinant human OPG with concentrations ranging from 0·01 to 5·0 ng/ml were used as standards. Uncomplexed RANKL was measured using the human soluble RANKL ELISA from Biomedica (A-1210 Vienna, Divischgasse, Austria). A series of standards ranging from 0 to 50 pmol/l and supernatant from the cell sonicates were assayed in duplicate. PBS was used as the 0 standard.

All values were corrected for protein. Protein concentrations were determined by the method of Bradford (Bradford 1976) on the cell lysates.

Indirect immunolocalisation of OPG and RANKL

The expression of OPG and RANKL protein was assessed qualitatively by immunolocalisation. The HCC1 and HOB cells were grown in Lab-Tek chamber slides (Nunc, Nottingham, UK). Following fixation in 4% paraformaldehyde (PFA) for 10 min, they were washed three times in Tris–HCl buffer (pH 7·6). Non-specific activity was blocked with 20% normal rabbit serum for 30 min. The cells were incubated with primary goat anti-human polyclonal antibodies against either OPG or RANKL (Santa Cruz Biotechnology Inc, Santa Cruz, USA) for 1 h at 1:200 dilution, washed three times and incubated with the second antibody for 45 min (biotinylated rabbit anti-goat IgG). After washing, the slides were stained with streptavidin ABC complex/alkaline phosphatase conjugate (Dako, Cambridge, UK) for 30 min. The cells were visualised with Sigma Fast Red TR/naphthol AS-MX substrate in tablet form and counterstained with Harris' haematoxylin. The slides were examined by bright field

microscopy and photographed on Kodak 64K film. To check specificity of staining, negative control slides were processed without either primary antibodies, or secondary antibody, or incubated with goat immunoglobulins.

OPG and RANKL mRNA expression

Total cellular RNA was isolated from the HCC1 and HOB cells following incubation in serum-free DMEM with vehicle or E_2 or RAL for 24 h. RNA was extracted using the Trizol reagent (Invitrogen) and purified according to the manufacturer's instructions. cDNA was synthesised from 4 μg of total RNA in a 40 μl reaction containing random hexanucleotides and Moloney murine leukaemia virus reverse transcriptase (Invitrogen).

Real-time quantitative PCR assay for OPG and RANKL mRNA expression

Real-time quantitative PCR analysis was done using a 5700 Sequence Detector (PE Applied Biosystems, Foster City, CA, USA), which is a combined thermal cycler and fluorescence detector with the ability to monitor the progress of individual PCR reactions optically during amplification. Amplification reactions were set up in 25 μl reaction volumes containing amplification primers and SYBR Green PCR Master Mix (PE Applied Biosystems). A 1 μl volume of cDNA was used in each amplification reaction. Preliminary experiments were carried out for primer concentration optimisation. Primer sequences are detailed below and were derived using Primer Express Software (PE Applied Biosystems). Sequence data were obtained from the GenBank Sequence Database (accession numbers U94332 (OPG), AF019047 (RANKL)).

Primer sequences

Beta-actin
Forward primer: 5′-CCCAGCCATGTACGTTGCTA
Reverse primer: 5′-AGGGCATACCCCTCGTAGATG

OPG
Forward primer: 5′-CGTCAAGCAGGAGTGCAATC
Reverse primer: 5′-CCAGCTTGCACCACTCCAA

RANKL
Forward primer: 5′-TCGTTGGATCACAGCACATCA
Reverse primer: 5′-TATGGGAACCAGATGGGATGTC

Amplifications were performed in 96-well reaction plates designed to prevent light scattering (PE Applied Biosystems). Calibration curves were run in parallel in triplicates for each analysis. Each sample was analysed six times during each experiment. The experiments were carried out at least twice on each cell line. Amplification data were analysed using the Sequence Detector System Software (PE Applied Biosystems). The results were normalised to β-actin and expressed as percentage of controls.

Statistical analyses

The statistical significances of differences between the control and treated cells were determined by Student's *t*-test. The critical value for significance was $P<0{\cdot}05$. Values are given as means ± S.E.M.

Results

The effect of E_2 (10^{-8}–10^{-6} M) and RAL (10^{-8}–10^{-6} M) on IL-6 secretion

In the SaOS-2 cells, IL-6 production was significantly reduced following treatment with E_2 at all concentrations tested. In contrast, RAL led to a significant decrease in IL-6 at a concentration of 10^{-7} M only (72·2 ± 5·8% of vehicle-treated controls, $P<0{\cdot}01$). In the HOB cells, IL-6 was significantly reduced in the presence of E_2 (10^{-8}, 10^{-7}, 10^{-6} M) compared with controls (87 ± 3·6%, 61·7 ± 7·8%, 63·4 ± 6·9%, $P<0{\cdot}01$). RAL led to a reduction in IL-6 in the HOB cells at concentrations of 10^{-7} and 10^{-6} M only (82·9 ± 3·9% and 84·6 ± 4·2%, $P<0{\cdot}05$). At all concentrations tested, the addition of E_2 to the HOB cells resulted in a more profound inhibition of IL-6 production than RA ($P<0{\cdot}05$). In the HCC1 cells, we observed a reduction in IL-6 production after treatment with RAL only at concentrations of 10^{-8} and 10^{-7} M (78 ± 4·0% and 67·7 ± 2·4%, $P<0{\cdot}05$). The results are shown in Fig. 2. In the HOB cells, IL-6 production was stimulated (five- to tenfold) in response to lipopolysaccharide (LPS 10 mg/ml). Co-treatment with E_2 (10^{-8} and 10^{-7} M) attenuated the stimulated IL-6 production of controls (77 ± 5·3% and 82 ± 3·2%, $P<0{\cdot}05$). Co-treatment with RAL (10^{-8} M) also resulted in a reduction in IL-6 of controls (87 ± 4·0%, $P<0{\cdot}05$). There was no significant change in IL-6 following treatment with E_2 or RAL at lower concentrations (10^{-12}–10^{-9} M).

Intracellular IL-1α, IL-1β and RANKL

IL-1α and IL-1β could not be detected in the conditioned medium in any of the cell lines by the specific ELISA. Intracellular IL-1α and IL-1β could be detected and were well above the detection limit of the assay in the HCC1 cells only. A significant reduction in IL-1α was seen following treatment with E_2 (10^{-7}, 10^{-6} M) (70·8 ± 2·9% and 78·2 ± 4·8% of vehicle-treated controls, $P<0{\cdot}01$). Similar results were observed with RAL at 10^{-8} and

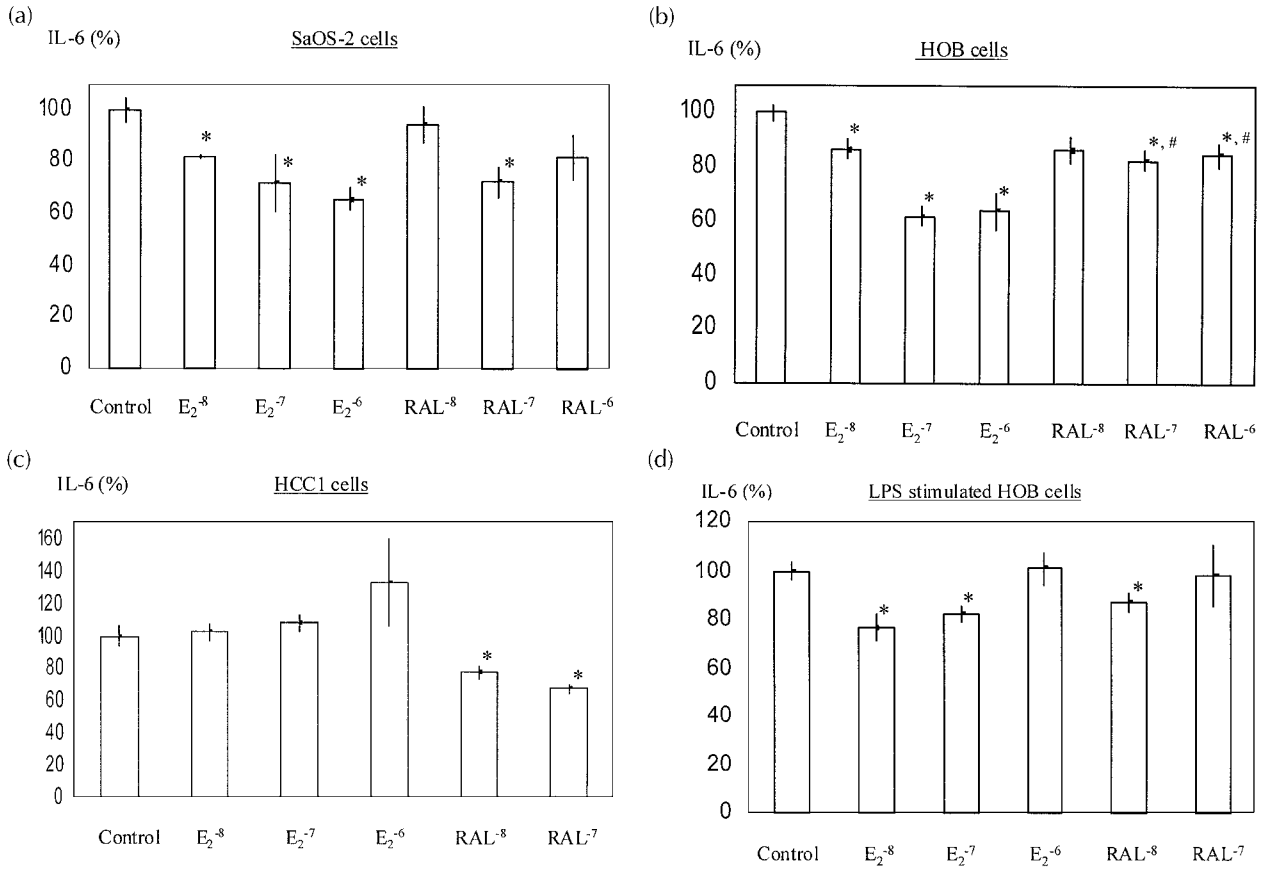

Figure 2 IL-6 production in the cell culture medium expressed as a percentage of control (vehicle-treated cells) following treatment with E_2 (10^{-8}–10^{-6} M) and RAL (10^{-8}–10^{-6} M). The results are the means ± S.E.M. of three different experiments carried out in triplicate. (a) SaOS-2 cells, (b) HOB cells, (c) HCC1 cells, (d) HOB cells following stimulation with LPS (10 mg/ml). *P<0·05 compared with control, #P<0·05 compared with E_2-treated cells.

10^{-7} M, as shown in Fig. 3. IL-1β was also significantly reduced after treatment with E_2 (10^{-7}, 10^{-6} M) and RAL (10^{-8}–10^{-6} M) (Fig. 3). No changes were observed at lower E_2 and RAL concentrations (10^{-12}–10^{-9} M). RANKL could only be detected in the HCC1 cells following sonication. The mean ± S.E.M. in the vehicle-treated cells was 6·05 ± 0·4 pmol/l, which was at the lower end of the assay range. Values expressed as a percentage of control for E_2 (10^{-8} M, 10^{-7} M) were 106 ± 30% and 100 ± 4% respectively. A small reduction in RANKL was seen following treatment with E_2 (10^{-6} M) (80·5% ± 4·5), although this was not significant. RAL (10^{-8} M) did not lead to any change in RANKL (114 ± 14%). Treatment with RAL (10^{-7}, 10^{-6} M) led to a significant reduction in RANKL compared with vehicle-treated cells (66 ± 6·4% and 74 ± 3%, P<0·05). RANKL could not be detected in the HOB cell sonicates.

OPG protein secretion

OPG protein concentration in the conditioned medium decreased significantly in a dose-dependent fashion in both the HOB and HCC1 cells following treatment with E_2 (10^{-8}–10^{-6} M) and RAL (10^{-8}–10^{-6} M) at supra-physiological concentrations only. However the decrease was less than that observed in dexamethasone-treated cells (10^{-9}–10^{-7} M). The results are shown in Fig. 4. No changes were observed at lower E_2 and RAL concentrations (10^{-12}–10^{-9} M).

OPG and RANKL protein expression by immunolocalisation

Pink staining for OPG and RANKL was detected in both the HCC1 and HOB cells. Basal expression of OPG was higher than RANKL, as shown in Fig. 5. Basal RANKL protein expression in the cells was too low to allow accurate semi-quantitative assessment (by cell counting) of RANKL protein expression following treatment with E_2 and RAL.

Real-time PCR quantitative analysis of OPG and RANKL mRNA

OPG and RANKL mRNA expression normalised to actin and the RANKL/OPG ratio was expressed as percentages

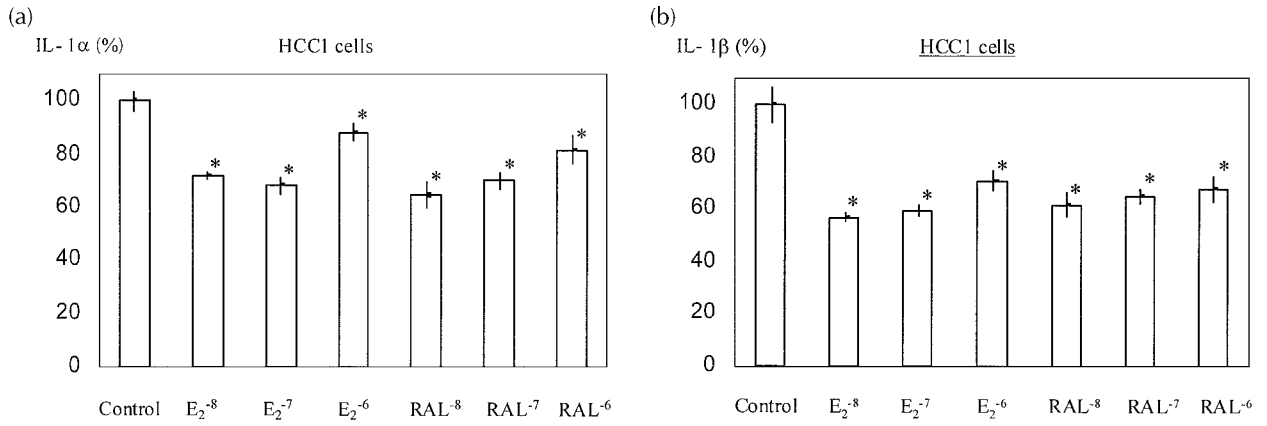

Figure 3 Intracellular IL-1 production expressed as a percentage of control (vehicle-treated cells) following treatment with E_2 (10^{-8}–10^{-6} M) and RAL (10^{-8}–10^{-6} M) in the HCC1 cells. The results are the means ± S.E.M. of three different experiments carried out in triplicate. (a) IL-1α production (b) IL-1β production. *P<0·05 compared with control.

of controls in the HOB and HCC1 cells and is shown in Fig. 6. There was no change in OPG mRNA steady state levels in the HCC1 cells following treatment with E_2 and RAL (10^{-8}–10^{-6} M). The results for E_2 and RAL (10^{-6} M) are shown in Fig. 6. In contrast, we observed a significant reduction in RANKL mRNA expression at RAL concentrations of 10^{-7} and 10^{-6} M and RANKL/OPG ratio at RAL (10^{-6} M) in the HCC1 cells. The mean ± S.E.M. percentage reduction in RANKL mRNA expression and RANKL/OPG ratio in the HCC1 cells following treatment with RAL (10^{-6} M) was 64·9 ± 5·8% and 56·9 ± 20% compared with controls (P<0·01) (Fig. 6). RANKL mRNA expression was significantly lower following treatment with RAL (10^{-6} M) compared with E_2 (10^{-6} M) (P<0·05) in the HCC1 cells. No significant reduction in RANKL/OPG ratio was seen following E_2 (10^{-6} M) treatment in the HCC1 cells. RAL (10^{-6} M) treatment also led to a reduction in RANKL mRNA and the RANKL/OPG ratio in the HOB cells (72 ± 5·5% and 65·6 ± 5·0%, P<0·01). There was a significant reduction in OPG mRNA expression following E_2 in the HOB cells. In contrast, there was no significant change in either RANKL mRNA or the RANKL/OPG ratio in the HOB cells versus control following E_2 treatment, although the results were different compared with RAL (Fig. 6).

Discussion

In the present study we have shown that both E_2 and RAL treatment down-regulate the production of bone-resorbing cytokines IL-6, IL-1α and IL-1β by HOB cells. In addition, we also observed an inhibition in OPG protein production/secretion by E_2 and RAL. RANKL protein, mRNA expression and the RANKL/OPG mRNA ratio were more profoundly decreased following treatment with RAL compared with E_2, suggesting that RAL and E_2 may have different effects on RANKL expression. The results also indicate that the effects of RAL on HOB cells may favour a reduction in bone resorption through modulation of the RANKL/OPG system.

In the studies reported here, treatment with E_2 at physiological and supra-physiological doses resulted in a reproducible reduction in IL-6 production by HOB cells. Previous studies of the effect of E_2 on IL-6 production in osteoblasts have been equivocal (Kassem *et al.* 1996). This has been attributed, in part, in some human *in vitro* systems to the heterogeneity of the osteoblastic cell systems and to low ER expression. In this study, in addition to primary osteoblastic cultures, a homogeneous clonal cell line was used (Davies *et al.* 2002). Steady state levels of ERα mRNA expression were found to be similar in both culture systems. In the HOB cells, E_2 inhibited basal as well as stimulated IL-6 production. This effect was more marked than with RAL. In contrast, however, in the clonal osteoblastic cell line, HCC1, only RAL had an inhibitory effect on IL-6 production. One explanation for this finding is the origin of the cell line, which was obtained from the bone marrow aspirate of an adult male subject (Ogston *et al.* 2002). It is unlikely that the differences in the effects of E_2 and RAL on IL-6 production in this cell line are due to differences in the number of ERαs, as we observed no differences in the mRNA steady state levels between the HOB and HCC1 cells. However, it is plausible that our findings may be due to differential activation of ER subtypes by E_2 and RAL. This differential effect of RAL and E_2 on IL-6 production has also been reported (Taranta *et al.* 2002) in murine osteoblasts, although in contrast to our study they observed a reduction in IL-6 at lower concentrations of RAL. Other bone-resorbing cytokines also mediate part of the

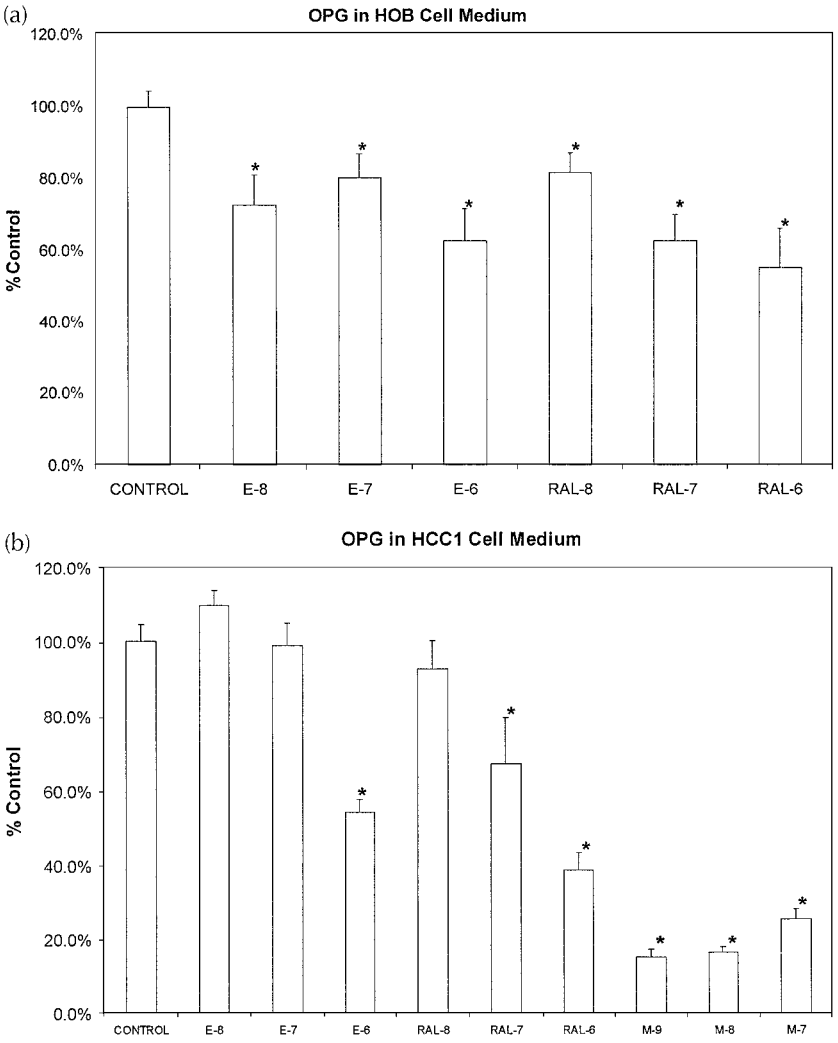

Figure 4 OPG secretion in the cell culture medium expressed as a percentage of control (vehicle-treated cells) following treatment with E_2 (10^{-8}–10^{-6} M), RAL (10^{-8}–10^{-6} M) and dexamethasone ('M', HCC1 cells only) (10^{-9}–10^{-7} M). The results are the means ± S.E.M. of three different experiments carried out in triplicate. (a) HOB cells, (b) HCC1 cells. *P<0·05 compared with control.

oestrogenic action on bone and this includes IL-1α and IL-1β. Constitutive levels of IL-1α and IL-1β were low in the HOB cells. The pattern of reductions in the pro-resorbing cytokines IL-1α and IL-1β was similar following treatment of the HCC1 cells by either E_2 or RAL. The inhibitory effect of E_2 on IL-1 production could, at least in part, explain how E_2 down-regulates osteoclast activity, as previously reported (Pacifici 1998). Our data also confirm this effect with regards to RAL.

It is now thought that the ratio of RANKL/OPG determines osteoclast differentiation and activation, and that these two downstream factors serve as the final effectors in the modulation of bone resorption (Hofbauer *et al.* 2000). The RANKL/OPG system is also believed to be a mediator of E_2 anti-resorptive actions. Several investigators have shown that E_2 increases the production of OPG (Hofbauer *et al.* 1999, Saika *et al.* 2001). In our study, we observe a reduction in OPG in the primary HOB cultures and in the clonal cell line HCC1 following treatment with E_2 at concentrations ranging from 10^{-8} to 10^{-6} M. This effect was also seen with RAL. Changes in OPG of this order of magnitude (30–40%) have previously been shown to be of physiological and clinical significance in *in vivo* studies (Lindberg *et al.* 2001, Khosla *et al.* 2002). One explanation for the lack of OPG stimulation observed in our study is the lower ER numbers in our cell culture model, as in the studies mentioned above the extent of OPG protein secretion was dependent on ER receptor

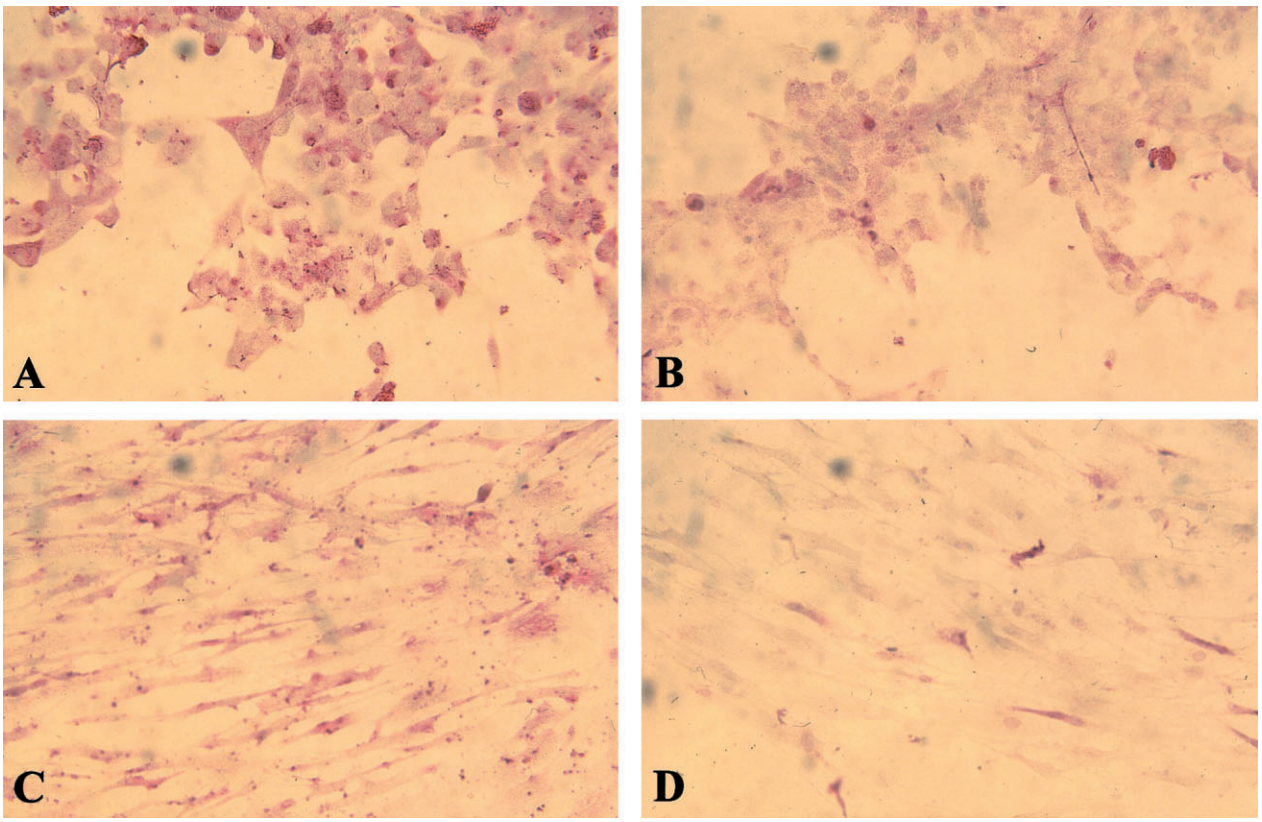

Figure 5 OPG and RANKL protein expression by immunolocalisation in the HCC1 and HOB cells. (A) OPG expression in the HCC1 cells. (B) RANKL expression in the HCC1 cells. (C) OPG expression in the HOB cells. (D) RANKL expression in the HOB cells.

numbers and maximal stimulation was shown in cells over-expressing ERα. Indeed, the magnitude of OPG induction by E_2 was shown to be relatively small in primary osteoblast cultures and in untransfected ST-2 cells, a mouse bone marrow stromal cell line. Secondly, our results could be attributed to differences in culture conditions. Thirdly, differences in ERβ expression in our cell culture system may account for our findings. Indeed, Saika *et al.* (2001) have recently shown a negative regulation by the E_2–ERβ complex on OPG expression. It is also plausible that a reduction in the upstream cytokines such as IL-6 and IL-1 leads to inhibition of OPG secretion as these cytokines have been shown to stimulate OPG production *in vitro* (Hofbauer *et al.* 1998, Vidal *et al.* 1998*b*), although data regarding IL-6 remain conflicting (Brändström *et al.* 1998). These results would be compatible with the hypothesis that E_2's effects on OPG may be indirect (Riggs *et al.* 2002) and are mediated at least in part by the modulation of other cytokines, as previously suggested for TGF-β (Oursler *et al.* 1991, Hughes *et al.* 1997). This interaction between various cytokines has previously been reported to explain the anti-resorptive effects of E_2. Increased bone marrow levels of IL-1 in ovariectomised mice have been shown to be, at least in part, a result of increased TNF-α production (Cenci *et al.* 2000). Fourthly, the inhibitory effect of E_2 on OPG is supported by the findings of Yano *et al.* (1999), who showed an increase in OPG in post-menopausal women that was postulated to be a compensatory response to increases in bone resorption seen in this population. Our *in vitro* findings of a reduction in OPG following E_2 and RAL treatment would be consistent with a reduction in bone turnover and slowing of bone remodelling. Indeed, a recent *in vivo* study (Lindberg *et al.* 2001) has shown the decrease in bone turnover following E_2 treatment in orchidectomised mice to be associated with a 30% decrease in OPG mRNA expression as confirmed in the present study in the HOB cells, although the authors did not investigate OPG protein expression. Our study also suggests that the mechanisms of inhibition of OPG secretion by E_2 and RAL are cell specific and in the case of RAL occur at the post-transcriptional level as, in contrast to Saika *et al.* (2001) and Lindberg *et al.* (2001), we observed no change in OPG mRNA expression. Similar findings of a lack of effect of E_2 and RAL on OPG mRNA expression have been reported (Vidal *et al.* 1998*a*, Taranta *et al.* 2002). The divergence of OPG regulation would imply that RAL may have an inhibitory effect, directly or indirectly at the

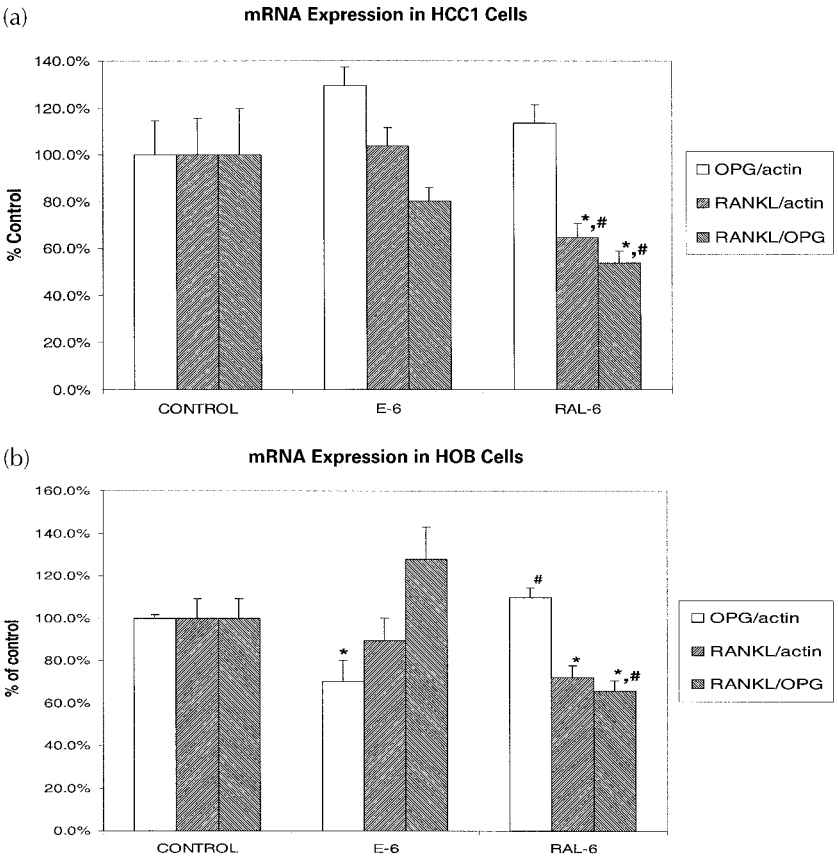

Figure 6 mRNA expression of OPG and RANKL and the OPG/RANKL ratio expressed as a percentage of control (vehicle-treated cells) following treatment with E_2 and RAL (10^{-6} M) in the HCC1 cells (a) and in the HOB cells (b). The results are the means ± S.E.M. of three different experiments carried out in triplicate. *P<0·05 compared with control, #P<0·05 compared with E_2-treated cells.

post-transcriptional level, on the assembly of the protein and/or secretion of the homodimeric form. This remains to be investigated.

RANKL expression at the mRNA and protein level was much lower than OPG in the HOB and HCC1 cells. The number of HOB and HCC1 cells that stained positive for RANKL was small. RAL led to a significant reduction in RANKL mRNA in both the HCC1 and HOB cell lines. In addition, RANKL protein expression was also seen to decrease with RAL in the HCC1 cultures. In contrast, RANKL mRNA and protein expression did not change with E_2 in the HCC1 cells and HOB cells. This has previously been reported (Saika *et al.* 2001, Taranta *et al.* 2002), although Lindberg *et al.* (2001) reported an increase in RANKL mRNA expression following E_2 treatment. Our data further confirm the differences in the mechanisms of action of RAL and E_2 at the molecular level (Lonard & Smith 2002). The differential effects between E_2 and SERMs regulation of gene transcription may be attributed, at least partly, to their different interaction with ERα and ERβ. RAL activates transcription with both ERα and ERβ. In contrast, oestrogen interacts with ERα to activate transcription, whereas with ERβ it inhibits transcription (Compston 2001). Another explanation is ligand-specific conformational changes in the ligand-binding domain of the ER which can affect gene transcription, as previously shown in the case of RAL and tamoxifen (Brzozowski *et al.* 1997, Shiau *et al.* 1998). Other cell-type and promoter-specific differences in co-regulator recruitment may also explain the divergent effects of E_2 and RAL (Shang & Brown 2002).

Because the interaction between RANKL and RANK depends on the balance between RANKL and OPG, its decoy receptor, we looked at the RANKL/OPG mRNA ratio. We observed a significant reduction in the RANKL/OPG mRNA ratio by RAL which would favour reduction in bone resorption. Our data therefore show that RAL may act via the RANKL/OPG system, at least in part, for its anti-resorptive effect. In contrast, with E_2 we observed no change in the RANKL/OPG mRNA

ratio in the HCC1 cells and in the HOB cells we saw an increase in the RANKL/OPG mRNA ratio compared with RAL, again demonstrating the differential effects of the two ligands on different cell cultures, as discussed above. Our results would indicate a relative increase in the RANKL/OPG ratio with E_2, as we saw a reduction in OPG but no change in RANKL. Thus, as previously documented (Lindberg *et al.* 2001), our observations would suggest that these may be secondary changes in order to restore bone remodelling to normal, rather than a direct effect of E_2 on OPG and RANKL. Other additional regulatory pathways may therefore be involved in E_2's anti-resorptive effects and may involve, in part, modulation of RANK expression by osteoclasts (Shevde *et al.* 2000).

In conclusion, although E_2 and RAL share common pathways in the regulation of bone resorption by HOB cells, this study shows that the two agents also have divergent cell-specific effects, particularly on the RANKL/OPG pathway. Further studies are needed to investigate and compare the effects of these two agents at the molecular level on the multiple regulatory factors involved in bone remodelling.

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