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Biochemical and Biophysical Research Communications 322 (2004) 162–167
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Vitamin A differentially regulates RANKL and OPG expression in human osteoblasts☆

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Received 28 June 2004
Available online 4 August 2004

Abstract

All-*trans*-retinoic acid (ATRA) induces bone resorption, but the molecular mechanisms are unknown. We have studied the effect of ATRA on osteoprotegerin (OPG) and receptor activator of NF-κB ligand (RANKL) expression in human MG-63 osteosarcoma cells and primary osteoblast-like cultures. ATRA dose-dependently down-regulated protein levels of OPG in MG-63 cells, with a maximum (−56%) observed at a dose of 10^{-6} M. This effect was confirmed with quantitative real-time PCR, where OPG mRNA was decreased after 4 h (−68%) in primary cultures and after 8 h (−87%) in MG-63 cells. The reduction in OPG expression was inhibited by a retinoic acid receptor (RAR)-antagonist and was mimicked by a RARβ,γ-agonist, indicating that the ATRA effect is mediated by these receptors. In primary cultures we found a threefold induction of RANKL mRNA expression. Thus, the RANKL/OPG ratio was markedly increased, suggesting a potential mechanism of ATRA-induced bone resorption.

Keywords: Vitamin A; Osteoprotegerin; Bone resorption; Retinoic acid; Osteoporosis; Receptor for activation of NF-κB ligand; Retinoic acid receptor; RAR-antagonist; Osteoblast; Bone

Bone tissue is maintained by continuous formation and resorption. This remodeling process, determined by the net effect of the activity of the bone-forming osteoblasts and the resorbing osteoclasts, is physiologically controlled by a complex network of endocrine hormones and local cytokines [1,2]. The receptor activator of nuclear factor-κB (RANK), RANK ligand (RANKL), and osteoprotegerin (OPG) constitute the essential regulatory components in the paracrine signaling necessary for osteoclast differentiation, activation, and apoptosis and is a suggested connection pathway between osteoblasts and osteoclastogenesis [3].

RANKL, expressed on the surface of stromal bone marrow cells and osteoblasts, binds to its cognate receptor RANK present on the surface of osteoclastic progenitors [4,5]. Through this cell–cell interaction osteoclastogenesis is activated [6,7]. OPG, another receptor for RANKL, is expressed in bone by osteoblastic cells [8–11]. The secreted, soluble OPG can bind to RANKL, thereby blocking its interaction with RANK and neutralizing its osteoclast activating effects [9].

Osteoporosis, the most common metabolic bone disease in the Western society, reflects an imbalance in the remodeling process in favor of bone resorption, leading to decreased bone mineral density and a tendency to fracture. Among the nutritional factors of importance for the pathogenesis, vitamin A is gaining increasing interest [12,13]. In laboratory animals hypervitaminosis A causes accelerated bone resorption, bone fragility, and spontaneous fractures [14–17] and a high intake of dietary vitamin A is associated with reduced bone mineral

☆ Funding sources: Swedish research council, No. K2002-72X-12671-05A, Torsten and Ragnar Söderberg Foundation.
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0006-291X/$ - see front matter © 2004 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2004.07.092

density and increased risk for hip fracture in humans [18–20]. In vitro, vitamin A increases osteoclast proliferation and activity and stimulates bone resorption [16,21,22].

In most tissues, the active vitamin A metabolite all-*trans*-retinoic acid (ATRA) binds and activates the retinoic acid receptor (RAR), forming a complex which acts as a transcription factor in the nucleus [23]. The three different RAR subtypes, i.e., RARα, RARβ, and RARγ, are all expressed in osteoblasts [24–26] but until now it has not been defined whether the bone-resorptive effect of ATRA is mediated via any of these receptors. Overall, the molecular mechanisms by which ATRA affects bone tissue are poorly known. In the present study, we describe the effect of ATRA on OPG and RANKL expression in the human osteosarcoma cell line MG-63 as well as in primary cultures of human osteoblast-like cells.

Materials and methods

Cell culture materials. Cell culture media, PEST (penicillin 5000 U/ml, streptomycin 5 mg/ml), L-glutamine, PBS, trypsin–EDTA, fetal bovine serum (FBS), and ATRA were purchased from Sigma, Stockholm, Sweden. ATRA was dissolved in 95% ethanol in a dark room under flow of nitrogen. The 0.5 mg/ml (1.66 mM) stock solution was stored in −70 °C and shielded from light until use. The high affinity pan-RAR-antagonist (AGN 194310) and the RARβ,γ-agonist (AGN 190299) [27], which were kindly provided by Dr. Chandraratna, Allergan, Irvine, CA, were dissolved in dimethyl sulfoxide (DMSO) and used at a concentration of 10^{-6} M.

Cell culture. The methods used for isolation and culture of the human osteosarcoma cell line MG-63 and primary human osteoblasts have been reported previously [28–30]. Primary cultures were isolated from bone fragments taken from patients undergoing surgery. Briefly, trabecular bone was cut into pieces (2–3 mm) and thoroughly rinsed and vortexed in PBS five times. Cells were incubated in a humidified CO_2 incubator at 37 °C and the medium was changed two times a week until confluence was achieved. Both cell types were cultured in α-MEM supplemented with 10% FBS, 2 mM L-glutamine, and antibiotics. The osteoblastic phenotype of cells in the primary culture was verified by use of biochemical markers as previously described [30]. Prior to stimulation with ATRA, cells were incubated in serum-free medium for 24 h. ATRA, at final concentrations ranging from 10^{-6} to 10^{-10} M, or vehicle (ethanol, not exceeding 0.1%), pan-RAR-antagonist (10^{-6} M) or RARβ,γ-agonist (10^{-6} M) was added in fresh serum-free medium. For measurement of osteoprotegerin secretion, 500,000 MG-63 cells were seeded in triplicates in 57 cm^2 dishes. After 24 h medium aliquots were collected and the number of cells in each well was counted manually. For RNA isolation, cells were seeded in duplicates in 75 cm^2 flasks at a density of 650,000 cells/flask (MG-63) and 150,000 cells/flask (primary cultures). Cultures were harvested 2, 4, 8, and 24 h after stimulation with ATRA.

Measurement of osteoprotegerin secretion. The levels of OPG were analyzed using an ELISA, as described previously [31]. Briefly, a MaxiSorb microtiter plate was coated with mouse anti-human OPG antibody. Samples or the standard protein recombinant human OPG was added and incubated for 2 h at room temperature. The bound proteins were detected with biotinylated goat anti-human OPG antibody. After developing, the plate was read at 450 nm in a microplate reader (Labsystems iEMS reader MF). Detection limit was 30 pg/ml, intra-assay variation was 7.2%, and inter-assay variation was 22%. Protein concentrations were normalized to the number of cells and expressed as pg/ml per 10^6 cells.

RNA isolation and quantitative real-time PCR analysis. In our initial OPG mRNA experiments Northern blots were performed. Due to the low RANKL expression levels in primary osteoblast cultures, we chose quantitative real-time PCR (qRT-PCR) analysis for these experiments. To be able to compare the OPG and RANKL data, we then repeated the OPG experiments with qRT-PCR, and since the results were the same as in the previous Northern blots, we have chosen to only present the qRT-PCR results.

Total ribonucleic acid was isolated using RNeasy Midi kit, VWR, Stockholm, Sweden. RNA (1 μg/sample) was reversibly transcribed to cDNA with Superscript II (Invitrogen, Sweden) and used in real-time PCR analysis using TaqMan 7700 (Applied Biosystems, Sweden). RNA quality was analyzed with Agilent Bioanalyzer. All RNA samples from one experiment were transcribed at the same time and each cDNA was analyzed in duplicates. Fluorescent FAM-labeled probes and gene-specific primers, spanning over exon–intron boundary, for RANKL and OPG were purchased as Assay-On-demand from Applied Biosystems. A JOE-labeled housekeeping-gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), was used as an endogenous control. Standard curves for the various genes were achieved through 10^{-2}–10^{-8} dilution series of a verified PCR product with a concentration of 10 ng/μl.

Statistics. The mRNA levels are expressed as mean arbitrary units ± intra-assay variation of duplicate analyses. Each experiment was repeated for assessment of inter-assay variation and differed by less than 10%. For protein production measured with OPG ELISA, statistical significance was calculated from three separate experiments and evaluated by student's t test with $p < 0.01$ denoted as (**) and was considered as statistically significant.

Ethics. The study was approved by the Uppsala University Ethics Committee (Registration No. UPS 03-561).

Results

ATRA down-regulates OPG protein secretion

To assess the effect of ATRA on OPG protein secretion, MG-63 cells were treated with doses ranging from 10^{-10} to 10^{-6} M in serum-free media. ATRA decreased OPG protein in a dose-dependent way, with a maximum effect (44% of control, $p < 0.01$) at 10^{-6} M ATRA (Fig. 1).

OPG transcriptional levels are down-regulated by ATRA through RAR

To investigate the effect at the transcriptional level, a series of quantitative real-time PCR experiments was performed. After treatment with 10^{-6} M ATRA in a time-course experiment, mRNA levels in MG-63 cells were decreased with a maximum effect after 8 h (data not shown). A dose–response of ATRA on OPG expression is shown in Fig. 2, where MG-63 cells have been treated with 10^{-8}–10^{-6} M ATRA for 8 h. ATRA inhibited OPG mRNA levels dose-dependently. At 10^{-6} M, OPG mRNA had decreased dramatically (13% of control). The addition of 10^{-6} M

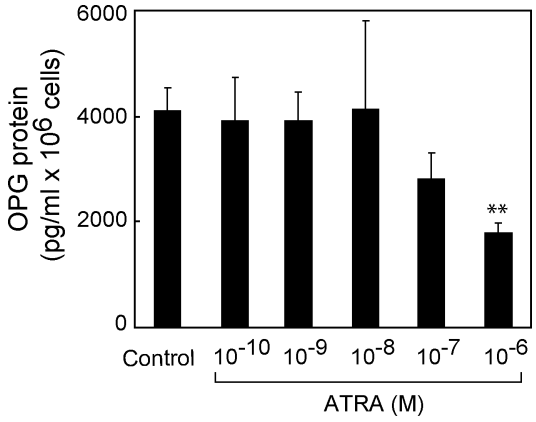

Fig. 1. Dose–response effects of all-*trans*-retinoic acid on OPG protein secretion in MG-63 cells. Cells were serum-deprived for 24 h before stimulation with either all-*trans*-retinoic acid (ATRA) or ethanol (control). After 24 h of stimulation, aliquots of medium were collected and the concentration of OPG protein was determined with ELISA. Protein concentrations were normalized to the number of cells, counted manually. Values are from one representative out of three independent experiments and expressed as means ± SD.

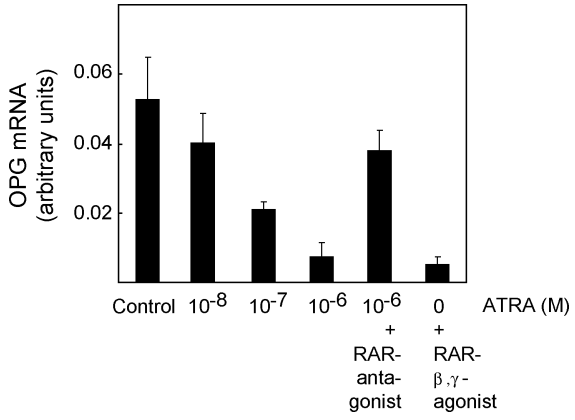

Fig. 2. Dose-dependent regulation of the OPG transcript in the human osteosarcoma cell line MG-63 after treatment with all-*trans*-retinoic acid (ATRA) for 8 h. Cells were grown in serum-free medium for 24 h before stimulation with ATRA, ethanol (control), or ATRA together with 10^{-6}M of pan-RAR-antagonist or RARβ,γ-agonist, all in serum-free medium. Total RNA was isolated from the harvested cells, transcribed to cDNA, and quantified in real-time PCR analysis using TaqMan 7700. The amount of OPG mRNA was compared to the amount of the housekeeping gene GAPDH mRNA. Values are expressed as means ± intra-assay variation of duplicate analyses.

pan-RAR-antagonist, which inhibits the action of RARα, β, and γ, could abolish the down-regulatory effect of 10^{-6}M ATRA. Furthermore, an agonist for RARβ and γ diminished OPG mRNA to a similar extent as ATRA.

Our findings in MG-63 cells were confirmed in primary human osteoblasts. ATRA treatment decreased the OPG expression in a time-course experiment with a maximum effect (to 32% of control) after 4 h (Fig. 3A). As in the osteosarcoma cells, the pan-RAR-antagonist inhibited and the RARβ,γ-agonist imitated the ATRA response (Fig. 3B). Neither RAR-antagonist alone nor DMSO affected expression (data not shown).

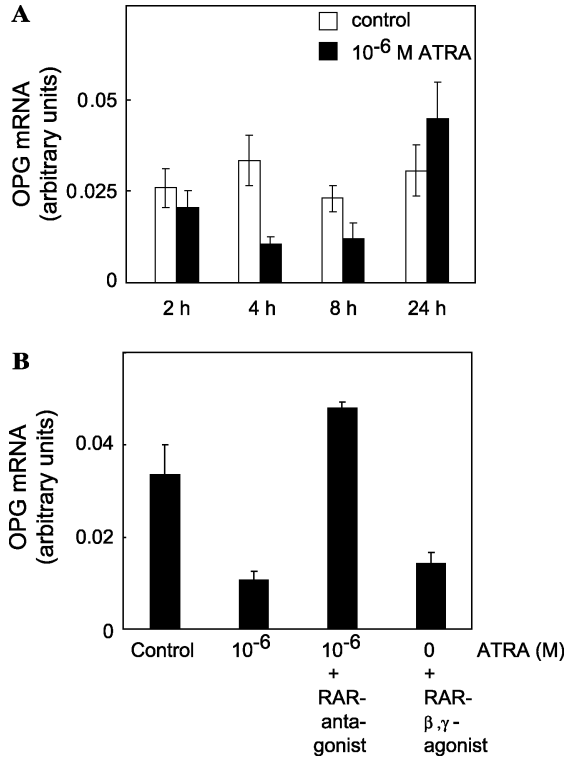

Fig. 3. Regulation of the OPG transcript in human primary osteoblast cultures after treatment with all-*trans*-retinoic acid (ATRA). Cell treatment and analysis of transcription levels with real-time PCR as in Fig. 2. (A) Time-course stimulation with 10^{-6}M ATRA or ethanol (control). (B) Stimulation with vehicle, 10^{-6}M ATRA, ATRA together with 10^{-6}M of pan-RAR-antagonist, or 10^{-6}M of RARβ,γ-agonist for 4 h. Values are expressed as means ± intra-assay variation of duplicate analyses.

RANKL transcriptional levels are up-regulated by ATRA

We next examined the effect of ATRA on RANKL expression. Consistent with previous studies [32], no RANKL expression was detected in MG-63 cells (data not shown). In primary cultures, RANKL mRNA expression was induced after 10^{-6}M ATRA exposure, with a maximum after 8 h (threefold induction; Fig. 4A). The modulation of RANKL mRNA was RAR-dependent (Fig. 4B).

ATRA increases the RANKL/OPG ratio

To see the net effect of RANKL and OPG in our in vitro system, we calculated the ratio of the two after ATRA treatment in primary cultures. The mRNA ratio was six times higher after 8 h of ATRA treatment compared to control. After 24 h, the ratio had almost returned to its initial value.

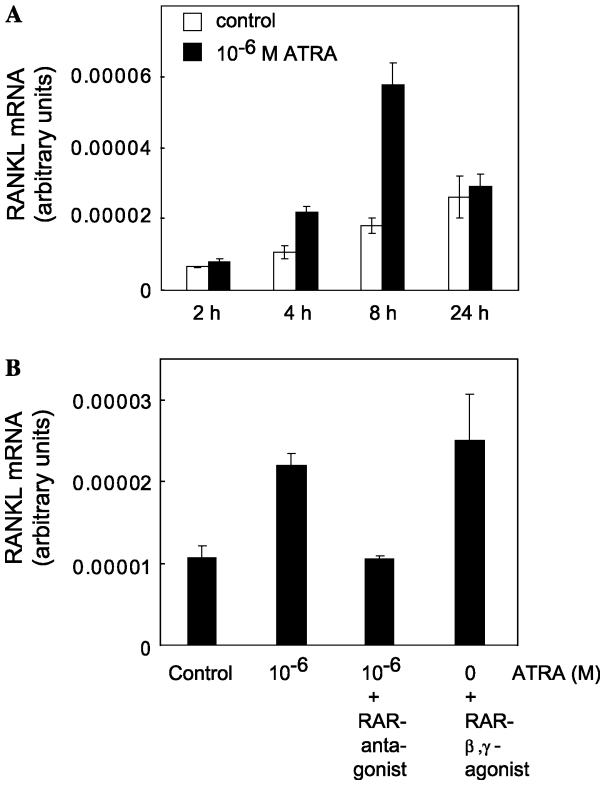

Fig. 4. Analysis of RANKL transcriptional levels with real-time PCR in human primary osteoblast cultures stimulated with all-*trans*-retinoic acid. (A) Time-dependent regulation of RANKL mRNA in cultures stimulated with 10^{-6}M all-*trans*-retinoic acid (ATRA) or ethanol (control). (B) Stimulation with 10^{-6}M ATRA, ethanol (control), ATRA together with 10^{-6}M pan-RAR-antagonist, or RARβ,γ-agonist for 4 h. Values are expressed as means ± intra-assay variation of duplicate analyses.

Discussion

The molecular mechanisms of vitamin A action on bone cells still are incompletely understood. In the present study we demonstrate that OPG is down-regulated and RANKL is up-regulated in osteoblastic cells by ATRA, the active metabolite of vitamin A. The importance of OPG and RANKL as regulators of osteoclastogenesis is well known. Over-expression of OPG in transgenic mice generates an osteopetrotic phenotype [8], whereas the OPG knock-out mice have a severe, early onset osteoporosis [33,34]. Soluble recombinant RANKL can increase osteoclast formation and activity in vitro, is a potent inducer of bone resorption and osteoporosis in mice, and is associated with systemic hypercalcemia [6,7]. Thus, the differential regulation of these two proteins may be an important mechanism by which vitamin A induces bone resorption.

The cytokine effect on osteoclast formation and activation is determined principally by the relative ratio of RANKL/OPG in the bone marrow microenvironment [7,35]. Interestingly, the ratio of serum RANKL and OPG levels has been shown to be of prognostic significance in multiple myeloma; an increased ratio is associated with a higher risk for osteolytic lesions and mortality [36]. An increased RANKL/OPG ratio has also been reported in other patients with severe osteolysis [37]. In our in vitro system, ATRA affected RANKL and OPG in osteoblasts in opposite directions, which resulted in a markedly increased RANKL/OPG ratio, supporting our hypothesis that ATRA-induced bone resorption is mediated via the RANK–RANKL–OPG system.

The resorptive effect of vitamin A is considered, at least in part, to be caused by a direct effect on bone tissue [16,38]. Although it has previously not been demonstrated, the most likely signaling pathway is via the retinoic acid receptors. In our study, the effects of ATRA on OPG and RANKL expression were RAR-dependent, since they were abolished by a pan-RAR-antagonist and mimicked by a RARβ,γ-agonist, but we cannot really determine from these experiments whether the changes in mRNA levels result from regulation of transcription or a change in message stability. To confirm that these are indeed transcriptional effects mediated by RARβ and/or γ, experiments with a reporter assay using RANKL and OPG upstream regulatory sequences will be needed.

Both vitamin A and glucocorticoids induce bone resorption. The ATRA effects that we have found are similar to those reported for glucocorticoids [39]. We observed maximum effects at the mRNA level after 4–8 h, and the effects were transient. Hofbauer et al. described a dose- and time-dependent inhibition of OPG mRNA levels by glucocorticoids in all human osteoblastic systems tested, including human primary osteoblast cultures and MG-63. Concurrently dexamethasone stimulated RANKL mRNA steady-state levels by 2- to 4-fold in immortalized fetal osteoblasts, and the effect was transient with a maximal response at 6 h [39]. In contrast, these investigators [40] found no consistent effect on OPG mRNA expression of ATRA at a dose of 10^{-8}–10^{-7}M for 48 h. This may appear as conflicting to our results, but their experiments were performed with a serum-free medium containing bovine serum albumin. Serum albumin binds ATRA and has been shown to reduce its bioavailability [41–43]. Our results are fully consistent with a study in human breast cancer T47D cells [44]. In that study, a maximal, RAR-dependent up-regulation of cytokeratin mRNA was observed with 10^{-6}M ATRA after 8 h, and wash-out experiments indicated that continuous exposure to ATRA was required to maintain high keratin expression.

The expression of OPG and RANKL is developmentally regulated [45]. OPG increases during osteoblast differentiation [3,45], whereas RANKL expression is inversely related to the degree of differentiation. Comparing the two human osteoblastic culture systems we

used, the OPG expression was more prominent in MG-63 cells than in primary bone cultures. Together with the lack of RANKL expression in MG-63 cells, this suggests that MG-63 behave as a more differentiated cell in our culture conditions. Thus, although we only tested two different cell systems, our results suggest that ATRA inhibits OPG mRNA levels in osteoblastic cells regardless of their stage of differentiation.

In conclusion, we find that ATRA acts on osteoblastic cells to increase the RANKL/OPG ratio. A local increase of RANKL and a decrease of OPG levels in the bone microenvironment may be an important component of the paracrine mechanisms by which vitamin A induces bone resorption and increases the risk of fractures.

Acknowledgments

This work was supported by grants from the Swedish research council (K2002-72X-12671-05A), and from Torsten and Ragnar Söderberg Foundation. We thank Dr. Olof Nilsson for providing bone biopsies, Elin Grundberg and Helena Brändström for preparation of primary cultures, Anna-Lena Johansson for skillful technical support, and Dr. Chandraratna, Allergan, Irvine, CA, for providing RAR-antagonist (AGN 194310) and RARβ,γ-agonist (AGN 190299).

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