CHAPTER 7

Effects of Arachidonic Acid, Docosahexaenoic Acid, Prostaglandin E_2 (PGE_2), Oestrogen and Parathyroid Hormone on Osteoprotegerin (OPG) and RANKL Secretion by MG-63 and MC3T3-E1 Osteoblast-like Cells

7.1 Introduction

Bone is continuously remodeled through resorption by osteoclasts and the subsequent synthesis of bone matrix by osteoblasts.[6] (Refer to 2.4). The remodeling cycle is finely regulated by a variety of systemic and local factors e.g. oestrogen, parathyroid hormone (PTH), $1,25(OH)_2D_3$, growth factors and cytokines.[6,34,35] (Refer to 2.5). Cell-to-cell contact between osteoblasts (or bone marrow stroma cells) and hematopoietic osteoclast precursors (present in bone marrow, spleen, and peripheral blood) is required for osteoclast formation.[7] (Refer to 2.3.3.2). During the past decade various research groups have identified some of the proteins involved in the interaction between cells of osteoblastic and osteoclastic lineage. These proteins belong to the families of tumor necrosis factors and receptors [8-12] and comprises *RANKL* (Receptor activator of nuclear factor-κB ligand) and its cognate receptor *RANK* (Receptor activator of nuclear factor-κB), as well as a decoy receptor *osteoprotegerin* (OPG).

RANKL is a protein expressed on the osteoblast cell membrane that binds to its cognate receptor RANK present on the osteoclast progenitor membrane. Binding of RANKL to RANK activates NFκβ and c-jun N-terminal protein kinase (JNK), which is associated with osteoclastic differentiation and activation.[13] Three distinct variants of RANKL have been identified: 1) a transmembrane cell bound variant,[9] 2) a soluble (cleaved) form[9,65] and 3) another secreted form produced by activated T cells.[66] RANKL expression in osteoblasts and stromal cells can be induced by PTH,[124] PGE_2,[138] interleukin-1α,[366] $1,25(OH)_2D_3$,[138] vitamin A metabolites[367] and

glucocorticoids.[368] It has recently been reported by Wu *et al* (2005) that apart from its role in osteoclastogenesis, RANKL also acts as a survival factor in osteoclasts by downregulating Fas-mediated apoptosis and Fas expression in mature osteoclasts,[369] thereby protecting the number of functional osteoclasts available for bone resorption.

Osteoprotegerin (OPG*),* a secreted glycoprotein, is a member of the tumor necrosis factor receptor family and is produced by cells of the osteoblast lineage as well as other cells in the marrow.[10,11] Binding of RANKL and RANK can be prevented by OPG binding to RANKL. If the binding between RANK and RANKL is interrupted by OPG, the osteoclast precursor cannot differentiate and fuse to form mature resorbing osteoclasts. OPG therefore acts as a decoy receptor in the RANKL-RANK signaling system thereby inhibiting osteoclastogenesis.[9,10] Apart from inhibiting osteoclastogenesis, OPG is also involved in suppressing osteoclast survival.[11,70] The presence of OPG in the bone microenvironment therefore limits the number of mature osteoclasts and could thereby have a determining role in resorption rate and bone mass. Various mediators modulate OPG secretion. OPG secretion is stimulated by oestrogen,[105] phytoestrogens,[320,368] IL-1β[132] and TGF-β[14] but inhibited by IL-1α,[366] PGE_2,[139,142] PTH,[125] glucocorticoids[142,368] and vitamin A metabolites.[365]

The importance of the RANKL/RANK/OPG system has been investigated extensively. Studies of bone metabolism in genetically altered animals presented some interesting findings. Overexpression of OPG in transgenic mice results in severe osteopetrosis with a large increase of mineralised trabecular bone as the number of osteoclasts is decreased and bone remodeling is favoured towards bone formation.[10] In contrast, adult OPG knockout mice lacking OPG exhibit severe osteoporosis due to increased osteoclast formation and activation and therefore bone resorption.[371] From these studies it is clear that OPG has an important regulating effect on the number of mature osteoclasts, resorption rate and bone structure.

A number of *in vivo* studies support the importance of the OPG/RANKL ratio for maintenance of healthy bone. Mice injected daily with recombinant OPG for seven days had a threefold increase in trabecular bone mass at the proximal tibial metaphysis. This finding shows that OPG treatment had an effect on bone formation

within a short period of time.[10] Ovariectomized (OVX) animals are often used as a model for postmenopausal bone loss. Administration of recombinant OPG to OVX rats protected the animals from bone loss, suggesting that OPG prevented OVX-induced bone loss.[10] In postmenopausal women, Bekker *et al* (2001) showed that a single subcutaneous dose of OPG causes rapid and sustained inhibition of bone resorption as indicated by bone resorption marker changes.[372]

Under normal physiological conditions, the differentiation of osteoclast progenitors to mature resorbing osteoclasts in the bone marrow depends on the balance between RANKL-RANK signaling and the levels of OPG produced by stromal cells and osteoblasts.[131] Hofbauer *et al* (2000) proposed a 'convergence hypothesis' for the regulation of osteoclast functions by cytokines.[14] The regulation of RANKL and OPG by various pro-resorptive and anti-resorptive agents suggests that their effects may converge at the level of RANKL and OPG, which then functions as the final effector system to modulate differentiation and activation of osteoclasts. For example, the stimulation of RANKL by PTH, PGE_2 and glucocorticoids[124,138] and the inhibition of OPG by these same agents[125,139,368] may mediate the pro-resorptive effects of these agents. The ratio of OPG/RANKL therefore could present a new therapeuticaly approach for osteoporosis treatment. Figure 7.1 illustrates Hofbauer's convergence theory.[14]

Polyunsaturated fatty acids (PUFAs) have been implicated in bone homeostasis both *in vitro*[29,168,223] and *in vivo.*[25,28,29,31,373] There is increasing evidence that lack of certain PUFAs in the diet can induce bone loss,[21,23,24] while dietary supplementation of some PUFAs have been shown to be beneficial for bone.[25-28] Clinical studies have shown, for instance, that supplementation of calcium, γ-linolenic acid and eicosapentaenoic acid (EPA) in the diets of elderly women decreases bone turnover and increases bone mineral density.[26,27] *In vivo* studies have shown that supplementation with PUFAs such as γ-linolenic acid, EPA and DHA, could decrease bone turnover in OVX animals by decreasing bone resorption.[25,29]

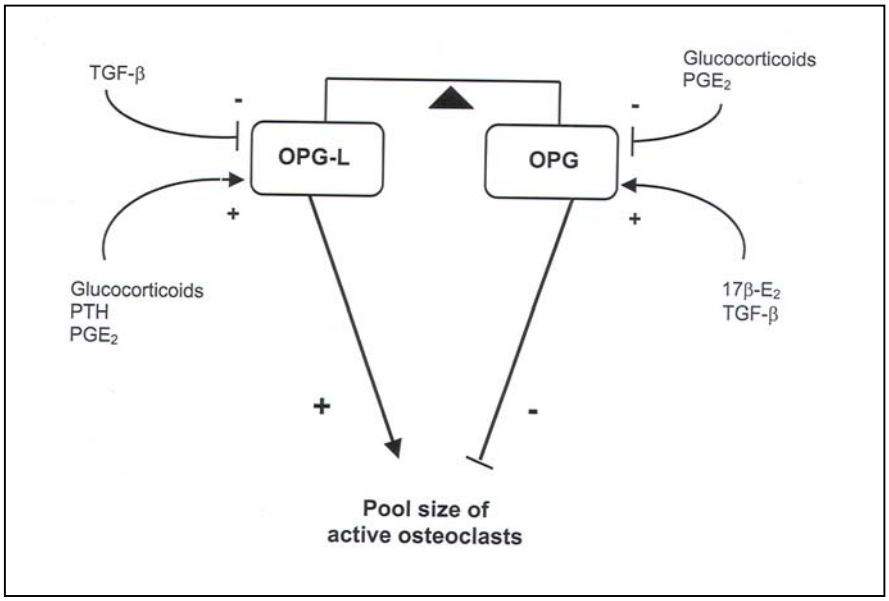

Figure 7.1 The 'Convergence hypothesis' for the regulation of osteoclast functions by cytokines.

This hypothesis proposes two levels of regulation of osteoclast functions. A variety of "upstream" cytokines and hormones alter the pool size of active osteoclasts by converging at the level of OPG-L (RANKL) and OPG. These two "downstream" factors serve as the final effectors for osteoclastogenesis and also affect osteoclast activation and osteoclast apoptosis. At steady state, there is a "balance" of levels of OPG-L (RANKL) and OPG levels that maintain a pool size of active osteoclasts that supports normal levels of bone resorption. When a change in one or more upstream factors tilts the balance toward a functional excess of OPG-L (RANKL), the pool size of active osteoclasts increases; when the balance tilts toward a functional excess of OPG, the pool size decreases. 17β-E_2, 17β-estradiol; PGE_2, prostaglandin E_2; PTH,parathyroid hormone; TGF-β, transforming growth factor β.
(From: Hofbauer LC, Khosla S, Dunstan CR, Lacey DL, Boyle WJ, Riggs BL. The roles of osteoprotegerin and osteoprotegerin ligand in the paracrine regulation of bone resorption. J Bone Miner Res 2000;15:2-12[14]) With permission of the American Society for Bone and Mineral Research.

The molecular mechanisms whereby PUFAs affect osteoclastogenesis and bone resorption are not fully understood and are currently under investigation. Omega-3 PUFAs may be metabolised to compounds such as the newly identified *resolvins* that are produced through COX metabolism followed by transcellular neutrophil lipoxygenase processing.[374] These were reported to have anti-inflammatory properties similar to the known cytokines, also induced or modulated by the PUFAs.[374]

Sun *et al* (2003) investigated the action of n-6 and n-3 PUFAs on bone resorption by feeding ovariectomised mice diets containing 5% corn oil (rich in n-6 PUFAs) or fish oil (rich in n-3 PUFAs).[29] Apart from measuring bone mineral density, they also measured RANKL expression in activated spleen lymphocytes from these animals.

Analysis for RANKL showed increased $RANKL^+$ T cells in corn-fed mice whereas fish-oil fed mice showed no change in $RANKL^+$ T cells. The increased $RANKL^+$ T cells in corn-fed mice correlated closely with bone mineral density loss, whereas fish oil decreased bone loss by preventing changes in RANKL surface antigen on T cells, thereby demonstrating a bone protective effect of n-3 PUFAs.[29] Sun *et al* (2003) also investigated the effects of selected n-3 and n-6 PUFAs on *in vitro* osteoclastogenesis by culturing primary murine bone marrow cells in the presence of $1,25(OH)_2D_3$ and examining TRAP (tartrate-resistant acid phosphatase) activity[29] which is considered to be a marker of osteoclast maturation.[29,375] Compared to $1,25(OH)_2D_3$ alone, both EPA and DHA (n-3 PUFAs), alone or in combination caused a significant decrease in osteoclast maturation compared with the n-6 PUFAs linoleic acid (LA) and AA.[29] These results demonstrate an inhibitory effect of n-3 PUFAs on osteoclastogenesis *in vitro*.

Although PUFAs have been implicated in bone homeostasis, their effects on OPG and RANKL secretion by osteoblasts have not been investigated. PUFAs may affect the osteoblastic RANKL-OPG system via PGE_2 modulation. AA (n-6 PUFA) is a substrate for PGE_2 synthesis in various cell types[170] and PGE_2 is the most abundant prostanoid among prostaglandins in bone.[134] PGE_2 has been shown to stimulate osteoclastogenesis and bone resorption[318] by inducing RANKL on osteoblastic cells[131,138] and inhibiting OPG synthesis in primary human bone marrow cells.[139] Although the n-3 PUFAs are precursors of PGE_3, that is equally potent to PGE_2 in bone, their conversion is less effective than for PGE_2, thereby resulting in less total PGE_2.[177,178] DHA is not a substrate for prostaglandin synthesis but could interfere with PGE_2 synthesis by replacing AA in the cell membrane, thereby limiting the amount of AA available for PGE_2 production.[172,176]

To determine whether PUFAs and the bone active hormones oestrogen and PTH affect OPG secretion *in vitro*, MG-63 and MC3T3-E1 osteoblasts were exposed to these compounds. PGE_2, a product of AA metabolism in osteoblasts (refer to Chapter 4), and previously implicated in bone homeostasis,[134,143] was included in this study. Since the OPG/RANKL ratio is important for regulation of the bone microenvironment, RANKL secretion by MC3T3-E1 cells, after exposure to the above-mentioned components was also investigated.

7.2 Materials and Methods

Reagents and Materials

Sigma Chemical Co (St. Louis, MO, USA) supplied L-glutamine, crystal violet, trypan blue, arachidonic acid, docosahexaenoic acid, β-estradiol (oestrogen), parathyroid hormone fragment 1-34, PGE_2 and dextran coated charcoal. Heat-inactivated fetal calf serum (FCS) was purchased from Highveld Biological (Pty) Ltd. (Sandringham, SA). DMEM was obtained from Sterilab Services (Kempton Park, SA) and gentamycin from Gibco BRL (Invitrogen Corp., Carlsbad, CA, USA). All other chemicals were of analytical grade and purchased from Sigma Chemical Co (St. Louis, MO, USA). Sterile cell cluster plates were supplied by LASEC (Johannesburg, SA).

Cell cultures and maintenance

MG-63 (human osteoblast-like, osteosarcoma-derived) cells were purchased from the American Type Culture Collection (ATCC), Rockville, MD, USA. Nontransformed MC3T3-E1 mouse calvaria fibroblasts (established from the calvaria of an embryo/fetus C57BL/6 mouse) described to differentiate to osteoblasts,[285] were purchased from Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ), Braunschweig, Germany. MCF-7 human breast carcinoma cells, a positive control for oestrogen receptors,[309,310] were obtained from Highveld Biological (Pty) Ltd, (Sandringham, SA).

Cell cultures were maintained in DMEM (with 10% heat-inactivated FCS) at 37ºC in a humidified atmosphere of 95% air and 5% CO_2. All cell cultures were supplemented with 2 mM L-glutamine and gentamycin (25 µg/ml). Fatty acid stock solutions were stored in small aliquots at -70ºC and the working solutions freshly prepared each time prior to their use. The ethanol concentration did not exceed 0.2%. Previous studies in our laboratory showed no toxic effects of the ethanol vehicle at this concentration.

Determination of osteoprotegerin concentrations in conditioned media

After trypan blue exclusion, MG-63 and MC3T3-E1 cells were seeded in sterile 24-well culture plates at a density of 50 000 cells/well in DMEM (without phenol red) containing 5% charcoal stripped FCS. After cells had attached firmly for 24 hours, culture medium was replaced with fresh medium. Vehicle (0.2% ethanol), PUFAs (AA and DHA)(2.5 to 20μg/ml) or hormones (oestrogen and PTH) (10^{-8} M) and PGE_2 (10^{-8} M) were then added. In some cases the cyclo-oxygenase inhibitor, indomethacin (1μM final concentration) was added to the growth medium 45 minutes prior to the addition of test substances. After 24 hours conditioned media were harvested and stored at -70ºC until analysed.

Following removal of medium, cell numbers were determined by crystal violet staining as previously described (refer to Chapter 3). In short, cultures were fixed with 1% glutaraldehyde, stained with 1% crystal violet, and the dye extracted with 0.2% Triton X-100. Absorbance (O.D.) was read on an ELX800 Universal Microplate Reader (Bio-Tek Instruments Inc., Analytical Diagnostic Products, Weltevreden Park, SA) at a wavelength of 570nm; 0.2% Triton X-100 in water was used as a blank. Crystal violet is a basic dye that stains cell nuclei.[298] Spectrophotometer readings of colour intensity are therefore an indication of DNA content and therefore cell numbers. Results were analysed using a linear standard curve established from known cell numbers (Refer to Figure 3.2). Three independent experiments were conducted (n=4).

Quantification of osteoprotegerin concentrations in MG-63 conditioned media

Levels of OPG in the conditioned media were determined using an adaptation of the sandwich ELISA protocol developed by Brändström, *et al* (2001).[139] A MaxiSorb microtiterplate (NUNC™ Brand Products, Roskilde, Denmark) was coated with 2 μg/ml monoclonal mouse anti-human OPG capture antibody (R&D Systems Inc, Minneapolis, MN, USA) and incubated overnight at 4ºC. The plate was then blocked with PBS containing 1% BSA, 5% sucrose and 0.05% NaN_3 for one hour at room

temperature. (**) Samples (diluted 1:100 in DMEM), and standards (ranging from 31.25 to 5000 pg/ml) (human recombinant OPG, Amgen Inc, Thousand Oaks, California, USA) diluted in dilution buffer (PBS containing 1% BSA and 0.02% NaN_3) were added and incubated for two hours at 37ºC. Cell culture medium was used as blank. (**) Thereafter 0.2 μg/ml biotinylated goat anti-human OPG detecting antibody (R&D Systems) was added. After two hours incubation at 37ºC (**), peroxidase-conjugated streptavidin (DAKO A/S, Glostrup, Denmark) (1:2000 dilution in PBS containing 0.05% Tween^{R}20 (MERCK, Schuchardt, Germany)) was added and incubated for 30-40 minutes at room temperature. (**) Ready-to-use 3'3',5,5' tetramethylbenzidine (TMB) liquid substrate (Sigma-Aldrich, Inc., Saint Louis, MO, USA), was added and the plate incubated at room temperature with shaking (300 rpm) for a period of 10 to 20 minutes until colour developed. The reaction was terminated by the addition of 0.9M H_2SO_4. Optical density was read at 450/620 nm using an ELX800 Universal microplate reader (Bio-Tek Instruments Inc., Analytical Diagnostic Products, Weltevreden Park, SA). Results were analysed using a linear standard curve. (Refer to figure 3.6). (**) Between each step of the ELISA the plate was washed three times with PBS containg 0.05% Tween. Intra and interassay variability was 5% and 11% respectively.

Quantification of osteoprotegerin concentrations in MC3T3-E1 conditioned media

For measurement of murine OPG, a sandwich ELISA protocol similar to the one used for the detection of human OPG was developed. A monoclonal anti-mouse OPG antibody (R&D Systems Inc, Minneapolis, MN, USA) was used as capture antibody, while a biotinylated anti-mouse OPG antibody (R&D Systems Inc, Minneapolis, MN, USA) was used as detection antibody. Undiluted samples and standards (31.25 to 5000 pg/ml) (recombinant mouse OPG/Fc chimera) (R&D Systems) were prepared as described above. Intra and interassay variability was 6.5% and 16% respectively.

Quantification of free sRANKL (secreted RANKL) concentrations in MC3T3-E1 conditioned media

A commercial sandwich ELISA kit (Biomedica Medizinprodukte GmbH & Co KG, Vienna, Austria) was used for the quantitative determination of free sRANKL in the conditioned media of the MC3T3-E1 cells. Due to the high cost of the commercial kit only one experiment was conducted (n=4). In short, recombinant murine OPG was used as 'capture antibody' while a polyclonal biotinylated anti-mouse sRANKL antibody was used as detection antibody. Recombinant mouse sRANKL ranging from 12.5 to 100 pmol/liter was prepared in DMEM and used as standards. Cell culture medium was used as blank. Streptavidin-horseradish peroxidase was used as conjugate and TMB liquid as substrate for colour development. The reaction was terminated by addition of H_2SO_4. Optical density was read at 450/620 nm using an ELX800 Universal microplate reader (Bio-Tek Instruments Inc., Analytical Diagnostic Products, Weltevreden Park, SA). Results were analysed using a linear standard curve that was constructed from the standard values. RANKL concentrations were then expressed as (pg/ml)/10 000 cells. (1 pg/ml = 0,05 pmol/liter). Intra and interassay variability was 4.2% and 9% respectively.

Detection of oestrogen receptors in MG-63 cells

Following trypan blue exclusion, 250 000 MG-63 cells were seeded onto sterilised coverslips in 6-well cluster plates and left to adhere. MCF-7 human breast carcinoma cells, a positive control for oestrogen receptors,[309,310] were treated similarly. After 24 hours, the culture media were replaced and the cells incubated for another 24 hours. At the end of the experiment, the media were discarded and the cells fixed in 10% formaldehyde (v/v)(in 2 mM EGTA in PBS) for 10 minutes at room temperature. The coverslips were then transferred to a staining dish and rinsed thrice for 5 minutes with PBS at room temperature. Thereafter cells were permeabilised in ice cold 97% methanol (containing 2 mM EGTA in PBS) at –20°C for 4 minutes. After rinsing the cells thrice for 5 minutes with PBS, they were incubated for 1 hour at 37°C, in each of the following: primary monoclonal antibody against oestrogen receptor (developed in rabbit (1:1000 in PBS)) (Sigma-Aldrich, Inc., Saint Louis, MO, USA); goat anti-rabbit IgG peroxidase conjugate

(1:200 in PBS) (Cappel™ Research Reagents, ICN, Aurora, Ohio, USA) and ExtrAvidin®-peroxidase conjugate (1:15). 3 x 5 minutes washing steps separated incubations. Antigen detection was achieved by adding 3,3' diaminobenzidine as substrate (60 mg in 200 ml PBS, containing 0.1% hydrogen peroxide). Coverslips were left in the dark for 5 minutes and subsequently mounted with a glycerol-based mounting fluid. Photographs were taken with 400 ASA film with a Nikon camera attached to a Nikon Optiphot microscope (Nikon, Tokyo, Japan).

Statistics

For each of the cell lines tested three independent experiments were conducted (n=4). Statistical analysis was performed using Statistics for Windows software (version 2, Tallahassee, Florida, USA). Student's paired *t*-test was use to evaluate differences between the sample of interest and its respective control. For analysis of dose responses, means of groups were compared by one way ANOVA and significance was determined by post-hoc testing using Bonferroni's method. A *P* value of less than 0.05 was considered to be statistically significant.

7.3 Results

7.3.1 Effects of arachidonic acid, prostaglandin E_2, and parathyroid hormone on osteoprotegerin secretion in MG-63 and MC3T3-E1 cells

Effects of arachidonic acid, prostaglandin E_2 and parathyroid hormone on osteoprotegerin secretion in MG-63 cells

Figure 7.2 depicts the effects of vehicle (0.2% ethanol)(control), AA (2.5 to 20 μg/ml), PGE_2 (10^{-8} M) and PTH (10^{-8} M) on OPG secretion by MG-63 cells. Compared to control, AA dose-dependently inhibited OPG secretion by 25% to 40%. Pre-incubating the cells with the unselective COX-inhibitor indomethacin prior to AA exposure slightly attenuated the inhibitory effect of AA, especially at lower AA concentrations (2.5 to 5 μg/ml). PGE_2 inhibited OPG secretion by 40%, while PTH had no effect.

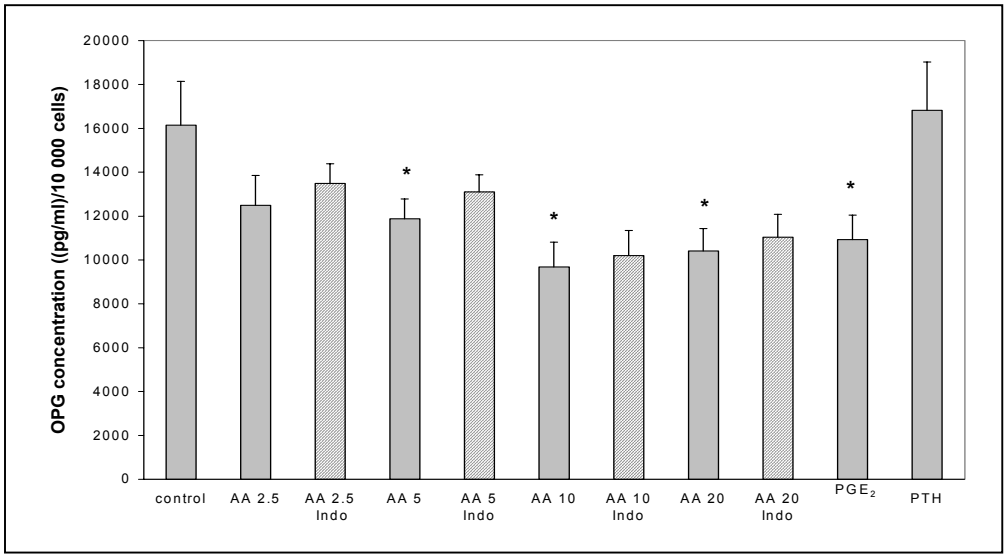

Figure 7.2 Effects of arachidonic acid, prostaglandin E_2, and parathyroid hormone on osteoprotegerin secretion by MG-63 cells.

Analysis of OPG levels from MG-63 cells that were cultured for 24 hours in the presence of vehicle (0.2% ethanol)(control), AA (2.5 to 20 μg/ml), PGE_2 (10^{-8} M) and PTH (10^{-8} M). Indomethacin (Indo)(1μM) was added 45 minutes prior to AA-treatment. OPG protein secretion was measured by ELISA from conditioned medium and standardised for cell number as described in the Materials and Methods. Three separate experiments were conducted; data are from a representative experiment. * Significant difference from control, $P<0.05$, n=4.

Effects of arachidonic acid, prostaglandin E_2, and parathyroid hormone on osteoprotegerin secretion in MC3T3-E1 cells

Figure 7.3 depicts the effects of AA (2.5 to 20 μg/ml), PGE_2 (10^{-8} M) and PTH (10^{-8} M) on OPG secretion by MC3T3-E1 cells. Compared to control, AA inhibited OPG secretion by 10% to 30%. Pre-incubating the cells with the unselective COX-inhibitor indomethacin prior to AA-exposure (AA concentrations of 2.5 to 10 μg/ml) abolished the inhibitory effect of AA on OPG secretion completely, and increased OPG secretion to levels higher than that of the control values. Indomethacin did not abolish the inhibiting effect of 20 μg/ml AA on OPG secretion. PGE_2 inhibited OPG secretion by 40% while PTH had no effect.

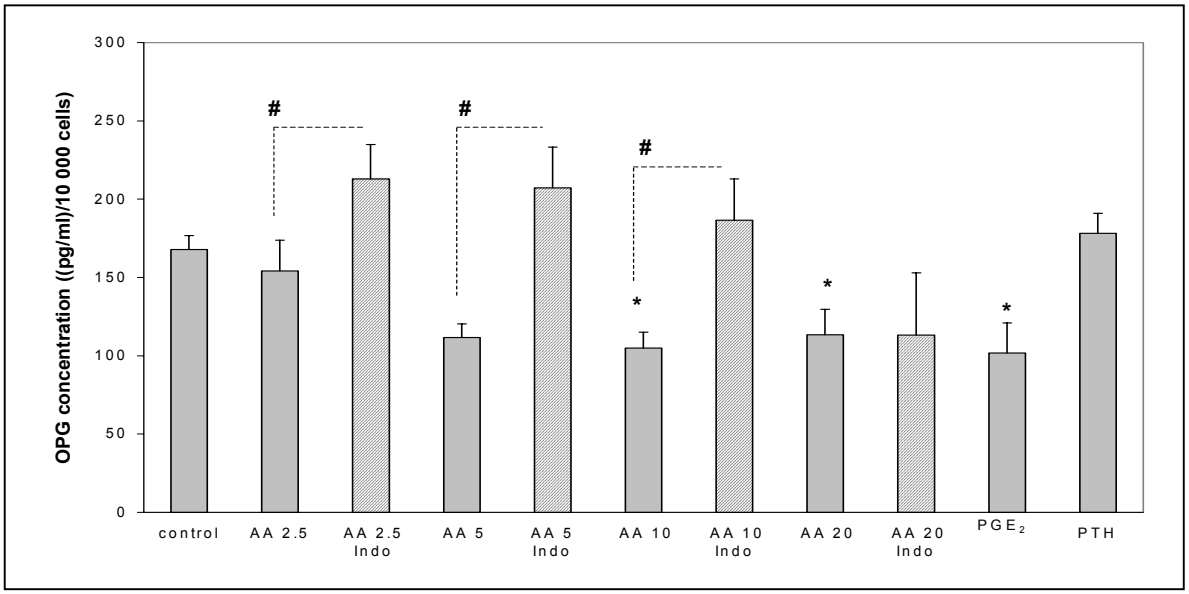

Figure 7.3 Effects of arachidonic acid, prostaglandin E_2, and parathyroid hormone on osteoprotegerin secretion by MC3T3-E1 cells

Analysis of OPG levels from MC3T3-E1 cells that were cultured for 24 hours in the presence of vehicle (0.2% ethanol)(control), AA (2.5 to 20 μg/ml), PGE_2 (10^{-8} M) and PTH (10^{-8} M). Indomethacin (Indo)(1μM) was added 45 minutes prior to AA-treatment. OPG protein secretion was measured by ELISA from conditioned medium and standardised for cell number as described in the Materials and Methods. Three separate experiments were conducted; data are from a representative experiment. * Denotes $P<0.05$ compared with the control cultures (n=4). # Denotes $P<0.05$ compared with the corresponding indomethacin-treated culture (n=4).

7.3.2 Effects of docosahexaenoic acid and oestrogen on osteoprotegerin secretion in MG-63 and MC3T3-E1 cells

Effects of docosahexaenoic acid oestrogen (E2) on osteoprotegerin secretion by MG-63 cells

Figure 7.4 depicts the effects of DHA (2.5 to 20 μg/ml) and oestrogen (E2) (10^{-8} M) on OPG secretion by MG-63 cells. Compared to control, DHA inhibited OPG secretion by 20% to 50%. DHA at concentrations of 10 to 20 μg/ml caused the largest inhibition (50%). Indomethacin pre-incubation attenuated the inhibitory effect of 10 μg/ml DHA. Oestrogen (10^{-8} M) inhibited OPG secretion by 50%.

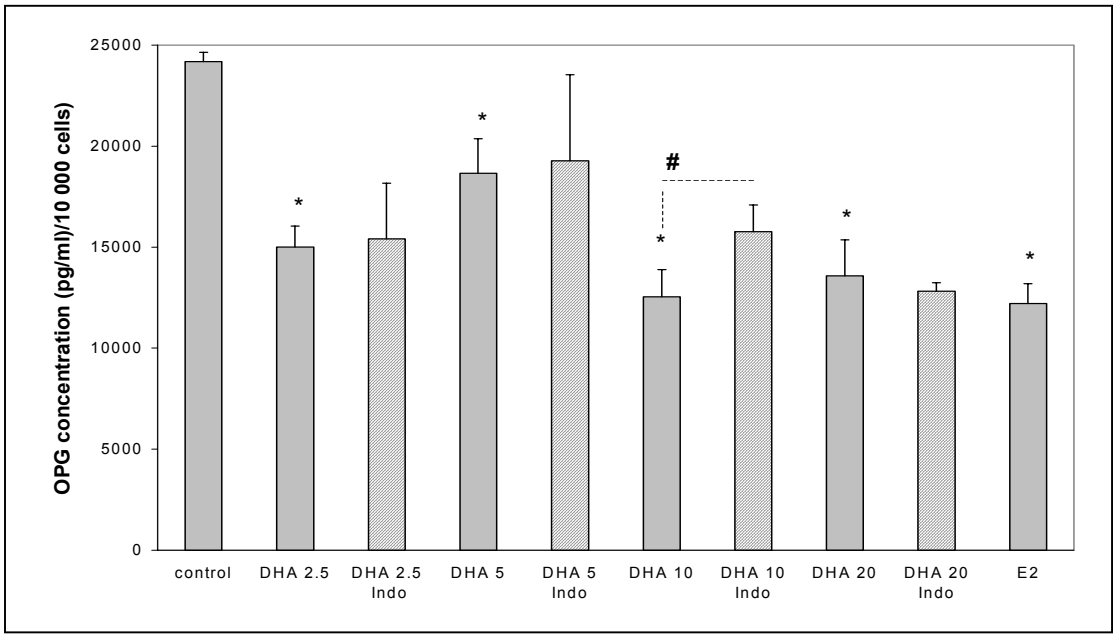

Figure 7.4 Effects of docosahexaenoic acid and oestrogen on osteoprotegerin secretion by MG-63 cells.

Analysis of OPG levels from MG-63 cells that were cultured for 24 hours in the presence of vehicle (0.2% ethanol)(control), DHA (2.5 to 20 μg/ml) and oestrogen (E2) (10^{-8} M). Indomethacin (Indo)(1μM) was added 45 minutes prior to DHA treatment. OPG protein secretion was measured by ELISA from conditioned medium and standardised for cell number as described in the Materials and Methods. Three separate experiments were conducted; data are from a representative experiment. * Significant difference from control, $P<0.05$, n=4. # Denotes $P<0.05$ compared with the corresponding indomethacin-treated culture (n=4).

Effects of docosahexaenoic acid and oestrogen on osteoprotegerin secretion by MC3T3-E1 cells

Figure 7.5 depicts the effects of DHA (2.5 to 20 μg/ml) and oestrogen (E2) (10^{-8} M) on OPG secretion by MC3T3-E1 cells. Compared to control, DHA dose-dependently inhibited OPG secretion by 12% to 30%. Pre-incubating the cells with indomethacin prior to DHA-exposure (2.5 to 10 μg/ml) abolished the inhibitory effect of DHA on OPG secretion completely, and increased OPG secretion to levels slightly higher than that of the control values. Indomethacin did not abolish the inhibitory effect of 20 μg/ml DHA on OPG secretion. Oestrogen (10^{-8} M) inhibited OPG secretion by 50%.

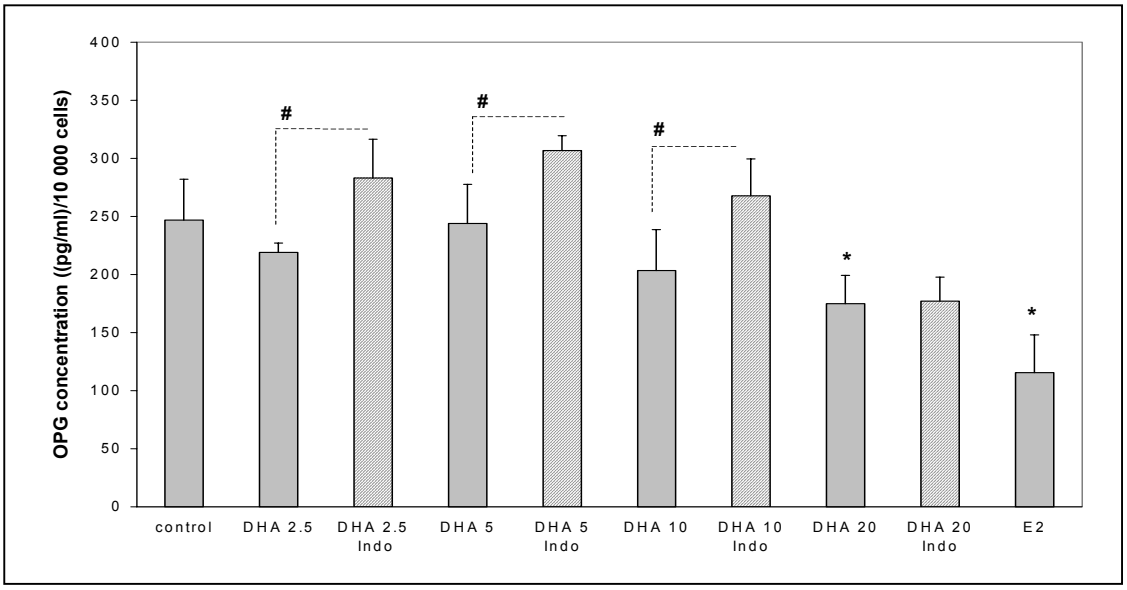

Figure 7.5 Effects of docosahexaenoic acid and oestrogen on osteoprotegerin secretion by MC3T3-E1 cells

Analysis of OPG levels from MC3T3-E1 cells that were cultured for 24 hours in the presence of vehicle (0.2% ethanol)(control), DHA (2.5 to 20 μg/ml) and oestrogen (E2) (10^{-8} M). Indomethacin (Indo)(1μM) was added 45 minutes prior to DHA treatment. OPG protein secretion was measured by ELISA from conditioned medium and standardised for cell number as described in the Materials and Methods. Three separate experiments were conducted; data are from a representative experiment. * Denotes $P<0.05$ compared with the corresponding control cultures (n=4). # Denotes $P<0.05$ compared with the corresponding indomethacin-treated culture (n=4).

7.3.3 Effects of arachidonic acid, docosahexaenoic acid, prostaglandin E_2,, parathyroid hormone and oestrogen on RANKL secretion in MC3T3-E1 cells

Figure 7.6 depicts the effects of AA (2.5 to 20 μg/ml), PGE_2 (10^{-8} M) and hormones (PTH and oestrogen) (10^{-8} M) on RANKL secretion by MC3T3-E1 cells. The concentrations of RANKL secreted by these cells at control conditions and lowest AA concentration (2.5 μg/ml) were too low to be detected by the sRANKL ELISA procedure used. AA at concentrations of 5 to 20 μg/ml, however, dose-dependently increased RANKL secretion. Indomethacin pre-incubation attenuated the stimulatory effect of AA on RANKL secretion, especially at 20μg/ml AA. PGE_2 (10^{-8} M) stimulated RANKL secretion to levels slightly higher than that of 20 μg/ml AA. Of the agents tested, PTH (10^{-8} M) stimulated RANKL secretion the most. Cells exposed to DHA and oestrogen did not secrete high enough RANKL levels to be detected with the commercial ELISA protocol we used and are therefore not included in the graph.

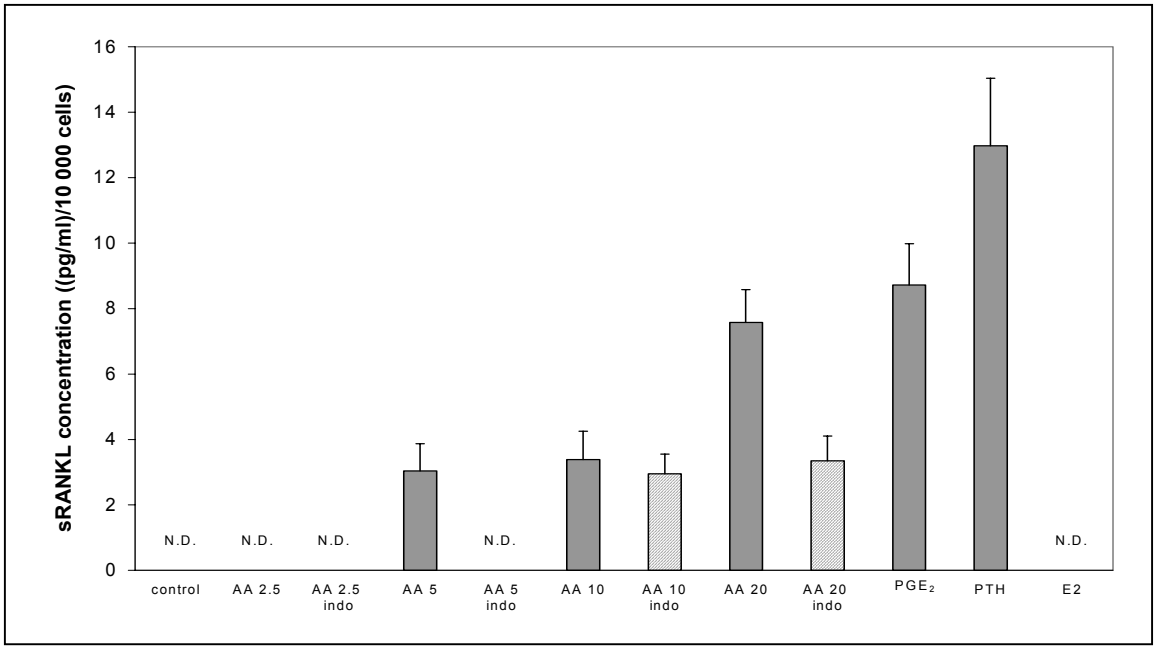

Figure 7.6 Effects of arachidonic acid, prostaglandin E_2,, parathyroid hormone and oestrogen on RANKL secretion by MC3T3-E1 cells

Analysis of levels from MC3T3-E1 cells that were cultured for 24 hours in the presence of vehicle (0.2% ethanol)(control), AA (2.5 to 20 μg/ml) PGE_2 (10^{-8} M) and PTH (10^{-8} M). Indomethacin (Indo) (at 1μM final concentration) was added 45 minutes prior to AA treatment. RANKL secretion was measured by ELISA from conditioned medium and standardised for cell number as described in the Materials and Methods. (n=4) (N.D. not detected)

7.3.4 Effects of arachidonic acid, prostaglandin E_2 and parathyroid hormone on the osteoprotegerin/sRANKL ratio in MC3T3-E1 conditioned media

The effects of AA, PGE_2 and PTH on the OPG/sRANKL ratio in MC3T3-E1 cells are shown in Figure 7.7. Although OPG was secreted by cells exposed to vehicle (0.2% ethanol) and 2.5μg/ml AA, sRANKL could not be detected in these conditions and the OPG/sRANKL ratio could not be determined. Exposure to higher AA concentrations (5 to 20μg/ml) dose-dependently decreased the OPG/sRANKL ratio, with 20μg/ml AA causing a decrease of more than 50% compared to 5μg/ml AA. Compared to 5μg/ml AA, PGE_2 (10^{-8} M) and PTH (10^{-8} M) decreased the OPG/sRANKL ratio by 75%.

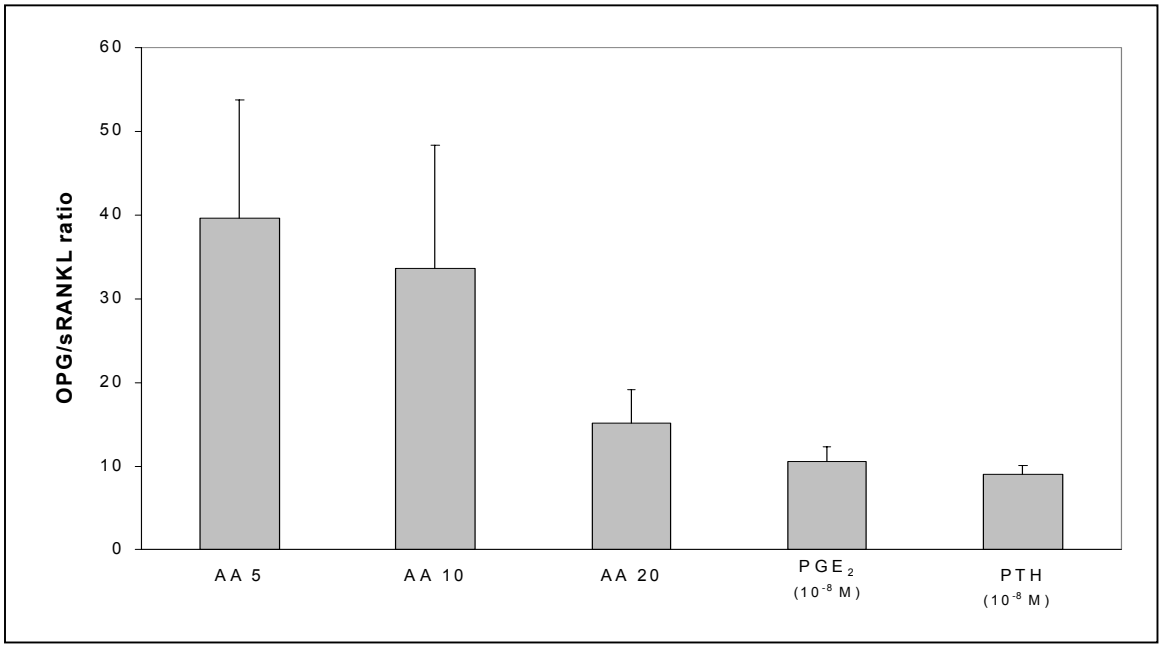

Figure 7.7 Effects of arachidonic acid, prostaglandin E_2, and parathyroid on the osteoprotegerin/sRANKL ratio in MC3T3-E1 cells.

MC3T3-E1 cells were cultured for 24 hours in the presence of vehicle (0.2% ethanol)(control), AA (2.5 to 20 μg/ml), PGE_2 (10^{-8} M) and PTH (10^{-8} M). RANKL and OPG secretion were measured by ELISA from conditioned media, standardised for cell number as described in the Materials and Methods, and expressed as OPG/sRANKL ratio.

7.3.5 Detection of oestrogen receptors in MG-63 cells

As the MG-63 cells did not respond to oestrogen as would have been expected of osteoblastic cells expressing large numbers of oestrogen receptors,[108] we tested these cells for the expression of oestrogen receptors (ER). MCF-7 human breast carcinoma cells, known to express high numbers of ER,[309,310] were used as a positive control. Figure 7.8 A depicts the expression of oestrogen receptors in the nuclei of MCF-7 carcinoma cells. Figure 7.8 B shows the absence of ER in MG-63 cells.

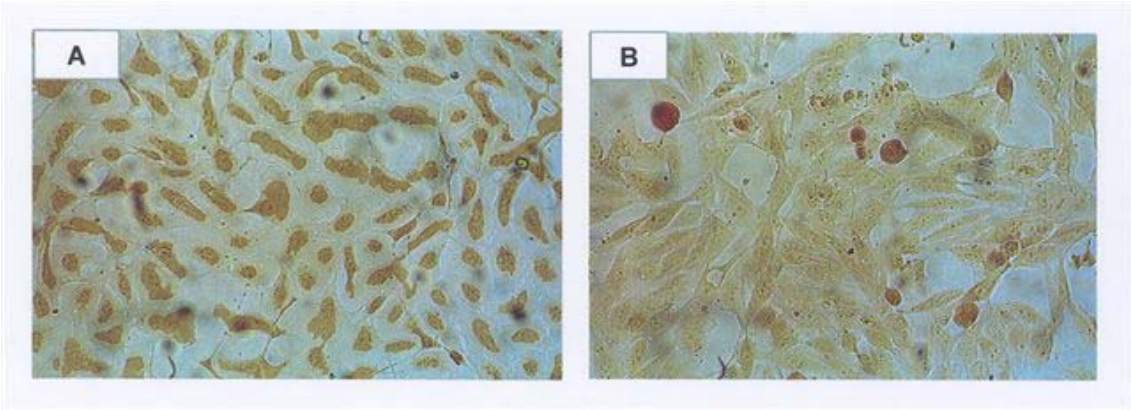

Figure 7.8 Oestrogen receptor detection in MCF-7 human breast carcinoma cells and MG-63 human osteosarcoma cells.

Cells were incubated in DMEM (without phenol red) supplemented with 5% charcoal stripped FCS for 24 hours. Thereafter medium was changed, the cells cultured for another 24 hours and oestrogen receptors detected as described in the Materials and Methods. **A:** MCF-7 cells staining positive for oestrogen receptors; **B:** MG-63 cells staining negative for oestrogen receptors. (Original magnification: 100 x)

7.4 Discussion

Apart from having an effect on bone formation, osteoblasts are also coupled with osteoclasts through the release of various cytokines including macrophage colony-stimulating factor (M-CSF) and RANKL.[9] Most pro-and anti-osteoclastogenic cytokines act primarily through the osteoblast to alter levels of RANKL and OPG, the balance of which determines overall osteoclast formation.[8,14,124] (Refer to 2.3.3.2).

Prostaglandins, especially PGE_2, produced by osteoblasts from its fatty acid precursor arachidonic acid, have pronounced effects on bone. (Refer to 2.11.3) Depending on the concentration and experimental model, both anti-resorptive and pro-resorptive effects of prostaglandins have been reported.[134] Several studies confirmed the importance of PGE_2 in osteoclast formation and bone resorption.[135,137,257] PGE_2 has been shown to inhibit OPG synthesis in various cell cultures such as primary human bone marrow cells [139] and tissues such as mouse calvaria[142] and to stimulate expression of mRNA for RANKL.[138] PGE_2 thus lowers the OPG/RANKL ratio thereby favouring osteoclastogenesis and bone resorption. Many bone-active agents that induce bone resorption such as PTH[313,314] and cytokines such as IL-1 and IL-6 are prostaglandin mediated,[133,153,155,157] since the COX- blocker indomethacin partially inhibits their action.

Prostaglandins act through specific prostanoid receptors thereby changing the levels of second messengers.[170] (Refer to 2.11.1). At least four distinct receptors for PGE_2 with different signaling pathways have been identified: EP_1 is coupled to Ca^{2+} mobilisation; EP_2 and EP_4 with stimulation of cAMP production; and EP_3 mainly with the inhibition of cAMP production.[235,236] The expression patterns of PG receptors differ in various cell types, differentiation status of these cells, tissues, and species. It has been shown that PGE_2 possibly down-regulates the expression of OPG mRNA in primary human bone marrow stroma cells through a PKA-cAMP-dependent pathway.[376] Making use of EP agonists[280] and antagonists[278,281] it was shown that PGE_2 acts on mouse calvaria cultures mainly via the EP_2 and EP_4 receptors to induce cAMP. Others confirmed the importance of the EP_2 receptor in osteoclastogenesis by showing that knockout of the EP_2 receptor in EP_2 -/- mice resulted in defective responses of osteoblastic cells to PGE_2 *in vitro*.[278]

We speculated that PUFAs indirectly affect bone resorption through modulation of the OPG/RANKL ratio via PGE_2 synthesis. To determine whether PUFAs affect OPG secretion *in vitro,* MG-63 and MC3T3-E1 osteoblasts were exposed to the n-6 PUFA AA and the n-3 PUFA DHA. To determine whether the effects of PUFAs could be attributed to modulation of PGE_2 synthesis or not, cells were pre-incubated in some experiments with the unselective COX-blocker indomethacin.[252]

7.4.1 Effects of arachidonic acid, docosahexaenoic acid and prostaglandin E_2 on osteoprotegerin secretion

Effects of arachidonic acid and prostaglandin E_2 on osteoprotegerin secretion in MG-63 and MC3T3-E1 cell lines

OPG concentrations in the harvested medium of the MG-63 human osteosarcoma-derived osteoblastic cell line were significantly higher than that of the murine MC3T3-E1 osteoblastic cell line (Figure 7.2 and Figure 7.3). This might be attributed to species differences or could be due to differences in the differentiation status of these cell lines as it has been shown that OPG expression is developmentally regulated and increases during osteoblast differentiation.[14,377] The MC3T3-E1 cell line is regarded as a pre-osteoblast,[285] which could explain the lower OPG concentrations, detected.

Results from our study (Figure 7.2 and Figure 7.3) showed that AA suppressed OPG secretion in a dose-dependent manner in both cell lines, possibly via PGE_2 production, as PGE_2 alone also significantly reduced OPG secretion. In the MG-63 cell line pre-incubation with indomethacin, prior to AA exposure, partially reversed OPG inhibition, thereby confirming that the inhibitory effect of AA on OPG secretion could be PGE_2-mediated.

In the MC3T3-E1 cell line indomethacin pre-treatment completely abolished the inhibitory effects of 2.5 to 10 μg/ml AA on OPG secretion and even enhanced OPG concentrations to levels surpassing that measured in control conditions, thereby suggesting that indomethacin stimulated OPG secretion in these conditions (Figure 7.3). However, the culture medium contains FCS-derived AA[56] that could be

metabolised to low levels of PGE_2 that could inhibit OPG secretion even in control conditions. Although it is customary to omit FCS from culture media when testing for secreted mediators such as OPG,[132,139,367] we chose not to do this as both MG-63 and MC3T3-E1 cell lines do not tolerate PUFAs in FCS free conditions. Research in our laboratory reported detectable levels of PGE_2 after four hours of incubation in control conditions (refer to Chapter 4) in the presence of FCS. Our results are supported by those of O'Brien *et al* (2001) who demonstrated higher levels of OPG in medium from mouse calvaria cultured in the presence of indomethacin than with PGE_2.[142] They also reported significant amounts of OPG secretion even in the presence of PGE_2,[142] thereby confirming our results.

In both cell lines, indomethacin pre-treatment could not attenuate the inhibitory effect of 20μg/ml AA on OPG secretion to the same degree as it did at lower AA concentrations. We speculate that this might be explained by the inability of indomethacin to completely block PGE_2 synthesis at high AA concentrations as reported in Chapter 4 and confirmed by others.[319] Alternatively, high concentrations of AA *per se* could have an inhibitory effect on OPG secretion independently of PGE_2 synthesis.

Effects of docosahexaenoic acid on osteoprotegerin secretion in MG-63 and MC3T3-E1 cell lines

DHA, one of the n-3 PUFAs, is not a substrate for prostaglandin synthesis but inhibits PGE_2 synthesis[172] possibly by replacing AA in the cell membrane thereby limiting the amount of AA available for PGE_2 production.[176] MG-63 and MC3T3-E1 osteoblast-like cells were exposed to DHA to determine whether DHA affects OPG secretion. Cells were also pre-incubated with the unselective COX-blocker indomethacin[252] to determine whether the effects of DHA could be attributed to modulation of PGE_2 synthesis.

In the MG-63 cell line DHA suppressed OPG secretion significantly even at low DHA concentrations, which suggested that DHA per se had an inhibitory effect in this cell line. Indomethacin pre-treatment had little effect on OPG secretion suggesting that the low levels of AA provided by the FCS in the medium did not interfere with OPG

synthesis in this model. In the MC3T3-E1 cell line DHA suppressed OPG secretion slightly, which was significant only at the highest DHA concentration (Figure 7.5). Indomethacin pre-treatment, however, affected OPG secretion significantly. It therefore seems that AA supplied by the FCS in the culture medium affected OPG secretion, probably via PGE_2 synthesis in this cell line. The mechanism whereby DHA inhibited OPG secretion in both cell lines is not known and will have to be investigated.

In an *in vivo* study, Atkinson *et al* (1997) supplemented the diets of rats with oil rich in DHA.[33] Although the oil did not contain detectable levels of EPA, significantly elevated EPA levels were reported in the membrane phospholipids of a variety of tissues suggesting considerable retroconversion of DHA back to EPA.[33] EPA is a substrate for cyclooxygenase that catalises the formation of PGE_2 and PGE_3[172] and it has been shown that PGE_3 stimulates bone resorption with a potency similar to that of PGE_2 in cultured fetal rat bones and neonatal rat calvaria.[177] EPA, however, is only one-tenth as effective for PGE_3 synthesis as AA for PGE_2 synthesis,[177] suggesting that replacing AA with EPA in osteoblasts could have a bone protective effect. One could therefore speculate that DHA exposure could, via retroconversion of DHA to EPA, result in the formation of low levels of PGE_3, which could inhibit OPG secretion.

7.4.2 Effects of arachidonic acid, docosahexaenoic acid and prostaglandin E_2 on RANKL secretion and the osteoprotegerin/RANKL ratio in MC3T3-E1 osteoblasts

Factors that affect bone resorption may affect RANKL and/or OPG secretion, the balance of which determines osteoclastogenesis and ultimately affects bone resorption rate.[14] The biological activity of RANKL *in vitro* and *in vivo* has been characterised. When combined with M-CSF, RANKL stimulates osteoclast development and activates mature, pre-existing osteoclasts.[9] Apart from cell-bound RANKL present on osteoblast membranes, soluble RANKL (sRANKL) is synthesised as a membrane-anchored precursor, which is then released from the plasma membrane by a metalloprotease. Soluble RANKL demonstrates potent osteoclastogenic activity.[65]

In order to determine whether the compounds we tested impacted on RANKL as well as the OPG/sRANKL ratio, this study was extended to include measurements of RANKL secretion. The expression of OPG and RANKL is developmentally regulated.[377,378] While OPG increases during osteoblast differentiation,[14,377] RANKL expression is inversely related to the degree of differentiation.[377] It has been reported that MG-63 cells do not express RANKL and as such could be regarded as a more differentiated cell line.[367] Therefore in our study, testing for RANKL secretion was limited to the MC3T3-E1 cell line only.

Effects of arachidonic acid and prostaglandin E_2 on RANKL secretion and osteoprotegerin/RANKL ratio in MC3T3-E1 cells

The levels of soluble RANKL in the harvested culture media of MC3T3-E1 cells were very low and in some cases could not be detected by the commercial sRANKL ELISA protocol we used. Others reported low levels of RANKL mRNA expression in MC3T3-E1 cells,[124,125] which could explain the low levels of RANKL secreted by these cells. No sRANKL could be detected in the media harvested from MC3T3-E1 control cells exposed to vehicle (0.2% ethanol) only. AA, however, stimulated RANKL secretion in a dose-dependent fashion in this model (Figure 7.6). Sun *et al* (2003) demonstrated increased RANKL+ T cells in corn oil fed mice, thereby confirming that oils high in n-6 PUFAs such as AA, could stimulate RANKL expression in lymphocytes.[29]

PGE_2 also enhanced RANKL secretion in our model. Results from our study suggested that the AA-stimulated secretion of RANKL could be PGE_2-mediated, as indomethacin pre-treatment attenuated this effect significantly. Our results confirm those of Nakashima *et al* (2000) who have shown that PGE_2 increases RANKL mRNA and protein expression in murine osteoblast stromal cells.[138] Making use of EP agonists[280] and antagonists[278,281] it was shown that PGE_2 acts on mouse calvaria cultures mainly via the EP_2 and EP_4 receptors to induce cAMP and expression of RANKL in osteoblastic cells. PGE_2 could possibly stimulate RANKL secretion via binding to the EP_4 receptor in MC3T3-E1 cells as mRNA for the EP_4 receptor has been detected in these cells.[229] In the MC3T3-E1 cell line AA affected RANKL and

OPG in opposite directions, which resulted in a markedly decreased OPG/RANKL ratio. Whether the change in the OPG/RANKL ratio is sufficient to increase osteoclastogenesis and eventually enhance bone resorption has to be investigated.

The effects of PGE_2 on osteoclastogenesis are complicated. A study by Wani *et al* (1999) showed that apart from the osteoblast-mediated effect of PGE_2 on osteoclast formation, PGE_2 also synergises with RANK in inducing osteoclastogenesis in cultures not containing osteoblasts probably through a direct action on the osteoclastic haemopoietic precursors.[282] The response depended on the presence of exogenous soluble RANKL, as PGE_2 alone had no effect.[282] It has recently been shown that the PGE_2-mediated osteoclastogenic effect is brought about mainly through EP_2 and EP_4 receptors on osteoclast precursors.[283] In another study, Ono *et al* (2005) reported a biphasic effect of PGE_2 on osteoclast formation from spleen cell cultures treated with M-CSF and RANKL.[379] They demonstrated an initial inhibitory effect on osteoclast formation, possibly mediated by the EP_2 and EP_3 receptors, and a later stimulatory effect, mediated by the EP_2 receptor and possibly also acting on T-cells.[379]

Effects of docosahexaenoic acid on sRANKL secretion and the osteoprotegerin/RANKL ratio in MC3T3-E1 cells

No sRANKL could be detected in the culture media of DHA-exposed MC3T3-E1 cells. It therefore seems that DHA did not stimulate RANKL secretion, in contrast to AA that dose-dependently stimulated RANKL secretion. This observation is supported by the findings of Sun *et al* (2003)[29]: Although not in an osteoblast cell model, it was demonstrated that treatment of mice with fish oil (that contains high levels of n-3 PUFAs such as EPA and DHA) had no affect on RANKL expression in stimulated T cells. In contrast, corn oil (that contains high levels of n-6 PUFAs) stimulated RANKL expression.[29]

As RANKL secretion could not be detected after DHA exposure, the OPG/RANKL ratio could not be calculated. However, since OPG was detected and is known for its anti-resorptive properties, the absence of sRANKL after DHA exposure suggests a possible bone-protective role for DHA.

7.4.3 Effects of parathyroid hormone on osteoprotegerin and RANKL secretion and the osteoprotegerin/RANKL ratio

PTH has dual activities in bone. Continuous PTH administration results in enhanced bone resorption. Intermittent PTH therapy, while having a net anabolic effect on bone, stimulates both bone formation and bone resorption.[34] Depending on the model used, disparate effects of PTH on OPG and RANKL synthesis as well as the OPG/RANKL ratio have been reported. In murine bone marrow cultures, PTH stimulates RANKL and inhibits OPG expression thereby adversely affecting the OPG/RANKL ratio.[117] The effect on the OPG/RANKL ratio was evident after only a few hours.[124,125] In a similar study using murine osteoblasts, no stimulatory effect of PTH on RANKL expression could be demonstrated.[125] As PTH also had no effect on OPG expression it did not affect the OPG/RANKL ratio in this model.[125] In rats, subcutaneous administration of a single injection of PTH induced a rapid and transient decrease in OPG mRNA expression in both metaphyseal and diaphyseal bone. Decreased OPG expression was evident by one hour and mRNA levels returned to baseline within 24 hours.[123] In addition, it was suggested that intermittent PTH treatment enhances osteoblast differentiation through an insulin growth factor I (IGF-I) dependent mechanism, while continuous PTH treatment enhances osteoclastogenesis through reciprocal increases in RANKL and decreases in OPG.[117]

In our model, PTH did not affect OPG secretion in either the MG-63 or MC3T3-E1 cell lines at the PTH concentration tested. The inability of PTH to stimulate OPG secretion in MC3T3-E1 cells has been confirmed by Suda *et al* (1999)[131] as well as Lee and Lorenzo (1999)[125] who demonstrated that PTH did not affect OPG mRNA expression in these cells. However, in our laboratory, in the MC3T3-E1 cell line PTH stimulated RANKL secretion significantly which resulted in a decreased OPG/RANKL ratio (Figure 7.6 and Figure 7.7). Our results confirm the work of others who demonstrated PTH-mediated stimulation of RANKL expression in murine bone marrow cells,[111,122] which was mainly dependent on the cAMP/PKA pathway.[122,123] As PTH did not enhance PGE_2 synthesis in the MC3T3-E1 cell line (refer to results from Chapter 4) stimulation of RANKL secretion by PTH is probably not PGE_2-mediated in this model.

7.4.4 Effects of oestrogen on osteoprotegerin and RANKL secretion

The molecular mechanisms of oestrogen action on bone are not completely understood. The principal effect on bone is a decrease in bone resorption.[103,105,380] Oestrogens regulate the function of osteoblasts and osteoclasts through high affinity oestrogen receptors (ER) located in the nucleus.[35] Recent reports suggested the involvement of OPG in the paracrine mediation of oestrogen effects on bone. *In vitro* oestrogen treatment dose- and time-dependently stimulates OPG secretion by human osteoblasts[103,105,370] and was highest in osteoblasts expressing the largest number of oestrogen receptors.[105] Oestrogen was also found to stimulate OPG mRNA expression and OPG protein secretion in mouse bone marrow stromal cells[103] and primary human osteoblasts specifically through activation of the ER-α.[103,104] Withdrawal of oestrogen after a 5-day pretreatment period, mimicking the event occurring *in vivo* at menopause, dramatically down-regulates the expression of OPG in mouse bone marrow stromal cells.[103] In contrast, results from Simonet *et al* (1997) have shown that administration of recombinant OPG inhibits OVX-associated bone loss in rats.[10]

Although a stimulatory effect of oestrogen on OPG synthesis has been demonstrated in various osteoblastic models, results from our study could not confirm this observation. In our study, oestrogen inhibited OPG secretion in both MG-63 and MC3T3-E1 cell lines significantly (Figure 7.4 and Figure 7.5). This observation was unexpected. Oestrogen mediates its effects via oestrogen receptors (ER) expressed in the nucleus.[35] We suspected that the cell lines we used might be ER-deficient and therefore used an immunocytochemistry protocol to test for the presence of the receptors. Compared to the MCF-7 human breast carcinoma cells, known to express high numbers of ER,[309,310] MG-63 cell line tested negatively for ER expression. This observation could explain the lack of response of this cell line to oestrogen treatment as reported in the preceding chapters. Exposure of primary human osteoblasts to high levels of oestrogen up-regulates ERα expression[104] but we were unable to demonstrate this effect in the MG-63 osteosarcoma-derived cell line. Although the presence of ER in the MC3T3-E1 cell line was not tested for, results from the current study suggest the absence of these receptors.

The commercial sRANKL ELISA protocol was not sensitive enough to detect any RANKL secreted into the culture media of oestrogen-exposed MC3T3-E1 cells. The OPG/RANKL ratio therefore could not be calculated in these conditions.

7.4.5 Conclusions

A large number of stimulators and inhibitors of osteoclast formation converge on the RANKL/RANK/OPG pathway, making this an appropriate target for therapeutic intervention.[14] A logical therapeutic approach would be to search for ways to either increase OPG formation or decrease RANKL formation by bone cells, tilting the scale towards decreased osteoclast formation and less bone resorption thereby protecting bone.

Some PUFAs have been shown to protect bone by demonstrating anti-resorptive properties.[26,27] Our study demonstrated that PUFAs could affect OPG secretion in MG-63 and MC3T3-E1 osteoblast-like cells. AA, one of the n-6 PUFAs, inhibited OPG secretion dose-dependently in both cell lines. Indomethacin pre-treatment prior to AA-exposure attenuated the inhibitory effect of AA especially in the MC3T3-E1 murine osteoblasts suggesting that the AA-induced inhibition of OPG could possibly be mediated via PGE_2 synthesis. Although the MC3T3-E1 cells secreted very low levels of RANKL into the cultured media, AA dose-dependently stimulated RANKL secretion in the MC3T3-E1 cells thereby affecting the OPG/RANKL ratio in a negative way, supporting various reports that AA and PGE_2 do cause bone resorption.

DHA suppressed OPG secretion but to a smaller extent than AA. This could however be due to endogenous PGE_2 production, as DHA itself is not a substrate for PGE_2 synthesis. No sRANKL could be detected after exposing the MC3T3-E1 cells to DHA. Since OPG was detected in the media and is known for its anti-resorptive properties, the absence of sRANKL could suggest that DHA could be protective to bone.

Exposure of both MG-63 and MC3T3-E1 cell lines to PTH did not affect OPG secretion, an observation confirmed by Lee *et al* (1999).[125] However, PTH enhanced

RANKL secretion in the MC3T3-E1 cells thereby decreasing the OPG/RANKL ratio. These results confirm that PTH may cause bone resorption in a dose-dependent manner. Although it has been documented that oestrogen stimulates OPG secretion in various bone marrow and osteoblastic cell cultures, our study did not confirm this observation. The MG-63 cell line however, tested negatively for oestrogen receptor expression, this finding could explain the lack of response to oestrogen in this model. Some of the variable actions of oestrogen on osteoblasts may be attributed to osteoblastic cell lines expressing different numbers oestrogen receptors as well as different ratios of REα and ERβ.[101]

The expression of OPG and RANKL has been shown to be developmentally regulated[377,378] and it has been hypothesised that undifferentiated marrow stromal cells with a high RANKL/OPG ratio can initiate and support osteoclastogenesis, while the mature osteoblastic phenotype, that mostly express OPG, acquire an osteogenic phenotype.[377,378] In our MC3T3-E1 model, secreted RANKL levels were very low and could not be detected in all the samples. Others have reported low levels of mRNA RANKL expression in this cell line,[124,125] which suggests that the MC3T3-E1 cell line might not be a suitable model for investigating RANKL modulation.