Differential effects of arachidonic acid and docosahexaenoic acid on cell biology and osteoprotegerin synthesis in osteoblast-like cells

by

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SUMMARY

The purpose of the study was to elucidate the mechanisms by which polyunsaturated fatty acids (PUFAs) prevent bone loss. MG-63 human osteoblasts and MC3T3-E1 murine osteoblasts were exposed to the n-6 PUFA arachidonic acid (AA) and the n-3 PUFA docosahexaenoic acid (DHA) as well as oestrogen (E2) and parathyroid hormone (PTH) and the effects thereof tested on a variety of biological parameters characteristic of osteoblasts. These parameters included prostaglandin E_2 (PGE_2) synthesis, proliferation, differentiation to mature mineralising osteoblasts as well as osteoprotegerin (OPG) and receptor activator of nuclear factor κB ligand (RANKL) secretion.

Results showed that AA stimulates PGE_2 production significantly in both cell lines. Stimulated PGE_2 production by MC3T3-E1 cells however, was significantly higher, which might be attributed to auto-amplification by PGE_2 itself in this cell line. Pre-incubation of the MG-63 cells with cyclo-oxygenase (COX)-blockers inhibited PGE_2 production significantly, suggesting that both COX enzymes were involved in PGE_2 synthesis.

The number of functional osteoblasts is important for bone formation therefore *in vitro* osteoblastic cell proliferation was investigated. In contrast to the hormones E2 and PTH, both AA and DHA inhibited proliferation significantly. The AA-mediated anti-proliferative effect is possibly independent of PGE_2 production, as PGE_2 *per se* had little effect on proliferation. DHA inhibited proliferation of MG-63 cells more severely, which might be attributed to the osteosarcoma nature of the MG-63 cells. The anti-proliferative effect of these PUFAs might be attributed to modulation of cell cycle progression or anti-mitotic effects of PUFA peroxidation products. Morphological studies showed apoptotic cells after DHA exposure in MG-63 cells.

There is a reciprocal relationship between reduced proliferation and the subsequent induction of cell differentiation *in vitro*. High basal levels of alkaline phosphatase (ALP) activity, a marker of the mature mineralising osteoblastic phenotype, were detected in MC3T3-E1 cells. Long-term exposure to AA inhibited ALP activity in these cells. This process might be PGE_2-mediated. Exposure to PUFAs, however, did not compromise the ability of the MC3T3-E1 cells to differentiate to mature mineralising osteoblasts.

In contrast with MC3T3-E1 cells, MG-63 cells demonstrated low basal ALP activity and were unable to differentiate to mature mineralising osteoblasts. In the absence of osteogenic-inducing supplements, PUFAs induced adipocyte-like features that might be due to the expression of high levels of PPARγ in this cell line. Lipid-filled vacuoles were absent in the MC3T3-E1 cells suggesting that the MC3T3-E1 cell line may not express PPARγ mRNA.

The study furthermore demonstrated that PUFAs are able to modulate OPG and RANKL secretion in osteoblasts. AA inhibited OPG secretion dose-dependently in both cell lines, this could be PGE_2-mediated. AA dose-dependently stimulated soluble RANKL (sRANKL) secretion in 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. No sRANKL could be detected after exposing the MC3T3-E1 cells to DHA suggesting that DHA could be protective to bone.

In conclusion, contrary to *in vivo* evidence, this *in vitro* study could not indisputably demonstrate protective effects of PUFAs on the osteoblastic cell lines tested.

KEY WORDS:

Osteoblasts, polyunsaturated fatty acids, arachidonic acid, docosahexaenoic acid, prostaglandin E_2, proliferation, differentiation, alkaline phosphatase activity, mineralisation, transdifferentiation, osteoprotegerin (OPG), receptor activator of nuclear factor κB ligand (RANKL).

OPSOMMING

Die doel van die studie was om die meganismes waardeur poli-onversadigde vetsure (POVS) beenverlies voorkom te verklaar. MG-63 menslike osteoblaste en MC3T3-E1 muis-osteoblaste is blootgestel aan die n-6 POVS aragidoonsuur (AS) en die n-3 POVS dokosaheksaenoësuur (DHS) sowel as estrogeen (E2) en paratiroïedhormoon (PTH) en die effekte daarvan op 'n verskeidenheid biologiese parameters kenmerkend aan osteoblaste getoets. Hierdie parameters sluit in prostaglandien E_2 (PGE_2) sintese, proliferasie, differensiasie na volwasse mineraliserende osteoblaste sowel as osteoprotegerien (OPG) en reseptor aktiveerder van nukluêre faktor κB ligand (RANKL) sekresie.

AS het PGE_2-produksie in beide sellyne betekenisvol gestimuleer. Gestimuleerde PGE_2-produksie was aansienlik hoër by die MC3T3-E1-selle wat moontlik toegeskryf kan word aan outoversterking deur PGE_2 in hierdie sellyn. Voorafblootstelling van die MG-63-selle aan sikloöksegenase (SO)-blokkers het PGE_2-produksie betekenisvol geïnhibeer, wat op die betrokkenheid van beide SO-ensieme by PGE_2-sintese kan dui.

Aangesien die aantal funksionele osteoblaste belangrik vir beenvorming is, is die *in vitro* proliferasie van osteoblaste bestudeer. In kontras met die hormone E2 en PTH, het beide AS en DHS proliferasie betekenisvol geïnhibeer. Die inhiberende effek van AS op selproliferasie is waarskynlik onafhanklik van PGE_2-produksie, aangesien PGE_2 op sigself min effek op selproliferasie gehad het. DHS het proliferasie van MG-63-selle meer geïnhibeer as dié van die MC3T3-E1-selle, wat moontlik aan die tumorigeniese aard van die MG-63-selle toegeskryf kan word. Die anti-proliferatiewe effekte van POVS kan moontlik aan modulering van selsiklusprogressie, of andersins aan antimitotiese effekte van POVS-peroksidasieprodukte toegeskryf word. Morfologiese studies het die teenwoordigheid van apoptotiese selle na DHS-blootstelling by MG-63-selle aangetoon.

Daar bestaan 'n omgekeerde verwantskap tussen 'n afname in proliferasie en die daaropeenvolgende induksie van seldifferensiasie *in vitro*. Hoë basaalvlakke van alkaliese fosfatase (ALF)-aktiwiteit, 'n merker vir die volwasse mineraliserende osteoblastiese fenotipe, is by die MC3T3-E1-selle waargeneem. Langdurige blootstelling aan AS het ALF-aktiwiteit in hierdie selle geïnhibeer, wat moontlik PGE_2-gemedieerd kan wees. Die vermoë van die MC3T3-E1-selle om na volwasse

mineraliserende osteoblaste te differensieer, is egter nie deur blootstelling aan POVS benadeel nie.

In teenstelling met die MC3T3-E1-selle het die MG-63-selle lae basaalvlakke vir ALF-aktiwiteit getoon en hulle was nie in staat om na na volwasse mineraliserende osteoblaste te differensieer nie. In die afwesigheid van osteogenese-induserende supplemente het POVS adiposiet-agtige eienskappe geïnduseer, wat moontlik aan die uitdrukking van hoë PPARγ-vlakke in hierdie selle toegeskryf kan word. Die afwesigheid van lipiedvakuole by die MC3T3-E1-selle dui daarop dat hierdie sellyn moontlik nie PPARγ bRNS uitdruk nie.

Die studie het verder getoon dat POVS daartoe in staat is om OPG en RANKL-sekresie in osteoblaste te moduleer. AS het OPG-sekresie in beide sellyne op ‘n dosisafhanklik wyse geïnhibeer wat moontlik PGE_2-gemedieerd kan wees. AS het verder op ‘n dosisafhanklike wyse die sekresie van oplosbare RANKL (oRANKL) in MC3T3-E1-selle gestimuleer en dus die OPG/RANKL verhouding negatief beïnvloed. Hierdie bevinding ondersteun verslae dat AS en PGE_2 beenresorpsie kan veroorsaak. Geen oRANKL is na DHS-blootstelling aan MC3T3-E1-selle waargeneem nie wat daarop kan dui dat DHS moontlik beskerming aan been kan bied.

Opsommend, in teenstelling met *in vivo* studies, kon hierdie *in vitro* studie nie bo alle twyfel beskermende effekte van POVS op die osteoblastiese sellyne soos getoets, aantoon nie.

SLEUTELWOORDE:

Osteoblaste, poli-onversadigde vetsure, aragidoonsuur, dokosaheksaenoësuur, prostaglandien E_2, proliferasie, differensiasie, alkaliese fosfatase-aktiwiteit, mineralisasie, transdifferensiasie, osteoprotegerien (OPG), reseptor aktiveerder van nukluêre faktor κB ligand (RANKL).

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AA	arachidonic acid (C20,5c,8c,11c,14c-20:4)[n-6]
ALA	α-linolenic acid (C18,9c,12c,15c-18:3)[n-3]
ALP	alkaline phosphatase
α-MEM	alpha modification of Eagle's minimal essential medium
ANOVA	analysis of variance
β-GP	β-glycerophosphate
BMP	bone morphogenetic protein
BMP-2	bone morphogenetic protein-2
BSA	bovine serum albumine
BSS	balanced salt solution
caspases	cysteinyl aspartate-specific proteases
cAMP	cyclic AMP
Cbfa1	core binding factor α-1
cdks	cyclin-dependent kinases
CLA	conjugated linoleic acid
COX	cyclo-oxygenase
COX-1	cyclo-oxygenase-1
COX-2	cyclo-oxygenase-2
Col1a1	type I collagen
cPGES	cytosolic prostaglandin E synthase
cPLA$_2$	cytosolic phospholipase A$_2$
DAG	diacylglycerol
ddH$_2$O	deionised distilled water
DGLA	dihomo-gamma-linolenic acid (C20,8c,11c,14c-20:3) [n-6]
DHA	docosahexaenoic acid (C22,4c,7c,10c,13c,16c,19c-22:6)[n-3]
DMEM	Dulbecco's modified Eagle's medium
DMSO	dimethylsulphoxide
E2	oestrogen (17β-estradiol)
EDTA	disodium ethylene diaminetetraacetate
EFAs	essential fatty acids
EGTA	ethylene glycol-bis[beta-aminoethyl ether]N,N,N$_1$,N$_1$-tetra-acetate
ELISA	enzyme-linked immunosorbent assay
EP	prostaglandin E$_2$ receptor
EPA	eicosapentaenoic acid (C20,5c,8c,11c,14c,17c-20:5)[n-3]
ER	oestrogen receptor
Erk	extracellular signal-regulated kinase
FCS	fetal calf serum

FGFs	fibroblast growth factors
FITC	fluoroisothiocyanate
GLA	gamma linolenic acid (C18,6c,9c,12c-18:3)[n-6]
GPCRs	G protein-coupled receptors
H^+-ATPase	proton ATPase
H&E	Haematoxylin and eosin
hBMSc	human bone marrow stroma cells
hFOB	human fetal osteoblastic cell line
HOE	Hoechst no 33342
HOE/PI	Hoechst no 33342 and propidium iodide
IGFs	insulin-like growth factors
IGF-1	insulin-like growth factor-1
IGFBPs	insulin-like growth factor binding proteins
IL	interleukin
Indo	indomethacin
JNK	c-jun N-terminal protein kinase
LA	linoleic acid (C18,9c,12c-18:2)[n-6]
LO	lipoxygenase
MAP	mitogen-activated protein
MAPK	mitogen-activated protein kinase
MC3T3-E1	mouse calvaria osteoblast-like cell line
MCF-7	human breast carcinoma cell line
M-CSF	macrophage-colony stimulating factor
MEM	minimum essential medium with Earle's salts
MG-63	human osteoblast-like osteosarcoma-derived cells
mPGES	membrane-associated prostaglandin E synthase
mRNA	messenger RNA
MSCs	mesenchymal stem cells
n-3	omega-3. Family of polyenoic fatty acids with 3 or more cis-unsaturated centres separated by methylene groups and having first unsaturated center 3C from the methyl terminal.
n-6	omega-6. Family of polyenoic fatty acids with 2 or more cis-unsaturated centres separated by methylene groups and having first unsaturated center 6C from the methyl terminal.
N.D.	not detected
NF$\kappa\beta$	nuclear factor $\kappa\beta$
NSAIDS	nonsteroidal anti-inflammatory drugs
O.D.	optical density
OPG	osteoprotegerin
OPGL	osteoprotegerin ligand (RANKL)
Osx	osterix

OVX	ovariectomised
PBS	phosphate-buffered saline
PDGF	platelet-derived growth factor
PGs	prostaglandins
PGE_2	prostaglandin E_2
PGG_2	prostaglandin endoperoxide G_2
PGH_2	prostaglandin endoperoxide H_2
PGHS-1	prostaglandin endoperoxide synthase-1
PGHS-2	prostaglandin endoperoxide synthase-2
PGI_2	prostacyclin
PI	propidium iodide
PKA	protein kinase A
PKC	protein kinase C
PLA_2	phospholipase A_2
ρ-NP	para-nitrophenol
ρ-NPP	para-nitrophenylphosphate
PPAR	peroxisome proliferator activated receptor
PTH	parathyroid hormone
PTHrP	parathyroid hormone related peptide
PUFA	polyunsaturated fatty acid
PUFAs	polyunsaturated fatty acids
RANK	receptor activator of nuclear factor-κβ
RANKL	receptor activator of nuclear factor-κβ-ligand
RIA	radioimmunoassay
rpm	revolutions per minute
RXR	retinoid X receptor
SDF-1	stromal cell-derived factor-1
sRANKL	soluble secreted RANKL
SREBP	sterol regulating element binding protein
TBS	tris-buffered saline
TGF-β	transforming growth factor-β
TMB	3'3',5',5' tetramethylbenzidine
TNF	tumor necrosis factor
TNFα	tumor necrosis factor-α
TRAF-6	TNF receptor-associated factor-6
TRAP	tartrate-resistant acid phosphatase
UV	ultra violet
v/v	volume per volume
vit D_3	1,25-Dihydroxy vitamin D_3 (calcitriol)
w/v	weight per volume

CHAPTER 1

General Introduction

Motivation for the study

Osteoporosis, the most commonly occurring bone disease, is the leading cause of serious morbidity and functional loss in the elderly.[1] This disease is considered a major public health problem in the Western World and the prevalence thereof is increasing in the developing world. Statistics for the prevalence of osteoporosis in South Africa are not available. However, it is predicted that in the developed world one in three women and one in eight men over the age of 50 will suffer an osteoporotic fracture during their lifetime.[2] Although osteoporosis is generally considered a modern disease, forensic evidence shows that bone loss with age already occurred in ancient communities such as the Egyptians dating back to the XIIth Dynasty (1990-1786 B.C.).[3] Various factors have been identified as contributing to osteoporosis, including genetic factors; aging; oestrogen deficiency; low body mass; smoking; physical inactivity; medications such as glucocorticoids; and malnutrition.[4] Among dietary factors, heavy alcohol consumption, low energy intake, low intake of calcium and vitamin D and high protein consumption have been listed.[2,5]

The mature human skeleton is a metabolically active organ that is continuously resorbed and rebuilt by osteoclasts and osteoblasts. Osteoclasts and osteoblasts work together in a synchronised manner, such that bone resorption and formation are closely coupled, this results in the maintenance of a constant level of bone mass. Disruption of this coordination underlies many bone diseases, including osteoporosis.[6] Differentiation of osteoclasts is closely coupled with the function of osteoblasts through a variety of cytokines.[7] Some of the proteins involved in the interaction between cells of osteoblastic and osteoclastic lineage have been identified. These proteins belong to the families of tumor necrosis factors and receptors[8-12] and include *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). When RANKL, expressed on the osteoblast cell membrane, binds to its natural receptor RANK, present on the osteoclast progenitor membrane, osteoclastic differentiation and activation is initiated.[13] OPG is a soluble decoy receptor secreted by osteoblasts that competes with RANKL for binding to RANK, thereby preventing its osteoclastogenic effect.[10-11] The presence of OPG in the bone microenvironment thus limits the number of mature osteoclasts and therefore could have a determining effect on resorption rate and bone mass. The discovery of the OPG/RANKL/RANK system provides a completely new perspective on bone biology. A large number of stimulators and inhibitors of bone resorption, such as cytokines and hormones, have been shown to converge on the RANKL/RANK/OPG pathway, making this an appropriate target for therapeutic intervention.[14]

It is preferable to prevent osteoporosis rather than having to treat it. The bone mass attained early in life may be the most important determinant of skeletal health in later life.[15-16] A balanced diet, amongst others, is considered to be of utmost importance in the prevention of osteoporosis. Adequate consumption of specific nutrients, especially calcium and vitamin D in early life will optimise peak bone mass, and adequate intakes of these nutrients should continue through the remainder of life to maintain bone mass.[15,17]

Polyunsaturated fatty acids (PUFAs) of the n-6 and n-3 series are essential for life and health, they cannot be produced by animals and they (or some suitable precursor) must be obtained from plant or animal sources as part of the diet. PUFAs, especially the omega-3 (n-3) PUFAs such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) present in fish oil, are of paramount importance for health and disease prevention. The n-3 PUFAs have been shown to be beneficial in the prevention and treatment of a variety of medical conditions such as cardiovascular diseases, neurological disorders, inflammatory diseases, some cancers and rheumatoid arthritis.[18-20] During the past two decades, the effects of dietary long chain PUFAs on bone health received considerable attention.[16,21,22]

There is increasing evidence that lack of certain dietary PUFAs contribute to bone loss.[21,23-24] On the other hand, dietary supplementation of some PUFAs has been shown to be beneficial for bone.[25-28] Animal studies suggest that the n-3 PUFAs

EPA and DHA could reduce the risk of osteoporosis and fractures.[25,29] Human studies confirmed the beneficial effects of dietary PUFA supplementation. A controlled clinical study, for instance, found that supplementation of calcium, γ-linolenic acid and EPA in the diet of elderly women enhance calcium absorption, reduce calcium excretion, and have overall positive effects on bone mineral density.[27] It has also been shown that a reduction of the n-6/n-3 PUFA ratio could result in increased bone strength in animals[30-31] and in humans.[32] Considering these results, it may be possible that the consumption of diets rich in n-3 fatty acids could help to build and maintain a healthy skeleton in the human. As these products are part of the diet, they could be conveniently delivered as dietary supplements to a large population at an affordable cost.

Although results from clinical trials and *in vivo* animal studies suggest that specific PUFAs might benefit bone health, the cellular mechanisms of different PUFAs have not been clarified and need to be investigated. Changes in dietary PUFAs are reflected in the composition of various tissues, including bone cells such as the osteoblasts.[20,33] The cellular presence of specific PUFAs, therefore, could affect osteoblastic functioning via modulation of the synthesis of fatty acid products (e.g. prostaglandins), proliferation, differentiation and synthesis of proteins e.g. RANKL and OPG.

Purpose of the study

To elucidate the mechanisms by which PUFAs prevent bone loss by comparing the mechanism of action on osteoblasts of oestrogen, an anti-resorptive hormone and the action of parathyroid hormone, a pro-formation hormone in low concentrations, with PUFAs representative of the n-3 and n-6 polyunsaturated fatty acid families.

Method of investigation

An experimental study was conducted in which osteoblast-like cells (MG-63 human osteosarcoma cell line and MC3T3-E1 murine osteoblasts) in culture were exposed to PUFAs representative of the n-3 and n-6 polyunsaturated fatty acid families, and bone active hormones. The effects of these agents were tested on a variety of biological parameters characteristic of osteoblasts, e.g., prostaglandin E_2 (PGE_2) synthesis, proliferation, differentiation to mature mineralising osteoblasts as well as OPG and RANKL secretion.

Objectives

The objective of the study was to investigate whether arachidonic acid (AA) (representative of the n-6 PUFAs) and docosahexaenoic acid (DHA) (representative of the n-3 PUFAs), both of which have been shown to have *in vivo* effects on bone,[25,29,30,32] have differential effects on osteoblast-like cells *in vitro*. Experiments were conducted to determine how these PUFAs affected the following osteoblastic functions:

1. ***PGE_2 secretion***

   AA is the natural substrate for PGE_2 synthesis in many cell types including osteoblasts. DHA is not a substrate for prostaglandin synthesis but could interfere with PGE_2 synthesis.

2. ***Proliferation***

   PUFAs as well as their metabolites e.g. prostaglandins and oxidation products may be implicated in osteoblastic cell proliferation.

3. ***Differentiation to mature mineralising osteoblasts***

   Osteoblasts differentiate to mature mineralising osteoblasts when stimulated by osteogenic agents. PUFAs and their metabolites e.g. prostaglandins may affect mineralising properties of osteoblasts.

4. OPG and RANKL synthesis

Most pro-and anti-osteoclastogenic cytokines act primarily through the osteoblast to alter levels of RANKL and OPG; the relative balance of the latter determines overall osteoclast formation. It has been shown that PGE_2 inhibits OPG synthesis and stimulates expression of mRNA for RANKL. PUFAs may thus affect the OPG/RANKL ratio via manipulation of PGE_2.

CHAPTER 2

Literature Review

I Bone Homeostasis

2.1 Introduction

The mature skeleton is a metabolically active organ that undergoes continuous remodeling by a process that replaces old bone with new bone. Remodeling is necessary to maintain the structural integrity of the skeleton and to serve its metabolic functions as a store of calcium and phosphorus. This dual function often comes into conflict under conditions of changing mechanical forces or of nutritional and metabolic stress.[6] Osteoblasts that are responsible for bone formation originate from bone marrow stromal precursor cells that then differentiate into mature osteoblasts. Osteoclasts that are responsible for bone resorption originate from haematopoietic stem cells known as monocytes.[6]

The remodeling cycle is finely regulated by a variety of systemic and local factors e.g. oestrogen (E2), parathyroid hormone (PTH), $1,25(OH)_2D_3$ (vit D_3), growth factors and cytokines.[6,34-35] Bone formation and resorption are usually balanced and a constant level of bone mass is maintained. An imbalance between bone formation and resorption causes metabolic diseases such as osteoporosis and osteopetrosis (a family of diseases characterised by increased bone mass due to decreased bone resorption).[6]

2.2 Composition of bone

Bone tissue has three components: an inorganic bone mineral component, an organic matrix, and bone cells. In mature bone, inorganic bone mineral is deposited on a framework of organic support material known as osteoid. The mineral fraction of bone consists of calcium phosphate in the form of hydroxyapatite crystals

(Ca_{10} $(PO_4)_6(OH)_2$), and is responsible for about half of the bone mass. The organic matrix of bone contains 95% collagen. The remaining 5% of noncollagen organic matter, also known as ground substance, consists of a mixture of various proteoglycans, high-molecular-weight compounds comprised of carbohydrate and protein. The combination of organic and inorganic materials is responsible for bone's mechanical strength.[36]

Mature bone comprises two basic bone types, cortical or compact bone and cancellous or trabecular bone. Cortical bone makes up the shafts of long bones as well as the outer envelope of all bones. It has mainly supporting, protective and mechanical functions in the body. Trabecular bone, on the other hand, has a honeycomb structure well suited for a site for bone-forming cells and a large surface area that provides a reservoir for minerals. Trabecular bone is found in the inner parts of the vertebrae and pelvis and the ends of the long bones.[37] Figure 2.1 depicts the basic bone types as seen with an electron microscope.[38]

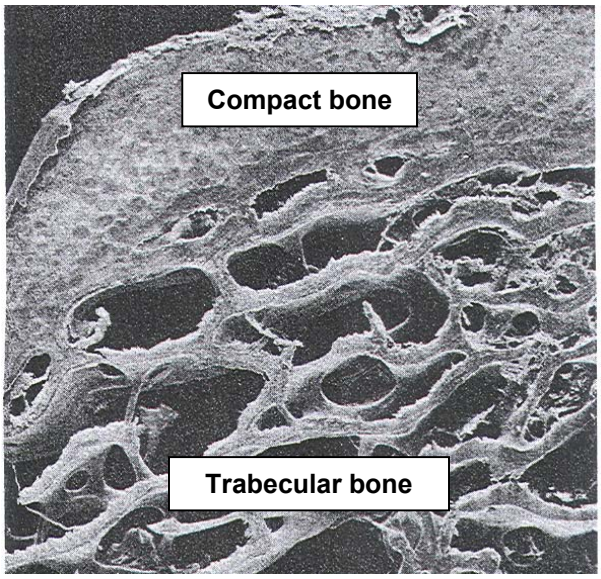


Figure 2.1 A scanning electron micrograph of compact and trabecular bone.
(Reprinted from: Moffet DE, Moffet B, Schauf CL. Human Physiology: Foundations and Frontiers. 2nd ed. St Louis: Mosby-Year Book, Inc; 1993. p. 577[38]) Copyright (1993), with permission from Elsevier.

2.3 Bone cells

There are three types of bone cells, osteoblasts, osteocytes, and osteoclasts. Osteoblasts, located side by side on the surface of bone, synthesise osteoid (organic matrix) and are therefore responsible for bone formation. As the deposition of osteoid and bone mineralisation continues, osteoblasts become surrounded by mineralised bone. At this stage, osteoblasts progressively lose their bone-forming capability and are termed osteocytes. Osteoclasts are multi-nucleated cells that break down osteoid by a process known as bone resorption.[36] Figure 2.2 depicts the location of the different types of bone cells.[39]

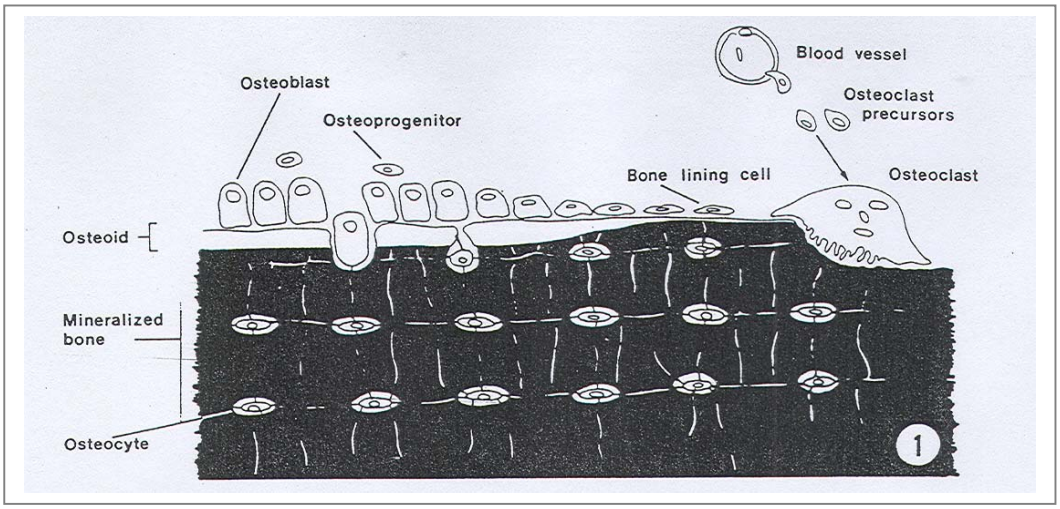


Figure 2.2 The origins and locations of bone cells.

(Marks SC, Popoff SN. Bone cell biology: The regulation of development, structure, and function in the skeleton. Am J Anat 1988;183:1-44)[39] ©1988 (Wiley-Liss, Inc., A Wiley Company) Reproduced with permission of John Wiley & sons, Inc.

2.3.1 Osteoblasts

Osteoblasts actively producing osteoid are cuboid-shaped and exhibit an abundant endoplasmic reticulum and Golgi complex, characteristic of cells synthesising proteins for export. Osteoblasts are responsible for synthesising, secreting, organising, and mineralising the bone matrix, or osteoid. They also produce a variety of regulatory factors including prostaglandins, cytokines and growth factors, some of which are incorporated into the developing matrix.[40] Mature osteoblasts are rich in

alkaline phosphatase that is believed to play an important role during mineralisation.[41]

Osteoblasts, not actively engaged in bone formation have a flatter appearance. Osteoblasts have numerous cytoplasmic processes that bring them into contact with neighbouring osteoblasts.[36] Once the osteoblast has differentiated and completed its cycle of matrix synthesis, it can either become a flattened lining cell on the bone surface, be buried in bone as an osteocyte, or undergo programmed cell death (apoptosis).[6,42]

2.3.1.1 Origin of osteoblasts

Bone marrow contains hematopoietic precursors, their progeny, and stromal cells. Stromal cells include adipocytes, fibroblastic cells, endothelial cells, and mesenchymal stem cells (MSCs). Mesenchymal stem cells are pluripotent and able to differentiate into several distinct cell types, including osteoblasts, adipocytes, fibroblasts and myoblasts.[43-44] The osteoblastic differentiation pathway from mesenchymal stem cells is illustrated in figure 2.3.[6]

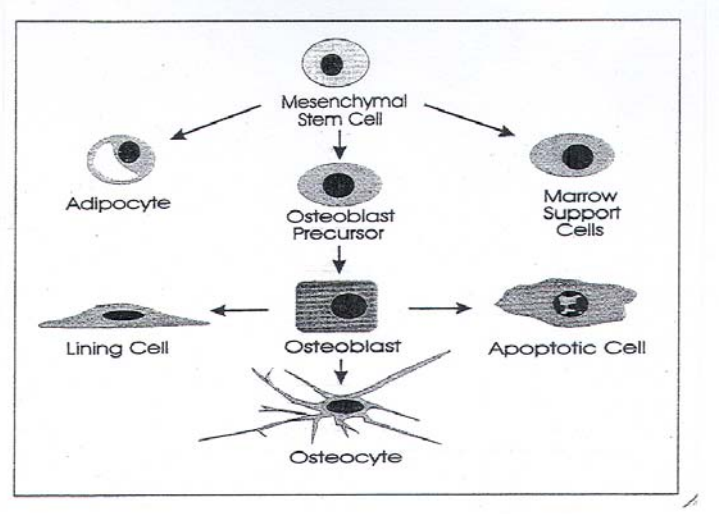


Figure 2.3 Origin and fate of osteoblasts.

The mesenchymal stem cell that gives rise to osteoblasts can also produce cells of other lineages. It is also possible that osteoblast precursors can differentiate into, or derive from adipocytes and marrow support cells. Osteoblasts can be buried as osteocytes, remain in the bone surface as lining cells, or undergo apoptosis. *(Reprinted with permission from Raisz LG. Physiology and pathophysiology of bone remodeling. Clin Chem 1999;45:1353-8)*[6]

2.3.1.2 Transcriptional control of osteoblast differentiation

The commitment of a mesenchymal stem cell to the osteoblastic lineage is regulated by specific transcription factors. Activated transcription factors bind to nuclear DNA and induce the expression of a new set of genes which ultimately change the characteristics of that cell.[45] Core Binding factor α-1 (Cbfa1, also known as Runx-2) is the earliest and most specific marker of osteoblastogenesis.[46-47] In addition to its critical role during osteoblast differentiation, Cbfa1 controls bone formation by differentiated osteoblasts.[46-49] Another transcription factor called Osterix (Osx), acting downstream from Runx/Cbfa1 was recently identified.[49] Figure 2.4 shows a model of the osteoblast differentiation pathway as regulated by transcriptional factors.[49]

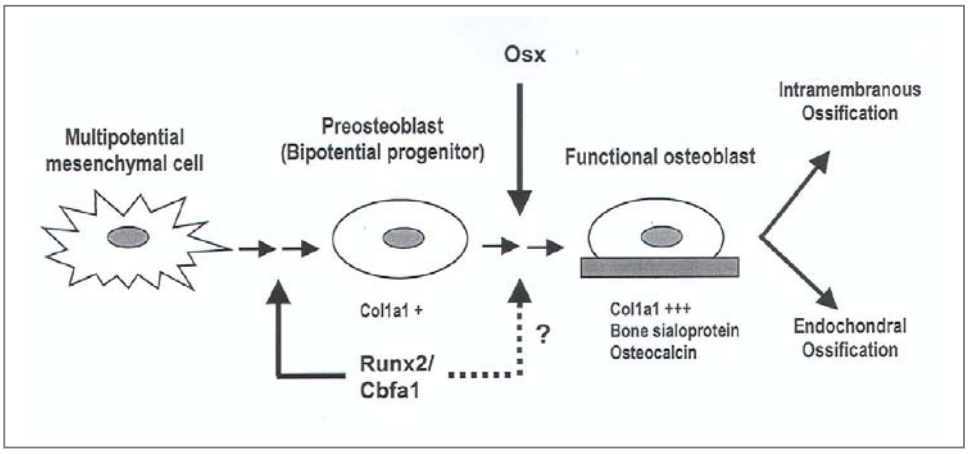


Figure 2.4 Model of the osteoblast differentiation pathway.

Multipotential mesenchymal progenitors first differentiate into preosteoblasts, a process for which Runx2/Cbfa1 is needed. These preosteoblasts are still bipotential; i.e. they have the potential to differentiate into both osteoblasts and chondrocytes. Preosteoblasts do not express osteoblast marker genes, except low levels of Col1a1 typical of mesenchymal cells. Preosteoblasts then differentiate into functional osteoblasts expressing high levels of osteoblast marker genes. This process requires Osx. *(Reprinted from: Nakashima K, Zhou X, Kunkel G, Zhang Z, Deng JM, Behringer RR, et al. The novel zinc finger-containing transcription factor Osterix is required for osteoblast differentiation and bone formation. Cell 2002;108:17-29.[49] Copyright (2002), with permission from Elsevier.)*

The differentiation potential of osteoblastic precursors is not yet fully understood. One theory is that multipotent bone marrow progenitor cells could differentiate into various phenotypes.[50] Plasticity between cell types has also been reported. For a cell to be converted from one cell type to another there must be suppression of differentiation of the original cell type with promotion of differentiation to the new

type. For example, it has been shown that both adipocytes and preadipocytes obtained from murine bone stromal cells express a number of osteoblastic markers.[51] Skillington *et al* (2002) proved that pre-adipocytes could be converted into fully differentiated osteoblasts in response to bone morphogenetic protein-2 (BMP-2) and retinoic acid signaling.[52] This observation is consistent with the hypothesis that a single progenitor cell gives rise to both adipocytes and osteoblasts and that conversion between these lineages, in response to exogenous growth factors, is possible.[51]

The early step in the commitment of a mesenchymal stem cell to the osteoblastic or the adipocytic lineage depends on activation and expression of Cbfa1, necessary for osteogenesis, and the peroxisome proliferator-activated receptor γ2 (PPAR-γ2) necessary for adipocytic differentiation respectively.[46,53] PPARγ is a member of the nuclear receptor family of transcription factors, a large and diverse group of proteins that mediate ligand-dependent transcriptional activation or repression.[54] The diverse spectrum of activities appears due to cell specificity of PPARγ function and the nature of the ligand.[50,55] The basic action of the nuclear hormone receptors such as PPAR is illustrated in figure 2.5.[54]

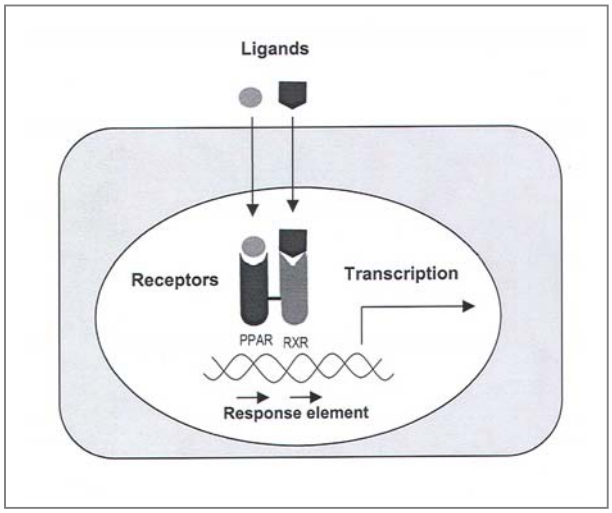


Figure 2.5 Basic action of nuclear hormone receptors.

Nuclear hormone receptors bind to a specific sequence in the promoter of target genes (called hormone response elements), and activate transcription upon binding of ligand. Several nuclear hormone receptors, including PPARγ, can bind to DNA only as a heterodimer with the retinoid X receptor (RXR), as shown. *(Kersten S, Desvergne B, Wahli W. Roles of PPARs in health and disease. Nature 2000;405:421-4).*[54]

With ageing and osteoporosis there is a decrease in osteoprogenitor cells with an accompanying increase in adipocytes in bone marrow.[44] This is due to altered differentiation of the common mesenchymal stem cell.[44,56-58] It has been shown that cells cultured from human trabecular bone are not only osteogenic, but able to undergo differentiation to adipocytes when treated with long chain fatty acids. The formation of differentiated adipocytes was dependent on increased expression of PPARγ2.[57] *In vitro* studies have shown that overexpression of PPAR-γ reduces osteoblastic differentiation of murine MSCs by inhibition of Cbfa1 expression.[59] PPARγ deficiency on the other hand, results in enhanced bone formation with increased osteoblastogenesis from bone marrow progenitors.[60] The pathway to adipocytes is of great importance since cells that have the potential to form osteoblasts can be diverted into the adipocytic lineage and are then no longer available for bone formation. Factors driving MSCs to differentiate down the two lineages therefore play important roles in determining bone density.

Although there is sufficient evidence for the theory that multipotent bone marrow progenitor cells could differentiate into various phenotypes such as osteoblasts and adipocytes, others differ. Bellows and Heersche (2001) for instance, reported that in fetal rat calvaria, the large majority of osteoprogenitors are committed and restricted to the osteoblastic lineage and that the large majority of adipocyte progenitors are committed and restricted to the adipocytic lineage, whilst the common osteoblast/adipocyte progenitor is only present in low numbers. This common osteoblast/adipocyte progenitor is suggested to be the source of the clonal cell lines that have been described to possess both adipogenic and osteogenic potential.[61]

2.3.2 Osteocytes

As the deposition of osteoid and bone mineralisation continues, osteoblasts become surrounded by mineralised bone, progressively lose their bone-forming capability and become osteocytes. Osteocytes form cell networks and can transduce mechanical stimuli from the periphery to the centre of bone.[36] Osteocytes are critical for maintaining fluid flow through bone, and changes in this fluid may provide the signal for the cellular response to mechanical forces such as impact loading.[6]

2.3.3 Osteoclasts

Osteoclasts are multi-nucleated cells that break down osteoid by a process known as bone resorption.[36] Active multinucleated osteoclasts adjacent to bone have a ruffled appearance that increases surface area and allows the cell to perform the task of bone resorption effectively. When stimulated, osteoclasts secrete H^+ and proteolytic enzymes into the extracellular space adjacent to the bone. The acidic environment increases the solubility of bone mineral, while the proteolytic enzymes attack the organic matrix of bone. Together, these two factors promote the process of bone resorption.[36]

2.3.3.1 Origin of osteoclasts

Osteoclasts are derived from haematopoietic cells of the monocyte-macrophage lineage. These stem cell precursors first undergo a phase of *determination,* acquiring the potential to become either osteoclasts or macrophages. The transcription factor PU.1 is required for the commitment of myeloid precursors to macrophage and osteoclast precursors.[62] Macrophage-colony stimulating factor (M-CSF) secreted by osteoblasts, through its action on the *c-fms* receptor on early but already committed progenitors, promotes proliferation, differentiation and survival of these cells.[62] A phase of lineage-specific differentiation follows when the early response gene *c-fos* permits differentiation into the osteoclast-lineage and away from macrophages.[62]

Cell-to-cell contact between osteoblasts (or bone marrow stroma cells) and osteoclast precursors is required for osteoclastogenesis.[7] Recently 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. RANKL (Receptor activator of nuclear factor (NF)-κβ ligand), a protein expressed on the osteoblast cell membrane, binds to RANK (Receptor activator of NF-κβ) a receptor located on the osteoclast membrane.[8] This cell-to-cell interaction initiates a signaling cascade downstream of RANK/RANKL. RANKL interacts with RANK to recruit tumor necrosis factor (TNF) receptor-associated factor-6 (TRAF-6), a member of a family of TNF signal transducers. TRAF-6 binds to binding sites in the intracellular domain of the RANK

receptor and then signals downstream through several signaling cascades, most notably those involving NFκβ, mitogen-activated protein (MAP) kinases, extracellular signal-regulated kinase (Erk), Janus N-terminal kinase (JNK), and p38.[62-64] 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]

A number of molecular 'markers' of the mature osteoclast, each with important roles to play in osteoclast function, have been identified. These include tartrate-resistant acid phosphatase (TRAP) activity, calcitonin receptors (CTR), vitronectin receptors (αvβ3 subunits) carbonic anhydrase II, cathepsin K and vacuolar proton ATPase (H^+-ATPase). However, the singular defining feature of the active osteoclast is its ability to resorb bone.[67] Figure 2.6 depicts the differentiation pathway of osteoclast progenitors into functionally active osteoclasts.[68]

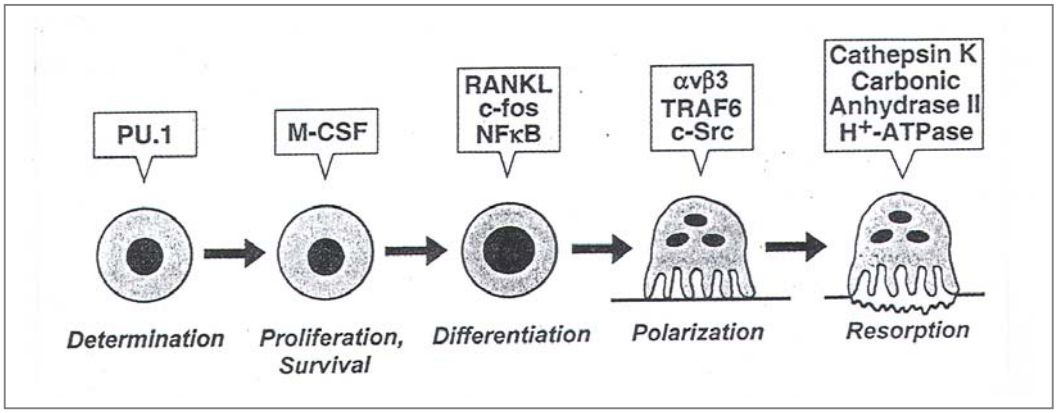


Figure 2.6 Differentiation of osteoclast progenitors into functionally active osteoclasts.

The transcription factors and cytokines required for each step of the pathway is indicated.

(Reprinted with permission from Teitelbaum SL. Bone resorption by osteoclasts. Science 2000;289:1504-8.[68]) Copyright (2000)AAAS.

Experimental data suggest that osteoclasts are not only derived from immature cells but also from mature cells of the monocyte-macrophage lineage when a suitable microenvironment is provided by bone marrow derived stromal cells.[69]

2.3.3.2 Regulation of osteoclast differentiation and activation by the osteoprotegerin-RANK-RANKL system

Cell-to-cell contact between osteoblasts (or bone marrow stroma cells) and osteoclast precursors is required for osteoclastogenesis.[7] When RANKL (expressed on the osteoblast cell membrane) binds to its receptor RANK (located on the osteoclast membrane) it not only stimulates osteoclastogenesis, but also activates these cells to become mature resorbing osteoclasts.[8] Macrophage-colony stimulating factor produced by osteoblastic cells, is also required for osteoclastic proliferation, differentiation and survival.[8,62]

Osteoprotegerin (OPG*),* a secreted member of the tumor necrosis factor receptor family, is produced by cells of the osteoblast lineage.[10] 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 acts as a *decoy* receptor in the RANK-RANKL signaling system inhibiting osteoclast formation.[10] Apart from inhibiting osteoclast formation, 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 has a determining effect on resorption rate and bone mass. The regulation of osteoclast differentiation and activation by the OPG-RANK-RANKL system is depicted in figure 2.7. [64]

Overexpression of the osteoblast-specific transcription factor Cbfa1 in human osteoblast-like osteosarcoma cells, results in a significant increase in the level of OPG secreted into the culture medium of these cells.[71] These results indicate that Cbfa1, in addition to its role in osteoblast differentiation and osteoblast maintenance, could also inhibit osteoclast formation and activity by stimulating OPG gene expression.

Tissues other than bone (e.g., lung, kidney, thyroid and endothelial cells) also produce OPG.[10,72-73] The physiological functions of OPG in these tissues are not clear yet. OPG is implicated as a potent survival factor for endothelial cells, thereby implicating OPG as a potential regulator of vascular homeostasis.[73]

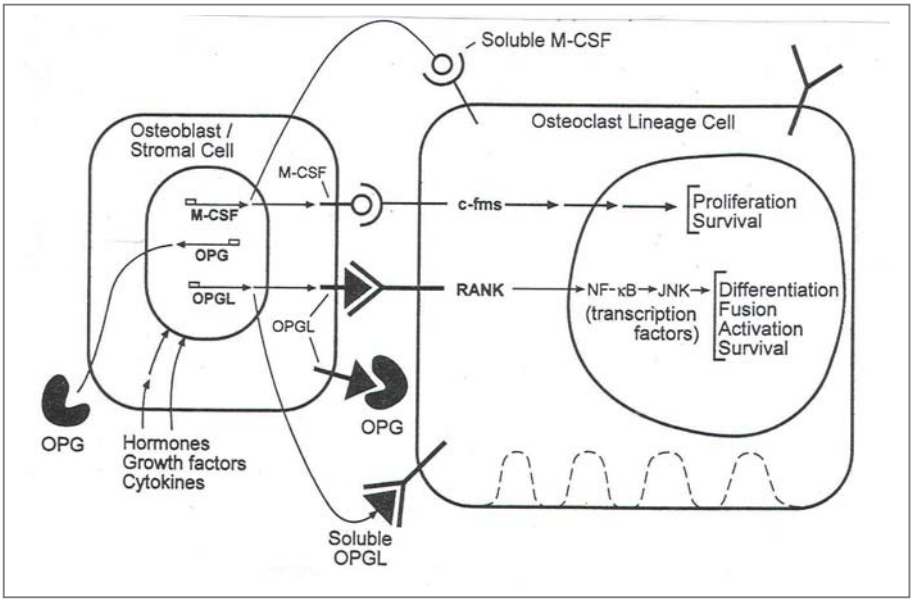


Figure 2.7 Interactions of osteoprotegerin, RANKL (OPGL) and RANK on the differentiation and activation of osteoclast precursors.

M-CSF and its receptor c-fms act as proliferation and survival factors for preosteoblasts. The subsequent differentiation of osteoclasts require RANKL (OPGL). Hormones, growth factors and cytokines, which direct the synthesis of membrane-bound (and secreted) OPGL and secreted OPG from osteoblasts and stromal cells, regulate bone resorption. The relative amounts of OPG and OPGL produced will dictate the osteoclast response. Binding of RANKL (OPGL) to RANK leads to activation of transcription factors. This binding results in the differentiation, fusion, and activation of osteoclasts. OPG serves as a secreted 'decoy' receptor, which opposes the RANKL-RANK interaction. *(Reproduced with permission from Kostenuik PJ, Shalhoub V. Osteoprotegerin: a physiological and pharmacological inhibitor of bone resorption. Curr Pharm Des 2001;7:613-35).*[64]

Local inflammation within bone due to metastasis, infections or fractures, or joint inflammation in arthritis, attracts T cells. It has been shown that activation of T cells might influence bone metabolism as these activated T cells produce RANKL, both in a membrane-bound and secreted form. It is therefore suggested that activated T cells could trigger osteoclastogenesis culminating in bone loss through RANKL.[66,74]

2.3.4 Cell proliferation and cell death

Since osteoblasts and osteoclasts have opposing effects on bone homeostasis, the number of functional osteoblasts and osteoclasts will affect bone's structure and strength. Cell number is dependent on the rate of replication and the number of cells dying through oncosis (toxic cell death) or apoptosis (programmed cell death).

Somatic cells alternate between periods of growth and division (mitosis). In the cell cycle the period of cell growth is known as interphase and is divided into three stages: **G_1**, a period of protein synthesis and organelle production; **S**, the period during which DNA is replicated in preparation for cell division; and **G_2**, a period of protein synthesis and final preparations for cell division. Cells can also enter from the G_1 phase in a rest phase, called the **G_0** phase. Cell replication takes place by means of mitosis (M phase) that is divided into prophase, metaphase, anaphase and telophase.[75] Stimulation of cell proliferation depends on the activity of the cell cycle. Cyclins and cyclin-dependent kinases (cdks) regulate cell cycle progression. Specific cyclin and cdk complexes are responsible for progression through each stage of the cell cycle.[76]

Cell death can either be caused by oncosis or apoptosis. Oncosis is a *passive* process that is induced by lethal chemical, biological or physical events resulting in cells being lysed or cell membranes ruptured with the resultant leakage of cytosol into the surroundings.[77] The escape of the cytosol releases kinins, lysosomal proteases and lipases into the tissue that stimulate inflammation.[78]

Apoptosis, or programmed cell death, is a biological process that eliminates unwanted cells. It therefore represents the *physiological* mode of cell death. The majority of nucleated cells appear to possess the genetic programming to undergo apoptosis.[79] Apoptosis is an *active* process which is controlled from within the cell by a large number of regulatory factors, but can be induced or inhibited by external factors through receptor-mediated mechanisms.[79-80] Wang *et al* (1999) defines apoptosis as "a gene-directed mechanism activated as a suicidal event to get rid of excess, damaged, or infected cells".[81] Apoptosis can be induced by activators such as tumor necrosis factor α (TNFα), oxidants, free radicals and bacterial toxins.[82] Presence or absence of specific growth factors, nutrients and hormones also affects

induction of apoptosis.[77,82] It has been shown that nitric oxide (NO) can promote apoptosis in some cells, whereas it inhibits apoptosis in other cells.[83]

Apoptosis is characterised by a sequence of morphologically recognisable events. Initially, an individual cell becomes detached from its neighbours and shrinks morphologically. Condensing of the chromatin and ruffling of the plasma membrane known as 'budding' then follows.[75,78,84] Cell fragments 'pinch off' as separate bodies (known as apoptotic bodies) that contain the condensed cytoplasmic proteins and intact organelles with nuclear fragments.[78,80] Adjacent cells recognise the apoptotic bodies and rapidly eliminate them through phagocytosis thereby avoiding an inflammatory response. Bratton and Henson (2005) suggested that oxidation of the dying cell's membrane lipids may provide important recognition signals to scavenger receptors on the phagocyte, thereby aiding phagocytosis of the apoptotic remnants.[85] The process of apoptosis occurrs quickly and cells undergoing this form of death disappear within hours without causing damage to surrounding cells or tissues.[78,80] Figure 2.8 shows the morphological events characteristic of apoptosis and oncosis.[84]

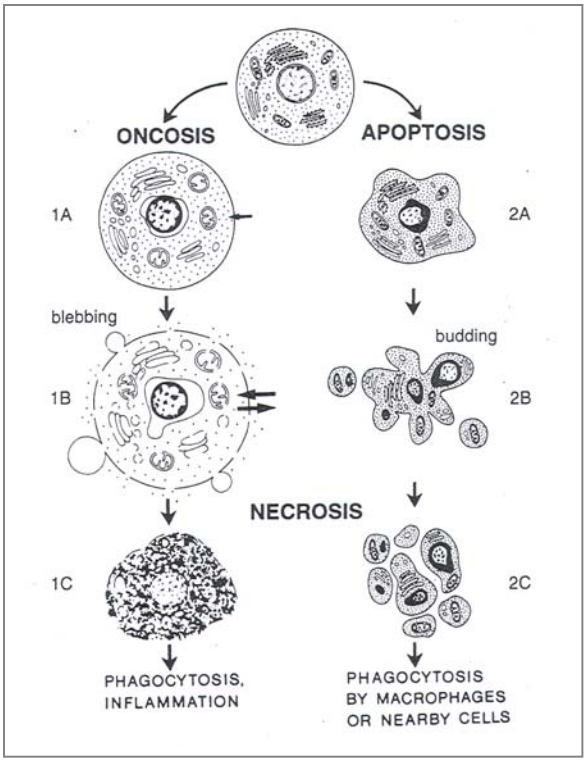


Figure 2.8 Illustration of the morphological events characteristic of apoptosis and oncosis. *(Majno G, Joris I. Apoptosis, oncosis, and oncosis. Am J Pathol 1995;146:3-15.)*[84]

Apoptosis is initiated via two main stimuli. The *extrinsic pathway* signals through a receptor-ligand mediated mechanism while the *intrinsic pathway* is triggered in response to DNA damage and is associated with the release of cytochrome c from the mitochondrial intermembrane space into the cytoplasm.[86] These mechanisms activate cysteine proteases (caspases) which are responsible for the characteristic morphological changes observed during apoptosis.[75] Two opposing mechanisms maintain homeostatic control of apoptosis. Fas ligand promotes cell death by binding to Fas, a cell membrane receptor, initiating a cascade of events leading to apoptosis. In contrast, the *Bcl-2* protein (a member of the *Bcl-2* family of pro- and anti-apoptotic mediators) prevents the release of cytochrome c from the mitochondrial membrane, thereby inhibiting mitochondrial-associated apoptosis.[78]

Jilka *et al* (1998) demonstrated that a large number of both human and murine osteoblasts undergo apoptosis and that growth factors and cytokines in the bone microenvironment can modulate this process *in vitro.*[42] It has been shown that transforming growth factor-β (TGF-β) as well as the cytokine interleukin-6 (IL-6) had antiapoptotic effects on osteoblasts as they were able to counteract the apoptotic effect of serum starvation.[42] Based on these results it was speculated that *in vivo* induction of apoptosis might be attributed to either an increased sensitivity to apoptosis-inducing agents or alternatively to decreased concentrations of anti-apoptotic growth factors and cytokines in the osteoblasts' immediate vicinity.[42] Apoptosis of osteoblasts might also be enhanced as a result of increased concentrations of pro-apoptotic factors such as tumor necrosis factor (TNF) or Fas ligand.[42] Lynch *et al* (1998) suggested that cell death by apoptosis is a fundamental component of osteoblast differentiation that contributes to maintaining tissue organisation.[87]

2.4 Bone remodeling

Bone is remodeled continuously during adulthood through the resorption of old bone by osteoclasts and the subsequent formation of new bone by osteoblasts. Bone remodeling, also known as bone turnover, takes place only on the surface of bone in closely coordinated local packets. As cancellous bone (trabecular bone) makes up

more than 80% of bone's surface, it is more metabolically active and more rapidly remodeled than cortical bone. [37]

Normally, bone remodeling proceeds in cycles in which osteoclasts adhere to bone and subsequently remove it by acidification and proteolytic digestion. Shortly after the osteoclasts have left, osteoblasts invade the area and begin the process of secreting osteoid thereby forming new bone, which is eventually mineralised. Thereafter, a distinct type of differentiated osteoblasts, the lining cells, cover the surface of the bone.[88]

The process of bone remodeling is coupled which means that bone formation is linked to bone resorption. The bone remodeling cycle consists of specific steps known as 1) activation, 2) resorption, 3) reversal, 4) formation and 5) mineralisation.[37] A schematic diagram of the remodeling cycle is depicted in Figure 2.9. [89]

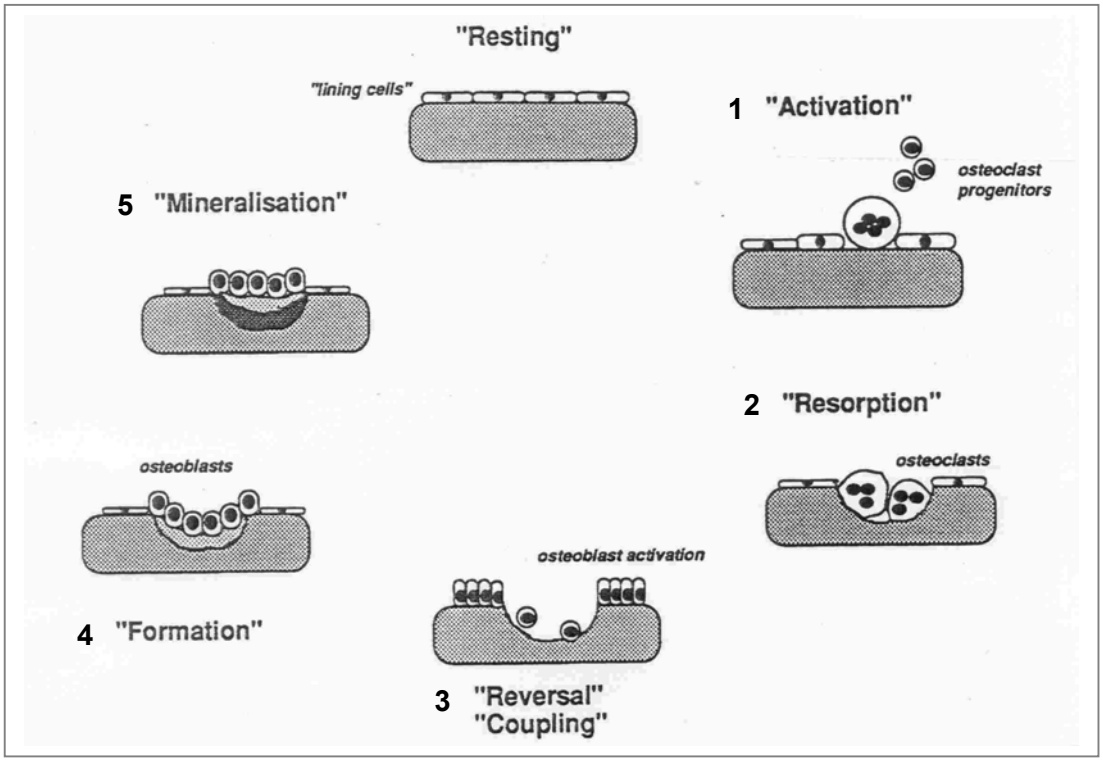


Figure 2.9 Schematic diagram of the bone remodeling cycle.
(Reprinted from Gowen M. Cytokines and cellular interactions in the control of bone remodelling. In: Heersche JNM, Kanis JA, editors. Bone and Mineral Research/8. Amsterdam Elsevier Science. 1994. p. 77-114.[89]) Copyright (1994), with permission from Elsevier.

2.4.1 Resorption

Bone resorption is a multistep process initiated by the proliferation of immature osteoclast precursors, the commitment of these cells to the osteoclast phenotype, and finally, degradation of the organic and inorganic components of bone. The cycle begins with recruitment of osteoclastic precursors from bone marrow monocyte precursors, which attach to the bone surface. Recent research[90] indicates that osteoclast precursors and mature osteoclasts have the capacity to modulate the activity of osteoblasts, and that, yet unknown membrane-bound signaling molecules are essential in inducing retraction of osteoblasts and the subsequent formation of a cell-free area for attachment of mature osteoclasts.

Osteoclast progenitors move from bone marrow to bone either through the circulation or by direct migration from the marrow. Signals targeting osteoclasts to bone and resorption sites are not well characterised and are currently under investigation. Small chemotactic cytokines (also known as chemokines), such as stromal cell-derived factor-1 (SDF-1) seem to be important in attracting osteoclastic precursors to resorption sites. SDF-1 is constitutively expressed at high levels within bone and is a ligand for its receptor CXCR4 that is constitutively expressed on circulating monocytes and pre-osteoclasts.[91]

After activation by the RANK-RANKL mechanism (Refer to Figure 2.7), the differentiated osteoclast polarises on the bone surface. Close physical contact between matrix and osteoclast is required for resorption, and it appears that the recognition of bone by osteoclasts is controlled by transmembrane integrins such as $\alpha v ß 3$ integrin. A ruffled border develops beneath the osteoclast, sealing the space beneath the cell. The osteoclast generates H^+, lactate, and proteolytic enzymes into this subcellular space, which cause a breakdown of the protein matrix and release calcium and other bone mineral constituents.[37,68,92] A recent publication[93] suggests that autocrine nitric oxide (NO) production by osteoclasts might be important in regulating attachment and motility of human osteoclasts *in vivo*. Figure 2.10 depicts bone resorption by osteoclasts.[92]

Figure 2.10. Osteoclastic bone resorption.

Multinucleated osteoclasts adhere to bone through $\alpha_v\beta_3$-integrins. Carbonic anhydrase II (CAII) can generate H^+ and HCO_3^-. HCO_3^- is extruded for Cl^- at the basolateral membrane. H^+ can be secreted through an H^+-ATPase on the apical membrane (ruffled border) into the resorption space and Cl^- is secreted through a Cl^- channel. HCl will dissolve matrix mineral. Phosphatases and cysteine proteinases - notably cathepsin K- will be released from lysosomes and degrade matrix proteins. Metalloproteinases that are released from secretory vesicles will also degrade matrix proteins *(Republished with permission of Nature Publishing Group from Goltzman D. Discoveries, drugs and skeletal disorders. Nature Rev Drug Discov 2002;1:784-96[92]; permission conveyed through Copyright Clearance Center, Inc.)*

2.4.2 Reversal

The regulatory mechanisms that stop osteoclastic activity are poorly understood. Raisz,[6,94] and Teitelbaum(2000)[68] list the following possiblities:

i) Osteoclasts have limited life spans. Anti-osteoporosis agents such as oestrogen (E2) could be involved in osteoclast apoptosis;

ii) Accumulation of calcium at high concentrations in the ruffled border area of the osteoclast results in inactivation;

iii) The release of TGF-β or related peptides from the matrix inactivates osteoclasts and attracts osteoblasts;

iv) During the reversal phase, osteoclasts disappear and macrophagelike cells appear on the bone surface. These cells could also release

factors such as TGF-β that inhibit osteoclasts and stimulate osteoblasts.

2.4.3 Formation and mineralisation

After the osteoclasts have excavated a resorption pit or lacuna, osteoblasts are recruited to this site where they synthesise, secrete, organise, and mineralise osteoid. Osteoid is composed predominantly of type I collagen and other noncollagenous proteins, such as osteopontin, osteonectin, and osteocalcin. Following its formation, osteoid normally undergoes rapid mineralisation with calcium and phosphorus.[37] Local and systemic factors control the replication and differentiation of successive waves of osteoblasts that replace the resorbed bone. Local factors that stimulate formation could be derived from osteoclasts, reversal cells, or marrow cells as well as from the bone matrix itself. Systemic hormones influence osteoblast replication and differentiation.[94]

2.5 Regulation of bone remodeling

Bone remodeling is regulated by both local and systemic factors, including hormones, growth factors and cytokines.[37,95]

2.5.1 Circulating hormones

Although various hormones are known to affect bone resorption and turnover, the most relevant to this presentation are oestrogen, PTH and $1,25(OH)_2D_3$ and will therefore be discussed.

2.5.1.1 Oestrogen (17β-estradiol)

The main cause of bone loss in postmenopausal osteoporosis is oestrogen deficiency,[96] which results in increased osteoclastogenesis causing an imbalance between bone formation and resorption.[97-98] Oestrogen treatment on the other hand, has long been known to inhibit bone loss in postmenopausal women.[99-100]

The principal *in vivo* effect of oestrogen on bone is a decrease in bone resorption, mostly by indirect actions such as regulation of growth factor and cytokine production in osteoblasts and their precursors, which, in turn, regulate osteoclast differentiation and activity.[101] In mature osteoblasts in culture, oestrogen has been shown to induce synthesis TGF-β, insulin-like growth factor-I (IGF-I), and IGF-binding proteins, and to inhibit synthesis of IL-1, IL-6, and IL-11.[97,101-102] Oestrogen, by regulating the levels of local growth factors and cytokines, indirectly manipulates the bone microenvironment thereby affecting bone metabolism.[98] Oestrogen acts on osteoblasts through high affinity oestrogen receptors (ER) located on the nuclear membrane.[35] The molecular mechanisms of oestrogen action on bone however, are not completely understood.

Recent reports demonstrated the involvement of OPG in oestrogen's paracrine-mediated effects in bone. *In vitro* oestrogen exposure dose- and time-dependently stimulates OPG secretion in human osteoblasts[103-105] and mouse bone marrow stromal cells;[103] OPG levels were highest in osteoblasts expressing the largest number of oestrogen receptors.[105] Oestrogen's protective effect on bone could well be explained by its stimulatory effect on OPG synthesis as OPG is known to be a potent inhibitor of osteoclast formation and activation. A local increase in OPG in the bone microenvironment may therefore be an important mechanism by which oestrogen reduces bone resorption. Oestrogen withdrawal, after a five-day pretreatment, mimicking the event occurring *in vivo* at menopause, dramatically down-regulates OPG expression in mouse bone marrow stromal cells.[103] As OPG specifically blocks RANKL-RANK interaction and therefore inhibits osteoclast differentiation and function,[10] down-regulation of OPG expression upon oestrogen withdrawal could increase osteoclastic bone resorption. Oestrogen, apparently, also

modulates M-CSF and RANK expression,[98,106] further enhancing the effects of OPG on the RANK-RANKL system.

In summary, it has been postulated that oestrogen inhibits bone resorption by inducing small but cumulative changes in multiple oestrogen-dependent regulatory factors.[101,107-109] Figure 2.11 illustrates a model of the interaction and coupling between osteoblasts and osteoclasts via OPG, RANKL (OPGL), and other growth factors and cytokines.[101]

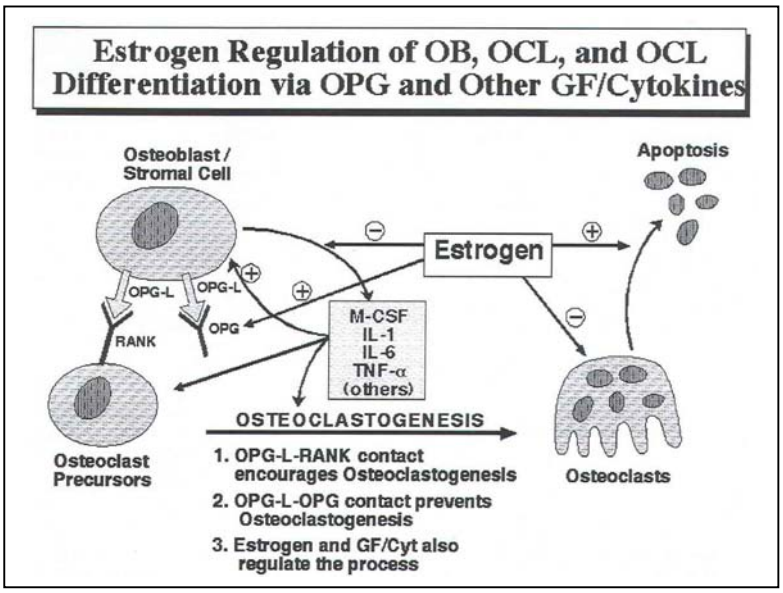


Figure 2.11. Oestrogen regulation of osteoblasts, osteoclasts, and osteoclast differentiation via osteoprotegerin (OPG) and other growth factors and cytokines.
(Spelsberg TC, Subramaniam M, Riggs BL, Khosla S. The actions and intractions of sex steroids and growth factors/cytokines on the skeleton. Mol Endocrinol 1999;13:819-28[101]) Copyright 1999, The Endocrine Society.

Although most of oestrogen's bone protective effects are modulated via osteoclast-mediated mechanisms, oestrogen has also been shown to act on osteoblasts. In a murine bone marrow cell model, oestrogen regulated early differentiation of osteoblastic progenitor cells in an ER-dependent way.[50,110] In another study,[50] oestrogen stimulated mRNA expression of Cbfa1 and down-regulated mRNA expression of PPAR-γ 2 in bone marrow stromal cells, which resulted in increased osteoblast numbers and decreased adipocyte numbers. It therefore seems that oestrogen not only inhibits bone resorption indirectly by regulating osteoclast numbers, but also affects bone formation by stimulating osteoblast differentiation from osteoblastic precursors in the bone marrow.

2.5.1.2 Parathyroid Hormone (PTH)

In humans, PTH is the major calcium-regulating hormone that maintains adequate levels of plasma calcium in times of dietary calcium deficiency by promoting bone resorption and inhibiting renal calcium excretion.[111-112] Calcium-sensing proteins, that register plasma calcium concentrations, are found in the parathyroid gland. When calcium concentrations decrease below normal, PTH is secreted into the circulation and proceeds to the osteoclasts where it enhances bone resorption, thereby releasing calcium. In addition, PTH acts on the proximal convoluted tubule cells of the kidney, thereby increasing the activity of 1α-hydroxylase which catalyses the formation of 1,25$(OH)_2D_3$ (calcitriol), the hormonal form of vitamin D_3 that stimulates intestinal calcium absorption. Apart from PTH's effect on vitamin D_3 hormone activation, it also stimulates the active reabsorption of calcium from the distal renal tubules. As soon as the serum calcium concentration exceeds the set point of the calcium-sensing system, it shuts down the parathyroid gland-induced cascade of events.[112]

PTH has complex effects on bone, depending on the mode of administration.[113-115] *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.[34,116-118] The mechanisms of these observed effects of PTH are not yet fully understood. However, it has been suggested that it could in part be explained by desensitisation of the PTH/PTH-related peptide (PTHrP) receptors.[119] G protein-coupled receptors (GPCRs) play a key role in regulating bone remodeling. Whether GPCRs exert anabolic or catabolic effects in bone may be determined by the rate of receptor desensitisation in osteoblasts. *Continuous* presence of PTH might attenuate the reponsiveness of GPCRs; in contrast to *intermittent* stimulation of the receptor, which permits prolonged activation of signaling pathways leading to net bone formation.[119] PTH, apparently also induces several growth factor genes, including those for IGF-I, IGF-II, and TGF-β, thereby indirectly affecting the bone microenvironment and bone quality.[120]

Parathyroid hormone effects on osteoprotegerin (OPG) and RANKL

Depending on the model used, disparate results have been reported on the effects of PTH on the OPG/RANKL ratio. Experimental evidence suggests that PTH administration rapidly and transiently inhibits the level of OPG mRNA in bone cells both *in vivo* and *in vitro.*[121-123] Regulation, however, is complex and depends on the differentiation status of the cells as well as the interval after stimulation when they were examined. In murine bone marrow cultures, PTH stimulates RANKL and inhibits OPG expression thereby adversely affecting the OPG/RANKL ratio.[121,122,124-125] Based on experimental results reported by Huang *et al* (2004),[111] it was suggested that PTH might induce a possible switch in the regulatory mechanism of osteoclastogenesis where OPG is inhibited early and RANKL is increased at later stages of osteoblast differentiation.

Parathyroid hormone effects on osteoblast numbers

The number of functioning osteoblasts available largely determines the *in vivo* bone formation rate. Experimental results reported by Jilka *et al* (1999) suggests that the increased osteoblast number, bone formation rate, and bone mass caused by daily PTH injections to mice is caused by an anti-apoptotic effect of this hormone on the osteoblasts.[126]

Using cultured murine marrow cells, [117] it was shown that *intermittent* PTH treatment increases mRNA for osteoblastic differentiation markers e.g., Runx2, alkaline phosphatase and type I procollagen. *Continuous* treatment on the other hand, resulted in production of large numbers of mature osteoclasts. Experimental evidence suggests that *intermittent* PTH treatment enhances osteoblast differentiation through an IGF-I dependent mechanism whilst *continuous* PTH treatment enhances osteoclastogenesis through reciprocal increases in RANKL and decreases in OPG.[117]

2.5.1.3 1,25-Dihydroxy vitamin D_3 (calcitriol)

Vitamin D_3 is a prohormone that can be obtained from the diet or formed in skin through ultraviolet irradiation of 7-dehydrocholesterol. It is biologically inert and must be metabolised to 25-hydroxyvitamin D_3 in the liver and subsequently to $1,25(OH)_2D_3$ (calcitriol) in the kidney upon PTH stimulation, as previously described (refer to 2.5.2.2). Calcitriol, the hormonal form of vitamin D_3, acts through a nuclear receptor to carry out its many functions, including active absorption of calcium and phosphorus in the intestine, calcium mobilisation in bone, and calcium reabsorption in the renal tubule.[112,127]

In vivo bone effects of calcitriol depend on its dose levels. It has been shown in rats as well as in an ovariectomised (OVX) mouse model that pharmacological doses of this hormone are required to induce bone resorption.[128-129] These high doses of calcitriol increase *in vivo* serum Ca^{2+} and expression of RANKL in the presence of PTH. Physiological doses of calcitriol on the other hand, do not stimulate bone resorption but rather inhibit bone resorption by inhibiting PTH-induced expression of RANKL mRNA.[128] At cellular level, it was shown that calcitriol accelerates *in vitro* osteoclastogenesis by upregulating RANKL gene expression in human osteosarcoma osteoblastic cells[130] and bone marrow stromal cells.[131] However, Hofbauer *et al* (1998)[132] reported that calcitriol up-regulated OPG mRNA expression as well as OPG protein synthesis in a human fetal osteoblastic cell line (hFOB) and normal trabecular osteoblastic cells.

2.5.2 Prostaglandin E_2

Osteoblasts produce prostaglandins from fatty acid precursors. Prostaglandins, especially PGE_2 derived from its precursor arachidonic acid, have pronounced effects on bone. Prostaglandins are likely to be local mediators *in vivo* because they do not circulate in significant amounts.[134] Depending on the concentration and experimental model, both anti-resorptive and pro-resorptive effects of prostaglandins have been reported.[134]

PGE_2 stimulates osteoclast formation in bone marrow cultures (10^{-10} to 10^{-5} M),[135-137] increases expression of mRNA for RANKL (10^{-6} M)[138] and down-regulates OPG in cultures of primary human bone marrow stromal cells (10^{-10} to 10^{-6} M).[139] These reported effects of PGE_2 on OPG and RANKL will ultimately have a detrimental effect on the OPG/RANKL ratio in the bone microenvironment and could ultimately lead to a decrease in bone mass as previously described.

Inflammatory conditions are associated with increased PGE_2 levels.[134,140] One could therefore speculate that the effects of PGE_2 on OPG (downregulation) and RANKL expression (upregulation) might be the cause of increased bone loss adjacent to inflammatory tissues, as is observed in rheumatoid arthritis and other diseases. Non-steroidal anti-inflammatory drugs (NSAIDS) e.g. indomethacin have been shown to inhibit bone loss *in vivo* and bone resorption *in vitro*, and this is associated with a loss of osteoclasts from the bone surface.[141] Increased OPG secretion was reported after PGE_2 inhibition by indomethacin in mouse calvaria *in vitro*.[142]

Prostaglandins have dual effects on bone formation. Several *in vivo* animal studies proved that PGE_2 administration increases bone formation.[143-145] In organ cultures, stimulation of DNA, collagen, and noncollagen protein synthesis is observed with low concentrations of PGE_2 whilst high PGE_2 concentrations inhibits collagen synthesis.[146] The stimulatory effects of prostaglandins may depend on their ability to stimulate endogenous growth factors such as IGF-I, bone morphogenetic protein-7 (BMP-7) and BMP-2.[147-149] For a detailed description of prostaglandins' effects on bone metabolism see 2.11.

2.5.3 Growth factors and cytokines

Growth factors are proteins that serve as signaling agents for cells. They are synthesised by osteoblasts, nonosteoblast skeletal cells, and marrow cells and function as part of a vast cellular communications network that influences critical functions such as cell division, matrix synthesis, and tissue differentiation.[150] Regulation of bone volume may in part depend on local growth promoting activities of these bone growth factors. Apart from IGFs, human bone cells in culture produce

IGF-binding proteins (IGFBPs), which have been shown to modulate IGF actions in bone.[45,151]

Large amounts of growth factors, of which IGF-II and TGF-β are the most abundant, are deposited in the mineralised matrix of bone. When these growth factors are released again during osteoclastic bone resorption, they may act on preosteoblasts thereby allowing for a site-specific replacement of bone that is lost to resorption. Systemic hormones such as PTH, oestrogen, progesterone and vit D_3 may modulate local bone formation, at least in part, through regulation of synthesis and release of bone growth factors.[6,94,101,151]

The mechanisms whereby growth factors exert their effects are not clear but Jilka *et al* (1998) presented evidence that growth factors such as TGF-β and IL-6 type cytokines prevent osteoblast apoptosis which suggests that osteoblast survival is regulated by factors produced in the bone microenvironment.[42] Growth factors have also been reported to impact on the OPG-RANKL system. Hofbauer *et al* (1998), for instance, have shown that BMP-2 increases OPG production in human osteoblast lineage cells.[132] Experimental data have shown that low TGF-β levels stimulate osteoclast differentiation by affecting the RANKL/OPG ratio while high TGF-β levels repress osteoclast differentiation by multiple mechanisms independent of the RANKL/OPG ratio or M-CSF expression regulation.[152]

Cytokines are extracellular protein messengers that regulate immune responses. TNF-α, TNF-β and IL-1 stimulate osteoclast recruitment and are potent stimulators of bone resorption.[94,153-155] At least part of the effects of IL-1 on bone resorption is prostaglandin mediated, since the prostaglandin synthetase blocker indomethacin partially inhibits them.[133,153,155] Hofbauer *et al* (1998) reported stimulation of OPG production by IL-1ß and TNF-α in osteoblast lineage cells,[132] which could ultimately inhibit osteoclastogenesis and osteoclast activation.

Interleukin-6 (IL-6) is a multifunctional cytokine, which, apart from its immunomodulatory effects, affects osteoclastic bone resorption. IL-6 is produced in nanomolar quantities by both stromal cells and osteoblastic cells and production thereof is stimulated by systemic hormones as well as various bone resorptive agents such as PTH, PGE_2, IL-1, and TNF.[88,156-158] IL-6 has been shown to stimulate

osteoclastogenesis in organ culture systems[133,156] as well as mesenchymal cells.[156] Evidence has recently been provided for cross-talk between PGE_2 and IL-6 signaling that enhance osteoclast differentiation via effects on the OPG/RANKL/RANK system in bone cells.[159]

2.6 Summary

Although bone is constantly being remodeled throughout life, the process of bone remodeling is normally tightly regulated. Two distinct types of bone cells mediate remodeling; the multinucleated osteoclast reponsible for bone resorption and the osteoblasts, which are bone forming cells. The process of bone remodeling is coupled which means that bone formation is linked to bone resorption. The process of bone remodeling proceeds through a number of steps, e.g. activation, resorption, reversal, formation and mineralisation that are summarised in figure 2.9.[89]

It is well known that cell-to-cell contact between osteoblasts (or bone marrow stroma cells) and osteoclast precursors are required for osteoclast formation. However, the precise mechanism by which pre-osteoblastic/stromal cells control osteoclast development, activation and subsequently bone resorption was unknown. The discovery of the RANK-RANKL-OPG system has solved this long-standing question in bone biology (summarised in Figure 2.7). As reported in the literature review, many bone-active agents that regulate osteoclastic bone resorption do so indirectly by controlling the production of RANKL or OPG by osteoblasts thereby affecting osteoclast maturation and activation. The balance between the osteoclast-promoting RANKL and the osteoclast-inhibiting OPG can therefore regulate the number and activity of osteoclasts. Figure 2.12 summarises some of the effectors of osteoclast formation, function, and apoptosis in the bone microenvironment.[109]

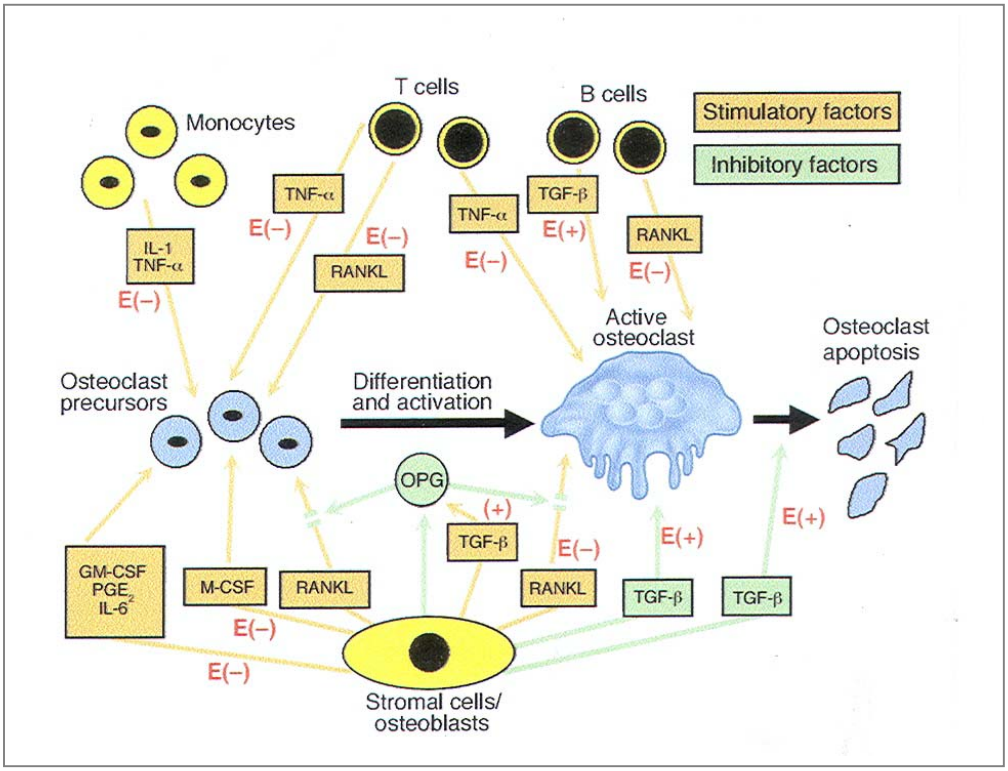


Figure 2.12. Regulation of osteoclast formation, function, and apoptosis by cytokines produced by bone marrow cells, osteoblasts, monocytes, T cells, and B cells.

Stimulatory factors are shown in orange and inhibitory factors in green. The effects of E (oestrogen) to enhance (+) and inhibit (-) the factors are shown in red. *(Republished with permission of The journal of Clinical Investigation Organisation from Bell NH. RANK ligand and the regulation of skeletal remodeling. J Clin Invest 2003;1120-2*[109]*) Permission conveyed through Copyright Clearance Center, Inc.*

In addition, Hofbauer *et al* (2000) proposed a *Convergence Hypothesis* for the regulation of osteoclast functions by cytokines.[14] According to this hypothesis, the regulation of RANKL and OPG by various systemic hormones, growth factors as well as cytokines are due to convergence at the level of RANKL and OPG, which then function as the final effector system to modulate differentiation and activation of osteoclasts. For example, the stimulation of RANKL by PTH and PGE_2,[124,138] and the inhibition of OPG by these same agents[125,139] may mediate the pro-resorptive effects of these agents. Figure 2.13 depicts this *Convergence Hypothesis* as proposed by Hofbauer *et al* (2000).[14]

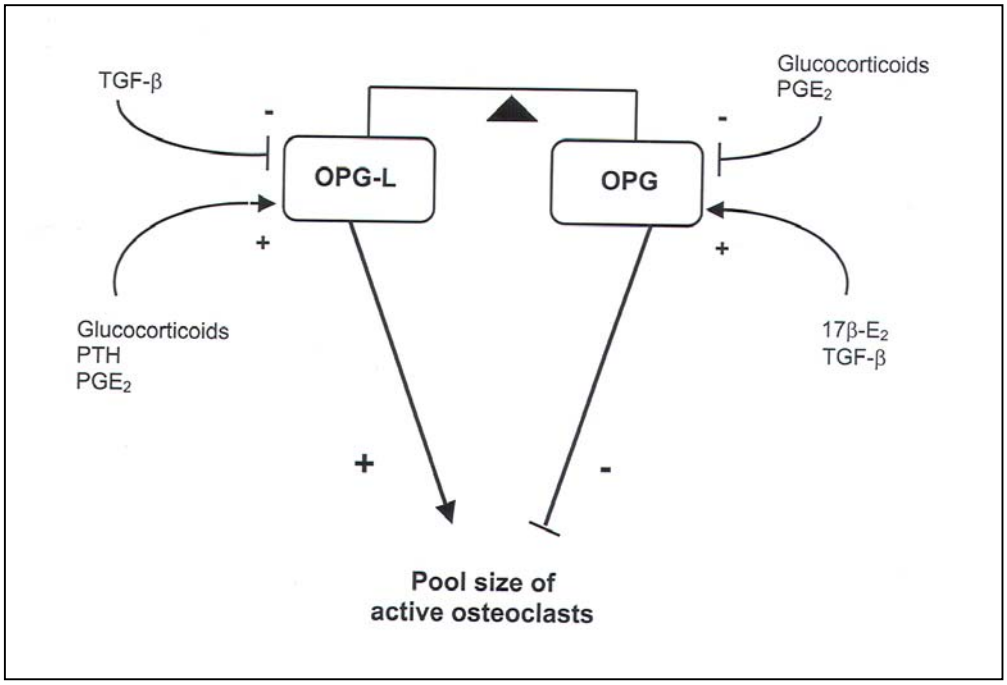


Figure 2.13 The 'convergence hypothesis' for the regulation of osteoclast functions by cytokines.

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.

II Polyunsaturated Fatty Acids

2.7 Types of polyunsaturated fatty acids (PUFAs)

Fats contain fatty acids, which vary widely in the number of carbons and the number of double bonds in the carbon chain. Saturated fatty acids have no double bonds, whereas cis-unsaturated fatty acids have one (monounsaturated) or more (polyunsaturated) double bonds. Lengthening of the chain and the introduction of additional double bonds beyond the first one occur from the carboxyl-terminal of the fatty acid. Based upon the position of the first double bond, polyunsaturated fatty acids (PUFAs) are further classified in families: omega-3, or n-3, fatty acids have the first double bond between carbon atoms three and four (counting from the methyl end) whilst omega-6, or n-6, fatty acids have the first double bond between carbon atoms six and seven.[160]

2.8 Metabolic pathways of essential fatty acids

Two PUFAs, linoleic acid (LA) (18:2 c n-6) and α-linolenic acid (ALA) (18:3 c n-3) are considered essential fatty acids (EFAs), as they cannot be synthesised by humans and must be provided in the diet. Human biosynthetic enzymes can only insert a double bond at the n-9 position or higher; but not in any position closer to the methyl end such as position 6 or 3 as in the case of LA and ALA.[22] LA is found in seeds of most plants except coconut, cacao, and palm and ALA is found in linseed and chloroplast of green leafy vegetables.[22] These two fatty acids are converted via a series of desaturation and elongation steps by the same enzyme systems to different fatty acids, which serve as precursors for the eicosanoids.[40,161] Generally, the desaturation steps are slow and rate limiting, while the elongation steps usually proceed rapidly (Figure 2.14).[23]

The n-3 and n-6 PUFAs are competitive inhibitors of each other's metabolism. Competition is not as apparent in the elongation steps, which are rapid and allows large amounts of both series of PUFAs to be metabolised by the same common enzyme systems but is apparent at the slow desaturation steps, where a large

amount of one type of PUFA will interfere with the metabolism of the other. It has been found that n-3 PUFAs are more effective at inhibiting desaturation of n-6 PUFAs than vice versa.[162]

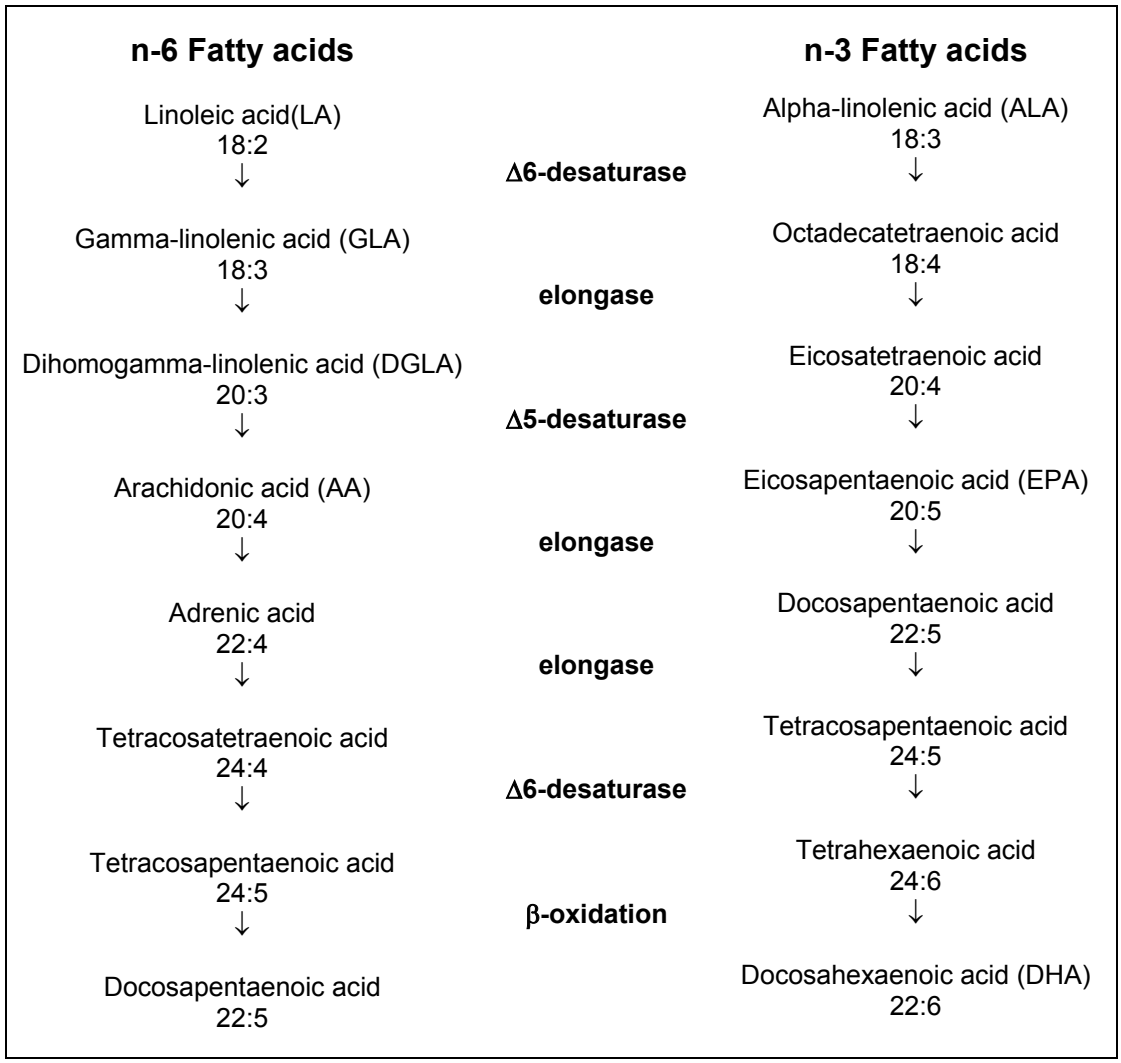


Figure 2.14. The elongation and desaturation pathways for n-3 and n-6 fatty acids.
(Reprinted from Kruger MC, Horrobin DF. Calcium metabolism, osteoporosis and essential fatty acids: a review. Prog Lipid Res 1997;35:131-51.[23]) Copyright (1997), with permission from Elsevier.

2.9 Cellular functions of polyunsatutated fatty acids

Dietary fatty acids can be oxidised to provide energy, stored in adipose tissue, or selectively incorporated into cell membranes. PUFAs have two fundamental physiological functions; they are present as structural phospholipids in high

concentrations in all membranes and are the primary precursors of eicosanoids. In addition to these roles, PUFAs can also affect cell function either by modulating intracellular signal transduction, modulating cell-cell interaction or modulating gene transcription.[22,163] These actions are initiated by phospholipases such as PLA_2 that releases PUFAs thereby enabling them to be metabolised to PUFA derivatives such as eicosanoids.[22]

2.9.1 Composition of membranes

Biological membranes surrounding cells and subcellular organelles exist primarily as lipid bilayers that are mainly composed of phospholipids and free cholesterol, which interface with a variety of proteins functioning as receptors, transporters, enzymes and ion channels.[161] Phospholipids contain a diverse range of PUFAs and manipulation of dietary lipids readily modify the fatty acid composition of membranes in both experimental animals and humans.[161,164-167] The presence of specific PUFAs may determine the biological properties of the membranes and the way cells respond to various stimuli. Because of unsaturation of the PUFAs they affect membrane properties such as fluidity, flexibility, and permeability which in turn affect functioning of such proteins as receptors, enzymes such as ATPases or ion channels.[162,163] It has been shown, for instance, that supplementation of the diet with evening primrose oil or fish oil containing considerable quantities of n-3 PUFAs increases the unsaturation index of intestinal brush border membrane vesicles and significantly enhances calcium transport.[168] A later study by Haag *et al* (2003) showed that n-3 PUFAs are involved in multiple signaling effects that affect ATPases in the basolateral membrane thereby enhancing calcium absorption.[169]

2.9.2 Eicosanoid synthesis

Essential fatty acids are the precursors of the eicosanoids, prostaglandins, leukotrienes, and other oxygenated derivatives, derived predominantly from the 20-carbon polyunsaturated fatty acids dihomogamma-linolenic acid (DGLA) (1 series prostaglandins), arachidonic acid (AA) (2 series prostaglandins) and eicosapentaenoic acid (EPA) (3 series prostaglandins). Eicosanoids are produced

via a cascade of steps starting with the cyclooxygenase (COX) or lipoxygenase (LO) enzymes. The main COX products comprise the classical prostaglandins, prostacyclin and the thromboxanes, while the main lipoxygenase products are leukotrienes (Figure 2.15).[163] Eicosanoids are produced locally as and when they are needed and have effects that are usually confined to the immediate vicinity of the cells in which they are produced.[160,162,170]

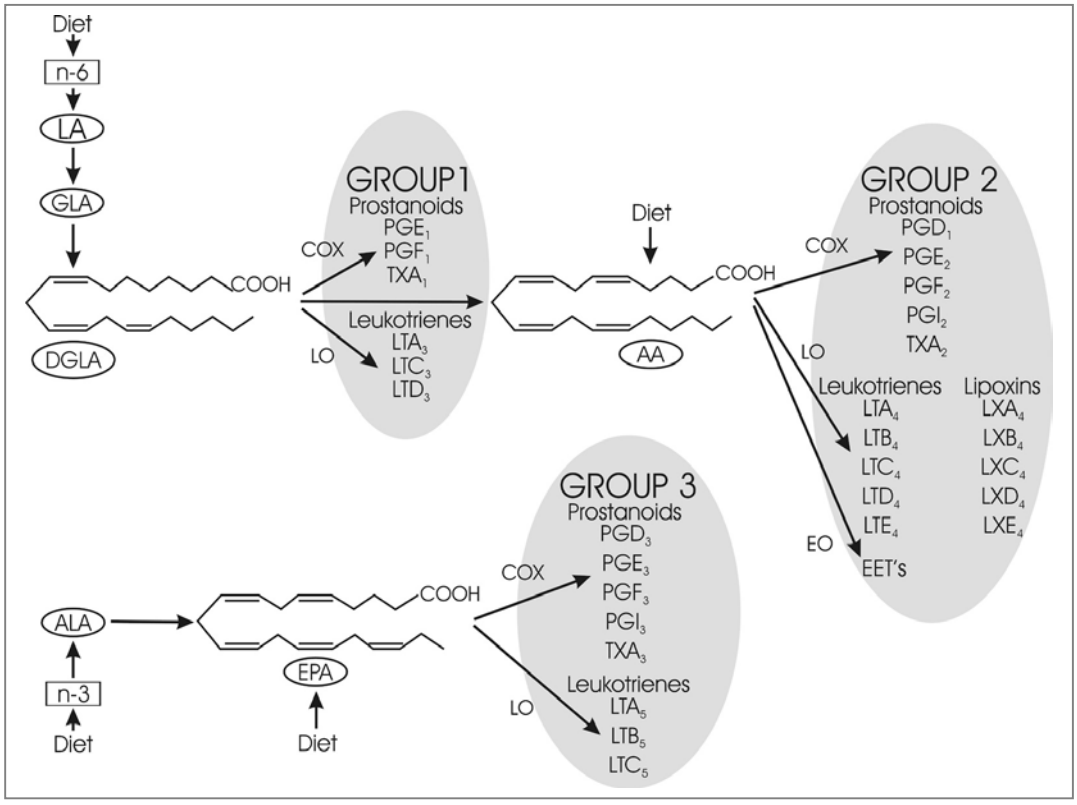


Figure 2.15. The synthesis of eicosanoids from polyunsaturated fatty acids.

LA: linoleic acid; GLA: gamma-linolenic acid; DGLA: dihomo-gamma-linolenic acid; AA: arachidonic acid; ALA: alpha-linolenic acid; EPA: eicosapentanoic acid; COX: cyclo-oxygenase,; LO: lipoxygenase;
EO: epoxygenase; PG: prostaglandin; TX: thromboxane; LT: leukotriene; PGI: prostacyclin; LX: lipoxin; EET: epoxyeicosatrienoic acid. *(Reprinted with permission from Haag M. Poly-unsaturated fatty acids: Their cellular role and clinical applications (Part 1). The Medicine J (SA) 2001;43:13-17.[163]) Copyright (2001), Medpharm Publications.*

Prostaglandins are considered fast-acting local hormones, often displaying biphasic properties. PGE_2, which is derived from AA, is thought to contribute to pro-inflammatory processes and high concentrations may inhibit bone formation.[171] Varying the ratio of the precursor fatty acids in the diet is an effective way to modify prostaglandin production in the body. Increasing the dietary content of the n-3

PUFAs EPA and DHA will inhibit the synthesis of 2 series eicosanoids derived from AA by inhibiting AA release from membranes by PLA_2 and its cascade through the cyclooxygenases and lipoxygenases.[30,160,172-176] Apart from replacing AA in cell membranes, EPA can be utilised as substrate for the synthesis of PGE_3 that is regarded equally potent as PGE_2 in bone resorption. EPA, however, is only one-tenth as effective for PGE_3 synthesis as AA for PGE_2 synthesis,[177,178] suggesting that replacement of AA by EPA could be beneficial. It has also been reported that PGE_3 has milder inflammatory effects compared with PGE_2.[22] PGE_2 synthesis can also be reduced by provision of the n-6 PUFA gamma-linolenic acid (GLA). Available evidence suggests that GLA increases the synthesis of DGLA but not AA, probably due to limited activity of Δ-5-desaturase.[171] In addition to reducing PGE_2 synthesis, dietary GLA can enhance production of PGE_1, which has anti-inflammatory effects.[160,171]

2.9.3 Second messengers

Membrane lipids not only serve a fundamental role in the structure of membranes but also play a critical role in the processes of signal transduction and cell regulation.[179] Phospholipase A_2 that is controlled by hormones and other signals, liberates fatty acids from the sn-2 position of phospholipids, and these can subsequently be used as precursors for eicosanoids. These lipid-soluble molecules can diffuse out of the cell and combine with receptors on neigbouring cells to exert a paracrine function. In addition, free fatty acids can also interact with a number of cellular proteins including phospholipases, G-proteins, ion channels and protein kinases.[18,23,179] PUFAs and their metabolic products have also been shown to be part of most of the second messenger signaling systems within the cell.[180] Reviews by Khan *et al* (1995),[179] Kruger and Horrobin (1997)[23] and Haag (2003)[18] listed the following:

1. PUFAs are regulators of protein kinase function and hence phosphorylation and activation.
2. DGLA, EPA and AA are substrates for oxygenated derivatives including eicosanoids such as the prostaglandins, leukotrienes, thromboxanes and hydroxy fatty acids, which perform a wide range of second messenger signaling functions. The different effects of the prostaglandins are due to

their effects on different signaling systems: prostaglandins of the n2-family transduce signals via a G_s protein, thus elevating cAMP levels, whereas those of the n-3 family use a G_I protein, which have the opposite effect. Prostaglandins of the 1 family use a phosphoinositide signaling system.

3. PUFAs are important constituents of the diacylglycerols (DAG) released from P-inositol during the course of inositol signaling. The diacylglycerols have been shown to be regulators of protein kinases and calcium signaling.
4. Cyclic nucleotide synthesis is under control of various PUFA-derived molecules, especially the prostaglandins.

Figure 2.16 presents the most important membrane second-messenger mechanisms with the red numbered circles showing the locations where PUFA effects have been demonstrated.[18]

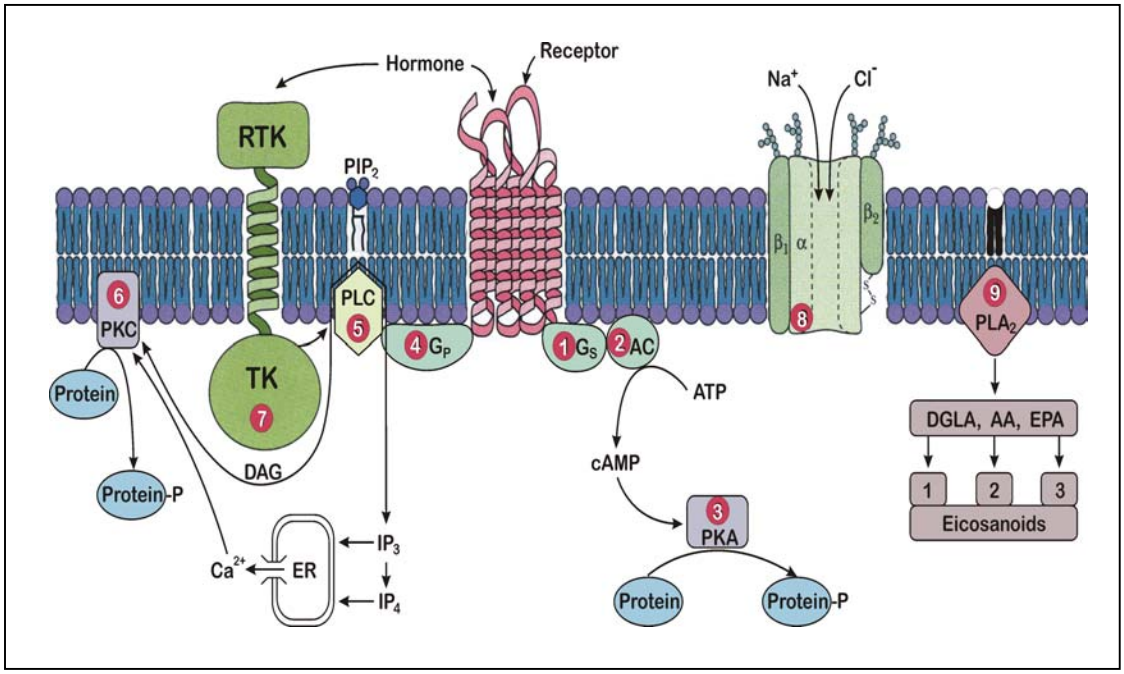


Figure 2.16. Role of polyunsaturated fatty acids in signal transduction.

1: G_s protein; 2: adenylate cyclase; 3: protein kinase; 4: G_p protein; 5: phospholipase C; 6: protein kinase C; 7: tyrosine kinase; 8: ion channel; 9: phospholipase A_2.
(Abbreviations: AA : arachidonic acid; DGLA: dihomo-gamma-linolenic acid; EPA: eicosapentaenoic acid; PL: phospholipid; PIP_2: phosphatidylinositol pyrophosphate; DAG: diacyl glycerol; ER: endoplasmic reticulum; IP_3 and IP_4: inositoltris- and tetrakosphosphates.) *(Reprinted with permission from Haag M. Essential fatty acids and the brain. Can J Psychiatry 2003;48:195-203.)*[18]

Differential effects of the n-6 and n-3 PUFAs on signal transduction have been reported.[180,181] Mirnikjoo *et al* (2001) reported that n-3 PUFAs inhibited the *in vitro*

activities of cAMP-dependent protein kinase, protein kinase C, Ca^{2+}/calmodulin-dependent protein kinase II, and mitogen-activated protein kinase (MAPK). They concluded that one mechanism by which n-3 fatty acids could affect cellular function is by inhibition of these second messenger-regulated protein kinases.[181]

2.9.4 Modulation of gene transcription

Ingestion of PUFAs will lead to their distribution to virtually every cell in the body with effects on membrane composition and function, eicosanoid synthesis, and signaling as well as the regulation of gene expression.[182] Dietary PUFAs and their derivates such as eicosanoids can act as signaling molecules involved in the regulation of gene expression by interacting with specific nuclear receptors within the cell's nucleus.[183] These nuclear receptors control the rate of gene transcription by binding to DNA at specific responsive elements. Depending on the nature of the transcription factor and its binding substrate e.g. PUFAs or their derivatives, genes associated with the production of functional proteins can either be stimulated or repressed.[160]

Peroxisome proliferator activated receptors (PPAR) are examples of nuclear receptors that may utilise long-chain PUFAs or their derivatives as substrates.[184,185] At present, three isoforms of PPAR have been cloned (PPARα, -β, and -γ) with tissue specific expression, ligand-specific activation, and the ability to heterodimerise with retinoid X receptors (RXR).[183] (Refer to figure 2.5).[54] PPAR can be found in all tissues of the body, but especially in the liver (PPARα), where they control the synthesis of lipids and in adipose tissue (PPARγ) where they control the differentiation of adipocytes.[160] PPARα is often considered a 'master switch' transcription factor[28] as it plays a role in the regulation of an extensive network of genes involved in glucose and lipid metabolism including fatty acid transport, fatty-acid-binding proteins and fatty acyl-CoA synthesis.[186] Various fatty acids have been shown to bind to and activate PPARs including n-6 PUFAs such as GLA, AA, LA and linolenic acid; n-3 PUFAs such as EPA and DHA; mono-unsaturated fatty acids such as oleic acid and elaidic acid; and saturated fatty acids e.g., palmitic and stearic acid.[184,187,188]

Whereas PPARα operates in the catabolism of fatty acids in the liver, PPARγ influences the storage of fatty acids in adipose tissue.[188] PPARγ is also part of the adipocyte differentiation program that induces the maturation of pre-adipocytes into adipocytes.[54,189] Activated PPARγ induces lipoprotein lipase and fatty acid transporters and enhances adipocyte differentiation as well as inhibiting NFκβ function and cytokine and COX-2 expression.[186] Apart from fatty acids themselves, PUFA metabolites such as prostaglandins have also been shown to be direct ligands for PPARγ and inducers of adipogenesis[190,191]

Osteoblasts derive from marrow stromal cell progenitors, which are capable of differentiating into several different cell types, including adipocytes.[43,44] Two forms of PPARγ are expressed in subclones of marrow-derived cell lines, PPARγ1 and PPARγ2.[59] Activation of PPARγ2 has been shown to induce adipogenesis in these cell lines but PPARγ1 does not have this function. In addition, PPARγ2 suppresses the expression of genes such as Cbfa1 involved in osteoblastogenesis.[55,59] Transcriptional activation of PPARγ2 is potentiated by various lipid-like compounds, including naturally occurring PUFAs.[189] Apart from osteoblast progenitors, PPARγ is also expressed in osteoblasts and activation of PPARγ by fatty acids, as well as various linoleic acid peroxidation products such as 9,10-epoxyoctadecenoic acid can induce transdifferentiation of these cells into cells expressing the adipocytic phenotype *in vitro*.[55,56]

The binding of free fatty acids to steroid hormone receptors can also modulate gene expression. It has been shown that free fatty acids and steroid hormones are involved in an intertwined regulatory loop: Free fatty acids can interact with cytoplasmic or nuclear steroid hormone receptors to modulate the binding of steroid hormones positively or negatively.[192] In turn, the steroid hormone, either bound to the receptor or unbound, can intervene in the synthesis and activities of different enzymes responsible for the release, reincorporation or the synthesis of fatty acids.[192]

The effects of fatty acids on gene expression have received considerable attention because it represents a direct route for fatty acids to regulate gene function.[182]

Omega-3 PUFAs for instance, have been shown to have rapid effects on gene expression such as the PPARs[28] and changes in mRNAs encoding several lipogenic enzymes can be detected within hours of feeding animals diets enriched with n-3 PUFAs.[182] These effects are sustained for as long as the n-3 PUFAs remain in the diet.[182]

2.10 Effects of polyunsaturated fatty acids on bone

Polyunsaturated fatty acids (PUFAs), especially the omega-3 (n-3) PUFAs such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) present in fish oil, are of paramount importance for health and disease prevention. The n-3 PUFAs, especially, have been shown to be beneficial in the prevention and treatment of a variety of medical conditions such as cardiovascular diseases, neurological disorders, inflammatory diseases, some cancers and rheumatoid arthritis.[18-20]

During the past two decades, the effects of dietary PUFAs on bone health received considerable attention.[16,21,22] It has been suggested for instance that PUFAs of the n-3 series, as well as the n-6 fatty acid GLA, may prove beneficial when consumed in appropriate amounts.[27] In addition, it has been shown that a reduction of the n-6/n-3 PUFA ratio could result in increased bone strength in animals[30-31] and in humans.[32] Changes in dietary PUFAs are reflected in the composition of various tissues, including bone cells such as the osteoblasts.[20,33] As PUFAs are substrates for different prostaglandins, some of the effects of PUFAs have been attributed to modulation of prostaglandin synthesis in bone.[40]

2.10.1 Essential fatty acid deficiencies and bone

Because EFAs are widely distributed in plant products in the diet, EFA deficiency is rare in humans. However, following dietary EFA deficiency, pathological fractures were reported in newborn rats.[193] Borland and Jackson (1931) reported that EFA-deficient animals were found to develop severe osteoporosis coupled with increased renal and arterial calcification.[194] Early studies dating back to 1946

reported that individuals with osteoporosis frequently also had ectopic calcification in other tissues, particularly intervertrabal discs, arteries and kidneys.[195] In osteoporosis, calcium is not simply lost from bone and from the body but some of the calcium is deposited in arteries and kidneys, where it is harmful.[196] Treatment of animals with EPA and GLA attenuated ectopic calcification, thereby suggesting that EFAs might be benificial for treatment of this condition.[197,198]

2.10.2 Nutritional *in vivo* human and animal studies

It has been shown in various animal and human models that supplementation of the diet with n-3 PUFAs such as EPA and DHA have beneficial effects for bone.[21,174,199-201] A controlled clinical study, for instance, found that supplementation of calcium, γ-linolenic acid and EPA in the diets of elderly women enhances calcium absorption, reduces calcium excretion, and have overall positive effects on bone mineral density.[27] Reinwald *et al* (2004) have shown that n-3 deficient weanling rats exhibited a marked increase in n-6 PUFAs and a corresponding decrease in n-3 PUFAs in bone. Diminished structural integrity was observed when mechanical properties of bone in these rats were measured.[202] Repletion with dietary n-3 PUFAs, however, restored the (n-6)/(n-3) PUFA ratio in the bone compartments of these animals and reversed the compromised bone modeling as well.[202] In a study designed to investigate the effects of varied amounts of dietary AA, while keeping DHA status and the total (n-6)/(n-3) ratio constant, it was shown that supplementation of the diet with AA (n-6) enhanced bone mass in piglets.[203]

A large number of studies reported the effects of dietary PUFAs on young growing animals. Norrdin *et al* (1990), however, cautioned that it should be taken into account that the growing skeleton is usually more responsive due to its greater activity concerned with lengthening of bone at the cartilaginous growth plates where bone modeling mainly depends on bone formation.[204] In the mature animal, on the other hand, cellular activity is primarily concerned with bone remodeling where bone resorption and bone formation alternate at specific bone sites and are responsible for internal turnover of the matrix.[204]

It has been shown that lowering of the dietary (n-6)/(n-3) fatty acid ratio could be beneficial for bone in animals and humans.[25,29,30,173,192,205] Weiss *et al* (2005) reported findings of a population-based cohort study, known as the Rancho Bernardo study, in which a large number of older, middle class residents in California participated. In this study, dietary data were obtained between 1988 and 1992 through food-frequency questionnaires. In addition, bone mineral density of the participating subjects was determined. Results from this study have shown that a higher n-6/n-3 PUFAs ratio is associated with lower bone mineral density at the hip in both sexes. These findings suggest that the relative amounts of dietary PUFAs might play a vital role in preserving skeletal integrity in older age.[32]

The mechanisms underlying the response of bone to dietary fatty acids are not fully understood. So far, increased calcium absorption and decreased urinary calcium loss[168,192,206] have been reported as well as alterations of eicosanoid metabolism[30,173,174,207] and growth factors such as insulin-like growth factor I.[173,174,205,207] In addition, the n-3 PUFAs have been shown to posess anti-inflammatory qualities that could also protect bone, especially in inflammatory conditions.[208,209]

2.10.2.1 Effects of dietary polyunsaturated fatty acids on calcium balance and bone status

Dietary fats could influence bone health by affecting intestinal calcium absorption and renal calcium excretion. Calcium absorption is increased and excretion decreased when standard diets are supplemented with specific oils. Addition of the PUFAs GLA and EPA to the diets of healthy[192,206] or ovariectomised rats[25] suppressed bone resorption and enhanced bone mass. Supplementation of the diet with evening primrose oil or fish oil high in n-3 PUFAs has been shown to increase the unsaturation index of intestinal brush border membrane vesicles, resulting in significantly enhanced calcium transport by these membranes.[168] Van Papendorp et al (1995)[26] supplemented the diet of osteoporotic patients with evening primrose and fish oil or olive oil (control) for 16 weeks. Patients supplemented with the PUFA rich oils showed an improvement in calcium absorption and stimulation of osteoblastic

activity indicated by a rise in osteocalcin and procollagen, both markers of bone formation.[26]

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, therefore demonstrating a bone protective effect of n-3 PUFAs.[29] The mechanism by which n-3 fatty acids prevent activation of RANKL is not known.

A few studies reported negative effects of n-3 supplementation on bone metabolism. Judex *et al* (2000) showed that 10% fish oil supplementation in the presence of modest vitamin E supplementation can lead to substantial degradation of morphological and mechanical properties of cortical bone of rapidly growing rabbits. [210] This observation indicates that supplementation of fish oil in large quantities, had detrimental effects on the skeleton of these animals.[210] In another study, feeding fish oil to weanling male rats showed no effect on biomechanical strength properties of femurs and vertebrae but in female rats reduced length growth and a lower vertrebral peak load was observed.[211]

2.10.2.2 Effects of dietary polyunsaturated fatty acids on prostaglandin secretion and bone status

Prostaglandins are metabolised from PUFAs and are considered fast-acting local hormones often displaying biphasic properties. PGE_2 derived from the n-6 PUFA AA, is the major prostaglandin in bone and has been shown to be a potent modulator of bone remodeling, affecting both bone resorption[136,137] and formation.[143,149] Excessive production of PGE_2 may affect bone modeling adversely, whereas a lower level of PGE_2 is believed to stimulate bone formation in animals fed diets containing moderate levels of n-6 PUFAs. [171] (Refer to 2.8)

Varying the ratio of the precursor fatty acids in the diet is an effective way to modify prostaglandin production in the body. Since n-3 and n-6 fatty acids serve as substrates for the same enzymes along the conversion pathways but are metabolised at different rates,[23] lowering the dietary (n-6)/(n-3) PUFA ratio can reduce PGE_2 production.[30,174,205,212] The n-3 PUFAs are precursors to PGE_3 that is equally potent to PGE_2 in bone resorption.[177] However, conversion is less effective than for PGE_2 from n-6 PUFA resulting in lower PGE_2 levels.[177,178] PGE_2 production can also be reduced by provision of the n-6 PUFA GLA.[171] In addition to reducing synthesis of PGE_2, dietary GLA can enhance production of PGE_1, which has anti-inflammatory effects that could also benefit bone.[160,171]

Weiler's group conducted several fatty acid nutritional studies on growing piglets.[175,212-215] In a short-term study (21-days) it was shown that modulation of the (n-6)/(n-3) PUFA ratio alters the bone fatty acid profile in piglets; however, the bone mass of these animals was not affected.[212] Higher plasma DHA levels were paralleled with lower bone resorption rates as assessed by urinary N-telopeptide. Furthermore, bone formation as indicated by plasma osteocalcin, was suppressed in these piglets with an elevated *ex vivo* PGE_2 release from bone.[212] In another study, Lucia *et al* (2003) compared the effects of dietary PUFAs with that of low dosage exogenous PGE_2 on bone metabolism in piglets.[175] Results from this study indicated that PGE_2 enhances osteoblast activity as indicated by increased plasma osteocalcin and reduced urinary calcium excretion. On the other hand, dietary PUFAs provided as AA and DHA resulted in reduced bone resorption as indicated by urinary N-telopeptide. It was concluded that dietary PUFAs and exogenous PGE_2 could both lead to enhanced mineral content in this growing piglet model, but through distinct mechanisms.[175]

Watkins *et al* (1996) have shown that feeding chicks menhaden oil (high in n-3 PUFAs) resulted in a higher serum ALP activity and an increase in the bone formation rate compared to those chicks given soy-bean oil (high in n-6 PUFAs).[207] The effect of PUFAs might be exerted via modulation of PGE_2 synthesis. It has been shown that PGE_2 inhibits ALP activity, as inhibition of PGE_2 synthesis resulted in higher ALP activity.[205,207] PGE_2 is thought to contribute to pro-inflammatory processes, and high concentrations thereof may inhibit bone formation. Watkins *et al*

(2001) suggested that by lowering the dietary (n-6)/(n-3) PUFA ratio, PGE_2 production could be reduced and bone formation therefore enhanced.[176]

Changes in the PUFA content of the diet have been shown to not only alter the experimental animal's tissue fatty acid profile but also that of its offspring.[216] Liu and Denbow (2001) have demonstrated that supplementation of quail hens' diet with fish oil high in n-3 PUFAs, significantly lowered *ex vivo* PGE_2 production of tibiae in newly hatched quail compared to those from hens fed control diets.[216] These results suggested that maternal dietary lipids might have the potential to influence bone metabolism of embryos by modifying the fatty acid composition of this tissue.[216]

2.10.2.3 Effects of dietary polyunsaturated fatty acids on insulin-like growth factor (IGF-I) and insulin-like growth factor binding proteins (IGFBPs)

It has been shown that PGE_2 at moderate levels may increase the production of bone-derived growth factors such as insulin-like growth factors (IGFs) in osteoblasts.[147,217] IGFs, especially IGF-I, are major bone-derived growth factors and are believed to function as both systemic and local growth factors for bone tissue. Once secreted and deposited in bone matrix, IGFs are released during osteoclastic bone resorptive activity, acting in an autocrine or paracrine fashion to stimulate new bone cell formation and matrix production.[218] IGF-I acts as a regulator of bone cell function as it stimulates the proliferation of pre-osteoblasts, thereby increasing the number of cells capable of producing bone matrix. In addition, IGF-I increases collagen expression while decreasing collagen degradation, causing an anabolic effect in bone tissue.[219,220] In osteoblasts, hormones such as growth hormone, PTH and oestrogen modulate IGF-I expression.[220] Apart from IGF-I, osteoblasts also synthesise extracellular high affinity IGF-binding proteins (IGFBPs), which modify the interaction of IGF-I with its receptors by prolonging IGF stability and by influencing ligand-receptor interaction.[45,221]

It has been suggested that dietary PUFAs, depending upon the type and amount ingested, may up-regulate or down-regulate IGF-I production in bone via their ability to modulate local concentrations of PGE_2.[174] PGE_2, produced from AA by

osteoblasts, stimulates IGF-I synthesis[147,174,220] as well as the expression of various IGF-binding proteins,[217,219,221] suggesting that PGE_2 could keep IGF available for stimulation of osteoblasts at a later phase of bone remodeling.[219] McCarthy *et al* (1991) suggested that the ability of PGE_2 to enhance osteoblastic IGF-I synthesis could explain its anabolic potential, and furthermore suggests a role for PGE_2 in coupled bone remodeling.[147] The anabolic effects of PGE_2 may occur through stimulation of endogenous IGF-I production by osteoblasts[94] or by increased bone cell responsiveness to IGF-I.[222] It has been shown by Li *et al* (1999) that feeding a fish oil-enriched diet to rapidly growing male rats increases the serum concentration of IGFBP-3, an important modulator of IGF-I and overall bone growth and development.[174]

2.10.3 Effects of polyunsaturated fatty acids on bone cells

Although a large number of studies of dietary PUFA effects on bone homeostasis have been published,[16,21,22] the cellular mechanisms of these fatty acids on bone have not been well investigated.

2.10.3.1 Effects of polyunsaturated fatty acids on early osteoblastic differentiation

Atkinson (1997) demonstrated that DHA feeding of weanling male Fisher rats had a substantial bone marrow enhancing activity, resulting in a two-fold increase in bone marrow cell number over n-6 PUFA fed animals.[33] Bone marrow contains various precursor cells including mesenchymal stem cells that are pluripotent and able to differentiate into several cell types including osteoblasts and adipocytes.[43,44] One could therefore speculate that higher numbers of bone marrow cells could increase the potential for osteoblastogenesis, provided the required transcription factors are expressed. Commitment of a mesenchymal stem cell to the osteoblastic lineage is regulated by specific transcription factors of which Cbfa1 has been identified as the earliest and most specific marker of osteogenesis.[46,48]

Watkins *et al* (2003) reported regulatory effects of PUFAs on Cbfa1 expression in fetal murine calvarial osteoblasts.[223] AA, EPA and LA stimulated Cbfa1 expression but conjugated linoleic acid (CLA) decreased protein levels for Cbfa1 after 14 days of treatment.[223] This preliminary study suggests that fatty acids may affect Cbfa1 expression. It was speculated that the observed stimulatory effects of AA and LA in this experimental model could be mediated by elevated PGE_2 production.[223] The possible involvement of PGE_2 in Cbfa1 expression is supported by findings of Zhang *et al* (2002) who reported significant inhibition of Cbfa1 expression in a COX-2 knockout mouse model, which was reversed by the addition of PGE_2.[224] In addition, it has been shown that PGE_2 induces expression of Cbfa1 as well as bone morphogenetic protein-2 (BMP-2) through activation of the EP_4 prostaglandin receptor.[149] Bone-morphogenetic proteins (BMPs) are members of the transforming growth factor-β superfamily and are considered important regulators of the differentiation of uncommitted mesenchymal cells into osteoblasts during both embryonic development and bone repair.[52,225] Zhang *et al* (2002) speculated that PGE_2 might induce BMPs and/or cooperate with BMPs to increase Cbfa1 and osterix, two essential transcription factors required for bone formation.[149] However, whether dietary PUFAs could affect BMP-2 expression via modulation of PGE_2 synthesis, is not known.

Osteoblastic precursors not only express Cbfa1 but also PPARγ, a transcription factor responsible for adipocyte differentiation.[46,53] It has been shown that activation of PPARγ, by fatty acids as well as a various linoleic acid peroxidation products, can induce adipogenesis and inhibit osteoblastogenesis in some osteoblastic precursors *in vitro.*[55,56] The modulation of osteoblast precursors is complicated and depends not only on the provision of PUFA metabolites but also on the expression of specific subscription factors in these cells.

2.10.3.2 Effects of polyunsaturated fatty acids on osteoclastogenesis

Sun *et al* (2003)[29] 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 which is considered to be a marker of osteoclast maturation.[29] 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.* It was therefore concluded that inhibition of osteoclastogenesis might be one of the mechanisms by which dietary n-3 PUFAs reduce bone loss in OVX mice.[29]

2.10.3.3 Effects of polyunsaturated fatty acids on cytokine expression

Cytokines, such as IL-1 mainly regulate immune responses; however, they have also been shown to stimulate osteoclast recruitment and are potent stimulators of bone resorption.[152] Priante *et al* (2002) conducted an experiment to investigate the effects of different fatty acids on the expression of cytokines involved in bone remodeling.[226] Osteoblasts were exposed to AA, EPA and oleic acid and cytokine mRNAs determined in MG-63 osteoblasts. The results showed that AA (25 to 100 μM) stimulated expression of IL-1α, IL-1β, TNF-α and M-CSF. EPA and oleic acid (25 to100 μM) on the other hand, had no stimulatory effects, but instead caused a significant inhibition of AA-induced cytokine mRNA expression.[226] Results from inhibitor studies suggested that a protein kinase C-dependent mechanism could account for the effects of AA on cytokine production.[226] Downregulation of resorptive cytokines such as TNF-α and IL-6 by n-3 PUFA in OVX mice was also reported by other research groups.[29,227]

2.10.3.4 Effects of polyunsaturated fatty acids on alkaline phosphatase (ALP) activity

Watkins *et al* (2003) reported increased ALP activity after n-3 PUFA treatment of MC3T3-E1 osteoblasts. It was speculated that EPA might achieve the stimulatory effect of EPA on ALP activity via inhibition of PGE_2 production.[223]

2.11 Prostaglandins in bone

With the exception of the red blood cell, prostaglandins are produced and released by nearly all mammalian cells and tissues, including bone.[170] Prostaglandins are considered local hormones that are not stored in cells but are synthesised and released immediately as required. Compounds of the 2-series of prostaglandins derived from AA are the principal prostaglandins in humans and are considered biologically most significant.[170] (Refer to figure 2.15). Although several prostaglandins are produced by osteoblasts, PGE_2 is the major prostaglandin produced by these cells.[228,229]

AA is stored in cell membrane phospholipids and release is brought about mainly through the actions of the hydrolase phospholipase A_2 (PLA_2). Cytosolic PLA_2 ($cPLA_2$) is constitutively expressed in bone cells, and many agents such as cytokines and growth factors increase levels thereof.[153] After AA has been released from membrane phospholipids, it is converted to prostaglandin endoperoxide G_2 (PGG_2) by prostaglandin G/H synthase (PGHS) in a cyclooxygenase reaction, and then reduced to prostaglandin endoperoxide G_2 (PGH_2) by PGHS in a peroxidase reaction.[170]

Two enzymes for PGH_2 encoded by separate genes have been identified. Cyclooxygenase-1 (COX-1) (also known as PGHS-1), is constitutively expressed in most tissues and performs a 'housekeeping' function to synthesise prostaglandins which regulate normal cell activity.[230] Cyclooxygenase-2 (COX-2) (also known as PGHS-2), is generally only expressed at very low levels in most tissues but can be rapidly and transiently induced to high levels by multiple factors e.g., chronic inflammation,[155] nitric oxide,[231] PGE_2 itself[232] and growth factors and cytokines such as TGF-α and IL-1.[228] Compared to COX-2, COX-1 is only moderately affected when stimulated with hormones and growth factors.[228] It has also been reported that COX-1 requires higher concentrations of AA for its optimal function than does COX-2, implying that the amount of AA supplied by $cPLA_2$ critically influences which COX enzymes are utilised.[233] Although COX-1 and COX-2 are both found in the endoplasmic reticulum as well as the nuclear envelope, COX-2 is more highly concentrated on the nuclear membrane than COX-1.[234]

2.11.1 Prostaglandin receptors

Following their intracellular synthesis, prostanoids exit the cell, act on the parent cell and/or neighbouring cells in an autocrine and/or paracrine fashion through specific prostanoid receptors, thereby affecting changes in the levels of second messengers.[170] PGE receptors belong to the G protein-coupled seven transmembrane domain family of receptors. There are at least four distinct receptors for PGE_2 with differential signaling pathways: EP_1 with 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. MC3T3-E1 mouse osteoblastic cells for instance have been shown to predominantly express EP_1 and EP_4 receptors,[229] primary cultures of murine osteoblasts express EP_2 and EP_4 receptors and human mesenchymal stem cells express EP_4 receptors.[149]

2.11.2 Regulation of prostaglandin production in bone

Prostaglandins are amongst the most important local factors in bone and their production is under the control of many hormones, such as the sex hormones, PTH, glucocorticoids and $1,25(OH)_2D_3$.[237] (Refer to 2.9.2). Other cytokines and local factors such as IL-1, TNF-α, and TGF-β also regulate PG production.[134,204,238,239]

2.11.2.1 Stimulation of prostaglandin production in bone

Many of the important regulators of bone metabolism under both physiological and pathological conditions have been shown to stimulate prostaglandin production:

Systemic hormones

PTH and PTH-related peptide are potent stimulators of PGE_2 secretion in cultured neonatal rat calvaria[237] (refer to 2.5.2) and it has been shown that PTH induces

COX-2 expression with little or no effects on COX-1 or $cPLA_2$.[240] Thyroid hormone and $1,25(OH)_2D_3$ also exhibit stimulatory effects on PGE_2 production.[240-242]

Auto-amplification of prostaglandin E_2

PGE_2 is known to enhance its own production by inducing COX-2 in bone[243-246] and it has been shown by Suda *et al* (1998) that this auto-amplified production is mediated via the EP_1 subtype of PGE receptors in mouse MC3T3-E1 osteoblasts.[232] It was suggested that PGE_2-auto-amplification could be important in extending the otherwise short-lived action of this prostaglandin in certain physiological conditions such as mechanical stress and fracture healing.[232] Recently, Sakuma *et al* (2004) demonstrated that PGE_2 is an inducer of COX-2 in cultured primary murine osteoblasts and attributed it to cAMP-dependent PKA activation involving the activation of both EP_2 and EP_4 receptors in this model.[246]

Cytokines and growth factors

Cytokines such as IL-1 and TNF-α are important local factors in bone metabolism and mediators in inflammatory processes. They are thought to play a role in bone loss associated with oestrogen withdrawal.[247] IL-1, IL-6 and TNF-α have been shown to stimulate PGE_2 production largely by stimulation of COX-2 expression[157,228,248,249] in osteoblastic and stromal cell cultures as well as cultured rat calvaria.[133,152,157,231,239,250] IL-1α is regarded as one of the most potent bone-resorbing factors involved in bone loss that is associated with inflammation. A recent study by Tanabe *et al* (2005) reported that IL-1α stimulates the formation of osteoclast-like cells via an increase in M-CSF and PGE_2 production as well as a decrease in OPG production by osteoblasts.[247]

IL-1 and TNF-α induce both COX and nitric oxide synthase, which results in the release of prostaglandins and nitric oxide (NO), respectively. Kanematsu *et al* (1997) demonstrated that NO could be involved in the increased production of PGE_2 through stimulation of COX pathways in murine MC3T3-E1 osteoblastic cells.[231] These researchers suggested that the interaction between NO and the COX

pathways might play an important role in the regulation of osteoblastic functions under physiological as well as pathological conditions.[231]

Growth factors such as TGF-α and TGF-β stimulate PGE_2 synthesis in neonatal mouse calvarial cell cultures[250,251] and mouse osteoblastic cells.[228] Furthermore, the stimulatory effect of IL-1 on PGE_2 biosynthesis is synergistically enhanced by the presence of fibroblast growth factor-1 in MG-63 cells.[249] It has also been shown that cell density affects PGE_2 production, as subconfluent cells displayed a greater reponse to IL-1 than confluent cultures, which could be associated with reduced IL-1 receptor expression in these confluent cultures.[249]

2.11.2.2 Inhibition of prostaglandin production in bone

Hormones

It has been shown that oestrogen and androgens inhibit PGE_2 production in primary osteoblasts, human osteosarcoma osteoblasts and organ culture,[106] while cortisol inhibits PGE_2 production in neonatal rat calvaria.[134,237] Glucocorticoids inhibit COX-2 mRNA and protein expression, which accounts for much of their effects on PG production in bone and other tissues.[153]

Pharmacological blockers

Nonsteroidal anti-inflammatory drugs (NSAIDs) inhibit prostaglandin synthesis. A well known example of this class, indomethacin, reversibly inhibits prostaglandin synthesis by competing with the substrate AA for the active site of the enzyme, thereby blocking both COX-1 and COX-2 activity.[230,252] NS-398, on the other hand, is known to selectively block only COX-2 mediated prostaglandin production.[252,253] Steroidal anti-inflammatory drugs, such as bethamethasone block prostaglandin synthesis by inhibiting PLA_2 activity, thereby interfering with mobilisation of AA.[254] It has also been demonstrated that the synthetic glucocorticoid dexamethasone inhibits prostaglandin synthesis.[140,214,255,256]

2.11.3 *In vitro* effects of prostaglandin E_2 on bone

PGE_2 is a potent modulator of bone remodeling and influences both bone resorption[136,137,257] and formation.[143,149,175] The effects of PGE_2 on bone are complicated and depend on the duration of exposure, concentration of PGE_2, and animal model or cell type.

Prostaglandins are believed to exert their divergent actions via different membrane receptors on the surface of the target cells.[236] Pharmacological and morphological data indicate that EP_1 promotes cell growth and suppresses cell differentiation, whilst EP_2 and EP_4 are responsible for decreased cell growth and increased osteoblastic differentiation.[229] In addition, it has been shown that EP_4 stimulates osteoblastogenesis and thereby stimulates *de novo* bone formation.[258] EP_2 receptors apparently have a major influence on biomechanical properties of bone in mice. It has been shown that absence of EP_2 receptors in the murine EP_2 knockout model result in weak bone strength properties compared to wild-type control mice.[259]

2.11.3.1 In vitro effects of prostaglandin E_2 on bone formation

The effects of *in vivo* PGE_2 administration on bone parameters were reported in a variety of animal models as previously described. However, prostaglandins could also affect bone formation through effects thereof on the circulation, tissue metabolism or the formation of intermediary factors.[204] It is therefore important to also investigate *in vitro* effects of PGE_2 on bone.

Prostaglandin E_2 effects on osteoblastic differentiation

Zwang *et al* (2002) investigated mineralisation in bone marrow stromal cultures obtained from COX-$2^{-/-}$ and wild type mice and reported that bone nodule formation was severely reduced in the knockout mice cultures.[224] Addition of PGE_2 to these cultures, however, completely reversed the defective osteogenesis, thereby demonstrating that COX-2-mediated PGE_2 synthesis is required for mesenchymal cells to differentiate into mineralising osteoblasts.[224] A recent report indicated that

activation of the EP_4 receptor by PGE_2 enhances differentiation of osteoblast progenitor cells.[260] Importantly, Zwang *et al* (2002) also suggested that the transcription factors Cbfa-1 and osterix are regulated by COX-2 via PGE_2, and that decreased expression of these transcription factors, necessary for bone formation, may contribute to defective bone repair in COX-2 knockout mice.[224] The importance of PGE_2 on osteoblastic differentiation was confirmed by demonstrating that PGE_2 activation of the EP_4 receptor enhances bone formation through induction of both Cbfa-1 and BMP-2 expression.[149,258]

Prostaglandin E_2 effects on osteoblast proliferation

Depending on the model and concentration of PGE_2 used, disparate results were reported on the effects of PGE_2 on osteoblastic proliferation. It has been shown that prostaglandins stimulate proliferation in less differentiated bone cells such as preosteoblasts.[261] In a later study, Woodiel *et al* (1996) reported an anabolic effect of PGE_2 on replication and differentiation in cultured fetal rat calvarial cells and concluded that these effects were likely to be mediated by an EP_2 receptor, which stimulates cAMP-dependent activation of PKA.[262] Exposing human osteoblastic osteosarcoma cells and primary neonatal mouse calvarial osteoblasts to relatively low PGE_2 concentrations inhibited proliferation of these cells.[263-265]

Biphasic growth effects of PGE_2 have also been reported. In MC3T3-E1 cells, lower PGE_2 concentrations inhibited proliferation while higher concentrations were shown to stimulate proliferation slightly.[266] In contrast, a dose-related biphasic effect has been reported in cultured human bone cells with stimulation at 10^{-9} M and inhibition at 10^{-6} M.[267] It was suggested that the stimulation of proliferation by low doses of PGE_2 in this model is mediated by an enhancement of phospholipase C, which results in both an increase in PKC activity and an increase in intracellular calcium influx.[267]

Prostaglandin E_2 effects on in vitro bone formation

Biphasic effects of PGE_2 on bone formation have been reported in cultured fetal rat calvariae. Doses of PGE_2 equal to, or greater than 1 μM inhibit collagen synthesis whilst physiological concentrations have stimulatory effects on bone formation.[146] Raisz and Fall (1990) showed that addition of PGE_2 reversed the inhibitory effects of cortisol on collagen synthesis to levels above untreated cultures and suggested that PGE_2 could be regarded as a local stimulator of bone formation, which could mediate responses to local stress.[146]

The mechanisms whereby PGE_2 affect bone formation are not clear but it has been shown in organ cultures of fetal rat calvariae and neonatal mouse calvariae that PGE_2 stimulates bone formation by increasing osteoblast numbers.[268] Scutt and Bertram (1995) demonstrated the existence of two populations of osteoblastic precursors, one highly adherent and the other non-adherent, in a rat bone marrow cell model.[269] They concluded that the transition between the non-adherent and adherent phenotypes could be PGE_2-mediated thereby explaining some of the anabolic actions of PGE_2 on bone.[269] PGE_2 has also been shown to enhance the production of local growth factors such as IGF-I, BMP-7 and BMP-2 in bone.[147-149] Paralar (2002) concluded that induction of bone formation upon systemic treatment with PGE_2 could in part, be due to local induction of growth factors.[148]

Prostaglandin E_2 effect on osteoblastic alkaline phosphatase (ALP) activity and mineralisation

Alkaline phosphatase (ALP) is a membrane-bound ectoenzyme that can hydrolyse organic phosphates on the outer surface of the cell.[41] An increase in ALP activity reflects the maturation from an earlier to a more mature stage of osteoblast differentiation. The level of ALP is therefore used in *in vitro* experiments as a marker of osteoblast differentiation and bone formation.[41] Divergent effects of PGE_2 on mineralisation properties have been reported, often in the same cell line. It has been shown that when endogenous PGE_2 was blocked by indomethacin in MC3T3-E1 cells, the maximal ALP activity was significantly increased, suggesting that PGE_2 suppresses ALP activity.[226,270,271] The impact of PGE_2 on ALP activity is confirmed

by the observation that exogenous PGE_2 significantly suppresses ALP activity in rat osteoblastic cells[272] as well as murine MC3T3-E1 osteoblastic cells.[231,266,271]

Biphasic effects of PGE_2 on ALP activity and bone formation have also been reported.[146,229] Low PGE_2 concentrations have been shown to stimulate ALP activity, whilst higher concentrations inhibit ALP activity and it was concluded that this effect could probably be due to the presence of multiple EP receptors.[229] Kanematsu *et al* (1997) speculated that low PGE_2 concentrations might stimulate ALP activity through EP_4 whilst high PGE_2 concentrations inhibit ALP activity through EP_1.[231]

Exposing cultured adult rat calvarial cells to PGE_2 stimulated the formation of mineralised bone nodules.[273-275] It was concluded that PGE_2 inhibits proliferation and stimulates differentiation of these calvarial osteoblasts by elevating the $[Ca^{2+}]_I$ through the activation of a phosphoinositide turnover.[275] Ho *et al* (1999) showed that PGE_2 stimulated ALP activity and type I collagen synthesis in rat osteoblasts in culture during the early stages of differentiation, implying that PGE_2 may be involved in the earlier stages of bone matrix maturation and subsequent bone mineralisation.[265]

2.11.3.2 Prostaglandin E_2 effects on bone resorption

PGE_2 is the most abundant prostanoid among prostaglandins in bone and has been believed to be the most potent bone resorber.[134,238] The ability of several hormones and cytokines to regulate bone resorption is to some degree dependent on endogenous prostaglandin synthesis.[231,237,239,276,277]

It has been demonstrated that prostaglandins, especially PGE_2 and PGE_1, stimulate resorption by recruitment of osteoclasts.[135,136] In addition, PGE_2 stimulates osteoclast-like cell formation and bone resorbing activity in mouse bone cell cultures, presumably through mechanisms involving osteoblasts.[240,257,277] It has been shown that PGE_2 stimulates osteoclast formation in bone marrow cultures,[135-137] increases expression of mRNA for RANKL[138] and down-regulates OPG in cultures of primary human bone marrow stromal cells.[139] These reported effects of PGE_2 on OPG and RANKL will ultimately have a detrimental effect on the OPG/RANKL ratio in the bone

microenvironment and could ultimately lead to a decrease in bone mass as previously described. Furthermore, it has been shown that COX-2 expression and the associated PGE_2 production are necessary for maximal resorption responses to $1,25(OH)_2D_3$ and PTH in marrow cultures from COX-$2^{-/-}$ knockout mice.[240,277]

PGE_2 exerts its actions via different PGE receptors on the surface of the target cells. Making use of different EP knock-out mouse models it has been shown that PGE_2 stimulates the formation of osteoclast-like cells *in vitro* and subsequent bone resorption by a cAMP-dependent mechanism via the EP_2[278] and EP_4 receptors.[279] These results were confirmed by others, who employed EP agonists[280] and antagonists[278,281] to show 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.

The effects of PGE_2 on osteoclastogenesis, however, are complicated and it has been shown that prostaglandins may modulate the process of bone resorption in three ways; 1) through osteoblasts via RANKL, as previously described 2) through osteoclast precursors and 3) through mature osteoclasts.[240] A study by Wani *et al* (1999) showed that apart from the osteoblast-mediated effect of PGE_2 on osteoclastogenesis, 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] This response depended on the presence of exogenous soluble RANKL, as PGE_2 alone had no effect.[282] It has recently been shown that the direct PGE_2-mediated osteoclastogenic effect is brought about mainly through EP_2 and EP_4 receptors on osteoclast precursors.[283] The effect of PGE_2 on *mature osteoclasts* might be an inhibitory mechanism that could oppose the activating effect of increased osteoclastogenesis.[257] This suggestion has been supported by the discovery that PGE_2 directly stimulates outwardly rectifying Cl^- channels by activation of a cAMP-dependent pathway through EP_2 and, to lesser degree EP_4 receptors, in rat osteoclasts. This pathway has been shown to contribute to the reduction of osteoclast cell area and loss of osteoclast motility, which is likely to reduce bone resorption.[284] Figure 2.17 represents a schematic diagram of the putative roles for prostaglandins in bone resorption.[240]

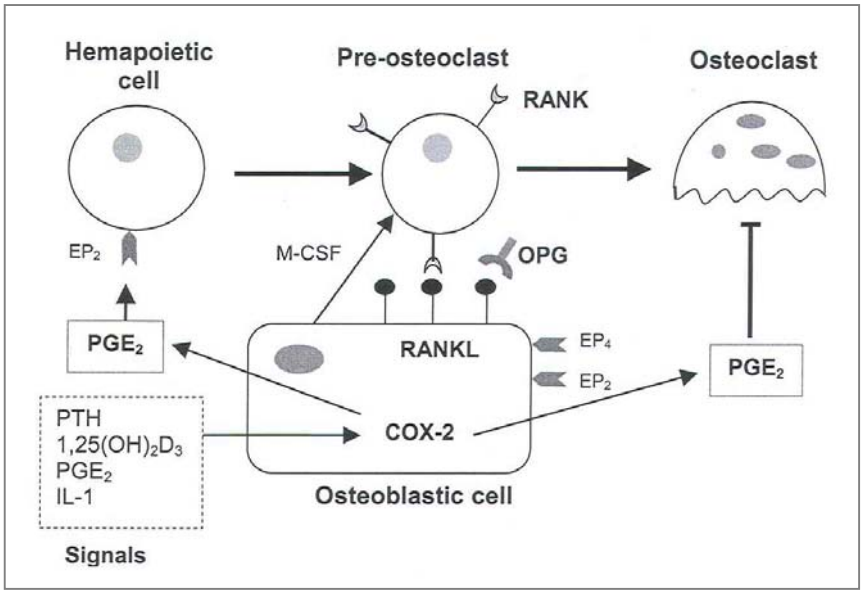


Figure 2.17. Schematic diagram of the putative roles of prostaglandin E_2 in bone resorption.

Prostaglandins have positive effects on formation of osteoclasts but may inhibit osteoclastic activity. Osteoblastic stromal cells express both COX-2 and RANKL. Interaction of RANKL with RANK, which is expressed by osteoclastic precursor cells, is required for precursor cells to differentiate into mature osteoclasts. COX-2 derived prostaglandins stimulate expression of RANKL, and enhance the stimulation of RANKL by other agonists. Prostaglandins can have a transitory effect on the activity of isolated mature osteoclasts. *(Reproduced with permission from Okada Y, Pilbeam C, Raisz LG, Tanaka Y. Role of cyclooxygenase-2 in bone resorption. J UOEH 2003;25:185-95.)*[240] *Copyright (2003) the UOEH.*

2.12 Summary

Systemic hormones and local factors such as eicosanoids, growth factors and cytokines, produced by bone, regulate the activity of bone formation and bone resorption. *In vitro* and animal data suggested that the effects of the PUFAs on bone could largely be mediated through modulation of PGE_2 production. Evidence from experimental studies have suggested that PGE_2, that derives from AA, may have a biphasic, dose-dependent effect on bone formation; stimulatory at low concentrations but inhibitory at higher concentrations.[40,146] It has also been shown that PGE_2 at low levels, may increase the production and action of major bone-derived growth factors such as IGFs[147] that are known to be powerful growth stimulators for bone.[151] High concentrations of PGE_2 on the other hand, have been shown to be associated with bone resorption.[257,277] Dietary supplementation of n-3 PUFAs such as EPA and DHA

inhibits PGE_2 synthesis[40] thereby protecting bone from the effects of high PGE_2 concentrations and could therefore be beneficial for bone. Watkins *et al* (2001) diagrammatically summarised the effects of PUFAs and PGE_2 on bone loss (Figure 2.18).[40]

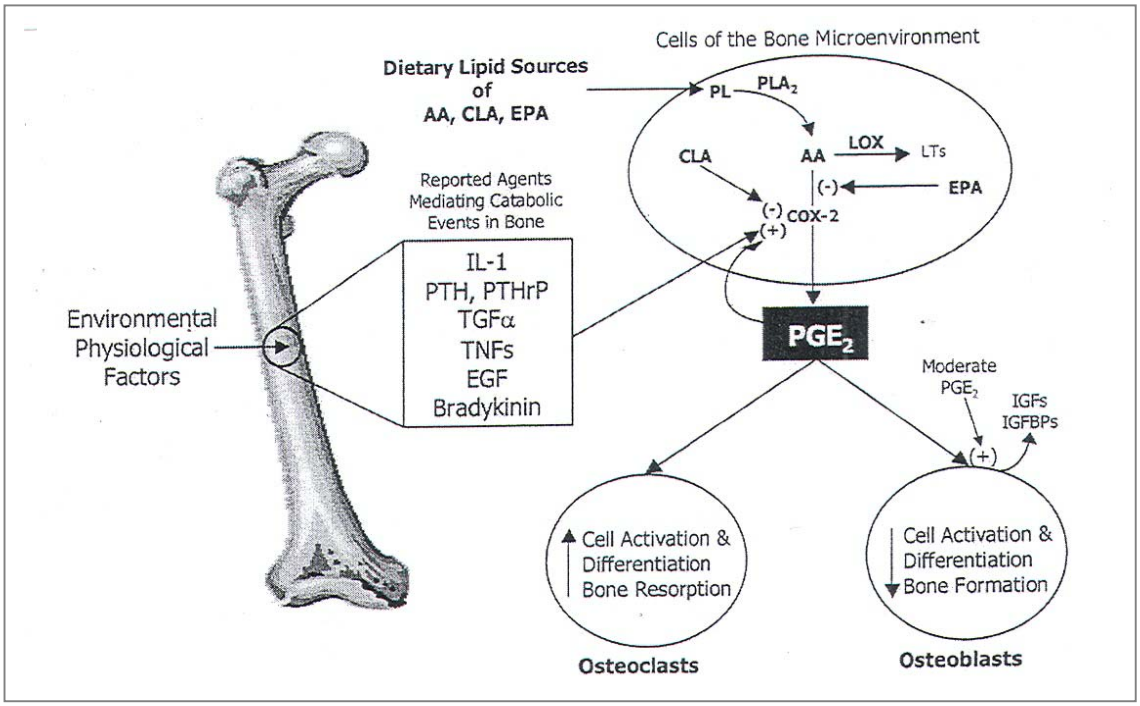


Figure 2.18. Speculative illustration of the possible effects of polyunsaturated fatty acids on bone loss.

This figure illustrates how CLA and EPA can decrease PGE_2 biosynthesised as a result of COX-2 induction by bone resorbing signals. The dietary fatty acids CLA and EPA offer novel opportunities to control potential detrimental effects of excess COX-2 derived PGE_2 on bone metabolism. (Abbreviations: CLA, conjugated linoleic acids; DGLA, dimomo-γ-linolenic acid; AA, arachidonic acid; EPA, eicosapentaenoic acid; COX-2, inducible cyclooxygenase; IGF/IGFBP, insulin-like growth factors/ IGF binding proteins.) *(Reprinted from Watkins BA, Lippman HE, Le Bouteiller L. Li Y, Seifert MF. Bioactive fatty acids: role in bone biology and bone cell function. Progr Lipid Res 2001;40:125-48.[40]) Copyright (2001), with permission from Elsevier.*

CHAPTER 3

General Cell Culture Procedures

This chapter deals with cell culture maintenance and general methodology. Chemicals and reagents used were of analytical grade and purchased from Sigma Chemical Co (St. Louis, MO, USA). Specialised reagents, kits and apparatus and the suppliers thereof are cited in the text. Detailed accounts of specific experimental protocols are given in the appropriate chapters.

3.1 Cell cultures

3.1.1 Cell lines

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.

3.1.2 Maintenance of cell cultures

Cells were grown at 37ºC in a humidified atmosphere containing 5% CO_2 in a Forma Scientific incubator (USA). Growth medium was replaced with fresh medium at one to three day intervals. All cell cultures were propagated in large tissue culture flasks until a confluent monolayer had formed. When confluent, the medium was discarded and the cells rinsed with phosphate-buffered saline (PBS). Cells were detached from the tissue culture flask by adding 3 ml 10% trypsin/EDTA (disodium ethylene diaminetetraacetate) for 2-5 minutes at 37ºC and gently shaking the flask. Three milliliters growth medium (see below) was then added to neutralise the trypsin. The

trypsin solution was removed by centrifugation at 200g for five minutes. Cells were subsequently resuspended in fresh medium and seeded into new flasks or cell culture cluster plates. When required, surplus cells plus 1.5 ml of the appropriate freeze medium per cryotube were frozen at -70ºC. The cooling rate during the freezing process is estimated at ± 1ºC/min. Cell lines were kept in continuous culture or frozen at -70ºC. Growth media and freeze media were used as recommended by the suppliers of the various cell lines. The composition of growth culture media and freeze media for the respective cell lines is given in Table 3.1.

Aseptic techniques were applied throughout, with all work being carried out in an Labotec laminar airflow cabinet (Labotec (Pty)Ltd., Halfway House, SA). All solutions used for cell culture maintenance or freezing were sterilised by passage through a 0.22 µm filter. Non-sterile equipment was steam sterilised in a Butterworth auto-clave (England).

Table 3.1. Composition of maintenance culture media and freeze media used.

Maintenance growth media	
MG-63 cells	90% Dulbecco's modified Eagle's medium (DMEM) 10% heat-inactivated fetal calf serum (FCS) 2 mM L-glutamine gentamycin (25 µg/ml)
MC3T3-E1 cells	90% alpha modification of Eagle's minimal essential medium (α-MEM) 2 mM L-glutamine 10% heatinactivated FCS gentamycin (25 µg/ml)
Freeze media	
MG-63 cells	95% DMEM 5% dimethylsulphoxide (DMSO)
MC3T3-E1 cells	70% α-MEM 20% heat-inactivated FCS 10% DMSO

3.1.3 Choice and preparation of cell culture media

Growth media recommended for cell culture maintenance and experimental conditions differ widely. The suppliers of MC3T3-E1 cells recommend the use of α-MEM for culturing, however, others use DMEM successfully.[252]

3.1.3.1 Preparation of cell culture media for oestrogen exposure

Although it is controversial whether the pH indicator phenol red has oestrogenic activity[286] or not[287] it was decided to use culture media without phenol red for all experiments investigating the effects of exposure of cells to low oestrogen levels. Whilst phenol red free α-MEM was not readily available and MC3T3-E1 and MG-63 cells both tolerated DMEM well (as tested in our laboratory), it was decided to use phenol red free DMEM, supplemented with 2 mM L-glutamine, for experiments investigating effects of oestrogen.

Using an adaptation of Horwitz et al's (1987) method,[288] dextran-coated charcoal was used to remove endogenous oestrogens from FCS as follows:
One gram dextran-coated charcoal was added to 100 ml FCS. The mixture was then placed in a shaking water bath at 45ºC for 30-45 minutes, keeping the charcoal in suspension. At the end of incubation the charcoal was removed by centrifugation (1500-2000 rpm for 30-45 minutes) and the supernatant carefully poured off. After cooling to room temperature, the FCS was filtered by passage through a 0.22 µm filter into sterile containers and stored at -20ºC until needed.

3.1.3.2 Media for growth (proliferation) studies

Ascorbic acid has been shown to stimulate proliferation of MC3T3-E1 cells.[289] As α-MEM contains ascorbic acid in its formula, it was decided to rather use DMEM, which is ascorbic acid free, for all experiments investigating proliferation. Fetal calf serum contains various growth factors, which reportedly also affect cell proliferation.[289,290] To limit the proliferative effect of high FCS levels, FCS content in

the culture media was limited to 5%. To minimize any differences in culture conditions, both MC3T3-E1 and MG-63 cell lines were treated in the same way.

3.1.3.3 Osteogenic supplemented media

It is customary to use osteogenic supplemented media when investigating mineralisation properties of cells.[291-293] This supplemented medium contains ascorbic acid, ß-glycerophosphate as well as the synthetic glucocorticoid dexamethasone. It was, however, decided to omit dexamethasone, as it was proven in rat bone marrow stromal cells that dexamethasone per se acts at multiple points in the differentiation process to stimulate osteoblastic maturation.[53,294-296] It has also been suggested that dexamethasone inhibits prostaglandin synthesis.[140,214,255,256] Since PGE_2 is a down-stream product of AA investigated in this study, it is important not to interfere with PGE_2 production. When an osteogenic medium was required, α-MEM supplemented with 50 μg/ml L-ascorbic acid and 10 mM ß-glycerophosphate was used.[291]

3.1.4 Trypan blue exclusion test for cell viability

Whenever cells were seeded for experiments cell viability was determined by making use of the trypan blue exclusion test. Cells in logarithmic growth phase (refer to figure 3.1)[297] were dissociated and stained 1:1 with 0.2% trypan blue in Hanks' balanced salt solution (Hanks' BSS). Unstained, and thus viable, cells were counted with a haemocytometer. Ten µl of the cell suspension was introduced under the slip of the haemocytometer on each side and cells in the eight 1 mm^2 squares were counted. The concentration of cells per ml of cell suspension was calculated by dividing the total by 4 and multiplying by 10^4.

3.2 Preparation of stock solutions

Table 3.2 indicates the preparation and storage conditions of compounds used throughout this study. Stock solutions were prepared according to the manufacturer's recommendations.

Table 3.2 Preparation and storage conditions of compounds used.

Compound	Preparation and storage of stock solution
Polyunsaturated fatty acids: Arachidonic acid (AA)* Docosahexaenoic acid (DHA)*	100 mg/ml in 100% ethanol. Aliquots stored at -70ºC in the dark.
Oleic acid*	100 mM in 100% ethanol. Aliquots stored at -70ºC in the dark.
PTH	10^{-4} M in distilled water + 0.1% bovine serum albumin (BSA). Aliquots stored at -70ºC.
Oestrogen (17-β estradiol)**	$2x10^{-3}$ M in DMSO. Kept at room temperature.
PGE_2 *	1 mg/ml in 100% ethanol. Stored at -20ºC.
PGE_2 antibody, lyophilised	Reconstituted in distilled water. Aliquots stored at -70ºC.
NS-398**	0.1 M in DMSO. Aliquots stored at -70ºC.
Indomethacin**	0.1 M in DMSO. Freshly prepared when needed.
Anti-COX-1 antibody, lyophilised	2500 μg/ml in distilled water. Aliquots stored at -70ºC.
Anti-COX-2 antibody, lyophilised	5000 μg/ml in distilled water. Aliquots stored at -70ºC.
Osteoprotegerin (OPG)	100 μg/ml in PBS + 0.1% BSA. Aliquots stored at -70ºC.
Anti-OPG capture antibodies	500 μg/ml in PBS. Aliquots stored at -70º C.
Anti-OPG detection antibodies	50 μg/ml in tris-buffered saline (TBS) + 0.1% BSA. Aliquots stored at -70ºC.
Ascorbic acid	50 mg/ml in sterile ddH_2O. Aliquots stored at -70ºC.
$1,25(OH)_2D_3$ (Calcitriol)*	50 mg/ml in 100% ethanol. Aliquots stored at -70ºC in the dark.

*** When diluted, ethanol concentrations in cell culture medium did not exceed 0.2% (v/v).**

****When diluted, DMSO concentrations in cell culture medium did not exceed 0.05% (v/v).**

Ethanol (final concentration 0.2%) and DMSO (final concentration 0.05%) per se had no toxic effects on cells tested in our laboratory.

3.3 Standardisation of a method for quantification of cell number

Cells seeded into culture do not divide initially. During this phase, known as the *lag-phase* of cell growth, cells are preparing for mitosis and there is little change in cell number. When cells divide, the increase in cell number is logarithmic. This phase is known as the *log phase* of cell growth. The final phase of cell growth is referred to as the *plateau* or *stationary phase.*[297] (Figure 3.1).[297]

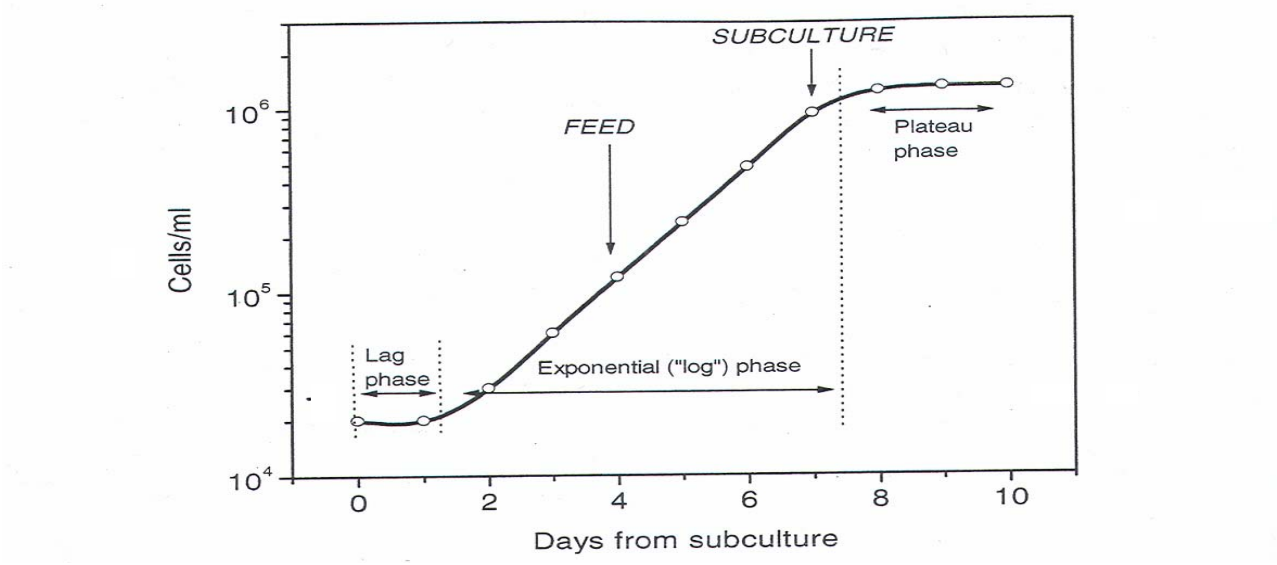


Figure 3.1 Growth curve and cell maintenance.

Semilog plot of cell concentration versus time from subculture, showing the lag phase, the exponential phase, and a plateau, and indicating times at which subculture and feeding should be performed. *(Freshney RI. Culture of animal cells: a manual of basic technique. 4th ed. New York: Wiley-Liss; 2000. p.184.)*[297] *©2000 (Wiley-Liss, Inc., A Wiley Company) Reproduced with permission of John Wiley & sons, Inc.*

For some experiments it is essential to quantify the number of cells in culture. By applying an adaptation of the crystal violet staining procedure[290,298,299] a method was developed to quantify cell number. Following trypan blue exclusion, MG-63 and MC3T3-E1 cells were seeded in quadruplicate at densities from 1.563 x 10^3 to 50 x 10^3 cells per well (in DMEM with 5% FCS) in 24-well cluster plates. After 24 hours with cells still in the lag phase, the experiment was terminated by fixing the cells in 300µl/well of 1% glutaraldehyde in PBS for 15 minutes. Thereafter 300µl/well crystal violet (1% in PBS) was added for 30 minutes. Subsequently the plates were

immersed in running tap water for 15 minutes. After the plates had dried, 500µl of 0.2% Triton X-100 was added to each well. The plates were then incubated at room temperature for 90 minutes and 150 µl of the liquid content transferred to 96-well microtiter plates. Absorbance (optical density) (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, which 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 (Figure 3.2). Intra and interassay variability was 4% and 11% respectively.

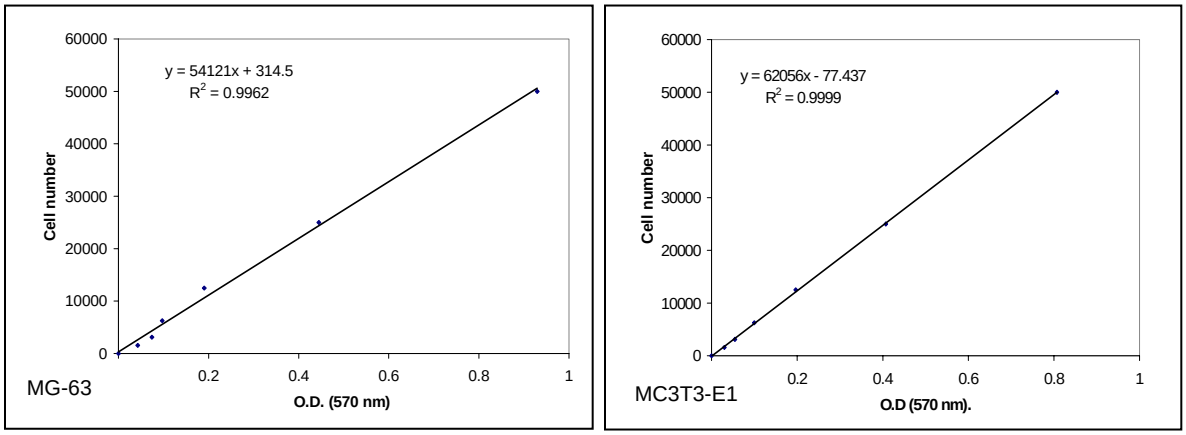


Figure 3.2 Correlation between crystal violet-derived absorbance and cell number.

Cells (MG-63 and MC3T3-E1) at densities ranging from 1.563 x 10^3 to 50 x 10^3 were seeded in 24-well cluster plates. After 24 hours the cultures were fixed with 1% glutaraldehyde, and subsequently stained with 1% crystal violet. Dye was extracted with 0.2% Triton X-100 and the absorbance read at 570 nm. Each data point represents the mean for quadruplicate cultures.

3.4 Prostaglandin E_2-related experiments

3.4.1 Radioimmunoassay (RIA) of prostaglandin E_2 in cell culture media

The amount of PGE_2 produced by cells and released into media was assessed using a competitive binding radioimmunoassay, adapted from a method described by Raisz and Simmons (1985).[237] In this assay, 100 µl unlabeled PGE_2 samples or

standards diluted in working buffer (0.01 M TRIZMA®BASE in 0.14 M NaCl, pH 7.6, containing 2 mg gamma globulin/ml) in duplicate were incubated overnight at 4ºC with radiolabeled PGE_2 ([^{3}H]-PGE_2, New England Nuclear™ Life Science Products, Inc., Boston, MA, USA) and PGE_2 antibody (provided by Dr Laurence Levine, Brandeis University, Waltham, Massachusetts, USA). The samples were put on ice and subsequently, 400µl dextran-coated charcoal (4 g/100 ml cold dH_2O) was added to each polystyrene tube. Free PGE_2 was removed by centrifuging for 15 minutes at 3000 rpm at 4ºC with a Rotixa 120R Hettich centrifuge (Tuttlingen, Germany). Thereafter, 400µl supernatant was transferred to a counting vial and 4.5 ml Liquiscent (NEN) scintillation cocktail (Packard Bioscience B.V., Groningen, The Netherlands) added. Samples were counted for 10 minutes each on the ^{3}H channel using a Beckman L55000CE Liquid Scintilation counter (Beckman instruments, Nuclear systems operations, Fullerton, CA, USA). The percentage of bound versus unbound [^{3}H]-PGE_2 was compared to a standard curve (region used for analysis: 150 to 1000 pg/ml) to calculate the amount of PGE_2 in the original samples (Figure 3.3). The intra and interassay coefficients of variation were 3% and 4.4% respectively.

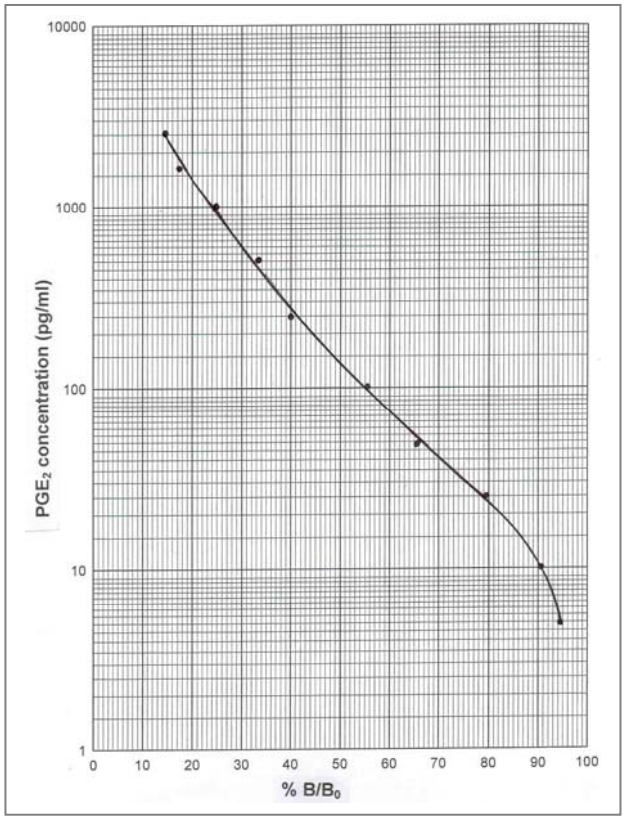


Figure 3.3 Example of a standard curve for the calculation of the amount of prostaglandin E_2 in cell culture samples using RIA.

3.4.2 Indirect immunofluorescence for detection of COX-1 and COX-2 in MG-63 cells

The presence of COX-1 and COX-2 in MG-63 cells after exposure to vehicle (0.2% ethanol) and AA were visualised by indirect immunofluorescence. Cells were seeded at 150 000/well onto heat-sterilised coverslips in 6-well culture plates. At the end of the experiment, after discarding the medium, cells were 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 five 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 four minutes and then rinsed thrice for five minutes with PBS. Primary antibody (25 μl mouse monoclonal antibody against human COX-1 or COX-2 (1:1000 in PBS))(Cayman Chemical Company, Ann Arbor, MI, USA) was added to each coverslip and incubated for 60 minutes at 37ºC in a humidified environment. Cells were rinsed thrice for five minutes with PBS. Subsequently, the cells were incubated with the secondary antibody (25 μl 1:100 biotin-conjugated anti-mouse IgG (Fab-specific, developed in goat) in Fluroisotiocianate-conjugate (FITC-conjugate) diluent) (Diagnostic and Technical Services, Johannesburg, SA.), for 60 minutes at 37ºC in a humidified environment. Following rinsing, cells were finally incubated with ExtrAvidin®-FITC conjugate (1:200 in FITC-conjugate diluent)(Sigma Chemical Co, St. Louis, MO, USA) for 60 minutes. The coverslips were then mounted with a glycerol-based mounting fluid after the final 3x5 minutes wash step. Photographs were taken with 400 ASA film on a fluorescent microscope (Nikon Optiphot microscope equipped with an episcopic-fluorescence attachment and an excitation-emission filter with an average wavelength of 495 mm for FITC).

3.5 Proliferation studies

Proliferation can best be evaluated over an extended period of time; therefore it was decided to evaluate the effects of the different agents over a 72 hour exposure period. Longer periods of time are not suitable as cells then often reach confluency, which causes contact inhibition.

MG-63 and MC3T3-E1 cells were seeded at 3000 cells/well in sterile 96-well culture plates (n=8). After 72 hours at the end of culture, after exposure to vehicle and test agents, proliferation was determined by making use of an adaptation of the crystal violet staining procedure.[300] Hundred µl crystal violet (1%, in PBS) per well was added to fixed cells for 30 minutes; thereafter the culture plates were immersed in running tap water for 15 minutes. After the plates had dried, 200 µl of 0.2% Triton X-100 was added to each well. The plates were incubated at room temperature for 90 minutes and subsequently 100 µl of the liquid content was transferred to 96-well plates. Absorbance (OD) 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. Colour intensity is an indication of DNA content, thus cell growth.[298] Results are presented as percentage relative to control. Three independent experiments (each conducted in eightfold) were conducted to validate this procedure.

3.6 Haematoxylin and eosin (H&E) cell staining

Previous studies detected morphological effects of PUFAs on cultured osteoblasts after 24 to 48 hours exposure.[301] Cells were therefore seeded onto heat-sterilised coverslips in 6-well culture plates. After 48 hours at the end of culture, after exposure to vehicle and test agents, the experiment was terminated by removing the coverslips from the cluster plates, inserting them into coverslip holders followed by exposure to Bouin's fixative (Accustan™ Bouin's solution, Sigma) for 30 minutes. Thereafter, cells were stained with haematoxylin and eosin (H&E) using the method of Kiernan (1990).[302]

Cells were left in 70% ethanol for one hour; rinsed with tap water; left in Mayer's haemalum for 15 minutes; rinsed in running tap water; rinsed with 70% ethanol and subjected to 1% eosine for two minutes. Thereafter, cells were dehydrated by rinsing twice for five minutes with each of the following: 70% ethanol; 96% ethanol and 100% ethanol. Finally, coverslips were rinsed twice with xylol for five minutes, mounted to microscope glass slides with rapid mounting resin (Entellan) and left to

dry. Photographs were taken with 400 ASA film with a Nikon camera (Nikon, Tokyo, Japan) attached to a Nikon Opthiphot microscope (Nikon, Tokyo, Japan).

3.7 Hoechst 33342 (HOE) and propidium iodide (PI) staining for detection of apoptosis

Various methods for the detection of apoptosis in cell cultures have been developed, including terminal deoxynucleotidyl Transferase Biotin-dUTP Nick End Labeling (TUNEL). HOE and PI staining for apoptosis detection has previously been standardised in our laboratory and was therefore applied in this study. Previous studies reported apoptotic effects within 24 to 48 hours of exposing cells to apoptotic agents.[42] Similar to H&E staining, testing for apoptosis was limited to 24 hours to 48 hours PUFA exposure.

Cells were seeded onto heat-sterilsed coverslips in 24-well cluster plates. At the end of culture, after treatment with vehicle and test agents, growth medium was discarded and the cells gently rinsed with PBS. Thereafter 500 μl/well Hoechst No 33342 (HOE) (0.5 μg/ml in PBS) was inserted into each well. After 30 minutes incubation at 37ºC, 125μl of a propidium iodide (PI) solution (0.5 mg/ml in PBS) was added directly to each well. Within five minutes, coverslips were mounted on microscope slides with mounting fluid (90% glycerol, 4% N-propyl-gallate, 6% PBS). Photographs were taken with 400 ASA film on a Nikon Optiphot microscope (Nikon, Tokyu, Japan) with UV-light and a blue filter.

While all cells take up HOE, only cells with intact cell membranes can exclude PI. Cells stained pink with PI are therefore classified as necrotic, while apoptotic cells only stain blue, indicating that these cells still have functional cell membranes capable of excluding PI, although they may have an aberrant appearance.[303]

3.8 Quantification of alkaline phosphatase (ALP) activity

Alkaline phosphatase (ALP) is a membrane-bound ectoenzyme that can hydrolyse organic phosphates on the outer surface of the cell. An increase in ALP activity reflects the maturation from an earlier to a more mature stage of osteoblast differentiation. The level of ALP activity is therefore used in *in vitro* experiments as a marker of osteoblast differentiation.[41] As $1,25(OH)_2D_3$ (vit D_3) induces cellular ALP activity,[241,292,299] 10^{-8} M vit D_3 was used as a positive control in some experiments.

At the end of the culture period, after treatment with vehicle and test agents, cells were washed with PBS and fixed in 300 μl/well 4% formaldehyde in PBS (v/v) for 15 minutes. Using a colorimetric assay, ALP activity was assayed as the release of ρ-nitrophenol (ρ-NP) from ρ-nitrophenylphosphate (ρ-NPP) substrate.[291] Fixed cells were incubated with 300 μl/well ALP assay buffer (5 mM ρ-NPP; 0.5 mM $MgCl_2$; 0.1% Triton X-100 in 50 mM TBS, pH 9.5) for 60 minutes at 37ºC. Thereafter 100 μl reaction product per well was transferred to a 96-well microtiter plate and 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 405 nm. Assay buffer was used as a blank. Cells were washed again with distilled water, and cell number determined using a standard curve established with crystal violet assay, as previously described. Three independent experiments were executed in quadruplicate. Specific ALP activity was quantified against a standard curve of 0-400 μM ρ-NP and expressed as nanomoles of ρ-NP per hour per 10^4 cells (Figure 3.4).

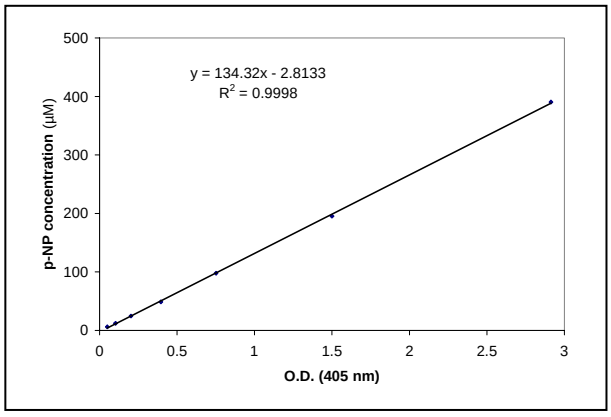


Figure 3.4 Standard curve for the calculation of alkaline phosphatase (ALP) activity in cell cultures using colorimetric para-nitrophenol hydrolysis.

3.9 Detection of mineralising properties

The terminal differentiation of osteoblasts *in vitro* is characterised by the formation of mineralising plaques.[304] To examine the capability of MG-63 cells and MC3T3-E1 cells to mineralise *in vitro*, formation of mineralising plaques were visualised by a modified von Kossa staining technique for phosphate detection (black) on fixed cell layers.[305,306]

Cells were rinsed thrice in 0.9% NaCl solution (pH 7.2) and fixed in 300 μl/well 4% (v/v) paraformaldehyde in 0.9% (w/v) NaCl solution at room temperature for 10 minutes. After fixation, the cells were rinsed thrice in ddH_2O, incubated with 400 μl/well 3% (w/v) $AgNO_3$ in the dark for 30 minutes, and exposed to ultraviolet light (254 nm) for 30 minutes. Cells were then washed again in ddH_2O and counterstained with Toluidine Blue for five minutes. Cells were then photographed making use of a Zeiss Axiovert 200 microscope attached to a Nikon DXM 1200 digital camera (Nikon, Tokyo, Japan). Alternatively, rinsing twice each with 70% ethanol, 96% ethanol, 100% ethanol and xylol cells were dehydrated. Coverslips were then mounted to microscope glass slides with resin, left to dry and photographed with a Nikon camera (Nikon, Tokyo, Japan) attached to a Nikon Optiphot microscope (Nikon, Tokyo, Japan) using 400 ASA film.

3.10 Assay of adipocytogenesis

3.10.1 Quantification of adipocytogenesis by Oil red O staining

Oil red O is a dye that is soluble in lipids and specifically stains triglycerides and cholesterol oleate but no other lipids. To determine whether osteoblasts accumulate lipid droplets in the cytoplasm when treated with PUFAs, an adaptation of the Oil red O staining procedure developed by Ramirez-Zacarias *et al* (1992) was performed on these cells.[307] Previous studies reported the accumulation of large quantities of triglycerides in the cytoplasm, after exposing osteoblasts to 100 μM oleic acid for a six-day period.[56]

At the end of the culture period, after treatment with vehicle and test agents, cells were rinsed with PBS, fixed in 300 μl/well 10% paraformaldehyde in PBS (v/v) for 15 minutes, and washed with PBS. Cells were then stained for 30 minutes with Oil red O by complete immersion in 300 μl/well working solution*. Thereafter, cells were rinsed briefly with 60% propanol, rinsed exhaustively with dH_2O and air-dried. In order to determine the extent of adipose conversion, 500 μl/well isopropanol was added to the stained cultures for 10 minutes. One hundred and fifty μl/well of the extracted dye was removed by gentle pipetting and transferred to a 96-well cluster plate. Using a GLR 1000 microplate reader (Genelabs Diagnostics, UK) absorbance was monitored spectrophotometrically at 490 nm. Isopropanol was used as blank. After washing with 70% ethanol, cell numbers were determined by crystal violet assay, as previously described. Three independent experiments were executed in quadruplicate to validate the assay.

*An Oil red O stock solution was prepared by dissolving 1.0 g of Oil red O in 100 ml isopropanol. After stirring overnight at room temperature, the solution was filtered through analytical filter paper. Oil Red O working solution was prepared by diluting the stock solution with dH_2O at a ratio of 7:3 and filtering twice before application.

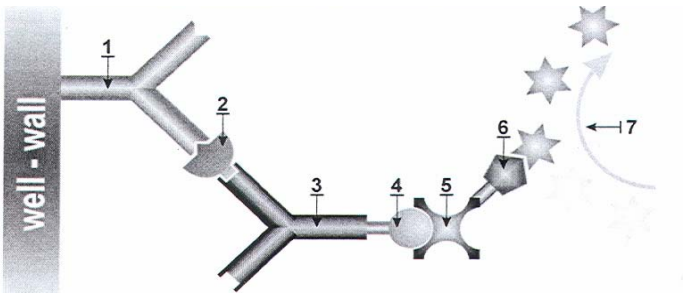
3.10.2 Microscopic visualisation of lipid accumulation

In order to visualise the accumulation of lipid droplets in cells after treatment with PUFAs, MG-63 and MC3T3-E1cells were seeded aseptically onto heat-sterilised coverslips in 6-well culture plates. At the end of culture, after exposure to vehicle and test agents, cells were rinsed with PBS, fixed in 10% paraformaldehyde in PBS for 15 minutes, and washed with PBS. The coverslips were then removed from the cluster plates, inserted into coverslip holders, stained with Oil red O for 30 minutes and rinsed briefly with 60% isopropanol. The cells were then counter-stained with hematoxylin for five minutes,[110] and mounted to microscope glass slides with rapid mounting resin and left to dry. Photographs were taken with 400 ASA film with a Nikon camera (Nikon, Tokyo, Japan) attached to a Nikon Optiphot microscope (Nikon, Tokyo, Japan). (Original magnification 400x)

3.11 Measurements of osteoprotegerin and RANKL secretion

3.11.1 Enzyme linked immunosorbent assay (ELISA) 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] The principle of the assay is explained in Figure 3.5.[308]



1 OPG capture antibody
2 Sample/standard
3 OPG detecting antibody
4 Peroxidase-conjugated streptavidin
5 TMB liquid substrate
6 Stop (H_2SO_4)
7 Enzyme catalyzed colour change

Figure 3.5 Principle of the osteoprotegerin ELISA assay.

(Reproduced with permission from Osteoprotegerin. Enzyme immunoassay for the quantitative determination of osteoprotegerin in EDTA plasma, heparin plasma, serum or cell culture supernatants. ELISA kit protocol. Cat. No. BI-20402. Biomedica Medizinprodukte GmbH&Co KG, Vienna.)[308]

A MaxiSorb microtiter plate (NUNC™ Brand Products, Roskilde, Denmark) was coated with 50 µl/well of 2µg/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 using 250µl/well PBS containing 1% BSA, 5% sucrose and 0.05% NaN_3 for one hour at room temperature. (**) Fifty µl/well of samples (diluted 1:100), 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) in duplicate were added and incubated for two hours at 37ºC. (**) Thereafter 50 µl/well of 0.2µg/ml biotinylated goat anti-human OPG detecting antibody (R&D Systems) was added. After two hours incubation at 37ºC (**), 50µl/well 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. (**) Two hundred µl/well 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 50µl/well of 0.9M H_2SO_4. Optical density was then 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 (Figure 3.6). (**) Between each step of the ELISA the plate was washed three times with PBS containing 0.05% Tween^{R}20. Intra and interassay variability was 5% and 11% respectively.

3.11.2 Enzyme linked immunosorbent assay (ELISA) quantification of osteoprotegrin 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) was used as detection antibody. Undiluted samples and standards (31.25 to 5000 pg/ml) (recombinant mouse OPG/Fc chimera) (R&D Systems) in duplicate were prepared as described above. Intra and interassay variability was 6.5% and 16% respectively.

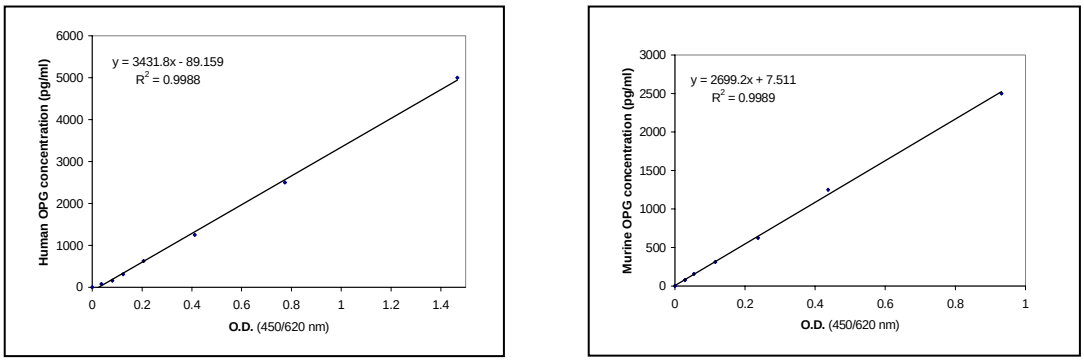


Figure 3.6 Examples of standard curves for the calculation of the amount of osteoprotegerin in the conditioned media from MG-63 cells and MC3T3-E1 cells.

3.11.3 Enzyme linked immunosorbent assay (ELISA) quantification of free soluble RANKL (sRANKL) 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. In short, recombinant murine OPG in duplicate 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 the 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.

3.12 Detection of oestrogen receptors in MG-63 cells

For the detection of oestrogen receptors, 250 000 MG-63 cells were seeded onto sterilised coverslips in 6-well plates and left to adhere for 24 hours. MCF-7 human breast carcinoma cells (Highveld Biological (Pty) Ltd, (Sandringham, SA)), as positive control,[309,310] were treated similarly. Cells were then fixed and permeabilised as previously described for COX-detection. Cells were incubated sequentially for one hour at 37°C in each of the following, with incubations separated by 3 x 5 minutes washing steps: 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). 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 five minutes and subsequently mounted with a glycerol-based mounting fluid. Photographs were taken with 400 ASA film with a Nikon camera (Nikon, Tokyo, Japan) attached to a Nikon Optiphot microscope (Nikon, Tokyo, Japan).

3.13 Statistics

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

CHAPTER 4

Effects of Arachidonic Acid, Oestrogen and Parathyroid Hormone on Prostaglandin E_2 (PGE_2) Production in MG-63 and MC3T3-E1 Osteoblast-like Cells

4.1 Introduction

Polyunsaturated fatty acids (PUFAs) as well as the bone modulating hormones, oestrogen (E2) and parathyroid hormone (PTH) have positive effects on bone homeostasis. Hormones may affect bone cell activity by stimulating or suppressing the release of locally acting factors such as the prostaglandins in the bone cell environment.[243]

PUFAs are converted via a series of desaturation and elongation steps to various longer chain PUFAs specifically arachidonic acid (AA) and eicosapentaenoic acid (EPA), which in turn can act as precursors for prostaglandins (PGs).[171] (Figure 2.15). Some studies suggested that PGs may mediate the anabolic effects of oestrogen in rats and mice,[311,312] while both PGs and oestrogen seem to target early osteoblast precursors in the bone marrow.[109] PTH induces PG synthesis by osteoblasts,[313,314] and PGs can induce bone resorption, an effect of PTH at physiological concentrations.[313] PTH may therefore exert some of its effects via induction of PG synthesis. Exposure of cultured mouse parietal bones to AA and PTH simultaneously amplified PG production compared to PTH exposure only.[315]

Two iso-enzymes, COX-1 (constitutive) and COX-2 (inducible) regulate PG synthesis (Figure 4.1). Two isoforms of the PGE synthase for PGE_2 biosynthesis have been cloned and characterized. Membrane-associated PGE synthase (mPGES) is thought to be an inducible enzyme whilst cytosolic PGE synthase (cPGES) is constitutively expressed.[316-318] cPGES is functionally coupled with COX-1 and mPGES is functionally coupled with COX-2.[149,233,317] PTH induces COX-2 expression with little

or no effects on COX-1 or $cPLA_2$.[240] PTH and PTH-related peptide are potent stimulators of PGE_2 secretion in cultured neonatal rat calvari.[237]

Following their intracellular synthesis, prostaglandins exit the cell, act on the parent cell and/or neighbouring cells in an autocrine and/or paracrine fashion through specific prostaglandin receptors thereby affecting changes in the levels of second messengers.[170] PGE receptors belong to the G protein-coupled seven transmembrane domain family of receptors. There are at least four distinct receptors for PGE_2 with differential signaling pathways: EP_1 with Ca^{2+} mobilization; EP_2 and EP_4 with stimulation of cAMP production; and EP_3 mainly with the inhibition of cAMP production.[235,236] Recently, Sakuma *et al* (2004), using primary cultures of murine osteoblasts, demonstrated that PGE_2 is an effective stimulator of cAMP production and an inducer of COX-2.[246] This effect was attributed to cAMP-dependent activation of protein kinase A and to be the result of the combined effects of activation of the EP_2 and EP_4 receptors.[246]

Nonsteroidal, antiinflammatory drugs (NSAIDs) inhibit synthesis of prostaglandins. A well known example of this class, indomethacin, reversibly inhibits prostaglandin synthesis by competing with the substrate AA for the active site of the enzyme, thereby blocking both COX-1 and COX-2 activity.[230,319] NS-398 on the other hand, is known to selectively block only COX-2 mediated prostaglandin production.[252,253] It is possible that modulation of PG synthesis in osteoblasts using selective NSAIDs may affect bone formation or resorption. Figure 4.1 illustrates the action of indomethacin and NS-398 on COX-1 and COX-2.

The purpose of the study was to determine whether AA alone or in combination with oestrogen and PTH modulates PGE_2 synthesis in osteoblast-like cells and to determine to what extent COX-1 and COX-2 contribute to the observed effects by employing specific blockers. DHA is not a substrate for prostaglandin synthesis[172] but could interfere with PGE_2 production by displacing AA in the cell membrane thereby limiting the amount of AA available for PGE_2 synthesis.[176] It has also been shown that DHA competes with AA for binding to COX-1 and therefore may be regarded as a strong competitive inhibitor for PGE_2 synthesis.[122] However, as PGE_2 synthesis is hardly detectable in basal (unstimulated) control conditions, the effect of DHA on PGE_2 synthesis was not investigated in our study.

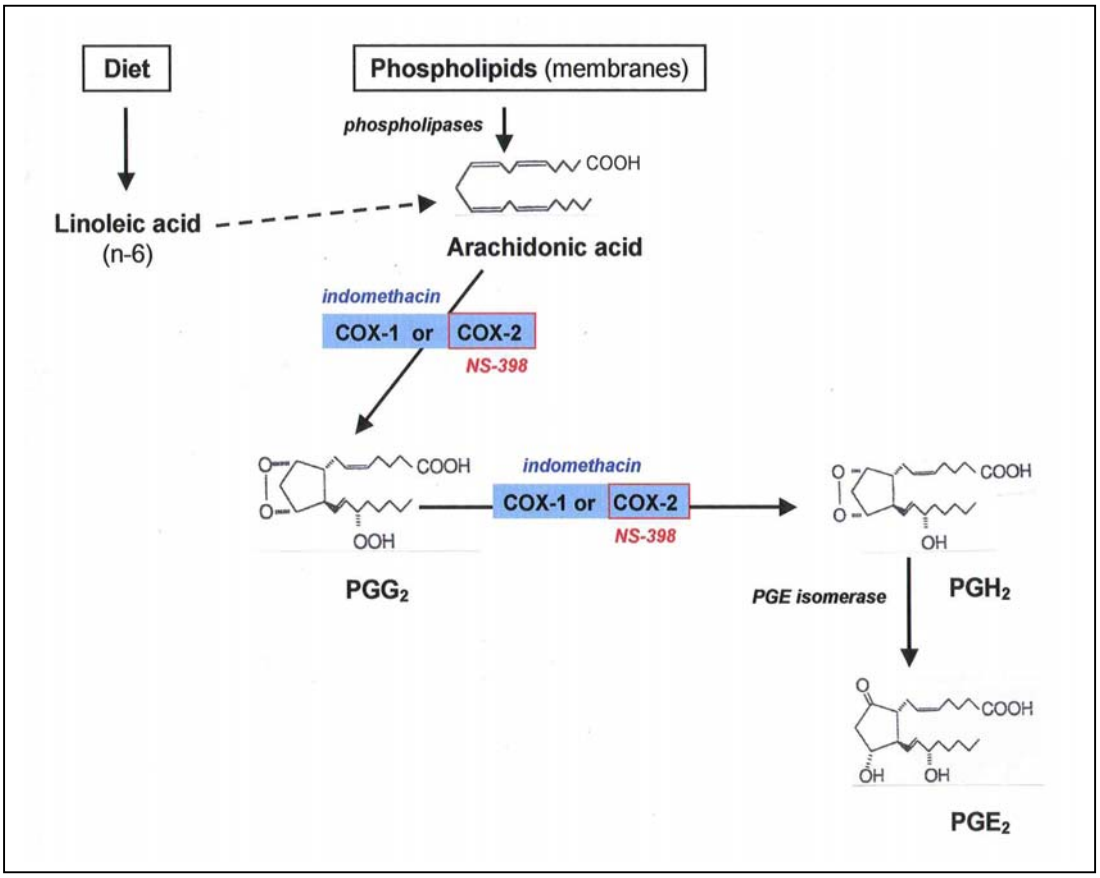


Figure 4.1. The prostaglandin pathway indicating the enzymes involved in the synthesis of prostaglandin E_2 from its substrate AA. Indomethacin is an unselective blocker of both COX-1 and COX 2, while NS-398 blocks only COX-2.

4.2 Materials and Methods

Reagents

Sigma Chemical Co (St. Louis, MO, USA) supplied DMEM, L-glutamine, crystal violet, trypan blue, arachidonic acid, oestrogen, parathyroid hormone fragment 1-34, indomethacin, gamma globulin, dextran-coated charcoal and PGE_2. Heat inactivated fetal calf serum (FCS) was obtained from Highveld Biological (Pty) Ltd. (Sandringham, SA). Gentamycin was supplied by Gibco BRL (Invitrogen Corp., Carlsbad, CA, USA). NS-398 was purchased from Cayman Chemical Company (Ann Arbor, MI, USA). All other chemicals were of analytical grade and purchased from Sigma Chemical Co (St. Louis, MO, USA).

Cell cultures

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.

Exposure of cell cultures to arachidonic acid, parathyroid hormone and oestrogen

Following trypan blue exclusion, MG-63 and MC3T3-E1 cells were seeded at densities of 40 000 cells per well in 24-well cluster plates. Cells were cultured in DMEM with 10% heat-inactivated FCS, as measurable PGE_2 is not produced by cells in serum-free media.[248] Regulation of PGE_2 biosynthesis in MG-63 cells is cell density-dependent. Subconfluent cultures displayed a greater response to cytokine stimulation than confluent cultures.[249] It therefore seems that signaling pathways resulting in PGE_2 biosynthesis are subject to complex regulation by cell-cell or cell-extracellular matrix interactions, therefore only subconfluent cell cultures were used in these experiments. After 24 hours, medium was changed and subconfluent cells exposed to vehicle (0.2% ethanol), PTH (10^{-7} M), oestrogen (E2) (10^{-6} M) or AA (20μg/ml) for four hours. Concentrations of the agents used, were determined from results of earlier dose-response trials performed in our laboratory. To determine the role of the PGHS enzymes COX-1 and COX-2 on PGE_2 synthesis, cells were incubated with the unselective COX-blocker indomethacin (1μM) or the COX-2 specific blocker NS-398 (0.1μM) which were added to the growth medium 45 minutes prior to the addition of AA and hormones.[248,253] Cell cultures were maintained 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). After 4 hours conditioned media were aspirated from cultured cells and stored at -70ºC until analysed. Following removal of medium, cell numbers were determined by crystal violet staining.[290,298] In short, cultures were fixed with 1% glutaraldehyde, stained with 1% crystal violet, and the dye extracted with 0.2% Triton X-100.

Absorbance (OD) 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, which 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 3.3). Three independent experiments were conducted in quadruplicate.

Radioimmunoassay (RIA) of prostaglandin E_2 in cell culture media

The amount of PGE_2 produced by the cells and released into the media was assessed using a competitive binding radioimmunoassay, adapted from a method described by Raisz and Simmons (1985).[237] In this assay, unlabeled PGE_2 samples or standards diluted in working buffer (0.01 M Tris HCl in 0.14 M NaCl, pH 7.6, containing 2 mg gamma globulin/ml) were incubated overnight at 4ºC with radiolabeled PGE_2 ([^{3}H]-PGE_2, New England Nuclear™ Life Science Products, Inc., Boston, MA, USA) and unlabeled PGE_2 antibody (provided by Dr Laurence Levine, Brandeis University, Waltham, Massachusetts, USA). The samples were put on ice and subsequently dextran-coated charcoal was added to each polystyrene tube. Free PGE_2 was removed by centrifugation for 15 minutes at 3000 rpm at 4ºC with a Rotixa 120R, Hettich sentrifuge (Tuttlingen, Germany). Thereafter, the supernatant was transferred to a counting vial and Liquiscent (NEN) scintillation cocktail (Packard Bioscience B.V., Groningen, The Netherlands) added. Samples were counted for 10 minutes each on the ^{3}H channel using a Beckman L55000CE Liquid Scintilation counter (Beckman Instruments, Nuclear Systems Operations, Fullerton, CA, USA). The percentage of bound versus unbound [^{3}H]-PGE_2 was compared to a standard curve to calculate the amount of PGE_2 in the original samples. (Refer to Figure 3.3). The intra and interassay coefficients of variation were 3% and 4.4% respectively.

Indirect immunofluorescence for detection of COX-1 and COX-2 in MG-63 cells

Following trypan blue exclusion, cells were seeded at a density of 200 000 cells per heat-sterilised coverslip in 6-well cluster plates and left to adhere for 24 hours. Fresh growth medium containing vehicle only (0.2% ethanol)(control) or 20 µg/ml AA was added to near confluent layers. Cells were exposed for four hours at 37ºC. At the end of the experiment, after discarding the medium, cells were fixed in 10% formaldehyde (v/v) in (2 mM EGTA in PBS) for 10 minutes at room temperature. The presence of COX-1 and COX-2 after exposure to AA were visualised by indirect immunofluorescence as follows:

The coverslips were transferred to a staining dish and rinsed three times with PBS at room temperature. Cells were then permeabilised in ice cold 97% methanol containing 2 mM EGTA in PBS at –20°C for 4 minutes whereafter they were rinsed thrice with PBS. Primary antibody (25 µl mouse monoclonal antibody against human COX-1 or COX-2 (1:1000 in PBS))(Cayman Chemical Company, Ann Arbor, MI, USA), was added to each coverslip and incubated for 60 minutes at 37ºC in a damp environment. After rinsing the cells with PBS, the cells were incubated with the secondary antibody (25 µl 1:100 biotin-conjugated anti-mouse IgG (Fab-specific, developed in goat) in FITC-conjugate diluent))(Diagnostic and Technical Services, Johannesburg, SA.), for 60 minutes at 37ºC in a damp environment. After rinsing, cells were finally incubated with ExtrAvidin®-FITC conjugate (1:200 in FITC-conjugate diluent) (Sigma Chemical Co**,** St. Louis, MO, USA) for 60 minutes. The coverslips were then mounted with a glycerol-based mounting fluid after the final wash step. Photographs were taken with 400 ASA film on a fluorescent microscope (Nikon Optiphot microscope equipped with an episcopic-fluorescence attachment and an excitation-emission filter with an average wavelength of 495 nm for FITC).

Statistics

Data presented are the results of three separate experiments (n=4). Statistical analysis was performed using Statistics for Windows software (version 2, Tallahassee, Florida, USA). Student's paired *t*-test was used to evaluate differences between the sample of interest and its respective control. A *P* value of less than 0.05 was considered to be statistically significant.

4.3 Results

4.3.1 Effects of cyclooxygenase blockers and arachidonic acid on prostaglandin E_2 production in MG-63 cells

The effects of the cyclooxygenase blockers indomethacin and NS-398 and AA on PGE_2 production in MG-63 cells are shown in Figure 4.2. Compared to control, incubation of MG-63 cells with either indomethacin or NS-398 resulted in almost 90% inhibition of PGE_2 production. Inhibition was similar for both treatments.

AA increased PGE_2 synthesis six- to seven-fold compared to control. Pre-incubation with COX-blockers prior to AA-treatment inhibited PGE_2 secretion. Indomethacin-mediated inhibition however, was greater than NS-398-mediated inhibition (60% compared to 20%) as indicated in Figure 4.2. Application of blockers did not result in the very low levels of PGE_2 synthesis observed when AA-unstimulated cells (controls) were exposed to these blockers.

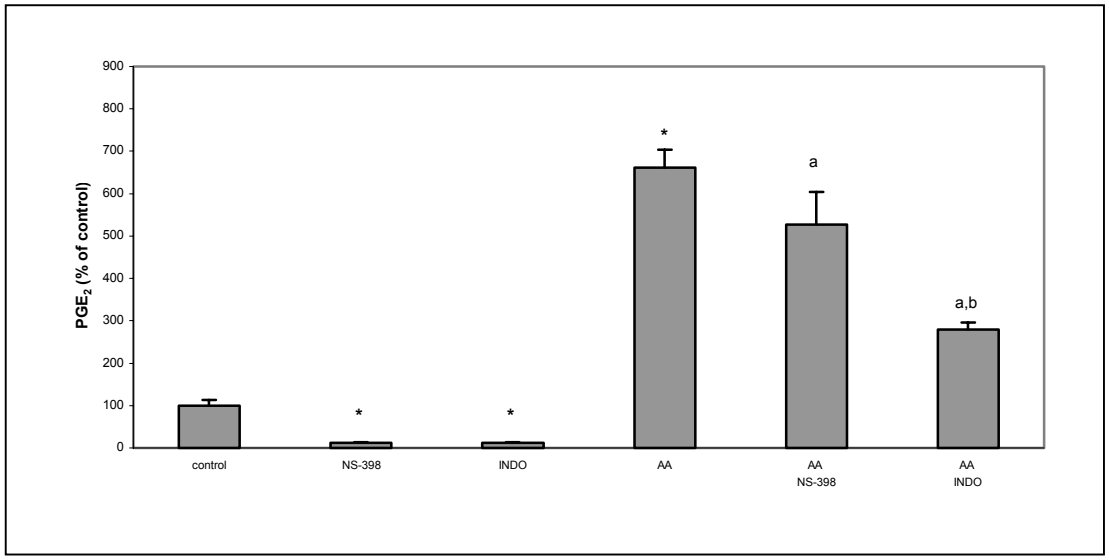


Figure 4.2 Effects of cyclooxygenase blockers and arachidonic acid on prostaglandin E_2 production in MG-63 cells.

MG-63 cells were treated with vehicle (0.2% ethanol) or AA (20 μg/ml) for four hours before sampling of the media. Indomethacin (INDO) (1 μM) and NS-398 (0.1μM) were added 45 minutes prior to AA- treatment. PGE_2 radioimmunoassay was performed as described in Materials and Methods and expressed as percentage of control. *Significant difference from control; **a** significant difference from AA; **b** significant difference from (AA + NS-398), $P<0.05$, n=4. Data are from a representative experiment. The experiment was repeated three times, each experiment yielding comparable data.

4.3.2 Indirect immunofluorescence staining for COX-1 and COX-2 in control and arachidonic acid-activated MG-63 cells

Figure 4.3 depicts the presence of COX-1 and COX-2 enzymes in control and AA-activated MG-63 cells as shown by indirect immunofluorescence staining. Exposing MG-63 cells to either vehicle (0.2% ethanol) or AA (20 μg/ml) in the presence of FCS, resulted in detection of both COX-1 (Figure 4.3 A and B) and COX-2 (Figure 4.3 C and D) enzymes. Morita *et al* (1995) reported that COX-1 and COX-2 are both located in the endoplasmic reticulum as well as the nuclear envelope.[234] COX-2 however, is more highly concentrated on the nuclear membrane than COX-1.[234] This phenomenon is confirmed in Figure 4.3. No difference in the densities of either COX-1 (Figure 4.3 A and B) or COX-2 (Figure 4.3 C and D) in control or AA-treated cells was found.

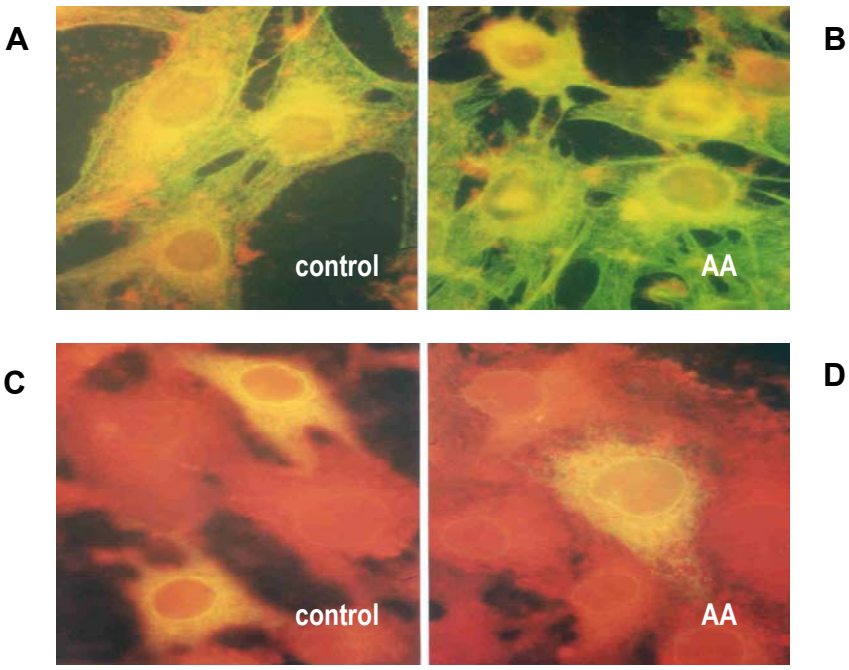


Figure 4.3 Immunofluorescent staining for COX-1 and COX-2 in control and arachidonic acid-activated MG-63 cells

After MG-63 cells were cultured with DMEM with 10% FCS for 24 hours, fresh growth medium containing vehicle only (control) or 20 μg/ml AA was added to near confluent layers. Cells were exposed for four hours at 37ºC. At the end of the experiment, cells were subjected to indirect immunofluorescence staining using mouse monoclonal antibody against human COX-1 (A and B) or COX-2 (C and D) and then biotin-conjugated anti-mouse IgG (Fab-specific, developed in goat) in FITC-conjugate diluent. Photographs were taken with 400 ASA film on a fluorescent microscope. (Original magnification: 400x).

4.3.3 Effects of arachidonic acid, parathyroid hormone and oestrogen on prostaglandin E_2 production in MG-63 and MC3T3-E1 cells

Effects of arachidonic acid and parathyroid hormone on prostaglandin E_2 production in MG-63 cells

Figure 4.4 depicts the effects of AA and PTH on PGE_2 production in MG-63 cells. Exposure to PTH (10^{-7} M) for four hours resulted in a 30% increase in PGE_2 production compared to control. Simultaneous exposure of these cells for four hours to AA and PTH did not increase PGE_2 secretion above the AA-stimulated PGE_2 production. It therefore seems that AA-PTH co-treatment had no synergistic effect on PGE_2 production in this model. Pre-incubation of MG-63 cells with the COX- blockers indomethacin or NS-398 prior to the combined PTH-AA treatment, yielded results similar to those observed when blockers were added prior to AA treatment only. Therefore no PTH-mediated COX stimulation could be observed under the conditions used in this assay.

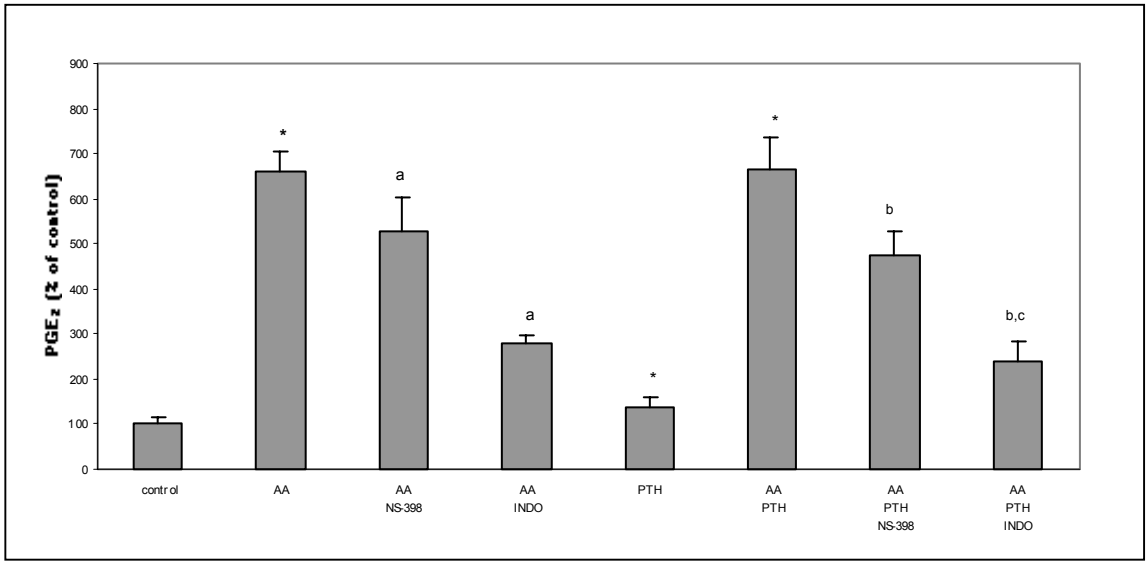


Figure 4.4 Effects of arachidonic acid and parathyroid hormone on prostaglandin E_2 production in MG-63 cells

MG-63 cells were treated with vehicle (0.2% ethanol), AA (20 μg/ml) and PTH (10^{-7} M) for four hours before sampling of the media. Indomethacin (INDO)(1μM) and NS-398 (0.1μM) were added 45 minutes prior to AA treatment. PGE_2 radioimmunoassay was performed as described in the Materials and Methods and expressed as percentage of control.
* Significant difference from control; **a** significant difference from AA; **b** significant difference from (AA + PTH); **c** significant difference from (AA + PTH + NS-398), $P<0.05$, n=4. Data are from a representative experiment. The experiment was repeated three times, each experiment yielding comparable data.

Effects of arachidonic acid and oestrogen on prostaglandin E_2 production in MG-63 cells

Figure 4.5 depicts the effects of AA and oestrogen (E2) on PGE_2 production in MG-63 cells. Exposing MG-63 cells to oestrogen (10^{-6} M) for four hours did not affect PGE_2 production compared to control. Exposing cells to AA and oestrogen simultaneously did not enhance PGE_2 production above that already observed after AA exposure only. Pre-incubation of MG-63 cells with the COX-blockers indomethacin or NS-398 prior to the combined oestrogen-AA treatment, yielded similar results to those observed when blockers were added prior to AA treatment only. Therefore, oestrogen was not able to enhance AA-stimulated PGE_2 synthesis under the conditions used in this assay.

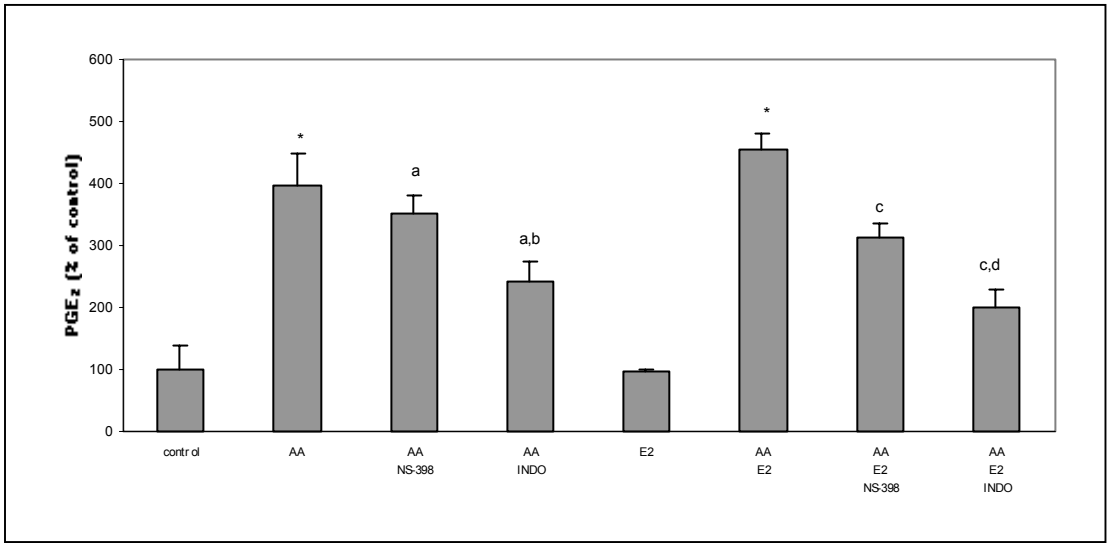


Figure 4.5 Effects of arachidonic acid and oestrogen on prostaglandin E_2 production in MG-63 cells

MG-63 cells were treated with vehicle (0.2% ethanol), AA (20 μg/ml) or E2 (10^{-6} M) for four hours before sampling of the media. Indomethacin (INDO)(1 μM) and NS-398 (0.1μm) were added 45 minutes prior to AA treatment. PGE_2 radioimmunoassay was performed as described in the Materials and Methods and expressed as percentage of control. * Significant difference from control, **a** significant difference from AA; **b** significant difference from (AA + NS-398); **c** significant difference from (AA + E2); **d** significant difference from (AA+E2+NS-398), $P<0.05$, n=4. Data are from a representative experiment. The experiment was repeated three times, each experiment yielding comparable data.

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

The MG-63 osteoblast is an osteosarcoma derived cell line. These cells have undergone an extended period of abnormal growth *in vivo*.[305] Concern has been expressed that cell lines derived from tumor tissue are different from their normal counterparts because cell regulatory mechanisms in permanently transformed cells might differ from those in normal cells. Therefore, one comparative experiment to examine PG synthesis following exposure to AA, and systemic hormones, oestrogen and PTH was conducted on normal murine osteoblast-like cells.

Figure 4.6 depicts the effects of AA, oestrogen and PTH on PGE_2 production in MC3T3-E1 murine osteoblasts. Exposing MC3T3-E1 cells to AA for four hours stimulated PGE_2 secretion significantly. PGE_2 synthesis in the MC3T3-E1 cells was amplified two and a half times over and above what was observed in the MG-63 cells after exposure to AA. Compared to control, AA-stimulated PGE_2 secretion was 16 fold in MC3T3-E1 cells, compared to six fold in MG-63 cells (Figure 4.2 and Figure 4.6). No stimulation of PGE_2 secretion was observed after exposure of the MC3T3-E1 cells to PTH or oestrogen.

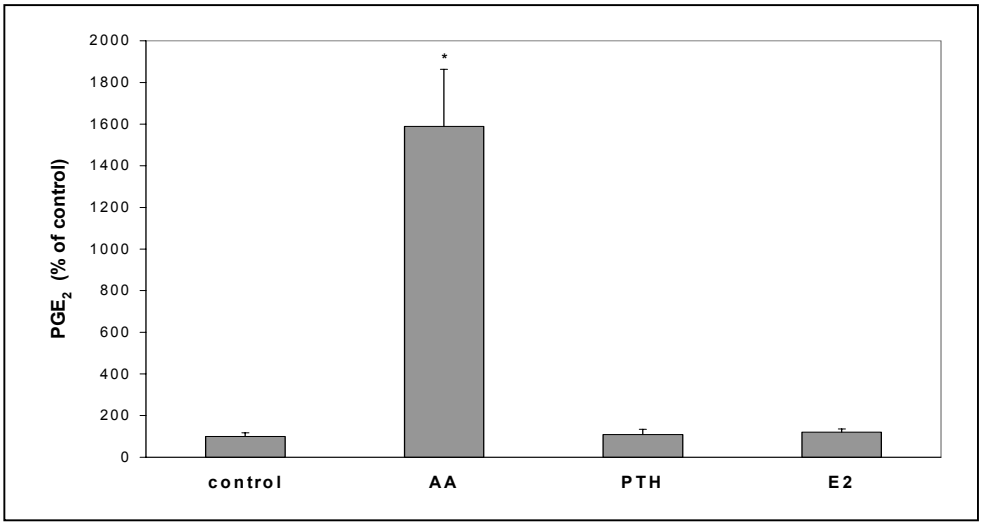


Figure 4.6 Effects of arachidonic acid, parathyroid hormone and oestrogen on prostaglandin E_2 production in MC3T3-E1 cells

Cells were treated with vehicle (0.2% ethanol), AA (20 μg/ml), oestrogen (E2) (10^{-6} M) and PTH (10^{-7} M) for 4 hours before sampling of the media. PGE_2 radioimmunoassay was performed as described in the Materials and Methods and expressed as percentage of control. * Significant difference from control, $P<0.05$, n=4. Data are from a representative experiment. The experiment was repeated three times, each experiment yielding comparable data.

4.4 Discussion

The aim of this study was to determine whether AA, oestrogen and PTH modulate PGE_2 synthesis in MG-63 and MC3T3-E1 osteoblast-like cell lines, and whether COX-1 or COX-2 is involved. Exposure to AA enhanced PGE_2 production significantly in both MG-63 and MC3T3-E1 cell lines (Figure 4.2 and Figure 4.6). Since AA is the natural substrate for PGE_2 synthesis[170] and PGE_2 the major prostaglandin produced by osteoblastic cells,[228,229] this observation was expected. The AA-stimulated production of PGE_2 by MC3T3-E1 cells however, was significantly higher than that in MG-63 cells (Figure 4.2 and Figure 4.6). This observation might reflect a difference in the stimulatory mechanisms involved in PGE_2 production in these cell lines. PGE_2 may autoamplify its own production by inducing COX-2 in bone.[243-246,314] Suda *et al* (1998) showed that autoamplified production of PGE_2 is mediated via the EP_1 subtype of PGE receptors in mouse MC3T3-E1 osteoblasts.[232] It is not known whether MG-63 cells express the EP_1 receptor.

Prostaglandins act on the parent cell and/or neighbouring cells in an autocrine and/or paracrine fashion through specific prostanoid receptors thereby affecting changes in the levels of second messengers.[170] There are at least four distinct receptors for PGE_2 with differential signaling pathways: EP_1 with 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. MC3T3-E1 mouse osteoblastic cells have been reported to predominantly express EP_1 and EP_4 receptors,[229] whilst human mesenchymal stem cells express EP_4 receptors.[149] (Refer to 2.11.1)

To elucidate whether AA increased PGE_2 via COX-1 and/or COX-2, COX-blockers were included before exposure to AA. Indomethacin is known to block both COX-1 and COX-2, while low concentrations of NS-398 are responsible for COX-2 specific inhibition.[248,253] Exposing MG-63 control cells to either NSAIDS inhibited PGE_2 production significantly. As both blockers are reponsible for COX-2 inhibition and the PGE_2 inhibitory effects by these blockers were similar, it is possible that the observed inhibition could be attributed mainly to COX-2 suppression. In our

experiment, cells were cultured in DMEM with 10% heat-inactivated FCS, as measurable PGE_2 was not produced by cells in serum-free media.[248] Fetal calf serum contains AA in its composition, however, the concentration thereof may be different for different batches.[56] It has been reported that COX-1 requires higher concentrations of AA for its optimal function than does COX-2, implying that the amount of AA supplied critically influences which COX isozymes are utilised.[233] One can therefore speculate that in basal conditions, with low AA concentrations present in the culture media, COX-2 could be the major cyclooxygenase responsible for AA metabolism.

In the MG-63 cells pre-incubation with either indomethacin or NS-398 prior to AA supplementation resulted in a decrease in PGE_2 production compared to AA-stimulated PGE_2 production (Figure 4.2). The degree of inhibition of the blockers however was not the same. The use of indomethacin resulted in a much larger inhibitory effect than NS-398, suggesting that both COX-1 and COX-2 were involved in AA-stimulated PGE_2 production. When PGE_2 production was stimulated by exogenous AA supplementation, pre-incubation with COX-blockers did not reduce PGE_2 production to the same low levels observed when these blockers were applied to basal culture conditions (Figure 4.2). Hamilton *et al* (1999) reported similar results and showed that increasing the supply of AA in rats *in vivo* reduces the effectiveness of NSAIDS as inhibitors of prostanoid production.[319]

In order to confirm the observation that both COX-1 and COX-2 isoenzymes are involved in AA-stimulated PGE_2 synthesis, indirect immunofluorescence was applied to determine the presence and location of the COX enzymes in MG-63 cells. Exposing MG-63 cells to either vehicle (0.2% ethanol) or AA in the presence of FCS, resulted in detection of both COX-1 and COX-2 enzymes. Morita *et al* (1995) reported that COX-1 and COX-2 are both located in the endoplasmic reticulum as well as the nuclear envelope.[234] COX-2 however, is more highly concentrated on the nuclear membrane than COX-1.[234] Our study confirmed this observation (Figure 4.3). Indirect immunofluorescence did not show any difference in the densities of either COX-1 or COX-2 in control or AA-treated cells, suggesting that the experimental conditions applied did not affect distribution of these enzymes. However, FCS itself can induce COX-2 expression.[234,248] The lack of effect of AA on COX distribution in our experiment may therefore be masked by the FCS-stimulated

effect on COX-2 expression. In order to confirm this observation, cells should also be cultured in FCS- free conditions.

Systemic hormones can influence prostaglandin production.[170,313] In order to determine whether the bone active hormones oestrogen and PTH affected PGE_2 production in osteoblast-like cells, MG-63 and MC3T3-E1 cells were exposed to these hormones. Although it was reported by others that oestrogen inhibits osteoblastic production of PGE_2 in primary osteoblasts, human osteosarcoma osteoblasts and organ culture,[108] oestrogen exposure did not affect PGE_2 production in either MG-63 or MC3T3-E1 cell lines in our study. However, it is well documented that oestrogen acts through oestrogen receptors (ER) located on the nuclear membrane, thereby affecting intracellular pathways.[35] It might be necessary to determine whether the cell lines used in our study express sufficient ER levels to be affected by oestrogen treatment. One can therefore speculate that the cells investigated in our study might be ER-deficient or may express very low levels of ER. Low ER expression levels in MC3T3-E1 cells have been reported[320] and could explain the inability of these cells to respond to oestrogen treatment.

In our study, PTH-treatment stimulated PGE_2 production significantly in MG-63 cells (Figure 4.4). Others confirmed this observation.[313-315] The mechanisms implicated in this observation are not clear. PTH stimulation of PGE_2 synthesis has been attributed to induction of COX-2[313,314] as well as increased release of incorporated AA from cell membranes.[321] PTH could act through cAMP, which can increase PG production in bone.[322] In our study, PTH however, had no stimulatory effect on PGE_2 production by MC3T3-E1 cells. Suda *et al* (1983) reported the presence of PTH receptors in this cell line,[285] however, subcloning of this original nontransformed cell line, may have resulted in loss or modification of the PTH receptor.

In conclusion, our study confirms that exogenously added AA (20 μg/ml) stimulates PGE_2 production significantly in both the MG-63 human osteosarcoma cell line as well as the nontransformed murine MC3T3-E1 osteoblast-like cell line. Stimulated PGE_2 production by MC3T3-E1 cells, however, was significantly higher than that of MG-63 cells. This observation might be attributed to autoamplification, as PGE_2 autoamplification is mediated via the EP_1 subtype of PGE receptors in mouse MC3T3-E1 osteoblasts.[232] Although others reported a stimulatory effect of PTH on

PGE_2 production in osteoblasts,[313-315] we could only demonstrate a stimulatory effect in the MG-63 cell line. Rickard (1999) reported that oestrogen inhibits PGE_2 production in osteoblast-like cells[108] but we were unable to confirm these effects in the cell lines used and experimental conditions. Variation in responses may reflect species differences, differences in the mechanism of cell line immortalisation, or differences in the degree of osteoblastic differentiation. Further work, including receptor studies, needs to be done to elucidate these responses or lack thereof.

Animal as well as human studies has shown that PUFAs such as AA (representative of the n-6 PUFA family) and DHA (representative of the n-3 PUFA family) affect the structure and strength of bone.[25-29,31] Experimental work was therefore included in this study to investigate the effects of these PUFAs on osteoblastic cell proliferation (Chapter 5) as well as differentiation of these cells into mature mineralising osteoblasts (Chapter 6).

CHAPTER 5

Effects of Arachidonic Acid, Docosahexaenoic Acid, Prostaglandin E_2, Oestrogen and Parathyroid Hormone on Cell Proliferation and Morphology of MG-63 and MC3T3-E1 Osteoblast-like Cells

5.1 Introduction

The mature skeleton is a metabolically active organ that undergoes continuous remodeling by a process that replaces old bone with new bone. During the remodeling cycle bone is resorbed by osteoclasts and then replaced again by osteoblasts through the process of bone formation.[89] The bone formation rate *in vivo* is largely determined by the number of mature functioning osteoblasts, which in turn is determined by the rate of replication of osteoblastic progenitors and the life-span of mature osteoblasts.[42,126] (Refer to 2.3.1 and 2.4).

Agents stimulating cell proliferation do this by binding to receptors with intrinsic tyrosine kinase activity.[323] These receptors share a common signal transduction pathway that, via a complex kinase cascade, leads to cell proliferation. One of the components involved in the cascade is mitogen-activated protein (MAP) kinase which has been shown to be essential in the proliferative response of several cell types.[324] Stimulation of cell proliferation depends on the activity of the cell cycle. Cyclins and cyclin-dependent kinases (cdks) regulate the progression through each stage of the cell cycle.[76]

Osteoblasts originate from bone marrow stromal precursor cells that then differentiate into mature osteoblasts.[6] Once the osteoblast has differentiated and completed its cycle of matrix synthesis, it can either become a flattened lining cell on the bone surface, be buried in bone as an osteocyte, or undergo programmed cell

death (apoptosis).[6] It has been shown that the majority of osteoblasts will eventually undergo apoptosis.[42] (Refer to 2.3.1).

Apoptosis is a biological process that eliminates unwanted or damaged cells. The majority of nucleated cells appear to possess the genetic programming to undergo apoptosis.[79] Apoptosis is an active process that is controlled from within the cell by a large number of regulatory factors, but can be induced or inhibited by external factors through receptor-mediated mechanisms.[79,80] Some of these mechanisms activate cysteine proteases (caspases) which are responsible for the characteristic morphological changes observed during apoptosis.[75] Known activators of apoptosis include tumor necrosis factor α (TNFα), oxidants, free radicals and bacterial toxins.[82] The presence or absence of specific growth factors and hormones also affect induction of apoptosis.[77,82] Apoptosis is characterised by a sequence of morphologically recognisable events. Initially, an individual cell becomes detached from its neighbours and morphologically shrinks. Condensing of the chromatin and ruffling of the plasma membrane known as 'budding' then follows.[75,78,84] Cell fragments 'pinch off' as separate small membrane-bound vesicles known as apoptotic bodies that contain the condensed cytoplasmic proteins and intact organelles with nuclear fragments.[78,80,325] Adjacent cells recognise the apoptotic bodies and rapidly eliminate them through phagocytosis thereby avoiding an inflammatory response. Apoptosis occurrs quickly and cells undergoing this form of death disappear within hours without causing damage to surrounding cells or tissues.[78,80] (Refer to 2.3.4).

Studies conducted over the past decade showed that bone active hormones such as oestrogen (E2)[25,99,100] and parathyroid hormone (PTH)[116-118] are benificial to bone. Supplementation of diets with polyunsaturated fatty acids (PUFAs) also showed promising effects on bone.[25,28,29,31,192] PUFA supplementation increases bone formation in animals[25,31,192] and an anti-resorptive effect has been observed in elderly women after three years of PUFA supplementation.[26,27]

To determine whether PUFAs and the bone active hormones oestrogen and PTH affect osteoblast cell proliferation *in vitro* in a similar manner, MG-63 and MC3T3-E1 osteoblasts were exposed to arachidonic acid (AA) (representative of the n-6 PUFA family), docosahexaenoic acid (DHA) (representative of the n-3 PUFA family), PTH

and oestrogen. 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. Apart from proliferation studies, morphological studies were conducted to determine whether exposing the cells to PUFAs, PGE_2, and hormones caused structural damage to the cells thereby yielding invalid results.

5.2 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, charcoal (dextran coated), propidium iodide and Hoechst no 33342. Heat inactivated fetal calf serum (FCS) was obtained from Highveld Biological (Pty) Ltd (Sandringham, SA). DMEM was obtained from Sterilab Services (Kempton Park, SA). Gentamycin was supplied by Gibco (Invitrogen Corp., Carlsbad, CA, USA). All other chemicals were of analytical grade and supplied by Sigma Chemical Co (St. Louis, MO, USA). Glass coverslips and 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. 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

final ethanol concentration in the culture medium did not exceed 0.2%. Previous studies in our laboratory showed no toxic effects of the ethanol vehicle at this concentration.

Cell culture media for growth (proliferation) studies

Ascorbic acid has been shown to stimulate proliferation of MC3T3-E1 cells. This effect appears to be mediated through the stimulatory effect of ascorbic acid on collagen synthesis.[289] As α-MEM contains ascorbic acid in its formula, it was decided to use DMEM, which is ascorbic acid free, for all experiments investigating proliferation. In our experimental conditions both cell lines tolerated DMEM well. Fetal calf serum contains various growth factors, which reportedly also affect cell proliferation.[289,290] To limit the proliferative effects of high FCS levels, FCS content in the culture media was limited to 5%.

Cell culture media for oestrogen exposure

Although it is controversial whether the pH indicator, phenol red, has any oestrogenic activity[289] or not,[287] it was decided to use DMEM without phenol red for the experiments investigating oestrogen's proliferative effects. Using an adaptation of Horwitz *et al's* (1978) method,[288] dextran-coated charcoal was used to remove endogenous oestrogens from FCS as follows:

One-gram dextran-coated charcoal was added to 100 ml FCS. The mixture was then placed in a shaking water bath at 45ºC for 30-45 minutes, keeping the charcoal in suspension. At the end of incubation the charcoal was removed by centrifugation (1500-2000 rpm for 30-45 minutes) and the supernatant carefully poured off. After cooling to room temperature, the FCS was filtered by passage through a 0.22 µm filter into sterile containers and stored at -20ºC until needed.

Proliferation studies

Proliferation can best be evaluated over an extended period of time; it was therefore decided to evaluate the effects of the different agents over a period of 72 hours. Longer periods are not suitable as cells then often reach confluency, which causes contact inhibition. MG-63 and MC3T3-E1 cells were seeded in sterile 96-well culture plates at a density of 3 000 cells/well after trypan blue exclusion. After cells had attached firmly for a period of 24 hours, culture medium was replaced with DMEM containing 5% FCS. Vehicle (ethanol, 0.2%), PUFAs (AA and DHA) ranging from 2.5 to 20μg/ml or hormones (oestrogen and PTH) and PGE_2 (concentrations ranging from 10^{-10} to 10^{-6} M) were then added. After 72 hours, with cells still in the log phase of proliferation (refer to Figure 3.1) the experiment was terminated by replacing growth medium with 1% glutaraldehyde in PBS for 15 minutes. For determination of proliferation, an adaptation of the crystal violet staining procedure[300] was applied as follows: Crystal violet solution (1%, in PBS) was added to the fixed cells for 30 minutes, thereafter the plates were immersed in running tap water for 15 minutes. After the plates had dried, 200 μl 0.2% Triton X-100 was added to each well and incubated at room temperature for 90 minutes, and 100 μl of the liquid content subsequently transferred to 96-well microtiter plates. Absorbance (O.D.) was read on an ELX800 Universal Microplate Reader (Bio-Tek Instruments Inc., Analytical Diagnostic Products, Weltevreden, SA) at a wavelength of 570nm. Triton X-100 (0.2% in water) was used as a blank. Crystal violet is a basic dye, which stains cell nuclei.[298] Spectrophotometer readings of colour intensity are therefore an indication of DNA content and therefore cell numbers. Results are presented as percentage relative to control. Three independent experiments were conducted (n=8).

Morphology study: Haematoxylin and eosin (H&E) cell staining

MG-63 and MC3T3-E1cells (200 000/well) were seeded aseptically onto heat-sterilised coverslips in six-well culture plates. Growth medium with vehicle (0.2% ethanol) (control), PUFAs (AA and DHA) ranging from 2.5 to 20μg/ml or hormones (oestrogen and PTH) and PGE_2 (concentrations ranging from 10^{-10} to 10^{-6} M) were then added to near confluent monolayers for 48 hours.

At the end of culture, the experiment was terminated by removing the coverslips from the cluster plates, inserting them into coverslip holders followed by exposure to Bouin's fixative (Accustan™ Bouin's solution, Sigma) for 30 minutes. Thereafter cells were stained by standard haematoxylin and eosin (H&E) staining procedures.[302] Cells were left in 70% ethanol for 60 minutes; rinsed with tap water; left in Mayer's haemalum for 15 minutes; rinsed in running tap water; rinsed with 70% ethanol and subjected to 1% eosin for two minutes. Thereafter, cells were dehydrated by rinsing twice for five minutes with each of the following: 70% ethanol; 96% ethanol and 100% ethanol. Finally, coverslips were rinsed twice with xylol for five minutes, mounted to microscope glass slides with rapid mounting resin (Entellan) and left to dry. Photographs were taken with 400 ASA film using a Nikon Opthiphot camera attached to the microscope.

Detection of apoptosis by Hoechst 33342 and propidium iodide staining (HOE/PI staining)

Previous studies reported observation of apoptotic effects within 24 to 48 hours of exposing cells to apoptotic agents.[42] It was therefore decided to limit this experiment to 48 hours. Apoptosis and oncosis were assessed by viability staining as follows: 40 000 cells were seeded in DMEM with 5% FCS onto heat-sterilised coverslips in sterile 24-well cluster plates and left to adhere for 24 hours. Culture medium, containing vehicles, AA and DHA (ranging from 2.5 to 20 μg/ml), PTH, oestrogen and PGE_2 (ranging from 10^{-10} to 10^{-6} M) was then added to subconfluent cells for 48 hours. DMEM without FCS was employed as positive control for apoptosis.[42] At the end of culture, after treatment with vehicle and test agents, growth medium was discarded and the cells gently rinsed with PBS. Thereafter 500 μl/well Hoechst No 33342 (HOE) (0.5 μg/ml in PBS) was inserted into each well. After 30 minutes of incubation at 37ºC, 125μl of a 0.5 mg/ml propidium iodide (PI) solution was added directly to each well. Within five minutes, coverslips were mounted on microscope slides with mounting fluid (90% glycerol, 4% N-propyl-gallate, 6% PBS). Photographs were taken with 400 ASA film on a Nikon Optiphot microscope with UV-light and a blue filter.

While all cells take up HOE (blue), only cells with intact cell membranes exclude propidium iodide (red). Viable cells' nuclei therefore stain blue, while the nuclei of cells with decreased membrane integrity become bright red.[303] Although apoptotic cells may have an aberrant appearance they stain blue, indicating that these cells still have functional cell membranes capable of excluding PI.

Statistics

For each of the agents tested (AA, DHA, PGE_2, PTH and oestrogen) three separate proliferation experiments were conducted (n=8). Statistical analysis was performed using Statistics for Windows software (version 2, Tallahassee, Florida, USA). The results were analysed with one way ANOVA followed by Bonferroni's post-hoc testing. $P<0.05$ was considered to be significant.

Effects of docosahexaenoic acid on the proliferation of MG-63 cells and MC3T3-E1 cells after 72 hours exposure

The effects of the n-3 PUFA DHA on the proliferation of MG-63 and MC3T3-E1 cells are depicted in Figure 5.2. Compared to control, DHA inhibited cell proliferation in both cell lines in a dose-dependent manner. Compared to the MC3T3-E1 cell line, the inhibitory effect of DHA on the MG-63 cell line was much more severe, causing an inhibition of more than 80% at 20 μg/ml.

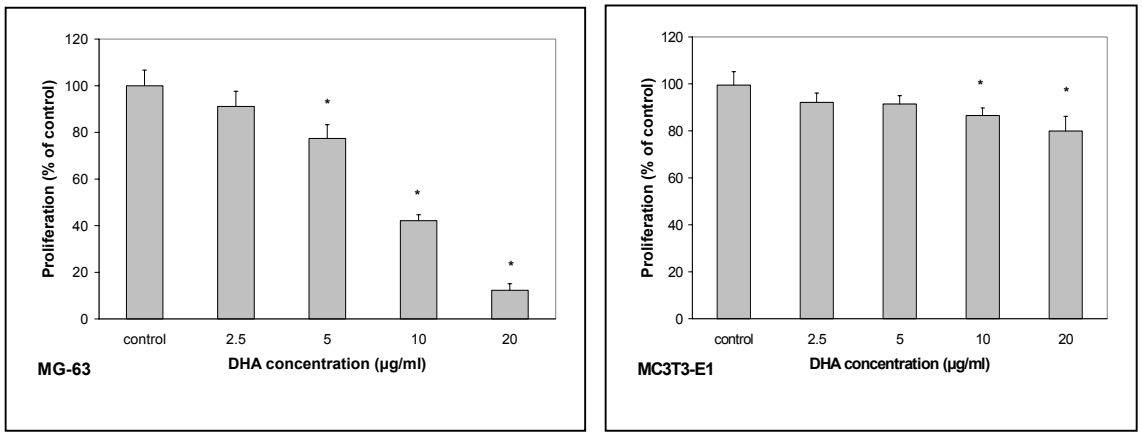


Figure 5.2 Effects of docosahexaenoic acid on MG-63 and MC3T3-E1 cell proliferation

Cells were seeded at 3 000 per well in 96-well plates in DMEM with 5% FCS, preincubated for 24 hours and treated for 72 hours with vehicle (0.2% ethanol)(control) or DHA (2.5 to 20 μg/ml). Cell number was determined by crystal violet staining as described in Materials and Methods and is presented as a percentage relative to control. Results shown are the mean ±SD, n=8. *Significant difference from control, *P*<0.05. Three independent experiments were conducted. Data are from representative experiments.

Effects of prostaglandin E_2 on the proliferation of MG-63 cells and MC3T3-E1 cells after 72 hours exposure

The effects of PGE_2 (10^{-10} to 10^{-6} M) on the proliferation of MG-63 and MC3T3-E1 cells are depicted in Figure 5.3. Compared to control, PGE_2 exposure caused a slight inhibition of cell proliferation in both cell lines. In the MG-63 cell line the greatest effect was observed at a relatively low concentration of 10^{-10} M (12% inhibition), while the proliferation of MC3T3-E1 cells were affected mostly at higher concentrations (10^{-8} to 10^{-7} M) (8% inhibition).

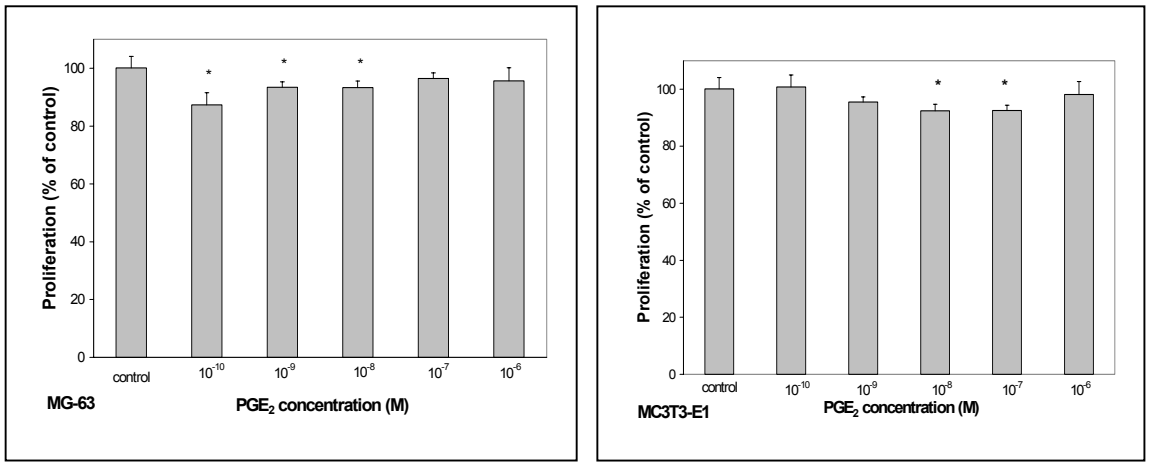


Figure 5.3 Effects of prostaglandin E_2 on MG-63 and MC3T3-E1 cell proliferation

Cells were seeded at 3 000 per well in 96-well plates in DMEM with 5% FCS, preincubated for 24 hours and treated for 72 hours with vehicle (0.2% ethanol)(control) or PGE_2 (10^{-10} to 10^{-6} M). Cell number was determined by crystal violet staining as described in Materials and Methods and presented as a percentage relative to control. Results shown are the mean ±SD, n=8. *Significant difference from control, $P<0.05$. Three independent experiments were conducted. Data are from representative experiments.

Effects of oestrogen (E2) on the proliferation of MG-63 cells and MC3T3-E1 cells after 72 hours exposure

Figure 5.4 shows the effects of oestrogen (10^{-10} to 10^{-6} M) on the proliferation of MG-63 and MC3T3-E1 cells. Exposing MG-63 cell to oestrogen caused slight inhibition of proliferation with the greatest effect at a concentration of 10^{-8} M (14% inhibition). Exposing MC3T3-E1 cells to oestrogen did not affect cell proliferation.

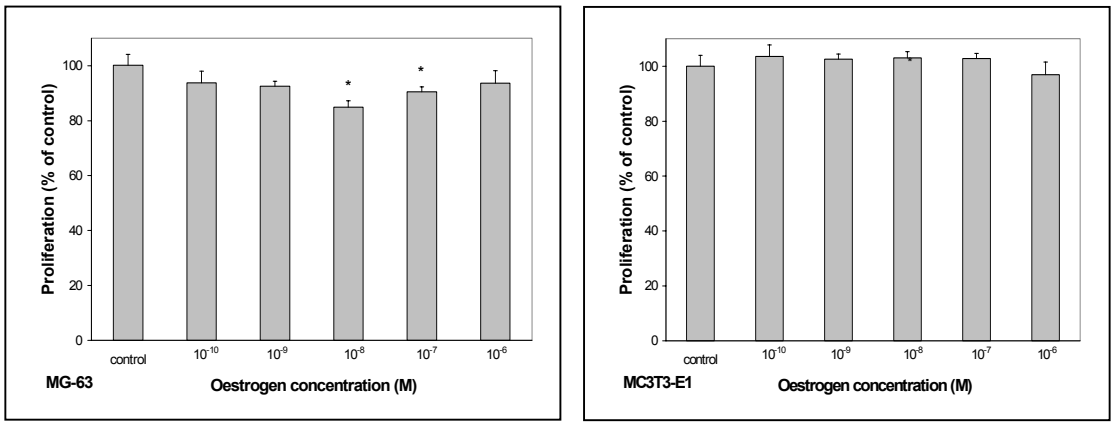


Figure 5.4 Effects of oestrogen on MG-63 and MC3T3-E1 cell proliferation

Cells were seeded at 3 000 per well in 96-well plates in DMEM with 5% FCS, preincubated for 24 hours and treated for 72 hours with vehicle (control) or oestrogen (10^{-10} to 10^{-6} M). Cell number was determined by crystal violet staining as described in Materials and Methods and is presented as a percentage relative to control. Results shown are the mean ±SD, n=8. *Significant difference from control, $P<0.05$. Three independent experiments were conducted. Data are from representative experiments.

Effects of parathyroid hormone on the proliferation of MG-63 cells and MC3T3-E1 cells after 72 hours exposure

The effects of PTH (10^{-10} to 10^{-6} M) on the proliferation of MG-63 and MC3T3-E1 cells are depicted in Figure 5.5. Exposure of both cell lines to PTH affected cell proliferation slightly, with the greatest effect at concentrations of 10^{-9} to 10^{-8} M (8% to 10% inhibition).

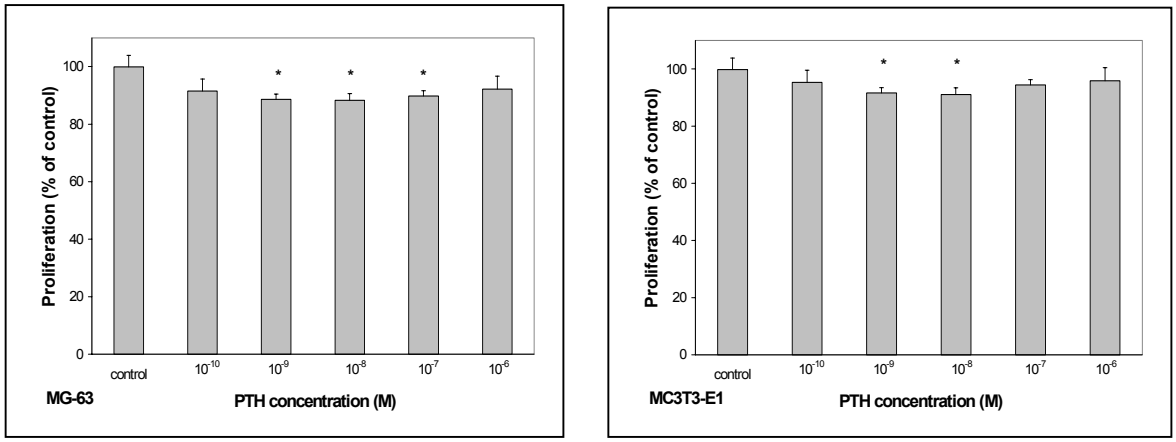


Figure 5.5 Effects of parathyroid hormone on MG-63 and MC3T3-E1 cell proliferation

Cells were seeded at 3 000 per well in 96-well plates in DMEM with 5% FCS, preincubated for 24 hours and treated for 72 hours with vehicle (control) or PTH (10^{-10} to 10^{-6} M). Cell number was determined by crystal violet staining as described in Materials and Methods and is presented as a percentage relative to control. Results shown are the mean ±SD, n=8. *Significant difference from control, $P<0.05$. Three independent experiments were conducted. Data are from representative experiments.

Results presented in Figures 5.3 to 5.5 indicate that PGE_2, E2 and PTH at 10^{-8} M had the greatest anti-proliferative effects on the cell lines tested. Based on this observation, it was decided to expose the cells to these agents at 10^{-8} M in all subsequent tests when the effects of PGE_2, E2 and PTH had to be tested.

5.3.2 Morphology study: Haematoxylin and eosin (H&E) cell staining

Haematoxylin and eosin staining was conducted to determine whether exposing the cells to PUFAs, PGE_2 and hormones caused structural damage to the cells.

MG-63 cells

No morphological damage or abnormalities were detected in the MG-63 cells after exposure to PGE_2, oestrogen, and PTH (results not shown). Figure 5.6 shows the effects of PUFAs on the morpholgy of MG-63 cells. Exposing the cells to vehicle (0.2% ethanol)(control) had no effect on cell morphology (Figure 5.6, A and B). Although 20 μg/ml of AA inhibited cell proliferation considerably (Figure 5.1) no structural damage to the cells was observed at this concentration (Figure 5.6, C and D). DHA had major effects on MG-63 cell proliferation as shown in Figure 5.2. The photographed field of these cells clearly shows that 48 hours DHA exposure affected the growth pattern of these cells (Figure 5.6 E). Although normal dividing cells are visible, the cells are less confluent than the control cells (Figure 5.6 A). Apoptotic cells are visible at higher magnification (Figure 5.6 F).

MC3T3-E1 cells

No morphological damage or abnormalities were detected in the MC3T3-E1 cells after 48 hours exposure to PGE_2 and the hormones oestrogen and PTH (results not shown). Exposing the control cells to vehicle (0.2% ethanol) or AA had no effect on cell morphology (Figure 5.7, A to D). Although 20 μg/ml DHA inhibited MC3T3-E1 cell proliferation by 40% (Figure 5.2), no morphological effects of this PUFA on these cells were observed (Figure 5.7 E and F). DHA exposure did not cause apoptosis of these cells and normal dividing cells were evident (Figure 5.7 F).

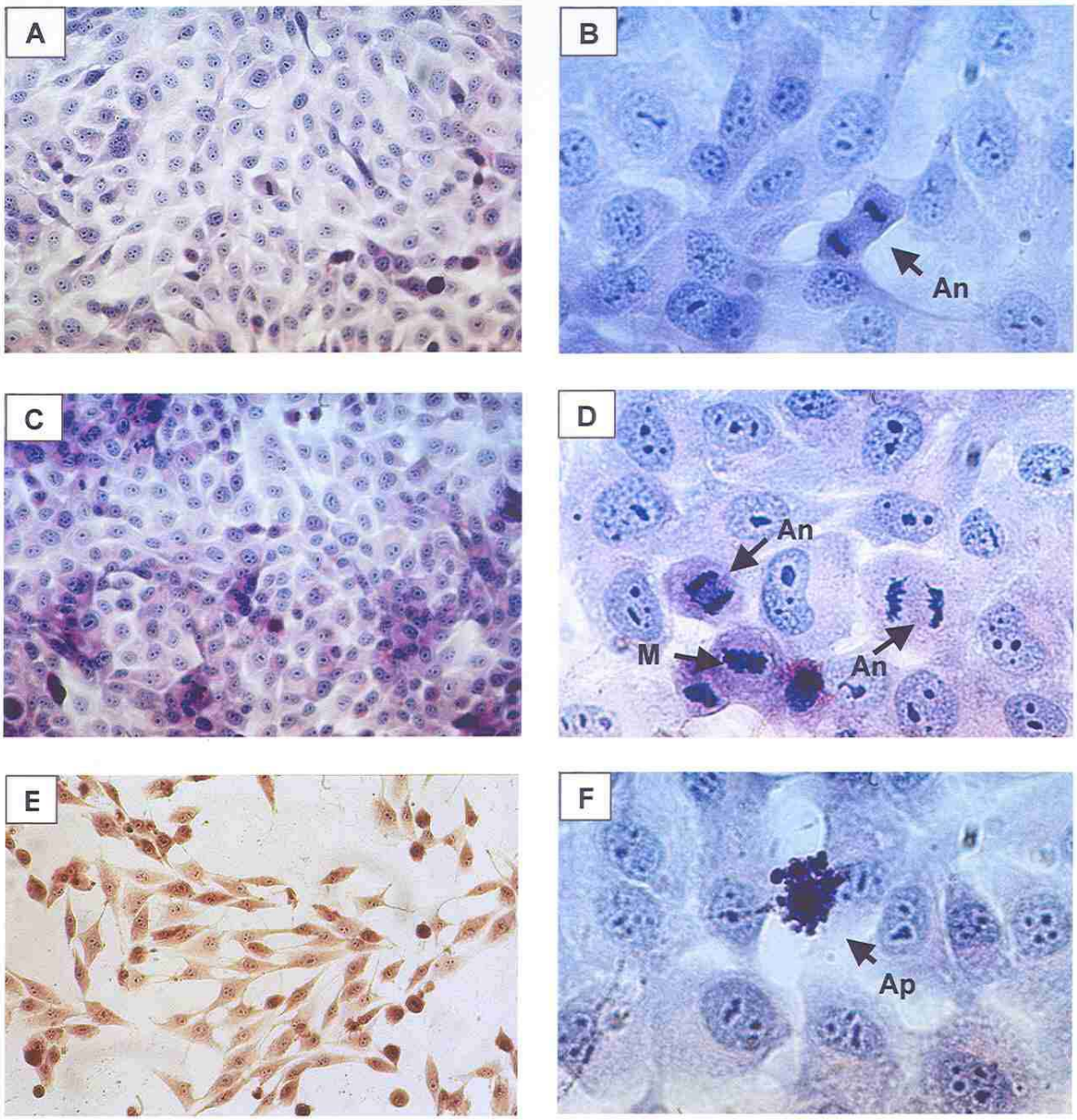


Figure 5.6 Photomicrographs of haematoxylin and eosin (H&E) stained MG-63 cells after 48 hours polyunsaturated fatty acid exposure

Cells were preincubated in DMEM with 5% FCS for 24 hours and subsequently exposed to vehicle (0.2% ethanol)(control) and PUFAs (AA and DHA) for 48 hours. H&E staining was then performed as described in Materials and Methods. **A:** control cells (exposed to vehicle only); **B:** control cells (black arrow indicates cells in anaphase); **C:** cells exposed to 20μg/ml AA; **D:** cells exposed to 20 μg/ml AA (arrows indicate mitotic cells, An-anaphase, M-metaphase); **E:** cells exposed to 20 μg/ml DHA; **F:** cells exposed to 20 μg/ml DHA (arrow indicates an apoptotic cell). (A, C and E: original magnification 100x) (B, D and F: original magnification 400x).

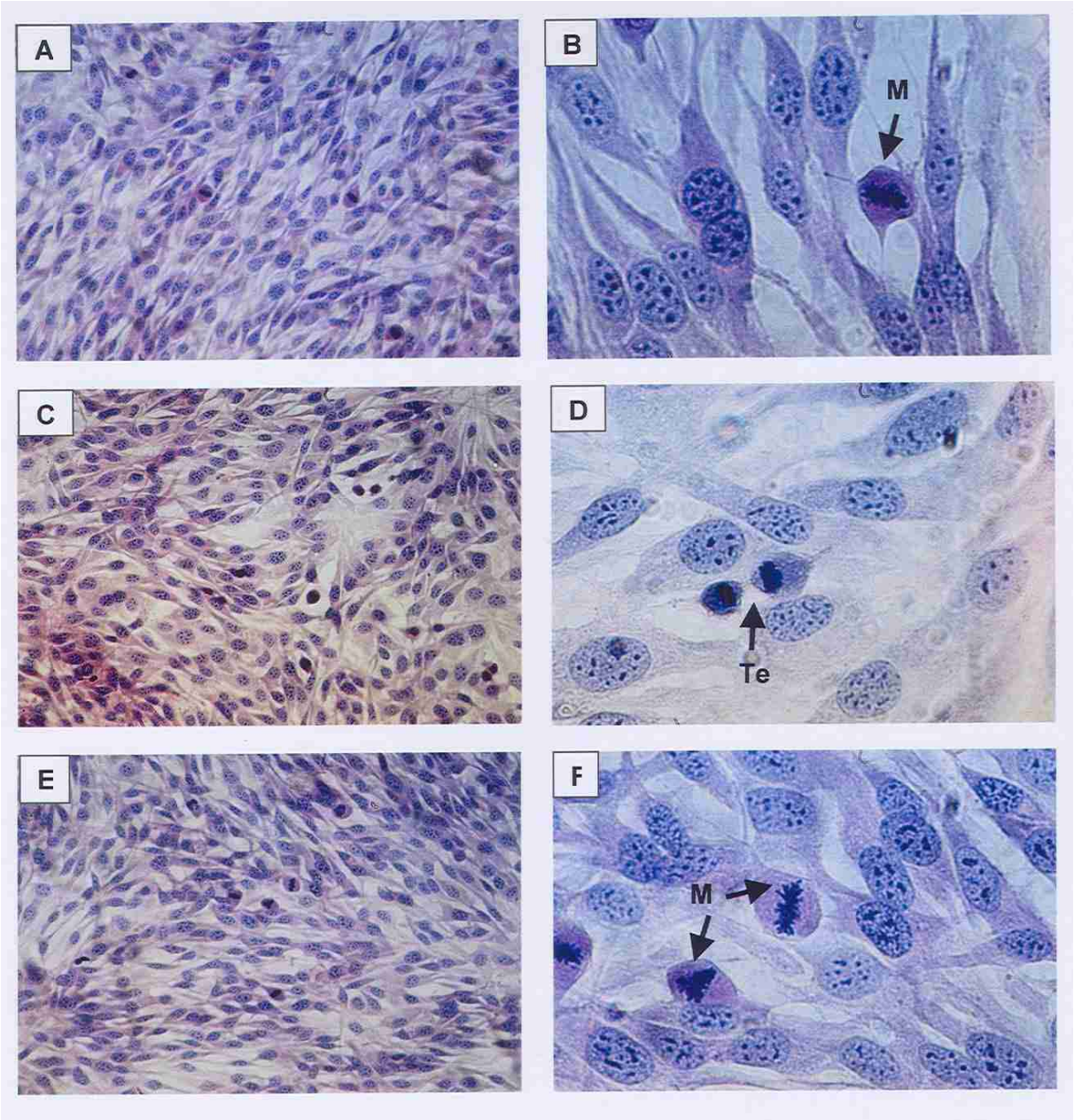


Figure 5.7 Photomicrographs of haematoxylin and eosin (H&E) stained MC3T3-E1 cells after 48 hours polyunsaturated fatty acid exposure

Cells were preincubated in DMEM with 5% FCS for 24 hours and subsequently exposed to vehicle (0.2% ethanol)(control) and PUFAs (AA and DHA) for 48 hours. H&E staining was then performed as described in Materials and Methods. **A:** control cells (exposed to vehicle only); **B:** control cells (the arrow indicates a mitotic cell in metaphase); **C:** cells exposed to 20μg/ml AA; **D:** cells exposed to 20 μg/ml AA (the arrow indicates a mitotic cell in telophase); **E:** cells exposed to 20 μg/ml DHA; **F:** cells exposed to 20 μg/ml DHA (arrows indicate mitotic cells in metaphase). (A, C and E: original magnification 100x) (B, D and F: original magnification 400x).

5.3.3 Hoechst 33342 and propidium iodide (HOE/PI) staining for detection of apoptosis

HOE/PI staining was performed to investigate the effects of AA, DHA, PGE_2 and hormones (oestrogen and PTH) on the viability of MG-63 and MC3T3-E1 cells. Neither PGE_2 nor the hormones affected MG-63 or MC3T3-E1 cell viability (results not shown). Exposure to vehicle (0.2% ethanol) did not compromise the membrane integrity of either MG-63 cells (Figure 5.8 A) or MC3T3-E1 cells (Figure 5.9 A) as evident by the cells' nuclei staining blue only.

Previous studies showed that depriving cells of FCS for a period of 24 to 48 hours causes apotosis.[42] Our study confirmed this observation as multinuclear apoptotic MG-63 and MC3T3-E1 cells could be detected after 48 hours of FCS-deprivation (Figure 5.8 B and Figure 5.9 B). Although AA inhibited cell proliferation in both cell lines (Figure 5.1), no apoptotic cells were detected after exposing these cells to 20μg/ml AA (Figure 5.8 C and Figure 5.9 C) thereby confirming the results obtained from H&E staining of these cells (Figure 5.6 D and Figure 5.7 D).

Exposing MG-63 and MC3T3-E1 osteoblasts to DHA inhibited cell proliferation significantly. To determine whether this observation could be attributed to apoptotic effects of DHA on these cells, HOE/PI viability staining was performed. In MC3T3-E1 cells no apoptotic cells were detected after 48 hours exposure to 20μg/ml DHA (Figure 5.9 D). However, exposing MG-63 cells to similar DHA concentrations induced the formation of multi-nucleated cells showing extensive nuclear blebbing (Figure 5.8 D), which may be an early step in apoptosis development,[326] thereby confirming results obtained by H&E staining (Figure 5.6 F).

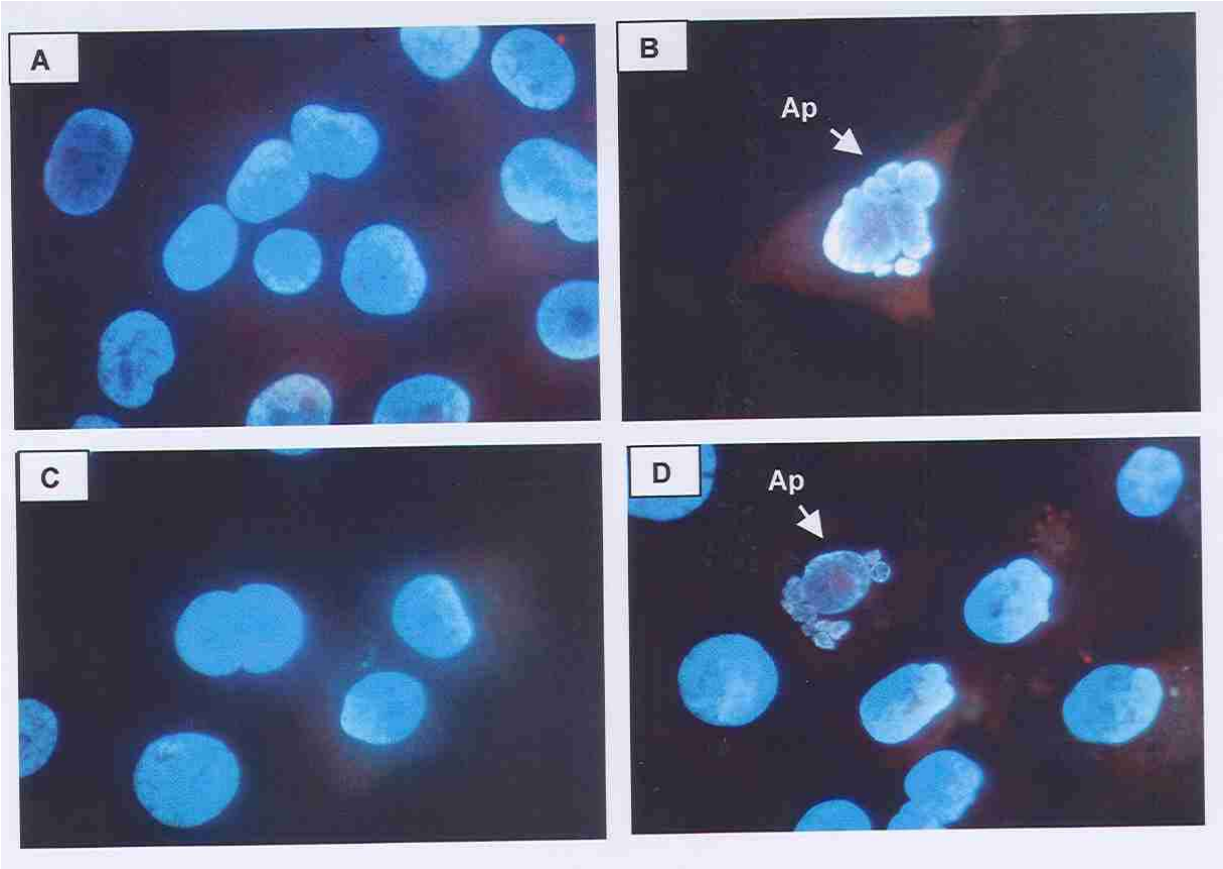


Figure 5.8 Photomicrographs of MG-63 cells after Hoechst and propidium iodide (HOE/PI) fluorescent staining for detection of apoptosis

Cells were preincubated in DMEM with 5% FCS for 24 hours and subsequently exposed to vehicle (0.2% ethanol) (control) and PUFAs (AA and DHA) for 48 hours. HOE/PI fluorescent staining was then performed and photomicrographs taken as described in Materials and Methods. **A:** Control cells (exposed to vehicle only); **B:** Multinucleated cell formation after 48 hours of FCS deprivation; **C:** Cells exposed to 20μg/ml AA for 48 hours; **D:** Cells exposed to 20μg/ml DHA for 48 hours (arrow indicates an apoptotic cell) (Original magnification of photomicrographs: 400x)

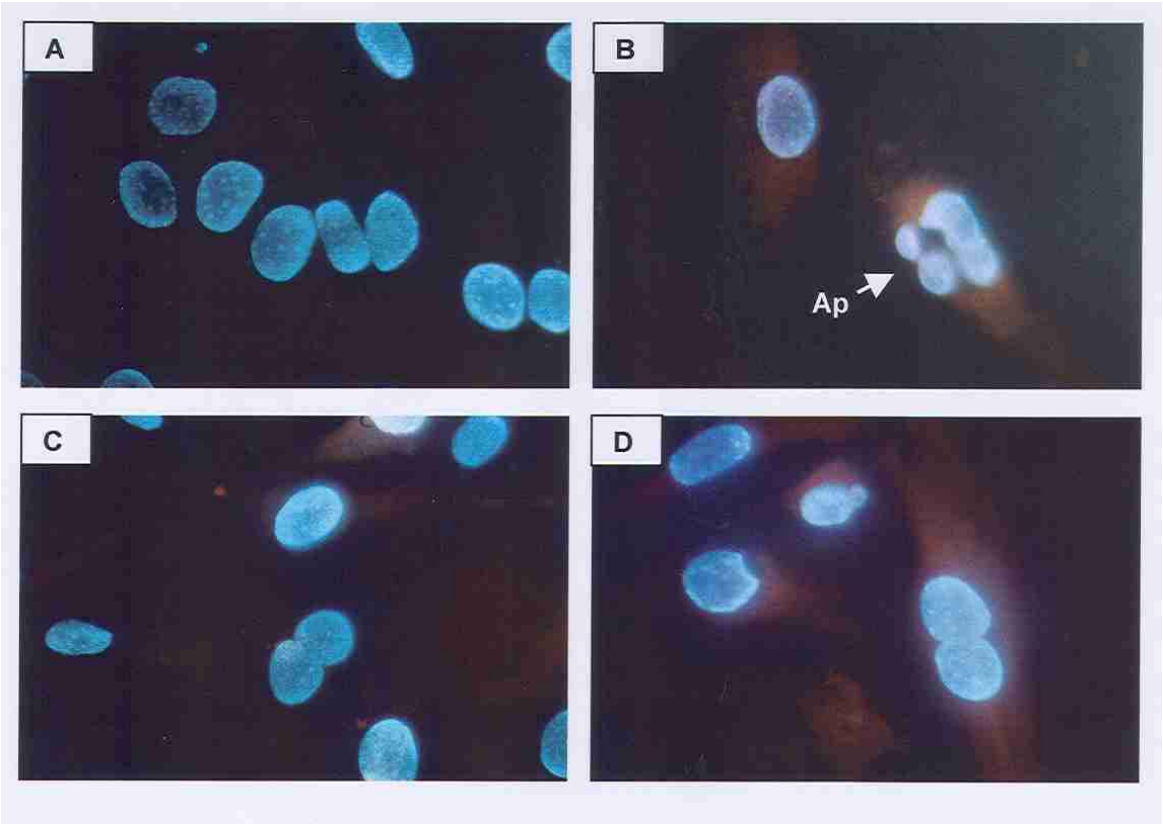


Figure 5.9 Photomicrographs of MC3T3-E1 cells after Hoechst and propidium iodide fluorescent (HOE/PI) staining for detection of apoptosis

Cells were preincubated in DMEM with 5% FCS for 24 hours and subsequently exposed to vehicle (0.2% ethanol) (control) and PUFAs (AA and DHA) for 48 hours. HOE/PI staining was then performed and photomicrographs taken as described in Materials and Methods. **A:** control cells (exposed to vehicle only); **B:** Cells after being deprived of FCS for 48 hours (arrow indicates a multinucleated apototic cell); **C:** Cells exposed to 20μg/ml AA for 48 hours; **D:** Cells exposed to 20μg/ml DHA for 48 hours. (Original magnification of photomicrographs: 400x)

5.3 Results

5.3.1 Proliferation studies

Effects of arachidonic acid on the proliferation of MG-63 cells and MC3T3-E1 cells after 72 hours exposure

The effects of the n-6 PUFA AA on the proliferation of MG-63 and MC3T3-E1 cells are shown in Figure 5.1. Compared to control, AA inhibited cell proliferation in both cell lines in a dose-dependent manner. High concentrations of AA (20 μg/ml) caused a 40 to 50% inhibition in the cell lines tested.

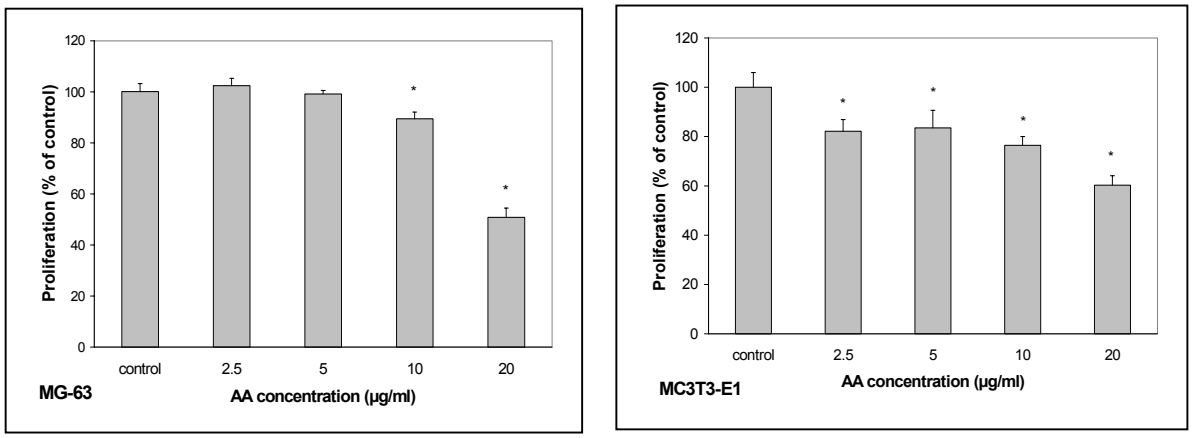


Figure 5.1 Effects of arachidonic acid on MG-63 and MC3T3-E1 cell proliferation

Cells were seeded at 3 000 per well in 96-well plates in DMEM with 5% FCS, preincubated for 24 hours and treated for 72 hours with vehicle (0.2% ethanol)(control) or AA (2.5 to 20 μg/ml). Cell number was determined by crystal violet staining as described in Materials and Methods and is presented as a percentage relative to control. Results shown are the mean ±SD, n=8. *Significant difference from control, $P<0.05$. Three independent experiments were conducted. Data are from representative experiments.

5.4 Discussion

5.4.1 Proliferation studies

In vivo studies have shown that dietary PUFAs could have beneficial effects on bone.[25,27-29,31,192] (Refer to 2.10.2). The cellular effects of PUFAs have, however, not been extensively investigated. As the number of functional osteoblasts is important for bone formation, the effects of PUFAs as well as the bone active hormones PTH and oestrogen on osteoblast proliferation were investigated in the present study.

Polyunsaturated fatty acids and prostaglandin E_2

Depending on the cell type, culture conditions and concentrations PUFAs have been reported to either stimulate[327] or inhibit[300,301,326,328-330] the proliferation of various cell types. The n-3 PUFAs eicosapentaenoic acid (EPA) and DHA have generally been described as inhibitors of cell proliferation,[328-333] while the n-6 PUFA AA has shown various effects depending on the origin of the cell type and experimental conditions.[327,330,332-334]

In our experimental model, AA as well as DHA dose-dependently inhibited proliferation in both cell lines (Figure 5.1 and Figure 5.2). DHA inhibited proliferation of the MG-63 osteosarcoma-derived cell line more than that of the murine MC3T3-E1 osteoblastic cells (80% inhibition versus 20% inhibition at 20 μg/ml) (Figure 5.2). The difference in response of these two cell lines may be explained by the fact that the MG-63 osteoblast cell line is osteosarcoma-derived, while the MC3T3-E1 osteoblastic cell line is a normal cell line. Others have confirmed the anti-proliferative effect of DHA in a variety of cancer cell lines.[329,331,333] Maurin *et al* (2002) confirmed the inhibitory effects of AA and DHA on cell proliferation in MG-63 osteoblasts as well as primary human osteoblasts.[330] The anti-proliferative effect of the PUFAs has been attributed to inhibition of the G1 to S phase transition of the cell cycle.[326,330,335] It was speculated that this effect may be due to inhibition of the expression or activity of some cyclins or cyclin-dependent kinases related to cell cycle progression.[330]

Further mechanisms whereby PUFAs may inhibit cell growth to be considered, may include the following: Incorporation of PUFAs in the cell membrane with subsequent modification in fluidity and permeability has been proposed which may induce some changes in cell behaviour.[336] PUFAs may also act as second messengers by promoting the transfer of signals from the cell surface to the nucleus, thereby affecting signalling mechanisms involved in cell proliferation.[300] Since tyrosine kinase activity is considered to be important for stimulation of cell growth,[323] one of the mechanisms whereby PUFAs inhibits cell growth might be through inhibition of tyrosine kinase activity. Joubert *et al* (1999) showed an unexpected increase in tyrosine kinase activity in two oesophageal cancer lines after PUFA exposure.[300] However, PUFAs may regulate tyrosine kinase activity in osteoblastic cells differently and this needs to be investigated.

As PGE_2 is synthesized from AA,[170] AA may exert its cellular effects via prostaglandin synthesis.[322,330] (Refer to 4.4). Results from our laboratory showed that both MG-63 and MC3T3-E1 cells produce considerable amounts of PGE_2 when exposed to 20 μg/ml AA (refer to Figure 4.2 and Figure 4.6). To determine whether the anti-mitotic effect of AA on these cell lines was mediated via PGE_2, the effect of PGE_2 exposure on cell proliferation in these cell lines was investigated. Compared to control, PGE_2 caused a slight inhibition of cell proliferation in both cell lines (Figure 5.3). In the MG-63 cells the greatest effect was observed at a relatively low concentration of 10^{-10} M (12% inhibition), while the proliferation of MC3T3-E1 cells was affected mostly at higher concentrations (10^{-8} to 10^{-7} M) (8% inhibition). Others confirmed the anti-proliferative effect of PGE_2 on osteoblast-like cells.[261,263-265] Although the mechanism by which prostaglandins inhibit cell proliferation is not fully understood, it has been shown that prostaglandins exert their effects through specific prostaglandin receptors (EP) located on cell membranes[235,236] It is not known which EP receptors MG-63 cells express but MC3T3-E1 cells predominantly express EP_1 and EP_4 receptors.[229] Prostaglandins can alter their target cells' transition through the different phases of the cell cycle. While PGE_2 causes an increase in S phase and decrease in G_1 phase in oesophageal carcinoma cells,[300] it inhibits proliferation of melanoma cells by blocking these cells in the G_2 phase of the cell cycle.[337] We suggest that in our model, the inhibitory effect of AA on osteoblast cell proliferation is possibly independent of PGE_2 production, as PGE_2 *per se* had little effect on proliferation in the cell lines tested. Joubert *et al* (1999) reported AA-mediated

inhibition of cell proliferation in oesophageal carcinoma cells. In their study addition of the cyclo-oxygenase blocker indomethacin did not abolish the inhibitory effect of AA on cell proliferation, thereby demonstrating that AA *per se* had an effect on these cells,[300] thereby supporting our observation.

Depending on the model and concentration of PGE_2 used, disparate results of the effects of PGE_2 on osteoblastic proliferation were reported.[261-267] The differentiation status of osteoblast-like cells also appears to affect the growth response of PGE_2 on these cells. Centrella *et al* (1994) demonstrated a stimulatory effect of PGE_2 on cell replication in less differentiated primary osteoblasts derived from fetal rat bone, but showed an inhibitory effect of PGE_2 on more differentiated bone cells.[261] In a later study, Woodiel *et al* (1996)[262] reported an anabolic effect of PGE_2 on replication and differentiation in cultured fetal rat calvarial cells and concluded that these effects were likely to be mediated by an EP_2 receptor, which stimulates cAMP-dependent activation of PKA.[262] Fujiedja *et al* (1999) demonstrated that PGE_2 inhibits cell proliferation in osteoblasts obtained from young rats but stimulates proliferation in osteoblasts from aged rats.[338] They concluded that the effect of PGE_2 on proliferation is exerted exclusively through the EP_1 receptor subtype in the cells from young rats and that this signal transduction pathway is not active in the cells from aged rats.[338] From these results it is clear that the effects of PGE_2 on cell proliferation are complex and influenced by differentiation status of the osteoblasts, PGE_2 concentration and expression of specific PGE_2 receptors.

The inhibitory effect of PUFAs on cell proliferation in the experimental model system could be due to the formation of PUFA peroxidation products in the culture media.[339,340] PUFAs are particularly susceptible to oxidation and is associated with the lipid peroxidation chain reaction.[341,342] Lipid peroxidation products can react with other molecules, such as proteins and DNA that are harmful to the cell. These peroxidation products could cause membrane damage changing signal transduction and cell metabolism.[341] Shiina *et al* (1993) reported inhibition of vascular smooth muscle cell proliferation by EPA that was reversed by the addition of anti-oxidants.[328] This observation was confirmed by Dommels *et al* (2003) who demonstrated that antioxidants such as vitamin E and vitamin C could partially reverse the AA- and EPA-induced decrease in proliferation in human coleorectal carcinoma cells.[339] Another study, however, showed that DHA had a dose-dependent inhibitory effect on

rat uterine stromal cell proliferation which was independent of lipid peroxidation, since it was not reversed by the addition of antioxidants, also no oxidation products of DHA occurred with the culture conditions applied.[327] An *in vivo* rat experiment investigated the influence of 12 weeks DHA supplementation on oxidative DNA damage in the bone marrow of young and aged rats.[342] Results from this study showed that in aged rats, but not young rats, excessive supplementation of DHA induces oxidative DNA damage in bone marrow and that decreased vitamin C synthesis in aged rats is involved in the mechanisms of DNA damage.[342]

Fatty acids and their products are also important ligands for the peroxisome proliferator activated receptors PPARγ.[188,343] (Refer to 2.3.1.2 and 2.9.4). DHA in particular has been shown to activate PPARγ.[343] Activation of PPARγ and PPARδ has been implicated in both inhibition and induction of cell proliferation. Therefore with both of these subtypes being expressed in osteoblastic cell lines, and in rat calvaria cells, the effects of PUFAs on osteoblasts may be mediated by the activation of PPARγ and needs to be investigated.[330]

Oestrogen

Depending on the cultures and conditions used, *in vitro* studies have shown inconsistent results regarding oestrogen's effect on osteoblast proliferation.[108,290,291] Cheng *et al* (2002) reported a small dose-dependent increase in proliferation in two human osteoblastic cell lines.[343] Exposing these cells to an oestrogen receptor modulator abolished the mitogenic effect of oestrogen.[344] It is well recorded that oestrogen acts through oestrogen receptors (ER) located on the nuclear membrane, thereby affecting intracellular pathways.[35,108] Difference in cell source, methods of isolation and culture conditions affect expression of ER in primary, immortalised and transformed osteoblastic cells.[108] Osteoblastic cell lines expressing artificially high levels of ER have been generated by stable transfection of the ERα gene. In these cells, oestrogen's response is dependent on the ER level, with a wider spectrum of responses produced by cells expressing higher ER levels.[108]

Beside oestrogen's classical receptor-mediated actions, it has been shown that steroids can produce responses in a variety of cells that are too rapid to be explained

by a genomic mechanism. These non-genomic mechanisms involve rapid and transient changes in ion fluxes across the plasma membrane.[108] It has been shown that oestrogen stimulates calcium ion influx and phosphatidyl inositol biphosphate metabolism in osteoblast-like cells.[345] Rapid effects of steroid hormones could also be mediated by interactions with components of various signal transduction pathways, including adenyl cyclase, MAPKs, and P13K.[346]

In our experimental model oestrogen slightly inhibited MG-63 proliferation (Figure 5.4), with a more pronounced effect at a concentration of 10^{-8} M (14% inhibition). Oestrogen exposure, however, had no effect on MC3T3-E1 cell proliferation (Figure 5.4). Others also could not demonstrate mitogenic effects of oestrogen on osteoblastic cells.[290,291] The expression of different numbers of ER might be one of the factors explaining the disparate effects of oestrogen on cell proliferation. Low ER expression levels in MC3T3-E1 cells have been reported[320] and could explain the inability of these cells to respond to oestrogen treatment.

Fohr *et al* (2000) investigated the effects of oestrogen on human osteosarcoma cell lines of various gender and differentiation status.[347] Oestrogen significantly increased proliferation of MG-63 and SaOS2 cell lines, but decreased proliferation of the HOS TE 85-cell line.[347] They concluded that the response of osteosarcoma cells to oestrogen is not related to the gender of the cell lines, but rather depends on its osteoblastic commitment.[347] In contrast to Fohr *et al*'s results, in our model oestrogen exposure slightly inhibited MG-63 cell proliferation (maximum inhibition up to 14%). The difference in outcome might be credited to different culture conditions, as Fohr *et al* applied serum free conditions for oestrogen exposure, while our experiment used culture media containing 5% FCS. Alternatively, the cells might express different numbers of oestrogen receptors. One can therefore speculate that the cells investigated in our study might be ER-deficient or expressing very low levels of ER. It is therefore necessary to determine whether the cells we used express sufficient ER levels to be affected by oestrogen treatment. However, other aspects of oestrogen cell signaling, such as possible non-genomic mechanisms may differ in the two cell lines tested.

Parathyroid hormone

Inconsistent results on the effects of PTH on proliferation have been reported. PTH stimulates[76,348] or inhibits[264,324,349] osteoblast cell proliferation. The outcome seems to depend on the cell lines used, cell density, PTH concentration, method of PTH administration, duration of exposure, and the presence of growth factors.[115,324,348,349] Jilka *et al* (1999) suggested that the anabolic effect of PTH *in vivo* could in part be attributed to PTH's ability to inhibit osteoblastic apoptosis, thereby prolonging the life-span of these cells.[126] In our model, continuous exposure of both cell lines to PTH for 72 hours inhibited cell proliferation slightly (Figure 5.5), with the greatest effect at concentrations of 10^{-9} to 10^{-8} M (maximum inhibition up to 10%). Others confirmed the inhibitory effect of PTH on osteoblastic cells.[324,349]

Several mechanisms for the PTH-modulated inhibitory effects on cell proliferation have been proposed: PTH interferes with the mitogenic pathway by inhibition of MAP kinase activity via a PKA-dependent pathway in osteoblastic osteosarcoma derived cells[264,324] and PTH *in vitro* prevents cell cycle transition from the G_1 to the S phase.[349]

5.4.2 Morphological studies

Morphological studies were conducted to determine whether exposing the osteoblastic cells to PUFAs, PGE_2, and hormones caused structural damage to the cells thereby yielding invalid results. Previous studies showed that PUFAs at high concentrations could be toxic to cells.[301,326,341] In our model, no morphological damage or abnormalities were detected after exposing MG-63 and MC3T3-E1 cells to AA (2.5 to 20μg/ml), PGE_2, oestrogen or PTH (ranging from 10^{-10} to 10^{-6} M). DHA exposure (2.5 to 20μg/ml) to MC3T3-E1 cells also did not harm these cells and normal dividing cells were evident. However, DHA at concentrations of 10 μg/ml to 20 μg/ml affected MG-63 cell density considerably and a number of apoptotic cells could be seen at high magnification (Figure 5.6F). Hoechst and propidium iodide fluorescent staining confirmed the presence of multi-nucleated cells with extensive nuclear blebbing (Figure 5.8D) that may be indicative of an early step in apoptosis development.[326]

As DHA is highly unsaturated, apoptosis may be due to the formation of DHA peroxidation products in the culture media and peroxides are known to enhance apoptosis.[82] MG-63 cells, being osteosarcoma-derived, could be more susceptible to DHA (and its peroxidation products) than normal cells such as MC3T3-E1 osteoblasts. Others confirmed the apoptotic effects of DHA on cancer cell lines.[329,333] Siddiqui *et al* (2001) demonstrated apoptotic effects of DHA on Jurkat leukemic cells and proved that survival of these cells were affected by induction of events upstream, leading to the activation of caspase-3 known to stimulate apoptosis.[333] According to Pompeia *et al* (2003) PUFA-modulated cell death seems to be associated with oxidative stress and lipid peroxidation that could trigger the release of cytochrome c from mitochondria resulting in apoptosis.[341]

A well-established strategy for inducing apoptosis in cell culture is to remove serum from the growth media.[42] Serum provides components such as proteins, amino acids, lipids, growth factors, vitamins, hormones and attachment factors, acts as a pH buffer, and provides protease inhibitors.[290] Free PUFAs are easily oxidised in culture media and peroxide levels may increase to cytotoxic levels in the medium.[328] Binding of PUFAs to albumin in serum protects cells from the cytotoxic effects of high concentrations of free fatty acids.[330,333,341,349] Ramesh *et al* (1992) demonstrated that albumin not only prevents the cytotoxic action of PUFAs, but also interfere with both the uptake of fatty acids and free radical generation in tumour cells.[350] In our laboratory, exposure of MG-63 cells to DHA in culture media supplemented with 10% FCS instead of 5% FCS, protected these cells against the apoptotic effects of high DHA concentrations.

Although AA[326,341,351] as well as PGE_2[352] have been reported to be inducers of apoptosis, we did not observe apoptotic cells in either MG-63 or MC3T3-E1 cell lines after 24 to 48 hours exposure to AA or PGE_2. However, apoptosis occurs quickly and cells undergoing this form of cell death disappear within hours.[78,80] Under *in vitro* cell culture conditions apoptotic bodies accumulate in the culture medium, since they cannot all be removed physiologically through phagocytosis by neighbouring cells.[325] This floating apoptotic cell debris may therefore not be detected when cell preparations are fixed and stained for morphological studies resulting in the underestimation of apoptosis in this cell model.

5.4.3 Conclusion

Results from our study have shown that oestrogen and PTH as well as PGE_2 slightly inhibited proliferation of MG-63 and MC3T3-E1 cells by up to 8% to 14%. In contrast, the PUFAs AA and DHA inhibited osteoblastic cell growth significantly (by up to 20% to 80%) at high concentrations. DHA also affected proliferation of the osteosarcoma-derived MG-63 osteoblasts more severely (by up to 80%) than MC3T3-E1 osteoblastic cells. As DHA is highly unsaturated and known to be oxidised in culture, we speculate that the anti-proliferative effects of DHA are probably mediated via production of peroxide products. Apoptotic cells were detected after exposure of MG-63 cells to DHA. This could be attributed to the apoptotic effects of DHA lipid peroxidation products that are known to stimulate apoptosis; cancer cells are also reported to be more susceptible to DHA than normal cell lines.

The initial phase of the osteoblast development is characterised by active replication of undifferentiated cells, however, confluent osteoblasts in culture can differentiate into mature mineralising osteoblasts when stimulated with osteogenic agents.[304] Stanford *et al* (1995) reported early changes in the onset of mineralisation when confluent osteoblast-like cells were cultured in an osteogenic medium for a relatively short culture period of 48 hours.[353] As there is a reciprocal relationship between reduced proliferation and subsequent induction of cell differentiation *in vitro,*[293,304,354] follow-up work was conducted to investigate whether inhibition of cell proliferation in our model is due to increased differentiation of osteoblasts to the mature mineralising osteoblastic phenotype. Results of these experiments are reported in Chapter 6.

CHAPTER 6

Effects of Arachidonic Acid and Docosa-hexaenoic Acid on Differentiation of and Mineralisation by MG-63 and MC3T3-E1 Osteoblast-like Cells

6.1 Introduction

The role of lipids in skeletal health is well documented. (Refer to 2.10.2 and 2.10.3). Some studies show that acidic phospholipids facilitate cartilage mineralisation in the growth plate, while prostaglandins, which are synthesised from fatty acids, affect bone formation in animals and affect insulin-like growth factors.[173] (Refer to 2.11.3). Long chain polyunsaturated fatty acids such as the n-6 PUFAs gamma-linolenic acid (GLA) and arachidonic acid (AA) as well as the n-3 PUFAs eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) all affect bone formation and resorption in both animal models and in humans.[25,27,31,173,192,203] Some suggested mechanisms of action for the PUFAs in these models include the following: modulation of calcium absorption from the intestine, increased bone mineralisation, enhancement of bone collagen synthesis, and reduction of urinary calcium excretion, thereby increasing the pool of available calcium for bone mineralisation in the body.[25-27,31,168,192]

The effects of PUFAs at cellular level are not fully understood. Some studies with n-3 PUFAs in rats show greater bone formation rates, and these actions seem to be linked with alteration in the functions of the osteoblast.[223] PUFAs may regulate protein expression in the osteoblast, and prostaglandin synthesis by modulating COX expression, but fatty acids may also affect pre-osteoblast-like cell differentiation into functional mineralising osteoblasts or alternatively into non-functional adipocytes.[293]

Osteoblastic cells are responsible for osteogenesis, which is characterised by a sequence of events marking *in vitro* maturation of the osteoblast phenotype. These

events involve active cell proliferation, followed by down-regulation of proliferation with accompanying upregulation of alkaline phosphatase (ALP) activity as marker for differentiation into functional osteoblasts. The final phase of osteoblastic development is characterised by formation of mineralised extracellular matrix.[41] Pre-osteoblasts may also differentiate into non-functional adipocytes, an alternative developmental pathway.[53,293] Figure 6.1 depicts the developmental sequence of the osteoblast.[41]

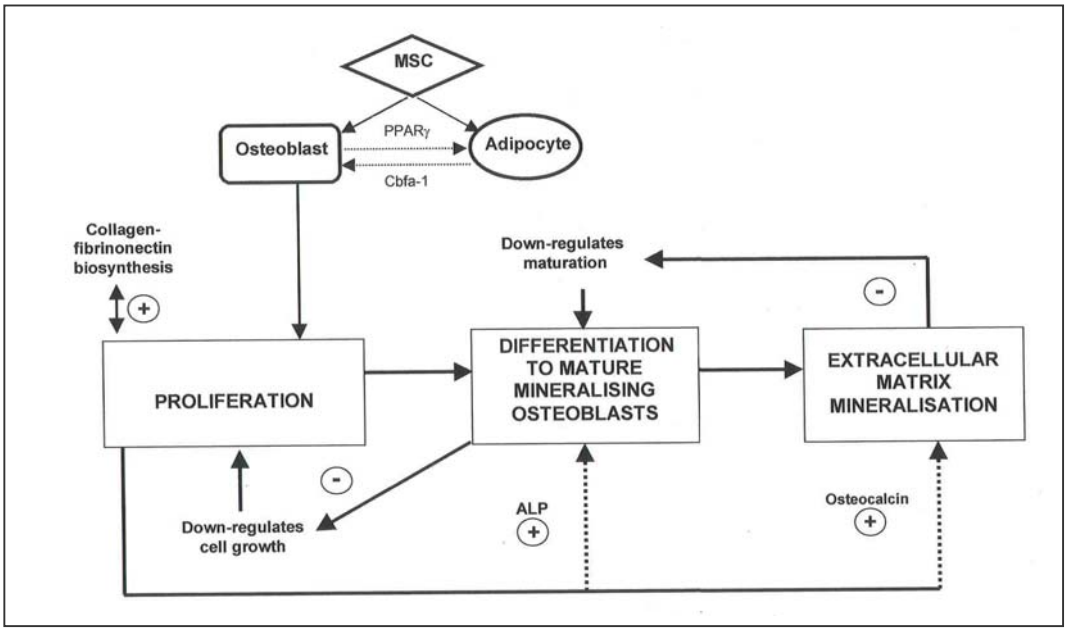


Figure 6.1 Stages in osteoblast development

Bone formation progresses through distinctive developmental stages that include commitment of mesenchymal stem cells (MSC) to the osteoblastic lineage, proliferation of osteoblast precursors, expression of the differentiated osteoblast phenotype, and ultimately, formation of mineralised extracellular matrix. Transdifferentiation between the osteoblastic and adipocytic lineages can occur, suggesting that plasticity exists between osteoblasts and adipocytes.

Proliferation supports the synthesis of a type I collagen-fibronectin extracellular matrix, which continues to mature and mineralise. Formation of this matrix down-regulates proliferation, and matrix mineralisation down-regulates the expression of genes associated with extracellular matrix maturation. As the cells stop proliferating and enter the maturation stage, expression of marker proteins such as alkaline phosphatase (ALP) becomes elevated. As the cells continue to differentiate and enter the mineralisation stage, the levels of proteins associated with maturation declines, and expression of osteocalcin and formation of hydroxyapatite are evident. *(Adapted from Stein GS, Lian JB. Molecular mechanisms mediating proliferation/differentiation interrelationships during progressive development of the osteoblast phenotype Endocr Rev 1993;14:424-42.[41]) Copyright 1993, The Endocrine Society.*

ALP is a membrane-bound ectoenzyme that can hydrolyse organic phosphates on the outer surface of the cell. ALP activity is therefore considered a marker of early

stage osteoblast differentiation.[41,355] Watkins *et al* (2003) have demonstrated that exposure of MC3T3-E1 cells, a murine osteoblast-like cell line, to AA and EPA affected ALP activity.[223] Compared to AA, exposure to EPA for five days increased ALP activity. This observation might be due to modulation of prostaglandin synthesis. Although Watkins *et al* (2003) reported a difference between the effects of AA and EPA, ALP activity in unexposed (control) cells was not reported.[223] Terminal differentiation of osteoblasts *in vitro* is characterised by formation of mineralising bone-like nodules.[304] *In vitro* mineralisation is usually induced by exposure of the cells to ascorbic acid and an organic phosphate source, ß-glycerol phosphate, to stimulate greater collagen production and cross-linking.[353] Once mineralisation starts, ALP activity decreases significantly.[291,293]

Another possible site of action for fatty acids is the modulation of the transcriptional factor PPARγ, a member of the peroxisome proliferator activated receptor family that is implicated in control of adipocyte differentiation.[56] Some bone cell lines, such as the MG-63 cells, express significant levels of PPARγ.[56] These receptors are activated by free fatty acids, their oxidation products, as well as some prostaglandins derived from fatty acids, that induce the differentiation of preadipocytes into adipocytic cells.[55,57,190,191,356] AA, which is a precursor for prostaglandins, has specifically been implicated in adipocyte differentiation.[56] With ageing there is a decrease in osteoprogenitor cells with an accompanying increase in adipocytes in bone marrow. This is due to altered differentiation of the common precursor cell, the mesenchymal stem cell (MSC).[44,56-58] When osteoblasts differentiate into adipocytes, ALP activity reduces significantly.[56]

Results from our laboratory have shown that AA and DHA can inhibit osteoblastic cell proliferation in a dose-dependent manner (Chapter 5). Since there is a reciprocal relationship between reduced proliferation and subsequent induction of cell differentiation *in vitro,*[293,304,354] it was decided to investigate the effects of AA and DHA as well as the hormones E2 and PTH on osteogenesis. The differentiation of MG-63 as well as MC3T3-E1 osteoblast-like cells into functional osteoblasts as indicated by alkaline phosphatase activity was investigated, while von Kossa staining was used to visualise bone nodule formation. Since fatty acids and their metabolites are ligands for members of the PPAR family, and are in part responsible for adipocyte differentiation,[55,57,191] measurement and visualisation of adipocyte

formation were included in this study. ALP activity versus Oil Red O staining was used as criteria to determine osteoblastic versus adipocytic differentiation.

6.2 Materials and Methods

Reagents and materials

Sigma Chemical Co (St. Louis, MO, USA) supplied L-glutamine, crystal violet, trypan blue, Oil Red O, arachidonic acid, docosahexaenoic acid, oleic acid, oestrogen, $1,25(OH)_2D_3$, parathyroid hormone fragment 1-34, ascorbic acid, β-glycerophosphate, ρ-nitrophenol (ρ-NP) and ρ-nitrophenylphosphate (ρ-NPP). Heat inactivated fetal calf serum (FCS) and α-MEM was obtained from Highveld Biological (Pty) Ltd (Sandringham, SA). Gentamycin was purchased from Gibco (Invitrogen Corp., Carlsbad, CA, USA). All other chemicals were of analytical grade and supplied by Sigma Chemical Co (St. Louis, MO, USA). Glass coverslips and 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. Cell cultures were maintained in α-MEM (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).

Osteogenic supplemented media

It is customary to use a supplemented medium when investigating mineralisation properties of cells.[291-293] Osteogenic supplemented medium contains ascorbic acid and ß-glycerophosphate to stimulate greater collagen production and cross-linking.[353] To enhance osteogenesis further, the synthetic glucocorticoid dexamethasone is often added. It was, however, decided to omit dexamethasone, as it was proven in rat bone marrow stromal cells that dexamethasone *per se* acts at multiple points in the differentiation process to stimulate osteoblastic maturation.[53,294,295,296] It has also been suggested that dexamethasone inhibits prostaglandin synthesis.[214,255,256] Since PGE_2 is a down-stream product of AA investigated in this study, it was important not to interfere with PGE_2 production. Therefore, when an osteogenic medium was required, α-MEM supplemented with 50 μg/ml L-ascorbic acid and 10 mM ß-glycerophosphate was used.[291]

Alkaline phosphatase (ALP) activity as marker of early differentiation

ALP is a membrane bound ectoenzyme that can hydrolyse organic phosphates on the outer surface of the cell. ALP is considered a marker of early stage osteoblast differentiation.[41,355] Following trypan blue exclusion, MG-63 and MC3T3-E1 cells were seeded at 10^5 per well in sterile 24-well plates with growth medium (α-MEM + 10% FCS) and pre-incubated for 48 hours until confluent. The medium was then changed to osteogenic supplemented medium, as well as vehicle (0.2% ethanol)(control), the PUFAs AA and DHA (2.5 to 20 μg/ml), oestrogen (10^{-8} M) and parathyroid hormone (PTH) (10^{-8} M) for a period of 48 hours.

At the end of culture, after treatment with vehicle and test agents, cells were washed with PBS and fixed in 4% formaldehyde in PBS (v/v) for 15 minutes. ALP activity was assayed as the release of ρ-nitrophenol (PNP) from the artificial substrate ρ-nitrophenylphosphate (ρ-NPP), using a colorimetric assay.[291] In short, fixed cells were incubated with ALP assay buffer (5 mM ρ-NPP; 0.5 mM $MgCl_2$; 0.1% Triton X-100 in 50 mM TBS, pH 9.5) for 60 minutes at 37ºC. (n=4). Thereafter 100 μl reaction product per well (in duplicate) was transferred to a 96-well microtiter plate

and absorbance (O.D.) read on an ELX800 Universal Microplate Reader (Bio-Tek Instruments Inc., Analytical Diagnostic Products, Weltevreden Park, SA) at a wavelength of 405 nm. Assay buffer was used as a blank. Cells were washed with distilled water, and cell number determined using a standard curve established with crystal violet assay.[290,298] 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, which 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. ALP activity was quantified against a standard curve of 0-400 μM ρ-NP and expressed as nanomoles of ρ-NP per hour per 10^4 cells. Three independent experiments were conducted in quadruplicate.

Onset of mineralisation as marker of osteoblast maturation

The terminal differentiation of osteoblasts *in vitro* is characterised by the formation of mineralising nodules.[304] Some osteoblastic cells appear to require a confluent state in order to generate a substantial calcification response implying a role for cell-cell contact in the mineralisation process.[353] To examine the capability of MG-63 cells and MC3T3-E1 cells to mineralise *in vitro*, cells were seeded at 10^5 per well on sterilised coverslips in sterile 24-well plates with growth medium (α-MEM with 10% FCS) and incubated for 48 hours until confluent. Medium was then changed to osteogenic supplemented medium, containing vehicle (0.2% ethanol) or PUFAs AA and DHA (2.5 to 20 μg/ml), oestrogen (10^{-8} M) and PTH (10^{-8} M). Once mineralisation starts, ALP activity decreases significantly.[291,293] Cells were therefore cultured in parallel plates for investigating formation of mineralised plaques as well as ALP activity.

After 14 days, with medium changes every three days, formation of mineralising plaques were visualised by a modified von Kossa staining technique for phosphate

detection (black) on fixed cell layers.[305,306] Cells were rinsed thrice in 0.9% NaCl solution (pH 7.2) and fixed in 4% (v/v) paraformaldehyde in 0.9% (w/v) NaCl solution at room temperature for 10 minutes. Cells were then rinsed thrice in ddH_2O, incubated with 3% (w/v) $AgNO_3$ in the dark for 30 minutes, and exposed to ultraviolet light (254 nm) for 30 minutes. Cells were then washed in ddH_2O and counterstained with Toluidine Blue for five minutes. Photographs were taken with a Zeiss Axiovert 200 microscope attached to a Nikon DXM 1200 digital camera (Nikon, Tokyo, Japan).

Oil Red O staining versus alkaline phosphatase activity as markers of osteoblastic transdifferentiation into adipocytes

Following trypan blue exclusion, MG-63 and MC3T3-E1 cells were plated in sterile 24-well cluster plates at 40 000/well, and cultured in α-MEM supplemented with 10% FCS until confluent. Medium was changed and vehicle (0.2% ethanol) or test agents AA (2.5 to 20 μg/ml) and DHA (2.5 to 20 μg/ml) were added. No osteogenic supplements were added to the medium to prevent forcing these cells along the osteogenic pathway. After three days, medium was changed and the cells cultured three more days in the presence of vehicle and test agents for a total of six days.[56]

Quantification of adipocytogenesis by Oil red O staining

Oil red O is a dye that is soluble in lipids and specifically stains triglycerides and cholesterol oleate but no other lipids.[52] As positive control for adipogenesis, the monounsaturated fatty acid, oleic acid (100 μM) was added to some cells.[56] To determine whether osteoblasts accumulate lipid droplets in the cytoplasm when treated with PUFAs, an adaptation of the Oil Red O staining procedure developed by Ramirez-Zacharias *et al* (1992), was performed.[307] At the end of culture, after treatment with vehicle and test agents, cells were rinsed with PBS, fixed in 10% paraformaldehyde in PBS (v/v) for 15 minutes, and washed with PBS. Cells were then stained for 30 minutes by complete immersion in Oil Red O working solution*. Thereafter, cells were rinsed briefly with 60% isopropanol, rinsed

exhaustively with dH_2O and air-dried. In order to determine the extent of adipocytic conversion, 500 μl/well of isopropanol was added to the stained cultures for 10 minutes. One hundred and fifty μl/well of the extracted dye was removed by gentle pipetting and transferred to a 96-well microtiter plate. Absorbance (O.D.) was read on a GLR 1000 microplate reader (Genelabs Diagnostics, U.K.) at a wavelength of 490 nm; isopropanol was used as blank. After washing with 70% ethanol, cell numbers were determined by crystal violet assay, as previously described. Three independent experiments were conducted (n=4). The amount of Oil Red O accumulated by the cells after PUFA treatment was compared to that of control cells (vehicle treated cells) and expressed as Oil Red O staining (% of control).

* Oil red O stock solution was prepared by dissolving 1.0 g of Oil Red O in 100 ml isopropanol. After stirring overnight at room temperature, the solution was filtered through analytical filter paper. Oil Red O working solution was prepared by diluting the stock solution with dH_2O at a ratio of 7:3 and filtering twice.

Alkaline phosphatase (ALP) activity

Apart from testing for the onset of adipogenesis as detected by Oil Red O staining, parallel cell cluster plates were cultured for the detection of changes in ALP activity. Vitamin D_3 ($1,25(OH)_2D_3$) is a well known enhancer of ALP activity and was used as positive control.[292,357] ALP activity was determined as previously described. (Refer to 3.8)

Microscopic visualisation of lipid accummulation

In order to visualise the accumulation of lipid droplets in cells after treatment with PUFAs, MG-63 and MC3T3-E1cells (150 000/well) were seeded aseptically onto heat-sterilised coverslips in 6-well culture plates. Twenty-four hours later, medium was changed and vehicle or test agents (AA and DHA) (2.5 to 20 μg/ml) were added to the culture. As positive control for adipogenesis, oleic acid (100 μM; ~ 30 μg/ml) was used.[56] After three days, medium was changed again and the cells cultured

three more days for a total of six days in the presence of vehicle and test agents. At the end of culture, cells were rinsed with PBS, fixed in 10% paraformaldehyde in PBS for 15 minutes, and washed with PBS. The coverslips were then removed from the cluster plates, inserted into coverslip holders, stained with Oil red O for 30 minutes, and rinsed briefly with 60% isopropanol. Cells were then counterstained with hematoxylin[110] and mounted to microscope glass slides with rapid mounting resin and left to dry. Photographs were taken with 400 ASA film with a Nikon camera (Nikon, Tokyo, Japan) attached to a Nikon Optiphot microscope (Nikon, Tokyo, Japan).

Statistics

Statistical analysis was performed using Statistics for Windows software (version 2, Tallahassee, Florida, USA). The results were analysed with one way ANOVA followed by Bonferroni's post-hoc testing. $P<0.05$ was considered to be significant.

6.3 Results

6.3.1 Alkaline phosphatase activity as marker of early differentiation

Effects of arachidonic acid, docosahexaenoic acid, parathyroid hormone and oestrogen (E2) on alkaline phosphatase (ALP) activity in MG-63 cells after 48 hours exposure

The effects of the PUFAs AA and DHA as well as the hormones PTH and E2 after 48 hours of incubation in osteogenic supplemented culture media are shown in Figure 6.2. Compared with control, incubation of MG-63 cells with a low concentration of AA (2.5 μg/ml) suppressed ALP activity by 30%, which was statistically significant. Exposure to higher AA concentrations did not affect ALP activity significantly. DHA slightly increased ALP activity; however, this increase was not statistically significant. Exposure to the hormones PTH or E2 (10^{-8} M) did not significantly affect ALP activity of the MG-63 cells in the conditions tested.

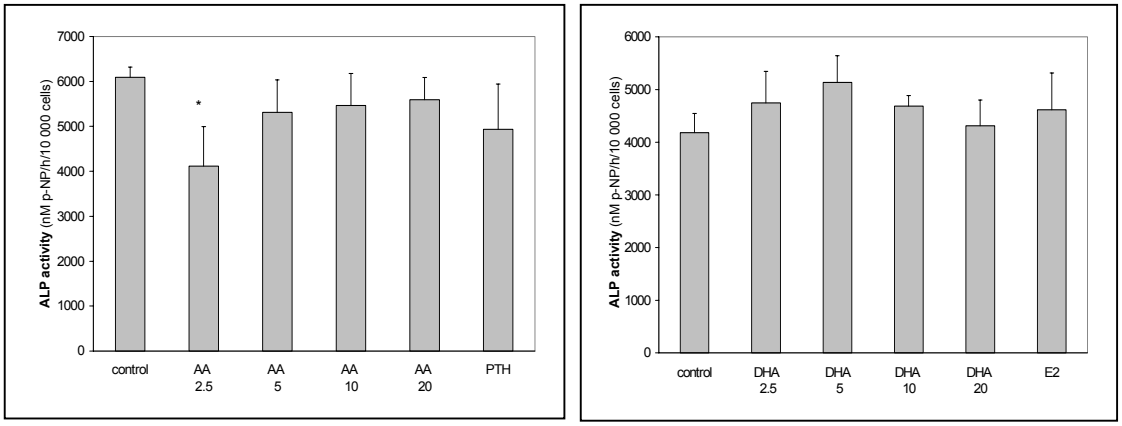


Figure 6.2 Alkaline phosphatase activity of MG-63 cells after 48 hours of exposure to polyunsaturated fatty acids and hormones

Cells were precultured in α-MEM with 10% FCS until confluent and subsequently exposed to osteogenic supplemented medium, containing 50 μg/ml ascorbic acid and 10 mM ß-glycerophosphate as well as vehicle (0.2% ethanol), AA (2.5 to 20 μg/ml), DHA (2.5 to 20 μg/ml), E2 (10^{-8} M) and PTH (10^{-8} M) for 48 hours. ALP activity was measured using the ρ-NPP assay and expressed as nanomoles of ρ-NP per hour per 10^4 cells. *Significant difference from control, $P<0.05$. Three independent experiments were conducted (n=4). Data are from representative experiments.

Effects of arachidonic acid, docosahexaenoic acid, parathyroid homone and oestrogen (E2) on alkaline phosphatase (ALP) activity in MC3T3-E1 cells after 48 hours exposure

The effects of the PUFAs AA and DHA as well as the hormones PTH and E2 after 48 hours incubation in osteogenic supplemented culture media are shown in Figure 6.3. Compared with control, AA did not affect ALP activity of the MC3T3-E1 cells under the conditions tested. Compared with control, DHA suppressed ALP activity in a dose-dependent manner, which was statistically significant at concentrations above 2.5 μg/ml. Whilst PTH (10^{-8} M) had no effect on ALP activity, E2 (10^{-8} M), although not statistically significant, suppressed ALP activity in these conditions.

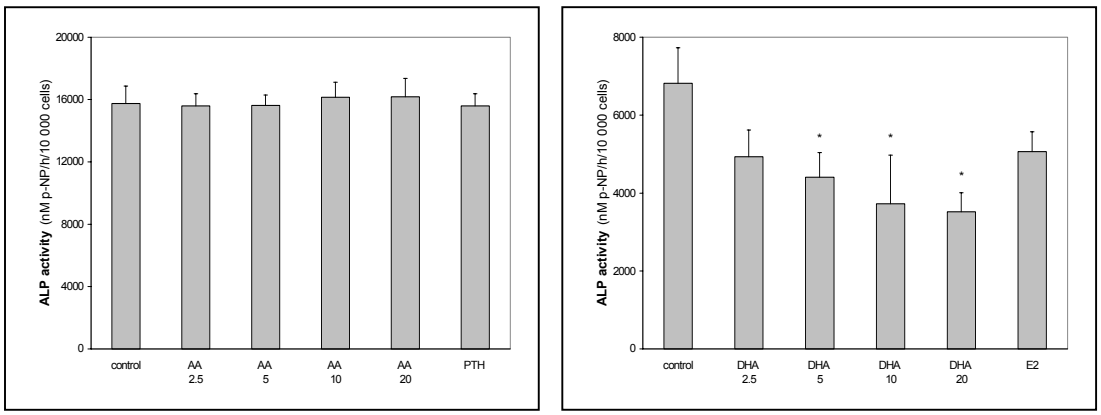


Figure 6.3 Alkaline phosphatase activity of MC3T3-E1 cells after 48 hours of exposure to polyunsaturated fatty acids and hormones

Cells were precultured in α-MEM with 10% FCS until confluent and subsequently exposed to osteogenic supplemented medium, containing 50 μg/ml ascorbic acid and 10 mM ß-glycerophosphate as well as vehicle (0.2% ethanol), AA (2.5 to 20 μg/ml), DHA (2.5 to 20 μg/ml), E2 (10^{-8} M) and PTH (10^{-8} M) for 48 hours. ALP activity was measured using the ρ-NPP assay as described in Materials and Methods and expressed as nanomoles of ρ-NP per hour per 10^4 cells. *Significant difference from control, $P<0.05$. Three independent experiments were conducted (n=4). Data are from representative experiments.

6.3.2 Onset of mineralisation as marker of osteoblast maturation

Differentiation of osteoblasts into mature mineralising osteoblasts is a time-dependent process. Exposure of cells for longer periods of time is therefore more

likely to show effects. Differentiation of MC3T3-E1 cells into mature mineralising osteoblasts has been been reported from 5 to 16 days post confluency.[352,356] Detection of mineralising properties after 14 days exposure to vehicle and test agents, was therefore included in this study. It has been shown that once mineralisation starts, ALP activity decreases significantly.[291,293] Cells were therefore cultured in parallel plates for investigating formation of mineralised plaques as well as ALP activity.

Effects of arachidonic acid, docosahexaenoic acid, parathyroid hormone and oestrogen on alkaline phosphatase (ALP) activity in MG-63 cells after 14 days exposure

The effects of the PUFAs AA and DHA as well as the hormones PTH and oestrogen after 14 days incubation in osteogenic supplemented culture media are shown in Figure 6.4. Compared with control, AA dose-dependently stimulated ALP activity in MG-63 cells, which was significant at concentrations of 10 and 20 μg/ml (by up to 20% to 50%). Compared with control, neither DHA nor the hormones (PTH and oestrogen) affected ALP activity significantly in this cell line under the experimental conditions tested.

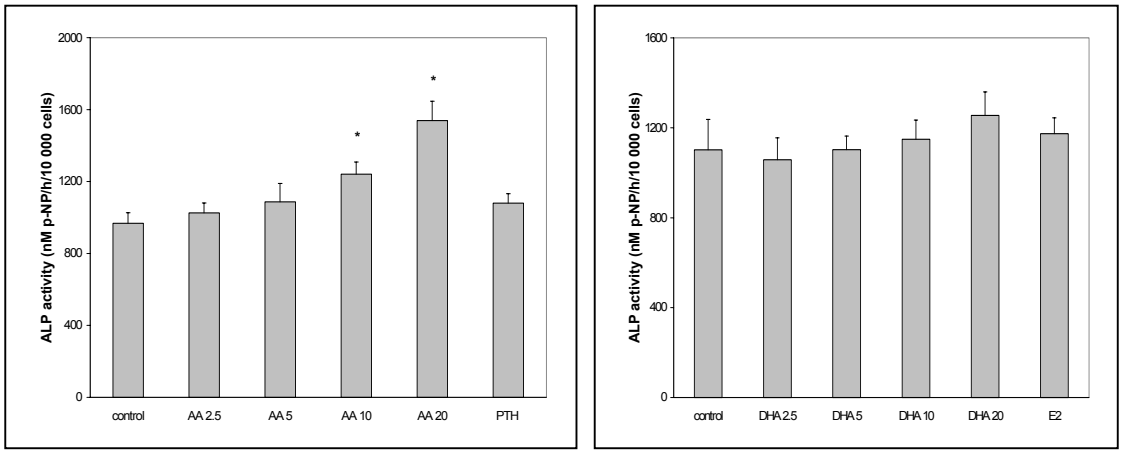


Figure 6.4. Alkaline phosphatase activity of MG-63 cells after 14 days of exposure to polyunsaturated fatty acids and hormones.

Cells were precultured in α-MEM with 10% FCS until confluent and then exposed to an osteogenic supplemented medium (containing 50 μg/ml ascorbic acid and 10 mM ß-glycerophosphate) as well as vehicle (0.2% ethanol), AA (2.5 to 20 μg/ml), DHA (2.5 to 20 μg/ml), E2 (10^{-8} M) or PTH (10^{-8} M) for 14 days. ALP activity was measured using the ρ-NPP assay and expressed as nanomoles of ρ-NP per hour per 10^4 cells. *Significant difference from control, $P<0.05$, n=4.

Effects of arachidonic acid, docosahexaenoic acid, parathyroid hormone and oestrogen on alkaline phosphatase (ALP) activity in MC3T3-E1 cells after 14 days exposure

The effects of the PUFAs AA and DHA as well as PTH and oestrogen on MC3T3-E1 cells' ALP activity after 14 days incubation in osteogenic supplemented culture media are shown in Figure 6.5. Compared with control, AA significantly suppressed ALP activity in a dose-dependent fashion. DHA also suppressed ALP activity, which was statistically significant at a concentration of 20 μg/ml. Compared with control, neither of the hormones, PTH or oestrogen, affected ALP activity in this cell line under the experimental conditions tested.

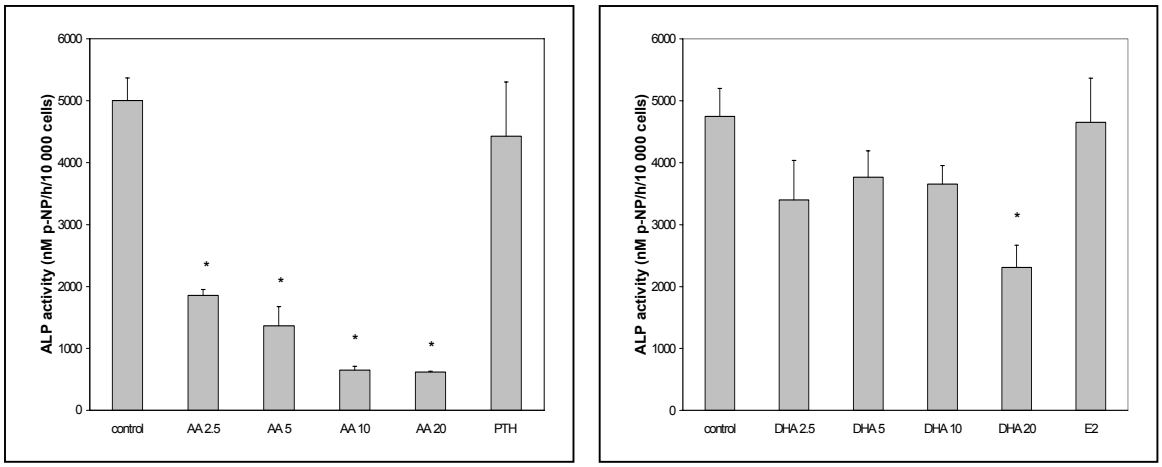


Figure 6.5. Alkaline phosphatase activity of MC3T3-E1 cells after 14 days of exposure to polyunsaturated fatty acids and hormones.

Cells were precultured in α-MEM with 10% FCS until confluent and then exposed to an osteogenic supplemented medium (containing 50 μg/ml ascorbic acid and 10 mM ß-glycerophosphate) as well as vehicle (0.2% ethanol), AA (2.5 to 20 μg/ml), DHA (2.5 to 20 μg/ml), E2 (10^{-8} M) or PTH (10^{-8} M) for 14 days. ALP activity was measured using the ρ-NPP assay and expressed as nanomoles of ρ-NP per hour per 10^4 cells. *Significant difference from control, $P<0.05$, n=4.

Visualisation of mineralised plaques in MG-63 and MC3T3-E1 cells after 14 days exposure to arachidonic acid, docosahexaenoic acid, oestrogen and parathyroid hormone

The terminal differentiation of osteoblasts in *vitro* is characterised by the formation of mineralised plaques.[304] Following von Kossa staining, black mineralised vesicles were detected microscopically in the matrix of the MC3T3-E1 cell culture after 14 days exposure to either vehicle (0.2% ethanol), AA, DHA, E2 (10^{-8} M) or PTH (10^{-8} M) (Figure 6.6 A-E). Exposure of MC3T3-E1 cells to DHA and PTH resulted in formation of more prominent mineral plaques, but it was not quantified.

In the MG-63 cell line, no mineralisation was detected by von Kossa staining after 14 days exposure to either vehicle or test substances (AA, DHA, E2, PTH) in our experimental conditions (Figure 6.6 F).

6.3.3 Oil Red O staining versus alkaline phosphatase activity as markers of osteoblastic transdifferentiation into adipocytes

Fatty acids and their metabolites are ligands for members of the PPAR transcriptional factor family, and are in part responsible for adipocyte differentiation.[55,57,191] (Refer to 2.3.1.2). Measurement and visualisation of adipocyte formation were therefore included in this study. ALP activity versus Oil Red O staining was used as criteria to determine osteoblastic versus adipocytic differentiation.

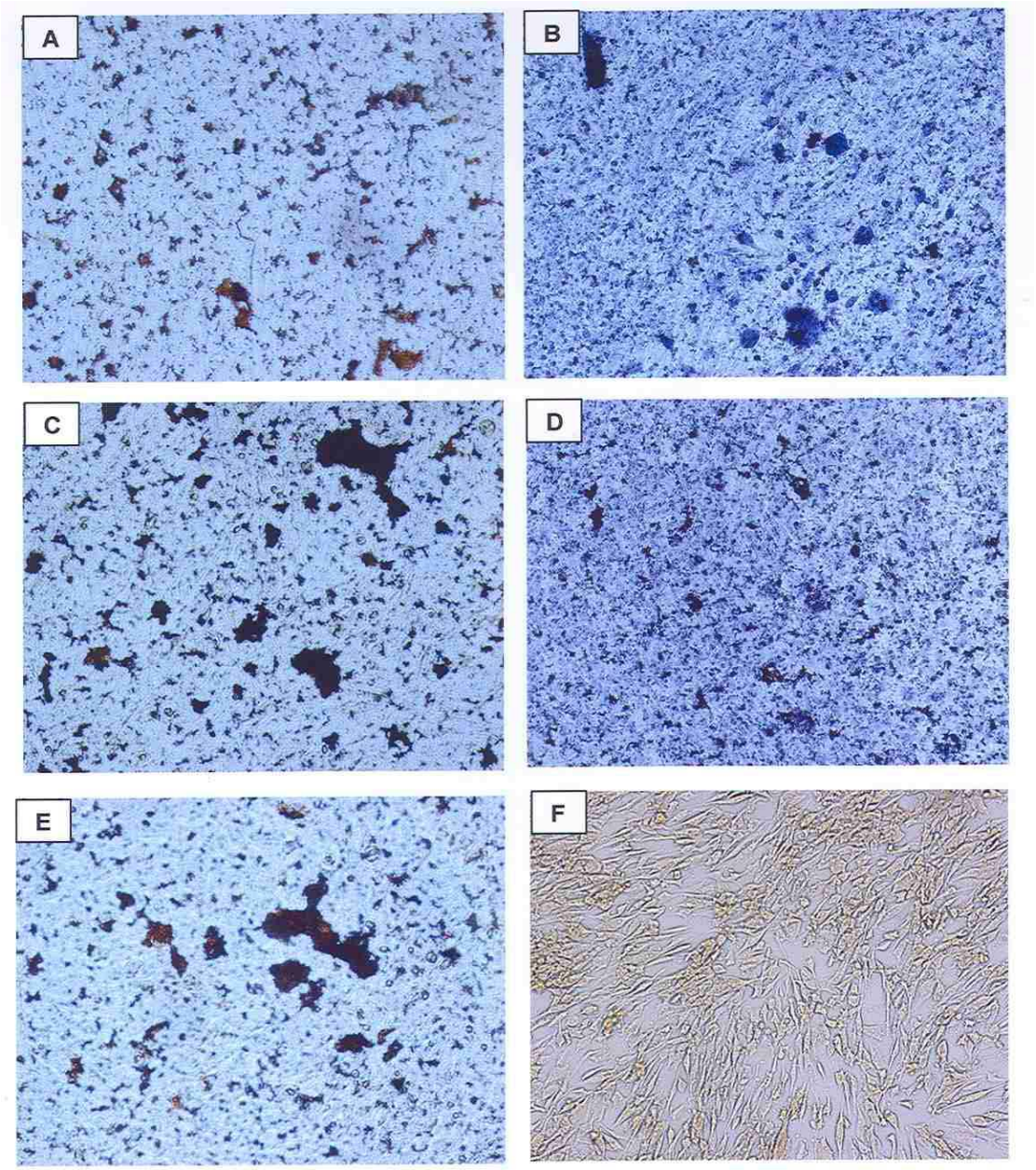


Figure 6.6 Photomicrograph of mineralised nodules.

Cells were grown to confluency in α-MEM with 10% FCS, thereafter medium was supplemented with osteogenic medium, containing 50 μg/ml ascorbic acid and 10 mM β-glycerophosphate as well as vehicle (0.2% ethanol)(control) and test agents (AA, DHA, E2 and PTH). Cultures were maintained *in vitro* for 14 days with medium changes every third day. At the end of culture, cells were fixed, stained with the von Kossa technique for mineralisation (black), and counter stained with toluene blue as described in Materials and Methods. **A:** MC3T3-E1 cells exposed to vehicle (0.2% ethanol); **B:** MC3T3-E1 cells exposed to 20 μg/ml AA; **C:** MC3T3-E1 cells exposed to 20 μg/ml DHA; **D:** MC3T3-E1 cells exposed to E2 (10^{-8} M); **E:** MC3T3-E1 cells exposed to PTH (10^{-8} M); **F:** MG-63 cells exposed to vehicle (0.2% ethanol).
(Original magnification: 80x).

Effects of arachidonic acid and docosahexaenoic acid on alkaline phosphatase (ALP) activity in MG-63 cells after six days

The effects of AA and DHA on ALP activity after six days incubation in medium *without* osteogenic supplementation are shown in Figure 6.7. Compared with control, AA suppressed ALP activity in a dose-dependent manner. Exposing the MG-63 cells to 20 μg/ml AA resulted in significant suppression of ALP activity, such as that no ALP activity could be detected at this dose. Compared with control, DHA had a biphasic effect on ALP activity, with stimulation at low concentration (2.5 μg/ml) and inhibition at high concentration (20 μg/ml). As no osteogenic supplements were added to the medium in this experiment, ($1,25(OH)_2D_3$) (vitamin D_3) which is a known stimulator of ALP activity was used as positive control.[292] Compared with control, vitamin D_3 exposure (10^{-8} M) enhanced ALP activity significantly by almost 40%, confirming that ALP activity could be stimulated under these conditions.

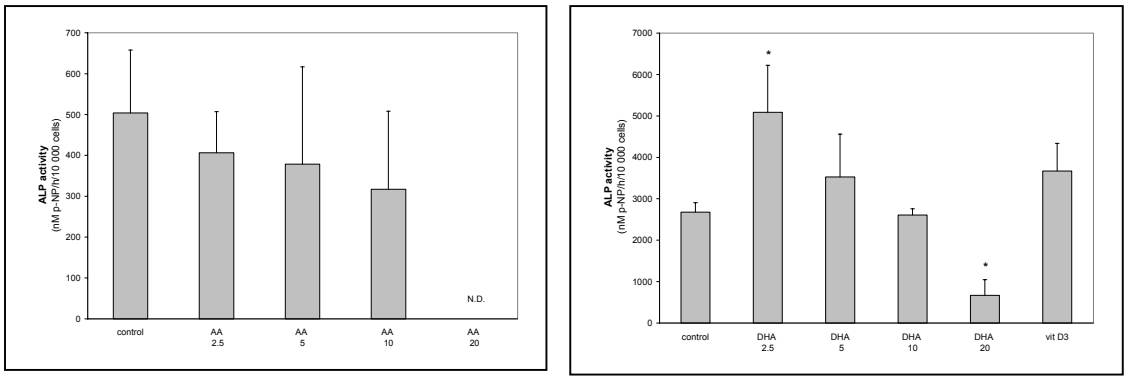


Figure 6.7 Alkaline phosphatase activity of MG-63 cells after six days of exposure to arachidonic acid and docosahexaenoic acid.

Cells were precultured in α-MEM with 10% FCS until confluent and subsequently exposed to vehicle (0.2% ethanol), AA (2.5 to 20 μg/ml) or DHA (2.5 to 20 μg/ml) for six days. Vitamin D_3 (10^{-8} M) was used as positive control for stimulation of ALP activity. ALP activity was measured using the ρ-NPP assay and expressed as nanomoles of ρ-NP per hour per 10^4 cells. *Significant difference from control, *P*<0.05. Three independent experiments were conducted (n=4). Data are from representative experiments. (N.D. not detected)

Effects of arachidonic acid and docosahexaenoic acid on alkaline phosphatase (ALP) activity in MC3T3-E1 cells after six days

Figure 6.8 depicts ALP activity of MC3T3-E1 cells after six days exposure to AA and DHA (2.5 to 20 μg/ml). The mean ALP activity in the presence of AA and DHA appeared to follow the trends (inhibition for AA /biphasic increase for DHA) at low concentration (2.5 μg/ml), followed by a decrease at higher concentrations (5 to 20 μg/ml) for both PUFAs in MG-63 cells. These changes were not statistically significant. Vitamin D_3 (10^{-8} M) significantly stimulated ALP activity up to 300%.

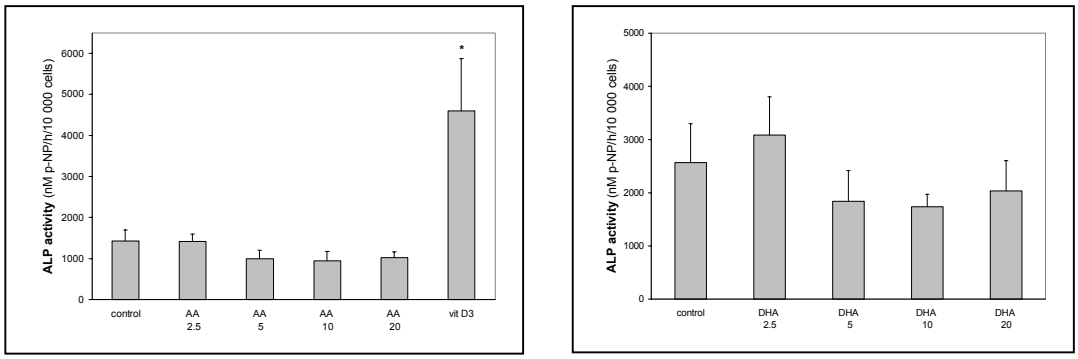


Figure 6.8. Alkaline phosphatase activity of MC3T3-E1 cells after six days of exposure to arachidonic acid and docosahexaenoic acid.

Cells were precultured in α-MEM with 10% FCS until confluent and subsequently exposed to vehicle (0.2% ethanol), AA (2.5 to 20 μg/ml) or DHA (2.5 to 20 μg/ml) for six days. Vitamin D (10^{-8} M) was used as positive control for stimulation of ALP activity. ALP activity was measured using the ρ-NPP assay and expressed as nanomoles of ρ-NP per hour per 10^4 cells. *Significant difference from control, $P<0.05$. Three independent experiments were conducted (n=4). Data are from representative experiments.

Effects of arachidonic acid and docosahexaenoic acid on Oil Red O staining in MG-63 cells after six days

Oil Red O is a dye that is soluble in lipids and specifically stains triglycerides and cholesterol oleate but no other lipids.[307] It was therefore used to determine whether osteoblasts accumulate lipid droplets in their cytoplasm when treated with PUFAs. This staining procedure is regarded adequate for the assessment of adipocytic phenotype differentiation and is considered equally accurate and sensitive as other procedures based on glycerophosphate dehydrogenase activity.[307] Exposure to the monounsaturated fatty acid oleic acid (100 μM) was used as positive control for adipogenesis.[56] Figure 6.9 shows that compared with control, AA concentrations less than 20 μg/ml inhibited lipid accumulation. DHA at low concentrations (2.5 to 5 μg/ml) inhibited lipid accumulation, while 10 mg/ml DHA had no effect. Exposure to high concentrations of AA (20 μg/ml), DHA (20 μg/ml) or oleic acid (100 μM) increased lipid accumulation significantly in these cells.

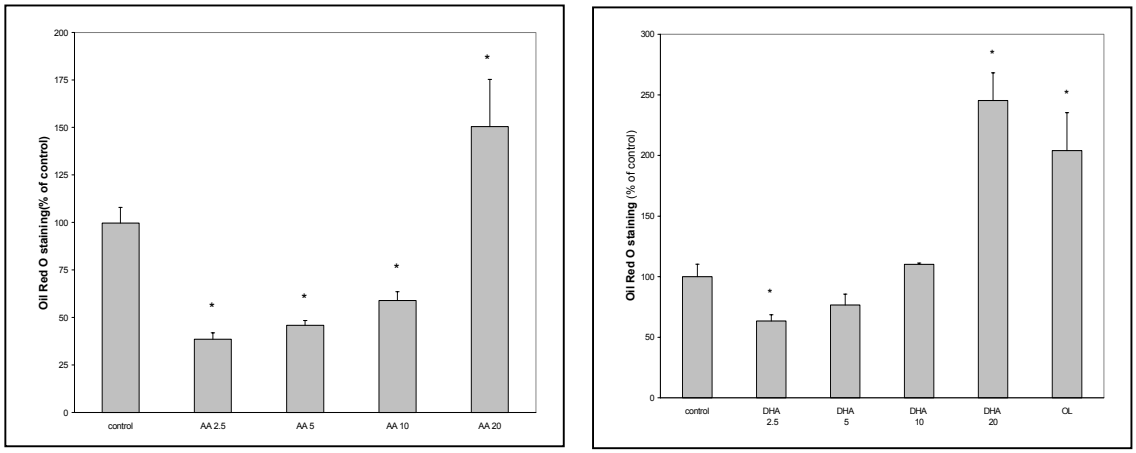


Figure 6.9 Quantification of Oil Red O staining of MG-63 cells after six days of exposure to arachidonic acid and docosahexaenoic acid.

Cells were precultured in α-MEM with 10% FCS until near confluent and subsequently exposed to vehicle (0.2% ethanol), AA (2.5 to 20 μg/ml) or DHA (2.5 to 20 μg/ml) for six days. Oleic acid (100 μM) was used as positive control. Oil Red O staining of cells was determined as described in Materials and Methods and expressed as % of control. *Significant difference from control, $P<0.05$. Three independent experiments were conducted (n=4). Data are from representative experiments.

Hardly any oil droplet accumulation was observed after Oil Red O staining of MC3T3-E1 cells. (Refer to Figure 6.10 F). Quantification of lipid staining was therefore not performed on this cell line.

Visualisation of Oil Red O staining in MG-63 cells and MC3T3-E1 cells after six days exposure to arachidonic acid, docosahexaenoic acid and oleic acid

Figure 6.10 depicts Oil Red O staining of lipids in MG-63 and MC3T3-E1 cells after six days exposure to vehicle and fatty acids. Compared with MC3T3-E1 cells, MG-63 cells in general were more capable of accumulating lipid vesicles in their cytoplasm and cytoplasmic lipid vesicle accumulation could even be detected in a few MG-63 control cells (Figure 6.10 A). Exposing MG-63 cells to oleic acid (100 μM), AA (20 μg/ml) and DHA (20 μg/ml) resulted in accumulation of large numbers of cytoplasmic lipid vacuoles indicating an adipogenic phenotype (Figure 6.10 B-D). Adipogenic conditions caused a change from the normal fibroblastic morphology to rounding of cells (Figure 6.10 C,D). No lipid vesicles could be detected in MC3T3-E1 cells exposed to vehicle (Figure 6.10 E), AA or DHA (results not shown). Exposure of MC3T3-E1 cells to oleic acid, which is regarded as an adipogenic agent[56] resulted in the accumulation of a small number of lipid vesicles in a few cells only (Figure 6.10 F).

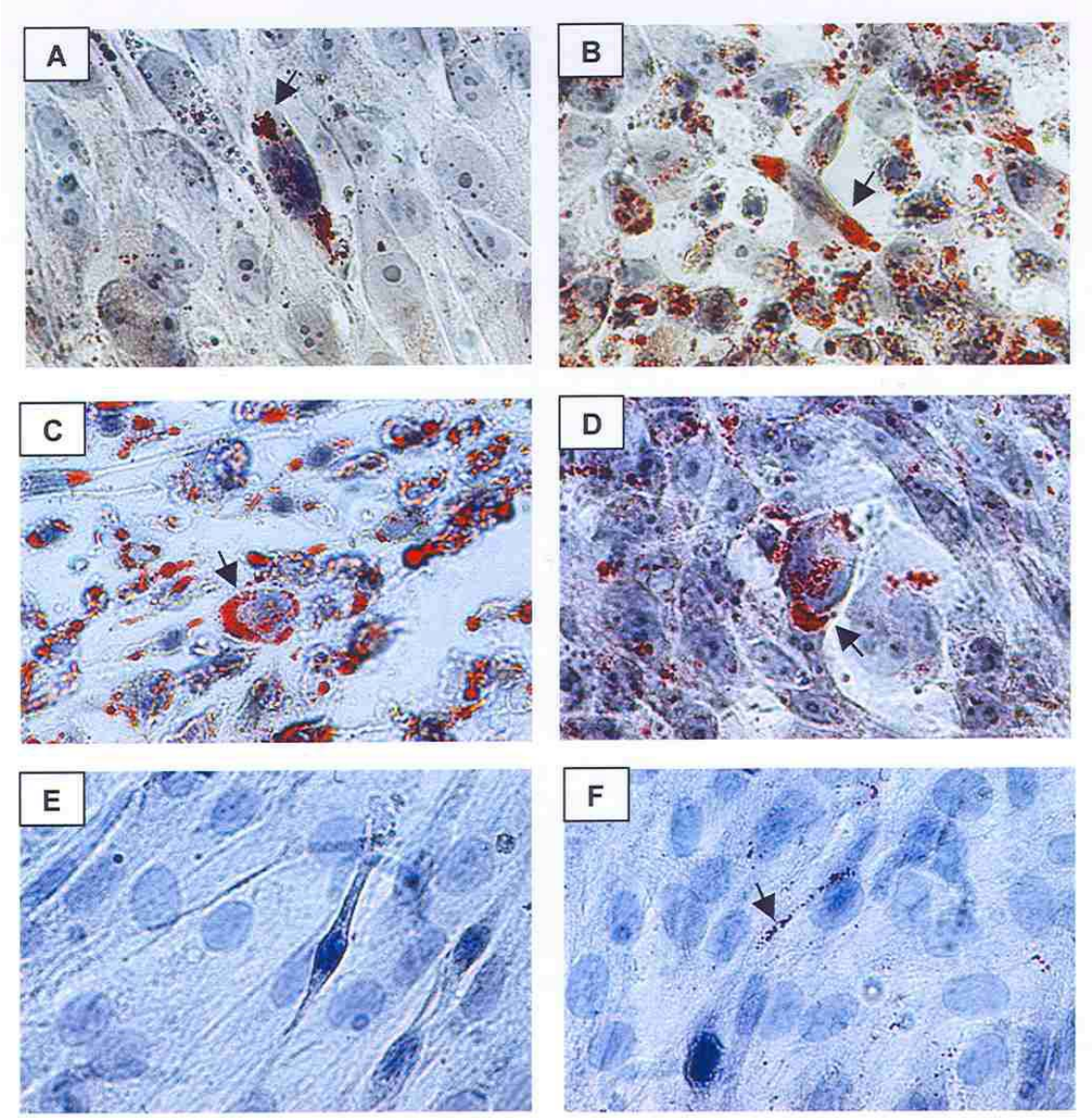


Figure 6.10 Photomicrographs of Oil Red O staining in MG-63 and MC3T3-E1 cells.

Cells were grown to near confluency in α-MEM with10% FCS, thereafter medium was changed and cells exposed to vehicle (0.1% ethanol)(control), AA (20 μg/ml) or DHA (20μg/ml) for six days. As positive control for adipogenesis, oleic acid (100 μM) was used. Oil red O staining was performed as described in Materials and Methods. **A:** MG-63 cells exposed to vehicle (0.2% ethanol) only; **B:** MG-63 cells exposed to 100 μM oleic acid, **C:** MG-63 cells exposed to 20 μg/ml AA; **D:** MG-63 cells exposed to 20 μg/ml DHA;**E:** MC3T3-E1 cells exposed to vehicle (0.2% ethanol) only; **F:** MC3T3-E1 cells exposed to 100 μM oleic acid. Arrows indicate lipid vesicles stained red by Oil Red O staining. (Original magnification: 400x)

6.4 Discussion

The aim of this study was to investigate whether the n-6 PUFA AA and the n-3 PUFA DHA as well as the hormones PTH and oestrogen affected osteogenesis in osteoblasts. The differentiation of MG-63 and MC3T3-E1 osteoblast-like cells into mature functional osteoblasts (as indicated by alkaline phosphatase activity and mineralised nodule formation) (refer to 2.10.3.1 and 6.1), or alternatively, the transdifferentiation of these cells into adipocytes (refer to 2.3.1.2), therefore non-functional osteoblasts, was investigated.

6.4.1 Alkaline phosphatase (ALP) activity as marker of early differentiation

An increase in bone specific ALP activity reflects the maturation from an earlier to a more mature stage of osteoblast differentiation. Mineralisation requires ALP to hydrolyse organophosphates thereby releasing inorganic phosphate, which appears to be the actual initiator of mineralisation.[359] It has been shown that inhibition of ALP activity by levamisole totally blocks the mineralisation response stimulated by organophosphates.[353] ALP activity is therefore used in *in vitro* experiments as a marker of osteoblast differentiation.[41,353]

Early changes in the onset of mineralisation have been reported when confluent osteoblast-like cells were cultured in an osteogenic medium for a relatively short culture period of 48 hours.[353] In order to evaluate whether AA and DHA affected such early differentiation of osteoblasts, ALP activity of confluent osteoblast-like cells in an osteogenic supplemented environment was investigated. In our experiments, exposing confluent MG-63 and MC3T3-E1 osteoblasts to AA for a relatively short period of 48 hours in osteogenic conditions did not result in a significant change in ALP activity (Figure 6.2 and Figure 6.3). We were therefore unable to show an effect of AA or its metabolites on osteoblastic differentiation as determined by ALP activity.

DHA treatment did not affect ALP activity of MG-63 cells significantly. (Figure 6.2). MC3T3-E1 cells, however, demonstrated a dose-dependent decrease in ALP activity

when exposed to DHA. (Figure 6.3). As osteoblasts continue to differentiate and enter the mineralisation stage, levels of proteins such as ALP associated with maturation declines, while expression of osteocalcin and formation of hydroxyapatite become evident.[41] The observed decline in ALP activity may therefore be attributed to either inhibition by DHA, or could be due to osteoblasts maturing into mineralising osteoblasts.

6.4.2 Onset of mineralisation as marker of osteoblast maturation

Osteoblasts not only synthesise bone matrix, but mineralise it as well. (Refer to 2.4.3). The final stage of osteoblastic maturation is marked by the expression of several matrix proteins, including osteocalcin, and the deposition of a calcium phosphate layer.[296] Bone nodule formation occurs gradually in several postconfluent osteoblastic cell lines when supplemented with an osteogenic medium containing ascorbic acid and β-glycerophosphate over an extended period of time. The extent of mineralisation depends on the osteoblastic cell type as well as culture conditions.[291-293,305] *In vitro*, as differentiation proceeds, the level of ALP activity rises and in the presence of β-glycerophosphate will generate free inorganic phosphate which,[360] apart from being an important constituent of the mineral itself, appears to be an important signaling molecule for mineralisation.[359] As osteoblasts continue to differentiate and enter the mineralisation stage, levels of proteins such as ALP associated with maturation declines, while expression of osteocalcin and formation of hydroxyapatite become evident.[41] The result of the differentiation process is the formation of hydroxyapaptite mineral that occurs through the formation of small vesicles that bud from the plasma membrane and accumulate calcium and phosphate, or alternatively, result from nucleation of collagen, regulated by associated noncollagenous matrix proteins.[359]

MG-63 cells, which are widely used as a model for human osteoblasts, are derived from human osteosarcoma tissue. These cells display several osteoblastic traits that are typical of a relatively immature osteoblast, including stimulation of ALP activity and responding to vitamin D_3 treatment, which proved them suitable for examining the early stages of osteoblast differentiation.[361] In our experiment, compared with

control, AA dose-dependently stimulated ALP activity in these cells, with 20 μg/ml of AA stimulating ALP activity by 50% after 14 days. (Figure 6.4). The ALP activity of the MG-63 control cells, however, was five times lower than that observed in the controls of the MC3T3-E1 cell line under similar conditions (Figure 6.4 and Figure 6.5). Low ALP activity in MG-63 cells was also reported by others.[299] DHA as well as PTH and oestrogen did not affect ALP activity in this cell line after 14 days.

Although AA exposure (2.5 to 20μg/ml) enhanced ALP activity significantly, the MG-63 cells were unable to produce any matrix mineralisation as detected by von Kossa staining after 14 days. Exposure to vehicle (0.2% ethanol), DHA, oestrogen or PTH produced similar results. The lack of mineralising properties by MG-63 cells is supported by the findings of other authors[248,299] and might be linked to the low ALP activity detected in this cell line.[299] Concerns have also been raised that osteosarcoma-derived osteoblasts may exhibit a deregulated proliferation/differentiation relationship, which might affect their response to various bone active agents.[291,292,360] It may therefore be advisable to use a nontransformed human cell line for mineralisation studies. These cells, however, normally have a short lifespan and may be difficult to maintain in the long term.

MC3T3-E1 murine osteoblast-like cells are considered immediate osteoblast precursors as these cells constitutively express high levels of ALP and calcify in basal conditions.[285,354] In our experimental conditions, compared with MG-63 control cells, MC3T3-E1 control cells exhibited five times higher basal ALP activity after 14 days (Figure 6.4 and Figure 6.5), thereby confirming the results of others.[285] It has been shown by Igarishi *et al* (1997) that the ALP activity of control MC3T3-E1 cells increases after seeding, reaches a maximum on day 15, and decreases thereafter up to day 21.[363]

Compared with control, AA (2.5 to 20μg/ml) dose-dependently inhibited ALP activity in the MC3T3-E1 cell line after 14 days (Figure 6.5). AA is the natural substrate for PGE_2 synthesis[170] and has been implicated in osteoblastic differentiation. It has been reported that PGE_2, at high concentrations, inhibits ALP activity,[266,271] while blocking of endogenously produced PGE_2 by COX blockers increases ALP activity.[271,361] It could therefore be speculated that the inhibitory effect of AA on ALP activity in our experiment, might be PGE_2-mediated. DHA exposure, although not as

pronounced as AA, also inhibited ALP activity in the MC3T3-E1 cells after 14 days. (Figure 6.5). The mechanism whereby DHA inhibits ALP activity is not clear and needs to be investigated. Watkins *et al* (2003) reported that compared with AA, the n-3 PUFA EPA increased ALP activity in MC3T3-E1 cells and attributed it to a PGE_2-lowering effect.[223] These authors, however, did not include a control in their study, making it impossible to compare our results with theirs.

Differentiation of MC3T3-E1 cells into mature mineralising osteoblasts has been reported from 5 to 16 days post confluency.[354,358] In our experiment, 14 days treatment of MC3T3-E1 cells with vehicle, PUFAs (AA and DHA) as well as oestrogen and PTH resulted in the observation of mineralised vesicles in the matrix. These vesicles could, however, only be detected by microscope. Although AA exposure inhibited the ALP activity in the MC3T3-E1 cells significantly, the high basal ALP activity of these cells might be sufficient to allow for mineralisation to still take place. Exposure to DHA and PTH resulted in the formation of more prominent mineralised plaques. However, as mineralisation was not quantified, we could not confirm whether the observed effect was statistically significant. Mineralisation is an ongoing process therefore it may be necessary to expose the cells for longer periods in order to detect differences in mineralisation.

In our study, long-term (14 days) exposure to PTH or oestrogen did not affect ALP activity in either of the MG-63 and MC3T3-E1 osteoblast-like cells, confirming the results of others.[110,291] Isogai *et al* (1996), however, have shown that, depending on the differentiation status of the osteoblast-like cells, PTH could exert diverse effects on the phenotype expression in these cells.[364] They demonstrated that PTH stimulates ALP activity at a preconfluent state but inhibits both ALP activity and osteocalcin production at a postconfluent state in primary osteoblast-like cells isolated from newborn mouse calvaria.[364] In our model, ALP activity was investigated in cells grown to confluency, which could explain the lack of response to PTH.

6.4.3 Oil Red O staining versus alkaline phosphatase activity as markers of osteoblastic transdifferentiation into adipocytes

Osteoblasts and adipocytes both originate from mesenchymal stem cells (MSCs). It has been shown that osteoblastic cells are not only osteogenic but also able to undergo differentiation to adipocytes when treated with long chain fatty acids.[56] The formation of differentiated adipocytes was dependent on increased expression of PPARγ2.[57] PUFAs and their metabolites are ligands for PPAR,[57,190,191,356] which have been implicated in the control of adipocyte differentiation.[54,57] (Refer to Figure 6.1). As osteoblasts transdifferentiate into adipocytes they lose their ALP activity.[56] ALP activity versus Oil Red O staining was therefore used as a criterion to determine osteoblastic versus adipocytic differentiation after PUFA exposure.

To investigate the effects of AA and DHA on the onset of adipogenesis whilst not forcing these cells along the osteogenic differentiation pathway, osteogenic supplements were omitted from the culture medium. Vitamin D_3 induces cellular ALP activity[241,292,299] and was therefore used as a positive control in some experiments. In our study, vitamin D_3 treatment for six days inhibited cell proliferation and significantly enhanced ALP activity in both MG-63 and MC3T3-E1 osteoblastic cells (Figure 6.7 and Figure 6.8), suggesting that ALP activity could be stimulated in basal conditions, in the absence of osteogenic enhancing supplements.

In the MG-63 cells, large quantities of triglycerides accumulated in the cytoplasm of these cells after oleic acid treatment (Figure 6.10 A-D), confirming results reported by Diascro *et al* (1998).[56] Exposure of MG-63 cells to high concentrations of AA and DHA (20 μg/ml) resulted in accumulation of large quantities of cytoplasmic lipid vesicles, which was paralleled by a significant inhibition of ALP activity. Diascro *et al* (1998) reported that MG-63 cells express significant levels of PPARγ mRNA and speculated that the expression of adipocytic markers by these cells could be attributed to these cells being tumor-derived cells.[56] Nuttal *et al* (1998) however, reported that osteogenic cells derived from explants of normal adult human trabecular bone are also capable to differentiate into adipocytes under defined culture conditions, thereby proving that plasticity exists among cells of the stromal

lineage.[57] Our findings suggest that PUFA treatment might cause MG-63 cells to transdifferentiate into adipocytes, therefore non-functional osteoblasts.

Though exposing MC3T3-E1 cells to high concentrations of PUFAs dose-dependently inhibited ALP activity, no lipid filled vacuoles could be detected in this cell line. Apart from a few small lipid vesicles, oleic acid treatment was unable to stimulate cytoplasmic triglyceride accumulation by MC3T3-E1 cells. It is not known whether MC3T3-E1 cells express PPARγ mRNA, but adipogenesis will not occur when cells express low levels of PPARγ mRNA.[59,60] Shin et al (2005) recently reported that retrovirus-mediated *overexpression* of PPARγ in MC3T3-E1 osteoblasts resulted in reduced ALP activity and that these cells could be induced to transdifferentiate into mature adipocytes.[365] This observation supports our speculation that MC3T3-E1 cells *normally* express low levels of PPARγ, thereby explaining the inability of these cells to transdifferentiate into adipocytes after PUFA exposure. The observed inhibition of ALP activity in the MC3T3-E1 cells in our model, therefore could not be attributed to transdifferentiation of these cells into adipocytes, but could be due to differentiation and maturation of these cells into mineralising osteoblasts.

6.4.4 Conclusions

Culturing MC3T3-E1 cells in osteogenic supplemented media resulted in ALP activity detection that is considered one of the markers for osteoblastic differentiation. In the osteogenic supplemented model, short-term (48 hours) exposure to AA did not affect ALP activity but long-term (14 days) exposure to AA (2.5 to 20μg/ml) significantly inhibited ALP activity in this cell line. DHA exposure (2.5 to 20μg/ml) also inhibited ALP activity in this cell line, which was evident after both short-term (48 hours) and long-term exposures (14 days). Although long-term exposure to these PUFAs inhibited ALP activity, these cells were still able to produce mineralised plaques in the matrix, suggesting that the ability of the MC3T3-E1 cells to differentiate to mature mineralising osteoblasts was not compromised by PUFA exposure.

The MG-63 cells, which are osteosarcoma-derived, are considered osteoblast-like cells exhibiting various osteoblastic traits.[361] In contrast to the MC3T3-E1 cells, MG-63 cells demonstrated a much lower basal ALP activity. In the osteogenic supplemented model, short-term exposure to either AA or DHA had no significant effect on the ALP activity in this cell line, as did long-term exposures to DHA. High concentrations of AA, however, increased ALP activity significantly in these cells after 14 days but the ALP stimulatory effect was not sufficient to allow the MG-63 cells to differentiate into mature mineralising osteoblasts and no mineralised plaques could be detected.

Exposure to high concentrations of PUFAs in culture conditions *without* osteogenic inducing supplements resulted in the observation of adipocyte-like features in the MG-63 cell line as evidenced by the accumulation of Oil Red O positive cytoplasmic lipid vacuoles (Figure 6.10 B-D). This observation might be attributed to MG-63 cells expressing high levels of PPARγ,[56] which is known to regulate adipogenesis when binding to PPARγ ligands such as PUFAs, PUFA metabolites e.g. prostaglandins, or PUFA oxidation products.[55,57,191] The accumulation of lipid vacuoles in the cytoplasm of these cells was paralleled by a decrease in ALP activity in the MG-63 cells (Figure 6.7). Although exposure to high concentrations (20 μg/ml) of AA and DHA inhibited ALP activity slightly in the MC3T3-E1 cell line (Figure 6.8), these PUFAs were unable to induce adipocyte-like features in these cells as evidenced by the absence of any Oil Red O positive vacuoles (Figure 6.10 F), suggesting low expression levels of PPARγ. The physiological significance of osteoblastic transdifferentiation into the adipogenic phenotype and the control thereof needs to be elucidated by *in vivo* experiments.

Osteoblasts and osteoclasts work together in a synchronised manner and differentiation of osteoclasts is closely coupled with the function of osteoblasts through a variety of cytokines.[7] Most pro- and anti-osteoclastogenic cytokines and hormones act primarily through the osteoblast to alter levels of RANKL and OPG; the relative balance of the latter determines overall osteoclast formation.[14] It has been shown that PGE_2, derived from AA, modulates OPG synthesis[139] as well as the expression of mRNA for RANKL.[131] It could therefore be speculated that apart from their effects on cell proliferation and differentiation, PUFAs might affect the

OPG/RANKL ratio via manipulation of PGE_2. We therefore investigated the effects of AA and DHA on OPG and RANKL synthesis in osteoblast-like cells. The results from this study are reported in Chapter 7.

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]

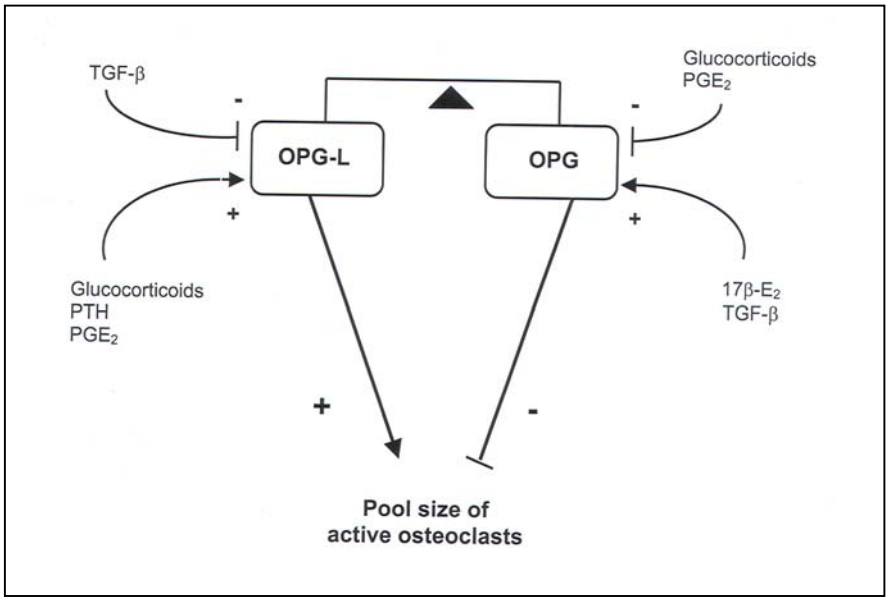


Figure 7.1 The 'Convergence hypothesis' for the regulation of osteoclast functions by cytokines.

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.

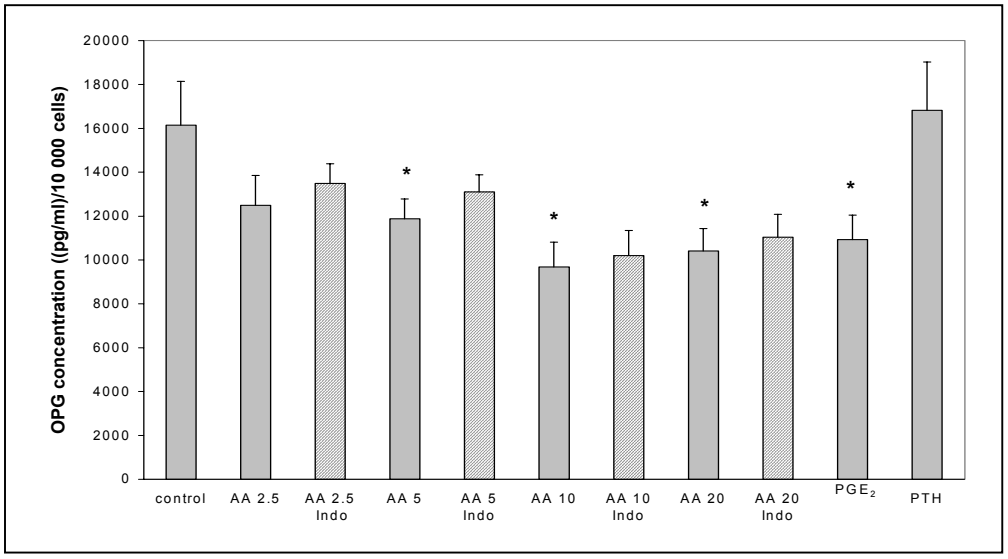


Figure 7.2 Effects of arachidonic acid, prostaglandin E_2, and parathyroid hormone on osteoprotegerin secretion by MG-63 cells.

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.

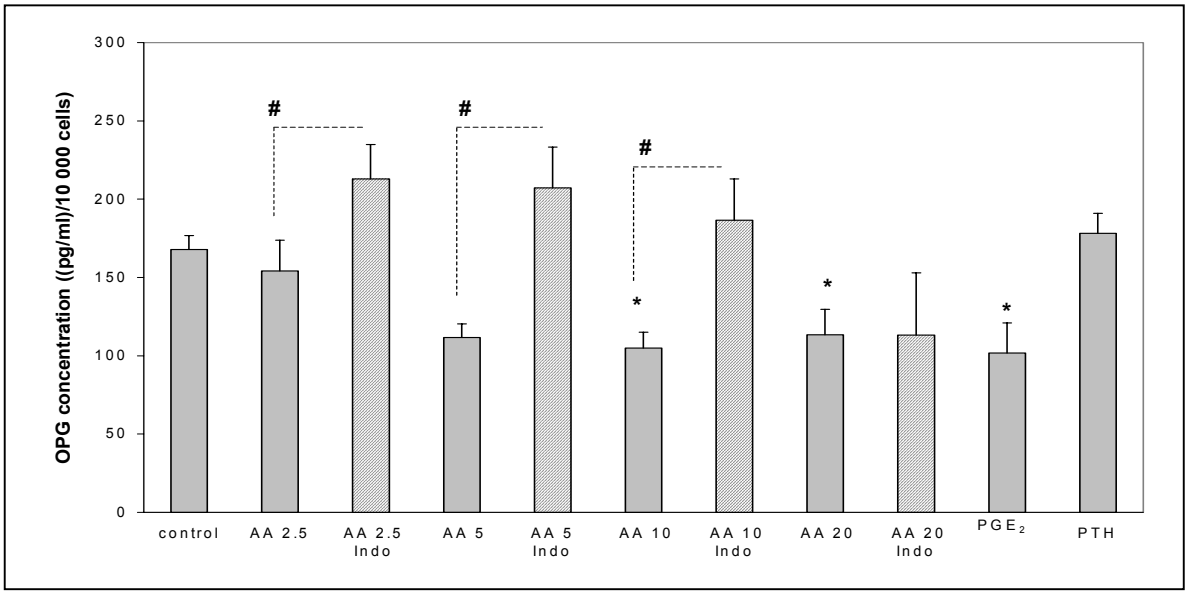


Figure 7.3 Effects of arachidonic acid, prostaglandin E_2, and parathyroid hormone on osteoprotegerin secretion by MC3T3-E1 cells

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%.

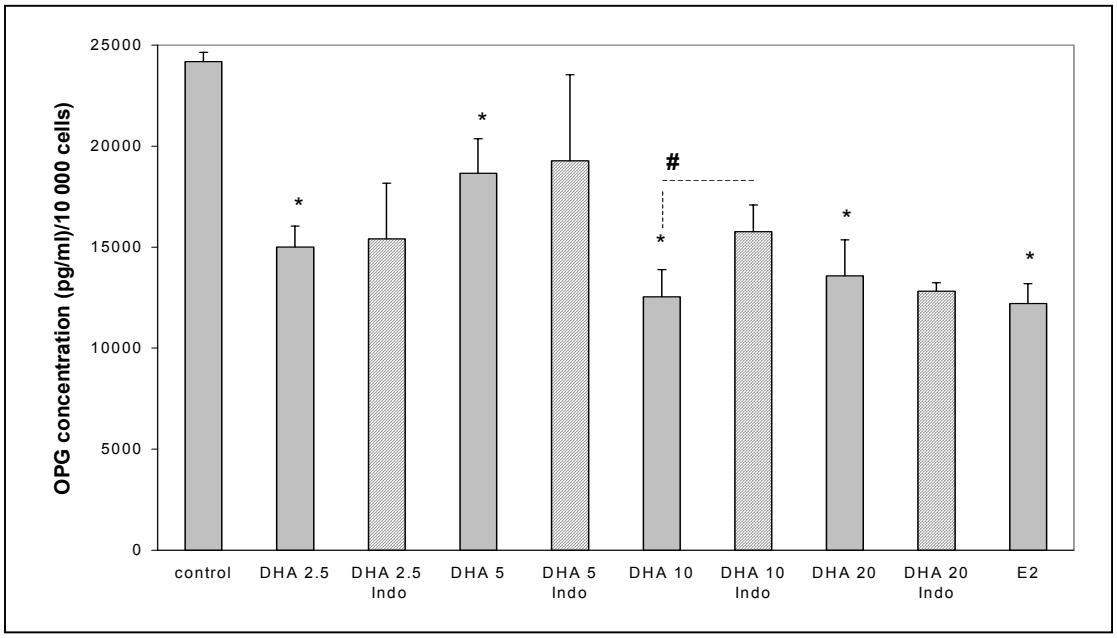


Figure 7.4 Effects of docosahexaenoic acid and oestrogen on osteoprotegerin secretion by MG-63 cells.

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%.

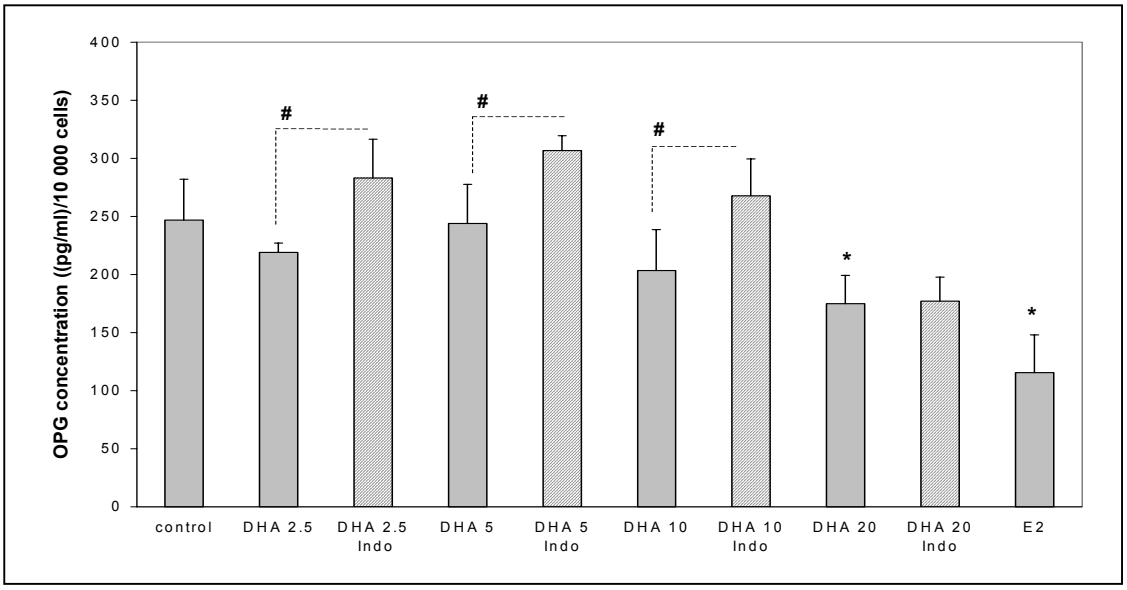


Figure 7.5 Effects of docosahexaenoic acid and oestrogen on osteoprotegerin secretion by MC3T3-E1 cells

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.

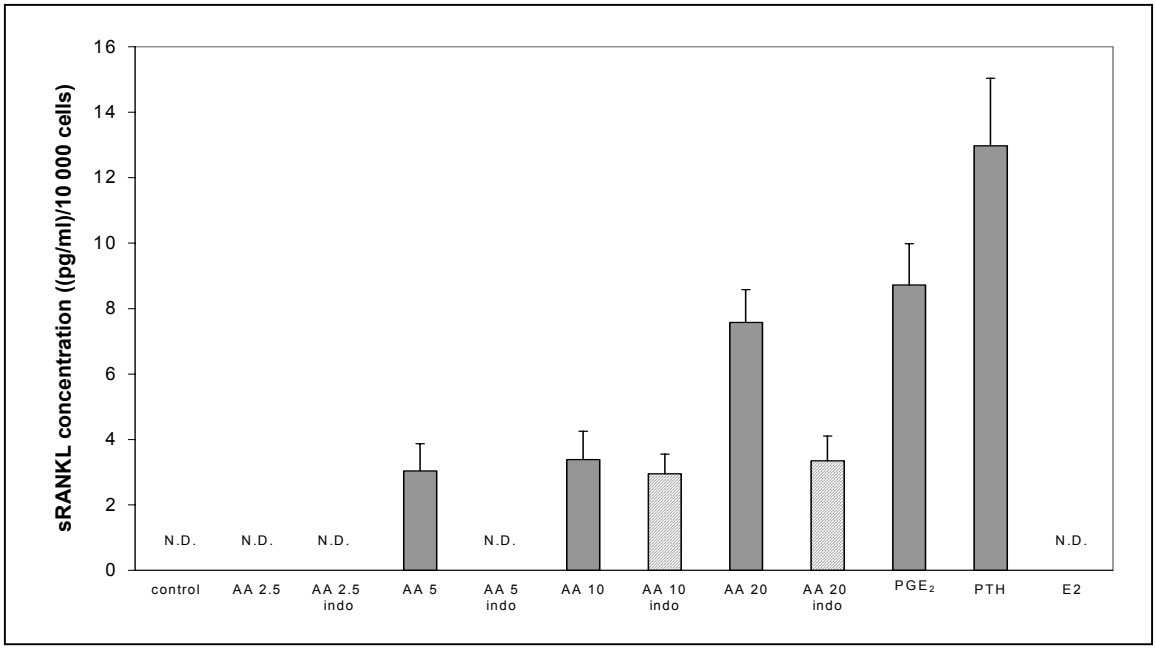


Figure 7.6 Effects of arachidonic acid, prostaglandin E_2,, parathyroid hormone and oestrogen on RANKL secretion by MC3T3-E1 cells

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%.

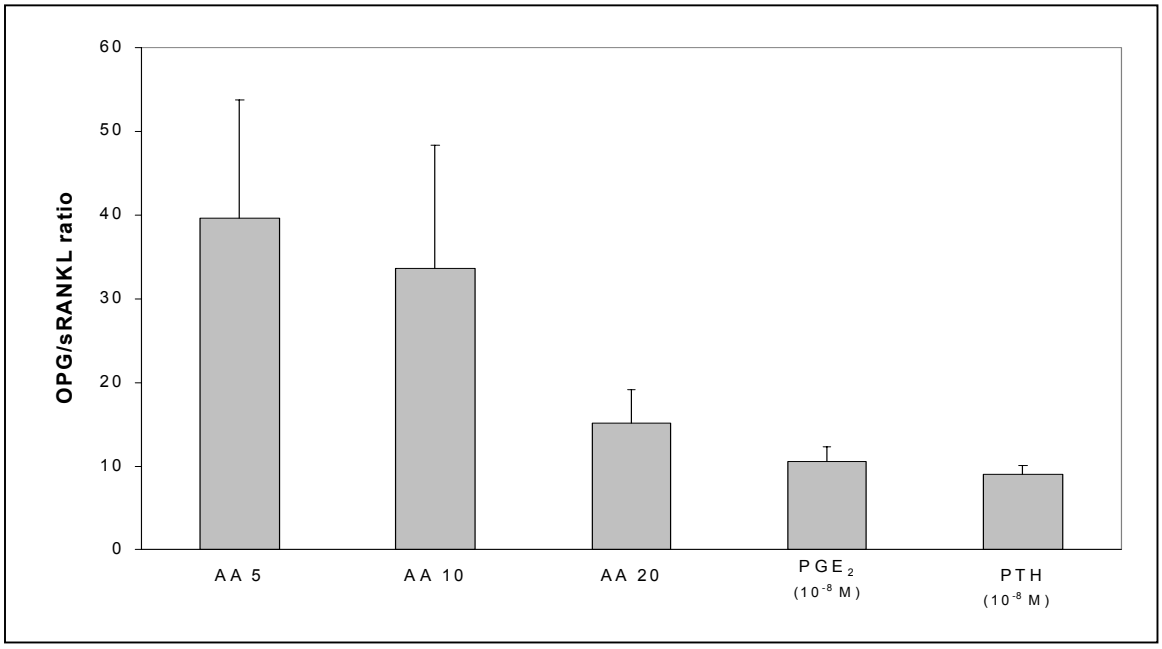


Figure 7.7 Effects of arachidonic acid, prostaglandin E_2, and parathyroid on the osteoprotegerin/sRANKL ratio in MC3T3-E1 cells.

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.

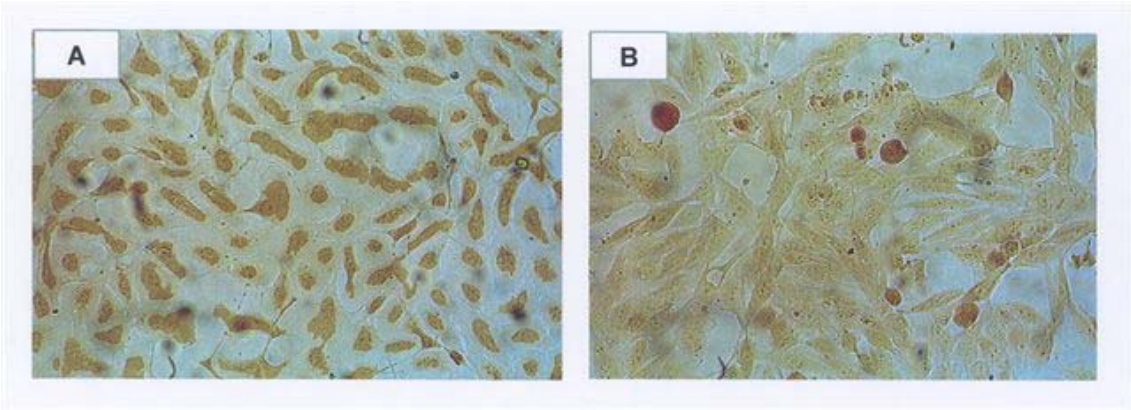


Figure 7.8 Oestrogen receptor detection in MCF-7 human breast carcinoma cells and MG-63 human osteosarcoma cells.

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.

CHAPTER 8

Conclusions and Further Research

Conclusions

To investigate the effects of PUFAs on osteoblastic functioning, MG-63 human osteosarcoma-derived osteoblasts and MC3T3-E1 murine osteoblasts in culture were exposed to AA (representative of the n-6 PUFAs) and DHA (representative of the n-3 PUFAs) as well as the bone active hormones PTH and oestrogen. The effects of these agents were tested on a variety of biological parameters characteristic of osteoblasts, including PGE_2 synthesis, proliferation, differentiation to mature mineralising osteoblasts as well as OPG and RANKL secretion.

1. Results from this study showed that exogenously added AA (20 μg/ml) stimulates PGE_2 production significantly in both the MG-63 and MC3T3-E1 cell lines. Since AA is the natural substrate for PGE_2 synthesis, this observation was expected. Stimulated PGE_2 production by MC3T3-E1cells however, was significantly higher than that of MG-63 cells, which might be attributed to auto-amplification by PGE_2 itself in this cell line. Pre-incubation of the MG-63 cells with either the unselective COX-blocker indomethacin or the COX-2 selective blocker NS-398 prior to AA exposure, inhibited PGE_2 production significantly, suggesting that both COX enzymes were involved in PGE_2 synthesis in our model. Results from our study demonstrated a stimulatory effect of PTH (10^{-7} M) on PGE_2 synthesis in the MG-63 cell line. The mechanism by which PTH stimulates PGE_2 synthesis is not clear but could be attributed to the possible induction of COX-2. AA (20 μg/ml) and PTH (10^{-7} M) co-exposure did not stimulate PGE_2 synthesis to levels higher than that already observed after AA (20 μg/ml) exposure only, suggesting the absence of a synergistic mechanism for these compounds in the MG-63 cells under the conditions analysed in this study.

2. As the number of functional osteoblasts is important for bone formation, the effects of the various agents on *in vitro* osteoblastic cell proliferation were investigated. In this study, oestrogen and PTH (10^{-10} M to 10^{-6} M) slightly inhibited proliferation in both MG-63 and MC3T3-E1 cell lines. In contrast, the PUFAs AA and DHA (2.5 to 20 μg/ml) inhibited cell growth significantly at high concentrations. We conclude that in our model, the inhibitory effect of AA on cell proliferation is possibly independent of PGE_2 production, as PGE_2 (10^{-10} M to 10^{-6} M) *per se* had little effect on proliferation in the cell lines tested. Furthermore, our results have shown that DHA affects proliferation of the MG-63 osteoblasts more severely than MC3T3-E1 cells. The difference in response of these cell lines may be explained by the fact that the MG-63 osteoblast cell line is osteosarcoma-derived, while the MC3T3-E1 osteoblastic cell line is a normal cell line.

   The anti-proliferative effect of the PUFAs could be attributed to inhibition of the expression or activity of some cyclins or cyclin-dependent kinases related to cell cycle progression; this needs to be verified by further experimental work. The inhibitory effect of PUFAs on cell proliferation could also be due to the formation of PUFA peroxidation products in the culture media, which could harm proteins and DNA or cause membrane damage thereby changing signal transduction that could ultimately affect cell proliferation. In the current study, morphological studies have shown the presence of apoptotic cells after DHA exposure in MG-63 cells, which could be attributed to the presence of DHA lipid peroxidation products. Apoptosis in the MG-63 cells might be explained by the nature of the MG-63 cell line, as cancer cells have been shown to be more susceptible to DHA than normal cell lines.

3. A reciprocal relationship between reduced proliferation and subsequent induction of cell differentiation *in vitro* has been shown. Since our results demonstrated that AA and DHA (2.5 to 20 μg/ml) inhibit cell proliferation in a dose-dependent manner, follow-up work was conducted to investigate whether inhibition of cell proliferation in this model is due to increased differentiation of osteoblasts to the mature mineralising osteoblastic phenotype. Exposing MC3T3-E1 cells to either vehicle or test agents resulted in the detection of markers of osteoblastic differentiation such as

ALP activity. In the osteogenic supplemented model, long-term (14 days) exposure to AA significantly inhibited ALP activity in this cell line, which might be PGE_2-mediated, as PGE_2 has been shown to inhibit ALP activity. DHA exposure also inhibited ALP activity in the MC3T3-E1 cells, which was evident after both short- (48 hours) and long-term (14 days) exposures. The mechanism whereby DHA inhibits ALP activity is not clear and needs to be investigated. Although long-term exposures to the PUFAs inhibited ALP activity, the MC3T3-E1 cells were still able to produce mineralised plaques in the matrix suggesting that the ability of these cells to differentiate to mature mineralising osteoblasts was not compromised by PUFA treatment.

In our experimental conditions, however, compared to the MC3T3-E1 cells the MG-63 cells demonstrated a much lower basal ALP activity. Long-term exposures to DHA had no significant effect on ALP activity, but high concentrations of AA enhanced ALP activity significantly. This ALP stimulatory effect, however, was not sufficient to allow the MG-63 cells to differentiate into mature mineralising osteoblasts and no mineralised plaques could be detected. The lack of mineralising properties of the MG-63 might be linked to the low ALP activity exibited by these cells. Results from this study suggest that the inhibition of osteoblastic proliferation by AA and DHA in our model could not be attributed to increased differentiation of the cells into the mature mineralising osteoblastic phenotype.

4. In culture conditions *without* osteogenic inducing supplements, exposure to high concentrations of PUFAs induced adipocyte-like features in the MG-63 cell line as evidenced by the accumulation of Oil red O positive cytoplasmic lipid vacuoles. The accumulation of lipid vacuoles in the cytoplasm of these cells was accompanied by an inhibition of ALP activity. This observation might be attributed to MG-63 cells expressing high levels of PPARγ mRNA which is known to regulate adipogenesis when binding to PPARγ ligands such as PUFAs, PUFA metabolites e.g., prostaglandins or PUFA oxidation products. Our findings suggest that PUFA treatment in specific culture conditions might cause MG-63 cells to transdifferentiate into adipocytes, therefore non-functional osteoblasts. Although exposure to high AA and DHA concentrations caused a slight inhibition of ALP activity in the MC3T3-E1 cell line, these PUFAs were unable to induce adipocyte-like features in these

cells as evidenced by the absence of Oil red O positive vacuoles. This observation suggests that the MC3T3-E1 cell line may not express PPARγ mRNA.

5. Our study demonstrated that PUFAs are able to modulate OPG secretion in osteoblast-like cells. AA (2.5 to 20 μg/ml) inhibited OPG secretion dose-dependently in both cell lines. Indomethacin pre-treatment attenuated the inhibitory effect of AA on OPG synthesis, especially in the MC3T3-E1 osteoblasts, suggesting that AA-induced inhibition of OPG could possibly be mediated via PGE_2 synthesis. DHA (2.5 to 20 μg/ml) 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. Although the MC3T3-E1 cells secreted very low levels of sRANKL into the cultured media, AA dose-dependently stimulated sRANKL secretion thereby affecting the OPG/RANKL ratio in a negative way, supporting various reports that AA and PGE_2 do cause bone resorption. No sRANKL could be detected after exposing the MC3T3-E1 cells to DHA. Since OPG was detected in the culture media and is known for its anti-resorptive properties, the absence of sRANKL suggests that DHA could be protective to bone. The expression of OPG and RANKL has been shown to be developmentally regulated 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. In our MC3T3-E1 model, secreted sRANKL levels were very low and could not be detected in all the samples suggesting that the MC3T3-E1 cell line might not be a suitable model for investigating sRANKL modulation.

6. Although both MC3T3-E1 cells and MG63 cells are considered osteoblasts, these cell lines have certain shortcomings. Compared to the normal MC3T3-E1 cell line, the transformed osteosacoma-derived MG63 cell line was more susceptible to anti-proliferative effects of PUFAs and apoptosis. This cell line also demonstrated low levels of ALP activity, was unable to differentiate into the mature mineralising osteoblastic phenotype and in certain conditions transdifferentiated into the adipocytic phenotype, all properties not common to normal

osteoblasts. Osteosarcoma-derived cells, such as the MG-63 cells, have undergone an extended period of abnormal growth *in vivo*. The cell regulatory mechanisms of these cells might therefore differ from those in normal cells. The MG-63 cells therefore may exhibit a deregulated proliferation/differentiation relationship, which might affect their response to various bone active agents. Our results suggest that the MG-63 cell line might not be a suitable model for investigating normal osteoblastic properties such as proliferation and mineralisation.

The production of sRANKL is developmentally regulated and is mainly secreted by undifferentiated marrow stromal cells and early primary osteoblastic cells. In our laboratory the levels of secreted sRANKL by MC3T3-E1 osteoblasts were almost undetectable, suggesting that this cell line might not be a suitable model for investigating sRANKL modulation.

In summary, results from this study showed that AA stimulated PGE_2 production in both MG-63 and MC3T3-E1 cell lines and that both AA and DHA inhibited cell proliferation as well as differentiation of these cells into mature mineralising osteoblasts. Furthermore, AA and DHA inhibited OPG secretion in both cell lines, but had differential effects on sRANKL secretion in the MC3T3-E1 cell line. AA stimulated sRANKL secretion thereby affecting the OPG/RANKL ratio in a negative way. DHA, on the other hand, did not stimulate sRANKL secretion suggesting that it could be protective to bone. As the PUFA concentrations (2.5 to 20 μg/ml) applied in this study is regarded to be within the physiological ranges of serum free fatty acids reported for humans and mice,[192] one could speculate that the PUFA effects reported in this *in vitro* study might also be reflected in *in vivo* studies.

In conclusion, contrary to *in vivo* evidence, protective effects of the PUFAs could not clearly be demonstrated in the *in vitro* cell models used in this study. More research is needed to elucidate the cellular mechanisms of action of the various PUFAs on bone.

The research presented in this dissertation therefore prove that

1) polyunsaturated fatty acids affect the cellular processes of osteoblasts specifically PGE_2 synthesis, proliferation, differentiation to mature mineralising osteoblasts and induction of adipocyte-like features
2) polyunsaturated fatty acids modulate the secretion of OPG and sRANKL by osteoblasts by decreasing OPG secretion and differentially stimulating sRANKL secretion.

Implications for Further Research

The following areas have been identified for further research:

- The effects of the PUFAs on cell proliferation in the presence of anti-oxidants as some of the observed inhibitory effects of PUFAs on cell proliferation could be due to the formation of PUFA peroxidation products in the culture media.
- The effects of the PUFAs on osteoblastic cell differentiation in non-transformed cells such as primary human/rat osteoblasts or conditionally immortalised cell lines as transformed cells such as the MG-63 cells might exhibit a deregulated proliferation/differentiation relationship.
- The effects of the PUFAs on OPG and sRANKL secretion in less differentiated cell lines such as primary bone marrow stroma cells and primary human/rat osteoblasts. The expression of OPG and RANKL has been shown to be developmentally regulated.
- The effects of the PUFAs on RANKL mRNA expression and synthesis of cell bound RANKL, as the secreted sRANKL levels in our study were very low and could not be detected in all the samples.
- The effects of the PUFAs on the modulation of growth factors, e.g. insulin like growth factor and BMP-2 as *in vivo* studies have suggested the possible involvement of growth factors.
- The effects of the PUFAs on the modulation of early transcription factors such as Cbfa-1 and PPARγ in pre-osteoblasts.
- The possible contribution of second messenger systems to the observed effect of PUFAs on bone cells.

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Addendum I

List of congresses where parts of the work were presented

1. Coetzee M, Kruger MC
Arachidonic acid and bone active hormone induce prostaglandin E_2 synthesis in osteoblasts.
Presentation: 10th Bone and Mineral Meeting, SEMDSA, Sandton, April 2001.

2. Coetzee M, Kruger MC
Arachidonic acid and bone active hormone induce prostaglandin E_2 synthesis in osteoblasts.
Presentation: New Zealand Endocrine Society Meeting, Wellington, May 2001.

3. Coetzee M, Kruger MC
Arachidonic acid and bone active hormone induce prostaglandin E_2 synthesis in osteoblasts.
Presentation: Study group, Department of Internal Medicine, University of Uppsala, Sweden, June 2002.

4. Coetzee M, Haag M, Kruger MC
The effects of essential fatty acids and oestrogen on the secretion of osteoprotegerin, a novel bone protective protein, by osteoblasts.
Poster: Faculty of Health Sciences, Faculty day, University of Pretoria, August 2003.

5. Coetzee M, Haag M, Kruger MC
The effects of essential fatty acids and oestrogen on the secretion of osteoprotegerin, a novel bone protective protein, by osteoblasts.
Poster: 31st Annual Congress of the Physiology Society of Southern Africa, Potchefstroom, September 2003.

6. Coetzee M, Haag M, Kruger MC
Exposure of MC3T3-E1 osteoblasts to polyunsaturated fatty acids modulates prostaglandin E_2 synthesis and secretion of osteoprotegerin.
Poster: 12th National Osteoporosis Foundation Symposium, SEMDSA, Sandton, 9-12 April 2005.

7. Coetzee M, Haag M, Kruger MC
Exposure of MC3T3-E1 osteoblast-like cells to arachidonic acid and docosahexaenoic acid modulates prostaglandin synthesis and secretion of osteoprotegerin.
Poster: 2nd Joint Meeting of the European Calcified Tissue Society and the International Bone and Mineral Society, Geneva, Switzerland, 25-29 June 2005.

Addendum II

List of abstracts and articles published from this work

1. Coetzee M, Kruger MC
Arachidonic acid and bone active hormone induce prostaglandin E_2 synthesis in osteoblasts.
Abstract: JEMDSA 2001; 6:22.

2. Coetzee M, Kruger MC
Arachidonic acid and bone active hormone induce prostaglandin E_2 synthesis in osteoblasts.
Abstract: The Endocrine Society of Australia proceeding 2001; abstract 15, p 32.

3. Coetzee M, Kruger MC
Osteoprotegerin-Receptor Activator of Nuclear Factor-κB Ligand ratio: a new approach to osteoporosis treatment? [Review].
Southern Medical Journal 2004;97:506-11.

4. Coetzee M, Haag M, Kruger MC
Exposure of MC3T3-E1 osteoblasts to polyunsaturated fatty acids modulates prostaglandin E_2 synthesis and secretion of osteoprotegerin.
Abstract: JEMDSA 2005;10:35-6.

5. Coetzee M, Haag M, Kruger MC
Exposure of MC3T3-E1 osteoblast-like cells to arachidonic acid and docosahexaenoic acid modulates prostaglandin synthesis and secretion of osteoprotegerin.
Abstract: Bone 2005;36:S276.

6. Coetzee M, Haag M, Claassen N, Kruger MC
Stimulation of prostaglandin E_2 (PGE_2) production by arachidonic acid, oestrogen and parathyroid hormone in MG-63 and MC3T3-E1 osteoblast-like cells.
Prostaglandins, Leukotrienes and Essential Fatty Acids 2005;76:423-430.