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]

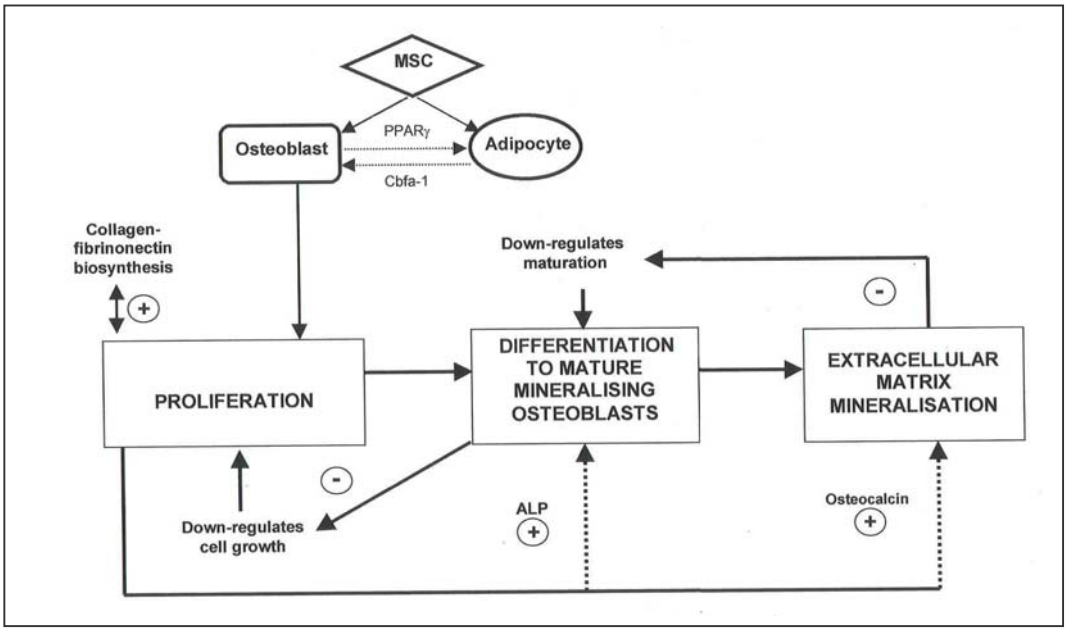

Figure 6.1 Stages in osteoblast development

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.

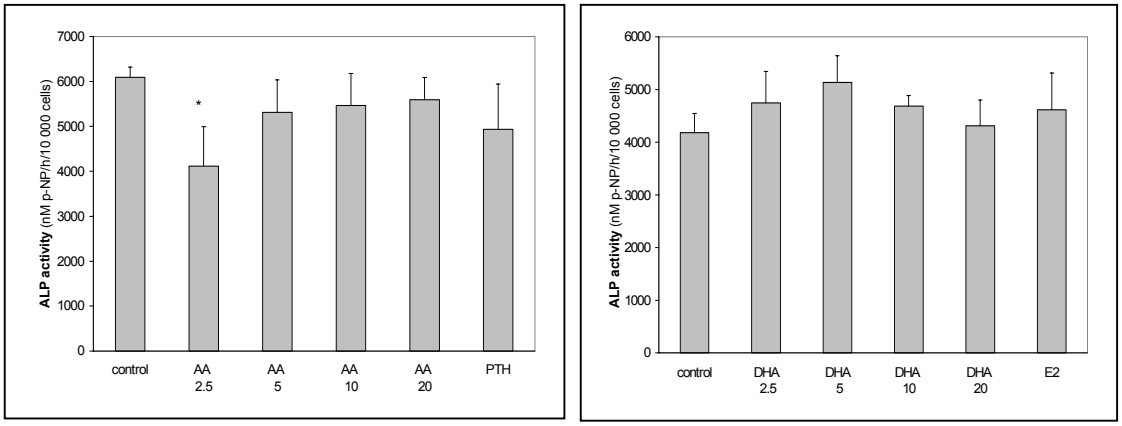

Figure 6.2 Alkaline phosphatase activity of MG-63 cells after 48 hours of exposure to polyunsaturated fatty acids and hormones

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.

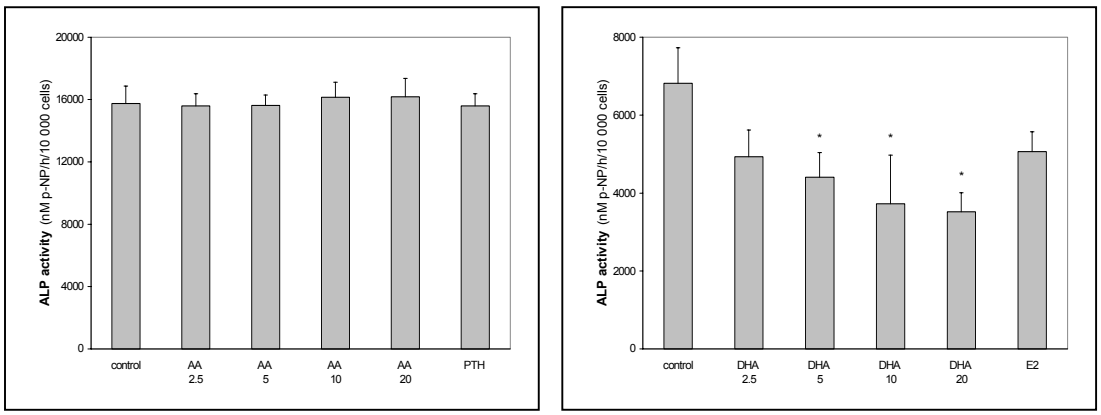

Figure 6.3 Alkaline phosphatase activity of MC3T3-E1 cells after 48 hours of exposure to polyunsaturated fatty acids and hormones

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.

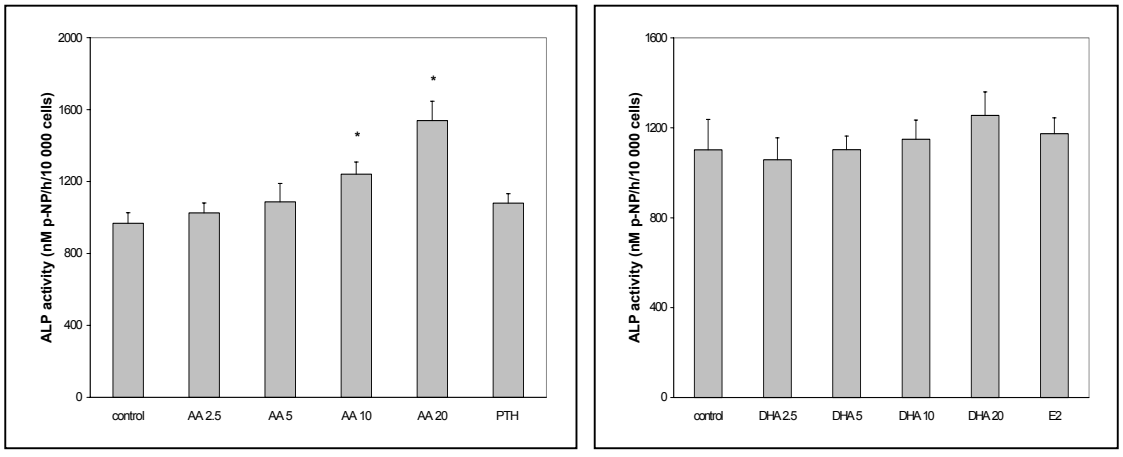

Figure 6.4. Alkaline phosphatase activity of MG-63 cells after 14 days of exposure to polyunsaturated fatty acids and hormones.

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.

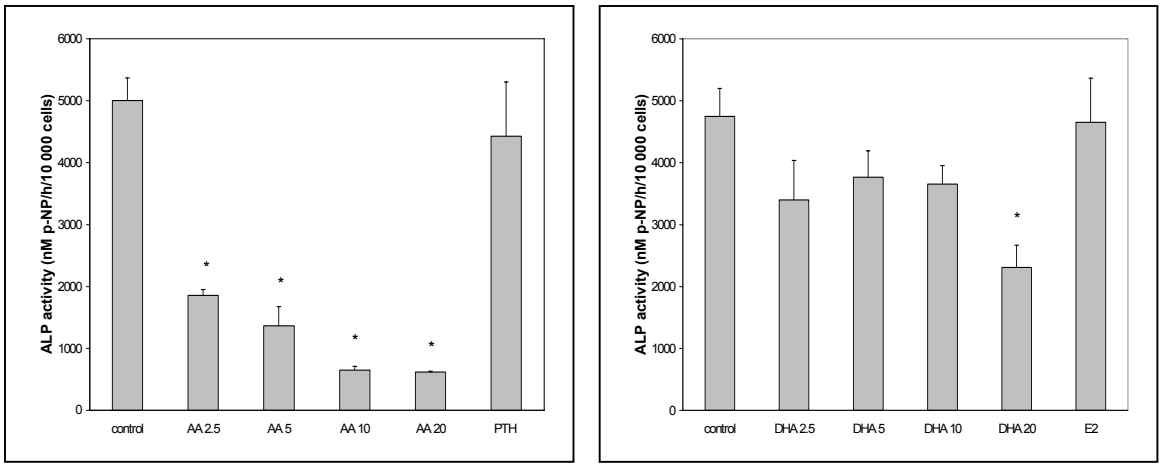

Figure 6.5. Alkaline phosphatase activity of MC3T3-E1 cells after 14 days of exposure to polyunsaturated fatty acids and hormones.

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.

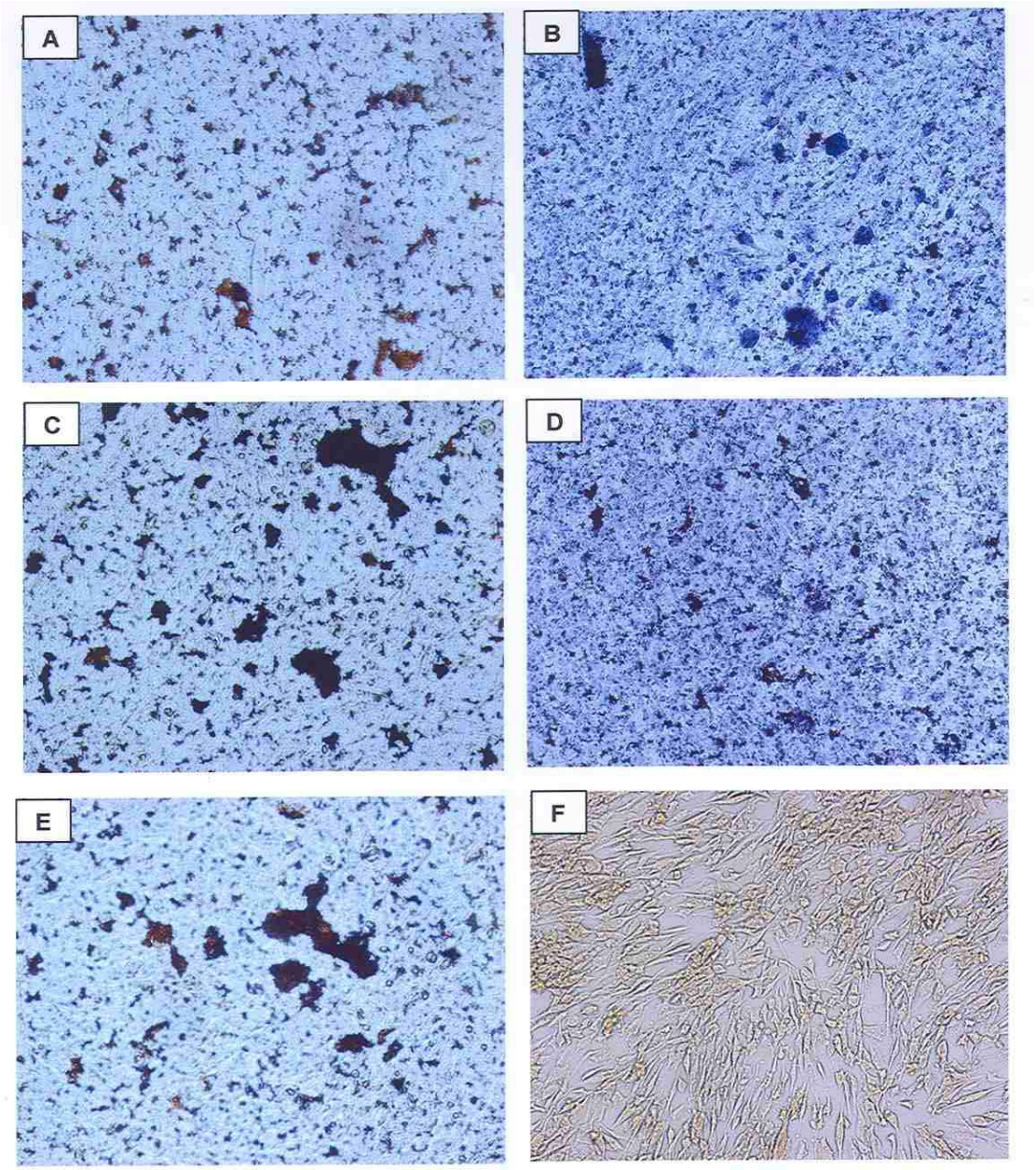

Figure 6.6 Photomicrograph of mineralised nodules.

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.

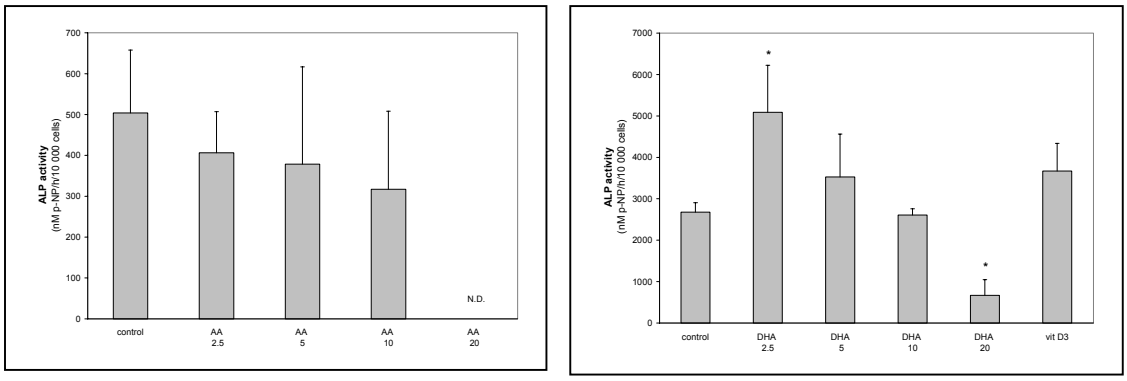

Figure 6.7 Alkaline phosphatase activity of MG-63 cells after six days of exposure to arachidonic acid and docosahexaenoic acid.

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%.

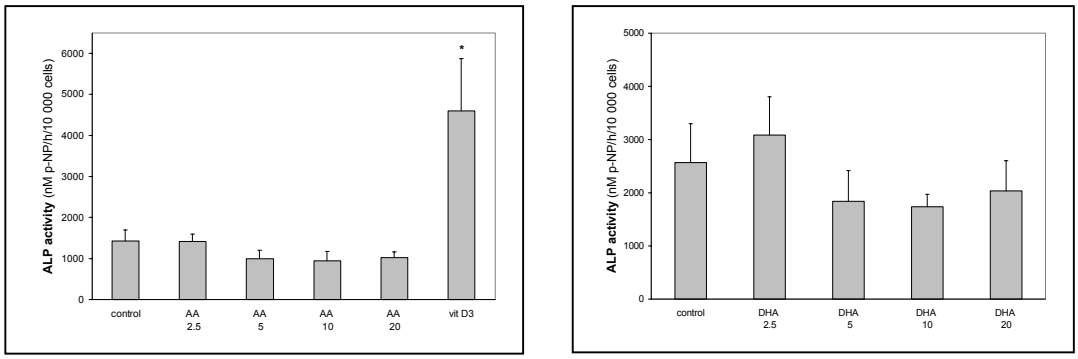

Figure 6.8. Alkaline phosphatase activity of MC3T3-E1 cells after six days of exposure to arachidonic acid and docosahexaenoic acid.

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.

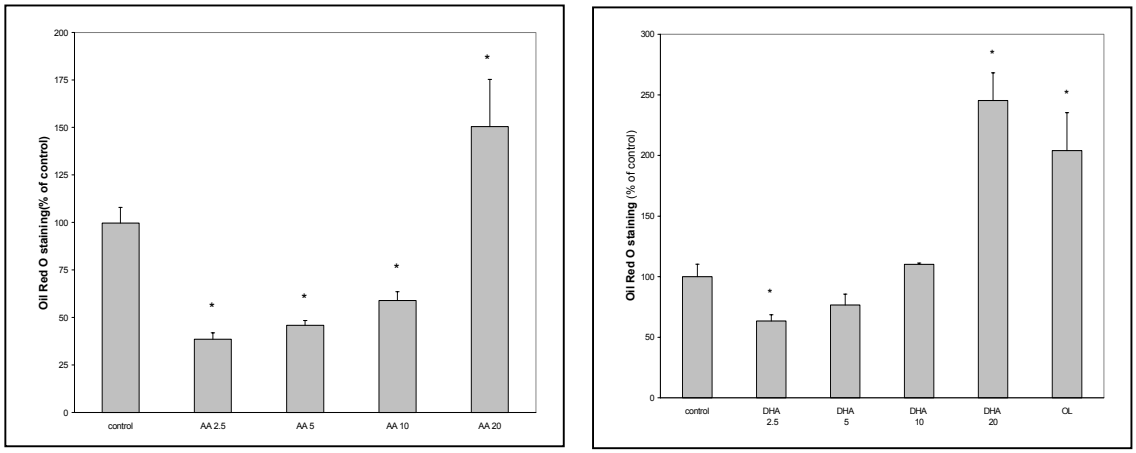

Figure 6.9 Quantification of Oil Red O staining of MG-63 cells after six days of exposure to arachidonic acid and docosahexaenoic acid.

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).

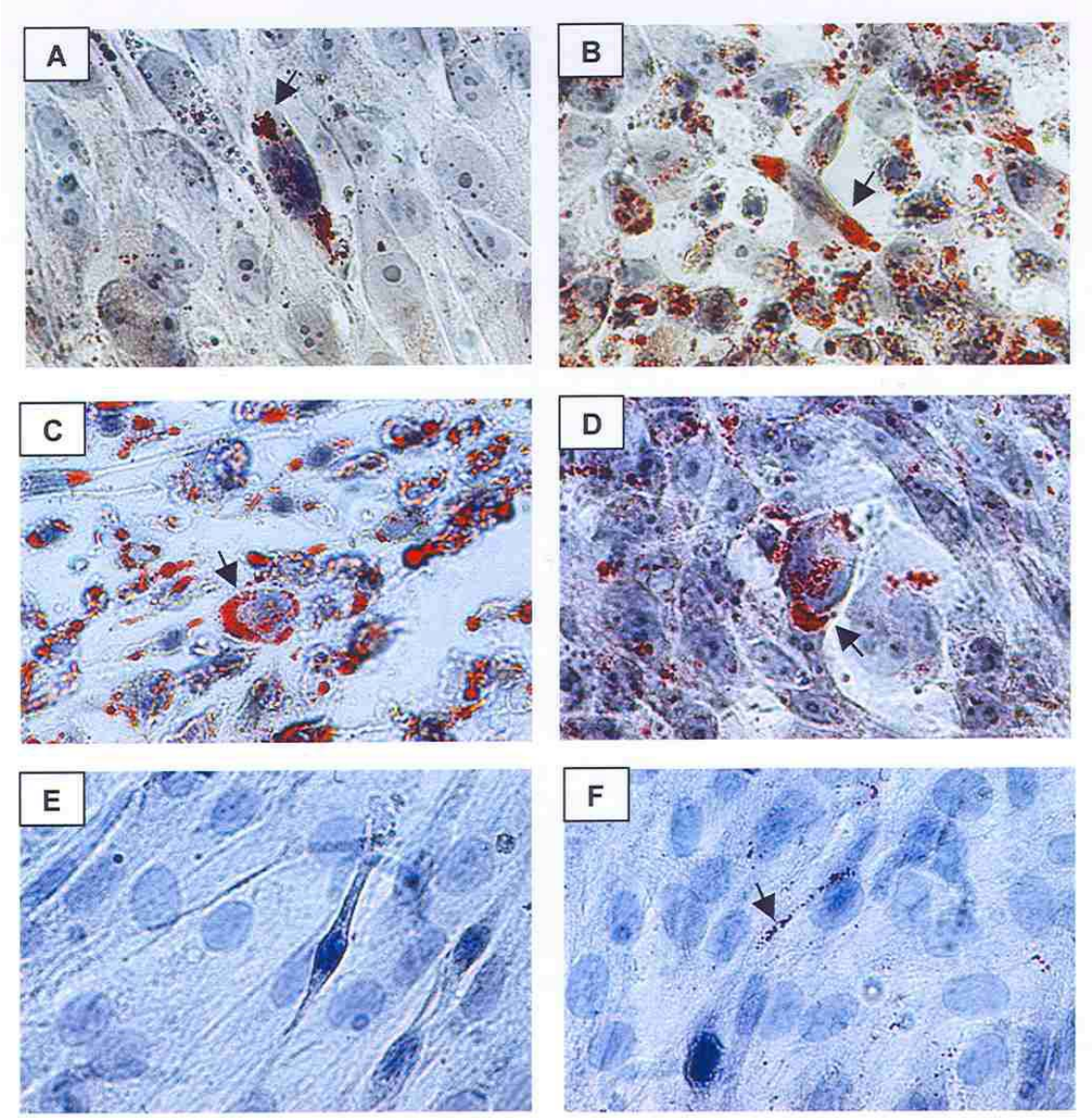

Figure 6.10 Photomicrographs of Oil Red O staining in MG-63 and MC3T3-E1 cells.

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.