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^TM 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.
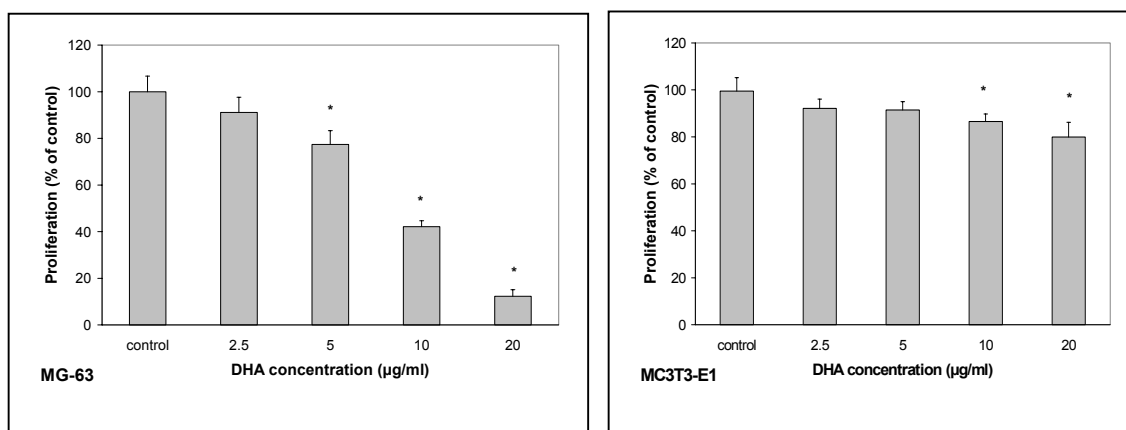

Figure 5.2 Effects of docosahexaenoic acid on MG-63 and MC3T3-E1 cell proliferation

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).
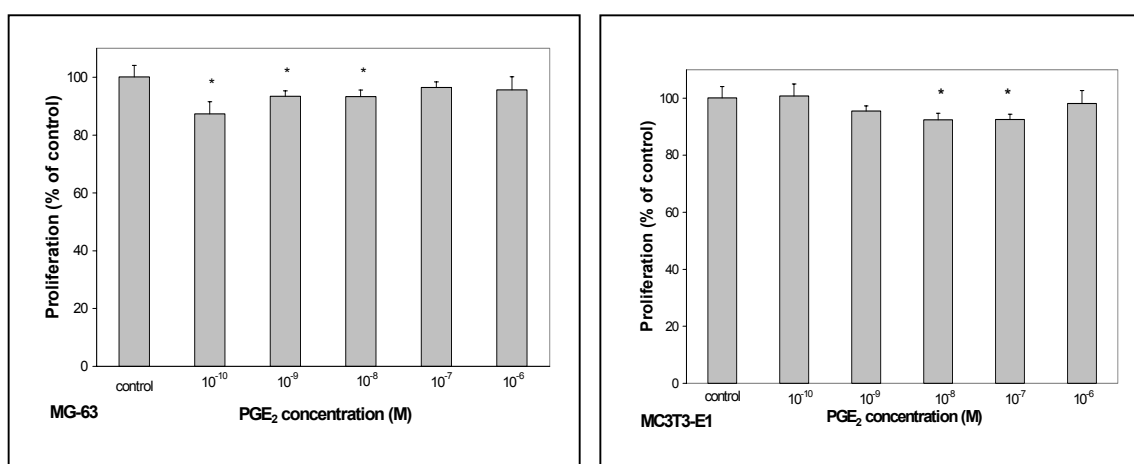

Figure 5.3 Effects of prostaglandin E$_2$ on MG-63 and MC3T3-E1 cell proliferation

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.
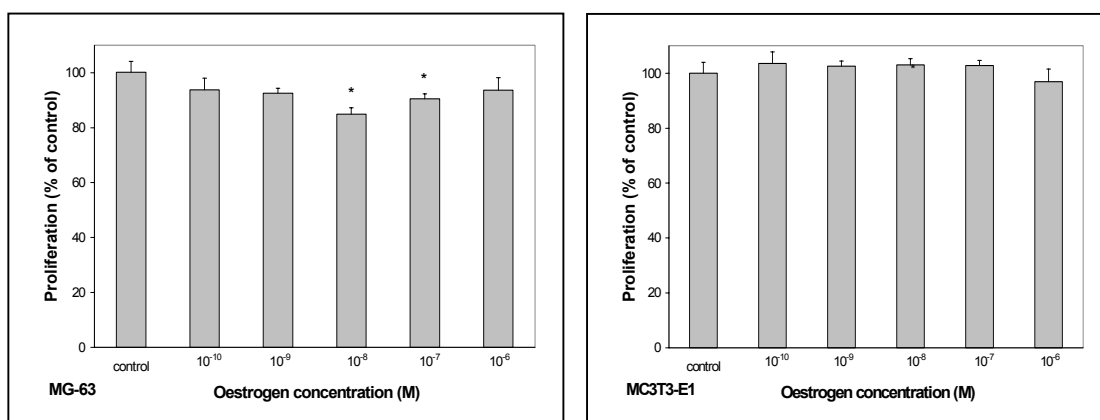

Figure 5.4 Effects of oestrogen on MG-63 and MC3T3-E1 cell proliferation

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).
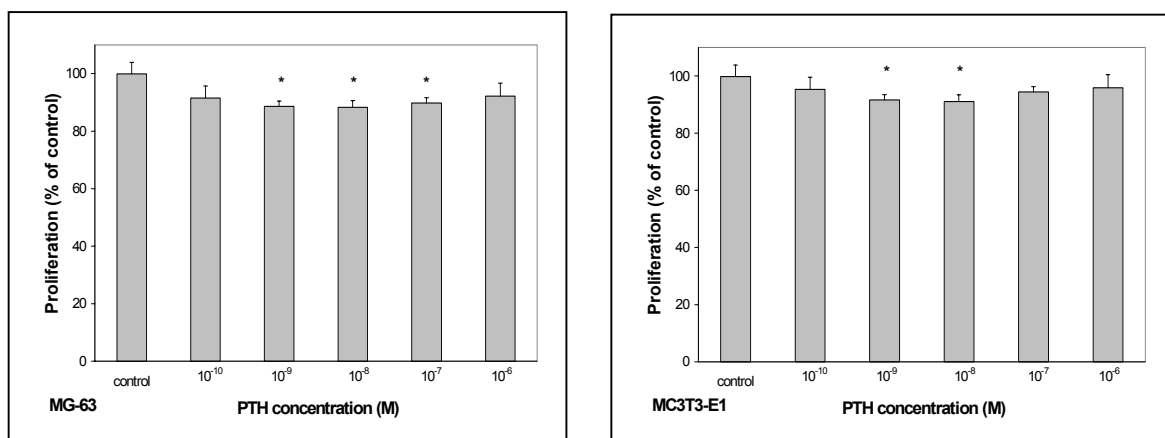

Figure 5.5 Effects of parathyroid hormone on MG-63 and MC3T3-E1 cell proliferation

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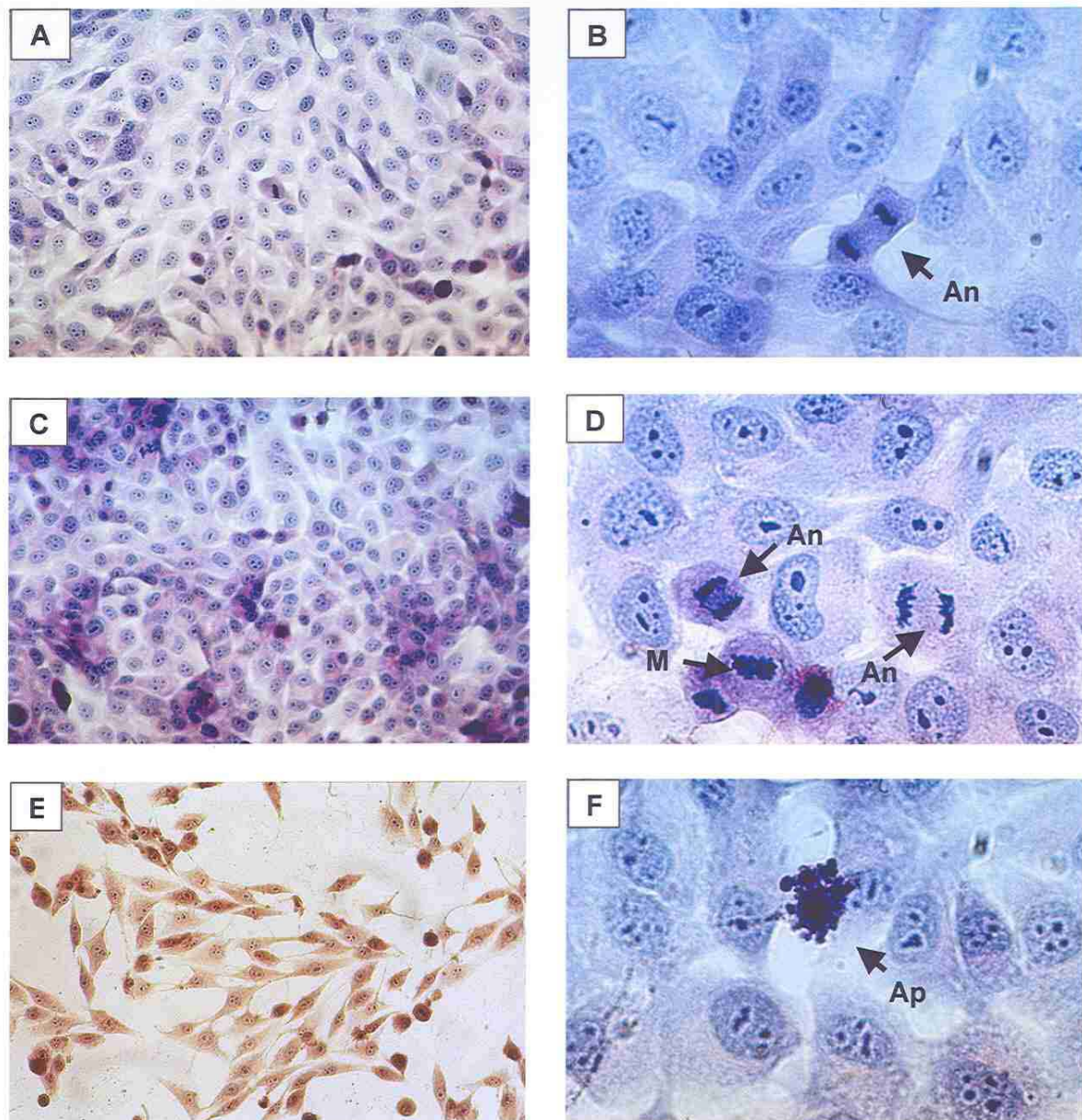
Figure 5.6   Photomicrographs of haematoxylin and eosin (H&E) stained MG-63 cells after 48 hours polyunsaturated fatty acid exposure

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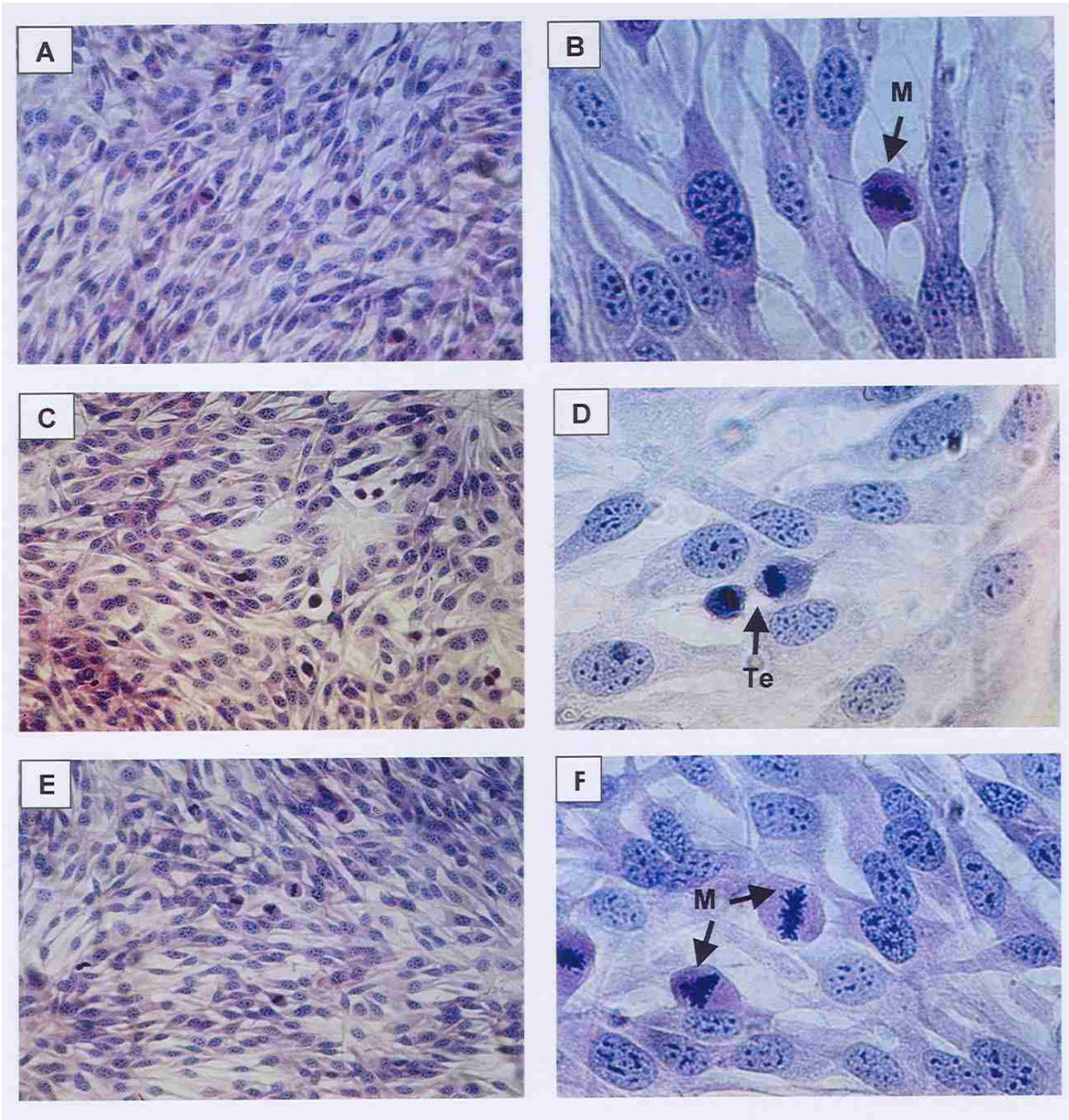
Figure 5.7 Photomicrographs of haematoxylin and eosin (H&E) stained MC3T3-E1 cells after 48 hours polyunsaturated fatty acid exposure

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).

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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 $20\mu g/ml$ 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 $20\mu g/ml$ 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).
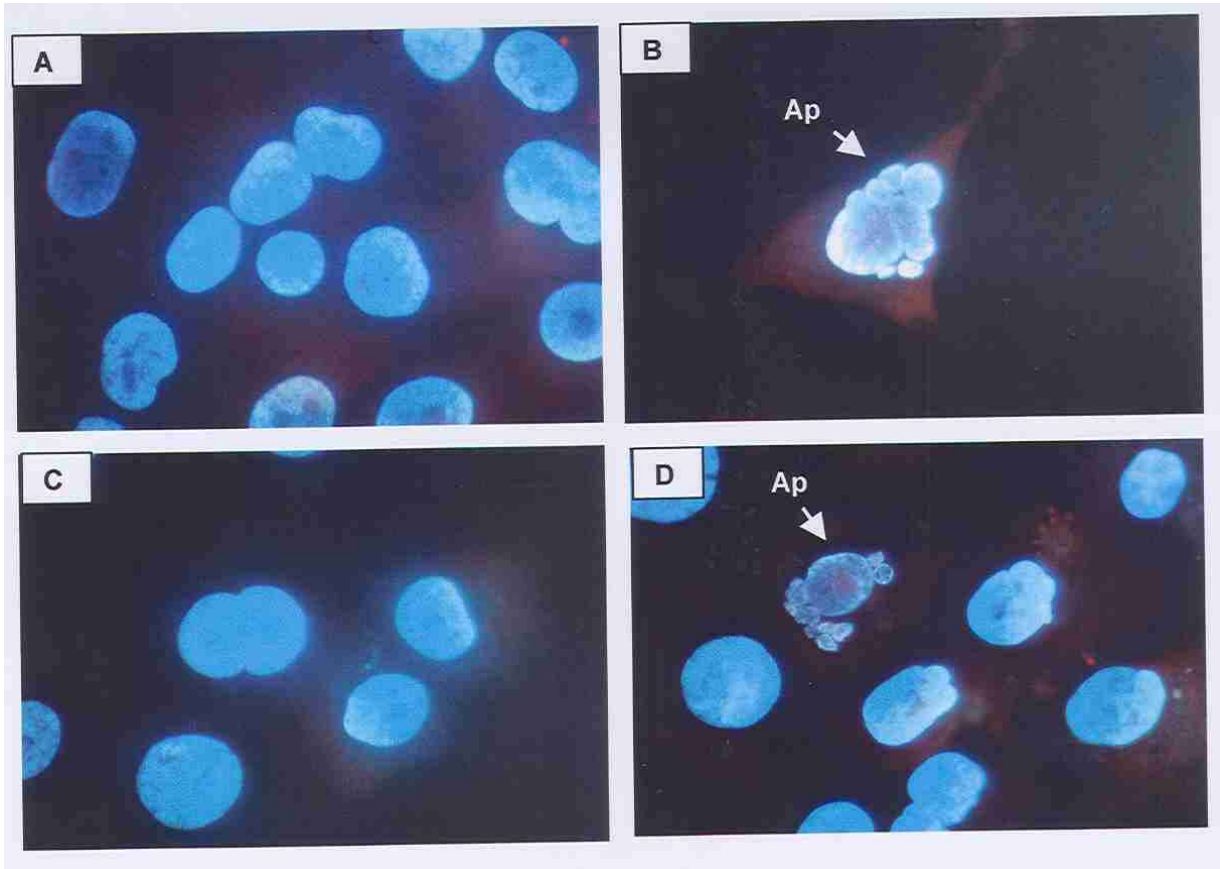
Figure 5.8 Photomicrographs of MG-63 cells after Hoechst and propidium iodide (HOE/PI) fluorescent staining for detection of apoptosis

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 $20\mu g/ml$ AA for 48 hours; **D:** Cells exposed to $20\mu g/ml$ DHA for 48 hours (arrow indicates an apoptotic cell) (Original magnification of photomicrographs: 400x)
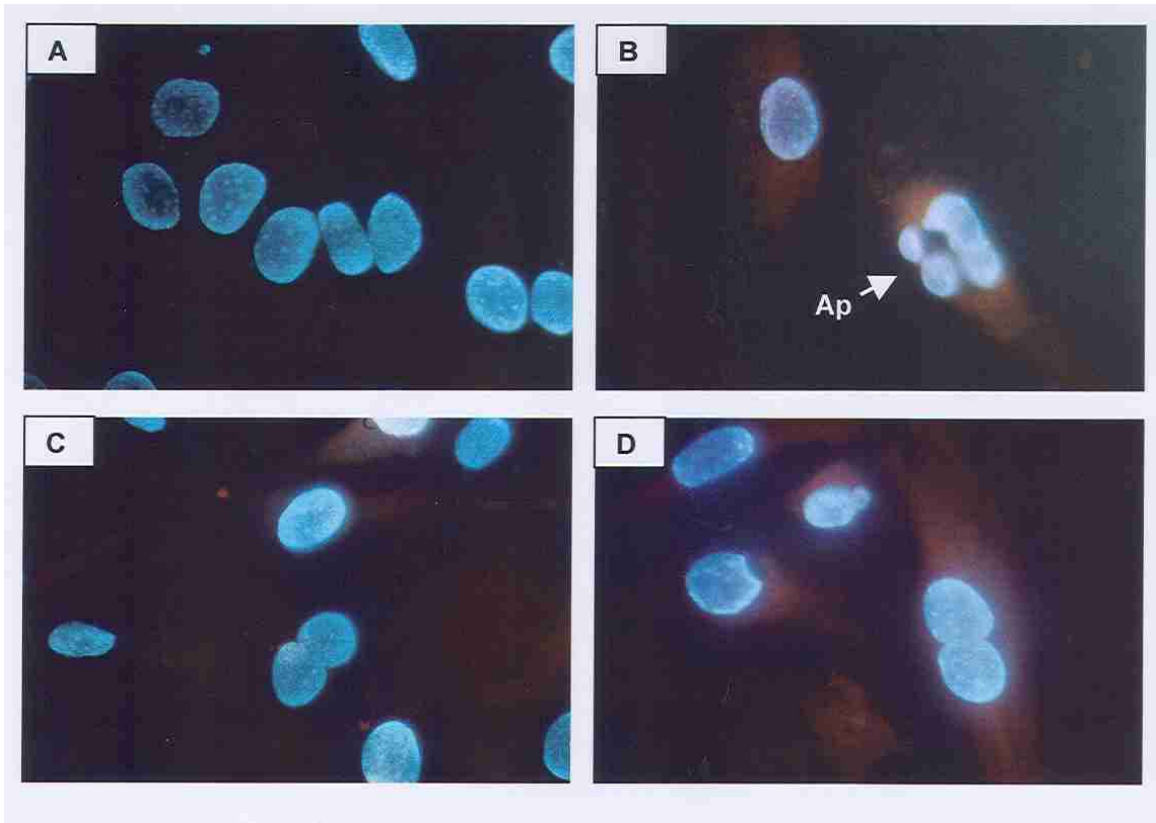
Figure 5.9 Photomicrographs of MC3T3-E1 cells after Hoechst and propidium iodide fluorescent (HOE/PI) staining for detection of apoptosis

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.
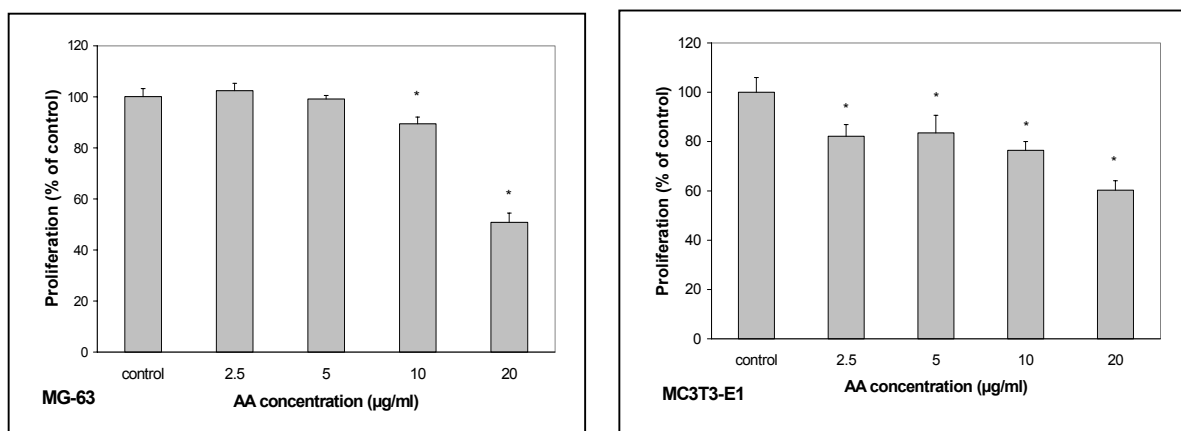

Figure 5.1 Effects of arachidonic acid on MG-63 and MC3T3-E1 cell proliferation

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

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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]

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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.