CHAPTER 8

Conclusions and Further Research

Conclusions

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

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

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

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

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

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

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

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

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

5. Our study demonstrated that PUFAs are able to modulate OPG secretion in osteoblast-like cells. AA (2.5 to 20 μg/ml) inhibited OPG secretion dose-dependently in both cell lines. Indomethacin pre-treatment attenuated the inhibitory effect of AA on OPG synthesis, especially in the MC3T3-E1 osteoblasts, suggesting that AA-induced inhibition of OPG could possibly be mediated via PGE_2 synthesis. DHA (2.5 to 20 μg/ml) suppressed OPG secretion but to a smaller extent than AA. This could, however, be due to endogenous PGE_2 production, as DHA itself is not a substrate for PGE_2 synthesis. Although the MC3T3-E1 cells secreted very low levels of sRANKL into the cultured media, AA dose-dependently stimulated sRANKL secretion thereby affecting the OPG/RANKL ratio in a negative way, supporting various reports that AA and PGE_2 do cause bone resorption. No sRANKL could be detected after exposing the MC3T3-E1 cells to DHA. Since OPG was detected in the culture media and is known for its anti-resorptive properties, the absence of sRANKL suggests that DHA could be protective to bone. The expression of OPG and RANKL has been shown to be developmentally regulated and it has been hypothesised that undifferentiated marrow stromal cells with a high RANKL/OPG ratio can initiate and support osteoclastogenesis, while the mature osteoblastic phenotype, that mostly express OPG, acquire an osteogenic phenotype. In our MC3T3-E1 model, secreted sRANKL levels were very low and could not be detected in all the samples suggesting that the MC3T3-E1 cell line might not be a suitable model for investigating sRANKL modulation.

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

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

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

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

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

The research presented in this dissertation therefore prove that

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

Implications for Further Research

The following areas have been identified for further research:

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