Chapter 4

Discussion

1. Osteoblast cell culture on titanium surface

1.1. Osteoblastic cell culture

Mouse osteoblast cell line, MC3T3-E1, is an appropriate cell line for this study, because it has shown normal patterns of growth curves, expressed osteoblastic phenotypes and secreted PGE_2 (Fig.27-38). This cell line is a useful *in vitro* system for study expression of osteoblastic phenotypes in relation to the different differentiation stages of osteoblasts. The MC3T3-E1 cells are pre-osteoblastic cells derived from calvaria of new born mice. The cells differentiate along the osteoblastic pathway in the presence of ascorbic acid and are capable of recapitulating osteogenic differentiation *in vitro*[52,58]. The cell line is well documented for its capacities to proliferate and differentiate while expressing osteoblastic phenotypes including ALP activity, osteocalcin and calcium production [58,103]. Expressions of ALP activity and osteocalcin, which are markers of osteoblastic differentiation in an early and late differentiation stage are clearly demonstrated (Fig.31-36). Additional advantages of using osteoblast cell line instead of primary cells are that MC3T3-E1 has higher homogeneity than primary cells and is able to maintain characteristic phenotypes of osteoblasts over a number of generations[58]. It provides an homogeneous population of osteogenic cells in a large quantity[104].

Expression patterns of markers of osteoblastic phenotypes are temporally regulated and related to growth of cells. Sequences of developmental stages of osteoblastic differentiation are proliferation (days 4-10), bone matrix formation/maturation (days 10-16) and mineralization stages (days 16-30). During the proliferative period, expressions of type I collagen and ALP were found in low levels. The highest level of type I collagen was found in a late matrix maturation stage. The highest level of ALP activity was found in an early matrix formation (day 10) but decreased during the matrix maturation stage (day 16). During the mineralization period (days 10 – 16), the expression of osteocalcin was high up until 28 days[105].

In this study, the highest level of ALP activity was found on day 10 (on treatment day 5 in the *log* phase). In the meantime, osteocalcin was expressed early on day 7 (on treatment day 5 in the *static* phase) and reached the highest level on day 11 (on treatment day 5 in the log phase), decreasing on day 18 (on treatment day 5 in the plateau phase). It is postulated that a reduction of the osteocalcin level on day 18 was because an extracellular matrix was mineralized in the plateau phase. These findings suggested that MC3T3-E1 cells in this study were highly differentiating cells with a high differentiation/mineralization potential after growth in terms of their ability to express osteoblast related proteins[52]. To verify this assumption, levels of calcium content should be detected.

1.2. Serum free culture medium condition

Fetal bovine serum (FBS) contains several growth factors necessary to maintain growth and function of cells in a cell culture system[7,106]. To avoid interfering effects of growth factors contained in the supplemented serum with inhibitory effects of medications on a cyclooxygenase function, cells were cultivated in a serum free culture medium for 1 – 5 days during treatment periods as recommended in most studies investigating effects of NSAIDs *in vitro*[32-34]. Advantages of serum free culture medium in this study was not only avoiding interference of growth factors but also providing a low protein binding condition for the drug, thus, potency of the drug might not be obscured.

In this study, the level of cell viability on a titanium surface in serum free culture medium was stable on culture days 1 – 6 (Fig. 18) or during treatment days 1 – 5 (Group E, Fig. 28 – 30). Therefore, it can be seen that cells are able to grow in a limited growth condition of serum free culture medium for at least 6 days. This assumption is supported by FDA green staining of vital cells in serum free culture medium on treatment day 5 in the *static* experimental phase (Fig.26) and SEM images of cells on titanium surfaces in all experimental phases on treatment days 1 and 5 (Fig. 20-25).

To repair cellular damage caused by the trypsinization process, the trypsinized cells were incubated in culture medium with 10% bovine serum for 24 hours after cell seeding before they were subjected to serum free culture medium for medication treatment[107]. In some

studies, the condition of culture medium during treatment was not clearly stated[10,108], thus serum free conditions might effect behavior of cells during the treatment period.

1.3. Cells-titanium surface interaction

Study of growth and differentiation of osteoblasts on a titanium surface in vitro was selected as a study model to investigate effects of NSAIDs on growth and differentiation of osteoblasts on the surface of titanium implants *in vivo*. Growth of cells on titanium disks is a standard study model for studying interaction between cells and metal surfaces and response of cells on titanium surface of implants to the external stimuli[109-113]. MC3T3-E1 cell line is widely used in biocompatibility testing and studying effects of matrix interaction and external stimuli on growth and differentiation of osteoblasts[64,66,114,115].

This study demonstrated that growth patterns of osteoblasts were influenced by cell-surface interaction on smooth titanium surfaces (acid pickling preparation). According to the growth curves of cells on cell culture plates and titanium disks, the growth rate of cells in the *log* phase, or a phase of rapid cell growth, on titanium disks was lower than cells on culture plates, as it was shown that a slope of growth curve of cells on titanium surfaces was less than a slope of cells on cell culture plates (Fig. 17). However, osteoblasts were able to differentiate on an acid pickling titanium surface in a similar fashion to cells on a cell culture plate, as it can be observed from a similar pattern for levels of osteocalcin of cells on titanium disks and culture plates (Fig. 35). This may be because the growth rate of cells was modulated by cell-substrate interaction, causing changes in intracellular, or extracellular ion concentrations[116], or integrin-matrix interactions [117], and because the osteoblasts in this study were grown on an acid pricked titanium surface, not a rough surface. It was reported that surface roughness and alumina oxide surface concentration had an effect on osteoblastic proliferation and function[118]. Another difference was found in the levels of PGE_2 in the *log* phase, that the levels of PGE_2 of cells on culture plates in the *log* phase was markedly higher than cells on a titanium surface (Fig. 38). A difference of PGE_2 level supports a postulation that growth and function of osteoblasts might be influenced by cell-material surface interaction[10,108,111-113].

Fluorescence staining observing under CLSM and SEM images proved to be a useful tool for visualizing attachment and morphology of cells on the opaque surface of titanium disks. An advantage of using fluorochrome diacetate (FDA) as a fluorescence staining not only enables a visualization of cells on titanium disks, but also demonstrates viability of cells with its green staining on vital cells[119-121].

SEM images demonstrated good attachment and growth of cells on titanium surfaces. Similarly to previous reports on growth of MC3T3-E1 on smooth titanium surfaces[122-125], osteoblasts attached and spread-out on titanium surfaces with multiple peripheral filopodia and cytoplasmic extensions and became polygonal shape, in this study. Cells established cellular interconnection and grew in multi-layers in the *log* and *plateau* phases (Figs. 20-25).

In comparison, a spreading of cytoplasmic processes and growth of cells in serum free culture medium was not in full. Growth of cells in serum free culture medium was significantly less than cells in culture medium supplemented with serum (Fig.20-25). This was probably due to the fact that serum free culture medium lacked protein deposition on titanium surfaces rather then resulting in limiting cell adhesion on titanium surfaces[106] and growth of cells were hindered by a deprived condition of serum free culture medium. This evidence suggested that the supplemented serum promoted growth and attachment of cells and serum free culture medium limited growth of cells, particularly growth of cells in the *static* phase (Fig.20-21).

SEM images of cells in culture medium with and without serum in *log* and *plateau* phases (Fig. 22-25) implied that adverse effects of serum free culture medium on growth and morphology of cells were less in osteoblasts growing in multilayers in a later stage of cell growth. This could be explained by arguing that extracellular matrix promoted intercellular contact and cell growth, and growth factors in the matrix functioned as autocrine in stimulating growth of osteoblasts[59], therefore, in the later stage of cell growth osteoblasts could establish intercellular contact and function in serum free culture medium.

2. Effects of specific COX-2 inhibitors upon osteoblasts on titanium surface

2.1. Effects of specific COX-2 inhibitors on growth and differentiation of osteoblasts

Effects of NSAIDs on proliferation and differention of osteoblasts are controversial. This study found that a high concentration of celecoxib 9 μM inhibited growth of osteoblasts, which was different from previous studies[9,10]. Boyan and co-workers[10] found that specific COX-2 inhibitors, NS-398, 1 and 10 μM, did not have any effects on proliferation of osteoblasts on smooth surface titanium disks[10], whereas Evan and Butcher[9] reported that indomethacin 0.1-1 μM inhibited proliferation of osteoblasts on cell culture plates.

Regarding the effects on differentiation of cells in this study, indomethacin and celecoxib in Groups A - C did not have any effects on expressions of ALP and osteocalcin, while Evan and Butcher[9] found that indomethacin 0.1 – 1 μM enhanced differentiation of osteoblasts in an early stage by increasing production of ALP and collagen type I[9]. This study agreed with Coetzee and coworkers[32] that specific cox-2 inhibitors, NS-398 0.1 μM and indomethacin 1 μM, inhibited PGE_2 production of MG-63 and MC3T3-E1, as well as Boyan and coworker[10] that NS-398, 1 and 10 μM, did not inhibit osteocalcin production on smooth surface titanium disks. Various conditions including a variation of medication treatment time, conditions of culture medium, with and without serum during the treatment period, and stages of differentiation of osteoblasts possibly contribute to discrepancy of the results. According to the current study, NSAIDs did not interfere with progress of osteoblastic differentiation into a terminal stage, as it could be seen that a decrease of level of osteocalcin was not found (Fig.36).

Dose dependent effects of conventional and specific COX-2 inhibitors NSAIDs are clearly demonstrated in most studies[9,32,33]. This study found that celecoxib inhibited proliferation of cells in dose and time dependent manner and its effects are most potentiated at the plateau phase or when high number of osteoblasts is exposed with the treated medications (Fig.27). This finding supports previous *in vivo* studies where specific COX-2 inhibitors in dose and time dependent manner delayed fracture healing and bone ingrowth into the porous implants[5,6,38,126] by inhibiting proliferation of cells.

Although animal studies clearly demonstrated inhibitory effects of specific COX-2 inhibitors, celecoxib and rofecoxib on bone healing[6], and bone ingrowth into porous chrome-cobalt implant[38], and titanium implants[5,126], it should be noted that supra-therapeutic doses of NSAIDs were administered in those studies. Thus, the results may not be able to directly imply in clinical situation, but suggest a potential risk of medications in high doses and possibly in long

term administrations. Nevertheless, there has been no study directly investigating the effects of specific COX-2 inhibitors NSAIDs in therapeutic doses on osteointegration of titanium implants.

This study could identify dose and time dependent effects of the NSAIDs on growth of cells in serum free culture medium for 5 day only because in serum free culture medium cell viability was stable on culture days 1 – 6 before it sharply decreased on day 7 (Fig.17). This was because a duration of cell culture was limited by a deprived condition of serum free culture medium. A longer exposure time to NSAIDs should be conducted in an animal study model, where an ongoing osteogenesis is directly exposed to different levels of NSAIDs in plasma for a longer period of time, for 1 – 3 months, to mimic a therapeutic situation in humans.

It should be noted that celecoxib used in this study was prepared from commercial celecoxib powder (Celebrex®), thus, a mixture of other inert ingredients in celebrex was expected to present in the stock solutions of celecoxib. However, those inactive gradients should pose no direct effects on the active function of celecoxib on inhibition of a cyclooxygenase-2 enzyme, as they are inert substrates[28].

2.2. Influence of stages of osteoblastic cell growth on effects of specific COX-2 inhibitors upon osteoblasts on titanium surface

Osseous healing around dental implants is suggested to occur in four stages. Stage I is the sprouting of vascular and early osteogenic phase or formation of fibrin clot and differentiation of precursor cells of osteoblasts occurring on the walls of the prepared osteotomy during days 3 -7 following implantation: Stage II is an early bone formation stage occurring 2 weeks after implantation, corresponding to the formation of bone trabeculae: Stage III is a bone growth stage occurring 4 weeks following implantation. The primary woven bone is replaced by a secondary mature bone and more new bone is formed: Stage IV is a bone maturation stage extending from 6 to 8 weeks following implantation. Bone remodeling is nearly complete. New mature bone is formed on the surface of the implant[85].

It is clearly demonstrated that osteointegration involves a complex series of events that are tied to discrete phases of osteogenesis including osteogenic differentiation, matrix production, mineralization and remodeling[93], thus growth and differentiation of osteoblasts are

prime factors determining success or failure of osteointegration. This study, therefore, investigated effects of celecoxib on growth and differentiation of osteoblasts in different stages of cell growth on the surface of titanium disks (acid pickling surface).

This study demonstrated influence of stages of cell growth on effects of celecoxib on growth and differentiation of osteoblasts by adding the medications in culture medium at different stages of cell growth. The stages of cell growth were defined according to the natural growth curve of cells in *static*, *log* and *plateau* phases (Fig.10), which are comparable to three stages of osteoblastic differentiation including proliferation, matrix formation and mineralization of matrixes[49,50]. Osteoblasts in proliferative and matrix formation stages are pre-osteoblasts characterized by expressions of type I collagen and ALP activity. Mature osteoblasts secrete osteocalcin and mineralize extracellular matrix[49,50,103]. The osteoblast, MC3T3-E1, in each stage of cell growth in this study were mature osteoblasts secreting osteocalcin in the static and log phases and was postulated to mineralize extracellular matrix in plateau phases.

In most of the previous studies, osteoblasts were treated with the medications during days 1-4 after cell seeding[9,10,32,33], which is comparable to the *static* experimental phase in this study. Only a study of Ho *et al*., 1999[32] where osteoblasts were treated with conventional NSAIDs, indomethacin, in different stages of cell growth on culture-days 2, 10, 15 and 20[32]. The authors found that effects of indomethacin on growth and differentiation of osteoblasts at different culture times were different. Our study supports Ho *et al*., 1999[32] where effects of specific COX-2 inhibitors on growth of cells were influenced by different stages of cell growth, as it was found that specific COX-2 inhibitors inhibited cell growth in only the *log* and *plateau* phases, not in the *static* phase (Fig.27-30)[32].

Because osteoblastic differentiation is temporally regulated, it is closely related to stages of cell growth[105]. Thus, one might be able to confer that a high dose celecoxib of 9 μM could pose deteriorating effects on osteointegrations.

Additionally, the inflammatory process including vasodilation and angiogenesis regulates early stages of bone healing in phase I of implant healing, hence factors disturbing the inflammatory process such as inhibitory effects of NSAIDs on cyclooxyganase pathway in an early inflammatory stage[127,128] may be another factor that compromises healing of an implant.

Therefore, not only effects of specific cox-2 inhibitors on growth and differentiation of osteoblasts, but also its inhibitory effects on inflammatory cytokine production can be causes of delay bone healing. This assumption is also needed to be further investigated in an *in vivo* study model.

3. Possible mechanisms of inhibitory effects of specific COX-2 inhibitors on growth and differentiation of osteoblasts on a titanium surface

How specific COX-2 inhibitors inhibit differentiation and proliferation of osteoblasts has not been clearly elucidated. It is generally postulated that inhibitory effects of NSAIDs on proliferation was caused by an inhibitory effect on production of PGE_2, but the results are debatable[8,10,32-34]. A reduction of cell growth might be a result of increasing of intracellular calcium concentration and induction of apoptosis of cells[94,129].

3.1. An inhibitory effect on PGE_2 synthesis

Numerous cytokines and growth factors, including prostanoid products (PGs and TXAs) are released during the inflammatory phase in early stages of implant insertion[127,128]. These cytokines play important roles in regulating proliferation and differentiation of osteoprogenitor cells and angiogenesis. PGE_2 is one of the important cytokines released during early phases of wound healing and bone repair. It plays important roles in maintaining bone homeostastis by regulating bone formation and resorption[21]. An application of specific COX-2 inhibitors NSAIDs is aimed to decrease pain and inflammation by inhibiting the function of enzyme cycloxygenase II resulting in a reduction in prostanoid products, particularly PGE_2. This effect may lead to adverse effects on bone formation and turn over on the titanium surface.

As it is known that conventional NSAIDs inhibit functions of both COX-1 and -2 and affinity of specific COX-2 inhibitors to COX-2 (IC_{50}) is much higher than to COX-1 (IC_{50} ratio COX-2/COX-1 $\leq$ 0.01)[15]. Significant decrease of PGE_2 levels in this study clearly demonstrated that treated indomethacin and celecoxib were able to inhibit the function of cyclooxygenase pathway by blocking the cyclooxygenase enzymes.

In this study a reduction of PGE_2 synthesis was found in all experimental groups, Groups A-D, but not in all experimental phases only in the *static* and *plateau* experimental phases

(Fig.38), whereas significant changes of markers of osteoblastic differentiation, ALP activity and osteocalcin, were not found (Fig. 31, 36). In addition, a significant decrease of cell growth was found in the experimental *log* phase, while the level of PGE_2 was not significantly decreased. The results strongly suggested that effects of specific COX-2 inhibitors NSAIDs on cell growth were not directly dependent on a reduction of PGE_2 synthesis. Moreover, inhibitory effects of celecoxib on PGE_2 synthesis was influenced by stages of cell growth, as decreasing of PGE_2 synthesis was not found in the *log* phase. This might be because cells in *log* phases were in an active stage with a high proliferating capacity and external stimuli had less effect on cell function and cytokine production[64].

Thus, the results of this study do not support the hypothesis that adverse effects of specific COX-2 inhibitors NSAIDs during treatment of OA or RA are caused by a reduction in PGE_2 synthesis, but this study supports the study of Chang and coworkers, that the effect of celecoxib on differentiation of osteoblasts on a smooth titanium surface is not via a reduction of PGE_2 production[33]. It may be because osteoblastic differentiation is a complex event and multifactorial and PGE_2 may not be the only factor regulating osteoblastic differentiation[130]. The results led to a postulation that differentiation of osteoblasts did not critically depend on effects of PGE_2.

3.2. An inhibitory effect on expression of bone morphogenetic proteins (BMPs)

Specific COX-2 inhibitors may influence osteogenesis by posing adverse effects on expression and signaling production of strong osteoinductive proteins and bone morphogenetic proteins. A comprehensive study of Zhang and coworkers reported a significant role of expression of COX-2 mRNA in osteogenic differentiation of mesenchymal stem cells and osetoblastic differentiation of pre-osteoblasts into mature osteoblasts and that an expression of Cox-2 mRNA is a down stream regulator of osteinductive effects of BMP-2[8]. BMP-2 has been shown to induce expressions of COX-2 in osteoblasts and the induction contributes to an effect of BMP-2 on osteoblastic differentiation[7]. Damrongsri and coworkers demonstrated an important role of the COX-2 enzyme in inducing expressions of BMP-6 and new bone formation. NS-398, 3 mg/kg/day injected intraperitoneally for 14 days significantly reduced expression of BMP-6 mRNA and impaired new bone formation. The results suggested an important role of COX-2 in inducing expressions of BMP-6 and new bone formation[131].

It can be seen that inhibitory effects of specific COX-2 inhibitors on osteogenesis can be either via inhibition of PGE_2 production or interference with the function of strong osteogenic proteins, BMPs[7, 8]. However, in this study celecoxib in therapeutic doses, 1.5 – 9 μM, did not show any effects on differentiation of osteoblasts, although, adverse effects of high dose specific COX-2 inhibitors on bone healing and bone growth into porous implants are found in animal study models[5,6,38,126]. This might be because of low concentrations and a short incubation time of NSAIDs in this study. Duing to the fact that the effects of specific COX-2 inhibitor and conventional NSAIDs are dose and time dependent, thus to estimate adverse risk of the medication on osteointegration, dose and duration of usage must be considered.

4. Dose and time dependent effects of specific COX-2 inhibitors and clinical application

It is well reported that non-specific NSAIDs, indomethacin, delays bone healing *in vitro* and *in vivo*[8,10,32-34]. The therapeutic dose of indomethacin is 50-200 mg or 284 ng/ml in plasma[37]. The therapeutic doses of celecoxib are 100 – 200 mg/day for OA, 200 – 400 mg/day for RA, 400 mg/day for colon carcinoma and 200 mg/day for acute pain. Celecoxib must be taken for at least 2 weeks to achieve analgesic effects and the medication will be continuously prescribed for 6 – 12 or 24 weeks[87,132]. With a half life of 12 hours, plasma levels of therapeutic doses of 100, 400 and 800 mg/day are equivalent to 1.7, 3.1 and 9.2 μM, respectively[127]. Therefore, the concentrations of the treated indomethacin and celecoxib used in this study were comparable to their therapeutic plasma levels.

McAdam *et al* reported that only a high dose celecoxib of 800 mg/day caused inhibitory effects on the platelet function and production of thromboxane A_2 [127]. The result of this study supports McAdam and coworkers[127] that only high doses of celecoxib causes adverse effects on proliferation of cells (Fig.27-30). Based on the results of this study that proliferation of cells was inhibited by celecoxib 9 μM on treatment day 5 in *log* and *plateau* phases. An administration of regular therapeutic doses of celecoxib of 100 – 400 mg/day for not more than 5 days should pose no adverse effects on bone healing and osteointegration. It may be postulated that an undertaking of a high dose of celecoxib more than 9 μM or 800 mg/day of more than 5 days could

do damage to osteointegration process by inhibiting proliferation of cells in proliferative and mature stages of osteointegration.