CHAPTER IV

DISCUSSION AND CONCLUSIONS

Study I

The study I demonstrated that bone regeneration could be obtained and studied by the model of bone regeneration using titanium chamber at rat femoral bone. According to the biological principle of guided tissue regeneration, the exclusion of undesired cells by barrier membranes can maintain an empty space to allow for ingrowth of cells with the capacity of regenerating the desired tissue. For successful bone healing and the formation of new bone, ingrowth of connective tissue which occurs rapidly may disturb or totally prevent osteogenesis from taking place in the defect or wound area (Linde et al., 1993). Many different barriers have been used to improve bone healing. Polytetrafluoroethylene (ePTFE) membranes and bioabsorbable polylactic material were used as barrier membranes for studying bone regeneration (Linde et al., 1993; Schmid et al., 1997). It was postulated that the barrier should be permeable for exchange of fluid. However, permeability of the membrane is not necessary in the guided tissue regeneration of the new bone. In addition, membrane collapse and subsequent lack of space maintaining for the ingrowth of bone seemed to have been responsible for negative outcome (Lamerigts et al., 1997). Therefore, the maintenance of a clot-filled space above the wounded bone surface appears to be a prerequisite for successful bone formation in this wound space (Dahlin et al., 1989).

Titanium has been suggested as the material of choice for implant fabrication because of its biocompatibility, high corrosion resistance and well integrated by host tissues (Branemark et al., 1977; Albrektsson et al., 1981). No carcinogenic potential has been observed at implant site when titanium was used in fabricating the implant. Many studies of bone regeneration model used titanium as a material of chamber (Kalebo and Jacobsson, 1988; Kaigler and Lang, 1989; Zhou et al., 1995; Aspenberg et al., 1996; Lamerigts, Aspenberg et al., 1997). One of the problems associated with studying guided tissue regeneration is quantifying the amount of bone regenerated. Membranes often collapse even when placed over standardized defects and the volume size and geometry of the defect varies considerably among animals. To address this issue we designed a new titanium tube model with fixed size volume and geometry enabling better quantitative kinetic assessment of bone fill, cell density and molecular expression. The model has the advantage of enabling the assessment of both transcortical and supracortical osseous regeneration and provides an excellent biopsy sample free from non-regenerating tissue for molecular biology studies. Titanium chambers in this study are not commercially available, however, they are inexpensive and are not difficult to prepare. Self-curing acrylic resin is used for sealing both ends of the chamber, therefore, a locally protected tissue environment is created and a selective osteogenesis within a defined defect can be accomplished by preventing fibroblasts from the connective tissue from migrating into the defect. The inner dimensions of the chamber allowed for the approximate size of 18 mm^3 of the tissue specimen which is enough for histochemistry or immunohistochemistry or molecular biology studies.

Mesenchymal stem cells capable of regenerating osteogenic tissue are present throughout the marrow cavity among adherent marrow stromal cells localized near the endosteal bone surface as well as in a subpopulation of nonadherent cells (Long, Williams and Mann, 1990). The blood clot that was used to fill the chamber space immediately before placing on the defect is essential (Linde, Thoren et al., 1993) and appears to have two principal functions. First, because blood is replete with factors capable of stimulating chemotaxis of osteogenic progenitor cells and it also release growth and differentiation stimuli which induce the local osteogenic cascade of migration, proliferation, differentiation and matrix secretion (Bab and Einhorn, 1994). Second, in addition to its function in stimulating osteogenic cell growth, the blood clot provides "mechanical guidance" essential for the migration of osteogenic cell (Bab and Einhorn, 1994). Other studies using an ePTFE membrane dome on calvaria have also reported that obtaining a well-organized blood clot was essential for regeneration (Linde, Thoren et al., 1993).

In this experimental model system, the amount of bone regeneration was found to be dependent on the length of the healing period. New bone formation was noticed at day 7 and increased through day 28 in both cortical defect area and supracortical defect area. Intense angiogenesis and concomitant proliferation of primitive mesenchymal cells was preceded bone neoformation. However, the rate of bone regeneration was different at different time intervals. It appears that osteoblastic proliferation and cancellous bone formation took place rapidly in the first two weeks, then slowly in the last two weeks of experimental period. It may reflect the rate of bone formation which higher for bone regeneration process at first and then stable which representing bone remodeling process. However, we do not know about the

remodeling process beyond 28 days after surgery.

To characterize the tissue healing dynamics, BrdU was injected to each animal before sacrifice. BrdU is a thymidine analogue that is also incorporated into nuclear DNA. Monoclonal antibody (MAb) directed against BrdU has been developed and can identify BrdU-containing nuclei as indicating DNA replication. BrdU has been used in immunohistochemical studies of cellular proliferative of normal and neoplastic tissue both *in vivo* and *in vitro* and cell positive for the BrdU stain are indicated of S-phase cells. The advantage of the BrdU technique is a less hazardous technique compared to radioactive thymidine indicating mitotic activity. At day 3, BrdU-positive cells were mainly found in the cortical defect area. This finding represents the active area of cellular proliferation which corresponded to a later bone regeneration in the cortical defect area of the samples at day 7. High proliferative activity was also observed at the supracortical area beneath the titanium chamber in the samples at day 7 which co-localized in the area that exhibited a large amount of bone regeneration in the supracortical area at day 21. Therefore, the detection of S-phase cells may reflect the proliferation of osteoprogenitor cells or osteoblast precursor cells which migrate from bone marrow space and then proliferate and differentiate to mature bone-forming cells at the cortical defect area and, subsequently, in the supracortical area. Therefore, mitotic signal or cytokines released into the cortical and supracortical area may act as proliferative substance for osteoprogenitor cells. Chemotactic signals from the breakdown of the cut cortical bone and from blood clot may act as chemotactic substance which led osteoprogenitor cells to migrate to cortical and supracortical area and differentiate to bone forming cells (Bab and Einhorn, 1994).

The type of bone being formed by applying the principle of guided tissue regeneration has been investigated in several animal studies (Kostopoulos and Karring, 1994; Schenk et al., 1994; Hammerle et al., 1995). In these animal experiments, a similar pattern of bone formation was observed. Initially, trabeculae of woven bone proliferated into the defect. The space provided by the barrier was filled with a newly formed connective tissue matrix prior to formation of mineralized bone which is similar to that in our model. In addition, common to all these experiments are the findings that volume of bone mass increased with time.

Complete bone fill depends on critical space, properties of the barrier and the reliability of the peripheral sealing between the borders of the barrier and the adjoining bone surface. The result of this study demonstrates that it is possible to regenerate bone into areas where there has been no bone before. In day 7, new bone primarily filled the cortical defect area, the previously prepared defect, with the amount of 40.32 % of the total defect area. In addition, bone ingrowth can be found in the supracortical area with the amount of 12.31 % of the total area in day 7 and reached 56.35% in day 28. Therefore, by reaching and exceeding the level of the surrounding host bone, true regeneration of bone had occurred. Bone formation did not come to halt at this point but proceeded above the border of the bone surface, thereby altering the genetically determined form of the skeleton. This formation of new bone beyond the skeletal borders by applying the method of guided tissue regeneration has been demonstrated in previous studies in animals (Linde, Thoren et al., 1993; Schmid et al., 1994; Lundgren et al., 1995; Schmid, Hammerle et al., 1997) and in humans (Hammerle et al., 1996).

The formation of prostaglandins requires the catalytic activity of cyclooxygenase (COX) which converts arachidonic acid to PGH_2, from which all other prostaglandins are formed. COX-2 is the highly inducible isoenzyme of COX and COX-2 activity can be rate-limiting in prostaglandin formation. Unlike COX-1 that is expressed constitutively, COX-2 can be induced by a variety of cytokines and stimuli. Transient elevation of COX-2 has been observed in inflammation and may contribute to both inflammatory and anti-inflammatory process (Gilroy et al., 1999). Some types of prostaglandins such as PGE_2, PGD_2, and Δ^{12}-PGJ_2 have been shown to stimulate bone formation under certain conditions (Koshihara and Kawamura, 1989; Tasaki, Takamori et al., 1991; Akamine et al., 1992). Interestingly, COX-2, PGD_2 and 15-deoxy Δ^{12}-PGJ_2 have been found to be increased at 48 h. and associated with decrease of inflammation (Gilroy, Colville-Nash et al., 1999). In this study, COX-2 immunoexpression were found in a very high level at day 3 and preceded the subsequent increase in bone volume (day 7-28). These associations may reflect COX-2 activity and a primary action of these anti-inflammatory prostaglandins in the early stage of bone regeneration. These prostaglandins may on act on bone marrow osteoprogenitor stromal cells because it has been shown that rat bone marrow stromal cells response to prostaglandin *in vitro* with an increase in cell number, collagen accumulation, total alkaline phosphatase activity and calcium deposition (Scutt and Bertram, 1995).

PDGF-B and IGFs are among the growth factors which stimulate bone cell replication and also differentiation of osteoblast (Solheim, 1998). PDGF stimulates cell proliferation in cultures of intact calvariae and osteoblast-enriched cells

(Pfeilschifter et al., 1990). Subperiosteal injection of PDGF in femurs stimulated mesenchymal cell proliferation in periosteum and new bone mass resulted of intramembranous ossification (Bolander, 1992). PDGF increases a cell population capable of synthesizing bone collagen and, as a consequence, bone matrix. Therefore, early increase of PDGF-B in day 3 during the early phase of bone regeneration may increase the number of multipotent immature cells which play critical roles in differentiation and matrix deposition at the later phase. PDGF was also found in immediate injury response 2 days post fracture and decrease from day 3 (Bourque, Gross and Hall, 1993; Solheim, 1998). Interestingly, the pattern of COX-2 expression corresponded with and that of PDGF-B expression which both have peak at day 3. It has been shown that PDGF induced expression of COX-2 protein in fibroblasts and osteogenic cells (Wadleigh and Herschman, 1999; Castano, Bartrons and Gil, 2000). However, expression of PDGF transcript and/or polypeptides has been reported in osteosarcoma, whereas culture medium conditioned by primary tissue explants of fetal rat bones contain undetectable level of PDGF (Harsh et al., 1990). Therefore, PDGF production may be limited or well controlled to particular time point or particular stage of differentiation of cells and may require the influence of a stimulatory agent present in specific cells. Some types of prostaglandin that are products of COX-2 such as cyclopentenone may induce PDGF expression as in the results of study III (see below).

IGF-I and -II have been suggested as important growth factors in the regulation of bone regeneration (Linkhart, Mohan et al., 1996). Locally produced IGFs are important in responses of bone cells to various stimuli. IGFs have been identified in bone matrix, but the ratio varies among species. IGF-II is the most

abundant form in bone matrix in most species, including humans, whereas IGF-I is predominant in rodent (Bautista, Mohan and Baylink, 1990). IGFs stimulate preosteoblastic cell replication, which increase cell population capable of synthesizing bone matrix. They also have an effect on differentiated function of the osteoblast which increasing bone collagen synthesis (Wakisaka et al., 1998). IGF-I is the bone-derived growth factor that can cause the appearance of markers of the differentiated osteoblast phenotype, including type I collagen, alkaline phosphatase activity and osteocalcin (Wakisaka, Tanaka et al., 1998). Therefore, the increase of IGF immunoexpression in day 21 and 28 may reflect the differentiation phase of the osteoblast precursor into mature cell after the initial event of chemotactic attraction of osteoblasts or their precursors and the second event of proliferation of osteoblast precursors. The increase in expression of IGFs in our study is consistent with the anabolic effects of these growth factors on bone formation *in vivo*. The observed increase in IGF expression postoperatively agrees with previous *in vivo* experiments in membranous osteotomy healing in the rat mandible (Steinbrech et al., 1999), however, a difference in the time of the peak IGF expression was observed in that model. This observation may be because of differences of bone site, nature of osteotomy and/or specificity of our IGF antibody which can detect both IGF-I and –II. IGF-I also stimulates differentiation as well as activation of osteoclasts which may be also another explanation of continued elevated of IGF expression in late healing (Steinbrech, Mehrara et al., 1999). Moreover, IGFs that are produced by bone cells may be fixed and storage in bone matrix by means of IGF-binding proteins (IGFBPs) which binds with high affinity to both hydroxyapatite and IGFs (Mohan, 1993).

BMP-6 is one of the members of bone morphogenetic proteins (BMPs) which are members of the transforming growth factor-β (TGF-β) superfamily. They are important in both extraskeletal and skeletal organogenesis, bone generation and regeneration (Wozney, 1992; Reddi, 1998). It has been shown that BMP-6 induces the differentiation in bone marrow stromal cells by stimulation of alkaline phosphatase activity (Yamaguchi et al., 1996). It is likely that several BMPs play a role in sequence at different stages of osteoblast differentiation. Interestingly, of the BMPs studied in spine fusion using autogenous bone graft, BMP-6 mRNA was the earliest BMP to be expressed and had a peak on day two after the surgery (Morone et al., 1998). A similar sequence and timing of BMP-6 expression was observed in an *in vitro* model of membranous bone formation using rat calvarial osteoblasts and the mRNA level also correlated with the levels of mature BMP-6 protein (Boden et al., 1997). These data suggested that BMP-6 might play a unique role in an early stage of osteoprogenitor cells to initiate membranous bone formation (Gitelman et al., 1995). BMP-6 also stimulates osteoblastic differentiation in rat calvaria-derived osteoblast-like cell (Hughes et al., 1995) and is a more potent inducer of membranous bone formation than BMP-2 (Boden et al., 1996). Even short exposure of cells to BMPs can result in terminal differentiation of osteoblasts in long term cultures (Boden, Hair et al., 1997). Therefore, the high expression of BMP-6 in day 3 may reflect the important role of BMP-6 in the early period of bone regeneration which stimulated osteoprogenitor cells in bone marrow stromal cells. This stimulation appear to be less important but still involve in bone regeneration from day 7 to 28 since the immunoexpression of BMP-6 is gradually decrease with the time. Although other growth factors not measured in this study may play a role in early osteoblast

differentiation, BMP-6 may represent a critical component of the osteoblast differentiation pathway.

The results of the study I clearly demonstrate that it is possible to create new bone tissue with the use of this chamber device and techniques without using a bone substitute. Bone regeneration can achievable using an experimental barrier with fix geometry, biocompatibility, sufficient stiffness, stability, total occlusiveness and capability of reliable and peripheral sealing. This report described an additional *in vivo* model for the assessment of bone-healing which using simple and inexpensive materials together with less complicate surgery procedures in tissue preparation. All of the animals recovered rapidly, within one day after operation, and resumed normal activities. In addition, both femoral bones could be used in the same animal without adverse effect so they can provide the control and test site in the same animal. The present technique was developed as an instrument for quantifying bone regeneration when local as well as systemic influencing factors may be analyzed both retarding and promoting factors on bone regeneration may be studied. The bone regeneration above the periosteal surface allowed the tissue to use for molecular biology techniques without contamination with other tissue. The model enabled bone and tissue regeneration as early as 7 days.

The animal model established in this study is potentially useful because it permits quantitative characterization of the time-dependent changes of bone regeneration. This model is also suitable for studying the regulatory role of various factors, including cytokines and growth factors, that have been suggested as regulatory agents in bone regeneration.

Study II

The study II was inspired by a previously observations in study I and in other studies concerning the potential of COX-2 and some type of prostaglandins to be anabolic bioregulators in the early stage of new bone regeneration and result in induction of some growth factor expression. NS-398 [N-(2cyclohexyloxy-4-nitrophenyl)methanesulfonamide], a selective inhibitor of COX-2, significantly inhibited new bone regeneration at day 14 in supracortical area. The same tendency was also found in cortical defect area with no statistically significant. Less mature bone in both cortical and supracortical defect area was also found in animals treated with NS-398. Several studies have demonstrated that COX-2 and prostaglandins are important for bone formation induced by mechanical strain. Prostaglandins are important early mediators of the response of bone cells to mechanical stress (Klein-Nulend et al., 1997). Mechanical stress also induced the expression of COX-2 but not COX-1 in primary bone cells from both human and mouse (Joldersma et al., 2000). Moreover, COX-2 inhibitor can block bone formation induced by mechanical strain (Forwood, 1996).

It is of interest that COX-2, PDGF-B, and BMP-6 expression was decreased in animals treated with NS-398. It has been shown that prostaglandin such as PGE_2 can increase intracellular cAMP level in osteoblast-like cells (Fang, Kujubu and Hahn, 1992; Smith, 1992) and cAMP response element (CRE) is critical for COX-2 promoter activity (Kirtikara et al., 2000). Therefore, the decrease in cAMP level may result in decrease in COX-2 promoter activity and COX-2 gene expression and

protein synthesis. Recently, it has been demonstrated that COX-2 promoter also contain a peroxisome proliferator response element (PPRE) that is responsible for the induction of COX-2 expression seen with peroxisome proliferator compounds binding to peroxisome proliferator activated receptors (PPARs) (Meade et al., 1999). Interestingly, cyclopentenone prostaglandin such as Δ^{12}-PGJ_2 and 15-deoxy- Δ^{12}-PGJ_2 are also ligand of PPAR. It is possible that the low level of cyclopentenone prostaglandin by inhibition of COX-2 activity may result in decreased in COX-2 expression. Cyclopentenone prostaglandin may also provided itself as a "signal" to induce PDGF-B and BMP-6 expression. Therefore, inhibition of COX-2 activity decreased PDGF-B and BMP-6 expression.

Surprisingly, significant increase in IGF expression has been found in animal treated with NS-398. CRE was also identified at IGF promoter and is required for activation by cAMP in osteoblasts (Thomas et al., 1996). However, inhibition of COX-2 did not decrease IGF expression so it is presumably that the dominant prostaglandin released in the defect may not be prostaglandin E series which induced intracellular cAMP. The possibility that cyclopentenone may be the major prostaglandin released in the defect is not unusual and is likely based on the recent study showing that cyclopentenone prostaglandins such as PGJ_2 and PGA_1 significantly repressed IGF expression (Bui and Straus, 1998). However, the level of IGF receptors and IGF binding proteins (IGFBPs) which mediated IGF expression can not be ruled out.

The effect of COX-2 inhibitor in inhibition of BMP-6 expression was confirmed by RT-PCR. In addition, NS-398 also significantly decreased BMP-2

mRNA level in sample from supracortical defect. Same tendency but not significant different was found in both PDGF-A and PDGF-B mRNA level in supracortical defect. However, the mRNA level from cortical defect were not correlated with that of supracortical defect. It should be noted that samples from supracortical area were all new bone formation with no contamination from other cells or tissue. In contrast, samples collected from cortical defect area also included bone marrow tissue containing different kind of cell. Therefore, tissue from suprascortical area is more suitable to use for molecular biology studies in new bone regeneration.

Study III and IV

In an attempt to access the role of cyclopentenone in mediating bone regeneration *in vivo*, Δ^{12}-PGJ_2, one of the cyclopentenone prostaglandin, has been used in the model of bone regeneration previously described in study I. The results presented here demonstrate that Δ^{12}-PGJ_2 acts as a bone regeneration agonist in our *in vivo* model system. The dose-response relationship indicates that this molecule is a relatively potent (oxymoron) inducer of new bone in a single dosage application. The second key observation is that this cyclopentenone, which is known to be produced when bone is under mechanical stress of bone, is an inducer of PDGF-A and -B and BMP-2 and -6 synthesis in a dose-dependent manner.

In this model, bone regeneration following guided bone regeneration techniques was demonstrated. Titanium chamber provide a fixed geometry barrier and physical support which maintain space where osteogenesis is to be take place and exclude competing non-osteogenic cells from the defect. These chamber also create a

space for collagen sponge carrier filled with Δ^{12}-PGJ_2.

The biological activity of Δ^{12}-PGJ_2 differs from that of the PGD_2. The former lacks antiaggregatory activity for human blood platelets and does not lead to the relaxation of smooth muscle (Fukushima, Kato et al., 1982). Δ^{12}-PGJ_2, has an effect on bone formation. It acts directly on human osteoblasts to cause stimulation of hydroxyapatite deposit and ALP activity (Koshihara and Kawamura, 1989; Koshihara, Amano et al., 1991). It has been shown that Δ^{12}-PGJ_2 strongly stimulates calcification with an efficacy almost equal to that of $1,25(OH)_2D_3$ (Tasaki, Takamori et al., 1991). However, other derivatives of PGD_2, such as PGJ_1, PGJ_2 and 12-metyl-PGJ_2, have no effect on calcification (Koshihara, Amano et al., 1991). Osteocalcin accumulated on cell layer during calcification, calcium and phosphorus were remarkably increased by Δ^{12}-PGJ_2 and PGD_2 in human osteoblastic cells (Fukushima, Kato et al., 1982) and, by Northern blot, the amount of type I procollagen (a_1) mRNA is increased time dependently when stimulate with Δ^{12}-PGJ_2 in human osteoblastic cells (Tasaki, Takamori et al., 1991). In ovariectomized rats, subcutaneous administration of a slow-release preparation of PGD_2 partially prevented the ovariectomy-induced decrease of femoral bone mineral density in osteopenic femur (Takagi, Yamamoto et al., 1993). Long term treatment with indomethacin, a non-steroidal anti-inflammatory drug well known for inhibiting prostaglandin production, leads to a decrease in trabecular bone mass and in compressive strength of the lumbar vertebra (Saino et al., 1997). PGD_2 also stimulates DNA synthesis in MC3T3-E1 (Tsushita et al., 1992). Because PGD_2 and PGJ_2 are converted to Δ^{12}-PGJ_2 in the presence of plasma, most of the biological activity exerted by the parent compounds is also exerted by their metabolite. Thus, it is likely that Δ^{12}-PGJ_2 is the ultimate

compound that exerts biological activity of PGD_2.

The role of prostaglandins in bone resorption and bone formation remains a puzzle. A number of studies have indicated that the response to prostaglandins was variable and depended upon dosage, animal model and site of evaluated bone formation (Jee et al., 1985; Norrdin, Jee and High, 1990). Administration of PGE_2 *in vivo* stimulates bone formation (Akamine, Jee et al., 1992; Jee et al., 1992; Mori, Jee and Li, 1992). Local delivery of PGE_1 enhances the formation of new bone (Marks and Miller, 1994) and PGE_2 leads to the formation of subperiosteal fibrous lamella new bone in a dose dependent manner. However, some studies have shown that the prostaglandins of the E series are the most potent of bone resorbers (Raisz, Alander and Simmons, 1989; Raisz and Woodiel, 1989). The fact that the prostaglandin E series can change to D and then J series by enzymatic isomerization may explain this apparent discrepancy. Prostaglandins of the E series act as catabolic prostanoids and the D/J cyclopentenones act as anabolic prostanoids which are physiologically in balance.

The concept that these prostanoids may serve as early signals for growth factor transcription and synthesis in bone is supported by the fact that the mechanism of action of these prostanoids, which form a distinct cyclopentenone subclass, differs from that of prostanoids of the E and F series. Specifically, E and F series prostanoids bind to extracellular receptors and activate cells by triggering an increase in intracellular cAMP (Rawlinson et al., 1991). Cyclopentenones prostaglandin enter the cell, bind to nuclear receptors and act as a cis-acting transcriptional regulatory factor (Forman, Tontonoz et al., 1995). However, the cellular and molecular mechanisms by

which Δ^{12}-PGJ_2 promote osteogenesis is still unclear and little is known about the growth factors that participate in this process. The ubiquitous presence of prostaglandins-metabolizing enzymes make these mediators unstable and their action short-lived. It is likely that Δ^{12}-PGJ_2 may promote their effects on bone regeneration via stimulating the local production of paracrine and autocrine bone growth factors.

PDGF is the product of two genes that express independently. They encode two distinct PDGF chains, PDGF-A and -B, from chromosome 7 and chromosome 22 respectively. These two peptides shared 56% amino acid sequence homology and can combine to form disulfide-linked dimers of PDGF-AA, PDGF-BB, or PDGF-AB approximately MW 30,000 (Hannink and Donoghue, 1989). The three PDGF isoforms are mitogens for a variety of cells and display different potencies: PDGF-BB>PDGF-AB>PDGF-AA. This has been attributed in part to the ability of PDGF-BB to bind to both the PDGF-a and b receptors, whereas PDGF-AA only occupies the a-receptor (Canalis, Varghese et al., 1992). In particular, PDGF is a potent mitogen for cells of connective tissue origin and PDGF-BB and PDGF-AA are important regulators of human osteoblast migration and participate in the recruitment of osteoblasts during bone remodeling (Lind et al., 1995). PDGF can also act as a paracrine or autocrine regulator of bone cells (Graves et al., 1989). PDGF can directly increase DNA replication and matrix protein synthesis by both differentiated and undifferentiated bone cells and stimulates DNA and protein synthesis (Graves and Cochran, 1990). A single dose of PDGF-BB stimulates bone formation in a critical size bone growth defect in calvaria bone (Vikjaer et al., 1997) and a single application of PDGF and IGF results in a significant improvement in bone growth in periodontal defects (Howell et al., 1997). In addition, normal osteoblasts have been shown to

express the PDGF-A gene (Graves, Valentin-Opran et al., 1989; Rydziel, Shaikh and Canalis, 1994) and PDGF-B gene (Rydziel and Canalis, 1996). Bone cells also possess cell surface receptors capable of binding and transduction of PDGF activity (Yu et al., 1997). PDGF alone or combined with IGF enhances bone formation (Mitlak et al., 1996) and periodontal attachment apparatus (Lynch et al., 1991; Giannobile et al., 1996). Exogenous PDGF also has a stimulatory effect on fracture healing (Nash et al., 1994). The expression of PDGF-A and its receptor in forming human bone indicates the autocrine and paracrine effects of osteogenesis regulation (Horner et al., 1996). These evidences suggest that bone-derived PDGF have the potential to act as a bone stimulating factor for bone cells and to play an important role in bone metabolism and remodeling. Different cell or tissue may express equivalent or different amount of both chains and the level of expression of two chains is cell specific. Because of this variability in the expression of both chains, it is possible that gene regulation of A and B chain differs. However, the increase of the level of PDGF itself may induce, in part, PDGF-A or -B chain (Bhandari, Grandaliano and Abboud, 1994).

Recent studies have indicated that mechanical strain can induce proliferation of osteoblastic cells (MC3T3-E1) and are accompanied with an increase in PDGF-A mRNA and antibodies against PDGF can inhibit physical stimulation-induced proliferation of MC3T3-E1 cell (Wang et al., 1997). It has been suggested that mechanical stimulation can change the conformation of the cell membrane, thereby exposing phospholipids to the action of phospholipases. The free arachidonic acid produced would be available for the prostaglandin synthases (Hong and Levine, 1976). Since PDGF has previously been identified as an important bone growth factor

involved in repair and regeneration, this finding establishes Δ^{12}-PGJ_2 as a potential signal for PDGF synthesis and bone regeneration. The fact that this compound binds to a transcriptional factor prompted us to explore whether the PDGF promoter, which regulates PDGF transcription, contains the sequence for binding of this transcriptional factor. Δ^{12}-PGJ_2 and its metabolite, 15-deoxy-Δ^{12}-PGJ_2, can bind to a protein transcription factor called peroxisome proliferator activated receptor or PPAR (Forman, Tontonoz et al., 1995). This nuclear transcription factor is ligand-activated which can form heterodimer with 9-*cis*-retinoic acid receptor (RXR) and binds to a specific DNA sequence, a transcriptional response element called peroxisome proliferator response element (PPRE) (Tugwood et al., 1992; Green and Wahli, 1994). The PDGF-A promoter contains the PPRE-liked sequence for the related motif of PPRE (Takimoto et al., 1991). Recent studies indicated that some hypolipidemic drugs are also ligands for PPAR (Forman, Chen and Evans, 1997). Interestingly, animals that received lipid-lowering drug therapy demonstrated significantly increased macrophage PDGF-B. Thus it appears likely that Δ^{12}-PGJ_2 is a transcriptional activator for osseous PDGF secretion. This statement awaits experimental validation, but the presence of this PPRE in the promoter of this growth factor is suggestive of a regulatory role. In other cell types, PPRE activation results in a cellular shift to an anabolic phenotype. For example it enhances triglyceride deposition in adipocytes (Chawla et al., 1994; Tontonoz, Hu and Spiegelman, 1994; Latruffe and Vamecq, 1997). Thus, in bone Δ^{12}-PGJ_2 may be an important anabolic signal. The observation that PDGF expression is enhanced, while there is no effect on IGF synthesis suggests that this activation displays some specificity. Interestingly, the PPRE sequence does not appear in the IGF promoter (Hall et al., 1992). Other cyclopentenone derivatives might bind to PPAR and lead to a better PPRE activation

than that observed with Δ^{12}-PGJ_2, therefore other compounds in this family should be tested.

It should be emphasized that the concept of cyclopentenone derivatives being part of a transcriptional regulatory pathway is quite new. Although many cells such as endothelial cells, fibroblasts, macrophages and adipocytes have well-characterized PPAR proteins (α, β, and γ family) (Latruffe and Vamecq, 1997), none have been described so far for osteoblasts. Thus, the study of PPAR proteins in osteoblast lineage may provide more evidences and new clues to the mechanisms involved in the bone regeneration of these cells. The concept that this new class of prostanoids (cyclopentenone) has anabolic effects on bone that can be triggered by mechanical stress suggests that this pathway may have a major importance in bone physiology and pathology. This hypothesis is supported by a wide range of studies, which have demonstrated an anabolic effect of Δ^{12}-PGJ_2 (Fukushima, Kato et al., 1982; Koshihara and Kawamura, 1989; Koshihara, Amano et al., 1991; Tasaki, Takamori et al., 1991).

IGFs play an important role in the local regulation of bone formation (McCarthy, Centrella and Canalis, 1989; Linkhart, Mohan et al., 1996). IGFs are the most abundant growth factors produced by bone cells and stored in bone. Much evidence shows that IGFs can increase bone formation both *in vivo* and *in vitro* (McCarthy, Centrella et al., 1989). In bone cultures, IGF-I can induce the proliferation of preosteoblastic cells and stimulate collagen synthesis in differentiated osteoblasts (Mundy, 1993; Rosen, Donahue and Hunter, 1994; Giannobile, Hernandez et al., 1996). In this study, we demonstrated that Δ^{12}-PGJ_2 did not effect IGF expression

during bone regeneration in this model. It has been suggested that hormones such as parathyroid hormone (PTH), growth hormone (GH), cortisol and some other growth factors such as basic fibroblast growth factor (bFGF) regulate IGF-I and -II. TGF-β, PGE_2, and PDGF-BB did not alter IGF-I levels in osteoblast culture system (McCarthy, Centrella and Canalis, 1990; Canalis, Centrella and McCarthy, 1991). These may reflect, in part, in steady level of IGFs expression in this study. However, the increase of new bone formation at high concentration of Δ^{12}-PGJ_2 may be associated with the increase of PDGF production. These growth factors are known to be the mitogenic (Davidai et al., 1992) and chemotactic agent (Tsukamoto et al., 1991; Hughes, Aubin and Heersche, 1992) for osteoblasts and to stimulate osteopontin expression in marrow stromal cells (Tanaka and Liang, 1995). Therefore PDGF may increase the cell numbers locally and recruit osteoprogenitor cells and osteoblasts into the defect by chemotaxis. PDGF are not considered to be a differentiation factor. In contrast, IGFs, which are known to be the differentiation factor for osteoprogenitor cells, were not found in significantly increased levels in the defect. However, a recent study suggested that PDGF could enhance IGF-I receptor RNA expression and the activity of the IGF-I receptor promoter (Rubini et al., 1994). Therefore, we may speculate that Δ^{12}-PGJ_2, by increasing PDGF production, could induce the proliferation, migration and differentiation of osteoprogenitor cells by increasing the expression of IGF receptors, therefor increasing the responsiveness to IGF. However, some insulin-like growth factors binding proteins (IGFBPs) which can bind to IGFs and modulate IGF action (Clemmons, 1997) as well as other growth factors such as BMP and TGF-β may also be involved in this process.

BMPs are group of at least 20 members of the TGF-β superfamily which plays an important role in bone formation in vertebrates (Hogan, 1996; Wozney and Rosen, 1998). BMPs are produced by bone marrow stromal cells and by osteoblasts and they are stored in bone in an inactive form. It has been shown that BMP-2 and -6 stimulated osteoblast differentiation and BMP-6 seemed to have an effect during early stage of differentiation (Gitelman, Kirk et al., 1995; Boden, Hair et al., 1997). The differentiation stage of multipotent cell population was found to be an important determinant of the effect of BMP (Yamaguchi, Ishizuya et al., 1996). Several studies have shown that BMPs do not stimulate mature osteoblasts. These results indicate that the osteogenic influence of BMP is directed towards immature and multipotent cells. Mature cells seem to loose their responsiveness. BMP-2 increased production of collagen and alkaline phosphatase in osteoblastic cell lines (Yamaguchi, Ishizuya et al., 1996) and mesenchymal progenitors in bone marrow (Gori et al., 1999). It has been shown that BMP-2 expression is enhanced when rat bone marrow cells are induced to become osteoblasts (Bi, Simmons and Mainous, 1999; Oreffo et al., 1999). Rat and mouse bone marrow have shown responsiveness to rhBMP-2 by an increase in osteoblastic parameters (Rickard et al., 1994). It also has been shown that BMP-6 induced the differentiation in bone marrow stromal cells by stimulation of alkaline phosphatase activity (Yamaguchi, Ishizuya et al., 1996). BMP-6 also stimulates osteoblastic differentiation in rat calvaria-derived osteoblast-like cells (Hughes, Collyer et al., 1995). Therefore, the significant increase in BMP-2 and -6 expression may involve in the mechanism of Δ^{12}-PGJ_2 induced bone regeneration. It is unlikely that BMP-2 induced BMP-6 since it has been demonstrated that BMP-6 expression was not increased by BMP-2 treatment in fetal rat calvarial cultures (Boden, Hair et al., 1997). Moreover, Δ^{12}-PGJ_2 induced protein disulfide isomerase (PDI) gene

expression which is a key regulator for protein folding and disulfide formation (Odani et al., 1996). It may involve in increasing of protein folding and dimerization of growth factors such as PDGFs and BMPs.

Cancellous bone density and structure of alveolar ridge are important for endosseous implant stability and prognosis. Loss of endosseous implants were report in a high number with implants in type-4 bone or less dense alveolar cancellous bone. Therefore, increasing in supporting bone around implants is crucial to the stability of the implants. Bone regeneration of implant is depended on migration of mesenchymal cells from bone marrow and from endosteal surface. In this study we evaluated peri-implant supporting bone by measuring new bone formation at endosteal surface as well as bone in the thread area. The major part of the new bone formed around the implants was bone formed from the endosteal surface growing towards and gradually enclosing the implants. Δ^{12}-PGJ_2 significantly increased both thickness and marginal level of extended bone from endosteal surface of new bone formation. These results may describe the quality of supporting bone and represented the long-term support and resistance to breakdown for the implants better than bone-to-implant interface (Meraw and Reeve, 1999).

In summary, this model of bone regeneration reveals that Δ^{12}-PGJ_2 can enhance new bone formation and that process is correlated with PDGF, BMP-2 and -6 expression. These results suggest that Δ^{12}-PGJ_2 may influence bone formation directly or indirectly by enhancing local PDGF, BMP-2 and -6 production. Moreover, a single application of Δ^{12}-PGJ_2 at the time of implant insertion can stimulate supporting bone regeneration around the implant. This compound is a potential new therapeutic agent

for the regeneration of bone. It could be used as a support treatment to increase amount and quality of supporting bone-surrounding implants as well as improve new bone formation in periodontal disease and accelerate the healing of extracting sockets resulting in increasing more predictable procedure of those treatments.

CONCLUSIONS

Based on the results obtained in this series of investigations it may be concluded that:

- Bone regeneration could be obtained and studied by the model of bone regeneration using titanium chamber at rat femoral bone without using of bone substitute.

- The titanium chamber can be used to isolate the defect from surrounding soft tissue, protecting it from ingrowth from undesired tissue and creating an environment that is more conductive to bone regeneration.

- The fixed geometry of rigid titanium exclusion stabilizes blood clot and maintains space above the wounded bone surface. These are important for successful outcome of new bone formation above the wounded bone.

- The fixed geometry titanium chamber also provide a tissue specimen of new bone regeneration above the periosteal surface which can be used for immunohistochemistry analysis or molecular biology studies without

contamination of pre-existing bone or other tissue.

- COX-2, arachidonic acid metabolites, PDGF, and BMP-6 may have important roles in the early period of bone regeneration.

- Inhibiting COX-2 during the process of bone regeneration decreases BMP-6 synthesis and area of new bone regeneration.

- Δ^{12}-PGJ_2 can induce significant new bone formation in this model system in a single dosage application. This cyclopentenone also induces the synthesis of PDGF-A and –B and BMP-2 and –6 in a dose dependent manner suggesting that Δ^{12}-PGJ_2 may influence bone formation directly or indirectly by enhancing these local growth factors production.

- A single application of Δ^{12}-PGJ_2 at the time of implant insertion can stimulate supporting bone regeneration around the implant. Δ^{12}-PGJ_2 may be a potential new therapeutic agent for bone regeneration

- It has been established that BMP are potent osteoinductive morphogens. Our new data would suggest that COX-2 activation in bone may result in the local production of Δ^{12}-PGJ_2 that acts to induce new bone formation by enhancing the transcription and synthesis of BMPs. This new pathway may explain the anabolic effects of COX-2 activation that has been observed but not previously characterized at a molecular level. Thus, that induce BMP synthesis and a cascade of osteogenic growth factors and bone-specific proteins.