Chapter 1

INTRODUCTION

An overview

1. Introduction

Oral and maxillofacial surgery frequently encounters defects in bone due to disease, trauma, tumours or physiologic bone loss. Autologous bone grafting is the standard method for bone repair. Limitations of an available amount of bone and secondary donor site defects are disadvantages of this method (Manson et al., 1986). Artificial bone substitute such as hydroxyapatite (Bucholz, 1987; Bucholz et al., 1987), demineralized human and bovine bone (Einhorn et al., 1984; Glowacki et al., 1981) have been used to repair bony defects but their osteoconductive activity is not sufficient to induce new bone formation in large surgical site defects.

Bone-tissue engineering improves bone regeneration by initiating and stimulating host bone regeneration process to morphologically and functionally restore skeletal defects. The procedures aim to overcome limitations caused by insufficient local osteoprogenitor cells or an ineffective bone regeneration process in skeletal defects. One strategy is to deliver osteogenic proteins or growth factors with and without living osteoblasts into skeletal defects to initiate and stimulate bone regeneration process (Bruder et al., 1998a; Caplan and Bruder, 1966). Bioresorbable scaffold is required as a carrier of implanted cells, or delivering vehicles of osteogenic proteins, into skeletal defects (Caplan and Bruder, 1966; Lane et al., 1999). Thus, the development of implantable porous matrixes, osteogenic cell culture of bone marrow and incorporation of

bone morphogenetic proteins or osteogenic growth factors on the scaffolds have been extensively studied in scope of bone-tissue engineering. It is advantageous, when skeletal defects can be repaired by autogenous osteogenic stem cells. Transferring living cells is particularly necessary in surgical sites with limited blood supply or poor tissue beds. These implanted cells are expected to act as osteogenic inducer cells and as an osteogenic precursor (Figure 3).

Activated platelets release at least four growth factors relating to wound and bone healing: the platelet derived growth factor (PDGF); the transforming growth factor – β1 (TGF-β1), the insulin-like growth factor I (IGF-1) and the epidermal growth factor (EGF) (Gunsilius et al., 2000; Landesberg et al., 2000; Marx et al., 1998). It is proposed that platelet-rich plasma (PRP) delivers growth factors contained in platelets and these growth factors activate proliferation and osteoblastic differentiation of mesenchymal stem cells and cells of osteoblastic lineage (Manson et al., 1986; Marx et al., 1998).

2. Concepts of the study

Based on a concept of bone-tissue engineering that aims to activate bone regeneration by reconstructing tissue *in vitro* and transfering the reconstructed tissue into the designed site to activate tissue regeneration *in vivo* (Schaffer et al., 1998), this study aims to develop a method to transfer living cells seeded on a three-dimensional scaffold into defect sites. Therefore it is necessary to be able to cultivate osteoblast-like cells and implant them *in vivo*.

Aiming at using autologous mesenchymal stem cells to repair bony defects, osteogenic differentiation of mesenchymal stem cells in bone marrow was studied. Cell culture of rat bone marrow and methods to induce osteogenic differention of mesenchymal stem cells were developed. Expressions of osteoblastic phenotypes were characterized. Bone morphogenetic protein-2 (BMP-2), 1α,25 dihydrocholicalciferol (VD3), and TGF-βs have various effects on growth and differentiation of osteoblast-lineage in dose and time dependent manners (Gori et al., 1999; Hutmacher et al., 1999; Lecanda et al., 1997; Rickard et al., 1994), thus, these growth factors were applied in bone marrow cell cultures to modify growth and differentiation of undifferentiated and differentiated mesenchymal stem cells.

Bone marrow and differentiated mesenchymal stem cells were seeded on an Innert collagenous bovine bone matrix (ICBM) for three-dimensional cell culture and implantated in ectopic sites in immunodeficient mice. This was based on five concepts: (1) secreting bone matrix is a unique characteristic of osteoblasts (Maniatopoulos et al., 1988), (2) bone marrow mesenchymal stem cells are able to differentiate to osteoblasts *in vitro* and *in vivo* (Ashton et al., 1980; Goshima et al., 1991a; Goshima et al., 1991c), (3) scaffold provides a three-dimensional structure for growth and differentiation of cells, (4) seeding cells on a three dimensional scaffold enables living cells to be implanted (Bruder et al., 1998c; Bruder et al., 1998b; Goshima et al., 1991a; Negishi et al., 2000; Schaffer et al., 1998) and (5) ICBM has good biocompatibility and is not osteoconductive (Kubler et al., 1998).

In the second part of the study, when bone marrow cells and osteogenic induction procedures were well established, bone marrow cell culture was applied to test effects of platelet-rich plasma to growth and differentiation of bone marrow cells. In a clinical study, it is postulated that growth factors released from PRP stimulate proliferation and differentiation of implanted mesenchymal stem cells and local osteoblastic lineages resulting in the increase of rates and amounts of bone regeneration (Marx et al., 1998). In order to clarify the effects of PRP on mesenchymal stem cells and pre-osteoblastic cells *in vitro* and further improve its clinical application, effects of PRP on differentiated and un-differentiated mesenchymal stem cells are studied by using three-dimensional cell culture and implanting the reconstructs in nude mice.

Literature review

1. Mesenchymal stem cells in bone marrow and osteogenic differentiation

1.1. Pluripotential stem cells in bone marrow

Adult tissue contains stem cells which can be renewable after trauma, disease or aging (Thomson et al., 1998). Pluripotent stem cells with the ability to renew and differentiate to a variety of cell types are found in bone marrow. Theise and coworkers. (Theise et al., 2000a) showed that when transplanting bone marrow of female mice into irradiated male mice, they would find that transplanted bone marrow cells incorporate into regenerating hepatocytes of recipient mice after two months of bone marrow transplant. The evidence is also demonstrated in humans that hepatocytes can be derived from extrahaptic circulating stem cells, probably being of a bone marrow origin. Sex chromosomes of bone marrow transplant donors are detected in hepatocytes of recipients (Theise et al., 2000b). Jackson and coworkers (Jackson et al., 1999) demonstrated that muscle contains populations of cells with characteristics of bone marrow-drived hematopoietic stem cells. It supports a proposal that tissue-specific progenitors may have differentiation potential outside of their tissue of origin.

Bone marrow contains both hematopoietic and non-hematopoietic, or mesenchymal stem cells. Mesenchymal stem cells reside in stromal fibroblastic units containing fibroblastic c ells, reticular cells, adipocytes and osteogenic cells (Owen, 1980; Owen and Friedenstein, 1988). They have a capacity for self-renewal and differentiating into various types of mesenchymal cells, fibroblasts, myoblasts, chondrocytes, adipocytes and osteoblasts (Otsuka et al., 1999; Owen and Friedenstein, 1988). Mesenchymal stem cells are characterized by their abilities to attach and form fibroblastic colonies in monolayer cultures. After passages, morphologies of cells become more homogenous with spindle-shaped or fibroblast-like cells (Ashton et al., 1980; Friedenstein et al., 1970).

Pittenger and coworkers (Pittenger et al., 1 999) were able to isolate and expand human mesenchymal stem cells. These expanded cells could differentiate into multi-lineages of adipocytes, chondroblasts and osteoblasts under specialized cell

culture conditions. Colter and coworkers (Colter et al., 2000) identified subpopulations of cells in cultures of mesenchymal stem cells, which were small and proliferative and larger cells being precursors of more mature cells in the same cultures. These cells formed recycling stem cells in which large precursor cells are predominant during the lag phase and the proliferation of small cells is increased during log phase growths. The ability to rapidly expand human mesenchymal stem cells (hMSCs) in culture is important in using the cells for cell and gene therapy.

Isolated colony forming units - fibroblast (CFU-F) are shown to be pluripotential. They differentiate into osteoblasts, chondrocytes, adipocytes and myotubes *in vitro* and *in vivo* (Ashton et al., 1980; Hou et al., 1999; Nilsson et al., 1999; Pereira et al., 1995; Pereira et al., 1998). Adipocyte and osteoblast differentiation pathways are closely associated (Gimble et al., 1996). Horse bone marrow cells can be induced to differentiate into chondroblasts by serial exposures to TGF-β1 and IGF-I. Pretreatment with 5 ng TGF-β1 increases proliferation of horse mesenchymal cells and IGF-I incorporated on a three-dimensional structure increased formation of procollagen type II mRNA in cell culture (Worster et al., 2001).

Prockop (Prockop, 1997) hypothesizes that bone marrow contains both mesenchymal stem cells and osteoblast precursors, which derive from a continual remodeling of an array of thin spicules of trabecular bone within marrow. Kuzenetsov and coworkers demonstrated that all implanted muti-colonies induce ectopic bone formation whereas 58.8% of single-colonies implanted in immunodeficient mice induced new bone formation. The study demonstrated heterogenous osteogenic capacities of CFU-F derived staines. Low osteogenic potential colonies require dexamethasone and ascorbic acid to induce their osteogenic differentiation (Kuznetsov et al., 1997).

Bone marrow enhances bone and cartilage regeneration. Autologous bone marrow injected into tibial nonunion stimulates callus formation resulting in an improvement of fracture healing (Connolly et al., 1991). Repairing cartilage defects can be enhanced by an implantation of cultured mesenchymal stem cells. A significant higher amount of new cartilage formation is found when cultivated rabbit bone marrow of rabbits is injected into full thickness defects of articular cartilage (Im et al., 2001).

1.2. Osteogenic differentiation of bone marrow *in vitro*

It is clearly demonstrated that marrow derived cells contain osteogenic mesenchymal stem cells which can be grown and expanded in culture. Cells appear to have at least bipotential for adipocyte and osteoblast differentiations. In the presence of dexamethasone, β-glycerophosphate, asocorbic acid and fetal bovine serum, mesenchymal stem cells in bone marrow differentiate into osteoblast-like cells and are able to maintain osteoblastic phenotypes in a cell culture system for more than a month (Friedenstein et al., 1987; Haynesworth et al., 1992b; Owen and Friedenstein, 1988; Toquet et al., 1999; Yoshikawa et al., 1997).

After initial plating, mesenchymal progenitor cells enter the proliferative phase characterized by minimal expression of terminal differentiation markers and synthesis of collagenous extracellular matrix. Once matrix synthesis begins, osteoblast marker genes are activated in a clear temporal sequence. Alkaline phosphatase (ALP) and the parathyroid hormone related protein receptor (PTHrP) are induced at early times while bone sialoprotein (BSP) and osteocalcin appears some time later (Wang et al., 1999). Osteoblast-associated markers, ALP and receptor to parathyroid hormone (PTH) are upregulated prior to a cessation of proliferation, except osteocalcin, which is upregulated only in the post-operative phase. A mineralized bone nodule represents the end osteogenic differentiation state of cells (Aubin, 1998).

1.2.1. Dexamethasone and osteogenic differentiation

A supplementation of dexamethasone in the culture medium of bone marrow cells is essential for osteogenic differentiation of mesenchymal stem cells in bone marrow (Gundle et al., 1995; Maniatopoulos et al., 1988). Maniatopoulos and coworkers (Maniatopoulos et al., 1988) reported that mineralized cell nodules with characteristics similar to bone are formed only in bone marrow cell culture in presences of β-glycerophosphate (10mM) and dexamethasone (10nM). These findings are supported by Gundle and coworkers (Gundle et al., 1995), that a presence of dexamethasone in culture medium is an absolute requirement for osteogenic differentiation of bone marrow cells. Bone marrow cells cultivated in the absence of dexamethasone (10^{-8} M) do not induce osteogenic formation in a diffusion chamber. Dexamethasone promotes osteogenic differentiation of mesenchymal stem cells and

osteoprogenitor cells in an early state of osteoblastic differentiation into osteoblast-like cells (Abe et al., 2000; Beresford et al., 1994). Supplementations of 10 nM dexamethasone and 50 μg/ml of ascorbic acid in culture medium of human bone marrow cells increase ALP activity of differentiated cells (Oreffo et al., 1999). Ascorbic acid promotes osteogenic differentiation of undifferentiated cells (stromal cell line ST2) and osteoblast precursor cells (MC3T3 mouse calvaria-derived cell line) by inducing a formation of type I collagen matrix, which is essential for the induction of osteoblast differentiation (Franceschi et al., 1994; Franceschi and Iyer, 1992; Otsuka et al., 1999).

1.2.2. 1α,25 dihydroxycholecalciferol (VD3) and osteogenic differentiation

VD3 promotes osteogenic differentiation of osteoblasts in an early state (Beresford et al., 1994). In cell culture of rat and mice bone marrow VD3 promotes osteblastic differentiation of stromal cells over adipocytic differentiation of these cells (Beresford et al., 1994; Gimble et al., 1996; Kelly and Gimble, 1998). Cells of osteoblastic lineage possess receptors for VD3. VD3 stimulates the production of several noncollageous proteins including osteopontin and osteocalcin (Suda et al., 1990). VD3 is an essential factor for osteocalcin expression (Rickard et al., 1994).

VD3 (10^{-10} – 10^{-7} M) increases mRNA expression and secretion of an insulin-like growth factor binding protein -2, -3 and -4 (IGFBP-2, -3, and -4) of human bone marrow stromal cells. The authors hypothesise that an increase in production of IGFBP-2, -3 and -4 may be an important mechanism mediating effects of calcitriol on bone marrow stromal cell differentiation (Kveiborg et al., 2001; Rickard et al., 1994). VD3 reverts *in vitro* age-related changes in osteoblast functions. VD3 (10^{-8} M) reduces proliferation and increases mRNA levels of alkaline phosphatase and osteocalcin of human osteoblastic cell lines (MK-7389) in early passage and reverse reductions of ALP and osteocalcin mRNA levels in late-passage of these cells (Kveiborg et al., 2000). The effects of VD3 can be enhanced by co-incubation with transforming growth factor-β1 (TGF-β1) (Liu et al., 1999).

1.2.3. BMP-2 and osteogenic differentiation in cell culture

BMP-2 is a potent stimulator of osteoblast differentiation and bone formation in human cells. BMP-2 induces ALP activity and production of parathyroid hormone (PTH) receptor of human bone, muscle and skin cells (Gori et al., 1999; Lecanda et al., 1997). BMP-2 inhibits myotube formation of human muscle cells (Kawasaki et al., 1998). Katagari et al., 1994 (Katagiri et al., 1994) report that BMP-2 induces osteogenic induction of myoblastic cells but a continuous presence of BMP-2 is required to maintain osteoblastic characteristics of these cells.

BMP-2 induces both osteoblastic and adipocytic differentiations of undifferentiated mesenchymal cells (Ji et al., 2000; Lecanda et al., 1997). An association between BMP-2 and up and down regulations of genes associated with osteoblast and adipocyte phenotypes are found (Ji et al., 2000). BMP-2 and dexamethasone induce osteogenic differentiation of muscle cells and adipose tissue, resulting in an increase in ALP activity of these cells (Lecoeur and Ouhayoun, 1997).

BMP-2 promotes expression of osteoblast phenotypes and most bone matrix proteins of immature osteoblasts and mature osteoblasts in human bone marrow stromal cells, where the expressions are sufficient to induce optimal matrix mineralization (Cassiede et al., 1996; Fromigue et al., 1998; Hanada et al., 1997). The effects are dose and exposure time dependent, at 25 – 100 ng/ml, and being evident after 1-7 days of treatment (Gori et al., 1999; Lecanda et al., 1997; Puleo, 1997). BMP-2 stimulates osteocalcin production in both human bone marrow stromal cells and osteoblasts. The stimulatory effect in an osteoblast is stronger than the effects on stromal cells. Expressions of BMP-2 and BMP-4 receptors are found in both human bone marrow stromal cells and human osteoblasts (Lecanda et al., 1997). BMP-2 induces maximum osteogenic differentiation when it is combined with dexamethasone (Rickard et al., 1994).

After the first week of a long term treatment with BMP-2 (50 ng/ml for 21 days) growth of bone marrow cells in the first passage is reduced but ALP activity is increased. At the beginning of the third week, mRNA expression and protein levels of osteocalcin and calcium content are increased. A transient treatment with BMP-2 (50 ng/ml for 1 to 7 days) decreases cell growth but increases ALP activity and

expressions of ALP, osteopontin and osteocalcin mRNA. Despite the cessation of the treatment for 2-3 weeks matrix mineralization is still markedly increased. A retention of the expressions of osteoblastic traits is exposure time dependent (Hicok et al., 1998). BMP-2 enhances expressions of BMP-3 and BMP-4 during mineralization phase of primary culture of fetal rat calvaria cells. The enhancement associates with expressions of bone cell differentiation markers. BMP-2 may act in a paracrine fashion in concert with other BMPs to induce and stimulate osteogenic induction (Chen et al., 1997).

1.3. Osteogenic differentiation of bone marrow *in vivo*

1.3.1. Bone marrow cell on a three-dimensional scaffold

Bone marrow cells are able to form bone *in vivo*. *In vivo* bone formation is a definite osteogenic differentiation marker of bone marrow cells. It can be tested by implanting bone marrow in a diffusion chamber or in a heterotopic site. Implantations of differentiated bone marrow or suspension of total bone marrow induces bone and cartilage formations in the diffusion chamber (Ashton et al., 1980; Bab et al., 1988; Friedenstein et al., 1970). Implanted fresh and differentiated bone marrow cells induce new bone formation when they are seeded on macroporous hydroxyapatite (HA), Tricalcium phosphate (TCP) ceramics, biphasic HA/TCP ceramic (Bruder et al., 1998c; Bruder et al., 1998b; Dennis et al., 1992; Goshima et al., 1991a; Goshima et al., 1991c; Kadiyala et al., 1997; Ohgushi et al., 1993), poly DL-lactic-co-glycolic acid foams (Ishaug-Riley et al., 1997), and fibrin gel (Yamagiwa et al., 2001).

It is postulated that mesenchymal stem cells in bone marrow can differentiate either into osteoblasts or chondroblasts and that hypoxia promotes cartilage formation (Ashton et al., 1980). Numbers and the osteogenic potential of marrow osteoprogenitor cells are age dependent. They decrease with age (Bab et al., 1988; Egrise et al., 1992; Tsuji et al., 1990). Cultured bone marrow contains more osteogenic mesenchymal cells or displayed higher osteogenic potential than fresh bone marrow (Goshima et al., 1991a).

The osteogenic capacity of the implants depends on the density of mesenchymal cells on the surface of the ceramic and the number of cell culture passages. Three cubic millilitres (3 mm^3) of ceramic must contain at least $3x10^4$ cells

or $1x10^4/mm^3$. The seeding cell concentration should be greater than $5x10^5$ cells/ml. The osteogenic capacity of cultured cell decreases with the number of passages (Dennis et al., 1992; Goshima et al., 1991a; Goshima et al., 1991b; Goshima et al., 1991c).

An osteogenic induction of cultivated rat bone marrow on poly DL-lactic-co-glycolic acid foams has been reported. Rat bone marrow cultivated in a mineralized culture medium supplemented with dexamehtasone in the first passage is seeded on polymer foams, $6.83x10^5$ cell/cm^2, and is further cultivated for 7 days. The constructs are implanted into a rat mesentery. As much as 11% of the volume of the foam is filled with new bone formation. The authors speculate that this not only comes from transplanted cells but also from cells in growth from adjacent bone induced bone formation (Ishaug-Riley et al., 1997).

1.3.2. Bone marrow cells in skeletal defect

Skeletal defects can be repaired by implanted bone marrow cells seeded on a three-dimensional scaffold. The ability of mesenchymal stem cells loaded on a hydroxyapatite ceramic to repaire critical-sized segmental defects in femurs of adult dogs (Bruder et al., 1998b) and adult athymic rats (Bruder et al., 1998c) has been reported. At 12 weeks post-implantation, Bruder and coworkers (Bruder et al., 1998c) demonstrated bone ingrowth in 45.8% of an available space of implanted hydroxyapatite/β-tricalcium phosphate ceramic loaded with human mesenchymal stem cells and in 30.3% of an available space of a control ceramic carrier without cell seeding in rat femur defects. Hydroxyapatite/beta-tricalcium phosphate ceramic loaded with dog autogenous mesenchymal stem cells were implanted in critical size segmental femoral defects of dogs. The reconstructs were filled with woven and lamella bone in a greater amount and rate than in a group of an implantation without cells. A larger collar bone around the implant and an integration of the callus from an implant with a callus from a host periosteum are found only in a group of implant with cell loading at 16 weeks post-implantation (Bruder et al., 1998b). Casabona and coworkers (Casabona et al., 1998) proposed a reconstruction of a preform-composite osteomyocutaneous flap. New bone formation is found in a similar shape to implanted porous hydroxyapatite ceramic scaffolds seeded with bone marrow cells.

The authors suggest that with a myocutaneous flap transferring technique, this construct can be transferred to skeletal defects for osteomyocutneous flap reconstruction.

1.3.3. Identifying the source of bone forming cells

The question arises whether newly formed bone is derived from host or donor cells. Bone formation on the surface of implants is a progressive process starting at 2 weeks and lamellar bone was found at 8 weeks after implantation. The authors hypothesize that implanted cells survive for 14 days after implantation and they play an important role in inducing bone formation during the first 3-4 weeks post-implantation and the bone remodelling process is taken over by host cells in the second stage of bone formation during 7-8 weeks post-implantation (Goshima et al., 1991b). Goshima and coworkers (Goshima et al., 1991b) aimed to identify bone forming cells. The authors implanted differentiated quail bone marrow stromal cells in nude mice and identified the location of quail cells after implantation by identifying a quail-specific nucleolar marker. Implanted cells are largely responsible for the observed osteogenesis during the three to four weeks after implantation but in the second phase, during 8-12 weeks, host cells act predominantly.

Using immuhistochemical staining with antibody directly against human osteonectin and in situ hybridization for human-specific *alu* sequences, demonstrated that new bone in subcutaneous transplanted hMSCs seeded on a HA/TCP and differentiated human bone marrow cells in a fibrin clot is formed by implanted cells of human origin without a replacement from host cells for at least 45 days and 28 days after transplantation, respectively (Kuznetsov et al., 1997; Yamagiwa et al., 2001). A role of transplanted cells as a source of bone forming cells in an ectopic site is further supported by Breitbart and coworkers (Breitbart et al., 1998) who reports that there was a contribution of implanted periosteal cells to a new bone formation during 4 – 12 weeks post-implantation. Bromodeoxyuridine cellular labelling of cultured periosteal cells loaded on a polyglycolic polymer was detected at 4 and 12 weeks after they were implanted in a 15 mm calvarial defect in a rabbit.

2. Models of the bone regeneration process

2.1. An overview

Bone regeneration repairs skeletal defects with bone tissue. The process involves the proliferation and differentiation of osteoprogenitor cells to osteoblasts or chondroblasts. Osteoblasts secrete a bone matrix and chondroblasts lay down a cartilaginous matrix to form a template which subsequently is replaced by a bone matrix. The events are regulated by interactions of multiple growth factors (Bolander, 1992; Frost, 1989; Yoo and Johnstone, 1998).

It is clearly demonstrated that there are osteoprogenitor cells in skeletal defects and heterotopic sites. There are two types of osteoprogenitor cells, determine osteoprogenitor (DOPC) and inducible osteoprogenitor cells (IOPC). Determined osteoprogenitor cells can differentiate into osteoblasts without osteoinductive protein. They reside in marrow, a cambium layer of periosteum, endosteum and adjacent host osteoblasts. These cells differentiate spontaneously to osteoblasts when transplanted heterotopically (Caplan, 1991; Triffitt, 1987; Yoo and Johnstone, 1998). Inducible osteoprogenitor cells are pluripotential mesenchymal stem cells residing in bone marrow and skeletal muscle (Hollinger, 1993; Yoo and Johnstone, 1998). These cells require an exogenous signal or osteoinductive protein to initiate their differentiation process. Osteogenic induction in skeletal muscle and subcutaneous tissue is a dramatic example of inducible osteoprogenitor cells in mesenchymal cells (Reddi, 1995). Pluripotential mesenchymal cells can be induced to differentiate into various cell types including: osteoblasts, chondroblasts, reticular cells, myoblasts, fibroblasts and adipocytes (Caplan and Bruder, 1966).

2.2. Bone regeneration process

The events that demonstrate existence of osteoprogenitor cells and represent bone regeneration processes are autogenous bone grafting, fracture healing, and heterotopic bone induction induced by a demineralized bone matrix (DBM) and bone morphogenetic proteins (BMPs). Cancellous bone graft induces bone regeneration through osteoconductive and osteoinductive processes. In the ostoconductive process, osteoblasts of host bone migrate into the graft. In the osteoinductive process,

inductive factors in the bone graft convert osteoprogenitor cells into osteoblasts. These two processes initiate a healing cascade starting with proliferation of osteoprogenitor cells, proliferation of mesenchymal stem cells and differentiation of these cells to osteoblasts and or chondroblasts to lay down a bone matrix or cartilaginous matrix, respectively. Then the bone matrix is mineralized and remodeled. The cartilagenous matrix is calcified and replaced by a bone matrix. In the last stage, hemopoietic marrow differentiates and unorganized woven bone trabeculae are replaced with organized lamellar bone. Osteoblasts and osteoclasts perform coupling functions of bone formation and resorption, which regulate bone remodeling process (Bolander, 1992; Hollinger, 1993; Lane et al., 1999; Yoo and Johnstone, 1998).

Osteogenic precursor cells can be induced to differentiate into osteoblasts or chondroblasts. When osteogenic precursor cells differentiate directly to osteoblasts and osteoblasts lay down a bone matrix, the process is called an intramembranous bone formation. When the precursor cells differentiate to chondroblasts and a bone matrix is laid down to replace the cartilaginous template, the process is called endochondral bone formation (Rabie et al., 1996; Solheim, 1998). The basic regulating factors of these two processes are differentiation pathways of osteoprogenitor cells into osteoblasts or chondroblasts.

Plasticity of differentiation pathways between chondrocytes and osteoblasts are suggested. Hypertrophic chondrocytes are able to down-regulate cartilage markers and up-regulate osteoblast markers (Aubin et al., 1995). Scammell and Roach (Scammell and Roach, 1996) proposed a differentiation plasticity between osteoblast and chondroblast lineages in mineralization of callus. The authors found that hypertrophic chondrocytes change phenotype and become bone-forming cells which secrete bone matrix to replace cartilaginous matrix. This bone is termed 'lacunar' bone, which possesses particular characteristics of being woven bone, containing acid phosphatase activity and having a strong antigenicity for bone sialoprotein. The authors suggest that direct replacement of cartilage callus by the formation of lacunar bone is a rapid mechanism and the process increases the strength of fracture callus.

Differentiation pathways of progenitor cells are influenced by osteoinductive substances and the hypoxic level in the bone regeneration area (Solheim, 1998). Various growth factors including TGF-β, fibroblast growth factors (FGF), IGF-I and -II, PDGF and BMPs, are secreted by mesenchymal and hematopoietic cells and stored in bone matrix (Baylink et al., 1993; Bostrom et al., 1999; Canalis et al., 1993).

Oxygen tension is another factor influencing differentiation and proliferation of osteoblasts. It is reported that the hypoxic level increases proliferation and decreases alkaline phosphatase (ALP) activity and collagen synthesis. High oxygen tension decreases cell proliferation and increases ALP activity and collagen synthesis of fetal rat calvaria osteoblast-like cells (Tuncay et al., 1994). These results are supported by the effects of hyperbaric oxygen on bone healing. Hyperbaric oxygen increases osteoid formation and accelerates union of autologous free bone grafts (Sawai et al., 1996). Hypoxia induces changes in the expression of molecules involving in osteoblastic differentiation and extracellular matrix formation such as the expression of IGF-II mRNA (Steinbrech et al., 2000b).

Shapiro and coworkers (Shapiro et al., 1982), report a correlation between concentrations of nicotininamide adenine dinucleotide (NADH) and NAD^+ to states of chondrocyte maturation and initiation of mineralization. The authors find an accumulation of NADH in hyperthrophic chondrocytes, where a tissue zone is hypoxic and mineralization is first seen. In proliferating cartilage, the concentration of NADH is decreased. High concentration of NADH suggests that mitochondria of hypertrophic condrocytes is in a resting state, in which there is loss of mitochondrial calcium (Ca^{2+}) and phosphate (Pi) ions initiating mineralization vesicles. These findings suggest that cell ion discharges and mineral deposition are associated with hypoxia related events.

2.2.1. Fracture healing

Fracture repair recapitulates a cascade of osseous repair involving proliferation and differentiation of osteoprogenitor cells and co-operations of growth factors (Bolander, 1992; Hollinger, 1993). Osteoprogenitor cells are DOPC and IOPC. DOPC reside in the cambial layer of the periosteum, endosteum and dura.

IOPC are pericytes in the endothelium of invading capillaries and nonspecific mesenchymal cells in skeletal muscle (Brighton et al., 1992; Owen, 1980).

Joyce and coworkers (Joyce et al., 1990b) clearly demonstrate features of cellular change in a femoral fracture in rats. An immediate response to injury within 48 hours is that hematoma and granulation tissue are formed and periosteum cells begin to proliferate. During day 3-7 of the fracture, intramembranous bone formation is found below proliferating periosteal cells situated near to the fracture site. On day 7, chondrogenesis is seen with chondrocytes and a cartilaginous matrix is found very close to the fracture gap in soft connective tissue. After 9-12 days, cartilagenous matrix is ossified. Extracellular matrixes surrounding hypertropic chondrocytes is calcified and invaded by blood vessels and osteoblasts. An ossification process progressively replaced all cartilage matrixes. The process is followed by bone remodeling to restore the normal structure.

This report agrees with Bolander (Bolander, 1992), who categorises fracture repair into 4 stages: immediate injury response, intramembranous ossification of the external callus, chondrogenesis of the internal callus, and infiltration of capillaries into a cartilage matrix and ossification of cartilaginous matrixes. Boyan and coworkers (Boyan et al., 1999) demonstrate that there are mesenchymal progenitor cells in fibrous tissues in fracture sites. They are multi-potent mesenchymal cells which can be induced to form bone and cartilage *in vivo*.

2.2.1.1. Growth factors and fracture healing

Fracture repair recapitulates the pathway of normal bone development. Complex interactions of local mediators cause migration, condensation, proliferation and differentiation of mesenchymal stem cells into bone forming cells (Bostrom, 1998; Bostrom and Asnis, 1998). It is now clear that numerous growth factors play roles in complex processes of bone regeneration. The involvement of growth factors in osteogenesis is demonstrated by expression of growth factors in fracture healing.

Expressions of IGFs, TGF-βs and BMPs are identified to verify roles of these proteins, in the bone healing process. Endothelial and mesechymal cells in granulation tissue of human normal fracture healing express IGF-II mRNA. In cartilage and bone formation stages, osteoblasts and non-hypertropic chondrocytes

express mRNAs of both IGF-I and -II. In the bone remodeling stage, some osteoclasts express IGF-II mRNA. These findings reflect a predominant role of IGF-II in human bone matrixes and roles of IGFs in local regulations of human fracture healing (Andrew et al., 1993b).

TGF-β1 mRNA expression varies with healing stages. The expression is high during chondrogenesis and endochondral ossification. TGF- β1 localized in hematoma as early as 24 hours after fracture can derive from platelets. In the latter stages of bone repair, chondrocytes and mesenchymal cells secrete TGF- β1. TGF-β1 is an important factor in the induction of chondrogenesis and osteogenesis (Bostrom and Asnis, 1998).

In an early state of intramembranous bone fracture healing, osteoblasts, osteoclasts and primitive mesenchymal cells within the fracture callus express BMP-2, BMP-4 and BMP-7. The intensity of staining is gradually decreased as bone healing progresses. The levels of expressions return to a baseline level when the mature lamellar bone is formed. These results suggest involvement of morphogenetic proteins in the process of intramembranous bone repair (Spector et al., 2001).

2.2.2. Osteogenic induction of demineralized bone matrix (DBM) and bone morphogenetic protein-2 (BMP-2)

2.2.2.1. Osteogenic induction of demineralized bone matrix (DBM)

Demineralized bone matrix (DBM) and bone morphogenetic proteins (BMPs) induce osteogenesis mostly through endochondral bone formation and in a minor extension through intramembranous bone formation (Kubler et al., 1998; Murata et al., 1998; Omura et al., 1998; Yoshida et al., 1998). The bone matrix contains osteoinductive and various growth factors. DBM induces heterotopic bone formation through endochondral bone formation. Mesenchymal stem cells differentiate to chondroblasts on day 5. On day 7-9 blood vessels invade the cartilagenous area, at the same time chondroblasts grow to be hypertrophic chondroblasts and the cartilaginous matrix is calcified. On day 10 osteoblasts lay down a bone matrix replacing the cartilaginous matrix. Bone ossicles are formed and bone marrow are developed (Reddi and Huggins, 1972).

Sources of DBM, intramembranous or endochondral bones, determine routes of the bone healing process. DBM prepared from endochondral bone induces bone healing through endochondral bone formation, whereas intramembranous DBM induced new bone formation through osteogenic ossification without cartilage formation. A combination of endochondral and intramembranous DBM induce new bone formation through an endochondral route. This phenomenon is also found when a mixture of autogenous endochondral and intramembranous bone are grafted in skeletal defects (Rabie et al., 1996).

In calvaria defect, DBM induces osteogenic differentiation of undifferentiated mesenchymal stem cells of dura and connective tissue covering the defect. Most of the mesenchymal cells are stimulated to proliferate and differentiate principally to osteoblasts and in a minor extension to chondroblasts. This study confirms that there is osteogenic induction protein in DBM (Wang and Glimcher, 1999). DBM induces higher amounts of cartilage formation in subcutaneous site than in a calvarial defect of rats. It is hypothesized that mesenchymal cells have specific receptors for osteoblast or chondroblast inductive proteins (Wang and Glimcher, 1999). The implantation of intramembranous DBM and a corticocancellous bone graft enhanced bone healing in a calvarial defect in rabbits when compared to an implantation of corticocancellous bone without DBM. The result suggests that osteogenic proteins in DBM enhance proliferation and differentiation of osteogenic stem cells. Bone formation progresses through the intramembranous process and cartilage formation is not found. A combination of DBM and cancellous bone induce greater amounts of new bone formation than the amount of bone formed by cancellous bone grafts without DBM (Rabie et al., 2000).

2.2.2.2. Osteogenic induction of bone morphogenetic protein – 2 (BMP-2)

BMPs are groups of proteins found in DBM. By molecular cloning, at least six related members of BMPs (BMP-2 through BMP-7) are identified. Based on the primary amino acid sequence homology they are a member of the TGF-β superfamily (Wozney, 1992). BMP-2 is able to induce osteogenic differentiation in both the determined and inducible osteoprogenitor cells. The osteogenic induction effect of

BMP-2 is much stronger than the effect of DBM. This may be because of a higher concentration of osteoinductive protein in BMPs (Reddi, 1995). BMP-2 induces osteogenic differentiation of mesenchymal cells in muscles and subcutaneous tissue, which principally leads to endochondral bone formation. Intramembranous bone formation is found in a minor extent (Kim et al., 1997; Kubler et al., 1998; Kusumoto et al., 1997; Murata et al., 1998; Yoshida et al., 1998). rhBMP-2 recruits mesenchymal stem cells and triggers their differentiation into chondrocytes (Kubler et al., 1998). Marden and coworkers (Marden et al., 1994) reported that a recombinant human BMP-2 (rhBMP-2) incorporated on an ICBM is able to completely repair a critical size calvarial defect in a rat. The defect was time and dose dependent. ICBM with BMP-2 has a stronger osteogenic induction than DBM.

A. Factors influencing osteogenic activity of BMP-2

(1) Oxygen tension

Differences in oxygen concentration and vascularization within the implanted carrier contribute to differences in osteoinduction processes induced by BMP-2. Rich vascularization into the structure of a denatured atelocollagen sponge (DCFD-AS) impregnated with BMP-2 promotes ectopic intramembranous bone formation of BMP-2 (Omura et al., 1998). The postulation is further supported by evidence of intramembranous bone formation found on the outer surface of the endochondral bone, which are induced by the intramuscular implantation of rhBMP-2 lyophilized on a collagen scaffold (Kubler et al., 1998).

(2) Implantation site

The amount of ectopic bone formation is influenced by sites of implantation (Okubo et al., 2000) and the amount of implanted BMP-2 (Kubler et al., 1998). Okubo and coworker *(Okubo et al., 2000)*, found that the highest amount of bone induction occurred when 5 μg BMP-2 was implanted intermuscularly compared with subcutaneous and intrafatty implantations, respectively. The result of Okubo and coworkers (Okubo et al., 2000) supports the previous report of Yoshida and coworkers (Yoshida et al., 1998), that the amount of osteoinduction can be related to partial pressure of oxygen or the blood supply in implantation sites. Yoshida and coworkers (Yoshida et al., 1998) found a higher ectopic bone formation when

rhBMP-2 in atelopeptide type I- collagen is implanted intramuscularly than subcutaneously.

The amount and rate of heterotopic bone formation induced by rhBMP-2 are dose dependent. The size of ossicles and the amount and rate of bone formation directly correlates to the amount of rhBMP-2 implanted intramuscularly in mice (Kubler et al., 1998). Six hundred micrograms of recombinant human osteogenic protein-I (rhOP-I) was incorporated into xenogenic bone mineral (BioOss) and implanted intramuscularly in minipigs in order to prefabricate a vascularized bone graft for a microvascular transplantation (Terheyden et al., 1999).

Recently, the delivering of BMP-2 into an implantation site was modified to achieve a gradual and continuous release of the protein in an implantation site and reduce the amount of implanted BMP-2. BMP-2 is delivered into an *in vivo* environment by using gene transfer technology. The mesenchymal progenitor cell line C3H/10T $_{1/2}$ transfected with an adenovirus-mediated human bone morphogenetic protein-2 gene is able to differentiate to express osteoblast phenotypes. The cells express ALP activity and induce heterotopic bone formation in nude mice (Liu et al., 1999).

2.2.3. Scaffold and osteogenic differentiation

Interaction between cells and extracellular matrix regulates growth and differentiation of osteoblasts (Sampath and Reddi, 1981; Sampath and Reddi, 1984). When pre-osteoblasts or osteoblasts in an early differentiation state contact basement membrane laminin, terminal osteoblastic differentiation is initiated (Vukicevic et al., 1990b; Vukicevic et al., 1990a). Type I collagenous matrix provides a suitable substratum for mesenchymal cells to attach to (Sampath and Reddi, 1981). Delicate inter-cellular collageneous fibers and crosslinking of collagen promote intramembranous bone formation rather than endochrondral osteogenesis induced by BMP-2 (Omura et al., 1998). Geometry of bone matrix, such as an extensive surface area of an inert collagenous bovine bone matrix (ICBM) is a crucial factor determining its suitability as a substrate for anchorage-dependent cells, such as osteoblasts, to proliferate and differentiate (Sampath and Reddi, 1984).

DBM provides both a scaffold for cell attachment and soluble osteoinductive protein (BMPs) producing chemotaxis, proliferation and differentiation in the matrix structure of matrix. Reconstituting BMPs on ICBM has a strong osteogenic activity (Reddi, 1995). Reconstitution of osteogenin, an osteogenic protein extract, on ICBM restored biologic activity of osteogenin. Implantations of osteogenin reconstituted on ICBM and DBM are able to completely regenerate critical size calvarial defect in primates. Osteogenicity of osteogenin on ICBM is stronger than DBM. A large amount of bone trabeculae and bone marrow are found as early as day 30 in a group of osteogenin and at day 90 in a group of DBM. Essential roles of ICBM as a collagenous matrix providing an optimal substratum for cell anchorage and regulation of osteogenic protein releasing are emphasized (Ripamonti, 1993).

A carrier has a significant role in controlling the osteogenic process by providing a scaffold for osteoblastic cells to attach, proliferate, differentiate, form matrix (Kuboki et al., 1998) and to deliver osteogenic proteins (Hotz, 1998). A stable three-dimensional structure of a carrier is necessary for shaping the size and features of new bone formation as required for each skeletal defect (Ishaug-Riley et al., 1997; Wald et al., 1993). A denatured collagen sponge has been reported as a biocompatible carrier with a three dimensional structure and a favorable collagen fiber, which is directly mineralized during a mineralization process of new bone formation *in vivo* (Omura et al., 1998).

It is postulated that the structure and nature of the carrier influences the bone induction process induced by implanted BMP-2 (Kuboki et al., 1998; Murata et al., 1998). Growth and differentiation of osteoblast is controlled by surface contact between osteogenic cells and the ceramic, as well as pore size and inter-pore structure of the ceramic. Osteoblastic cells require a large pore size ranging from 150-400 micron and a connected interpore structure, so cells can attach, differentiate and proliferate on a ceramic surface with a sufficient nutrient flow or blood supply from a vascular in growth (Goshima et al., 1991a; Kuboki et al., 1998; Ohgushi et al., 1993; Ohgushi et al., 1996).

The surface or texture of the biomaterial modified the growth and differentiation of cells. Sintered porous hydroxylapatite (SPHA) discs with concavities on their planar surfaces are able to induce bone formation in heterotopic

and orthototic critical-sized calvarial defects of adult baboons without exogenous bone morphogenetic protein. The authors propose that it was the geometry of the substratum which regulated intrinsic osteoinductivity of biomaterial (Ripamonti et al., 2001). Effects of TGF-β1 and BMP-2 are modified by the surface of biomaterials. A significant increase of ALP activity of fresh bone marrow and CFU-F (a stromal precursor) are found only when cultured on a titanium or pure titanium alloy, not on a hydroxyapatite surface (Kim et al., 1997). The highest cellular differentiation and mineralization is found, when rat osteoblastic cell line is cultured in TGF-β1 on a surface of sinter HA, compared with the results on the surfaces of as-received HA and calcined HA (Ong et al., 1999). These results demonstrate that osteoblastic cells respond differently to different surfaces of biomaterials.

The rate and mechanism of growth factors released from scaffold regulates the effects of growth factors. The differences in phamacokinetics between carriers and their physicochemical nature are expected to affect biological activity of the implanted BMP-2. The rate of BMP-2 released from the implant is highly dependent on both the nature of the carriers and the implantation time. Collagenous carrier retains the highest fraction of the implanted dose of BMP-2 and gradually releases BMP-2 into the implantation site, whereas some mineral-based carriers retained a fraction of implanted BMP-2 within the implants (Uludag et al., 1999). PDGF-BB and TGF-β1 are continuously released at a physiological level into the culture system from an ethylene-vinyl copolymer. The release rate of growth factors can be controlled by a modification of porosity and coating of the polymer (Kim and Valentini, 1997). A gradual and constant release of PDGF from chitosan sponge controls the growth and the differentiation of cultivated osteoblasts on the structure of chitosan sponge and the amount and rate of bone formation in periodontal defects. The rate and magnitude of PDGF released depends on the concentration of PDGF impregnated on chitosan sponge (Park et al., 2000b) and chemical composition of sponge (Park et al., 2000a). Chondroitin sulfate-chitosan sponge enhances proliferation of osteoblasts on the sponge and the amount of chondroitin sulfate composition on the sponge controls the release rate of impregnated PDGF (Park et al., 2000a).

The importance of extracellular matrix to growth and differentiation is also found in the differentiation of endothelial cells. Configurations of fibrin clots determined by polymerized pH and the presences of thrombin or dextran influence migration and morphogenesis of endothelial cells. Basic fibroblast growth factor (bFGF) and vascular endothelial growth factor (VEGF) stimulate cell proliferation, whereas three-dimensional structure of fibrin gel regulates capillary morphogenesis (Nehls and Herrmann, 1996).

Fibrin gel has several advantages as scaffolding. Three-dimensional fibrin gel structure can serve as a useful scaffold for tissue engineering with a controllable degradation rate, excellent seeding effects and good tissue development. Degraded fibrin gel produces non-toxic by-products and a fast cell attachment on the scaffold provides a spatially uniform distribution of seeded cells within gel structure. Fibrin gel may promote cell migration and proliferation and matrix synthesis through the release of growth factors such as PDGF and TGF-β1. Moreover, the gel structure is a semi-permeable membrane which separates cells from the environment in the culture medium (Ye et al., 2000). A composite of fibrin gel and non-woven polyglycolic acid (PGA) mesh is introduced as a new matrix for cell seeding and a vehicle for cell transplantation. It is stated that fibrin gel is a support for cell attachment and proliferation within and on the periphery of the construct. Additionally, cell growth and differentiation can be controlled by entrapped or accumulated growth factors within fibrin gel (Ameer et al., 2002).

3. Platelet-rich plasma (PRP)

3.1. PRP and bone repair in oral and maxillofacial surgery

Platelet-rich plasma (PRP) is a plasma containing high concentration of platelets of at least 4 times higher than its concentration in whole blood (Marx et al., 1998). PRP has been used locally as a source of growth factors to accelerate wound healing in cosmetic (Man et al., 2001) and periodontal surgery (Petrungaro, 2001) and promotes bone regeneration (Greenhalgh, 1996; Hollinger and Kleinschmidt, 1990). Local application of PRP increases the amount and rate of bone formation in mandibular bone grafting (Maniatopoulos et al., 1988), sinus graft (Kassolis et al.,

2000; Lozada et al., 2001; Rosenberg and Torosian, 2000), alveolar ridge augmentation (Shanaman et al., 2001) and dental implantation (Anitua, 1999).

The application of PRP in enhancing osteogenesis in oral and maxillofacial surgery was introduced by Marx et al., 1998. The authors demonstrate that implantation of PRP increases radiographic density and the amount of bone formation of autogenous bone graft with PRP. Radiographic density of cancellous bone with PRP is 1.62 - 2.16 times to density of bone graft without PRP. The amount of bone formation is 74.0 % ± 11% in the PRP group and 55.1% ± 8% in the bone graft without PRP group. The concentration of platelets in PRP is 300% - 400 % higher than the concentration of platelets in whole blood. Expressions of TGF-β1 and PDGF on surface of platelets and the presence of their receptors on cancellous bone are demonstrated. The authors postulate that secreting growth factors from platelets stimulates the proliferation and osteogenic differentiation of mesenchymal stem cells and pre-osteoblasts in the endosteum of implanted cancellous bone. Moreover, the gel-like form of PRP enhances graft handling during surgical procedures and fibronectin in fibrin clot promotes adhesion and cell migration. The authors support a use of PRP as an autogenous product to enhance bone healing in bone grafting.

A hypothesis that degranulated platelets release multiple growth factors is supported by reports of Kronemann and coworkers (Kronemann et al., 1999) and Slater and coworkers (Kronemann et al., 1999; Slater et al., 1995). Slater and coworkers (Slater et al., 1995) demonstrated that human platelet concentrate increases the proliferation of human fetal osteoblast-like cells in two-dimensional cell culture. The authors suggest that released growth factors, PDGF, TGF-β1 and IGF-1, have synergistic effects on the proliferation of osteoblast-like cells. This study supports an idea that exogenous platelet growth factors are potential regulators of osteoblast proliferation and differentiation in bone grafting and fracture repair. Kronemann and corworkers (Kronemann et al., 1999) reports that activated platelets release products that increase the expression of the vascular endothelial growth factor mRNA (VEGF mRNA) and secretion of VEGF in smooth muscle cell culture. VEGF is a mitogen of endothelial and chemo-attractant *in vivo*. Gunsilius and coworkers (Gunsilius et al., 2000) report that platelet is a major source of soluble vascular endothelial growth factors in serum. It has been found that level of VEGF is higher in serum than in

plasma and the concentration of this growth factor is significantly correlated with the concentration of platelets in whole blood.

Carlson (Carlson, 2000) supports an application of PRP in bone grafting. Supplementing PRP in bone grafting is one way to locally apply growth factors into the bone graft to enhance bone regeneration. The author suggests that PRP has several advantages over BMP-2, such as PRP releases multiple growth factors and it is an autogenous product. These released growth factors create interactive osteogenic effects.

A concern over the safety of using bovine thrombin to initiate clots has been raised. It is mentioned that bovine thrombin interacts with human clotting factor V and produces postoperative coagulopathies. Landesberg and coworkers (Landesberg et al., 2000) suggest using ITA gelling agent instead of bovine thrombin. The authors report a similar morphology and nature of clots and the levels of secreted growth factors, PDGF-AB and TGF-β1, derived from PRP activated by ITA gel and bovine thrombin. Marx (in the discussion of (Landesberg et al., 2000)) disagrees with Landesberg and coworkers (Landesberg et al., 2000) because the evidence of interaction of bovine thrombin with human thrombin is low.

3.2. Clinical application of PRP

This review focuses on the application of PRP in bone grafting. Since a report of Marx and coworkers (Marx et al., 1998) using PRP in repairing bony defects has been widely adopted in dental and oral and maxillofacial surgery practices. PRP is applied in alveolar, periodontal and maxillary sinus defects, which are mostly related to dental implant placement. Anuita (Anitua, 1999) reports that PRP accelerates bone formation in alvelolar bone defect. A mixture of PRP and cancellous bone was used to fill extraction sockets in groups of 10 patients with mean age of 41 years (range 35-55 years). Twenty milliliters of blood was drawn from each patient then PRP was prepared using buffy coat technique. Between 10 – 16 weeks, biopsies of bone were obtained for histological examination. More mature bone with better organized trabecular and greater bone regeneration was found in the group with cancellous bone and PRP implantation. The authors hypothesize that reinforcing growth factors released from applied PRP improves bone regeneration.

PRR was used to promote bone healing combined with allogenic material with and without guide bone tissue regeneration (GBR). Kassolis and coworkers (Kassolis et al., 2000) report 89% clinically successful results of 36 dental implants inserted in augmented maxillary sinus and alveolar ridge. The sinus and alveolar ridges are augmented with a mixture of freeze- dried bone allograft (FDBA) and PRP. The defects are covered with GBR. PRP prepared by platelet pheresis has between a threefold and tenfold increase in platelet concentration compared with the concentration in whole blood. At 5-6 months after augmentation, histological specimens confirm the presence of vital bone formation near to the residual FDBA particles. The author states that using PRP may accelerate bone healing process and allow an earlier implant placement and loading, however, there is no control group in this study. The gel like form of PRP facilitates graft material handling.

Shanaman and coworkers (Shanaman et al., 2001) report primary results of three patients in which PRP was used in conjuction with demineralized freeze-dried bone allograft (DFDBA) and GBR to augment alveolar ridge for implant placement. Histology demonstrates sufficient new bone formation and the usual bone formation process, which are similar to an expected histology of implanted DFDBA with GBR. Adding PRP does not enhance the quality and quantity with new bone formation compared with new bone formation in guided bone regeneration studies without PRP.

A combination of PRP and bovine porous bone mineral (BPBM) with and without guide tissue regeneration (GTR) was used to repair interproximal bony defects in 21 patients with severe periodontitis. It was found that a mixture of PRP and BPBM supported new bone generation by reducing probing depth and increasing the attachment level. A difference between groups with and without GTR was not found. GTR added no clinical advantage to the implantation of a combination of PRP and BPBM in this skeletal repair (Lekovic et al., 2002).

Bilateral maxillary sinuses of three patients were augmented with a combination of PRP and BiOSS on one side and BiOSS only on the other side. The defects on both sides were covered with GTR. Similar to other reports of repairing skeletal defects with a combination of PRP and allogenic bone graft, Froum and coworkers (Froum et al., 2002) found that the addition of PRP to anorganic bovine

bone (BiOSS) did not enhance new bone formation. Fibrin formation consolidates and improves handling of BiOSS graft.

The first study conducted in an animal model was reported. Total of 30 Avana dental implants were placed in the iliac crests of 10 dogs. The study compared the progress of new bone formation and osteointegration in three study groups: control group with no treatment; grafting with particulate dentin-plaster of Paris; and grafting with a mixture of particulate dentin-plaster of Paris and PRP. Bone-implant contact in the group of PRP and dentin-plaster of Paris was significantly higher than the contact area in the other two groups. It suggested that PRP in a combination with particulate dentin-plaster of Paris enhances the rate and amount of new bone formation (Kim et al., 2002).

3.2.1. Comments on the clinical application of PRP in bone regeneration

It can be seen that most of reports are clinical reports of small patient numbers with descriptive histology of new bone formation. They give the impression that a combination of PRP with cancellous bone obviously improves bone healing, but a combination of PRP with allogenic bone graft poses no adverse effects. The advantage of PRP over the conventional method of GBR is not clearly demonstrated. There is only one report, from Kim and coworkers (Kim et al., 2002) which clearly demonstrates the effects of PRP in an animal study with statistical analysis. Therefore, much more scientific study is required to verify the effects and advantages of PRP in promoting bone regeneration.

3.3. PRP preparation methods and properties of PRP

The previous section clearly demonstrates that PRP has been used as a source of local growth factors to promote bone healing. It is expected that activated platelets in PRP will secrete high concentrations of bone forming growth factors, PDGF, TGF-β1, and IGF-1 into grafting sites. Preparation methods of PRP are varied and characteristics of PRP are not well defined.

PRP preparation methods can be categorized into three types, apheresis platelets (AP); buffy coat-derived platelets (BCP); and the tube method (TP).

Apheresis platelets are prepared by using a blood cell separator, in which platelets are separated by centrifuge force in a closed system. Red blood cells and other blood components are returned to the patient's blood circulation. BCP is obtained from a second centriguation of buffy coat obtained from the middle layer between the layers of platelet-poor plasma and red blood cells from the first centrifugation. TP prepares platelet concentrate by performing serial centrifugation of whole blood to separate plasma from red blood cells, then plasma is centrifuged to concentrate platelets and create platelet-poor plasama (Zimmermann et al., 2001).

There are also commercial devices using the general principle of centrifugation instead of autotransfusion to produce PRP. They can produce 5 – 6 ml PRP from 45 - 60 ml of whole blood (Weibrich et al., 2001; Weibrich et al., 2002a). O'Neil and coworkers (O'Neill et al., 2001) compared isolations of autologous platelet-rich plasma using Haemonetics cell saver 5 and Haemonetics MCS+ to prepare platelet gel. Haemonetics cell saver 5 uses centrifugation technology with different centrifugation forces and spinning time to separate platelet concentrate from whole blood. Haemonetics MSC+ use principle of apheresis platelets to prepare platelet concentrate and return red blood cells to the patient's circulation. Larger volumes of PRP and higher concentrations of platelets can be collected from Haemonetics MSC+. The authors state that the volume of PRP and concentration of platelet required during surgery determined method of PRP preparation (O'Neill et al., 2001).

Weibrich and coworkers (Weibrich et al., 2001) investigated correlations between platelet concentration in PRP and extraction methods, age, sex and platelet count in whole blood. The authors found that platelet separation by discontinuous plasmapheresis method (the methods used by blood banks) yielded a higher platelet concentration than from `buffy coat´ method. Platelet concentration from apheresis technique was significantly correlated with platelet concentration in whole blood, but the correlation is not found in platelets separated by buffy coat method. A significant correlation is found between sex and platelet concentration (Weibrich et al., 2001).

Properties of platelets are influenced by PRP preparation methods. Platelet aggregation in PRP prepared from buffy coat technique is increased and red blood cell contamination is high in platelets prepared by tube centrifugation technique (Racz and

Thek, 1984). Landesberg and coworkers (Landesberg et al., 2000) reported on the influence of centrifugation force on platelet concentration obtained from tube centrifugation technique. Effects of anticoagulant agents to the integrity of platelet plasma membrane are mentioned. The authors suggest that citrate should be used instead of Ethylenediaminetetra-acetic acid (EDTA) as an anticoagulant to prevent platelet damage and aggregation during platelet concentrate preparation.

Zimmermann and coworkers (Zimmermann et al., 2001) found different concentrations of platelet and white blood cell contamination obtained from different preparation methods. The lowest platelet concentration and the highest white blood cell contamination were found in the TP group compared with AP and BCP groups. However, concentrations of PDGF-AB, PDGF-BB and TGF-β1 were comparable in these three groups. The levels of these growth factors were decreased when the platelets were stored for 5 days under continuous mild agitation at 22 °C. The authors conclude that concentrations of growth factors released from platelets are influenced by platelet concentration, lactate production and WBC contamination.

Different preparation methods yield different platelet concentrations, WBC contamination and *in vitro* platelet activation. These factors should be taken into account when planning and to use PRP. Variations in platelet concentration obtained from different preparation methods and concentration of growth factors, PDGF-AB and TGF-β1 are demonstrated. However, a correlation between concentrations of platelets and the amount of growth factor released was not found. The authors commented that WBC contamination in platelet concentrate may contribute to the level of growth factors released from PRP and the precise prediction parameter of growth factors cannot yet be established (Weibrich et al., 2002b; Weibrich et al., 2002a).

4. Growth factors released from platelets

4.1. Platelet derived growth factor (PDGF)

Platelet derived growth factor (PDGF) is encoded in two forms, PDGF-A and PDGF-B, which have approximately 56% sequence homology. They are independently regulated by different genes located on chromosomes 22 and 7. Polypeptide chains A and B form three disulphide-linked dimmer AA, BB and AB.

PDGF-AA induce its signal via cell surface receptor αα, PDGF-BB via cell surface receptor ββ, and PDGF AB via cell surface receptor αβ-receptors. *In vitro*, PDGFs induce proliferation, chemotaxis, and matrix secretion of osteoblasts and chondrocytes. They also stimulate endothelial cell proliferation and angiogenesis. PDGF-BB has the most potent effects on osteoblasts, followed by PDGF-AB and PDGF-AA, respectively (quoted in (Horner et al., 1996)). PDGF-BB secreted by osteoclasts inhibits differentiation of mouse osteoblast precursor-like cell line MC3T3-E1. This evidence demonstrates a direct regulation of osteoclasts to osteoblasts and suggests a key role of PDGF-BB in bone remodeling (Kubota et al., 2002).

Involvement of PDGF in bone formation and remodeling is demonstrated by expressions of PDGF genes and proteins in ectopic bone and during normal human fracture repair. Andrew and coworker (Andrew et al., 1995) described an expression of PDGF-AA mRNA in early and late states of bone healing in mesenchymal cells, endothelial, chondrocytes, osteoblasts and osteoclasts. Mature chondrocytes strongly express PDGF-AA. The expression is also found in small blood vessels, but the αα-receptor is found only on hypertrophic chondrocytes and invading blood vessels. Widespread expression of PDGF-AA and its receptors in forming human bone suggests that PDGF-AA may exert autocrine and paracrine effects to regulate osteogenesis during skeletal development. Expression of PDGF-BB mRNA was more restricted to osteoblast during the stage of bone formation. (Andrew et al., 1995). PDGF-AA mRNA and protein are widely distributed throughout heterotopic and osteophytic bone (Horner et al., 1996).

Hock and Canalis (Hock and Canalis, 1994) report that exposure to PDGF-AA and –BB (1-100 ng/ml) increases DNA synthesis in fetal rat calvarial organ culture, but the effect on collagen synthesis is small and inconsistent. There is no evidence to demonstrate that proliferated cells differentiate to osteoblasts. PDGF-BB is more potent than PDGF-AA. Yu and coworkers (Yu et al., 1997) studied the effects of PDGF-BB (20 ng/ml) on osteogenic differentiation and proliferation in long term mineralized cell cultures of fetal rat calvarial osteoblasts. It was found that prolonged exposure to PDGF-BB increased cell proliferation but inhibited osteoblastic differentiation. ALP activity, type I collagen synthesis, osteocalcin expression and

bone nodule formation were decreased. The ability to stimulate proliferation of PDGF is decreased when cells reach maturity (Yu et al., 1997).

Mitogenic effect of PDGFs on osteoblast-like cells is further demonstrated by a report of Hsieh and Graves (Hsieh and Graves, 1998). In fetal rat calvarial cell culture, PDGF-BB had a positive effect through increasing cell proliferation and a negative effect on inhibiting osteoblastic differentiation. Continuous treatment with 20 ng/ml PDGF-BB stimulates DNA synthesis and cell replication during 0 – 21 culture-days, but decreased mineralization of bone nodule in 5 weeks of continuous culture. This indicates that prolonged exposure to PDGF-BB inhibits osteoblast differentiation. In a brief exposure (for 24 hours), cell proliferation is increased and the mineralized nodule area is 50% increased. The effects are dose and incubation time dependent. It is postulated that a brief exposure may enhance bone formation *in vivo* (Hsieh and Graves, 1998). PDGF–BB enhances mineralization of bone matrix by increasing the transportation of inorganic phosphate (Pi) into osteoblastic cells. This Pi transportation determines the level of intracellular Pi concentration and it is important for the calcification of bone matrix (Zhen et al., 1997).

In 24 hours organ culture of fetal rat calvarial, PDGF (30 – 100 ng/ml) stimulates DNA synthesis but has no effect on collagen and osteocalcin synthesizes (Canalis and Lian, 1988). When 4 μg of PDGF was continuously administered by osmotic pump into implanted DBM in gluteal muscle of rats over two weeks, it was found that the calcium deposition in new bone formation of DBM with PDGF was higher than the calcium content in a group of DBM without PDGF. The authors suggest that PDGF promotes bone formation (Risto et al., 1991). When a bolus of 50 ng PDGF was mixed with 25 mg of DBM powder and collagen and implanted subcutaneously, it was found that PDGF increases expression of type II collagen mRNA, ALP activity and calcium content of the implant in mature rats (250-350 g). The effect was not seen in young rats (70-100g). It is suggested that in a sub-optimal condition, bone formation can be enhanced by PDGF (Howes et al., 1988). PDGF was found to enhance bone formation in the critical size calvaria defect of rabbit. PDGF-BB (50 μg/ml) was mixed with 4.4% methylcellulose gel and implanted in 15 mm full thickness calvarial defect in eight rabbits. Eight defects in 8 rabbits were kept as control group without PDGF. The defects were covered with expanded

polytetrafluoroenthylene membranes (e-PTFE) to protect the periosteum from the defect site. After four weeks, the defects were not completely filled. The amount of bone infiltration in groups with PDGF-BB was higher, but less mature than new bone formed in the control group. The authors hypothesize that PDGF-BB chemotaxises and induces proliferation of mature osteoblasts from the peripheral border of the defect leading to new bone formation. The results demonstrate that a single dose of rhPDGF-BB stimulates bone formation in critical size defect (Vikjaer et al., 1997).

In order to eliminate a limitation from a short half-life of PDGF *in vivo* (less than 4 hours) resulting in difficulty in maintaining therapeutic concentration from a single dose injection, a carrier system was developed to maintain PDGF-BB at the therapeutic concentration. Park and coworkers (Park et al., 2000a) demonstrated that PDGF-BB loaded on chondroitin sulphate chitosan sponge was continuously released in a constant level. The release rate and concentration are controlled by the amount of chondroitin sulfate or the amount of PDGF loaded on each sponge. PDGF released from chondroitin sulphate chitosan sponge increased proliferation of pre-osteoblasts derived from rat calvarial cell culture over a period of 14 culture-days. The mitogenic effect of released PDGF was tested *in vitro* and *in vivo*. Stephan and coworkers (Stephan et al., 2000) studied the release and adsorption of PDGF-BB from a mineral-collagen matrix. It was found that PDGF-BB was adsorbed from the collagen matrix in a dose dependent manner and 30% of the growth factor was slowly released over 10 days. PDGF-BB (2.5 μM) impregnated on a mineral-collagen matrix increases the attachment and proliferation of osteoblast-like cells deriving from neonatal rat calvaria on the matrix compared with attachment and proliferation of cells on the matrix without growth factors.

Aiming to deliver a constant therapeutic dose of PDGF-BB and solve the short half-life (4 hours) problem of PDGF *in vivo*, 100, 200 and 400 μg PDGF-BB was incorporated into the chitosan sponge by soaking to allow a continuous release of PDGF-BB over 28 days. It was found that chitosan sponge incorporated with PDGF-BB enhances the attachment and proliferation of rat calvarial osteoblastic cells on the structure of the sponge. *In vivo*, PDGF-chitosan sponge increases new bone formation in calvarial defect of rats compared with bone formation in groups of chitosan sponge without PDGF, although the 8 mm in diameter defects are not

completely filled. Bone grows from the periphery to centripetally. The results suggest that incorporation of PDGF on chitosan sponge is an efficient delivery system and the system is biocompatible with osteoblastic cell growth. This system may be able to apply to deliver growth factors into periodontal defects (Park et al., 2000b). Giannobile and coworker (Giannobile et al., 1997) tried to improve growth factor delivery into periodontal defect by using gene technology. Ad2/PDGF-A gene was transfered to root lining cells (cloned cementoblast) to tranduce and modulate the activity of cementoblasts. The transduced gene was able to enhance cementoblast DNA synthesis and cementoblast proliferation at levels similar to or greater than a continuous supplementation of PDGF-AA. This study suggests that gene therapy can be a mode of growth factor delivery in periodontal tissue engineering.

4.2. Insulin like growth factors (IGF)

IGF-I promotes osteoblastic differentiation and proliferation of rat calvaria pre-osteoblasts. A continuous incubation of rat calvarial pre-osteoblasts in 0.2 – 5.0 nM IGF-I for 6 days markedly increases ALP activity with a slight increase in cell numbers. The result contrasts sharply with effect of crude PDGF, which markedly decreases ALP activity and increases cell numbers (Schmid et al., 1984). It is found that IGF-I (0.4-35 nM) increases proliferation of newborn calvarial rat cells cultivated in three-dimension cell culture. In this study, viscous culture medium containing 0.8% methylcellulose creates three-dimensional extracellular matrix for osteoblast-like cells. These cells express high level of ALP activity and response to PTH in serum-free culture medium (Ernst and Froesch, 1987).

In osteoblast cell culture, IGF-I functions as a local regulator. Fetal rat parietal bone cells secrete IGF-I in cell culture. IGF-I derived from a culture medium of these cells at 20 nM stimulates DNA and collagen synthesis by 42% and 26%, respectively in monolayer cultures of osteoblast from rat parietal bone cells (Canalis et al., 1988). IGF-I regulates proliferation of both osteoprogenitor cells and differentiated osteoblasts. In organ culture of fetal rat calvarial, after 24 hours exposure to 1-100 nM IGF-I, the proliferation of cells in osteoprogenitor, osteoblast and periosteal fibroblast zones is increased. An increase in cell numbers results in a significant increase in bone matrix apposition. However, there is no evidence to

support that their matrix apposition progresses to be mineralized bone matrix (Hock et al., 1988). An involvement of IGF-I in bone formation is demonstrated by expression of IGF-I during bone fracture healing. Expression of IGF-I mRNA is found throughout the period of bone healing. The peak of expression is found at day 7 and the level of expression decreases to 2.3 times higher than the level of expression in control mandible expression at day 9. Low levels of expression are found at day 5, 23 and 37 (Steinbrech et al., 1999).

4.3. Transforming growth factor-βs (TGF-βs)

TGF-β1 is a member of TGF-β superfamily comprising more than 30 distinct members. They are grouped in several sub-families including TGF-β, activin and bone morphogenetic proteins. The superfamily members regulate proliferation, differentiation, migration and apoptosis of many cell types (Dijke et al., 1996). The activities of TGF-β are diverse depending on cell types, differentiation states and assay conditions (Alevizopoulos and Mermod, 1997; Kassem et al., 2000). The TGF-β family consists of at least five genes (TGF-β 1 – 5) encoding distinct proteins in vertebrates. They are 25 kDa disulfide-linked dimers with more than 70% homology of amino acid sequences. TGF-βs exert their biologic effects by binding heteromeric complexes of type I and type II serine/threonine kinases receptors at the surface of targets cells (Alevizopoulos and Mermod, 1997).

TGF-β1 plays a vital role in bone remodeling and metabolism. It is produced by both bone marrow mesenchymal cells and osteoblasts and is deposited in high concentration in extracellular matrix (Centrella et al., 1994). TGF-β1 promotes the proliferation and formation of new woven bone *in vitro* (Noda and Camilliere, 1989). In cell culture, the effects of TGF-β1 are varied depending on culture condition, differentiation state and source of cells (Kassem et al., 2000).

The richest source of TGF-β1 *in vivo* is platelets. Human and murine platelets contain TGF-β1 only, but porcine platelets contain TGF-β1 and TGF-β2. TGF-βs are secreted from platelets and are found in a latent form in a crude extract of cells and tissues. TGF-βs in a latent form bind their molecules with binding proteins of 400 kDa, preventing TGF-βs binding to the receptors. The latent form can be activated by

treatment with strong acid and base, urea and heat *in vitro* (Nakamura et al., 1986; Okada et al., 1989).

4.3.1. TGF-β1 in rat calvaria organ culture

In organ culture of fetal rat calvaria incubation in 0.1-10 ng/ml TGF-β1 for 24 – 96 hours increases the rate of DNA synthesis. Type I collagen synthesis is inhibited in a long incubation of 96 hours in TGF-β1, but the synthesis is increased in a short incubation of 24 hours. The results suggest that TGF-β1 stimulate bone cell replication but has no direct effect on type I collagen synthesis (Centrella et al., 1986). TGF-β1 plays a role in bone remodeling by inhibiting the osteoclast function consequently reducing the bone resorption rate. In an organ culture of neonatal rat femur, TGF-β1 decreases bone resorption and inhibits bone resorption activated by interleukin 1 (IL1) and VD3. Inhibition may be a result of the inhibiting effect of TGF-β1 on proliferation of osteoclast precursor cells (Pfeilschifter et al., 1990).

4.3.2. TGF-β1 and bone marrow cells

It is found that TGF-β1 inhibits the mitogenic effect of 15 ng/ml PDGF which increases DNA synthesis of human bone marrow fibroblasts. An inhibition effect of TGF-β1 is dose dependent (Bryckaert et al., 1988). An incubation in 5 ng/ml TGF-β1 for 48 hours increases cell proliferation but decreases ALP activity but these differentiated cells are able to induce bone formation *in vivo* (Cassiede et al., 1996). A recombinant human TGF-β1 fusion protein with collagen binding domain promotes migration, growth and differentiation of bone marrow mesechymal cells. TGF-β1 fusion protein binding with type I collagen matrix is able to stimulate differentiation of rat bone marrow cells (Andrades et al., 1999). To control a gradual and continuous release of TGF-β1, TGF-β1 is incorporated into microparticles of poly-DL-lactide-co-glycolic acid (PLGA) and poly-ethylene glycol (PEG) microparticles. TGF-β1 is released in biphasic pattern. An initial burst of release is found during the first 24 hours followed by a linear steady release for the rest of 28 day course. Primary bone marrow cells at culture-day 10 responds to TGF-β1 in a dose dependent manner. After 3 days incubation in 5 – 10 ng/ml TGF-β1, ALP activity is inhibited, whereas

incubation in 1 ng/ml TGF-β1 markedly increases ALP. A continuous release of TGF-β1 at 1 ng/ml over a period of 21 days of culture enhances proliferation and osteoblastic differentiation of bone marrow cells. The total cell numbers, ALP activity and osteocalcin production are increased. This study demonstrates the inhibitory effect of TGF-β1 in a high dose (Lu et al., 2001).

The influence of the differentiation state of cells to the effects of TGF-β1 on proliferation and osteoblastic differentiation is reported. The study is conducted in cell culture of trabecular bone osteoblasts (hOB) and human mesenchymal cells (hMSC) from bone marrow representing mature and immature osteoblasts, respectively. Both types of cells express TGF-β1 and TGF-β1 receptor mRNAs. TGF-β1 (0.1 – 10 ng/ml) increases proliferation of both hMSC and hOB. ALP activity of hOB is increased in to a higher level than when in hMSC. TGF-β1 decreases production of the type I collagen of hMSC. A combining treatment of calcitriol and TGF-β1 markedly increases ALP activity and type I collagen production but inhibits osteocalcin production in hOB. These results demonstrate the determining effects of the differentiation state on the effects of TGF-β1. TGF-β1 increases proliferation of cells in early and intermediate osteoblastic differentiation states and increases cell response to calcitriol, but TGF-β1 inhibits the activating effect of calcitriol in osteocalcin production. A consequence of combining treatment with calcitriol is an increase in expressions of type I collagen and ALP activity (Kassem et al., 2000).

Kveiborg and coworkers (Kveiborg et al., 2001) find that an incubation of human bone marrow cells in TGF-β1 (0.1-10 ng/ml) for 12-72 hours results in an increase of IGF-I and IGFBP-3 mRNA expressions in osteoblast precursor cells. Referring to Kassem and coworkers (Kassem et al., 2 000), IGF-I and IGFBP-3 increase proliferation in similar levels to those observed in TGF-β1 treatment. The authors propose that TGF-β1 increases cell proliferation through an activation of the IGF-system (Kveiborg et al., 2001).

4.3.3. TGF-β1 and rat calvarial cells or pre-osteoblasts

Cell density and dose of TGF-β1 determine effects of TGF-β1 on growth and a synthesis of type I collagen in primary cell cultures of rat parietal bone. It is found that TGF-β1, 0.15-15 ng/ml, decreases DNA synthesis in low cell density but increases the synthesis in confluent cells. TGF-β1 in a dose dependent manner increases type I collagen synthesis and the effect is stronger in low proliferating cells. At high concentrations of 15 ng/ml, TGF-β1 increased type I collagen synthesis in all cell density. It is suggested that TGF-β1 can enhance osteoblastic differentiation of non-proliferating cells (Centrella et al., 1987). An influence of cell maturation to the effects of TGF-β1 is supported by Breen and coworkers (Breen et al., 1994). The authors stated that the effects of TGF-β1 on growth and differentiation are dependent on the state of the cell maturation and exposure time. TGF-β1 exerts an irreversible inhibiting effect on osteoblast differentiation when it is applied in a culture medium of fetal rat calvarial cells in the proliferative phase. A continuous incubation in TGF-β1 from 0.1 ng/ml leads to a biphasic growth response where cell proliferation is increased in a more mature state. The authors postulated that changes in the component of the extracellular matrix and gene expression regulate the inhibitory effect of TGF-β1 (Breen et al., 1994).

Prolonged exposure to TGF-β1 inhibits osteogenic differentiation and mineralization of matrixes in fetal rat cavarial cell cultures. TGF-β1 exerts an inhibitory effect on pre-mature osteoblasts. A removal of TGF-β1 restores differentiation function of cells. It is clearly demonstrated that TGF-β1 (2 ng/ml) inhibited bone nodule formation and impaired expression levels of bone formation related genes, type I collagen, ALP, osteopontin, osteocalcin and BMP-2, when applied in confluent cell cultures of fetal rat calvaria cells in first passage. The authors postulate that inhibition of BMP-2 mRNA expression might be a cause of osteoblastic differentiation inhibition of TGF-β1 in this culture system (Harris et al., 1994).

4.3.4. TGF-β1 *in vivo*

Noda and Camilliere (Noda and Camilliere, 1989) first reported that subperiosteal injection of platelet-derived TGF-β1 induces woven bone formation. Woven bone with rich vascularization is formed locally around an injection site when 50 ng - 1 μg in 20 μl TGF-β is subperiosteally injected into the parietal bone of a neonatal rat. It is found that injections of at least 5 days are required to induce bone formation and the effect is reversible. The stimulation in bone formation of TGF-β1 is specific. Subperiosteal injections of other growth factors, including PDGF and bFGF, do not produce local bone formation (Noda and Camilliere, 1989). The results from Noda and Camillier (Noda and Camilliere, 1989) stimulate an interest in the role and effect of TGF-β on bone formation.

Daily subperiosteal injection of 20-200 ng TGF-β1 in a 10 μl solution induced intramembranous and chondrogenous bone formation. TGF-β1 stimulates proliferation of mesenchymal cells within the inner cambrial layer of the periosteum in rat femurs. The ratio of cartilaginous to intramembranous bone formation decreased when the concentration of injected TGF-β1 is lower. The differentiation paths of mesenchymal cells into osteoblasts or chondrocytes may be determined by concentrations of TGF-β1. Endochondral ossification and remodeling of intramembranous bone occur after a cessation of the injection sometime between 1 and 3 weeks (Joyce et al., 1990b).

A single subperiosteal injection of low dose platelet-derived TGF-β1 (5 – 50 ng/25 μl) induces woven bone formation with periosteal cell proliferation. A subcutaneous or supraperiosteal injection of TGF-β1 inhibited lamellar bone formation by delaying the mineral apposition rate and bone formation rate. This result could be due to the local anabolic effect following a local injection in fetal rat calvarial. It is demonstrated that TGF-β1 has local effects on bone formation and it is varied according to the injection site and concentrations of TGF-β1 (Fujimoto et al., 1999).

The bone formation effect of TGF-β1 is tested in the skeletal defect site. TGF-β1 (1 μg) is combined with natural coral granules and fibrin gel and implanted

in rabbit calvarial defects. The maximum amount of new bone formation in rabbit calvaria defects is found in combination with TGF-β1 and coral granules compared to implantations of coral granules or fibrin gel without TGF-β1. Cartilage formation is not found at the 1st and 2nd month-implantations. It is postulated that TGF-β1 may stimulate proliferation of osteoblasts residing in edges of bony defects (Arnaud et al., 1994).

Zellin and coworkers (Zellin et al., 1998) hypothesize that the osteogenicity of TGF-β1 in calvaria defect repair derives from a stimulation of committed osteoprogenitor cells in periosteum. It is found that critical size bicortical defects are completely filled in the TGF-β1 (5-10 μg) implantation group whereas filling in the control defects is less than 50%. When access to the periosteum of grafting materials is protected a repairing effect of TGF-β1 is not found (Zellin et al., 1998).

4.4. Multi-growth factor interaction

Applications of multiple growth factors demonstrate additive or synergistic effects which are greater than the effect from single growth factors. Pfeilschifter and coworker (Pfeilschifter et al., 1990) report additive effects of IGF-I (1-10 nM), PDGF (0.1-10 nM) and TGF-β1 (0.1-10 nM) on a bone matrix formation of fetal rat calvarial in an organ culture. A combination of PDGF and TGF-β1 enhances bone matrix formation in a dose dependent manner whereas a high dose of IGF-I reduces its bone matrix formation effect. TGF-β1 is the most potent stimulator of bone matrix formation. The authors hypothesize that a different spectrum of functions of these growth factors, in which PDGF is more potent in proliferation of osteoblastic precursor cells and TGF-β1 is more potent in extracellular matrix formation, may contribute to these additive effects (Pfeilschifter et al., 1990).

IGF-I, PDGF-BB and TGF-β1 have synergistic effects on mitogenesis and collagenous and non-collagenous protein syntheses of bovine osteoblast-like cells. IGF-I fail to activate ALP activity when it is combined with PDGF-BB and TGF-β1 (Giannobile et al., 1997). A combination of IGF-I and TGF-β1 enhances bone healing. Healing of a tibia fracture in a Yuctan pig is accelerated by a local release of IGF-I and TGF-β1 from a poly (D, L-lactide) (PDLLA) coating titanium interlocking

nail. In the growth factor group, the following are found: a faster consolidation of the fracture, higher torsional stiffness and maximum load and a larger callus volume. The authors state that local application of IGF-I and TGF-β1 enhances bone healing and a PLLA coating nail could be used as a growth factor delivery system (Raschke et al., 2002).

The opposite effects of TGF-βs and BMP-2 on growth and differentiation of pre-osteoblasts has been reported (Fromigue et al., 1998; Spinella-Jaegle et al., 2001). It is agreed that BMP-2 promotes osteoblastic differentiation of human mesenchymal cells and the murine cell line MC3T3-E2 and C3H10T1/2 by increasing ALP activity and osteocalcin expression of these cells *in vitro*. TGF-β1 and TGF-β2 exert similar effects in promoting proliferation and collagen synthesis and inhibiting osteoblastic differentiation. TGF-βs inhibited osteocalcin expression and mineralization of the matrix (Fromigue et al., 1998; Spinella-Jaegle et al., 2001). Co-treatment of BMP-2 and TGF-β2 resulted in intermediate effects on proliferation and differentiation markers of hBMS. BMP-2 alleviates the proliferative effect of TGF-β2 when it is supplemented in TGF-β2 treated cells but TGF-β2 does not abolish the effects of BMP-2 on BMP-2 treated cells. This study suggests an importance of sequences for growth factor interaction (Fromigue et al., 1998)

TGF-β1 stimulates expression of PDGF-BB mRNA of fetal rat calvarial osteoblasts and has a synergistic effect with VD_3 on ALP activity. The effects are unique for TGF-β1 since they are not found with other growth factors. TGF-β1, 0.01-1.2 nM, increases expression of PDGF-BB mRNA of fetal rat calvaria osteoblasts. The effect is not found when cells are treated with PDGF, bFGF or IGF-I (Rydziel and Canalis, 1996). Liu and coworkers (Liu et al., 1999) reports synergistic effects of TGF-β1 and VD_3 on proliferation and differentiation of hBMSC. A combination of both factors synergistically enhances ALP activity whereas a treatment with TGF-β1 alone inhibits ALP activity in hBMSC. TGF-β1 inhibits VD_3 induced osteocalcin production (Liu et al., 1999).

Aims of the study

1. Objectives

1. To induce osteogenic differentiation of mesenchymal stem cells in rat bone marrow.
2. To identify types of differentiated mesenchymal cells and characterize stages of osteoblastic differentiation of rat bone marrow cells.
3. To study the effects of the bone morphogenetic protein – 2 (BMP-2), and 1α,25-dihydroxycholecaciferol (VD3) on an osteogenic differentiation of mesenchymal stem cells in primary and first passages of bone marrow cell culture
4. To induce heterotopic bone formation using implanted fresh and cultivated bone marrow cells.
5. To study the effects of PRP on growth and differentiation of differentiated and non-differentiated mesenchymal stem cells in rat bone marrow, *in vitro* and *in vivo*, respectively.

2. Hypothesis

1. Mesenchymal stem cells in the bone marrow of mature Spraq-Dawly rats can be induced to differentiate into osteoblast-like cells. These osteoblast-like cells express osteoblastic phenotypes *in vitro* and *in vivo*.
2. Bone marrow cells in this culture condition differentiate into at least three types of mesenchymal cells, adipocyte, chondroblast and osteoblast-like cells.
3. BMP-2 and VD3 enhance osteogenic differentiation of mesenchymal stem cells in bone marrow.
4. Fresh and differentiated bone marrow cells are able to induce heterotopic bone formation.
5. PRP accelerates proliferation and differentiation of pre-mature osteoblasts *in vitro* and promotes hetrotopic bone formation of implanted bone marrow.

3. An overview of the study

This study aims to study osteogenic differentiation of mesenchymal stem cells in rat bone marrow and the effects of platelet-rich plasma to growth and differentiation of these cells. The study is separated into two parts: Part I: bone marrow cell culture and Part II: the effects of PRP on proliferation and osteogenic differentiation of rat bone marrow cells.

Bone marrow cell culture is established to induce osteogenic differentiation and proliferation of mesenchymal stem cells. Expressions of phenotypes of differentiated mesenchymal cells, adipocyte, chondroblast, and osteoblast, are characterized. Bone morphogenetic proteins-2 (BMP-2), 1α, 25 dihydroxycholecalciferol (VD3) and transforming growth factor-β2 (TGF-β2) are applied in a culture medium to modify the growth and differentiation of the cultivated cells. The ability of these cells to form bone is tested by implanting fresh bone marrow and differentiated bone marrow cells subcutaneously in nude mice.

In the second part, the study focuses the investigations on effects of PRP on growth and differentiation of mesenchymal stem cells and osteoblast-like cells in early state of differentiation. Interactions from various osteogenic proteins and growth factors which may occur during bone remodelling process are excluded. PRP is applied on differentiated bone marrow cells in a three-dimensional cell culture and on fresh bone marrow cells to be implanted intramuscularly in nude mice. Growth and differentiation of cultivated cells and new bone formation in vivo are evaluated. Effects of PRP on growth and differentiation are compared with the effects of rh BMP-2.

The study aims to establish a model for studying growth and osteoblastic differentiation of mesenchymal stem cells and cells of osteoblastic lineage in vivo and in vitro. The results from this study will further enhance an application of autogenous mesenchymal stem cells in bone tissue engineering. Three-dimensional ICBM is introduced as scaffolds for cell seeding and to deliver implanted cells.

It is expected that these new techniques will be able to improve bone regeneration, restore function and anatomical defects of damaged tissue. The limitation of bone tissue reconstruction caused by an insufficient amount of

autogenous tissue for bone grafting and the morbidity of donor sites will be eliminated.