Chapter 4

DISCUSSION

Part I: Osteogenic induction of bone marrow of fully mature rats

Bone marrow from fully mature rats was able to differentiate into mature osteoblasts *in vivo* and *in vitro*, but to induce full osteoblastic differentiation of these cells *in vitro* requires a continuous exposure to BMP-2 or VD3. Implanted cells were a mixture of pre-osteoblasts and adipocytes. Expanded bone marrow cells contained high osteogenic ability and formed large amount of new bone formation *in vivo*. Osteogenesis was induced through intramembranous and endochondrogenesis processes. It is hypothesized that culture conditions, numbers of implanted osteoprogenitor cells and the age of donors influence osteogenic differentiation of bone marrow cells. It is possible to cultivate bone marrow from adult subjects for autogenous implantation and for transferring into skeletal defects. ICBM scaffold is a biocompatible three dimensional scaffold which enhances delivering of cells into skeletal size.

1. Osteogenic potential of bone marrow

1.1. Osteogenic cell culture of rat bone marrow

This study found that bone marrow cells of fully mature rats contained mesenchymal stem cells, which were able to differentiate into osteoblast-like cells and adipocytes under the influence of dexamethasone in culture medium (Cheng et al., 1994; Friedenstein et al., 1987; Haynesworth et al., 1992a; Haynesworth et al., 1992b). Mesenchymal stem cells were identified by an ability to attach, proliferate and form stromal fibroblastic unit or cell nodules (Ashton et al., 1980). These

fibroblast-like cells with elongated cytoplasm were able to attach and form fibroblastic cell nodules in a monolayer culture. Morphology of cells become homogenous with culture-time (Ashton et al., 1980; Friedenstein et al., 1970). As it has been reported that each fibroblastic unit has different osteogenic potential (Kuznetsov et al., 1997) and contains both mesenchymal stem cells and osteoprogenitors cells (Prockop, 1997), no attempt in this study was made to expand mesenchymal stem cells from single colony or lineage. Therefore, expressions of osteoblastic characteristics were expressions of cells from multiple clonals of mesenchymal stem cells. Difference in level of osteoblastic differentiation of each colony was not tested. However, osteogenic differentiation of mesenchymal stem cells in bone marrow as a whole was studied. Supplementations of dexamethasone or other growth factors were aimed to maximize osteogenic differentiation of all colonies in the system.

Mesenchymal stem cells in this study differentiated into osteoblast-like cells and adipocytes. An adipocyte is one of the mesenchymal cell lineage in the stromal fibroblastic unit (Owen, 1988; Owen and Friedenstein, 1988). Chondroblastic differentiation was not found in this study. This might be because the culture condition was not suitable for chondroblastic differentiation. A presence of TGF-β1, -2, or -3 in culture mediums is required to promote a chondroblastic differentiation (Barry et al., 2001; Sekiya et al., 2002; Worster et al., 2001).

1.2. Expression of differentiation markers and cell culture condition

It has been reported that dexamethasone is an essential factor for osteogenic differentiation of bone marrow in cell culture (Cheng et al., 1994; Gundle et al., 1995; Maniatopoulos et al., 1988). Ascorbic acid enhances osteogenic differentiation of undifferentiated cells by inducing a formation of type I collagen (Franceschi and Iyer, 1992; Otsuka et al., 1999). External phosphate in culture medium promotes mineralization of the extracellular matrix (Franceschi and Iyer, 1992; Maniatopoulos et al., 1988). In this study, bone marrow was cultivated in a culture medium supplemented with ascorbic acid, β-glycerophosphate and dexamethasone. It was found that cultivated bone marrow cells of fully mature rats in 20 nM dexamethasone expressed osteoblastic phenotypes of ALP activity and type I collagen as protein

products and mRNA expressions. A supplementation with BMP-2 or VD3 of more than 10 days was essential for expression of osteocalcin and *in vitro* mineralization.

1.2.1. Expression of ALP and adipocytes

ALP activity is a widely measured osteoblastic marker during the proliferative state (Franceschi and Iyer, 1992; Lecoeur and Ouhayoun, 1997; Rickard et al., 1994) This enzyme begins immediately following the cessasation of cell proliferation and reaches a maximum level during the phase of matrix maturation (Risteli and Risteli, 1993; Stein et al., 1990). It was found that expressions of ALP and type I collagen are markers of osteoblastic differentiation in an early state. Expression of ALP activity was found as early as 72 hours after cell seeding and expression of ALP mRNA was demonstrated on the 7^{th} culture-day. This early expression of an osteoblastic marker might be a result of an early supplementation of dexamethasone, which had been supplemented since cell seeding. The supplementations were earlier than in other studies where dexamethasone was supplemented after the first culture medium change or in the first passage (Maniatopoulos et al., 1988; Otsuka et al., 1999). In this study, dexamethasone, ascorbic acid and β-glycerophosphate were supplemented in a culture medium since cell seeding in the primary passage to ensure the earliest and maximum osteogenic differentiation of mesenchymal stem cells and osteoprogenitor cells in bone marrow.

Positive staining of ALP was found closely related to adipocytes or oil red O positive stained cells since the 3^{rd} culture-day. These spatial and differentiation time relationships support a proposal that differentiation paths of osteoblasts and adipocytes are closely related (Gimble et al., 1996) and mesenchymal stem cells residing in bone marrow are capable of differentiating to adipocytes (Otsuka et al., 1999; Owen, 1988). Extramedullary adipose cells incubated in a combination of dexamethasone and BMP-2 underwent osteoblastic differentiation and expressed ALP activity (Lecoeur and Ouhayoun, 1997).

1.2.2. Expression of type I collagen and BMP-2 mRNAs

The earliest expression of type I collagen was found in the BMP-2 group on the 7 culture-day followed by the expression in VD3 and dexamethasone groups on

the 14th culture-day in the primary passage and the expression was persistent throughout cell culture. Synthesis of type I collagen indicates the beginning state of osteoblastic differentiation. Type I collagen was abundantly produced during the proliferative stage (Risteli and Risteli, 1993). The earliest expression of type I collagen found in the BMP-2 group suggested that BMP-2 accelerates osteoblastic differentiation in an early state of a differentiation cascade.

Expression of BMP-2 mRNA was found early on the 7th culture-day in the primary passage and the expression was consistent through out cell culture-period. BMP-2 is able to induce osteogenic differentiation of mesenchymal stem cells in bone marrow and the pre-osteoblast to express mature osteoblastic phenotypes, which are *in vitro* mineralization and osteocalcin production (Hicok et al., 1998; Lecanda et al., 1997). Expression of BMP-2 suggests that osteoblasts in early and advance differentiation states produce BMP-2. This autogenous BMP-2 can act as autocrine and paracrine factors to induce and accelerate osteoblastic differentiation of cells (Bostrom, 1998; Bostrom et al., 1999; Jaiswal et al., 1997; Spector et al., 2001). However a production of BMP-2 and the expression of BMP-2 receptors were not detected in this study.

1.2.3. Expression of osteocalcin and *in vitro* mineralization

Expression of osteocalcin was found only in cell culture supplemented with BMP-2 or VD3. Osteocalcin is a non-collageneous protein secreted exclusively by osteoblasts in a fully differentiation state. Osteocalcin mRNA appears at the onset of mineralization of cell nodules (Aubin, 1998; Price, 1985). In this study an expression of osteocalcin was found related to *in vitro* mineralization of cell nodules, which is also a marker of the function of mature osteoblasts (Wada et al., 1998). *In vitro* mineralization was clearly detected in cell culture supplemented with BMP-2 and VD3 on the 21st culture-day.

It is clearly demonstrated that BMP-2 and VD3 are essential for osteogenic differentiation of bone marrow cells transforming into a terminal state of osteoblastic differentiation. This assumption is supported by previous reports that VD3 can induce osteocalcin production and mineralization of cell nodules (Hay et al., 1999; Liu et al., 1999). BMP-2 is a strong osteogenic inducer of cultivated bone marrow

cells. BMP-2 induces differentiation of non-differentiated mesenchymal stem cells to express osteoblastic phenotypes and immature osteoblasts to a terminal differentiation state (Cheng et al., 1994; Gori et al., 1999).

In cell culture of bone marrow derived from fully mature rats, a continuous exposure to either BMP-2 or VD3 is essential. In this study, bone marrow cells in the primary passage needed to be exposed to BMP-2 or VD3 for more than 10 days to induce bone marrow cells to express osteocalcin mRNA. This might be because of a reduction in the potential of osteogenic differentiation or a reduction in the number of mesenchymal stem cells in the bone marrow of aged rats. The rate of skeletal tissue repair and number of mesenchymal stem cells in bone marrow decreased in old animals and aged patients (Haynesworth et al., 1992b; Inoue et al., 1997). The numbers decreased from $1{:}1x10^4$ cells in new-borns to $1{:}\ 2x10^6$ cells in aged persons (Cheng et al., 1994; Haynesworth et al., 1992b; Nakahara et al., 1991).

1.2.4. Supplementations in culture medium

A combination of BMP-2 and dexamethasone increases the osteoiductive ability of BMP-2 (Cheng et al., 1994; Rickard et al., 1994), but a withdrawal of dexamethasone from the culture medium enhances osteogenic induction of VD3 resulting in an increase of osteocalcin production of bone marrow cells (Cheng et al., 1994). In this study, dexamethasone was withdrawn during VD3 supplementation on or at around the 7^{th} – 10^{th} culture-day, aiming to promote a maximum osteogenic effect of VD3.

It is also found in this study that TGF-β2 caused morphological change of differentiated mesenchymal cells and inhibits cell nodule formation (Harris et al., 1994). When bone marrow was cultivated in TGF-beta 2 since the cell seeding stage, cells demonstrated homogenous morphology of fibroblast-like cells with flattened and elongated cytoplasm and cells did not form cell nodules. Most of the differentiated cells had positive ALP staining. Few adipocytes were seen.

1.3. Part I: Implantation of bone marrow cells, *in vivo* study

Differentiated bone marrow cells cultivated in VD3 in the first passage were implanted in nude mice to test their terminal osteoblastic differentiation *in vivo*,

whether they would be able to form bone or not. These cells were pre-osteblasts expressing ALP and type I collagen mRNAs and ALP activity (Wang and Glimcher, 1999). The osteogenic capacity of cultivated cells was compared with the capacity of fresh bone marrow. It was found that differentiated bone marrow cells and fresh bone marrow were able to induce heterotopic bone formation in subcutaneous pockets of nude mice. Differentiated bone marrow had a higher osteogenic potential than implanted fresh bone marrow. Implanted cells survived and continued to differentiate into mature osteoblasts and secrete bone matrix.

1.3.1. Implanted cells

It is clearly demonstrated that fresh bone marrow and adherent stromal cells have osteogenic potential and they can be expanded *in vitro* and form bone in an ectopic site. Implanted cells are retained in an implantation site for more than 4 weeks after implantation. These cells proliferate, differentiate and secrete bone matrix. Host cells became involved in the bone formation process during the bone remodeling process (Goshima et al., 1991a; Goshima et al., 1991c; Kuznetsov et al., 1997). Based on results of the *in vitro* study, cultivated bone marrow cells express BMP-2 mRNA through out the cell culture-period and it is reported that osteoblasts secrete growth factors such as TGF-β1 and IGF-I and II (Jaiswal et al., 1997). It is hypothesized that implanted bone marrow cells played a major role in the bone forming process in this ectopic site and secrete BMP-2, TGF-β1 and IGF-I. These autogenous osteogenic proteins accelerated the differentiation process of implanted cells and also induced osteogenic differentiation of mesenchymal stem cells residing in subcutaneous tissue of the implantation site.

Implanted pre-osteoblasts should be able to initiate bone formation process at a faster rate than DBM or BMP-2 because new bone was formed mainly through intramembranous processes. Implanted cells conversed more directly into osteoblasts than into chondroblasts. Only a small area of cartilage was found. This pattern of bone formation is different from osteogenic induction process induced by DBM or BMP-2, in which mesenchymal stem cells are induced to differentiate to chondrocytes (Kim et al., 1997; Rabie et al., 2000; Wang and Glimcher, 1999; Yoshida et al., 1998). Thus, if implanted cells are combined with BMP-2, sources of bone forming

cells can derive from implanted cells and host cells. This will result in an increasing rate and amount of bone formation.

The amount of new bone formation depends on the state of differentiaton of cells and the numbers of differentiated cells. A small amount of new bone formation and slow bone formation in the fresh bone marrow group was obviously contrasted by cultivated cell groups, where large areas of woven bone and cartilage formations were found since the 18^{th} culture-day. Cultivated cells and fresh bone marrow might induce bone formation through different pathways. Fresh bone marrow was likely to induce new bone formation mostly through a chondrogenesis process. A slow bone formation process of fresh bone marrow was confirmed by a presence of cartilage and ossification of cartilage on the 45^{th} implantation-day, whereas cartilage was not seen in the other group. This finding supports an assumption that cultivated cells contain higher numbers of osteoprogenitor cells than whole components of fresh bone marrow (Goshima et al., 1991a; Kadiyala et al., 1997). However, it should be noticed that a poor efficiency of fresh bone marrow seeding on porous structure of ICBM scaffold might contribute to lower numbers of implanted mesenchymal stem cells. Osteogenic capacity of the implants depends on a density of mesenchymal cells on a ceramic surface (Goshima et al., 1991a; Goshima et al., 1991b)) or at least $1x10^3$ cells/cm^2 (Dennis et al., 1992).

1.3.2. New bone formation processes

A direct conversion of implanted cells to osteoblast in the intramembranous process is similar to osteogenic induction of DBM prepared from inramembranous bone (Wang and Glimcher, 1999). This can be seen from an early state of bone formation on the 18^{th} implantation-day in which plump osteoblasts were found embedded in a newly synthesized bone matrix and lined on the border of new woven bone trabeculae. On the 28^{th} and 45^{th} implantation-days, woven bone trabeculae were more mature with well organized bone trabeculae and well developed bone marrow. This suggests a rapid bone formation process induced by implanted osteoprogenitor cells through intramembranous bone formation. It is postulated that cartilage formed on the 18^{th} implantation-day had progressed through a rapid osteochondrogenesis,

therefore there was no cartilage formation to be seen on the 28th and 45th implantation-days.

A small area of cartilage was found in a group of implanted cultivated cells. This pattern of a small amount of cartilage formation is also found with implanted cells seeded on a hydroxyapatite ceramic. The authors suggested that a crack or minor brakeage induced cartilage formation, not implanted cells (Bruder et al., 1998c; Bruder et al., 1998b; Goshima et al., 1991a; Goshima et al., 1991b; Ohgushi et al., 1993). The source of chondroblast differentiation can derive from pluripotential progenitor cells in implanted cells and/or differentiated mesenchymal stem cells in subcutenous tissue of the host (Wang and Glimcher, 1999). Chondrogenesis potential was found only in first and second subcultivation of mesenchymal cell culture (Goshima et al., 1991a; Goshima et al., 1991b; Ohgushi et al., 1993). Oxygen tension or a lack of blood supply and nutrient (Omura et al., 1998), DBM (Rabie et al., 1996) or BMP-2 (Murata et al., 1998) promotes chondroblastic differentiation of mesenchymal cells. It is postulated that oxygen tension, a lack of blood supply and nutrients (Omura et al., 1998) and autogenous BMP-2 and TGF-β1 (Jaiswal et al., 1997; Murata et al., 1998; Si et al., 1997) induce small amounts of cartilage formation in implanted differentiated bone marrow cells.

1.3.3. ICBM scaffold

Three-dimensional structure and biocompatibility of scaffold promote growth and osteogenic differentiation of cells *in vivo* (Kuboki et al., 1998). Implantation of a cell pellet or injection of cell suspension of fresh and cultivated bone marrow induce no bone formation in a heterotopic site (Goshima et al., 1991a; Goshima et al., 1991b; Ohgushi et al., 1993).

ICBM is an ideal carrier for the delivery of mesenchymal stem cells. Anchorage dependent cells such as osteoblasts requires large surface area for cell to attach and differentiate and a type I collagen matrix provides a compatible surface for attachment and differentiation of osteoblasts (Ripamonti, 1993; Sampath and Reddi, 1981). It is observed from this study that bone marrow cells grow well within a structure of scaffold and the bone matrix was laid directly on the surface of scaffold.

ICBM was inert and did not induce tissue reaction. Tissue reaction to ICBM scaffold was not seen. ICBM has a natural framework and porosity of cancellous bone, which allows vascular infiltration and intercellular communication of cells on a matrix structure, as large numbers of vascular infiltration were found through-out the implantation period. Blood vessels provide a portal of entry for nutrition, hemopoietic environment and host-derived mesenchymal stem cells (Kadiyala et al., 1997).

A finding of bone formation mostly in the peripheral area of the scaffold was a result of insufficient infiltration of seeded cells during the cell seeding procedure. Cells should be suspended in a maximum volume of solution that ICBM can absorb. Cell solution should be added on several surfaces of scaffold, instead of only from the top surface of scaffold, in order to facilitate a deeper infiltration and homogenous distribution of cells within a structure of the scaffold.

ICBM was radiolucent. That means the amount and area of radiopacity of new bone formation or bone remodeling can be interpreted without an interference from ICBM. A stable three-dimensional structure of ICBM provides a malleable scaffolds and controllable handling for cell transplantation and growth factor delivery. Stability and porosity of ICBM scaffold facilitate delivering of cells into subcutaneous pockets and ICBM can be shaped to fit with each anatomical defect.

ICBM is an ideal scaffold for cell transplantation and osteogenesis, according to criteria proposed by Bruder et al., 1991 (Bruder et al., 1998b). There are two drawbacks, the resorption rate of ICBM was relatively slow and it is a natural product of bovine bone. There was no evidence of resorption of ICBM on the 45th implantation-day.

Part II: Effects of PRP on growth and osteogenic differentiation of rat bone marrow

1. Overview

This study was conducted in order to clarify the promoting effects of PRP on bone regeneration reported in many clinical studies. This part of the study aimed to investigate the effects of PRP on proliferation and differentiation of mesenchymal

stem cells and pre-osteoblasts. PRP was applied on pre-osteoblasts derived from cultivated bone marrow during 7^{th}-9^{th} culture-day in the primary passage and on fresh bone marrow. Osteogenic effects of PRP were compared with the effects of BMP-2.

1.1. PRP preparation

PRP was prepared from a pool of whole blood of mature male rats 1 hour before use in each experimental setting. PRP was prepared three times, two times for *in vitro* study (the ALP measurement and the WST and calcium content measurements) and one time for *in vivo* study. Therefore, qualities of PRP regarding platelet aggregation and survival in this study were not compromised with a storage condition and individual variation from each rat. PRP should be stored at room temperature (22°C), without packed cells, of not more than 6 hours to prevent platelet aggregation (Ho and Chan, 1995). Platelet concentrate stored at 22°C has a higher survival *in vivo* than platelets stored at 4°C (Pietersz et al., 1987).

PRP was prepared by the centrifugation gradient technique or by tube methods (Landesberg et al., 2000; Zimmermann et al., 2001). High centrifugation force was used to separate plasma from blood cells, then plasma was centrifuged with a low centrifugation force to concentrate platelets (Landesberg et al., 2000; Sonnleitner et al., 2000; Zimmermann et al., 2001). This method yielded a high white blood cell contamination (1.3 times higher than white blood cells in whole blood), because white blood cells were included in PRP during a pipetting of the upper part of red blood cells locating immediately under the white blood cell layer. It is suggested by Marx et al., 1998 (Marx et al., 1998) that this layer contains large numbers of newly synthesized platelets. Possible interferences from blood cells were excluded by conducting an experiment in negative control groups. It was found that the level of ALP activity in a negative control group was minimal and sharply decreased to nearly zero level after the 3^{rd} culture-day.

PRP preparation using tube or buffy coat methods are not complicated and suitable for handling small volumes of blood (70 - 80 ml), but because it is an open technique one should be cautious of contamination during preparation. It was found that larger volumes of PRP and a higher concentration of platelets could be obtained from platelet pheresis or an autotransfusion technique when compared to PRP

prepared using the open centrifugation technique (O'Neill et al., 2001). Different preparation methods yield: different platelets concentration, red blood cells, white blood cells, volume of PRP, brakeage of platelet during preparation and growth factor contents in PRP (O'Neill et al., 2001; Racz and Thek, 1984; Weibrich et al., 2001; Weibrich et al., 2002b; Weibrich et al., 2002a; Zimmermann et al., 2001). The amount of the required PRP determines the preparation method (Zimmermann et al., 2001). Weibrich and coworkers (Weibrich et al., 2002a) and Zimmermann and coworkers (Zimmermann et al., 2001) postulated that white blood cell contamination might contribute to the level of growth factors released from PRP, but precise parameter prediction of the amount of the released growth factor can not yet be established.

1.2. PRP preparation efficiency and released growth factors

Concentration of platelets in PRP was rarely stated in clinical reports, only total volumes of whole blood and PRP were mentioned. Preparation methods and centrifugation forces were varied (Anitua, 1999; Kassolis et al., 2000; Lekovic et al., 2002; Shanaman et al., 2001). Marx et al., 1998 and Kassolis et al., 2000 (Kassolis et al., 2000; Marx et al., 1998) reported there was more than a 4 fold increase in platelet concentration in PRP compared to the concentration in whole blood.

Concentration of platelets in PRP should be more than 4 times higher than the concentration in whole blood. In this study, concentration of platelets in PRP was 9 times higher than the concentration in whole blood. Approximately 18% (18.4%) of the total number of platelets from one rat were concentrated in 185-200 μl of PRP, which were higher than what was reported by Anitua, 1999 (Anitua, 1999) where there were less than 15% of total platelets in whole blood. Approximately 4% (3.8%) of the total platelets in one rat (or $2.5x10^8$ platelets) were added to one sample in the 100% PRP group.

It has been postulated that degranulated platelets released growth factors (Miyazono and Takaku, 1989) and various numbers of platelets were activated directly on the surface of the matrix and cells. When PRP was activated with 1000 unit Bovine thrombin in 20% $CaCl_2$ solution, 386.42±81.34 ng/ml of TGF-β1 or 0.06

ng/one million platelets were detected in supernatants. PDGF content in rat platelets was not measured because of the unavailability of detecting tools for rat PDGF.

The amount of growth factor released from the platelets is influenced by activating methods. Concentration of TGF-β1 obtained using the freezing and thawing method is higher than concentration of TGF-β1 released from thrombin and $CaCl_2$ activation (Weibrich et al., 2001). Basing on the thrombin and $CaCl_2$ activation method, PDGF content in platelets is equal to the content of TGF-β1. One million human platelets contained 0.06 ng of PDGF (Bowen-Pope et al., 1984; Landesberg et al., 2000) and 0.06 ng of TGF-beta1 (Landesberg et al., 2000). Therefore, it is assumed that rat platelets contain an equal concentration of PDGF to concentration of TGF-β1 which is 0.06 ng/million platelets.

It is estimated that in this study 14.7±2.02 ng of TGF-β1 and PDGF-BB were added on each sample in a PRP 100% group ($2.5x10^8$ platelets) of *in vitro* samples. *In vitro* samples were cultivated in 9.81±1.46 ng/ml TGF-β1 and PDGF-BB in culture medium. TGF-β1 was released from platelets in an inactive form because un-activated serum contained a small amount of TGF-β1 content (data not shown). A much lower concentration of IGF-I content in platelets was reported (Weibrich et al., 2002b). It is assumed that PDGF-BB played a major role in this *in vitro* study.

Platelet poor plasma was a product of a second centrifugation of PPP, which was separated from the upper part of the centrifugated plasma. This procedure ensured that PPP contain minimum amount of platelets. This expectation was confirmed by a low amount of TGF-β1 content in PPP (8.14 ng/ml). Clotted PPP functioned solely as a fibrin clot.

1.3. *In vitro* and *in vivo* concepts of study design

1.3.1. Target cells

This study focused on investigating the effects of PRP on proliferation and osteogenic differentiation of osteoprogenitor cells or pre-osteoblasts and mesenchymal stem cells. The differentiated rat bone marrow cells on the 7^{th}-8^{th} culture days in this study were mature osteoprogenitor or pre-osteoblasts. They expressed mRNAs of ALP, type I collagen and BMP-2 and alkaline phosphatase activity. Mesenchymal stem cells in bone marrow were implanted *in vivo* together

with other components in bone marrow such as hematopoietic stem cells and stromal tissue.

1.3.2. Concepts of experimental design

The design of this study facilitates a close contact between PRP and the mesenchymal stem cells in bone marrow to imitate a close contact between PRP and cancellous bone. In clinical studies PRP was mixed with cancellous bone forming paste of bone for an implantation (Anitua, 1999; Marx et al., 1998). An invented method of fresh bone marrow seeding allowed an event distribution of bone marrow, a mixing between PRP or other blood products with thrombin-calcium chloride solution within the structure of an ICBM scaffold. Regarding the *in vitro* study, cultivated cells were directly contacted with activated PRP and PPP, when PRP and PPP were directly dropped and activated on the scaffold. Both *in vitro* and *in vivo* studies activated PRP, PPP and whole blood created fibrin clots enclosing a three-dimensional structure of ICBM scaffold and cells. This fibrin gel acted as a fibrin shield that separated cells from the external environment and was able to absorb released growth factors and provide an additional extracellular matrix for cell growth and differentiation (Bhanot and Alex, 2002).

It is possible that growth factors were secreted directly on cell surfaces in large volumes, then an excess volume was absorbed and kept in collagen fibers of ICBM scaffold and in the fibrin clot. This environment provided a close and constant contact between cells and growth factors which should be gradually released from the scaffold and clot. This setting created a blood clot environment, which is comparable to the natural condition of blood clotting forming immediately after skeletal fracture and bone grafting (Hollinger and Wong, 1996; Joyce et al., 1990c; Joyce et al., 1990a). It is proposed that blood clot in skeletal fractures release growth factors from platelets and decrease oxygen tension and pH condition (Bolander, 1992; Bostrom, 1998; Bostrom et al., 1999; Bostrom and Asnis, 1998).

Clotting PRP produces a fibrin clot. Fibrin gel maximized matrix and cell interaction by enhancing close intercellular contact, which is essential to growth and differentiation of osteoblasts to form bone-like tissue (Bellows et al., 1986; Bhargava et al., 1988). This assumption is supported by this study, that the rate and level of cell

proliferation and ALP activity in the PPP group were higher than the rate and level of activity in the dexamethasone group. Fibrin gel in the PPP group was biologically passive because PPP contained very low numbers of platelets and concentration of growth factors. PRP groups were markedly different from the results of the PPP group. This evidence suggests that ALP activity and the cell proliferation rate in PPP group were under the influence of fibrin gel. Fibrin cross-linked matrix of fibrin gel provides a three-dimensional scaffolding for cells to migrate and proliferate (Bhanot and Alex, 2002). Fibronectin in fibrin gel facilitated an attachment and growth of differentiated bone marrow cells on the surface of hydroxyapatite (Dennis et al., 1992). It is demonstrated in the neutral red vital stain and ALP staining that cultivated cells grow and differentiate well on a fibrin network and fibrin network facilitated the growth of cells within a structure of the scaffold and around the external surface of scaffold.

1.4. Three-dimensional scaffold

Stable and porous structures of ICBM scaffolds facilitate a delivering of bone marrow cells into the intramuscular site. The intramuscular site was chosen as an implantation pocket because it is rich in vascular supply (Omura et al., 1998). There was no evidence of mineralization and new bone formation on or within the structure of the ICBM scaffold in the control group. This demonstrated that the ICBM scaffold has no osteoconductive property and was biologically inert.

These results confirm comments from previous reports that a type I collagen matrix in the structure of an ICBM is favorable for attaching of mesenchymal cells (Sampath and Reddi, 1981) and geometry of the bone matrix of ICBM is suitable for attachment and growth of anchorage dependent cells such as osteoblasts (Sampath and Reddi, 1984). These effects can also be seen in morphological staining of the *in vitro* study demonstrating dense attachment, active proliferation and morphological differentiation of cells on structure and within the matrix.

A three-dimensional collagen matrix promotes osteogenic differentiation by providing three-dimensional space for cells to create cell to cell contact. The structure promotes attachment, proliferation, differentiation and secretion of an extracellular matrix of cells on the structure of the matrix. (Casser-Bette et al., 1990; Shea et al.,

2000). The ICBM matrix had a high affinity to preosteoblasts. Morphological staining of neural red vital stains and ALP staining demonstrates an extensive cell attachment, proliferation and differentiation of cells in all groups of this study.

1.5. PRP and osteogenesis

Various attempts have been applied to improve bone healing by accelerating the rate of new bone formation and maturation. An osteogenic inducer with or without osteoprogenitor cells was implanted into the bone grafting site to accelerate bone healing (Bruder et al., 1998c; Bruder et al., 1998b; Bruder and Fox, 1999; Kadiyala et al., 1997). PRP is introduced as a vehicle to deliver a high concentration of growth factors released from the platelets into the skeletal defect to promote a bone regeneration process (Carlson, 2000; Marx et al., 1998).

Activated platelets secrete various growth factors relating to the bone formation process including TGF-β1, PDGF-BB (in murine platelets), IGF-I and EGF from their alpha granules (Kronemann et al., 1999; Miyazono and Takaku, 1989; Slater et al., 1995). These growth factors are also secreted by osteoblasts (Baylink et al., 1993; Canalis et al., 1991; Globus et al., 1989; Mohan and Baylink, 1991). They are stored in bone matrix (Baylink et al., 1993), expressed in fracture site during the fracture healing process (Andrew et al., 1993a; Andrew et al., 1993b; Bostrom and Asnis, 1998; Steinbrech et al., 2000a) and secreted from degranulated platelets (Hwang et al., 1992; Pircher et al., 1986). Interactions of these growth factors including BMP-2 control healing process of bone grafting and fracture healing. They activate proliferation and differentiation of local osteoprogenitor cells leading to the laying down of the bone matrix and mineralization (Boden, 1999; Bolander, 1992; Bostrom et al., 1999; Canalis et al., 1993; Solheim, 1998).

It is reported that PRP accelerated bone graft healing and increased bone density (Marx et al., 1998). Many clinical studies and one study in an animal model reported advantages of using PRP in repairing skeletal defects (Anitua, 1999; Kassolis et al., 2000; Kim et al., 2002). They found that PRP increased the rate and amount of new bone formation and gel-like form of PRP improved graft handling. Positive effects of PRP on bone regeneration were hypothesized resulting from biological effects of growth factors released from platelets on osteoprogenitor cells and

mesenchymal stem cells (Anitua, 1999; Carlson, 2000; Froum et al., 2002; Kassolis et al., 2000; Kim et al., 2002; Landesberg et al., 2000). This study aims to verify the hypothesis above.

2. Effects of PRP in three-dimensional cell culture, *in vitro* study

It can be seen that this culture system provided an optimal environment for growth and differentiation of osteogenic cells and facilitated a supplementation of PRP in cell culture. A low level of cell proliferations in 6.25% and 25% PRP groups might be a result of an un-optimal or small amount of fibrin gel on the structure of the matrix. Smaller amounts of the PRP product, less than 10 µl, was added on each matrix of these groups instead of 38 – 40 µl of PRR in 100% PRP or40 µl of PPP in PPP groups.

ALP activity was detected as a marker of early osteoblastic differentiation or pre-mature osteoblasts, whereas *in vitro* mineralization was considered as a parameter of osteoblastic differentiation in the mature stage (Aubin et al., 1995; Aubin, 1998). It is obvious in this study that BMP-2 is the most potent osteogenic inducer. BMP-2 induced the highest rate and amount of ALP activity and the highest level of calcium content with low proliferation rate. It agrees with previous reports that BMP-2 is a strong osteogenic inducer being able to induce differentiation of mesenchymal stem cells and promote osteogenic differentiation of osteoprogenitor cells and pre-osteoblasts into terminal differentiation state *in vitro* and *in vivo* (Cheng et al., 1994; Gori et al., 1999; Hicok et al., 1998; Lecanda et al., 1997; Murata et al., 1998; Yoshida et al., 1998).

It is possible that the released growth factors were rapidly adsorbed in collagen fibers of the ICBM matrix and slowly released into culture medium resembling resorption and releasing patterns of PDGF impregnated in anorganic bovine collagen matrixes (Stephan et al., 2000) and chitosan sponge (Park et al., 2000a; Park et al., 2000b). BMP-2 lyophilized on an ICBM scaffold was expected to be gradually released from the scaffold in a similar manner. It can be seen from curves of cell proliferation and ALP activity that the proliferation rate and ALP activity progressed rapidly during the 3^{rd}-9^{th} culture-days and reached a plateau phase on the 9^{th} – 12^{th} culture-days. It is estimated that cells were exposed to a high

concentration of growth factors (9 ng/ml of TGF-β1 and PDGF-BB) or BMP-2 (300 ng) lyophilized on the scaffold or not higher than 100 ng/ml in the culture medium during the first three culture-days and to a lower concentration of growth factors continuously released from ICBM matrix until the 6^{th}-9^{th} culture-days. This assumption is supported by a hypothesis that growth factors were released from platelets as a bolus of growth factors and were kept within the clotting environment for a gradual release during the early stage of the bone healing process (Andrew et al., 1993a; Bolander, 1992). It should be noticed that proliferation rate and ALP activity of cells progressed in reversed directions. This result suggests an inverse relationship between proliferation and differentiation of cells.

Inhibition effects of PRP on ALP activity and activation of cell proliferation in a dose dependent manner are clearly demonstrated in PRP 100% group. High concentration of PRP inhibits osteoblastic differentiation and accelerates proliferation of pre-osteoblast-like cells or osteoblast precursor cells *in vitro*. PRP 25% and 6.25% did not show any marked effect on cell proliferation or differentiation.

Levels of ALP of each group corresponded to levels of their calcium contents. The BMP-2 group had the highest level of calcium content followed by the levels in PPP, 6.25% PRP, 25%PRP, and dexamethasone groups, respectively. It is clearly demonstrated that the maximum effects of PRP on ALP activity or osteogenic induction was far less than activity induced by 300 ng BMP-2 lyophilised on a matrix. Osteoblasts in the BMP-2 group had the highest ALP activity and level of calcium content.

It is speculated that PDGF, IGF, EGF and TGF-β1 were released as a bolus of growth factors from activated platelets and absorbed on the structure of the ICBM and the clot to be continuously released into culture medium. These growth factors inhibited osteoblastic differentiation and promoted proliferation of pre-osteoblast cells. The inhibitory effect of PRP is supported by a report of (Schmid et al., 1984). The authors reported that crude growth factors derived from lysate platelets decreased alkaline phosphatase activity and increased proliferation of rat calvaria osteoblasts in a dose dependent manner.

It is postulated that in this experiment major effects of PRP were derived from interactive effects of PDGF, IGF-I, EGF and TGF-β1. They are strong mitogenic

agents and inhibit osteogenic differentiation of cells in the early state of osteogenic differentiation. The effect from the TGF-β1 *in vitro* study is expected to be a minor extension because it was secreted in an inactive form and the amount of activated form in the culture medium was not detected.

PDGF has strong mitogenic factors. Prolonged exposure to PDGF inhibits osteoblastic differentiation (Hsieh and Graves, 1998; Yu et al., 1997). The effects of TGF-β1 divert depending on the dose and differentiation state of cells. On mesenchymal cells in bone marrow, TGF-β1 exhibits dose dependent inhibition effects. TGF-β1 inhibit osteogenic differentiation in high doses (5-10 ng/ml), but promotes differentiation in low dose (1 ng/ml). In low doses, TGF- β1 promotes proliferation of cells in early and intermediate osteoblastic differentiation states (Kassem et al., 2000). TGF-β1 may increase proliferation of cells by regulating expression of IGF-I (Kveiborg et al., 2001). TGF-β1 promote osteogenic differentiation of non-proliferating cells and strongly promote type I collagen formation of proliferating cells (Centrella et al., 1987). Prolonged exposure to TGF-β1 inhibits osteoblastic differentiation and mineralization of cell nodules (Harris et al., 1994). Subperiosteal injection of TGF-β1 is able to induce woven bone formation (Noda and Camilliere, 1989). It can be seen that TGF-β1 inhibits differentiation of proliferating cells and enhances collagen matrix formation. EGF inhibits ALP activity and type I collagen synthesis of rat calvarial cell culture (Canalis, 1983). In organ culture, EGF increases DNA synthesis, stimulates proliferation of periosteal fibroblasts and decreases bone collagen synthesis (Ernst and Froesch, 1987).

IGF-I is the only factor that stimulate proliferation and differentiation of osteogenic cells (Ernst and Froesch, 1987; Hock et al., 1988; Schmid et al., 1984). IGF-I enhances osteogenic differentiation by increasing ALP activity, type I collagen synthesis and minor degree of cell proliferation of osteoblast-like cells in rat calvaria cell culture (Schmid et al., 1984)and fetal rat calvaria in organ culture (Canalis et al., 1988; Hock et al., 1988). Osteogenic induction activity of IGF-1 might be seen briefly in 6.25% and 25% PRP groups on the 9^{th} culture-day, when these groups demonstrated a higher ALP activity and lower cell proliferation than the activities of the PPP group. Synergistic effects of PDGF, TGF-β1 and IGF-I on mitogenesis and collagenous and noncollagenous protein syntheses were reported (Giannobile et al.,

1997; Pfeilschifter et al., 1990). IGF-I could not activate ALP activity in presence of PDGF and TGF-β1 (Giannobile et al., 1997). Interactions of these growth factors may underline effects of PRP on proliferation and differentiation of cells in osteoblast lineage. The effect of PRP on protein synthesis was not performed in this study.

A lack of osteoinductive property of PRP is supported by clinical reports that there is no additive effect of PRP on the bone regeneration process when it is implanted with inorganic bone matrix (without autogenous bone). The results are not superior to results from conventional method of inorganic matrix implantation with guided bone regeneration (GBR) (Froum et al., 2002; Lekovic et al., 2002; Shanaman et al., 2001), however, no negative effect was reported (Kassolis et al., 2000).

3. An implantation in nude mice, *in vivo* study

The inhibition effects of PRP on osteogenic differentiation and strong osteogenic induction of BMP-2 are clearly demonstrated in this part of the study. BMP-2 induced ectopic bone formation in a dose dependent manner and enhanced osteogenic differentiation of implanted bone marrow cells. An implantation of bone marrow and PRP induced the minimum amount of new bone formation. The stimulating effect of PPP was not clearly seen in this study. The amount of bone formation between groups of bone marrow and whole blood and bone marrow and PPP was not significant. A combination of one microgram of BMP-2 and bone marrow induces new bone formation in a higher amount than the amount of bone formed in 3 μg of BMP-2 group and bone marrow with whole blood and PPP groups.

The *in vivo* results support an inhibitory effect of PRP in the *in vitro* study. PRP has no osteoconductive effect and inhibits differentiation of the mesenchymal stem cells into osteoblastic lineage. It is postulated that high concentrations of PDGF, TGF-β1 and EGF inhibit osteogenic differentiation of mesenchymal stem cells. PDGF and TGF-β1 might impair the osteogenic function of IGF-I to activate osteoblastic differentiation (Giannobile et al., 1997; Pfeilschifter et al., 1990).

It is in agreement with previous reports that BMP-2 is able to induce osteogenic differentiation of non-specific mesenchymal cells residing in skeletal muscle and the effect is dose dependent (Kim et al., 2002; Kubler et al., 1998; Kusumoto et al., 1997; Yoshida et al., 1998). BMP-2 induce new bone formation

through the endochondral bone formation process (Kubler et al., 1998; Murata et al., 1998) but cartilage formation was not found in this study. It is possible that endochondral ossification was complete before 4 weeks after implantation and a rich vascularization of the intramuscular site promoted ossification of cartilage and intramembranous bone formation (Omura et al., 1998).

In groups implanted with 3 and 10 μg BMP-2, mature woven bone trabeculae were found surrounded by well differentiated bone marrow with hemopoietic cells and adipose tissue. Most of the bone matrix was laid down directly on the surface of scaffolds and tended to be on peripheral areas. It is postulated that BMP-2 released from ICBM scaffold induces osteoblastic differentiation of mesenchymal stem cells residing in skeletal muscle and these cells laid down bone matrix directly on the structure of the scaffold. Lyophilized BMP-2 on structure of ICBM was expected to be gradually released from collagen fibers of scaffolds into serum or tissue fluid and interact with adjacent mesenchymal stem cell residing in skeletal muscle. Lyophilized BMP-2 was expected to be gradually released from collagen fibers of scaffolds into serum or tissue fluid and interact with adjacent mesenchymal stem cell residing in skeletal muscle in a dose dependent fashion. A passively diffused BMP-2 induced additional osteogenic differentiation within pore space of scaffolds.

Providing that surface of collagen matrix and its structure was favourable to cell growth and differentiation, differentiated cells attached, grew and secreted its extracellular matrix directly on surface of collagen fibers. This leads to direct mineralization of matrix on surface of the scaffold. Additionally, Collagen matrix of ICBM scaffold could play a significant role in facilitating direct mineralization of structure of the scaffold. ICBM scaffolds form templates for osteogenic replacement and mineralization. Amorphous ground substance binds fibers of type I collagen. A staggering of double helix collagen molecule creates holes and pores within structure of the collagen. These pores and holes are initiation sites of matrix vesicles and heterogenous mineralization. This zone harbours 50% of hydroxyapatite crystal deposited in bone (Bostrom et al., 2000). Matrix formation occurs at the interface between osteoblasts and existing osteoid and mineralization occurs at junction of osteoid and newly mineralized bone or at mineralization front (Jee, 1996), therefore, bone was formed directly on surface of the scaffold.

The pattern of bone formation in bone marrow implantation groups was different from that of the BMP-2 group. In addition to differences in terms of locations of bone formation related to the structure of the scaffold, origins of bone forming cells and bone formation processes were different. Implanted cells formed new bone within pores of the scaffold and mainly through intramembranous bone formation. It was found that most of the bone formation in implanted bone marrow with whole blood or PPP was in a mature state and a small area of cartilage was found. In the PRP group, undifferentiated mesenchymal cells and unorganized woven bone trabeculae rimmed with osteoblast cell lining were obviously seen in a larger area compared to that of the other two groups. Cartilage was hypertrophic and undergoing ossification in the PRP group. Histological patterns of PRP specimen demonstrated bone formation in an earlier state than bone formation in whole blood and PPP groups. Histology of bone formation supports radiographic interpretation that PRP inhibits bone formation of implanted bone marrow in a heterotopic site. The evidence supports an assumption that PRP inhibit osteogenic differentiation of mesenchymal stem cells in bone marrow and has no osteoinductive effect.

This study confirms results in Part I of the study that osteoprogenitor cells and multipotential mesenchymal stem cells reside in bone marrow. These cells survived after transplantation and differentiated into osteoblasts and chondroblasts and formed bone and cartilage *in vivo*. It was reported that osteoblasts secreted BMP-2, TGF-β1, IGF-I and IGF-II (Baylink et al., 1993; Bostrom, 1998; Si et al., 1997). These osteogenic proteins might enhance osteogenic differentiation of bone marrow cells in whole blood and PPP groups. In the PRP group it is postulated that PRP inhibits osteogenic differentiation of mesenchymal cells residing in bone marrow. Therefore, these implanted cells could not produce autogenous osteogenic proteins as differentiated cells in whole blood and PPP groups could in the same period of implantation time. The lack of autocrine and paracrine effects might lead to further impediment on osteogenic differentiation of cells in the PRP group.

The ostegenic induction effect of BMP-2 on bone marrow cells can be seen in an implantation of bone marrow with 1 μg of BMP-2. In this groups, the amount of bone formation was higher than those of whole blood or PPP groups. This could be a result of osteogenic induction of BMP-2 on mesenchymal stem cells and

osteoprogenitor cells in bone marrow. BMP-2 is a strong osteogenic inducer and able to induce osteogenic differentiation of mesenchymal stem cells in skeletal muscle (Kubler et al., 1998; Murata et al., 1998). Bone formation of bone marrow with 1 μg of BMP-2 group should derive from both implanted cells and host cells. The result suggests that BMP-2 and implanted bone marrow has a synergistic effect on osteogenic induction *in vivo*. This application will enhance the bone formation process of implanted bone marrow and it is able to reduce the dose of implanted BMP-2 leading to a decrease in expenses and risks of using supplementing artificial growth factors.

4. New bone formation and mineralization processes

4.1. Bone formation

As we can see from the first and second parts of this study that implanted bone marrow cells induced new bone formation mostly through intramembranous bone formation, in which osteogenic precursor cells differentiated directly to osteoblasts. These osteoblasts laid down bone matrix and the matrix was subsequently mineralized and forms a network of immature trabeculae or woven bone. Osteochondrogenesis was found in few areas, where cartilaginous matrix was formed and gradually replaced by mineralized woven bone. Woven bone was replaced by secondary bone or removed to form bone marrow (Bostrom et al., 2000; Jee, 1988). However, a few osteoclasts were found in this study. It is postulated that the specimens were examined when bone remodelling process was completed, at implantation day 28^{th}, and osteoclasts were already disappeared. Additionally bone remodelling in this study was a slow and happened in a minor degree, therefore, few osteoclasts were invloved. What can be seen at 28^{th} and 42^{nd} implantation days was an inactive bone formation process with flatten osteoblast cell lining on few bone trabeculae.

4.2. Mineralization of bone matrix

Bone mineralization of hard connective tissue involves the matrix vesicle and heterogenous nucleation. Matrix vesicle exists only in relation to initial mineralization. The vesicle is a small membrane-bone structure that buds off from the cell to form an independent unit within the first formed organic matrix of hard tissue.

The vesicle provides within itself a microenvironment that permits the initial formation of a crystal of apatite. In heterogenous nucleation, concentrations of calcium and phosphate ions are increased in holes and pores of collagen fibers. Calcium, inorganic phosphate and anionic phospholipids bind to calcium to form CaPi-phospholipid complexes forming nucleation of mineralization (Ten Cate, 1994).

Mineralization of skeletal tissue has two distinct phases, formation of the initial mineral deposit at multiple discrete sites and accretion of additional mineral crystal on the initial mineral deposits or growth of mineralized vesicle. Additional mineral deposit is deposited on initial nucleation and on nuclei of existing hydroxyapatite already contained within the pore and holes of the collagen fiber. Mineralized crystal expand and agglomerate to form fully mineralized bone matrix (Jee, 1988)

Alkaline phosphatase (ALP) plays role in facilitating crystal growth. ALP is found at membrane of bone forming cells and within matrix vesicle. Intracellularly, it is postulated that ALP provides phosphate ions at mineralization sites by hydrolysing phosphate ions from organic radicals at an alkaline pH. The extracellular activity of the enzyme is to cleave pyrophosphate on surface of crystal. Pyrophosphate is formed when crystal contact with serum or tissue fluid and it prevents crytal growth. Therefore, it is suggested that ALP can enhance bone formation by inhibition of crystal growth retardation due to pyrophosphate (Ten Cate, 1994).

5. A proposed scenario of osteogenesis process in bone grafting supplemented with PRP

It can be seen from the results of *in vitro* and *in vivo* studies that PRP inhibits osteoblastic differentiation of osteogenic precursor cells. These results contrast with clinical reports that show PRP promotes bone regeneration process. Based on results from this study, there is no direct evidence to suggest that PRP promotes osteogenesis process. PRP is not an alternative to replace bone morphogenetic proteins as osteoinductive protein.

Our results are not consistent with clinical reports that PRP promotes bone regeneration process (Marx et al., 1998). This might be the results of different study design. In previous report, the authors report effect of PRP which was modified and

influenced by various factors during bone remodeling process during 6 months after implantation. Our study clearly demonstrated effects of PRP on bone healing during repairing stage which was an initial step of bone formation without effects of bone remodeling process (Table 4-1). PRP was applied directly on cells in an early state of osteogenic differentiation in a strict control environment of cell culture and intramuscular implantation.

PRP delivers growth factors into a defect site in the same way as blood clotting after skeletal fracture released growth factors. PDGF, TGF-β1, IGF-I and EGF released from platelets in blood clots and PRP stimulate migration and proliferation of mesenchymal stem cells in bone marrow and osteoprogenitor cells residing in endosteum, cambium layers of periosteum and osteoblasts of implanted cancellous bone and the local host bone (Caplan, 1991; Yoo and Johnstone, 1998). PRP supplementation will create a large pool of undifferentiated cells. Hematoma induces hypoxia and close capsules capturing secreted growth factors within a cloting gel enhancing macrophage migration, angiogenesis and proliferation of osteogenic cells (Bolander, 1992; Joyce et al., 1990a; Joyce et al., 1991; Tuncay et al., 1994). These cells will first encounter with BMP-2 secreted from macrophage (Champagne et al., 2002) then they will be subsequently induced by BMP-2 and growth factors secreted by osteoblasts and released from the bone matrix (Andrew et al., 1993b; Baylink et al., 1993; Bostrom, 1998; Lane et al., 1999; Si et al., 1997; Spector et al., 2001). TGF-β1 increases expression of PDGF-BB mRNA of fetal rat calvaria (Rydziel and Canalis, 1996), IGF-I in human bone marrow cells (Kveiborg et al., 2001) and has synergistic effects with VD3 (Eichner et al., 2002; Liu et al., 1999; Rydziel and Canalis, 1996). The interaction of growth factors is speculated to be similar to interaction of growth factors in the process of fracture healing (Bolander, 1992; Brighton et al., 1992; Hollinger, 1993; Owen, 1980). It is hypothesized that the increases in the amount and rate of bone formation in bone grafting reported in PRP studies are consequences of multi-interaction of growth factors and bone remodeling.

The above scenario may explain the effects of PRP in clinical study. Nevertheless, PRP can not be a substitution for BMP-2 as the osteogenic inducer. Osteogenic effects of PRP require orchestration of local and systemic osteogenic proteins and growth factors. The effects of PRP may be more obvious and direct in

soft tissue wound healing, whereas proliferation of fibroblasts and angiogenesis facilitate wound healing.

Table 4- 1 Demonstrating effects of PRP applied in this study in relation to bone healing cascade (Bone healing cascade was modified after Kalfas (Kalfas, 2001))

Early Inflammatory stage	Repair stage	Late remodeling stage

- Hematoma-Hypoxic
- Inflammatory cells and fibroblast infiltrate
- Granulation tissue formation
	- Ingrowth of vascular tissue
	- Implanted viable osteoblast
	  - Laying down collagen matrix
	  - Secretion of osteoid
	- Creeping substitution – replacing of nonviable bone with new viable bone
	  - Migration & differentiation of mesenchymal cells
	  - **Differentiating of primitive mesenchymal cells to osteoblasts to deposit osteoid**
	  - **Laying down collagen matrix**
	  - **Secretion of osteoid**
	- **Mineralization of osteoid to form soft callus and woven bone**
		- Replacing of woven bone
		- Formation of lamellar bone
		- Restore shape, structure and mechanical strength
		← Effects of PRP