โครงการ

ผลของเกร็ดเลือดเข้มข้นต่อการเจริญเติบโตและการพัฒนาของเซลล์สร้างกระดูกอาตมันที่พัฒนามาจากเซลล์ไขกระดูกของหนูตัวเดียวกัน

โดย

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กิตติกรรมประกาศ

คณะผู้วิจัยขอขอบคุณหน่วยงาน ผู้ทรงคุณวุฒิ เพื่อนร่วมงาน และเจ้าหน้าที่ทุกท่านที่ได้ให้การสนับสนุนการดำเนินงานวิจัยนี้ทั้งในรูปของทุนการดำเนินการวิจัย การเอื้อเฟื้ออุปกรณ์วิทยาศาสตร์ ให้คำแนะนำที่เป็นประโยชน์ และช่วยเหลือในการดำเนินงานต่างๆให้สำเร็จลุล่วงไปด้วยดีดังมีรายนามดังต่อไปนี้

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หัวหน้าโครงการวิจัย

รูปแบบ Abstract (บทคัดย่อ)

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(ชื่อโครงการ) ผลของเกร็ดเลือดเข้มข้นต่อการเจริญเติบโตและการพัฒนาของเซลล์สร้างกระดูกอัตมันที่พัฒนามาจากเซลล์ไขกระดูกของหนูตัวเดียวกัน

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(คำหลัก) Heterotopic bone formation, Platelet concentrate, Mesenchymal stem cells

บทคัดย่อ- วัตถุประสงค์ การศึกษานี้มีวัตถุประสงค์ที่จะค้นหาขนาดของสารโปรตีนที่มีผลต่อการเจริญเติบโตอาตมัน (growth factors, GFs) ในเกล็ดเลือดที่มีขนาดเหมาะสมในการกระตุ้นการเจริญเติบโตและพัฒนาของเซลล์ต้นกำเนิดแบบมีเซนไคม์ในไขกระดูกโดยทำการศึกษาในสัตว์ทดลอง **วัสดุและวิธีการทดลอง** ภายใต้ภาวะสลบเลือดและไขกระดูกจะถูกเก็บจากหนูแรทขาว (Spraque-Dawly rats) ขนาด 350 – 400 กรัม จำนวน 25 ตัว เกร็ดเลือดเข้มข้น (PC) และ พลาสมาที่มีเกร็ดเลือดต่ำ (PPP) จากนั้นทำการวัดความเข้มข้นของ GFs ที่ได้จากการแตกตัวของเกร็ดเลือดโดยวิธี ELISA ไขกระดูกที่เก็บได้จะถูกนำมาเลี้ยงในน้ำเลี้ยงเซลล์ที่มีความจำเพาะสำหรับเซลล์กระดูก เซลล์ไขกระดูก (BMSCs) ที่อยู่ในระยะการเลี้ยงที่ 2 (2^{nd} passage) จะถูกนำมาตรวจสอบการแสดงออกของคุณสมบัติของการเป็นเซลล์สร้างกระดูก แล้วจึงถูกนำมาใส่ลงบนตัวรองรับสามมิติที่ทำมาจากคอลลาเจนของกระดูกวัว (Insoluble collagenous bovine bone matrix, ICBM) เพื่อนำไปฝังในกล้ามเนื้อที่ขาหลังของหนู การศึกษานี้แบ่งเป็น 5 กลุ่มการทดลอง กลุ่ม A – C ตัวอย่างที่ฝังในกล้ามเนื้อด้านซ้ายของหนูคือ ICBM, BMSCs, PPP and GFs 1, 3 และ 10 ไมโครกรัม/มิลลิลิตร ตามลำดับ ส่วนด้านขวาฝังตัวอย่างควบคุม ICBM, BMSCs และ PPP ในกลุ่ม D ฝังตัวอย่างที่ประกอบด้วย ICBM BMP-2 และ PPP ในกล้ามเนื้อด้านซ้าย และฝัง ICBM และ BMP-2 ในกล้ามเนื้อด้านขวา กลุ่ม E ซึ่งเป็นกลุ่มควบคุมทำการฝัง ICBM และ PPP และ ICBM อย่างเดียวในกล้ามเนื้อด้านซ้ายและขวาตามลำดับ โดยในแต่ละกลุ่มมีหนู 5 ตัว ทำการวัดอัตราเร็วของการสร้างกระดูกโดยการวิเคราะห์การติดสารเรืองแสง และวัดปริมาณการสร้างกระดูกใหม่ด้วยเครื่องคอมพิวเตอร์สามมิติขนาดเล็ก (micro computer tomography, micro-ct) **ผลการศึกษา** การศึกษานี้พบว่าความเข้มข้นของ TGF-beta1 มีความสัมพันธ์อย่างมีนัยสำคัญกับจำนวนของเกร็ดเลือดใน PC BMSCs สามารถพัฒนามาเป็นเซลล์สร้างกระดูกในระยะตัวอ่อนและตัวแก่และสร้างกระดูกในกล้ามเนื้อได้ ปริมาณการสร้างกระดูกที่สูงที่สุดและอยู่ในระยะที่มีการพัฒนาของการสร้างกระดูกสูงสุดพบในกลุ่ม D ที่มีการฝัง BMP-2 ร่วมกับ PPP ตามมาด้วยการฝัง BMP-2 **สรุป** ตัวรองรับสามมิติที่เป็นรูพรุนที่ทำมาจากคอลลาเจนของกระดูกวัวและไฟบรินเจลที่ได้จากพลาสมาที่มีเกร็ดเลือดต่ำเป็นองค์ประกอบของของโครงร่างสามมิติที่สำคัญในการนำส่งเซลล์และสารโปรตีนเข้าสู่บริเวณที่ต้องมีการซ่อมแซมกระดูกและยังมีส่วนช่วยส่งเสริมการเจริญเติบโตของเซลล์ นอกจากนี้ไฟบรินเจลส่งเสริมการสร้างกระดูกของ BMP-2 การศึกษานี้ได้แสดงให้เห็นอย่างชัดเจนว่าเกร็ดเลือดสามารถทำหน้าที่เป็นแหล่งของสารโปรตีนส่งเสริมการเจริญเติบโตอาตมันของเซลล์กระดูกแต่ผลของขนาดของสารโปรตีนที่มีต่อการส่งเสริมการเจริญเติบโตของเซลล์กระดูกไม่สามารถระบุได้จากการศึกษานี้

คำหลัก เซลล์เนื้อเยื่อไขกระดูก สารโปรตีนส่งเสริมการเจริญเติบโตอาตมัน การสร้างกระดูกในกล้ามเนื้อ

Abstract— This study aimed to determine range of effective doses of autologous growth factors from platelet concentrate on osteoblastic differentiation of autologous differentiated rat bone marrow stromal cells (BMSc) in vivo. **Materials and Methods**: Under general anesthesia blood and bone marrow were collected from 25 male Spraque-Dawly rats (350-400 grams). Platelet concentrate (PC) and platelet poor plasma (PPP) were prepared using open tube centrifugation technique. Autologous growth factors (GFs) were obtained from activated platelet concentrate (PC). Amount of TGF-β1 in the GFs was quantified using ELISA. Bone marrow was cultivated in osteogenic culture medium. Osteoblastic differentiation of bone marrow stromal cells (BMSCs) in the 2nd passage was characterized. Differentiated BMSCs were seeded on collagenous bovine bone matrix (ICBM) and implanted intramuscularly in tight muscle of rats. The study was categorized into 5 groups, Groups A-C, ICBM, PPP and BMSCs with different concentrations of TGF-β1 in GFs, 1, 3 and 10 ng of TGF-β1 were implanted on left side and BMSCs without GFs were implanted on right sides. In Group D, ICBM and bone morphogenetic protein-2 (BMP-2) with and without PPP were implanted on right and left sides, respectively and Group E, ICBM with and without PPP were implanted on right and left sides, respectively. Rate of bone formation was investigated by detecting sequential fluorochrome deposition and amount of heterotopic bone formation were investigated using micro-computer tomography (micro-CT). **Results**: It was found that concentrations of TGF-β1 in platelet concentrate were moderately correlated to numbers of platelets. BMSCs differentiated into pre-and mature osteoblasts in vitro and formed heterotopic bone. The highest amount of new formed bone was found in Group D on right side, BMP-2 with PPP. **Conclusions**: A tissue construct of collagenous bone matrix and autologous fibrin was an effective carrier of cells and osteogenic proteins in vivo. Fibrin gel enhanced osteogenic induction potential of BMP-2. Platelets are a potential source of autologous growth factors to be applied in bone tissue engineering, but a range of effective doses of autogenous growth factors on heterotopic bone formation was not found.

Keywords—Bone marrow stromal cells, Autogenous growth factors, Heterotopic bone formation

Effects of autologous platelet concentrate on growth and differentiation of osteoblasts derived from bone marrow stromal cells of rat, an *in vivo* study

(ผลของเกร็ดเลือดเข้มข้นต่อการเจริญเติบโตและการพัฒนาของเซลล์สร้างกระดูกอัดมันที่พัฒนามาจากเซลล์ไขกระดูกของหนูตัวเดียวกัน)

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Introduction

Oral and maxillofacial surgery frequently encounters defects in bone due to disease, trauma, tumours or physiologic bone loss (Manson *et al*, 1986). One strategy to improve bone healing is to deliver living osteoblasts into skeletal defects with and without osteogenic proteins or growth factors to initiate and stimulate bone regeneration process (Bruder *et al*, 1998a;Caplan & Bruder, 1966). Therefore it is essential to be able to cultivate osteoblast-like cells and implant them *in vivo*. Interaction between cells and extracellular matrix on three-dimensional scaffold regulates growth and differentiation of osteoblasts (Sampath & Reddi, 1984). Bone morphogenetic proteins (BMPs) are known to be strong osteoinductive proteins (Kubler *et al*, 1998;Ripamonti *et al*, 2001).

It is proposed that platelet-rich plasma (PRP) delivers growth factors contained in platelets, 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) (Bruder *et al*, 1998a;Gunsilius *et al*, 2000;Landesberg *et al*, 2000;Marx *et al*, 1998). These growth factors activate proliferation and osteoblastic differentiation of mesenchymal stem cells and cells of osteoblastic lineage (Bruder *et al*, 1998a;Gunsilius *et al*, 2000;Landesberg *et al*, 2000;Manson *et al*, 1986;Marx *et al*, 1998). Platelet-rich plasma (PRP) has been widely applied in clinical cases and its effect on promoting bone formation is promoted in the literatures (Anitua, 1999;Carlson, 2000;Kassolis *et al*, 2000;Landesberg *et al*, 2000;Marx *et al*, 1998).

However, the experiments investigating effects of PRP to guide tissue regeneration with and without bone graft (Camargo *et al*, 2002;Lekovic *et al*, 2002;Shanaman *et al*, 2001) and experiment in non-critical size defect of rabbits (Aghaloo *et al*, 2002) raise the question whether the good clinical results are primarily based on an improved osseous regeneration or more likely due to the formation of a fibrin gel and the stimulation of the surrounding connective tissue leading to a faster and improved soft tissue closure.

As it is reported that growth factors released from platelets, PDGF, TGF-ß1 and IGF-I promote survival or inhibit apoptosis of osteoblasts (Hill *et al*, 1997;Jilka *et al*, 1998;Meleti *et al*, 2000). It might be possible that these growth factors in PRP

inhibit apoptosis of implanted osteoblasts resulting in promoting bone formation. There is no previous study exploring effects of platelet concentrate on survival of implanted osteoblasts.

A small range of effective threshold of platelet concentrate in medium level demonstrates positive effects on bone regeneration surrounding dental implant, not the highest concentration(Weibrich *et al*, 2003). When platelets are degranulated with freeze and thaw method, amount of released growth factors does not relate to concentration of platelets, age or gender of each donor. There might be other factors contributing to amount of growth factors in platelet concentrate(Weibrich *et al*, 2002a). Differences in growth factor contents and platelet count are found in platelet concentrate prepared from different methods(Weibrich *et al*, 2002b).

These recent publications suggest substantial variation in growth factors contents of platelet rich plasma and its clinical application. They question a concept of applying the highest concentration of platelets into the defect site and also the reliability of clinical application of platelet-rich plasma. Using "platelet concentrate" instead of PRP conveys more specific clinical application of various concentrations of platelets. The dose of platelet concentrate required to achieve the intended biological effects still remain unclear, as it should be determined by amount of growth factor contents. Applying a known amount of growth factors released from concentrate platelet in correlation to numbers of platelets and numbers of target cells or volume of defect site might be a useful indirect guideline for selecting effective dose of platelet concentrate.

The study aimed to (1) Investigate dose dependent effects of autologous growth factor contents released from platelet concentrate on rate and amount of new bone formation formed by implanted autologous osteoblasts derived from bone marrow stromal cells. The results will be compared with osteoinductive effects of BMP-2 and (2) to determine range of effective dose of TGF-β1 in growth factor contents promoting growth and differentiation of osteoblasts.

Materials and Methods

1. Preparation of insoluble collagenous bovine bone matrix (ICBM)

Bovine spongiosa was defatted in chloroform and methanol 3:1 solution and demineralised in 0.5 M HCL. Extracellular matrix protein was extracted in 4M Guanidien hydrochloride in 50 mM Tris-HCL, pH 7.4 at 4°C.

The matrix was cut in a designed size, 5x10 mm cylinder and lyophilized at -45°C for 24 hours. The scaffolds was sterilized in chloroform and methanol, 1:1 solution for 12 hours (Kubler *et al*, 1998).

2. Groups of study

The study was categorized into 5 groups, Groups A - C: ICBM with cells, PPP and supernatant of degranulated PC containing TGF-ß1 10, 5 and 1 µg, respectively; Group D: ICBM with 10 µg BMP-2 and Group E: ICBM without cells with and without PPP. In Groups A – D, results was compared with ICBM with cells and PPP which was implanted on the right hand side, while the experimental samples were implanted on left hand side. Numbers of rats in each group were 5 rats per group (Table 1).

3. Blood collection

Xylazine hydrochloride 5 mg/kg and Ketamine hydrochloride, 120 mg/kg were intraperitoneally injected to induce general anesthesia. Whole blood was drawn from orbital sinus, 2.0 ml per rat (≤ 10% of total blood volume) into sterile pasture pipette and immediately transfer into 15.0 ml tubes containing sodium citrate as anticoagulant (Animal care unit, 1996).

4. Platelet concentrate preparation

Platelet was subjected to centrifugation at 3000 g at RT for 10 minutes, then layers of plasma, white blood cells (WBC) and red blood cells (RBC) on the upper surface (buffy coat) were collected for further centrifugation. Plasma on the upper part was collected as *platelet poor plasma (PPP)*. The collected buffy coat was centrifuged at 300g in RT for 10 minutes. Concentrate platelets, white blood cells (WBCs) and upper part of red blood cells (RBCs) located in the middle layer of the centrifuge were separated as *platelet concentrate (PC)*. Plasma located on the upper part of the tube was separated as plasma poor platelet (Landesberg *et al*, 2000).

5. Characterization of platelet concentrate

5.1. Platelet counting

Thc numbers of RBCs, WBCs and platelets in whole blood, platelet concentrate and plasma poor platelet were counted using electronic blood counter. Platelet concentrate was diluted with isotone into 1:10 concentration for counting.

5.2. Platelet staining

Whole blood and platelet concentrate were also smeared on a glass slide and stained with May-Gruenwald and Giemsa stainings. A bright blue colour of small granules or platelets, a light browny red of larger round shape RBCs and various types of WBCs were observed under light microscope (Landesberg et al, 2000;Marx et al, 1998) (Table 2).

6. Activation of platelets and measuring of growth factor contents

Platelets were activated by repeating freezing and thawing procedure and centrifuged at 3000 rpm for 30 min. Supernatant of degranulated platelets and plasma poor platelet of each rat were kept separately at -70°C until use.

TGF-ß1 content in platelet concentrate was detected using ELISA assay (TGF-β1 detection kit, ELISA, R&D system). Average concentrations of platelets in whole blood and platelet concentrate and TGF-ß1 content of one million platelets from every donor were calculated. Concentrations of TGF-β1 in samples were correlated with numbers of platelets in the samples (Landesberg *et al*, 2000).

7. Bone marrow harvesting

Under general anaesthesia as stated in blood drawing section, in a supine position, skin of inner thighs was shaped and painted with disinfectant solution and draped. The femoral diaphysis was exposed and a small defect was created using drill and cutting bur (Bidic *et al*, 2003). Bone marrow was aspirated and kept separately in sterile container and transferred to cell culture laboratory.

8. Bone marrow cell culture

Bone marrow cells from each donor were separately cultivated in mineralized culture medium, DMEM-F12 supplemented with antibiotics, 10% fetal bovine serum, ascorbic acid and ß-glycerophosphate, and 20 nM dexamethasone and 20 nM 1,25 α cholecalciferol (VD3) (Cheng *et al*, 1994). Differentiated bone marrow stromal cells (BMSCs) at 80% confluence in first passage were randomly selected and characterized for expressions of osteoblastic phenotypes then at confluence, cells were seeded on 5x3 mm ICBM, $5x10^5$ cells/matrix. The construct was further

cultivated in serum free culture medium for 24 hours before they were implanted (Figure 4). Characterization of osteoblastic phenotypes of differentiated cells

The differentiated cells were detected for expressions of ALP activity and expressions of type I collagen and osteocalcin proteins in extracellular matrix (Aubin, 1998;Wang & Glimcher, 1999).

9. Preparing constructs for implantation

Fifty microliters of plasma poor platelet were added on every scaffold. Supernatant from degranulated platelets containing 1 – 10 ng/ml of TGF-ß1 was supplemented in each 50 µl of platelet poor plasma to create a composite of growth factor contents and plasma poor platelet. The composite was activated with thrombin and calcium chloride solution to form gel like substance and coated on all surface of the scaffold to form gel-like capsule. The constructs were cultivated in serum free culture medium for 1 day before implantation.

10. Intramuscular implantation in rats

Rats were anesthesized with intramuscular Ketamine (100 mg/kg) and Xylazine (5 mg/kg). Amoxycillin (200,000 IU/kg) were given intramuscularly for prophylaxis against infection.

Intramuscular pockets were created in calf muscles, on the upper inner tight, of rats on right and left sides. The construct was placed in the pocket, one sample per one pocket, as stated in a study design section. Muscles enclose implanted material and skin will be closed in layers using Vicryl 4-0 and 4-0 silk sutures, respectively. Topical antibiotic ointment was applied on the suture line.

Rats were kept overnight in recovery area before they were returned to their cages. Post-operatively, analgesic drug (NSIAD) was mixed in drinking water.

11. Fluorochrome labelling

On implantation day 5, 11, 17 and 23 the following fluorochromes were injected subcutaneously, xylerol orange (90 mg/kg), calcein (15 mg/kg), tetracycline (50 mg/kg), and xylerol orange (90 mg/kg) respectively.

12. Sacrification

At 4 weeks, rats were sacrificed by anaphylaxis with carbon dioxide and specimen were harvested and fixed in 10% neutral formalin.

13. Evaluations of new bone formation of implanted specimens (Table 3)

13.1. Micro-tomographic (micro-CT) analysis

All specimens were examined using Micro-CT (Skyscan, The BIOMAT unit, Faculty of Engineering, The National University of Singapore). Area, pattern and distribution of new bone formation within scaffold were examined using various sections and 3-D reconstruction of micro-CT. Amount of new bone formation and numbers and thickness of bone trabeculae were analysed (Kubler *et al*, 1998) (Table 3).

13.2. Histological analysis of implanted specimens

13.2.1. Preparation of histological specimens

Specimens were cut in coronal direction through middle part of the specimens. One half was undecalcified and embedded in a polymethylmethracrylate resin and another half was decalcified and embedded in paraffin.

In decalcified specimens, the specimens were fixed for 2-3 days in 10% neutral formalin and followed by decalcification in a EDTA-HCL. This protocol results in adequate decalcification, without loss of antigenic sites of immunohistologic studies (Mullink *et al*, 1985) (Table 3).

13.3. Undecalcified specimens,

Undecalcified sections were ground to 40 μm for *examining of fluorochrome labellig* under fluorescence microscope and 7 μm for bone cell staining using H&E, von Kossa and Giemsa staining examined under light microscope (Donath & Breuner, 1982).

14. Data analysis

14.1.1. Qualitative data

Histological finding of new bone formation and patterns of fluorochrome labelling. The examinations were qualitatively described.

14.1.2. Quantitative data

Concentration of platelet, amount and concentration of growth factor contents, volume of new bone formation, total bone volume, numbers of trabeculae and thickness of bone trabeculae were presented as average values (mean ±SEM)

Statistical analysis of those values were performed by analysis of variance (ANOVA), supplemented with either Fisher's PLSD or Scheffe's post hoc test when appropriate for multiple comparisons. A p value of <0.05 is regarded as significant.

Results

1. **Bone marrow cell harvesting**

Autogenous bone marrow harvesting, osteogenic induction of rat bone marrow cells *in vitro* and implantation of autogenous bone marrow cells were established. Differentiated bone marrow cells were successfully implanted into tight muscle of rats (Figures 1 - 3).

2. Characterizations of platelet concentrate

Numbers of platelets (plt) and white blood cells (WBC) and levels of TGF-beta1 were evaluated from total of 60 samples.

It was found that numbers of platelets in platelet concentrate, $42.07\pm24x10^3/\mu l$, was higher than numbers of platelets in whole blood with an average ratio of 7.7±4.5 times. Average concentration of platelets in PPP was 700 and 160 times less than concentration of whole blood in PC and whole blood respectively. Platelets smear demonstrated a dense distribution of platelets in platelet rich plasma (Figure 4). WBC contamination was found in every sample with an average number of $5.14x10^3$ cells/μl. Differential blood count was not performed in PC and PPP because of a high cost of laboratory service.

A release of TGF-beta 1 from degranulated platelets and lysed WBC was 244.97±21.62 ng/ml. A moderate correlation between numbers of platelets and concentrations of TGF-beta 1 was found ($r^2=0.621$, $p<0.001$), but correlation between concentration of TGF-beta 1 and numbers of WBC was not found ($r^2=0.069$, $p>0.05$) (Table 2).

This investigation demonstrates that autogenous growth factors applied in this study was obtained from platelet concentrate and contained TGF-beta 1. Because contamination of WBC was found in all samples, TGF-beta 1 released from lyzed WBC might partially contribute to amount of TGF-beta 1 released from degranulated platelets. However, significant correlation between numbers of WBC and concentration of growth factor was not found.

3. Bone marrow cell culture and osteogenic differentiation *in vitro*

Bone marrow stromal cells differentiated into mature osteoblast and adipocyte *in vitro*. Differentiated cells demonstrated positive staining of ALP activity (Figure 5) and von Kossa staining (Figure 6).

Co-differentiation of fats cells were found mostly in central part of cell nodules. Fat cells had lipid droplets (stained bright red) in their cytoplasm. Most of cells demonstrated positive staining for ALP activity but with different levels of intensity (Figure 7).

4. Alkaline phosphatase activity (ALP) of differentiated rat bone marrow cells in 2nd passage (RBM-P2) on two-dimensional surface of cell culture plate

Rat bone marrow cells in a second passage, $5x10^4$ cells, were seeded on 35 mm plate and cultivated in mineralized culture medium supplemented with 10 nM dexamethasone for 15 days. Levels of ALP activity were analyzed from triplicate samples.

RBM-P2 at 24 hours after cell seeding had high level of the activity around 700 unit/mg proteins. ALP activity reached the highest level on day 3 then it was gradually decreased. This demonstrates a rapid progress of osteoblastic differentiation of cells from pre-osteoblasts to be mature osteoblasts in the second passage. It is expected that during 6 – 15 culture-day an expression of marker of mature osteoblast such as osteocalcin should be seen (Figures 8).

5. Comparing effects of serum free culture medium to growth and osteoblastic activity of cells

In order to reduce tissue reaction of host to implanted construct, cells and collagenous matrix, cells were seeded on scaffolds in culture free medium and cultivated in serum free culture medium for 24 hour before implantation. In order to investigate effects of serum free culture medium to growth and differentiation of implanted cells, rat bone marrow cells in second passage, $5x10^4$ cells, were seeded on 5x3 mm collagenous scaffold in serum free culture medium. At 48 hours after seeding cell vitality (MTT assay) and ALP activity were measured and neutral red vital stained was performed to demonstrated attachment of cells on scaffold.

It was found that growth of cells in serum free culture medium was significantly less than cells in culture supplemented with 10% FBS (Figure 9). ALP activity of cells in serum free culture medium was not significantly different than the activity of cells in culture medium supplemented with serum (Figure 10). Therefore, it can be sure that an incubation of seeded cells in serum free culture medium can be used in preparing cells for implantation.

6. Attachment and distribution of cells on three-dimensional scaffolds, ICBM

Neutral red vital stain and FDA staining were performed to demonstrate attachment and distribution of cells on the scaffolds. Rat bone marrow cells in second passage, $5x10^4$ cells were seeded on the scaffolds and incubated in culture medium supplemented with 10% FBS for 24 hours. The staining demonstrates well attachment and growth of cells into inner porous structure of the scaffolds. It was found that attachment and growth of cells were limited mostly on upper surface and peripheral area of the scaffolds (Figures 11 -14).

When cells were seeded on the scaffolds with fibrin gel for implantation, SEM images demonstrated round shape-cells embedded in fibrin network within porous structure of the scaffolds (Figure 15).

7. Osteoblastic differentiation of BMSCs in third passage on three-dimensional scaffolds, ICBM

7.1. Expressions of alkaline phosphatase activity (ALP) and osteocalcin

Differentiated rat bone marrow cells of three rats (numbers 125, 160 and 182) in third passage were cultivated separately in mineralized culture medium supplemented with 10 nM dexamethasone for 27 days. Levels of ALP and osteocacin were detected every 3-4 days and on culture-days 16 and 27, respectively. The investigations of each rat were performed in three samples at each investigation time.

Level of ALP was gradually increased on culture-days 2 – 12, after that the increasing rate was accelerated. The highest level (539.16±117.85 μM/mg protein) was found on culture-day 23 (Figure 16). A pattern of expression of ALP activity of cells from each rat was similar, but levels of the expression were varied individually. On culture-day 23, level of ALP of rat numbers 125 was significantly higher than rat number 182 ($p<0.05$) (Figure 17).

7.2. Expression of osteocalcin

Expression of osteocalcin in culture medium was found on culture-day 16 and 27. Average level of osteocalcin in culture medium on days 27 (2.67±1.21 ng/mg protein) was higher than on day 16 (2.01±0.56 ng/mg), but it was not significantly different ($p>0.05$) (Figures 18 and 19). Individual variations of expression were found as it was found in expression of ALP activity (Figure 16).

8. Characterization of expression of osteoblastic phenotypes of implanted rat bone marrow cells

8.1. Levels of ALP activity

Autogenous differentiated rat bone marrow cells in third passage were implanted in 25 rats and cells from 18 rats (51 % of total samples) were investigated for expression of ALP activity.

It was found that levels of ALP expression were varied individually with a range from 8.6-71.6 μM/mg protein. Average level of expression was 25.5±3.72 μM/mg protein and median was 17.62 μM/mg protein (Figures 20 and 21).

8.2. Expression of osteocalcin

The implanted cells of 10 rats (28.6% of total samples), $5x10^4$ cells/rat, were cultivated on 35 mm cell culture plate in mineralized culture medium for 5 day. Low levels of expression of osteocalcin in culture medium were found in every sample with a range of 0.75-3.99 ng/mg protein with an average level of 2.1 ± 1.1 ng/mg proteins (Figure 22). Median of expression was 1.81 ng/mg proteins (Figure 23).

These finding demonstrates that implanted differentiated rat bone marrow cells were osteoblast-like cells and these cells were able to differentiate into mature osteoblasts.

9. Heterotopic bone formation

It was found that implanted bone marrow was able to form bone in heterotopic site, however amount of bone formation was small and it was individually varied. There was no evidence of new bone formation in a control group of implantation of ICBM without cells. Porous structures of scaffolds were filled with fibrous tissue, blood vessel and inflammatory cells (Figure 24). Infiltration of chronic inflammatory cells and multiple nucleated giant cells were found in all samples. Multinucleated

giant cells and islands of granulomatous tissue surrounding foreign body were found scattering within the scaffolds.

Bone formation capacity of implanted cells was inconsistent, but they did formed new bone (Figure 25). It is clearly demonstrated that Recombinant bone morphogenetic protein-2 (rhBMP-2) was able to induce ectopic bone formation and new bone formation process was in advance stage that differentiated bone marrow could be seen (Figure 26 and Table 4). Bone formation in groups of implanted cells and BMP-2 was intramembranous bone formation. Endochondral bone formation was not seen (Figures 25 - 27). Infiltration of chronic inflammatory cells and multiple nucleated giant cells were found in all samples, particularly on peripheral area of the scaffolds (Figure 28).

10. Micro computer tomography analysis (micro-CT)

Micro-CT analysis was performed using threshold level at 165 grey levels. Three-dimensional reconstruction and tomographic sections demonstrated dense bone formation on peripheral and outer surface of the scaffolds, particularly implanted BMP-2 group (Figures 29 – 31).

The ICBM scaffolds had a total volume of 58.88 mm^3, total pore volume 41.22 mm^3 and porosity (pore volume/total volume) 0.70 (Figure 29). It was found that fraction of bone formation in implanted cells group was minimum (0.025±0.006) and markedly less than bone formation in BMP-2 groups (0.133±0.1) ($p<0.01$). Amount of bone formation in groups of cells with PC and PPP was not significantly different ($p>0.05$). Amount of bone formation and numbers and thickness of bone trabeculae of implanted BMP-2 groups were significantly higher than implanted cells groups ($p<0.01$). Amount of bone formation of BMP-2 with PPP (0.203±0.014) was significantly higher than BMP-2 only (0.133±0.010) ($p<0.05$), but numbers and thickness of bone trabeculae were not significantly different ($p>0.05$) (Figures 30 - 32).

Greater amount of bone formation in implanted BMP-2 group was further supported by significantly higher numbers and thickness of bone trabeculae of BMP-2 group than implanted cell group ($p<0.05$), but there was no differences between implanted BMP-2 with and without PPP (Figures 33 and 34).

11. Fluorochrome labeling

Sequential fluorochrome labeling of xylenol orange (bright red) (day 5), calcein (bright green) (day 11), tetracycline (light blue) (day 17) and xylenol orange (bright red) (day 23) demonstrated sequential osteoid deposition. The largest and brightest band of fluorochrome deposition in all specimens was green color of calcein. Faint staining of red and blue were found in all specimens. This demonstrated that the most active bone formation period is during 11-17 days. Deposition of osteoid was found directly on surface of scaffolds and within porous structure on outer surface of scaffolds.

Discussion

In this study platelet concentrate was prepared using open tube centrifugation technique containing high concentrations of platelets of more than 7 times greater than whole blood. It is clearly demonstrated that platelet concentrate can be a source of autogenous growth factor to be applied in skeletal defects (Landesberg et al., 2000). 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 resulting in increasing of rates and amounts of bone regeneration (Marx *et al.*, 1998).

Application of PRP or platelet concentrate into skeletal defects is a mean to apply high concentration of those growth factors directly on osteoblasts and precursor cells residing in autogenous bone graft or surrounding tissue. These growth factors activate proliferation and osteoblastic differentiation of mesenchymal stem cells and cells of osteoblastic lineage resulting in increasing of rates and amounts of bone regeneration. Platelet concentrate should be at least 4 times higher than whole blood (Marx et al., 1998). In this study platelet concentrate was prepared using open tube centrifugation technique containing high concentrations of platelets of more than 7 times greater than whole blood. It is clearly demonstrated that platelet concentrate can be a source of autogenous growth factor to be applied in skeletal defects and concentrations of released growth factors contents were directly related to numbers of platelets (Table 2).

This study further developed an application of platelet concentrate by controlling amount of growth factors applied in the defects. Supernatant of

degranulated platelets with known amount of growth factors was added into platelet poor plasma (PPP). PPP functioned as carrier of growth factor contents and BMP-2.

The study model was applied to test dose dependent effects of platelet-rich plasma on growth and differentiation of implanted osteoblasts. Concentration of TGF-β1 in supernatant of degranulated platelets was chosen as a representative concentration of growth factor contents in the supernatant, because TGF-β1 had a prominent role in enhancing bone formation and detecting tool for other growth factors in rat blood was not available. According to Landesberg and coworkers (Landesberg *et al.*, 2000) it is assumed that levels of concentrations of other growth factors, PDGF and IGF-I, will be in parallel fashion as concentration of TGF-β1.

A study model was designed to facilitate *ex vivo* cells transfer into *in vivo* environment, resemble cell transferring feature in cancellous bone grafting, where osteoblasts attaching on internal porous structure of cancellous bone and allow direct supplementation of growth factors on implanted osteoblasts. Autologous adult mesenchymal stem cells were harvested and expanded *in vitro* and re-implanted *in vivo*. Differentiated osteoblasts were seeded on three-dimensional scaffolds, insoluble collagenous bovine bone matrix (ICBM). This construct allowed direct contact between supplemented growth factors and targets cells, osteoblasts, mimicking supplementation of PRP in autogenous bone grafting.

It is clearly demonstrated that bone marrow of adult rats contains mesenchymal stem cells that were able to differentiate into osteoblasts *in vitro* and in *vivo*. Differentiated BMSCs expressed osteoblastic phenotypes of pre-and mature osteoblasts, ALP activity, mineralized extracellular matrix and secreted osteocalcin (Figures 5 – 6) (Miniatopoulos et al., 1988) and could differentiate to adipocytes as co-differentiation of osteolasts and adipocyte was found (Figure 7).

Cell seeding on three-dimensional porous collagenous scaffolds enabled transferring of cell and growth factors into defect sites. Differentiated BMSCs were able to grow and differentiate well on ICBM, as it can be seen from CLSM images and expressions of ALP activity and osteocalcin (Figures 12 and 14).

Insoluble collagenous bovine bone matrix (ICBM) promoted growth and differentiation of osteoblasts. Implantation of differentiated bone marrow cells on ICBM in ectopic site 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 be osteoblasts *in vitro* and *in vivo* (Ashton *et al.*, 1980; Goshima *et al.*, 1991), (3) scaffold provides a three-dimensional structure for growth and differentiation of cells, (4) seeding cells on a three dimensional scaffold enables living cells implantation (Negishi *et al.*, 2000; Schaffer *et al.*, 1998) and (5) ICBM has good biocompatibility and has no osteoconductive property (Kuebler *et al.*, 1998).

The results suggest that numbers and osteogenicity of implanted cells were important factors contributing to new bone formation. It is also important to homogenously distribute cells into inner structure of porous scaffold. This is because porous structure of scaffolds will promote attachment and differentiation of cells leading to new bone formation. Importance of cell seeding efficiency and homogenous distribution of cells on porous scaffolds was emphasized. Static cell seeding in minimum amount of culture medium was not able to distribute cells into inner structure of scaffolds (Wald et al., 1993). Most of cells were found on outer surface and peripheral area of the scaffolds (Figures 11 – 13). Limitation of distribution of cells within porous structure and survival of the implanted cells might contribute to a very low amount of new bone formation of the implanted cells group. To induce new bone formation in heterotopic site, transplanted cells must function as bone cells by secreting and mineralizing extracellular matrix and secreting BMP-2 (Bruder et al., 1998).

It is postulated that non-survival of the implanted cells was caused by clumping of cells on surface of the scaffolds leading to insufficient nutrition and blood supply and lacking of cell-surface contact (Goshima et al., 1991; Ishaug-Riley et al., 1997). In addition, an immediate implantation of cells-scaffold construct might not be an optimal condition for cell transplantation, as cells did not attached very well on the scaffolds and were in a traumatic stage after tripsinization. In addition, cultivation of cells in culture medium without FBS might jeopardize cell viability, although it was shown that cells were able to grow on three-dimensional matrix in serum free culture medium (Figures 9 – 10). A non-survival and poor distribution of cells on ICBM were a cause of small amount of bone formation and formation of bone mostly on peripheral areas of the implanted cells group. This might explain that dose dependent effects of incorporated growth factors were not seen in this study. On

the other hand, doses of growth factors might be too low to enhance proliferation of the transplanted cells.

Intramuscular implantation is chosen because it provides rich blood supply to the specimens and it is a rich source of inducible precursor cells or mesenchymal stem cells. There is no osteoprogenitor cell in this area. These conditions provide an optimal environment for testing osteoinductive property of substances. It is a conventional implantation site for testing osteoinductive effect of BMP-2 (Kuebler *et al.*, 1998). The amount of new bone formation induced by implanted BMP-2 in intramuscular site is higher than in subcutaneous site. This is because intramuscular site provides rich blood supply to the specimens (Okubo *et al.*, 2000).

Transferring of osteogenic protein was a reliable method and applicable. A higher amount of new bone formation was consistently found in implanted BMP-2 group (Figres 30 -34). It was clearly shown that BMP-2 impregnated on ICBM was released into the surrounding tissue and induce osteoblastic differentiation of mesenchymal stem cells in skeletal muscles (Kuebler et al., 1998). Geometry and nature of scaffolds influence rate and amount of new bone formation (Ripamonti, 1993).

Interaction between cells and biomaterial surface and releasing pattern of growth factor from the scaffold regulate osteogenesis (Sampath & Reddi, 1984). It is postulated that fibrin gel functioned as additional ECM for growth and differentiation of cells in porous structure, prolonged release of BMP-2 and enabled a close contact between cells and growth factors. Amount of bone formation of BMP-2 with fibrin gel was significantly higher than BMP-2 alone (Figure 32). According to fluorochrome labeling, new bone formation was active during 1 – 3 weeks after implantation, as serial deposition of fluorochrome was seen (Figure 35). The most active period was found on day 11. Most of osteoid was secreted on day 11 as it could be seen from the strongest color of calcein in that period of time.

Limitations of the study

Investigations of effects of growth factor contents on apoptosis and expression of TGF-beta1 were not performed, because of low amount of new bone formation and dose dependent effects were not found. Limited volume of blood, high mortality rate, a contamination of cell culture, crowded facility and high cost were major obstacles.

A study design of blood collection, bone marrow cell harvesting and re-implantation of tissue engineer constructs was too complex for rats. The study should be conducted in larger size animals such as rabbits or goats, in order that a greater amount of blood can be collected and tolerance of animals for multiple operations is higher.

Concentrations of more than one growth factor should be detected to clarify effects of different growth factors contained in platelet concentrate. This study detected only TGF-beta 1 because there is no detection kits available for PDGF, IGF-I and VEGF in rats and cost of ELISA kits are very high.

Cell transferring technology has a high potential of clinical application, but cost of the procedure is high and time consuming. High mortality rate and contamination of cultivated cells are major problems leading to consuming of time and high cost.

Research facility plays important roles in success of the study. This complicated study design, involving multiple surgical procedures in one rat, requires a well equipped animal laboratory with clean room, biohazard hood, anesthetic machine and operating table. It had been very difficult to make thing work and led to high mortality rate of rat post-peratively.

There is not enough equipment for bone research in Thailand. There are no cutting and grinding machine and micro-CT available in Thailand. A researcher has to find a connection and laboratory in foreign country.

Further study

Further study should be conducted in a larger animal. Blood collection should be drawn in advanced and more than one time to maximize amount of growth factors. Multiple growth factors should be detected and considered in application of growth factor contents in the defects site. Ranges of concentrations of growth factors should be wider and use physiology dose as a base line.

Cell seeding methods should be changed to dynamic cell seeding and distribution of cells within porous structure should be ensured. The construct should be cultivated *in vitro* for at least 24 hours before implantation. Cultivation of cells in serum free medium should be avoided. A special medium for serum free culture

should be used and the construct should be washed in buffer saline before implantation.

Implantation can be in calvarial defects, because it is easy to access and a representative of skeletal defects of intramembranous bone.

Effects of growth factors in platelet concentrate on apoptosis of osteoblasts should be further investigated.

Conclusions

Ex-vivo tissue engineer construct of autogenous cells and growth factors was established. Dose dependent effects of growth factor contents from concentrate platelets in a range of 1 – 10 ng TGF-beta 1 was not found. It was postulated a low numbers of cell survival after implantation led to low amount of new bone formation and lack of dose dependent effects of the supplemented growth factors.

Transplanted bone morphogenetic protein is easy and reliable methods. Appropriate dose and releasing patterns are important factors determine amount of new bone formation. Fibrin gel enhanced bone formation capacity of BMP-2.

Ex-vivo tissue engineer construct of autogenous cells and growth factors could be fabricated by seeding cells with or without growth factors on three-dimensional scaffolds and fibrin network would enhance transferring efficiency of cells and osteogenic proteins.

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