การตอบสนองของสไตรมัลเซลล์จากไขกระดูกมนุษย์ที่มีต่อออสทีโอพอนทินและโครงร่างออสทีโอพอนทิน/คอลลาเจนจากผิวหนังวัว เพื่อศึกษาด้วยการเพาะเลี้ยงเซลล์

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THE RESPONSE OF HUMAN BONE MARROW STROMAL CELLS TO OSTEOPONTIN AND OSTEOPONTIN/BOVINE DERMAL COLLAGEN SCAFFOLD IN CULTURE

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ปภาดพงศ์ ศิริคุรุรัตน์ : การตอบสนองของสโตรมัลเซลล์จากไขกระดูกมนุษย์ที่มีต่อออสทิโอพอนทินและโครงร่างออสทิโอพอนทิน/คอลลาเจนจากผิวหนังวัว เมื่อศึกษาด้วยการเพาะเลี้ยงเซลล์. (THE RESPONSE OF HUMAN BONE MARROW STROMAL CELLS TO OSTEOPONTIN AND OSTEOPONTIN/BOVINE DERMAL COLLAGEN SCAFFOLD IN CULTURE) อ. ที่ปรึกษาวิทยานิพนธ์หลัก : ผศ.ทพ. สุพจน์ ตามสายลม, อ. ที่ปรึกษาวิทยานิพนธ์ร่วม : รศ.ทญ.ดร. สมพร สวัสดิสรรพ์, 64 หน้า.

การศึกษานี้มีวัตถุประสงค์เพื่อทดสอบการตอบสนองของสโตรมัลเซลล์ต่อออสทิโอพอนทิน คอลลาเจน และโครงร่างออสทิโอพอนทิน/คอลลาเจน ในแง่ของความเข้ากันได้ทางชีวภาพ การเหนี่ยวนำการเปลี่ยนแปลงจำนวนเซลล์ และการยึดเกาะของเซลล์ที่เพาะเลี้ยงในห้องปฏิบัติการ ทำการศึกษาโดยการเพาะเลี้ยงสโตรมัลเซลล์จากไขกระดูกมนุษย์ร่วมกับคอลลาเจนที่สกัดจากผิวหนังวัวและรีคอมบิแนนท์ออสทิโอพอนทินของหนู จากนั้นศึกษาการเพิ่มจำนวนเซลล์ด้วยวิธีสอบวิเคราะห์เอ็มทีทีในเซลล์ที่เพาะเลี้ยงด้วย 4 สภาวะ ได้แก่ สภาวะที่ไม่ได้รับสารใด ๆ ได้รับสารละลายคอลลาเจน สารละลายออสทิโอพอนทิน และสารละลายออสทิโอพอนทินผสมคอลลาเจน และศึกษาลักษณะการยึดเกาะของเซลล์ด้วยกล้องจุลทรรศน์อิเล็กตรอนชนิดส่องกราดทั้ง 4 สภาวะ รวมทั้งการยึดเกาะของเซลล์บนโครงร่างออสทิโอพอนทิน/คอลลาเจน ผลการศึกษาพบว่าสารละลายคอลลาเจน และสารละลายคอลลาเจนผสมออสทิโอพอนทิน กระตุ้นการเพิ่มจำนวนสโตรมัลเซลล์ โดยเพิ่มขึ้นเป็นร้อยละ 106.88 ± 3.85 และ 118.12 ± 6.14 ตามลำดับ ($P<0.05$) แต่สารละลายออสทิโอพอนทิน มีผลลดจำนวนเซลล์อย่างมีนัยสำคัญทางสถิติ โดยลดลงเหลือร้อยละ 63.15 ± 8.03 เมื่อเปรียบเทียบกับกลุ่มควบคุม ($P <0.05$) การศึกษาด้วยกล้องจุลทรรศน์อิเล็กตรอนชนิดส่องกราด พบว่าในกลุ่มควบคุมและกลุ่มที่ได้รับสารละลายคอลลาเจนผสมออสทิโอพอนทิน เซลล์มีการแผ่ตัวและยึดเกาะดี เมื่อเพาะเลี้ยงเซลล์ร่วมกับโครงร่างออสทิโอพอนทิน/คอลลาเจนที่สร้างขึ้น พบว่าเซลล์มีการยึดเกาะและแบ่งตัวได้ดี โดยสรุป สารละลายคอลลาเจนมีการกระตุ้นให้เกิดการเพิ่มจำนวนและการยึดเกาะของสโตรมัลเซลล์จากไขกระดูกมนุษย์ได้ดี โดยสารละลายคอลลาเจนผสมออสทิโอพอนทินให้ผลดีที่สุด รวมถึงโครงร่างออสทิโอพอนทิน/คอลลาเจนมีความเข้ากันได้ทางชีวภาพกับเซลล์ แสดงให้เห็นว่าโครงร่างที่สร้างขึ้นใหม่นี้ ให้ผลดีต่อสโตรมัลเซลล์และอาจมีประโยชน์ในการนำมาใช้เพื่อทำให้เกิดการงอกใหม่ของแผลกระดูก

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สาขาวิชา ปริทันตศาสตร์ ลายมือชื่ออาจารย์ที่ปรึกษาวิทยานิพนธ์หลัก.....สุพจน์ ตามสายลม.....

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PAPATPONG SIRIKURURAT : THE RESPONSE OF HUMAN BONE MARROW STROMAL CELLS TO OSTEOPONTIN AND OSTEOPONTIN/BOVINE DERMAL COLLAGEN SCAFFOLD IN CULTURE. THESIS PRINCIPAL ADVISOR : ASST.PROF. SUPHOT TAMSAILOM, THESIS COADVISOR : ASSOC.PROF. SOMPORN SWASDISON, 64 pp.

The aims of this study are to investigate the response including cell proliferation and cell attachment to osteopontin, collagen and mixed osteopontin/collagen substrates and to construct the osteopontin/collagen scaffold with evaluation of its biocompatibility to the cells *in vitro*. The study was performed by co-culturing human bone marrow stromal cells with bovine dermal collagen and recombinant rat osteopontin. MTT assay was employed to determine the cell proliferation resulted from the cells being exposed to four conditions; unexposed, exposed to collagen, exposed to osteopontin and exposed to mixed osteopontin/collagen. Cell attachment to these four conditioned surfaces and cell morphology on the scaffold were observed by a scanning electron microscope. The results showed that the number of cells exposed to the collagen and the mixed osteopontin/collagen significantly increased to 106.88 ± 3.85% and 118.12 ± 6.14% respectively ($P < 0.05$), whereas the number of cells exposed to the osteopontin significantly decreased to 63.15 ± 8.03% when compared to the control group ($P < 0.05$). Scanning electron microscopy demonstrated good cell attachment in collagen and the mixed collagen/osteopontin groups. When the cells cultured with osteopontin/collagen scaffold, there were cell proliferation and cell attachment into the scaffold. In conclusion, this study revealed that both collagen containing solutions enhance the human bone marrow stromal cell proliferation and attachment. The enhancement was increased with the addition of osteopontin. The osteopontin/collagen scaffold has good biocompatibility with human bone marrow stromal cells. These results suggest that this novel scaffold were advantageous to the stromal cells. Therefore, it might be worth introducing them to the field of bone regeneration.

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LIST OF ABBREVIATIONS

ARS	Alizarin red S
BMSCs	Bone marrow stromal cells
CD	Cluster of differentiation
CO_2	Carbon dioxide
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethylsulfoxide
ECM	Extracellular matrix
Gly	Glycine
h	Hour
HAc	Acetic acid
Hyp	Hydroxyproline
kDa	Kilodalton
mg	Milligram
min	Minutes
mL	Millilitre
mm	Millimetre
mM	Millimole
MTT	3-(4,5)-dimethylthiazol-2-yl)-2,5-diphenyl - tetrazolium bromide
NaCl	Sodium chloride
nm	Nanometre
nM	Nanomole
OPN	Osteopontin
PBS	Phosphate buffer saline
PDGF	Platelet-derived growth factor
Pro	Proline
RGD	Arginine-glycine-aspartic acid
rpm	Round per minute
SEM	Scanning electron microscopy

TGF	Transforming growth factor
αMEM	Minimum Essential Medium Alpha
µg	Microgram
µL	Microlitre
µm	Micrometre

CHAPTER I

INTRODUCTION

Background and rationale

From the lack of a widely acceptable choice for bone regeneration, a number of treatment options have been developed along the century. Autogenous bone grafting with its good biological properties such as adequate structural support, osteogenesis by virtue of surviving cells, osteoinductiveness and osteoconductiveness, is used as the gold standard treatment for bone regeneration. The autogenous bone usually consists of bone collagen, bone mineral and bone matrix proteins (DeLacure, 1994; Johnson et al., 1996). On the contrary, autogenous bone grafting contains donor site limitations which are inadequate bone volume, donor site morbidity, deformity and discomfort. As the results from those disadvantages and limitations, allografts and alloplasts have been developed as substitutes and used based on their osteoinductive and osteoconductive properties (van Heest and Swiontkowski, 1999). These materials, however, have inherent disadvantages on failure of complete bone ingrowth, extended inflammatory reaction, transmission of infections, uncontrollable resorption rate of the graft and encapsulation by connective tissue with little bone formation (Constantino and Friedman, 1994; Yukna, 1993; Virolainen et al., 1997). At present, there are a number of limitations of current bone regeneration techniques in various types of clinical conditions. Hence, the development of improved methods, such as tissue engineered-mediated bone regeneration, is necessary for achieving future viable therapeutic alternatives (Gronthos et al., 2006).

Tissue engineering approaching to the restoration and repairing of damaged tissues requires a scaffold material upon which cells can attach, proliferate, and differentiate hopefully into a functionally and structurally appropriate tissue for the body location where it is placed. In addition, ideal scaffold would allow for delivery of signaling molecules capable of coordinating cellular function (Wan et al., 2006).

Varieties of scaffold materials have been available with different physical properties. Each material associates with a specific and unique host response when implanted in a host. Scaffold materials, which can be either synthetically or naturally derived, have been used with different success rates to generate tissue-engineered bone formation both *in vitro* and *in vivo*.

Among the naturally derived scaffolds, collagen scaffold is the most commonly used. Type I collagen is a rational option for use in bony defect healing. It is the major structural protein of bone and the natural scaffold for osteoblast migration. The advantages of collagen use are the low immunogenic response across species, availability, abundance in nature, biodegradability, hemostatic promotion, cell-binding properties and easy manipulation into different forms (DeLustro et al., 1987; Watson, 1999). It has been used for soft and hard tissue repair (Delbalso and Adrain, 1976). Moreover, collagen associated with growth factors or other extracellular components such as glycosaminoglycans, chondroitin sulphate, heparan sulphate, and chemical modification could enhance its applicability as a biomaterial (Pieper et al., 1999; Pieper et al., 2000; van Susante et al., 2001)

Osteopontin (OPN) or bone sialoprotein-1 is a member of non-collagenous acidic hydrophobic phosphoprotein in extracellular matrix. OPN is expressed by various cell types and it is a multifunctional protein. OPN contains an arginine-glycine-aspartic acid (RGD) cell binding sequence, thus cells can bind OPN via multiple integrin receptors including the vitronectin receptor as well as various β1 and β5 integrins (O'Regan et al., 1999). OPN plays an important role in cell physiology including cell adhesion, signaling, differentiation, regulation of intracellular calcium level, and modulation of the immune response to infection and neoplasia (Denhardt and Guo, 1993). OPN can act as an adhesion substrate and migration stimulus by interacting with several integrins. The interaction leads to downstream signaling events that include calcium mobilization, protein phosphorylation, regulation of gene expression, and cell differentiation. The function of OPN in differentiation of primary osteoblast indicates that binding of OPN to integrin regulates intracellular signal

transduction and upregulates alkaline phosphatase and osteocalcin expression via focal adhesion kinase phosphorylation in osteoblasts (Yabe, Nemeto and Uemura, 1997).

Objectives

This study is a part of the main project on establishment of a tissue engineering scaffold for bone regeneration. The aims of this study are to investigate the response including cell proliferation and cell attachment of human bone marrow stromal cells (BMSCs) to collagen and OPN and to construct the OPN/bovine dermal collagen sponge and evaluate its physical properties and biocompatibility on the human BMSCs *in vitro*.

Hypothesis

1. OPN/collagen can enhance the proliferation and induce the attachment of these cells *in vitro*.

2. OPN/bovine dermal collagen sponge is biocompatible to human BMSCs.

Key words

Bovine dermal collagen

Osteopontin

Scaffold

Tissue engineering

Bone marrow stromal cells

Limitation

Due to costly expenses of human bone marrow study, this study is classified to investigate limited human bone marrow samples.

Benefits of study

This study will provide the essential information on the biological properties of OPN and the novel tissue-engineered scaffold and will be published in the peered review journal. Furthermore, this information would be a guide for future studies in animal experiment and clinical study.

CHAPTER II

LITERATURE REVIEW

Scaffold

The potential of tissue regenerative treatments requires integration of three elements: inductive morphogenetic signals (morphogens), responding progenitor/stem cells and the extracellular matrix scaffold (Nakashima and Reddi, 2003). Tissue engineering has been evolved to repair or replace diseased or damaged tissue by using controlled scaffolds. The role of the scaffold is to provide a matrix of a specific geometric configuration in which seeded cells may grow to produce the desired tissue or organ (Abukawa et al., 2006). The scaffold should have following ideal characteristics; (1) three-dimensional and highly porous with an interconnected pore network for cell growth and effective transportation of nutrients and metabolic waste, (2) biocompatible, (3) bioresorbable with a controllable biodegradation and resorption rate to match cell/tissue growth in vitro and in vivo, (4) suitable surface for cell penetration, attachment, proliferation, and differentiation, and (5) provide an environment in which the cells can maintain their phenotype and synthesize proteins and mechanical properties to match those of the tissues at the site of implantation (Hutmacher, 2000; Vats et al., 2003; Abukawa et al., 2006). To date, the ideal scaffold that meets all these criteria has not been developed.

Even though several types of scaffolds have facilitated in vivo bone formation, the optimal scaffold has yet to be defined. Biomaterials used as scaffolds for bone tissue engineering can be classified into two broad categories: synthetic and naturally derived. Synthetic scaffold materials have been developed for bone tissue engineering including hydroxyapatite base materials, tricalcium phosphate, polymer matrices such as poly(α-hydroxyesters), polyglycolide, polylactides, polycaprolactone (Chou et al., 2006), and poly(D,L-lactic-co-glycolic acid) (Calvert et al., 2000; Abukawa et al., 2003). They have been introduced for tissue repair. Naturally derived scaffolds such as

collagen, glycosaminoglycans, alginate, agarose, and chitin (Hutmacher et al., 1996; Abukawa et al., 2006) have long been developed to give good results in tissue regeneration. The advantages of natural materials are that they have low toxicity and low chronic inflammatory response. They can combine into a composite with other natural or synthetic materials and can be degraded by naturally occurring enzymes. The disadvantages, on the contrary, are the lack of desired structural rigidity for independent use in load-bearing regions, easy denaturation and often require chemical modification, which can lead to toxicity (Vats et al., 2003; Wan, Nacamuli and Longaker, 2006). Collagen has been routinely used as a substrate for bone engineering. Type I collagen has been specifically used to promote bone formation in rat mandibular defects. Collagen placed into the fractured region led to histologic bone bridging after six weeks of implantation in an experiment (Saadeh et al., 2001). Recently, chitosan has emerged as another natural scaffold for use in craniofacial repair. Chitosan, an acidic-aqueous soluble form of chitin, enhanced healing of canine mandibular defects after injection into the defect sites (Cho et al., 2004). Lee et al. (2000) developed chitosan/tricalcium phosphate sponges as the scaffolds for bone formation by three-dimensional rat osteoblast culture. Furthermore, Zhang and colleagues (2007) developed plasmid encoding platelet-derived growth factor B (PDGFB) gene releasing chitosan/coral composite scaffold as a regenerative material for periodontal regeneration. They found that human periodontal ligament cells had much proliferation properties on the gene-activated composite scaffold.

Natural or synthetic hydroxyapatite and tricalcium phosphate are ceramics. Ceramic biomaterials are structurally similar to the inorganic component of bone. They are biocompatible, osteoconductive and protein-free material but the disadvantages of them are brittle, long time degradation in vivo (Vacanti and Bonassar, 1999; Handschel et al., 2002) and uncontrollable pore size during conventional processing methods (Chu et al., 2002). In 2002, Boo and coworkers compared hydroxyapatite and tricalcium phosphate as scaffolds for bone engineering. In this study, scaffold was seeded with mesenchymal stem cells and implanted in subcutaneous sites. The result from histologic examination after eight weeks of implantation showed active bone formation in

both scaffolds. Moreover, Schleiphake and colleagues (2004) applied mineral-based scaffolds to the calvarial defects in rats. Using several formulations of calcium phosphate, peri-implant bone deposition was noticed around all scaffolds after the implantation for 52 weeks. By varying the content of calcium phosphate within the scaffolds, the rate of resorption was also noted to vary, reflecting a change in the biodegradability of the construct.

Besides, there are composite scaffolds made of incorporation of new fillers into the classic scaffolds, for example, incorporation of tricalcium phosphate, hydroxyapatite (Shikinami and Okuno, 1998) and basic salts (Agrawal and Athanasiou, 1997) into a polymer matrix. These inorganic fillers help control the desired degradation and resorption of the polymer matrix. A composite material would also increase biocompatibility and hard tissue integration in a way that ceramic particles, which are embedded into the polymer matrix, allow for increased initial spread of serum proteins compared to the more hydrophobic polymer surface. Additionally, association between the natural scaffold such as porous collagen and other extracellular components such as glycosaminoglycans, chondroitin sulphate (van Susante et al., 2001), heparan sulphate and physical or chemical modification could enhance its applicability as an effective biomaterial in vitro and in vivo (Pieper et al., 1999; Pieper et al., 2000).

Collagen

Collagen is the most abundant protein in animals, approximately 30% of all proteins in mammals. Collagen is the major protein of connective tissue, tendons, ligaments, and cornea. It forms the matrix of bones and teeth. The most commonly occurring collagens are type I, II and III, which form the long fiber bundles in many tissues. At least 27 different types of collagen have now been identified on the basis of morphology, amino acid composition, and physical properties (Boot-Handford et al., 2003).

The basic unit of collagen, tropocollagen, is a rigid rod-shaped molecule approximately 300 nm in length and 1.5 nm in diameter (Young and Heath, 2000). Collagen molecule contains three polypeptide chains, so called α chain. Each chain has 1000 amino acids and contains at least one stretch of the repeating amino acid sequence Glycine (Gly) -X-Y, where X and Y can be any amino acid but are usually proline (Pro) and hydroxyproline (Hyp) (van der Rest and Garrone, 1991). Therefore one third of all the amino acids in each collagen chain is Gly. Pro and Hyp follow each other frequently, and about 10% of the molecule has the sequence Gly-Pro-Hyp. Most collagens consist of two α1 chains and one α2 chain. An individual α chain is a left handed helix with approximately 3.3 residues per turn. The α chains are twisted together to form a right-handed superhelical structure (Patino et al., 2002a).

Type I collagen is found in fibrous supporting tissue, the dermis of skin, tendons, ligaments and bone, in a variable arrangement from loose to dense according to the mechanical support required. The tropocollagen molecules are aggregated to form fibrils strengthened by numerous intermolecular bonds. Parallel collagen fibrils are further arranged into strong bundles 2-10 μm in diameter which confer great tensile strength to the tissue (Young and Heath, 2000)

Collagen has been extensively used in the medical, dental, and pharmacological fields. Collagen is capable of being prepared into cross-linked compacted solids or into lattice gels. Resorbable forms of collagen have been used to dress oral wound or burn wounds, to close the grafts and extraction sites, and as a hemostatic agent (Colen and Mathes, 1983; Watson, 1999). Besides, it has been used to augment soft tissue, reconstruct tissue contour, correct scars (Oliver, 1987), and enhance tissue healing (Patino et al., 2002b). Furthermore, collagen has been utilized as the barrier membrane in periodontal and implant therapy to prevent epithelial migration and allows cells with regenerative capacity from periodontal ligament and bone marrow space to repopulate at the defect area.

Collagen is the main structural protein for tissue support and remodeling upon physical stress and during wound healing process in vertebrates. It also plays an essential role in providing a biologic scaffold for cellular activities that influence cell attachment, migration, and even differentiation (Yang et al., 2004). Collagen has been use widely in tissue engineering of human tissues for medical applications, including bone engineering, three-dimensional scaffolds for seeding of mesenchymal stem cells (Xiao et al., 2003; Juncosa-Melvin et al., 2005; Hou et al., 2007)

There are many protocols to improve the properties of collagen scaffold used in tissue engineering. Collagen can be modified to increased strength by a variety of methods including cross-linking by ultraviolet-irradiation, dehydrative methods or chemical methods, for example, glutaraldehyde (Jayakrishnan and Jameela, 1996).

Osteopontin

OPN or bone sialoprotein-1 is a negative-charged acidic hydrophilic phosphoprotein of approximately 300 amino acid residues with molecular mass between 44 and 75 kDa (O'Regan and Berman, 2000). OPN is a multifunctional protein secreted into all body fluids. Although highly expressed in bone, it is also expressed by various cell types including macrophages, activated T lymphocytes, chondrocytes, endothelial cells, smooth muscle cells and epithelial cells (O'Brien et al., 1994; Malyankar et al., 1997). OPN contains an RGD cell binding sequence, a calcium binding site ,and two heparin binding domains. These two domains may co-interact with RGD domain (O'Regan and Berman, 2000). Cells may bind OPN via multiple integrin receptors including the vitronectin receptor as well as various β1 and β5 integrins. OPN may be cleaved by thrombin resulting in the exposure of additional cryptic binding sites as well as the production of functional chemotactic fragments (O'Regan et al., 1999).

During human development, OPN is detected in high level that is not presented in the normal adult. OPN is upregulated at sites of tissue remodeling. It is also found in

the pathologically destructive tissues, for example, cancers and infarcted tissues. OPN can be associated with extracellular matrix (ECM), however, it does not perform a structural role within tissues as other ECM proteins.

OPN plays an important role in cell physiology including cell adhesion, signaling, differentiation, regulation of intracellular calcium level, and modulation of the immune response to infection and neoplasia (Denhardt and Guo, 1993). In the immune system during inflammation, OPN acts as a cytokine in T lymphocyte which leads to inhibition of expression of inducible nitric oxide synthase in both macrophages and primary renal tubular epithelial cells during infection response (Mazzali et al., 2002). OPN associates with pathogen resistance, and wound healing (Weber and Cantor, 1996). OPN involves in the recruitment and retention of macrophages and T cells to inflamed sites. It can act as an adhesion substrate and migration stimulus by interacting with several integrins. The interaction leads to downstream signaling events that include calcium mobilization, protein phosphorylation, regulation of gene expression and cell differentiation. Function of OPN in differentiation of primary osteoblast indicates that binding of OPN to integrin regulates intracellular signal transduction and upregulates alkaline phosphatase and osteocalcin expression via focal adhesion kinase phosphorylation in osteoblasts (Yabe et al., 1997). Furthermore, Liu and coworkers (1997b) found that OPN can trigger alkaline phosphatase gene expression via integrin-mediated focal adhesion kinase activation. Alkaline phosphatase expression at the transcription level induced by OPN was much higher than that induced by other ECM components. Therefore, OPN may be responsible for early differentiation in the osteoblastic cell line. OPN is also involved in bone remodeling and mineralization, angiogenesis, protection of cells from undergoing apoptosis, tumor metastasis, inflammation, wound healing process ,and tissue remodeling (Omigbodun et al., 1997).

Liu and coworkers (1997a) investigated the adhesion process of cultured osteoblastic cells by using immunofluorescence and biochemical techniques. Type I collagen, fibronectin, osteonectin, OPN, and bone sialoprotein were shown to play an

important role in the early differentiation of rat bone marrow-derived osteoblastic primary cells. Uemura and coworkers (2001) proposed that activated osteoblasts secreting ECM components such as OPN can be autocrine, which are recognized and bound by integrin $\alpha_v\beta_3$ on other osteoblasts to be activated. Moreover, OPN was shown to induce the in vivo osteogenesis of a bone marrow-derived osteoblast in the porous hydroxyapatite scaffold. In 1997, Yabe and Nemoto and Uemura studied the role of OPN, they were interested in cell adhesion and alkaline phosphatase assays of rat bone marrow osteoblastic cells on type I collagen and OPN surfaces. The rat bone marrow-derived osteoblastic primary cells was proved to adhere strongly to the OPN and to have high alkaline phosphatase activity on the OPN. They concluded that pre-osteoblasts differentiate into osteoblasts which can form bone by recognizing OPN.

Bone marrow stromal cells

Bone marrow is a reservoir of pluripotent progenitor cells for mesenchymal tissues. BMSCs consist of a variety of cells, for example, pre-osteoblasts, erythrocytes, adipocytes, immune cells and mesenchymal stem cells. Mesenchymal stem cells are thought to be multipotential cells that can either replicate or differentiate to lineages of specific tissue such as bone and cartilage. BMSCs, which are also known as mesenchymal cells and have been identified as a population of organized postnatal mesenchymal stem cells, not only acted as myelosupportive stroma, but also have potential to differentiate into osteoblasts, chondrocytes, adipocytes, cardiomyocytes, myoblasts, and neural cells (Beresford, 1989; Prockop, 1997; Bianco et al., 2001). Temporal expression of various receptors by BMSCs is critical for tissue-specific differentiation, for example, signal transduction mediated by different cytokines such as bone morphogenetic proteins determines osteoblast and adipocyte differentiation from the same population of the progenitor cells (Chen et al., 1998). To date, surface antigenic markers have been described that were used in the purification of mesenchymal progenitor cells. BMSCs were initially identified by their capacity to form clonogenic adherent cell clusters with fibroblastic morphology in vitro (Kuznetsov et al., 2001; Weissman, 2000). The first antibody used to identified mesenchymal stem cells is

STRO-1 (Dennis et al., 2002; Gronthos et al., 2003) but actually, many types of stem cell markers were utilized, such as CD10, CD13, CD14, CD34, CD45, CD73, CD90, CD105, CD117, CD133, CD144 (Jones et al., 2002; Tuli et al., 2003). BMSCs can now be efficiently retrieved from bone marrow aspirates.

CHAPTER III

MATERIALS AND METHODS

3.1 Materials

1. Tissue culture medium

   Minimum Essential Medium Alpha (αMEM) (Gibco BRL, USA)

   Antibiotic–antimycotic solution (Gibco BRL, USA)

   L-glutamine (Gibco BRL, USA)

   Fetal bovine serum (Gibco BRL, USA)

   Trypsin–EDTA (Gibco BRL, USA)

   L-ascorbic acid

   β-glycerophosphate

   Dexamethasone

2. Scanning electron microscopy (SEM) materials

   Osmium tetroxide (EMS, USA)

   Glutaraldehyde (EMS, USA)

   Phosphate buffer saline (PBS)

   Ethanol

   Coverslips

4. Alizarin red S (ARS) staining

   Alizarin red

   Ammonium hydroxide

   PBS

   Methyl alcohol

   Deionized water

5. CD105 staining

NCL-CD105 (mouse monoclonal antibody) (Novocastra™)

Protease (Dipase) (Sigma®, USA)

10% Formalin

EnVision+® System (Labelled polymer – HRP anti-mouse)
(DakoCytomation, USA)

DAB buffer

DAB solution

1% Hydrogen peroxide

6. Bovine dermal collagen extraction

0.5 M acetic acid (HAc)

10% (w/v) sodium chloride (NaCl)

7. MTT assays

MTT ((3-(4,5)-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide)
(Sigma®, USA)

Dimethylsulfoxide (DMSO)

Dulbecco's Modified Eagle Medium (DMEM) without phenol red

8. Equipments

Laminar flow unit (Microflow, MDH Ltd, England)

24 well plate (Falcon, USA)

35 and 60 mm tissue culture dish (Falcon, USA)

Scale (Metler Toledo, Switzerland)

Centrifuge (Clay-Adams, New York)

Inverted phase contrast microscope (Olympus CK2, Japan)

Light microscope (Olympus BH2, Japan)

Carbon dioxide (CO_2) incubator (TC2323, Shel Lab, USA)

Scanning electron microscope (Jeol JSM-5410 LV, Japan)

Shell freezer (Mini-Shell, USA)

Lyophilizer (Flexi-Dry MP, USA)

Centrifuge (6K15, Sigma)

Rongeur forceps

3.2 Methods

3.2.1 Collagen isolation and preparation

Fresh bovine skin was obtained from the slaughterhouse. To isolate type I collagen, bovine skin was prepared by using neutral salt and diluted acid extractions (Bazin and Delaumay, 1976). Initially, the tissue was cleaned with water, finely chopped after dissecting away the excessive fatty tissue, cut into small pieces, and put in 0.5 M HAc with stirring. The extractant was centrifuged at 11200 rpm 4°C for 1 h. The pellet was discarded and the collagen was precipitated from the supernatant by adding 10% (w/v) NaCl. Collagen precipitate was collected by centrifugation at 11200 rpm 4°C for 1 h. The collagen precipitate was dissolved in 0.5 M HAc and dialysed against 0.1 M HAc. It was finally shell frozen by cooled ethanol and lyophilized approximate 48 h. Bovine dermal collagen had the white sponge-like appearance. The collagen sponge was dissolved in 0.1 M HAc, the final concentration of collagen solution is 0.1 mg/mL.

3.2.2 OPN preparation

Recombinant rat OPN-generated from bacteria (kindly provided by Associate Professor Pi-Ling Chang, University of Alabama at Birmingham, USA), was dissolved in simple αMEM. OPN solution was heated at 37°C for 30 min to 1 h, aliquoted, sealed with paraffin and frozen at -80°C prior to use. The final concentration of OPN solution is 10 μg/ml which was suggested by Associate Professor Pi-Ling Chang as nontoxic concentration for human BMSCs-aspirate from iliac crest.

3.2.3 Sponge fabrication

In the pilot study, various proportions of mixed OPN solution with collagen solution from 1:9 to 9:1 were prepared. The OPN/collagen sponge in the proportions of 2:8 to 9:1 could not be used due to poor physical properties. Therefore, the proportion 1:9 of OPN/collagen was used for further study.

Mixed 1 mL of 10 μg/mL OPN solution with 9 mL of 0.1 mg/mL bovine dermal collagen dissolved in 0.5 M HAc. The OPN/collagen mixture was then dialysed against 0.1 M HAc. It was finally shell frozen by cooled ethanol and lyophilized approximate 24 h. The sponge was sealed with paraffin and stored at room temperature before testing.

3.2.4 Bone marrow samples and criteria of subjects

Bone marrow samples were collected from three physically healthy patients who needed the bone removal for treatment. The subjects were male or female with the age of 20 or more. Within three months prior to bone surgery, antimicrobial or corticosteroid drug users were excluded. Bone marrow was obtained at the time of torectomy procedure for prosthetic reasons in the Department of Oral and Maxillofacial Surgery , Faculty of Dentistry, Chulalongkorn University. Before collection of the human tissue samples, the process of tissue collection was approved by the Ethics Committee of Faculty of Dentistry, Chulalongkorn University and the bone marrow samples were collected with the signed informed consents from the subjects.

3.2.5 Bone marrow stromal cell culture

Small pieces of bone marrow samples were washed several times with simple αMEM to remove adherent erythrocytes and lipid. The samples were cut with Rongeur forceps into 1-2 mm^3 fragments, then placed in 35 mm tissue culture dishes. The culture medium was added into the culture dishes which were kept in the CO_2 incubator at 37 ^{o}C and absolute humidity. The medium was changed every three days. Nonadherent and hematopoietic cells were removed on the first day, during medium changes, leaving the human BMSCs for culture. The first to fourth passage of BMSC culture was identified the characteristics of stem cells by using CD105

immunohistochemical staining. The culture medium for the fifth passage was added with the osteogenic inducers. The human BMSCs at the fifth passage were used for all other experimental assays. Culture medium for BMSCs in the first to fourth passage was prepared by αMEM supplement with 10% fetal bovine serum, 1% antibiotic-antimycotic solution and 1% L-glutamine. Addition of 10 mM β-glycerophosphate , 50 μg/mL ascorbic acid and 100 nM dexamethasone as osteogenic inducers were used only for the BMSCs in the fifth passage (Jaiswal et al., 1997; Pittenger et al., 1999).

3.3 Observation and Measurements

3.3.1 Physical appearance of the OPN/collagen sponge

The physical appearance and the pore size of OPN/bovine dermal collagen sponge were observed in semithin plastic sections under light microscope and SEM.

3.3.2 Identification of the human BMSCs and osteoblastic cells

3.3.2.1 Identification of the human BMSCs

BMSCs in the fourth passage were identified the characteristics of the stem cells by using standard immunohistochemical staining. Briefly, the BMSCs culture was kept in 4 °C overnight. After washed several times with PBS and fixed with 10% formalin for an hour. BMSCs were digested with protease enzyme at 37 °C for 15 min. After washing twice with PBS, the culture was incubated with monoclonal antibody to CD105 at 4 °C overnight. Then Envision+ System was added to the samples at room temperature for an hour. The antigen – antibody reaction was visualized by adding DAB solution for 10 min. Staining was evaluated as either positive or negative under light microscope by two independent observers.

3.3.2.2 Identification of osteoblastic cells

Alizarin red S staining

Calcium-rich deposit by the 30-day-old human BMSCs in fifth passage was evaluated by the use of ARS staining (Putchler, Meloan and Terry, 1969; Gregory et al., 2004). Samples were washed with PBS, fixed with 4°C methyl alcohol for 10 min, and washed with distilled water. ARS solution was prepared by mixing Alizarin red, ammonium hydroxide, and deionized water in the proportion 1 g: 1 mL: 100 mL respectively and added in the culture plates for staining. Staining was evaluated as either positive or negative by two independent observers.

3.3.3 Response of human BMSCs to collagen, OPN and OPN/collagen substrate

3.3.3.1. Cell morphology and attachment study

The coverslips were cut into small pieces (3x3 mm), then placed in 35 mm tissue culture dishes. The coverslips in culture dishes were coated with 0.1 mg/mL collagen solution, 10 µg/mL OPN solution and mixed OPN/collagen solution (1:1 by volume). Non-coated coverslips were used as control. Human BMSCs from the fifth passage from each patient were seeded in culture dishes at the cell density of $5x10^4$ cells/mL. The culture was kept in the CO_2 incubator at 37°C and absolute humidity. The medium was changed every three days. Cell attachment and cell morphology of the cells on coverslips were observed by SEM at the 10-day culture.

3.3.3.2. Cell proliferation study

The cell proliferation of the human BMSCs exposed to collagen, OPN and OPN/collagen was measured by using MTT assay (Mosmann, 1983; Kasugai, Hasekawa and Okura, 1991).

Human BMSCs from the fifth passage were seeded in 24-well plate at the cell density of $3x10^4$ cells/mL. At day 2, culture medium supplemented with 0.1 mg/mL collagen solution, 10 µg/mL OPN solution and mixed OPN/collagen solution (1:9 by volume) were added in each groups as in figure 1. The culture medium without FBS served as control. The culture plate was incubated in the CO_2 incubator at 37 °C and absolute humidity for 3 days.

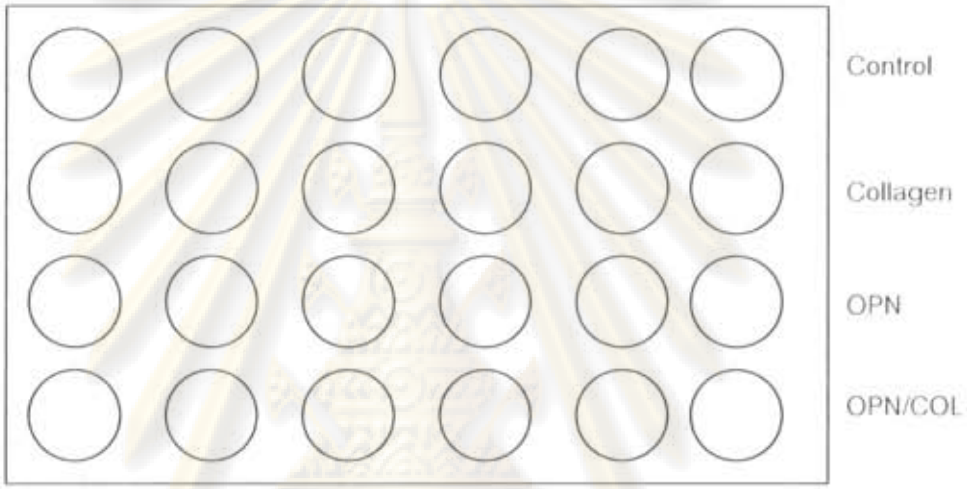

Figure 1 demonstrates each condition in 24-well plate for cell proliferation test.

MTT solution was prepared by dissolving MTT with PBS (5 mg in 1 mL of PBS). The solution was filtered through with 0.22 µm filter. For the MTT reaction, culture medium was replaced by 50 µL of MTT solution and 300 µL of DMEM without phenol red. The culture plate was incubated in the CO_2 incubator at 37 °C and absolute humidity for 4 h. DMSO 1000 µL was added into each well after removal of the MTT solution. The obtained purple solution was immediately measured by spectrophotometer at 570 nm wavelength. Each experiment was repeated 5 times by using the cells from three patients.

3.3.4 Response of human BMSCs to OPN/collagen sponge

For investigation of the cells in the sponge, OPN/collagen sponges were cut into 2x8x8 mm, then placed in 35 mm tissue culture dishes. Human BMSCs from the fifth passage from each patient were seeded in culture dishes with OPN/collagen sponge at the cell density of $5x10^4$ cells/mL. The culture was kept in the CO_2 incubator at 37 °C and absolute humidity. The medium was changed everyday. Observation of the cell attachment and cell morphology on the sponges was performed by SEM at the 5-day culture.

3.4 Analysis of the data

Descriptive analysis was used for observation of the sponge microstructure, cell morphology and cell attachment.

The One-sample Kolmogorov-Smirnov test was used to test the normal distribution of the data. The data of cell proliferation by MTT assay were not normally distributed, then the Kruskal-Wallis H test was performed to compare among groups. A critical level of 0.05 was employed. Thus, p-value less than 0.05 was considered statistically significant.

3.5 Ethical consideration

The protocol was approved by the Ethic Committee of the Faculty of Dentistry, Chulalongkorn University No. จธ. 64 / 2550.

CHAPTER IV

RESULTS

4.1 Physical appearance of the OPN/collagen sponge

OPN/bovine dermal collagen sponge had soft consistency and white sponge-like appearance (Figure 2). When put the sponge into the culture medium, the sponge absorbed the medium and was softer.

Figure 2 demonstrates the white sponge-like collagen prepared from bovine skin in different shapes.

When observed in semithin plastic sections under light microscope and SEM on the cross section of OPN/collagen sponge prepared, it was porous. The pores had polyhedral structure with the pore diameter ranged from 20 – 100 μm (Figure 3 and Figure 4).

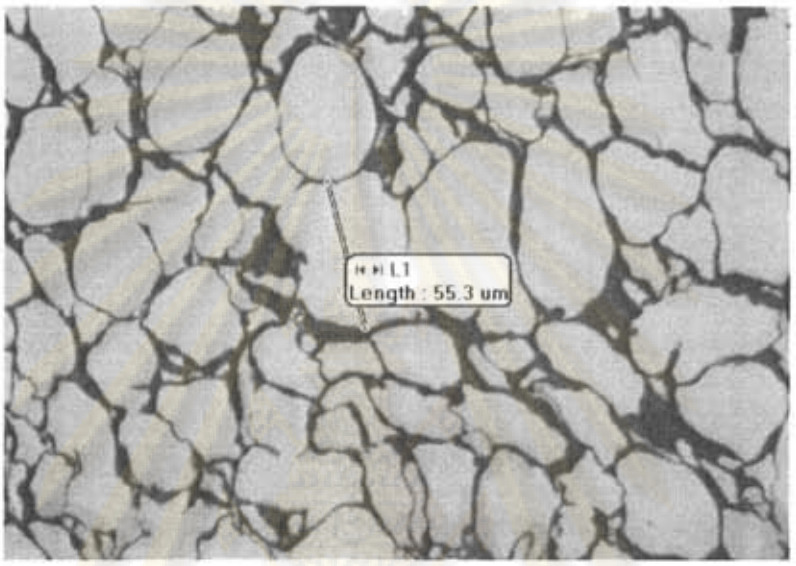

Figure 3 demonstrates the cross section of the porous OPN/collagen sponge. (Original magnification 10x)

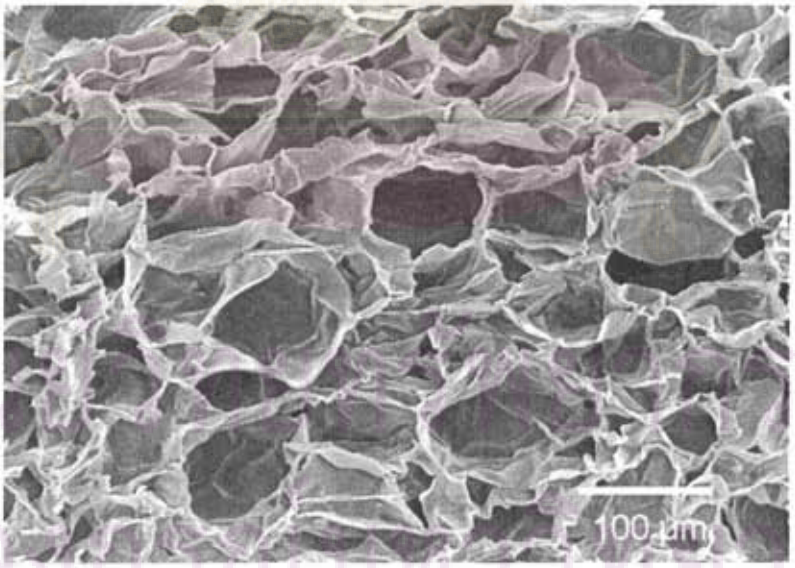

Figure 4 demonstrates the porous structure of the OPN/collagen sponge from SEM.

4.2 Identification of the human BMSCs and osteoblastic cells

In identification of stem cell by monoclonal antibody to CD105, the cultured cells in the fourth passage showed brown stained plasma membrane of the cells (Figure 5).

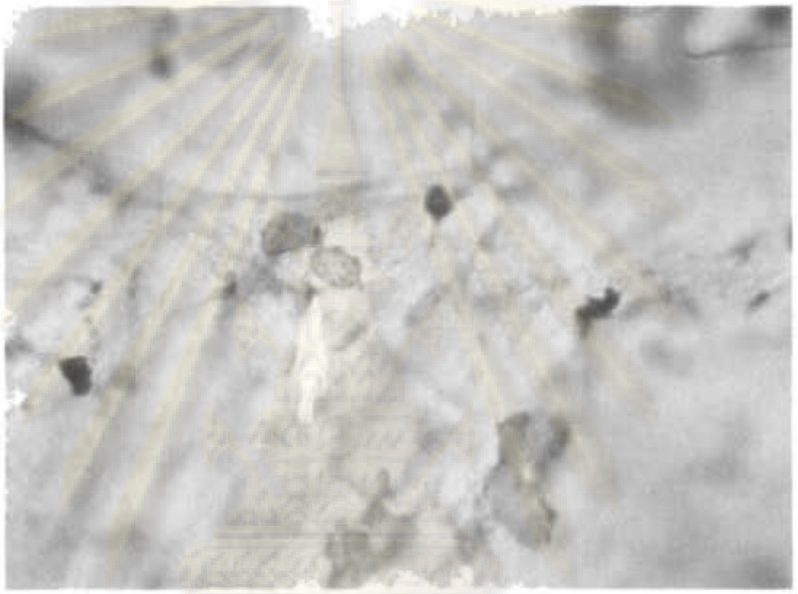

Figure 5 demonstrates the CD105 positive cells of BMSCs (arrow) (Original magnification 20X).

To confirm the type of the BMSCs, ARS staining was utilized to stain the cultured cells in the fifth passage. The staining showed formation and deposition of calcium salt, which was seen as the calcified nodule and positively stained by ARS (Figure 6).

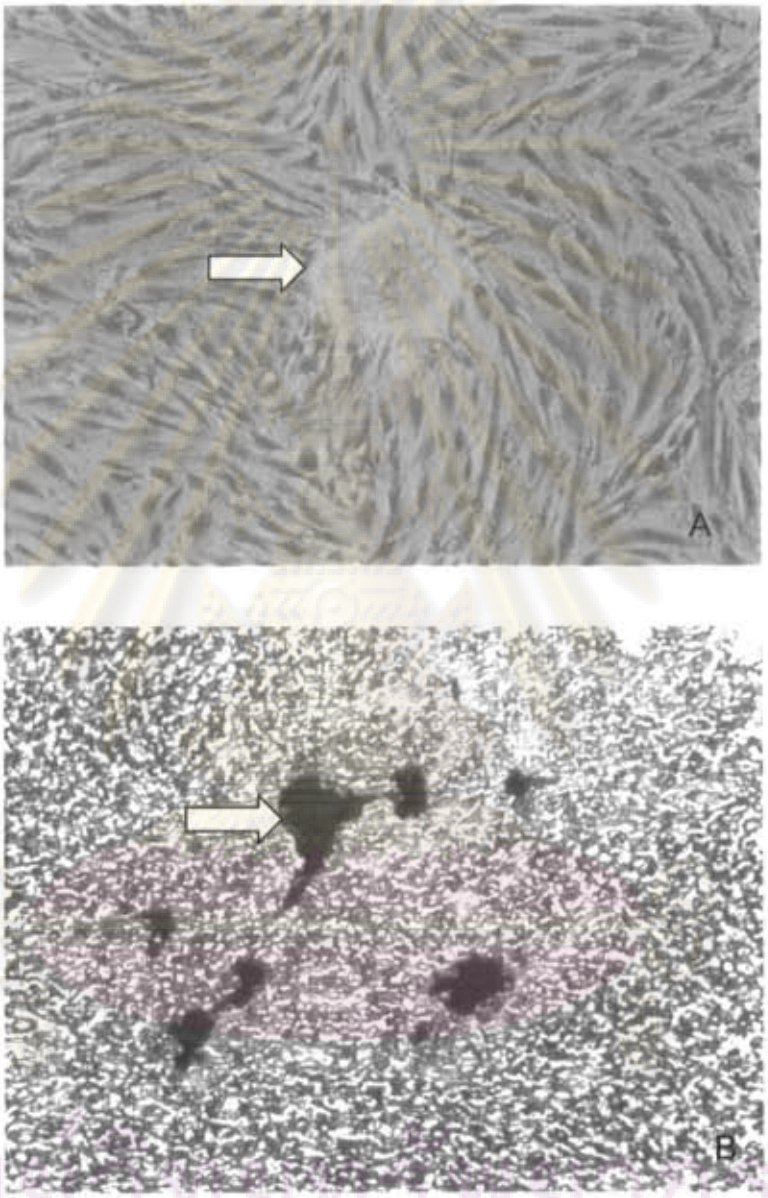

Figure 6 inverted phase contrast (A) and light (B) micrographs demonstrate the calcified nodules (arrow in A) in the 30-day-old BMSC culture and these nodules were positively stained by ARS (arrow in B) (Original magnification 40X).

4.3 Response of human BMSCs to collagen, OPN and OPN/collagen substrate

4.3.1 Cell morphology and attachment study

SEM was used in this study to observe cell attachment and population of attached cells. The SEM results showed the good spreading of cells and good cell attachment in the group of OPN/collagen coated coverslips. In the group of collagen coated coverslips and OPN coated coverslips, the results showed the good cell attachment, however, the spreading of cells was less than the group of OPN/collagen coated coverslips (Figure 7A, C, E and G). With the increased magnification, the control group and the group of OPN/collagen coated coverslips showed the good spreading of cells and good cell attachment. Extending cytoplasmic processes of the cells were generally found. This formed the attachment with the adjacent cells and surrounding contact area. The surface texture of the cells was smooth. In the group of collagen coated coverslips and OPN coated coverslips, there was less spreading of the cells compared to the first two groups. Extending cytoplasmic processes of the cells were rarely found. In addition, the bleb formation was found differently in size. There were also found large size of blebs and disrupted blebs, especially in the OPN coated coverslips group (Figure 7B, D, F and H).

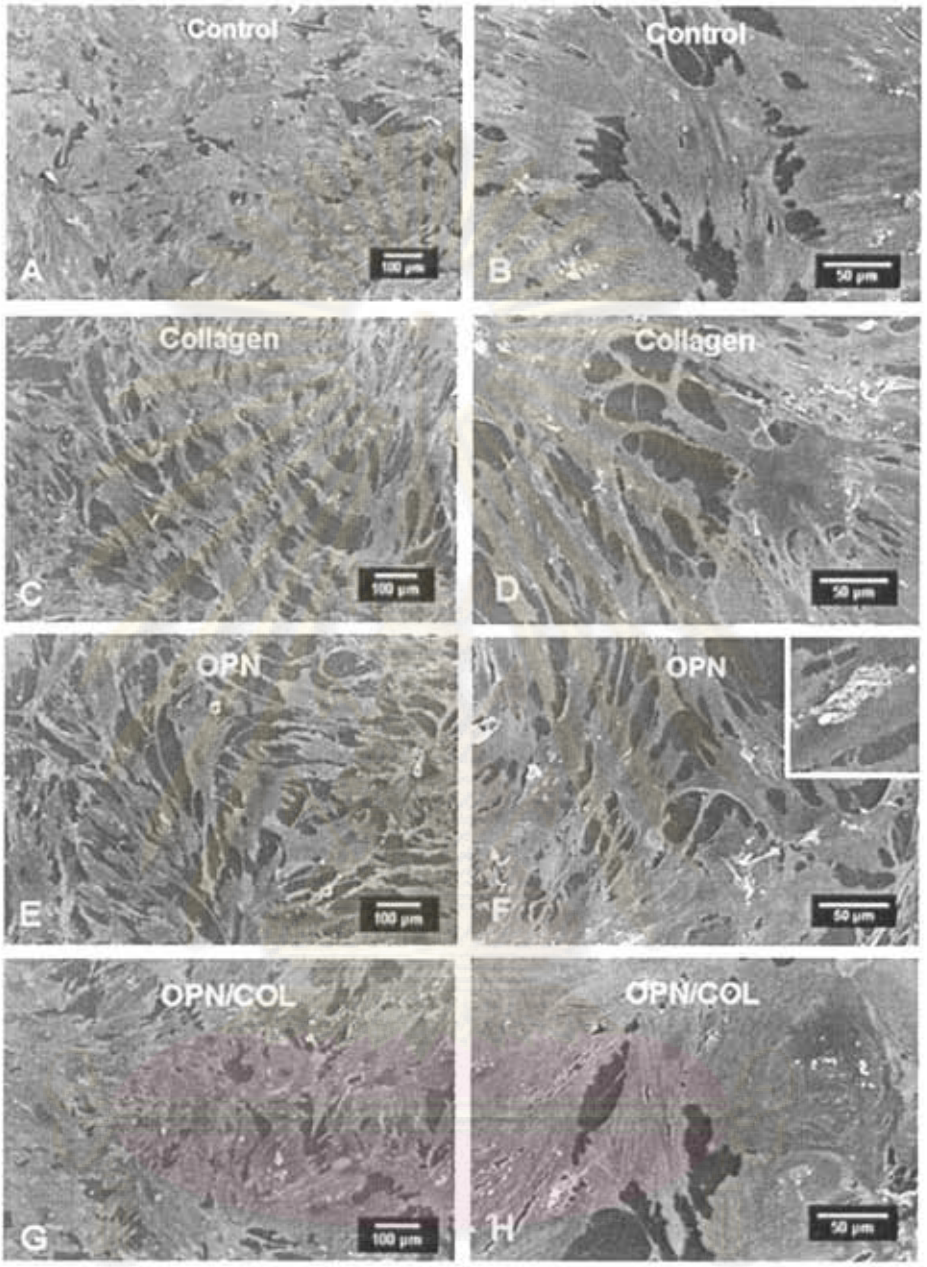

Figure 7 scanning electron micrographs at the low magnification (A, C, E, G) and higher magnification (B, D, F, H) show structures and attachment of cells grown on the surfaces coated with collagen (C, D), OPN (E, F) and mixed OPN/collagen (G, H) in comparison to the control group in which the cells were grown on the uncoated surface (A, B). The inset in F reveals disrupted blebs at the surface of the cell exposed to OPN.

4.3.2 Cell proliferation study

MTT assays demonstrated the increasing of BMSCs proliferation to 106.88 ± 3.85% and 118.12 ± 6.14%, respectively ($P < 0.05$), when the cells were exposed to the collagen solution and mixed OPN/collagen solution. Comparing to the control group, MTT assay of the cells exposed to the OPN solution demonstrated the decrease in cell number to 63.15 ± 8.03% ($P < 0.05$). Arranging the study groups and control group according to the capability of inducing BMSCs cell proliferation, mixed OPN/collagen solution had the highest capability followed by collagen group, the control group, and OPN group, respectively ($P < 0.05$) (Figure 8).

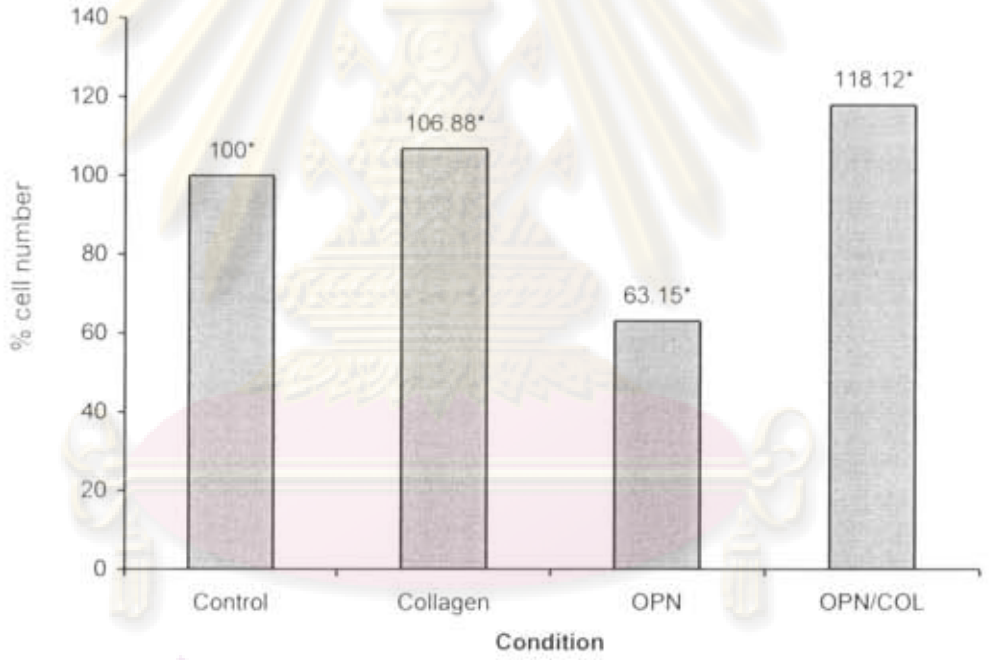

Figure 8 graph, plotted from the MTT study, demonstrates the percentages of cell number when the cells were grown in the 24-well plate supplemented with collagen, OPN or mixed OPN/collagen (OPN/COL) solution in comparison to the control group in which the cells were grown on the non-supplemented plate (* asterisks represent statistically significant difference among groups at $P < 0.05$).

4.4 Response of human BMSCs to OPN/collagen sponge

In exposure of BMSCs to OPN/collagen scaffold, there were proliferation and contact of BMSCs to the sponge (Figure 9).

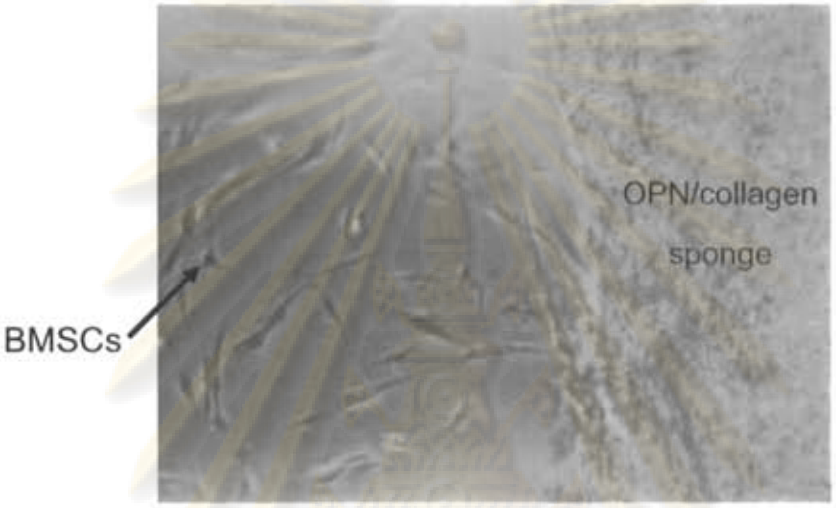

Figure 9 inverted phase contrast micrograph demonstrates the 5-day-old BMSCs culture with OPN/collagen sponge (Original magnification 40x).

With the SEM results of the fifth passage BMSCs cultured with OPN/collagen sponge in day 5, there were cell proliferation and cell attachment on the sponge and into the sponge (Figure 10).

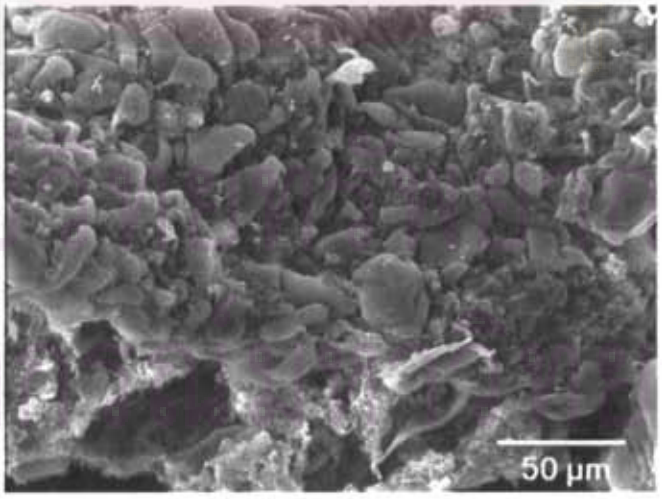

Figure 10 scanning electron micrograph demonstrates attached and proliferated BMSCs on OPN/collagen sponge at day 5.

CHAPTER V

DISCUSSION AND CONCLUSIONS

Discussion

The important role of the tissue-engineered scaffold is to provide a matrix of a specific geometric configuration in which seeded cells may grow and produce the desired tissue or organ (Abukawa et al., 2006). The ideal scaffold should have three-dimension and highly porosity with an interconnected pore network for cell growth and effective transportation of nutrients and metabolic waste, biocompatible, bioabsorbable with a controllable biodegradation time, allowed for cell penetration, attachment, proliferation, and differentiation (Hutmacher, 2000; Vats et al., 2003; Abukawa et al., 2006). Collagen is also one of the naturally derived scaffolds, which has been developed. Collagen has been widely used in human tissue engineering for medical applications. It plays an essential role in providing a biological scaffold for cellular activities that influence cell attachment, migration, and even differentiation (Yang et al., 2004). The recent development of collagen is focusing on bone engineering, three-dimensional scaffolds for seeding of mesenchymal stem cells (Xiao et al., 2003; Juncosa-Melvin et al., 2005; Hou et al., 2007). From the pilot study, when mixed OPN solution with collagen solution in the various proportions from 1:9 to 9:1, the OPN/collagen sponges in the proportions of 2:8 to 9:1 could not be used due to poor physical properties for being the scaffolds. The mixture of 1 mL of 10 µg/mL OPN solution with 9 mL of 0.1 mg/mL bovine dermal collagen was the most suitable ratio and this concentration was chosen to fabricate the OPN/collagen sponge. When evaluating the physical appearances of collagen sponge, the sponge prepared in this study is similar to collagen sponge available in the market. It has been previously reported that the cell attachment and viability in collagen-glycosaminoglycan scaffold were low when the mean pore size was higher than 100-150 µm and the cells increased their viability with decreasing pore sizes until no cells could fit into the pores (O'Brien et al., 2005). OPN/collagen scaffold in this study showed high porosity with the pore diameters of 20 - 100 µm which should be appropriate for cell survival, this three-dimensional scaffold

can provide a cellular matrix analog that will serve as a necessary substrate for the cells of the healing process to infiltrate and a physical support to guide the penetration, proliferation and differentiation of cells toward the targeted functional tissue.

The first antibody used for identification of mesenchymal stem cells was STRO-1 (Dennis et al., 2002; Gronthos et al., 2003). Later, many types of stem cell markers were reported, such as CD10, CD13, CD14, CD34, CD45, CD73, CD90, CD105, CD117, CD133, CD144 (Jones et al., 2002; Tuli et al., 2003). CD105 or Endoglin is an endothelial homodimeric membrane glycoprotein containg the peptide sequence RGD which is a recognition motif for adhesion receptors of the integrin family (Lastres et al., 1992). It has been proposed that Endoglin is a transforming growth factor (TGF)-β receptor (O'Connell et al., 1992) and differentiation of macrophage (Lopez-Casillas et al., 1991). CD105 is expressed on human macrophage, endothelial cells of capillaries, arterioles and venules in a variety of tissues (Lastres et al., 1992). In this study, CD105 was employed to characterize the mesenchymal progenitor cells from human bone marrow. The cultured cells in the fourth passage showed positive reaction at the plasma membrane of the cells. This staining pattern agreed with the previous studies and confirmed that the BMSCs isolated from the subjects and cultured in the laboratory in this study were mesenchymal stem cells (Jones et al., 2002; Tuli et al., 2003).

In 1995, Standford et al reported ARS staining could be used to detect mineral formation by detecting calcium mineral deposition in the ECM and what appeared to be intracellular paranuclear staining. Bodine, Trailsmith and Komm (1996) characterized human osteoblastic cell lines by ARS histochemical staining and demonstrated that these cells could produce mineralized nodules. Venugopal and colleagues (2007) utilized ARS staining to identify mineral calcium and phosphorous deposited on the surface of osteoblast cells on the polycaprolactone/hydroxyapatite - modified nanofibrous scaffolds. From the observation under inverted phase contrast microscope in our study, the BMSCs showed the property of cell aggregation or cluster forming. Furthermore, these cells induced by culture medium supplemented with osteogenic inducers represented their ability to form calcified nodules which positively stained by ARS. These results suggested that the cells in this study were bone-forming cells.

To study biocompatibility of the novel scaffold, both the inverted phase contrast microscope and SEM were used to evaluate the response of BMSCs to OPN/collagen sponge. The cell proliferation, contact and attachment to the sponge, which represented biocompatibility between OPN/collagen scaffold and BMSCs were observed. In the recent studies, ECM such as glycosaminoglycans, chondroitin sulphate, and heparan sulphate are being used increasingly as the tissue-engineered scaffold (Pieper et al., 1999; Pieper et al., 2000; van Susante et al., 2001). Moreover, gelatin is also used as the scaffold for bone regeneration (Rohanizadeh, Swain and Mason, 2008). With the advantages of ECM in good properties, flexibility, biocompatibility, and biodegradability, it has the potential to be used as a scaffold to support the osteoblasts and to promote bone regeneration in defective areas. These investigators also investigated cell penetration into the sponges using haematoxylin-eosin staining. Both composite and plain gelatin sponges demonstrated the ability to support cell growth and enhance cell penetration into the sponge pores (Rohanizadeh et al., 2008).

To simulate the environment associated with human bone regeneration, particular elements, for example, human BMSCs, which had been potentially differentiated into osteoblasts in the optimum environment and proper stimulation was shown in this study. In addition, collagen which has been used in various medical treatments, and the OPN which has the stimulation signal for development and differentiation of primary osteoblast to osteoblast, was also used in this study.

To study the proliferative effect possibly induced by collagen and OPN, MTT assay was performed in this study. Mosmann (1983) suggested that MTT assay is the suitable method to investigate cell survival and proliferation of cultured cells as well as evaluate products of vital cells under various conditions in vitro. This method identifies cell proliferation by counting vital cell number and evaluating products of vital cells under various conditions in the laboratory. The results from MTT assay in our study were initially analyzed for distribution of the data utilizing One-sample Kolmogorov-Smirnov test. The data was not normally distributed. The Kruskal-Wallis H test was performed to compare among each group. From the MTT assay and SEM observations,

mixed OPN/collagen solution showed the highest cell proliferation, which was statistically significant, followed by collagen solution. Mixed OPN/collagen solution also presented its capability to promote cell attachment. In contrast, OPN solution alone demonstrated the decrease in cell number. To clarify this result, the dynamic of cell development and proliferation in vital structure, and the roles of collagen and OPN must be seriously taken into consideration.

In multicellular organism, the major key of cell development is the cell ability in communication and reaction to the other cells and to the environment. These communications require appropriate ECM (Alberts et al., 2002). The mechanism of cell proliferation, firstly, depends on the optimal attachment of cells onto adhered surfaces or substrates. Then, the formation of cell - ECM interaction is occurred via integrin receptor. The better cell attachment will result in proper cytoskeleton organization and cell response. As the cells stabilize on the substrate, growth factor receptors are produced to react to the growth factors resulting in production of various kinds of proteins essential for cell development and differentiation. It was suggested to be multisteps in the biological processes in cells, however, it has not yet clearly explained (Juliano and Haskill, 1993). According to these processes, ECM plays an important role in cell attachment influencing the cell behavior and development.

Collagen is one of the ECM that is the most abundant structural proteins in human body. Collagen molecules have the particular part, which potentially promote cell adhesion, proliferation and differentiation of mesenchymal stem cells (Liu et al., 2004; Garcia and Reyes 2005). It has been used in the field of medical treatment for decades. OPN is also one of the ECM and presently is classified as the matricellular protein. Matricellular proteins, separated from other structural ECM, are related to reaction between cells, other ECM, and growth factors leading to growth and differentiation of cell more than promotion of cell attachment (Sage and Bornstein, 1991). Even though, the high level of OPN is presently reported in several tumor metastasis (Matsuzaki et al., 2007), OPN still has important roles in development of cells in relation to cell attachment, signal transduction, and development of osteoblast (Yabe

et al., 1997; Liu et al., 1997a; Liu et al., 1997b) including tissue repair and remodeling process (Kaartinen et al., 1999). OPN molecule also provides particular part, which promotes cell attachment via intregin receptor (O'Regan and Berman, 2000; Mazzali et al., 2002). Additionallly, it can stimulate alkaline phosphatase gene expression by the focal adhesion kinase phosphorylation pathway (Yabe et al., 1997). This leads to the differentiation of pre-osteoblasts to osteoblasts (Liu et al., 1997b).

In this study, the BMSCs showed good cell attachment in the collagen supplemented medium. When the cells were firmly attached, the cell proliferation was increased in both collagen and mixed OPN/collagen solutions. In the group of mixed OPN/collagen solution, the highest cell proliferation was observed. This highest proliferation may be related to the role of OPN in assisting the reaction between cells and growth factors and promoting cell proliferation. The concentration of OPN solution used in this study was proved in The United States with no toxicity to the human BMSCs, but in the group of OPN supplemented medium, the result showed the decrease in cell number compare to other groups. With the variety of cell response, racial or genetic differences may influence the results altered. This situation can be explained as the major involvement of OPN, which was associated with cell signaling and differentiation more than promotion of cell attachment and spreading (Sodek, Batista Da Silva and Zohar, 2006; Sodek, Ganss and McKee 2000). As a result, the decrease of cell attachment led to the decrease of cell growth. Another explanation for the decrease of cell number in this group was the high concentration of OPN which led to the inhibition of cell attachment. Even though, there was no obvious toxicity of OPN to cells found in this study, excessive volume of OPN may act as a negative signal to the cytoskeletal organization, which resulted in poor cell attachment. The effect of protein concentration on cell behavior was reported in some studies. For instance, TGF-β at the low concentration stimulated the production and secretion of PDGF. In contrast, with the high concentration, the TGF-β inhibited PDGF receptor (Gronwald, Seifert and Bowen-Pope, 1989). Even if there was decrease in cell number with concentrated OPN solution, the low concentration of OPN combined with collagen provided the increased cell number in this study. Considering along with the previous study which

demonstrated that porous hydroxyapatite coated with OPN induced BMSCs to produce forty percent higher bone formation in animal model compared to control group (Uemura et al., 2001), OPN might be worth developing as a morphogen for bone regeneration.

Conclusions

Both collagen and OPN/collagen solutions enhanced the human BMSC proliferation and attachment. The enhancement is increased with the addition of OPN. The novel OPN/collagen sponge has good characteristics and biocompatibility to human BMSCs. These results suggest that the mixture of collagen and OPN was advantageous to the BMSCs. Therefore, it might be worth introducing them to the field of bone regeneration.

Suggestions

From the result of this study, we founded that the OPN concentration used in OPN group (10 µg/mL) was excessive. Hence, the lower concentration, such as, 1:9 dilution of OPN solution should be used for comparing with the mixture of OPN/collagen solution in future study. Although, the OPN/collagen sponge developed in this study showed its beneficial in bone regeneration, the physical properties are not yet appropriate in clinical use since its consistency when moistened with culture medium is too soft. There should be improvement of the physical properties of the scaffold. Cross-linking with glutaraldehyde or ultraviolet light exposure might help increase the strength, stability and expand absorption period of the scaffold. Finally, the results of this study revealed that OPN/collagen had good biocompatibility, and increased capability to enhance human BMSC attachment and cell proliferation stimulation. Hence, these particular proteins, especially in sponge form, should be further developed and studied in animal and human to prove their safety and effectiveness in inducing bone regeneration.

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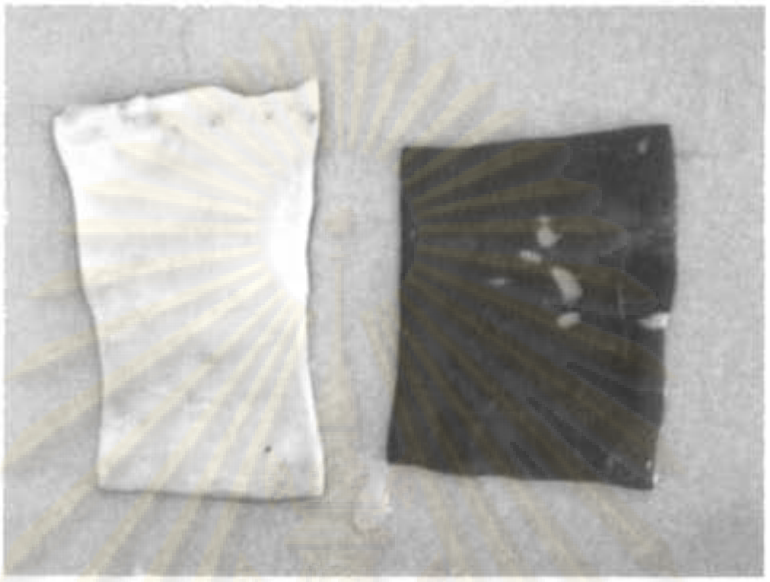
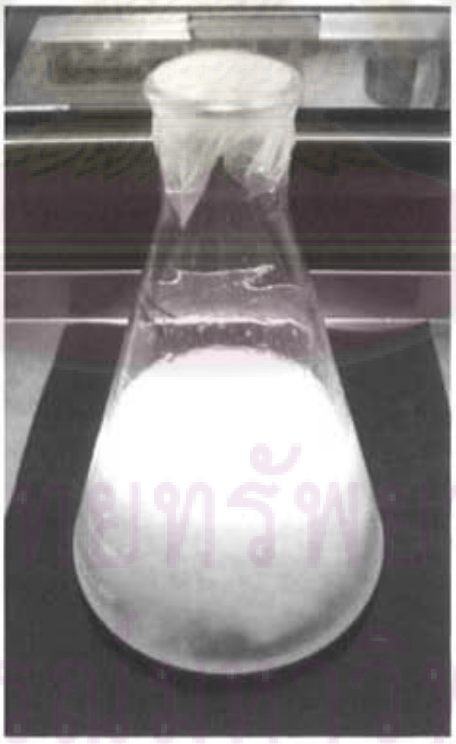
APPENDICES

Appendix A



The picture shows fresh skin dissected from bovine tail.



The picture shows bovine collagen (the white supernatant) from salt precipitation.

Appendix B

The table shows the optical density (OD) collected from cell proliferation testing by MTT

Subject	Test number	Optical Density (OD)			
		Control	Collagen	OPN	OPN_COL
Subject 1	1	.226	.246	.150	.269
	2	.228	.240	.169	.255
	3	.230	.240	.145	.257
	4	.232	.255	.138	.264
	5	.239	.260	.158	.260
Subject 2	1	.255	.241	.149	.268
	2	.258	.255	.146	.306
	3	.238	.248	.139	.297
	4	.210	.260	.131	.278
	5	.209	.258	.140	.262
Subject 3	1	.211	.259	.169	.289
	2	.244	.239	.152	.255
	3	.219	.244	.133	.299
	4	.245	.230	.139	.270
	5	.222	.240	.137	.277

Appendix C

Output from statistical analysis of cell proliferation test

Means

Case Processing Summary

	Cases					
	Included		Excluded		Total	
	N	Percent	N	Percent	N	Percent
OD * Group	60	100.0%	0	.0%	60	100.0%

Report

OD

Group	Mean	Std. Deviation	Minimum	Maximum
Control	.23173	.014786	.209	.258
Collagen	.24767	.009529	.230	.260
OPN	.14633	.011757	.131	.169
OPN+Collagen	.27373	.016808	.255	.306
Total	.22487	.049925	.131	.306

NPar Tests

One-Sample Kolmogorov-Smirnov Test

		OD
N		60
Normal Parameters[a,b]	Mean	.22487
	Std. Deviation	.049925
Most Extreme Differences	Absolute	.187
	Positive	.128
	Negative	-.187
Kolmogorov-Smirnov Z		1.449
Asymp. Sig. (2-tailed)		.030

a. Test distribution is Normal.

b. Calculated from data.

NPar Tests

Kruskal-Wallis Test

Ranks

	Group	N	Mean Rank
OD	Control	15	25.87
	Collagen	15	36.40
	OPN	15	8.00
	OPN+Collagen	15	51.73
	Total	60	

Test Statistics[a,b]

	OD
Chi-Square	49.886
df	3
Asymp. Sig.	.000

a. Kruskal Wallis Test

b. Grouping Variable: Group

Appendix D

บันทึกข้อความ

ส่วนราชการ งานบริการวิจัยและพัฒนา คณะทันตแพทยศาสตร์ จุฬาฯ โทร. 2188816 โทรสาร 2188810

ที่ ขร. 64/2550 วันที่ 24 ตุลาคม 2550

เรื่อง ผลการพิจารณาจริยธรรมการวิจัยในมนุษย์

เรียน รักษาการรองคณบดีฝ่ายวิจัย

คณะกรรมการพิจารณาจริยธรรมการวิจัยในมนุษย์ ได้พิจารณาตัดสินโครงการวิจัยที่ยื่นแสนอขอรับการพิจารณาจริยธรรมเรื่อง "การศึกษาสมบัติของสโตรมัลเซลล์จากไขกระดูกมนุษย์ที่มีต่อออสทีโอพอนทินและโครงร่างออสทีโอพอนทิน/คอลลาเจนจากผิวหนังวัวเมื่อศึกษาด้วยการเพาะเลี้ยงเซลล์" ผู้วิจัยหลัก ทพ. ปกาศพงศ์ ศิริคูรัตน์ มติของคณะกรรมการฯ คือ อนุมัติโดยไม่มีเงื่อนไข

จึงเรียนมาเพื่อโปรดพิจารณาดำเนินการต่อไปด้วย จักขอบคุณยิ่ง

(รองศาสตราจารย์ ดร. สุภัทรา อมาตยกุล)
กรรมการและเลขานุการ

ข้อมูลและรายละเอียดเกี่ยวกับการทำวิจัยที่ใช้ประกอบการพิจารณาเข้าร่วมโครงการ (Inform Consent) และ เอกสารยินยอมเข้าร่วมการวิจัย (Consent Form)

การวิจัยเรื่อง

การตอบสนองของสโตรมัลเซลล์จากไขกระดูกมนุษย์ที่มีต่อออสทีโอพอนทินและโครงร่างออสทีโอพอนทิน/ คอลลาเจนจากผิวหนังวัว เมื่อศึกษาด้วยการเพาะเลี้ยงเซลล์

เนื่องจาก ผู้ช่วยศาสตราจารย์ ทันตแพทย์สุพจน์ ตามสายลม และคณะ สังกัดภาควิชาปริทันตวิทยา คณะทันตแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย อยู่ในระหว่างการศึกษาวิจัยเกี่ยวกับการตอบสนองของ สโตรมัลเซลล์จากไขกระดูกมนุษย์ที่มีต่อออสทีโอพอนทินและโครงร่างออสทีโอพอนทิน/คอลลาเจนจาก ผิวหนังวัว เมื่อศึกษาด้วยการเพาะเลี้ยงเซลล์ ซึ่งในการทำวิจัยครั้งนี้ มีความจำเป็นต้องใช้กระดูกภายในช่องปากบริเวณทอรัสมาทำการศึกษา ในการนี้ คณะผู้วิจัยใคร่ขอความร่วมมือจากผู้ที่อาสาสมัครเข้าร่วมโครงการวิจัยจำนวน 3 ราย โดยผู้เข้าร่วมการศึกษาต้องมีสุขภาพแข็งแรง อายุ 20 ปีขึ้นไป เป็นเพศหญิงหรือเพศชาย หากเป็นเพศหญิงต้องไม่อยู่ระหว่างตั้งครรภ์ และผู้เข้าร่วมการวิจัย ต้องไม่ได้รับยาปฏิชีวนะหรือเสตียรอยด์อย่างน้อย 3 เดือนก่อนการผ่าตัด ระยะเวลาที่เข้าร่วมการวิจัย คือ ระยะเวลาที่ผู้ป่วยเข้ารับการผ่าตัดประมาณสามชั่วโมง คณะผู้วิจัยใคร่ขอความอนุเคราะห์จากท่าน ที่มารับการรักษาจากคลินิกภาควิชาศัลยศาสตร์ช่องปาก ซึ่งมีความประสงค์ที่จะผ่าตัดกระดูกส่วนนี้ออกเพื่อการรักษาอยู่แล้ว โดยการที่ท่านจะให้หรือไม่ให้ความอนุเคราะห์ครั้งนี้ไม่ได้ส่งผลใด ๆ ต่อวิธีการรักษาหรือส่งผลข้างเคียงต่อสุขภาพของท่านภายหลังการรักษา ทั้งนี้เพราะกระดูกที่จะนำไปศึกษานี้เป็นส่วนที่จะต้องถูกตัดออกอยู่แล้วในกระบวนการรักษาของทันตแพทย์ที่ให้ต่อท่าน ในกรณีที่ท่านยินยอมให้ความอนุเคราะห์ ทางคณะผู้วิจัยขอรับรองว่าจะเก็บข้อมูลเฉพาะของท่านเป็นความลับและจะใช้ข้อมูลจากกระดูกของท่านด้วยเหตุผลทางวิชาการเท่านั้น โดยผลจากการวิจัยนี้ จักเป็นประโยชน์ต่อการหาแนวทางในการรักษาโรคในทางการแพทย์เป็นอย่างยิ่ง

หากท่านมีข้อสงสัยอื่นใดเกี่ยวกับโครงการวิจัยนี้ กรุณาติดต่อที่ ผู้ช่วยศาสตราจารย์ ทันตแพทย์ สุพจน์ ตามสายลม ภาควิชาปริทันตวิทยา คณะทันตแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย หมายเลขโทรศัพท์ 02-2188850

และเพื่อให้การดำเนินงานในโครงการวิจัยนี้สมบูรณ์ตามกระบวนการการทำวิจัยในมนุษย์ ผู้ที่ได้อ่านและเข้าใจในคำอธิบายวัตถุประสงค์ของโครงการวิจัยแล้วและอาสาสมัครที่จะเข้าร่วมโครงการนี้ โปรดลงนามในหนังสือแสดงเจตนายินยอมเข้าร่วมการวิจัยด้วย

ก่อนที่จะลงนามในใบยินยอมให้ทำการวิจัยนี้ ข้าพเจ้าได้รับการอธิบายจากผู้วิจัยถึง วัตถุประสงค์ของการวิจัย วิธีการวิจัย อันตราย หรืออาการที่อาจเกิดขึ้นจากการวิจัย หรือจากยาที่ใช้ รวมทั้งประโยชน์ที่จะเกิดขึ้นจากการวิจัยอย่างละเอียด และมีความเข้าใจดีแล้ว

ผู้วิจัยรับรองว่าจะตอบคำถามต่างๆ ที่ข้าพเจ้าสงสัยด้วยความเต็มใจไม่ปิดบังซ่อนเร้น จนข้าพเจ้าพอใจ

ข้าพเจ้าเข้าร่วมโครงการวิจัยนี้โดยสมัครใจ ข้าพเจ้ามีสิทธิที่จะบอกเลิกการเข้าร่วมในโครงการวิจัยนี้เมื่อใดก็ได้และการบอกเลิกการเข้าร่วมการวิจัยนี้ จะไม่มีผลต่อการรักษาโรคที่ข้าพเจ้าจะพึงได้รับต่อไป ผู้ป่วยสามารถถอนตัวออกจากการวิจัยได้ทุกขณะ โดยไม่ต้องได้รับโทษ หรือสูญเสียประโยชน์ซึ่งพึงได้รับ

ผู้วิจัยรับรองว่าจะเก็บข้อมูลเฉพาะเกี่ยวกับตัวข้าพเจ้าเป็นความลับ และจะเปิดเผยได้เฉพาะในรูปที่เป็นสรุปผลการวิจัย การเปิดเผยข้อมูลเกี่ยวกับตัวข้าพเจ้าต่อหน่วยงานต่างๆ ที่เกี่ยวข้องกระทำได้เฉพาะกรณีจำเป็น ด้วยเหตุผลทางวิชาการเท่านั้น

ผู้วิจัยรับรองว่าหากเกิดอันตรายใดๆ จากการวิจัยดังกล่าว ข้าพเจ้าจะได้รับการรักษาพยาบาลโดยไม่คิดมูลค่า

ข้าพเจ้าได้อ่านข้อความข้างต้นแล้ว และมีความเข้าใจดีทุกประการ และได้ลงนามในใบยินยอมนี้ด้วยความเต็มใจ

ลงนาม.....ผู้ยินยอม

(.....)

ลงนาม.....พยาน

(รองศาสตราจารย์ ทันตแพทย์หญิง ดร.สมพร สวัสดิสรรพ์)

ลงนาม.....พยาน

(นางมาลี ตั้งพิสิฐโยธิน)

ลงนาม.....หัวหน้าโครงการวิจัย

(ผู้ช่วยศาสตราจารย์ ทันตแพทย์สุพจน์ ตามสายลม)

วันให้คำยินยอมเข้าร่วมวิจัย วันที่.....เดือน.....พ.ศ.....

ข้าพเจ้าไม่สามารถอ่านหนังสือได้ แต่ผู้วิจัยได้อ่านข้อความในใบยินยอมนี้ให้แก่ข้าพเจ้าฟังจนเข้าใจดีแล้ว ข้าพเจ้าจึงลงนาม หรือประทับลายนิ้วหัวแม่มือขวาของข้าพเจ้าในใบยินยอมนี้ด้วยความเต็มใจ

ลงนาม..... ผู้ยินยอม

(.....)

ลงนาม.....พยาน

(รองศาสตราจารย์ ทันตแพทย์หญิง ดร.สมพร สวัสดิสรรพ์)

ลงนาม.....พยาน

(นางมาลี ตั้งพิสิฐโยธิน)

ลงนาม.....หัวหน้าโครงการวิจัย

(ผู้ช่วยศาสตราจารย์ ทันตแพทย์สุพจน์ ตามสายลม)

วันให้คำยินยอมเข้าร่วมวิจัย วันที่.....เดือน.....พ.ศ.....

ในกรณีที่ผู้ถูกทดลองยังไม่บรรลุนิติภาวะ จะต้องได้รับการยินยอมจากผู้ปกครองหรือผู้อุปการะโดยชอบด้วยกฎหมาย

ลงนาม..... ผู้ยินยอม

(.....)

ลงนาม.....พยาน

(รองศาสตราจารย์ ทันตแพทย์หญิง ดร.สมพร สวัสดิสรรพ์)

ลงนาม.....พยาน

(นางมาลี ตั้งพิสิฐโยธิน)

ลงนาม.....หัวหน้าโครงการวิจัย

(ผู้ช่วยศาสตราจารย์ ทันตแพทย์สุพจน์ ตามสายลม)

วันให้คำยินยอมเข้าร่วมวิจัย วันที่.....เดือน.....พ.ศ.....

เอกสารยกเลิกการยินยอมเข้าร่วมวิจัย (Withdrawal Form)

การวิจัยเรื่อง

การตอบสนองของสโตรมัลเซลล์จากไขกระดูกมนุษย์ที่มีต่อออสทิโอพอนทินและโครงร่างออสทิโอพอนทิน/ คอลลาเจนจากผิวหนังวัว เมื่อศึกษาด้วยการเพาะเลี้ยงเซลล์

เหตุผลในการยกเลิกการยินยอมเข้าร่วมวิจัย

- ☐ ย้ายภูมิลำเนา
- ☐ ไม่สะดวกในการเดินทาง
- ☐ เหตุผลอื่น.....

ลงนาม.....ผู้ยกเลิกการยินยอม

(.....)

ลงนาม.....พยาน

(รองศาสตราจารย์ ทันตแพทย์หญิง ดร.สมพร สวัสดิสรรพ์)

ลงนาม.....พยาน

(นางมาลี ตั้งพิสิฐโยธิน)

ลงนาม.....หัวหน้าโครงการวิจัย

(ผู้ช่วยศาสตราจารย์ ทันตแพทย์สุพจน์ ตามสายลม)

วันยกเลิกการยินยอมเข้าร่วมวิจัย วันที่.....เดือน.....พ.ศ. .....

BIOGRAPHY

Mr. Papatpong Sirikururat was born in Hatyai, Songkhla on October 11th, 1982. He began his elementary and high school education from Senpong and Hatyai Wittayalai School, Songkhla. He earned his Doctor of Dental Surgery degree with first class honor from Chulalongkorn University in 2005. He served the government as a general dentist at Thapput Hospital, Phang-nga from 2005-2006. Presently, he practices in private dental clinics, Bangkok. At the same time, he attends the Master of Science Program in Periodontics, Department of Periodontology, Faculty of Dentistry, Chulalongkorn University. He was granted CHULALONGKORN UNIVERSITY GRADUATE SCHOLARSHIP TO COMMEMORATE THE 72nd ANNIVERSARY OF HIS MAJESTY KING BHUMIBOL ADULYADEJ in academic year 2006-2007.