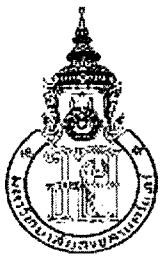

Characteristics of Adipose-derived Stem Cells Isolated from Buccal Fat Pads Using CD 271 Cell Sorting Technique

Monsikan Phopetch

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I hereby certify that this work has not been accepted in substance for any degree, and is not being currently submitted in candidature for any degree.

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ชื่อวิทยานิพนธ์ คุณสมบัติของเซลล์ต้นกำเนิดมีเซนไคม์ จากเนื้อเยื่อไขมันบริเวณแก้มที่ผ่านกระบวนการคัดแยกเซลล์ด้วยโปรตีนผิวเซลล์ CD 271

ผู้เขียน นางสาวมนสิการ โพธิ์เพชร

สาขาวิชา ศัลยศาสตร์ช่องปากและแม็กซิลโลเฟเชียล

ปีการศึกษา 2560

บทคัดย่อ

วัตถุประสงค์ เพื่อเปรียบเทียบคุณสมบัติของเซลล์ต้นกำเนิดจากเนื้อเยื่อไขมันบริเวณแก้ม ที่ผ่านกระบวนการคัดแยกเซลล์ที่มีโปรตีนผิวเซลล์ซีดี 271 ด้วยลูกปัดแม่เหล็ก (CD 271 Magnetic-Activated Cell Sorting, MACS) และวิธีดั้งเดิมคือพลาสติกยึดเกาะ (Plastic adherence)

วิธีการวิจัย ทำการเก็บเนื้อเยื่อไขมันบริเวณแก้มจากผู้ป่วยที่เข้ารับการผ่าตัดกระดูกใบหน้าและขากรรไกรจำนวน 10 คน เพื่อคัดแยกเซลล์ต้นกำเนิด โดยแบ่งเป็น 3 กลุ่มทดลอง ได้แก่ กลุ่ม A ใช้กระบวนการคัดแยกเซลล์ต้นกำเนิดวิธีดั้งเดิม กลุ่ม B และ C ใช้กระบวนการคัดแยกเซลล์ด้วยลูกปัดแม่เหล็ก โดยกลุ่ม B คือเซลล์ต้นกำเนิดที่มีโปรตีนผิวเซลล์ซีดี 271 ส่วนกลุ่ม C คือเซลล์ต้นกำเนิดที่ไม่มีโปรตีนผิวเซลล์ซีดี 271 จากนั้นประเมินคุณสมบัติของเซลล์ต้นกำเนิดแต่ละกลุ่ม โดยเปรียบเทียบความสามารถในการสร้างโคโลนี (Colony forming unit fibroblast : CFU-f) เปรียบเทียบการแสดงออกของโปรตีนผิวเซลล์ที่จำเพาะต่อเซลล์ต้นกำเนิดมีเซนไคม์ (Mesenchymal stem cells) ได้แก่ ซีดี90 (CD90), ซีดี73 (CD73) และซีดี105 (CD105) รวมถึงโปรตีนผิวเซลล์ที่จำเพาะต่อเซลล์ต้นกำเนิดเม็ดเลือด (Hematopoietic stem cells) ได้แก่ ซีดี 14 (CD14), ซีดี20 (CD20), ซีดี34 (CD34), และ ซีดี45 (CD45) โดยการวิเคราะห์ด้วยเครื่องโฟไซโตมิเตอร์ (Flow cytometry analysis) และใช้เซลล์สร้างเส้นใย (Fibroblast) เป็นกลุ่มควบคุม นอกจากนี้ยังเปรียบเทียบความสามารถของเซลล์ดังกล่าวในการเปลี่ยนแปลงไปเป็นเซลล์จำเพาะชนิดต่างๆ (Multi differentiation) ได้แก่ เซลล์ไขมัน เซลล์กระดูกอ่อน และเซลล์สร้างกระดูก

ผลการศึกษา เซลล์ต้นกำเนิดในกลุ่ม B มีความสามารถในการสร้างโคโลนีเมื่อเพาะเลี้ยงเซลล์เป็นเวลา 20 วัน ซึ่งไม่พบลักษณะดังกล่าวในเซลล์กลุ่ม A และ C เซลล์ทั้ง 3 กลุ่ม มีการแสดงออกของโปรตีนผิวเซลล์ที่จำเพาะต่อเซลล์ต้นกำเนิดมีเซนไคม์ได้ และมีการแสดงออกของโปรตีนผิวเซลล์ที่จำเพาะต่อเซลล์ต้นกำเนิดเม็ดเลือดในระดับต่ำ โดยไม่มีความแตกต่างอย่างมี

นัยสำคัญทางสถิติระหว่างกลุ่มทดลอง ในขณะที่เซลล์ในกลุ่มควบคุมมีการแสดงออกของโปรตีนผิวเซลล์ที่จำเพาะต่อเซลล์ต้นกำเนิดมีเซนไคม์อยู่ในระดับต่ำกว่ากลุ่มทดลองอย่างชัดเจน โดยมีความแตกต่างอย่างมีนัยสำคัญทางสถิติในการแสดงออกของซีดี73 และซีดี105 นอกจากนี้เซลล์ในกลุ่มทดลองยังมีสามารถในการเปลี่ยนแปลงไปเป็นเซลล์จำเพาะทั้งสามชนิดได้ไม่แตกต่างกัน

<u>สรุปผลการศึกษา</u> ซีดี 271 เป็นโปรตีนผิวเซลล์ที่เหมาะสมในการใช้คัดแยกเซลล์ต้นกำเนิดมีเซนไคม์ด้วยลูกปัดแม่เหล็กจากเนื้อเยื่อไขมันบริเวณแก้ม แม้ว่าการใช้โปรตีนผิวเซลล์เพียงชนิดเดียวอาจไม่เพียงพอสำหรับการคัดแยกเซลล์ต้นกำเนิดให้มีความบริสุทธิ์ อย่างไรก็ตามเซลล์ต้นกำเนิดที่คัดแยกด้วยวิธีดังกล่าวก็มีความสามารถในการเปลี่ยนแปลงไปเป็นเซลล์สร้างกระดูกได้ในห้องปฏิบัติการ ดังนั้นจึงสามารถนำเซลล์ดังกล่าวไปประยุกต์ใช้เพื่อการรักษาการสูญเสียกระดูกในผู้ป่วยได้

Thesis Title Characteristics of Adipose-derived Stem Cells Isolated from Buccal Fat Pads Using CD 271 Cell Sorting Technique

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Major program Oral and Maxillofacial Surgery

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ABSTRACT

Objective To compare the characteristics of ASCs isolated from intra-oral buccal fat pads using CD 271 Magnetic-Activated Cell Sorting (MACS) and plastic adherence (PA).

Materials and Methods The buccal fat tissue was harvested from ten patients whom undergone orthognathic surgeries. ASCs were isolated from the tissue using PA (Group A) and MACS; CD 271+ (Group B) and CD271- (Group C), (n=5/group). Colony forming unit fibroblasts (CFU-f) of the cells in each group were evaluated within 20 days of culture. The immune-phenotyping markers following the International Society for Cellular Therapy (ISCT) protocols including CD90, CD73 and CD105 as the positive markers and CD14, CD20, CD34, CD45 as the negative markers were analyzed by flow cytometry analysis. Gingival fibroblast was served as a negative control group. For multi-differentiation analysis, the cells of each group were cultured in the inductive medium. The adipogenic differentiation was assessed by Oil Red O staining. The chondrogenic differentiation was assessed by alcian blue staing. The osteogenic differentiation were assessed by ELISA to measure the level of alkaline phosphatase activity (ALP) and Osteocalcin (OCN).

Results CFU-f formed in the Group B cells, but were not detected in the other groups. The cells of groups A-C could express the immunophenotyping markers of mesenchymal stem cells including CD 73, 90 and 105. No statistical difference was detected among the groups. It was noted that CD 73 was detected at the highest levels followed by CD 105 and CD 90 respectively. The cells of the control group expressed

those markers remarkably less than the experiment groups (significant differences were found in CD 73 and CD 105, $p < 0.05$). In addition, the cells of all groups expressed hematopoietic stem cell markers including CD 14, 20, 34 and 45 at very low levels. The cells of groups A-C demonstrated adipogenic, chondrogenic and osteogenic differentiation when cultured in the inductive conditions. There was no significant difference of those properties among the groups.

Conclusion CD 271 is considered as a proper marker for sorting ASCs from buccal fat tissue. However, it cannot be use as the sole marker. Although the ASCs expressed CD 90 at the lowest levels, they still had osteogenic differentiation capacity. Therefore, they can be used as a stem cell source to repair bone defects.

Keyword Buccal fat stem cells, Adipose-derived stem cells, Stromal vascular fraction, Mesenchymal stem cell, CD271, Cell sorting.

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CHAPTER 1

INTRODUCTION

Background and rationale

Tissue engineering has become a popular alternative method in the field of reconstructive surgery. The bone tissue engineering triad includes scaffolds, cells, and signaling substances. A combination of scaffolds with bone forming cells as cell-scaffold constructs is a good strategy to enhance bone regeneration. Several studies (1-5) obtained better results in promoting new bone when the scaffolds were combined with several cell types, such as primary osteoblasts, mesenchymal stem cells (MSCs) from bone marrow (BM) (BM-MSCs), and dental pulp. Another source of MSCs is fat tissue from which adipose-derived stem cells (ADSCs) are obtained. ADSCs were found to express immunophenotyping markers similar to the BM-MSCs. Moreover, they can be differentiated toward various cell types, especially bone forming cells. Some studies (6-10) revealed that the buccal fat pads are suitable intra-oral sources of the ADSCs which provide a large amount of fat tissue that is easily harvested in routine intra-oral surgical fields. Plastic adherence (PA) capacity of stem cells is commonly used to isolate the ADSCs from other cell types since non-adherent cells can be washed out after periods of culture. Although this technique is very simple and cheap, the amount of stem cells obtained is only about 1 cell per 10^5 of adherent cells (11, 12). A new method to purify stem cell populations is magnetic-activated cell sorting (MACS) which uses antibody-binding. The specific markers are conjugated to iron oxide microbeads that can retain the desired cells in the column containers in a magnetic field, whereas unlabeled cells are eluted (13). Some studies (14-17) suggest using low-affinity nerve growth factor receptor (CD271) antibody as a positive selection marker of MSCs. The CD271 positive cells obtain

high purity of a MSC population that supports self-renewal capacity and multi-differentiation potential. However, there are no comparative results of characterization of the ADSCs isolated from the intra-oral buccal fat pads using the conventional PA method or MACS. In this study, CD271 was used as the specific marker of the MSCs and the positive and negative cells to the marker were included for the investigation.

Review of Literature

Mesenchymal stem cells

Mesenchymal stem cells are non-hematopoietic multipotent cells that have potency to differentiate into a variety of cells in mesenchymal lineage such as osteoblasts, chondrocytes, adipocytes myocytes, and neuron (18-21). MSCs are ideal for cell-based therapy of various inflammatory diseases, tissue repair and tissue engineering (22). MSCs can be utilized a number of pathways, including endocrine, paracrine, immunomodulation, direct differentiation, and induction of angiogenesis (21). The Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy (ISCT) clarifies nomenclature and definition of MSCs with the standard minimal criteria including (23).

1. Adherence to plastic in standard culture conditions
2. MSCs common markers positive (≥ 95%): CD73, CD90, CD105 and hematopoietic markers positive (≤ 2%): CD34, CD45, CD14 or CD11b, CD19 or CD79α, HLA-DR.
3. In vitro differentiation: osteoblasts, adipocytes, chondroblasts which are demonstrated by staining of cell culture

The methods of MSCs isolation

Plastic adherence method

Plastic adherence is the simplest method that is routinely used for the isolation of MSCs. This technique is based on the enzymatic digestion using collagenase, trypsin, or dispase. After digesting, the cell suspension will be passed through a filter to remove debris followed by plating on plastic culture plate allowing the cells to adhere for

48-72 hr. Afterwards, the non-adherent cells or hematopoietic cells will be excluded by washing. However, the adherent cells typically consist of heterogeneous mesenchymal population such as adipose stromal, endothelial cells, fibroblasts, lymphocytes, monocyte and macrophages (24-28). Some previous studies (11, 29, 30) demonstrated problems relating to low population of progenitor cells using this technique such as limited potential of self- renewal capacity and multi-differentiation. In addition, the heterogeneity of the cells would affect the proliferation potential (18, 31, 32).

Density based method

This technique is applicable for separation of bone marrow stem cells or peripheral blood. The MSCs will be separated by density gradient centrifugation with separation medium (usually sugar based). Although this technique is feasible, the differing densities of each cell type are unsuitable to separate the homogenous cell population. Therefore, it often use as a pre-enrichment step to remove unwanted cells prior to sorting by other methods such as plastic adherence. Meyerrose, et al (33) isolated mononuclear cells (MNCs) from lipoaspiration by density centrifugation 1,200 g for 10 min and 5 x 10^4 MNCs per cm^2 are plated in tissue-culture dishes, resulting in 10–20 CFU-F colonies per dish after 5 days. The initial cultures were highly heterogeneous, which contained 38.7%±12.4% CD45+ hematopoietic cells. Currently, there are many commercially available automated cell capturing system based on density gradient.

Antibody based method

Antibody-based methods are the most effective techniques for isolation of the specific cells with very high purity (34). This technique is based on the method of binding antibodies and cell surface antigens. It composes of 2 different conjugation techniques of fluorescence-activated cell sorting (FACS) and magnetic-activated cell sorting (MACS). The FACS technique uses the conjugation to fluorescent-labelled antibodies, whilst the MACS technique are conjugated to iron oxide microbeads. The limitation of separation depends on cell surface markers, which should be unique and specific to wanted cells. For example, the MSCs expression marker, CD 90 is also expressed by various cell types such as fibroblast, neuron, endothelial, hematopoietic stem cells (35-37). FACS can be tagged with multiple antibodies which create more

specific cell type, however, the isolation time is significantly slower than MACS (34). CD271 (Low-affinity nerve growth factor receptor, LNGFR or p75 Neurotrophin receptor, p75NTR) antibody has been suggested for positive selection of MSCs from bone marrow. Quirici, et al (16) obtained an average 30 ml of bone marrow from 11 healthy allogeneic bone marrow transplantation donors and separated MNCs with Ficoll-Paque gradient (specific gravity 1.077 g/mL). The isolation methods were performed by plastic adherent and selection of CD271 positive MSCs with MACS technique. The result showed the significant higher CFU-F in CD271 positive MSCs than plastic adherent MSCs (1584 colonies and 35 colonies per $1x10^6$ cells in LNGFR-MSCs and MNCs respectively). However, both of them expressed the same typical MSC phenotypic profiles including positive for CD73, CD90, CD105, CD146, CD44, CD29, CD166, HLA class I molecules and negative for the hematopoietic cell markers CD14, CD45, CD34 as well as class II HLA-DR (16). Correspondingly, Poloni, et al (15) demonstrated that the isolation of a MSCs by CD271 showed higher proliferation rate (6.89±1.57 logs) than isolation by plastic adherence (2.07±1.40 logs) without loss of multipotent differentiation capacity (15). In vivo studies, Yamamoto, et al (17) isolated the adipose tissue stromal vascular fraction from mouse subcutaneous adipose tissue. After culturing, the CD271 positive and negative cells were compared. The osteogenic differentiation of the CD271 positive cells showed higher Alizarin red staining than the CD271 negative cells (31.4 ± 7.0% and 15.0± 3.6% per the microscopic field respectively, $p < 0.05$). Moreover, the CD271 positive cells had very low contamination with hematopoietic cells, which was less than 1% measured using flow cytometry, while unseparated MNCs, which contained up to 13% (14). In addition, the cytokine releasing profiles showed significantly higher levels of IL-1β, IL-2, IL-3, IL-4, IL-8, IL-10, IL-12, G-CSF, granulocyte-monocyte colony-stimulating factor (GM-CSF), monocyte chemoattractant protein-1 (MCP-1), interferon-gamma (IFN- γ), and tumor necrosis-alpha (TNF-α) than plastic adherent MSCs ($P<0.05$) (14). Therefore, using of CD271 surface marker with MACS technique obtains the high purity of MSCs population that support self-renewal capacity, multi-differentiation potential and immunomodulatory effects of MSCs (14-17).

Adipose-derived stem cells (ADSCs)

Adipose tissue has been considered a good source of adult mesenchymal stem cells. The ADSCs are multipotent cells with the ability to differentiate into adipocytes, chondrocytes, and osteoblasts (8). The major mechanism would be due to the ability of ADSCs to secrete cytokines and growth factors and modulate stem cell niche of the host by recruiting endogenous stem cells to the affecting sites (38). For bone tissue regeneration, in vitro osteogenic differentiation of those cells can be induced using a culture medium supplemented with dexamethasone, β-glycerol phosphate and ascorbic acid. The differentiated cells can be characterized using specific gene or protein markers including alkaline phosphatase (ALP), bone sialoprotein (BSP) and osteocalcin (OCN) (8, 39). In vivo animal models, they also promoted bone regeneration by enhancing the growth and differentiation of bone forming cell in the critical sized calvarial defects (21,40). Thus, ADSCs have been showed significant osteogenic potential and also have promising results for repairing and regenerating injured tissues in vivo (25, 41). Methods for isolating ADSCs from the buccal fat pads are not complicate and they can be performed using basic facilities. Some previous studies (42-44) reported promising results of using ADSCs from buccal fat pads for bone tissue engineering both in vitro and in vivo. Niada, et al (43) isolated ASDCs from porcine buccal fat pads and subcutaneous adipose tissue and compared their osteogenic differentiation and growth on titanium and silicon disks. The results showed no difference in proliferation, viability, and clonogenicity of the cells between the two origins. They increased collagen formation and calcified extracellular matrix production, ALP activity, and OCN expression which indicating their ability to differentiate toward osteoblast-like cells. Likewise Broccaioli, et al (42) compared the features of human ADSCs from buccal fat pads and subcutaneous adipose in vitro, ADSCs from both sources could express the typical mesenchymal stem cell immunophenotype, and demonstrated clonogenic and multidifferentiation abilities. In addition, they adhered and proliferated well on some synthetic biomaterials including collagen membrane and polyglycol acid filaments. For a clinical trial, Khojasteh, et al (44) used the ADSCs from buccal fat pad combined with autogenous iliac bone graft (ICBG)

for augmentation of severe alveolar defects. New bone formation was assessed using cone beam computed tomography and histomorphometric analysis. The results demonstrated that the mean bone width change at the graft site was greater in the test group than in the control group without cells. Moreover the percentage of new bone formation of the test group was higher than the control group (65.32% and 49.21% respectively). Therefore the combining of the ADSCs with ICBG can accelerate the bone formation and reduce bone resorption in the severe alveolar defects. Moreover Yamada et al (45) assessed the relation of aging to the proliferation abilities and pluripotency of ADSCs in rat. The result showed slightly decrease proliferation of ADSCs with aging whereas multi-differentiated potency was maintain. Overall, ADSCs have significant advantages in the immunomodulation effect, osteogenic potential, and the availability even in elderly patients.

The stromal vascular fraction

For the adipose tissue, the isolation method was initiated by enzymatic digestion with collagenase, then the lipid and mature adipocyte were separated by centrifugation. The cell pellet at the bottom is called "stromal vascular fraction" or SVF which composed of various cell types including macrophages, various blood cells, pericytes, fibroblasts, smooth muscle cells, vascular endothelial progenitors and adipose-derived stem cells (46, 47). Population of ADSCs in SVF varies from less than 1 % to over 15 % depending on the method employed (46). Characteristics of SVF and ADSCs are summarized in table 1 according to the International Federation for Adipose Therapeutics and Science (IFATS) together with the International Society for Cellular Therapy (ISCT) in order to provide guidance for standardization between different research groups (48).

Table 1. Characteristics of SVF and ADSCs in term of cell viability, CFU-F, immunophenotype characteristic, and multilineage differentiation according to the International Federation for Adipose Therapeutics and Science (IFATS) together with the International Society for Cellular Therapy (ISCT).

Characteristic	SVF	ADSCs
Cell viability	> 70%	> 90%
CFU-f	> 1%	> 5%
Immunophenotype characteristic	Positive for CD13, CD29, CD44, CD73, CD90 (>40 %), and CD34 (>20 %)	Positive for CD13, CD29, CD44, CD73, CD90, and CD105 (>80 %)
	Negative for CD31 (<20 %), and CD45 (<50 %)	Negative for CD31, CD45, and CD235a (<2 %)
Multilineage differentiation	-	Capacity to differentiate into the adipogenic, osteogenic, and chondrogenic lineage

Hypothesis

CD 271 can be used as the specific marker for isolating of ADSCs from buccal fat tissue which generate the high purity of mesenchymal stem cells. Using of CD 271 with MACS method is more suitable than PA method for ADSCs isolation, which support the CFU-f, multi-differentiation capacities and display the MSCs common markers.

Objectives of the study

The characteristics of ADSCs isolated from intra-oral buccal fat pads using CD 271 Magnetic-Activated Cell Sorting (MACS) and plastic adherence (PA) were comparatively assessed as follows:

- Colony forming unit fibroblasts (CFU-f)
- The expression of immune-phenotyping markers of MSCs
- Multi-lineage differentiation into osteogenic, chondrogenic, and adipogenic differentiation.

Benefit of the study

To provide a scientific knowledge and find the suitable method for isolation of ADSCs from buccal fat pad for bone tissue engineering.

CHAPTER 2

MATERIALS AND METHODS

Patient enrollment

The protocol of this study were approved by the ethics committee, Faculty of Dentistry, Prince of Songkla University (EC5909-38-P-LR). Ten volunteers were recruited for harvesting of buccal fat pad. Those patients had undergone orthognathic surgeries to correct their skeletal discrepancies in the Oral & Maxillofacial Surgery clinic, Dental hospital, Faculty of Dentistry, Prince of Songkla University. The inclusion criteria of the subjects included ASA class I, ages more than 20 years old, weights more than 50 kg and at least 35% of hematocrit. The patients with systemic diseases including hereditary blood diseases, disorders of the blood and blood components, blood transmitted diseases and diabetes were excluded.

Isolating ADSCs from fat tissue

Each subject was undergone orthognathic surgeries as usual under general anesthesia. During the operations of Lefort I osteotomy of the maxilla or sagittal split osteotomy of the mandible, some parts of the fat pads were excised, then immediately placed into DMEM (Dulbecco's Modified Eagle Medium, Gibco, USA) and stored at 4° C until the isolation processes. The fat tissue was washed several times with sterile phosphate buffer saline (PBS) to remove contaminating debris and red blood cells, and then its volume (ml) was measured using 5-cc sterile disposable syringe. Afterwards, the tissue was minced into small pieces and enzymatically digested using 0.75% collagenase type I (Gibco, USA) in PBS at 37° C with gentle agitation for 60 min. The supernatants were collected, and then centrifuged at 400 g for 10 min to exclude the

remaining adipocytes and lipid droplets. The cellular pellets were resuspended in DMEM supplemented with 10% Fetal Bovine Serum (FBS; Gibco, USA), and then filtered through a 100 μm filter (Corning, USA).The ADSCs of the subjects were consecutively isolated using two different methods and divided into 3 groups including group A (PA), group B (CD271 positive cells), group C (CD271 negative cells) (n=5/ group). In group A (PA); the cell suspension was plated onto 6-well culture plates (Corning, USA) and cultured in a humidified atmosphere with 5% CO2 at 37°C (7). In group B and C; the cells were isolated using CD271 antibody for MACs (CD271 MicroBead Kit human, Miltenyi Biotec, Germany). In brief, the cell suspension was recentrifuged at 300 g for 10 min and the cell pellets were resuspended in 60 μL of buffer (PBS with 0.5% FBS, and 2 mM EDTA). The cell suspension was incubated in 20 μL of FcR Blocking Reagent and 20 μL of CD271 MicroBeads for 15 min at 4°C. Afterwards, the cells were washed by adding 1-2 mL of buffer, and then centrifuged at 300 × g for 10 min. Resuspended cell solution was made by adding 500 μL of buffer, and then it was loaded onto a column which placed in MiniMACS separator (Miltenyi Biotec, Germany). The magnetically labeled CD271 positive cells of group B were retained and eluted form the column after separating from the MiniMACS separator, whereas the unlabeled CD 271 negative cells of group C ran through. The cells of group B and C were collected and cultured in DMEM supplemented with 10% FBS in a humidified atmosphere with 5% CO_2 at 37°C. The cells of all groups at passage 1-4 were used for the following experiments.

Isolating and culture of human gingival fibroblast

The peri-coronal gingival tissue was collected during extraction of third molars of the patients. The tissue was rinsed with PBS, and minced into small pieces. Explant culture of the tissue was performed in DMEM supplemented with 10% FBS in a humidified atmosphere with 5% CO2 at 37°C. Subculture was performed when the

outgrowing fibroblasts were at 70-80% confluences (49).The cells of passage 2-3 were used for the flow cytometry analysis as the control group.

Determining the Characteristics of the cells

1. Colony-forming unit fibroblast (CFU-f) assays

The cells of each group at passage 1 were plated at a density of 100 cells/well in a 6-well plate to define the CFU-f. Within 20 days, the cells were fixed with 4% paraformaldehyde and stained with 0.1% toluidine blue (Sigma, USA). The CFU-f at least 50 cells or the colonies > 2 mm in diameters were observed under light microscope (Nikon, Japan) (2).

2. Flow cytometry analysis

The MSC immunophenotypes of the ADSCs were defined following the International Society for Cellular Therapy (ISCT) protocols (23). The analysis was performed using fluorochrome-conjugated monoclonal antibodies cocktail of MSC Phenotyping Kit human (Miltenyi Biotec, Germany), which composing CD73-APC, CD90-FITC, CD105-PE as the positive markers and CD14-PerCP, CD20-PerCP, CD 34-PerCP, CD45-PerCP as the negative markers. In brief, $5x10^5$ cells of passage 2-3 from each experiment groups and the control group were incubated in the antibodies cocktail and another $5x10^5$cells were incubated in the Isotype control cocktail for 10 min. After washing, the resuspend cell pellets were used for analysis by flow cytometry. At least 10,000 events were acquired for each sample using a fluorescent-activated cell sorting instrument (FACSCalibur, BD Biosciences) and the data was analyzed using CELLQUEST software (BD Biosciences).

3. Multi-differentiate potential of the ADSCs

3.1 Adipogenic differentiation

The cells at $1x10^4$/well were cultured in adipogenic induction medium comprised of DMEM supplemented with 10% FBS, 1µM dexamethasone, 10µg/mL insulin, 500µM 3-isobutyl-1-methyl-xanthine and 200µM indomethacin (Sigma, USA) for 21 days. The culture mediums were changed every 2 days. On day 21, the cells were fixed in 10% formaldehyde for 1 h and stained with Oil Red O solution (20mg/mL in isopropanol) (Sigma, USA) for 15 min (50). Lipid vacuoles were quantified by extracting with 100% isopropanol for 10 min and reading with microplate reader (Multiscan™Go, Thermo Fisher Scientific) at absorbance of 540 nm.

3.2 Chondrogenic differentiation

The cells of $5x10^5$ were centrifuged at 600 g for 5 min to form cell pellets and suspended in 2 ml chrondogenic medium (StemPro Chondrogenesis Differentiation Kit, Gibco) in 15 ml centrifugation tube (Corning, USA) for 21 days to induce chondrogenic differentiation (50, 51). Culture medium was changed every 3 days (51). Determination of expression of chondrogenic differentiation was performed after 21 days of culture using alcian blue staining (51).

3.3 Osteogenic differentiation

The cells of $1x10^4$ cells/well were cultured in the osteogenic medium (OS) (DMEM supplemented with 10% FBS, 5 mM beta-glycerophosphate, 100nM dexamethasone and 50 µg/ml ascorbic acid). Osteoblastic differentiation markers including alkaline phosphatase activity (ALP) and osteocalcin expression (OCN) at day 3, 7, 14 and 21 were assessed using ELISA (n = 5/group/time point). On the day of experiments, the cells were washed two times using PBS. After that, 200 µl of 1% Triton X-100 in PBS were added into each well, and then the cells were lysed by freezing and thawing for three cycles (30 min/cycle). The mixtures were transferred into the micro-

centrifuge tubes and centrifuged at 2000 x g for 10 min. The supernatants were collected as the cell lysis solutions and they were kept in -80 °C for the analysis of total cellular protein content, ALP activity and OCN expression. The quantification of total protein in the solutions were performed according to the manufacturer's instructions (Bio-Rad protein assay, USA) based on the method of Bradford. The absorbance at 750 nm were read using the micro-plate reader. The ALP activities were measured according to instructions using the commercial kit of Alkaline Phosphatase, AMP Buffer (Human, Germany) according to the recommendation of the International Federation of Clinical Chemistry (IFCC). Levels of the activity were calculated per one milligram of the total cellular protein [(U/L)/mg protein]. Quantification of OCN were performed according to the manufacturer instructions using the commercial kit of osteocalcin enzyme-linked immunosorbent assay (Biomedical Technologies Inc., USA). The solutions were read at 450 nm absorbance using the microplate reader and their concentrations were calculated with the serial diluted standard solution. The OCN levels were demonstrated as ng/mg protein (52).

Statistical analysis

The measured parameters were analyzed using statistics analysis software (SPSS, version 22.0, USA). One-way Analysis of Variance (ANOVA) followed by Tukey HSD were applied to compare the differences among the groups and time points. The levels of statistical significance was set at a $p < 0.05$.

CHAPTER 3

RESULTS

The average volume of buccal fat tissue harvested from the patients was 3.9 ± 2.6 mL.

CFU-F

CFU-F were detected only in Group B during 20 days of culture, whereas CFU-Fs were not detected in Groups A or C (Figure 1).

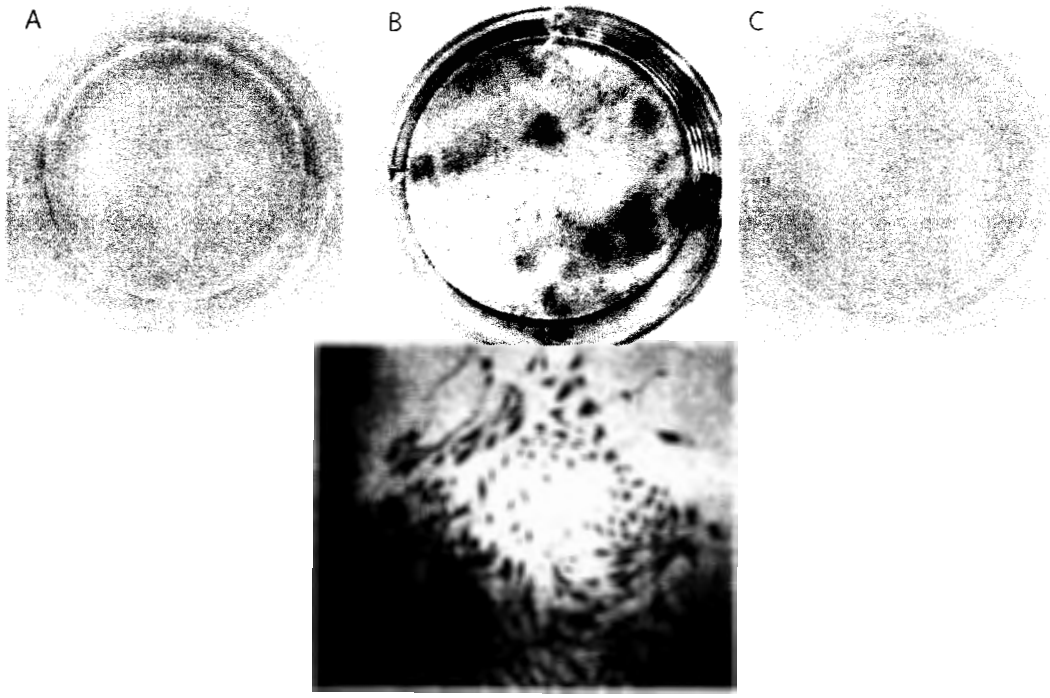

Figure 1. Toluidine blue staining of CFU-F after 20 days of culture. A: Group A plastic adherent cells (PA), **B**: Group B (CD271 positive cells) and **C**: Group C (CD271 negative cells). **D**: The magnified image of Group B (CD271 positive cells) CFU-F.

Flow cytometry analysis

Expression of the MSC immunophenotypes of the ADSCs is demonstrated in Figure 2 and Table 2. Among the groups, the profiles of the positive markers of MSC were not statistically different. The cells of all groups expressed CD73 at the highest levels, followed by CD105 and CD90. Expression of the hematopoietic markers of all groups was less than 1%. The gingival fibroblasts expressed CD73 and 105 significantly less than those of Groups A and B ($p < 0.05$).

Table 2. The percentages of immunophenotyping markers were demonstrated.

CD Markers (%) \ Groups		A (PA)	B (CD271+)	C (CD271-)	Gingival Fibroblasts (control)
MSCs markers	CD 90	54.5±27.4	48.7±16.7	58.1±12.6	56.4±9.3
	CD 105	78.7±12.1	60.6±9.7****	62.7±11.7*****	44.2±13.7##
	CD 73	88.8±5.3*	89.9±6.3**	86.7±13.5***	33.9±5.4#
Hematopoietic markers	CD 14, 20, 34, 45	0.71±0.4	0.72±0.4	0.99±0.1	0.2±0.17

The percentages of CD73 of groups A-C were significantly higher than CD90 (*p=0.03, **p=0.002, ***p=0.006). The percentages of CD105 of groups B and C were significantly higher than CD90 (****p=0.018, *****p=0.02). The percentages of CD90, CD105 and CD73 of the control group were not significantly different. CD 73 and CD 105 of this group were significantly less than those of group A and B (#p =0.002, ##p=0.007)

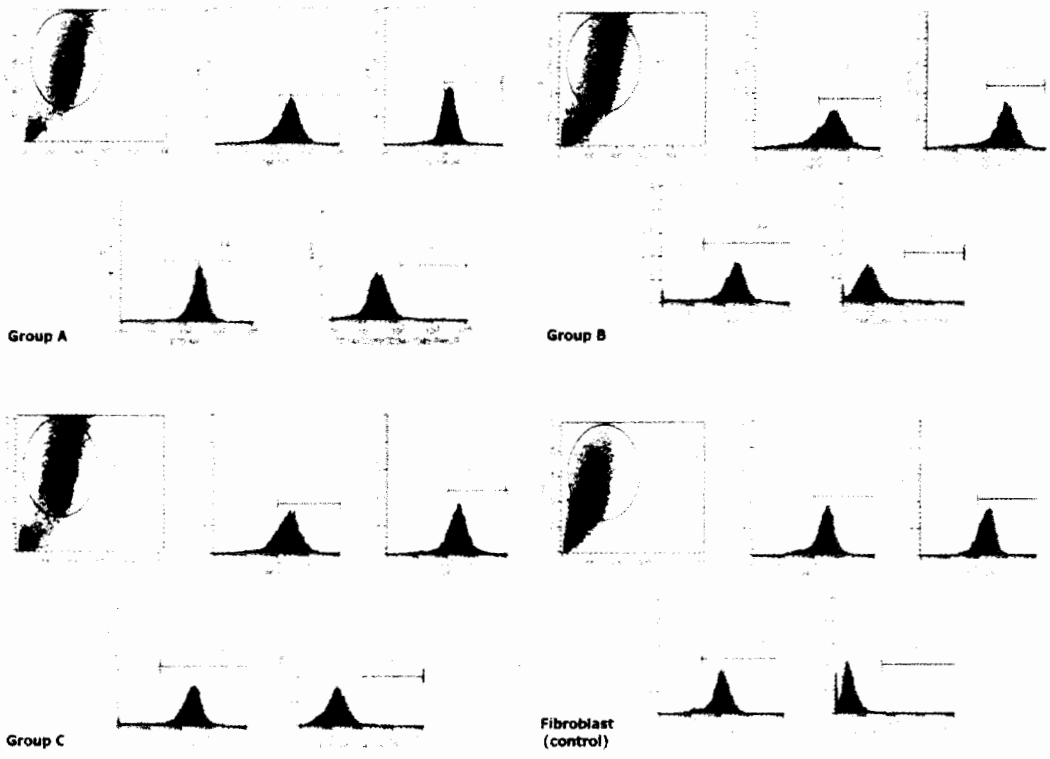

Figure 2. The pictures of flow cytometry analysis show the profiles of the MSC markers and the hematopoietic markers of the experiment groups.

Multi-differentiate potential of the ADSCs

Adipogenic differentiation

After 21 days of culture, lipid vacuoles were detected in red (Figure 3A). The quantitative measurement of the extracted lipid vacuoles is demonstrated in Figure 3B. There was no statistical difference among the groups ($p < 0.05$).

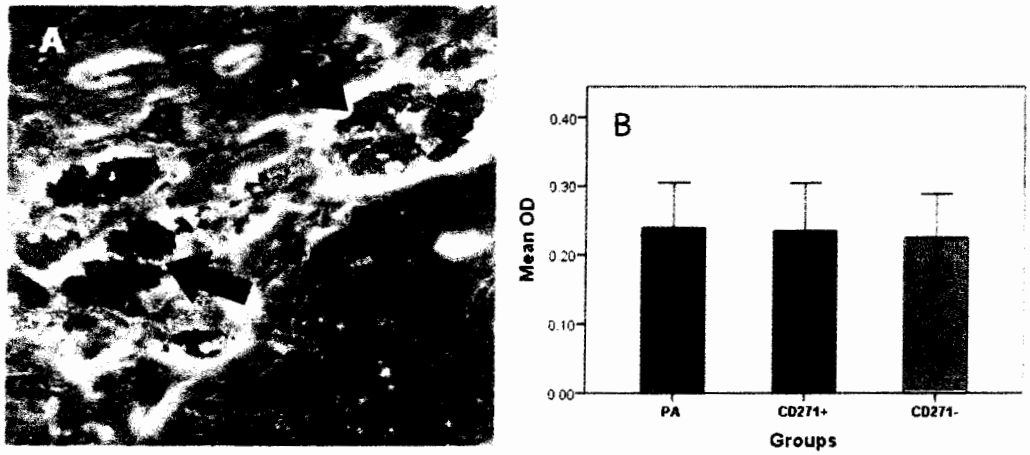

Figure 3. (A) Oil Red O staining demonstrates the lipid vacuoles seen in red (arrows). (B) The bar graphs demonstrate the OD levels of the solubilized Oil Red O. No significant differences were detected among Groups A-C.

Chondrogenic differentiation

After inductive culture, the cell pellets of Groups A-C could produce cartilaginous matrix (Figure 4).

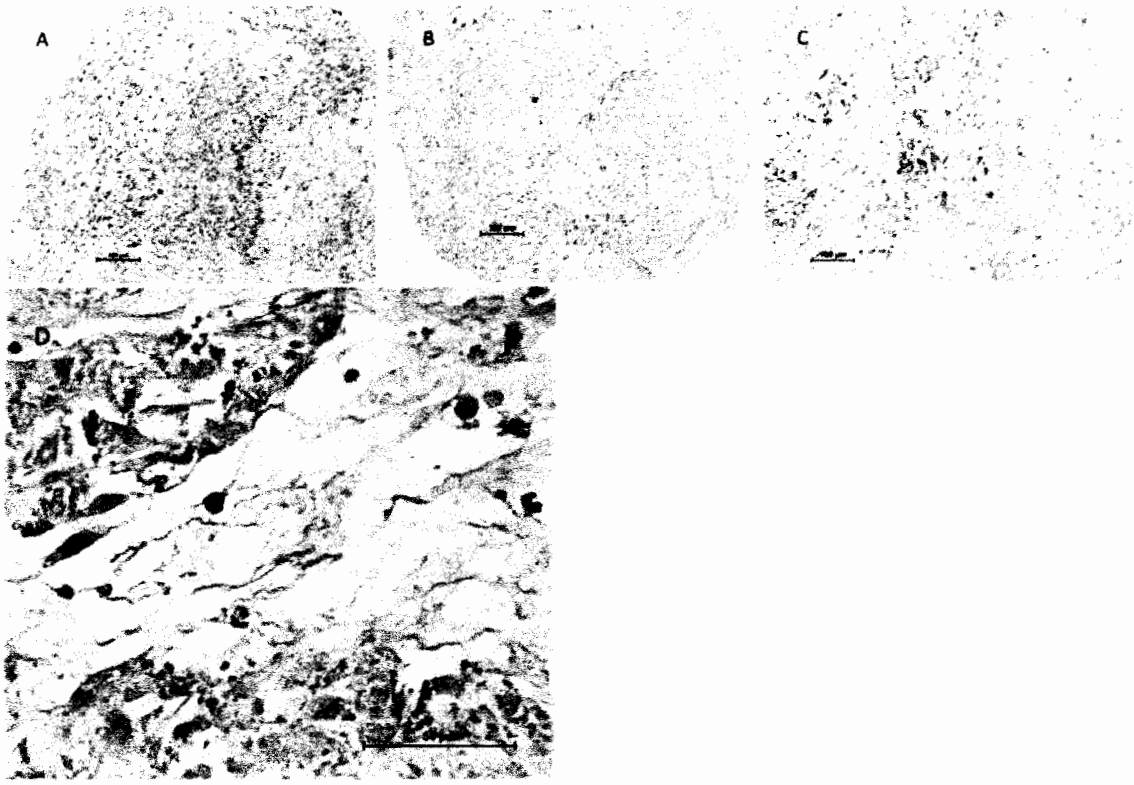

Figure 4. The histological images of the Alcian blue stained pellets of group A plastic adherent cells (A), group B CD271 positive cells (B) and group C CD271 negative cells (C). D; the magnified image of group A showed the deposition of GAGs (blue background) in the extracellular matrix,

Osteogenic differentiation

The ALP levels of Groups A-C are demonstrated in Figure 5. The levels of ALP of Groups A (PA) and B (CD 271 positive cells) seemed to be stable during the first 14 days, and then they remarkably increased at day 21. On day 21, the ALP level of Group A (PA) was significantly greater than the other groups ($p < 0.0001$). In Group C (CD 271 negative cells), the highest ALP was detected at day 3 and then the levels decreased on the following days. The OCN levels are shown in Figure 6. The levels of OCN in Groups A (PA) and B (CD 271 positive

cells) rapidly increased to reach the highest levels on day 7, and then decreased thereafter. The highest expression of OCN in Group C (CD 271 negative cells) was detected at day 3, and then the levels rapidly decreased on the following days.

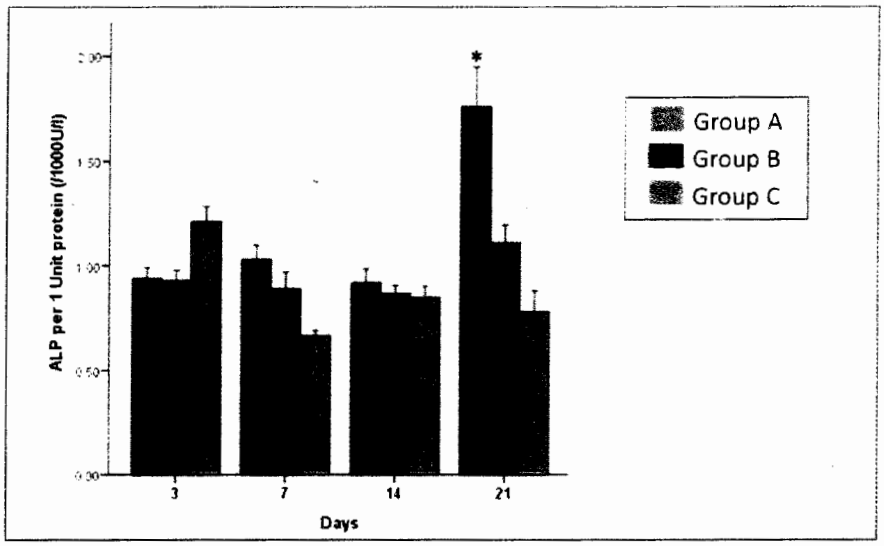

Figure 5. The bar graphs demonstrate ALP activities of group A (PA), group B (CD 271 positive cells) and group C (CD 271 negative cells). The data showed no statistically significant difference of the ALP levels among the groups on the first 14 days of culture. On day 21, the level of group A (PA) was significantly greater than the other groups (*$p<0.0001$) (n=5/group/time point).

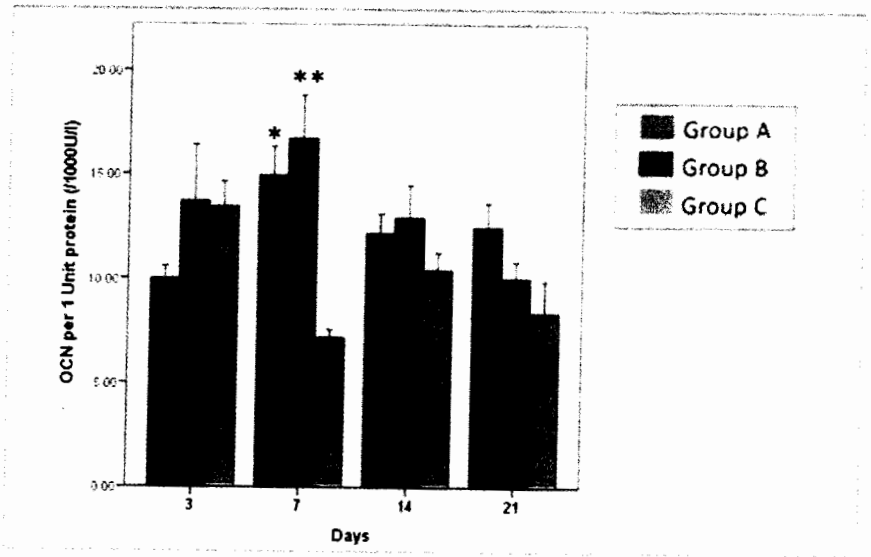

Figure 6. Bar graphs demonstrate the OCN levels of Groups A-C. The maximum levels of Groups A (PA) and B (CD 271 positive cells) were detected on day 7, whereas the maximum level of Group C (CD 271 negative cells) was detected on day 3. On day 7, the levels of Groups A (PA) and B (CD 271 positive cells) were significantly higher than group C (CD 271 negative cells) (*p=0.004, **p=0.001 respectively). (n=5/group/time point)

CHAPTER 4

DISCUSSION

In the field of bone tissue engineering, adipose tissue is an alternative potential source of mesenchymal stem cells, which have the ability to differentiate toward the lineage of osteoprogenitor cells (6, 53, 54). Buccal fat pads are excellent intra-oral sources of adipose tissue that provide greater volumes of tissue compared with dental pulp and periodontal ligament. Moreover, the harvesting technique of the tissue is easily performed under local anesthesia, which is less invasive compared with bone marrow sources. Plastic adherence (PA) is a conventional technique to isolate stem cells from tissue. The technique is routine and easy, but after enzymatic digestion, adipose tissue generates a pellet of stromal vascular fraction (SVF) which contains a heterogeneous cell population. At the cellular level, SVF is composed of mature adipocytes, fibroblasts, nerve cells, endothelial cells, immune cells, and preadipocytic cells (55, 56). Those cells usually have various protein and cytokine expression and differentiation potential (55). Therefore, methods to purify them are still interesting. Cell sorting with specific surface markers to isolate the cells can obtain a more homogenous cell population. MACS would be an optimum method for isolation of stem cells in clinical practices since it can be done as a chair-side procedure and the isolation processes can be finished within 2 h. Moreover, it remarkably reduces cultivation time, and avoids contamination of the cell culture reagents, when compared with the conventional plastic adherence method. However, the amounts of the stem cells from buccal fat tissue, which retained in the column of MACS might be low and they should be further assessed. Several markers have been used to isolate MSCs from various sources. Nevertheless, the specific markers are still not clear. CD271 is considered to be one of the most specific markers to isolate MSCs from bone marrow, dental pulp, and adipose tissue (14-16, 57-61). A recent study (60) stated that CD271 is the best single marker to isolate dental pulp mesenchymal stem cells with the greatest differentiation potential. However, no study has used this marker to isolate ADSCs

from buccal fat pads. Our study is the first to demonstrate the characteristics of the CD 271 positive ADSCs isolated from buccal fat pads using the cell sorting method in terms of expression of MSC markers and the capacity to exhibit trilineage differentiation. Their properties were compared with those isolated with conventional PA and the gingival fibroblasts.

The results of CFU-F assay revealed that self-renewal capacity of the cells was detected only in the CD 271 positive cells, whereas that property was not detected in the PA cells or in the CD 271 negative cells. These results corresponded with some previous studies (14, 15, 58, 62). Poloni, et al (15) compared numbers of CFU-Fs generated by human BM-MSCs isolated using Ficoll gradient and CD271 positive mononuclear cells isolated using MACS. After 14 days culture, the authors found higher numbers of CFU-Fs of the CD271 positive cells compared with the unsorted BM-MSCs. Kuçi, et al (14) demonstrated that CFU-F activity was found only in the CD271 positive cells, while no CFU-Fs were detected in the CD271 negative cells. Jarocha, et al (62) compared the capacity for CFU-F among various methods of isolating MSCs from bone marrow including PA, RosetteSep-isolation, and CD105 positive and CD271 positive selection. The results showed that the CD271 positive selection had the highest number of CFU-F colonies compared with the other groups. Quirici, et al (58) determined the clonogenic potential in three different populations from human adipose tissue including PA, CD271 positive cells, and CD34 positive cells. At less than 10 weeks of culture, no significant differences in the number of CFU-Fs among the groups were detected. However, after 20 weeks, the number of CFU-Fs in the PA group nearly disappeared, which was significantly less than in the CD34 positive and 271 positive groups. These results supported that the homogeneity of the cells could maintain the ability of self-renewal after long-term culture. Regarding our results, the heterogenicity of the PA and the CD271 negative cells was possibly due to contamination of hematopoietic cells, endothelial cells, erythrocytes, fibroblasts, lymphocytes, monocytes, and macrophages. Hence, the strong proliferation of cells could diminish the growth of progenitor cells (26, 48). In the future experiment, the ability to form CFU-F of the CD271 positive and negative

ADSCs after long-term culture should be investigated to prove that whether the selective isolation with the specific marker can support the better self-renewal capacity.

Based on the minimum criteria of ISCT, the cells that display the properties of plastic adherence (PA), positive expression of CD90, CD105, CD73, and negative expression of hematopoietic markers, and multi-differentiation capacity can be termed "MSC" (23). Our results demonstrated that the averages cell populations of PA, CD271 positive, and CD271 negative cells positively expressed CD90, CD105, and CD73, but less than 90%, while the hematopoietic markers including CD14, CD20, CD34, and CD45 negatively expressed without significant difference among the groups. The positive cell numbers were less than the ISCT criteria to identifying MSC, which purposed that the cell population ≥ 95% much express the CD73, CD90 and CD105 (23). However, our result corresponded to the consensus between ISCT and International Federation for Adipose Therapeutics and Science (IFATS) (48), which purposed that the ADSC should be positive to CD13, CD29, CD44, CD73, CD90, and CD105 (>80 %) and positive to CD31, CD45, and CD235a (<2 %). Whereas, the SVF should express the primary markers of stromal cells including CD13, CD29, CD44, CD73, CD90 (>40 %), and CD34 (>20 %), but express the negative markers of CD31 (<20 %) and CD45 (<50 %). The control group of fibroblasts could express the MSC markers, but the amounts were remarkably less than the experiment groups (significant differences were found in CD 73 and CD 105, $P < 0.05$). In addition, they expressed the hematopoietic markers less than the other groups. This character corresponded to previous studies (36, 37), which reported that the fibroblasts resembled many behaviors of MSCs such as cell morphology, self-renewing capacity, and cell surface protein expression, but they lacked multi-differentiation potency. Our results also demonstrated that the CD 271 positive cells were co-expressed with CD 105 (60.6±9.7%), CD 73 (89.9±6.3%), and CD 90 (48.7±16.7%), whereas they expressed the hematopoietic stem cell markers less than 1%. The profile of CD271 positive cells was similar to some previous studies which found that the CD271 positive cells co-expressed with CD90 49.6 ± 1.7% (17), and co-expressed with CD73 91% (63). In addition, the numbers of CD90 positive cells in our study were higher than those of the other previous

studies, which found that only 10-20% of CD271 positive MSCs co-expressed with CD90 (14, 16). Several surface markers have been investigated as co-expression markers of the CD271 positive cells. Some studies found that 82–85% of the CD271 positive cells from adipose tissue co-expressed with CD34 (58, 64). Maria, et al (65) reported the usefulness of using CD271 combined with CD45 to isolate fresh bone marrow MSCs. Mabuchi, et al (66) suggested that a combination of markers using CD271, CD90, and CD106 for the isolation achieved the most potent and genetically stable MSC. For differentiation of the cells, the results of our study demonstrated that the ADSCs could differentiate into three lineages including adipogenesis, chondrogenesis, and osteogenesis. Several previous studies investigated the correlations between the CD markers of the cells and their ability to differentiate. Some studies correspondingly demonstrated that MSCs, which are positive to CD271, 73, and 105, have the potency of chondrogenic diffentiation (67-70), whereas those that are positive to CD90, have more potency for osteogenic differentiation in both in vitro and in vivo (71-73). Arufe, et al (67) investigated the differentiation of CD73 positive and CD271 positive synovial membrane cells and found that the CD271 positive cells had higher potency of chondrogenic differentiation compared with CD73 positive cells. Ruth, et al (60) found that about 10.6% of cultured dental pulp cells were positive for CD271 and they had promising odontogenic and chondrogenic potential. The CD105 positive cells showed significantly greater chondrogenic potential in vitro even when cultured on tissue culture plastic, gel-embedded sheets (68) and biodegradable scaffolds (69). Kavan, et al (55) reported that CD90 positive ADSCs improved osteogenic differentiation over CD90 negative ADSCs, CD105 positive ADSCs, and unsorted cells. In vitro, the authors found that co-selection of CD105low/CD90high cells had more osteogenic phenotype compared with CD105low/CD90low cells. Our results showed that although the amounts of the CD 271 /CD90 positive ADSCs were remarkably less than those of the CD271/CD73 positive and CD271/CD105 positive ADSCs, they could differentiate into osteoblastic lineage after culturing in the osteoblastic inductive conditions. Interestingly, the ALP activity of the PA cells was significantly higher than the other group on day 21. It is possible that the expression of their surface marker was

changed after the periods of culture. Therefore, the CD markers of the cells in the early and late passages should be investigated to demonstrate their regulations of the stemness and multi-differentiation during long periods of culture.

It is known that adipose tissue composes of the various kinds of progenitor cells. The progenitor cells and stem cells can be generated from perivascular tissue, circulating bone marrow, and neural crest (NC) derived cells (74). Several literature reports hypothesize that some subsets of bone marrow, dental pulp, and adipose mesenchymal stem cells originate from the neural crest (75-79). Previous studies believe that the neural crest cells migrated into several tissue during the embryonic period then replaced by the non-NC cells after adulthood (80-82). Therefore, the amount of NC cells in BM-MSCs or ADSCs will be declined with age and resulting in a small population in adults. Wrage et al (83) found only 2% of ADSCs are NC-derived cells which not differentiated to neural cells under the culture conditions. Cuevas-Diaz Duran, et al (64) and Quirici, et al (58), reported that the amounts of CD271 positive cells isolated from fresh human adipose tissue were approximately 2.89% and 4.4% respectively. Correspondingly, Yoshihiro, et al (84) demonstrated that there was a low population of the NC cells in ADSCs and they exhibited a adipocyte-restricted differentiation potential, whereas chondrogenic potential was markedly attenuated. Another theory believes that the perivascular zone is the in vivo niche of mesenchymal stem cells which arise from a fibroblastic or pericytic origin. The cells are recognized as pericytes or perivascular cells which reside in the innermost layer of stromal cells contacting vessel endothelium (36, 84-86). CD146 is considered to be an early surface marker of MSCs derived from perivascular cells (87). CD146 positive perivascular cells can express general MSC surface antigens of CD73, CD90, and CD105, and they commonly express CD31, CD34, and CD45 less than 2% (54, 88, 89). On the other hand, CD146 is also highly expressed in MSCs, but not in dermal fibroblasts (89). Feng-Juan, et al (36) suggest that CD146 is another appropriate stem cell marker for universal detection of MSC populations from various tissues. The authors suggested that $CD271^{positive}$/ $CD146^{negative}$ cells are

bone-lining cells, whereas, CD271 ^{positive} /146 ^{positive} cells have perivascular localization. On the contrary, Yoshihiro, et al (84) identified p75NTR-positive NC-derived cells along the vessels in the trunk fat tissue and found that almost none of them were positive to the pericyte markers. Therefore, the amounts of CD146 should be investigated as the co-expressed marker of CD271 positive ADSCs in future experiments. The MACS method provides rapid isolation of the ADSCs from the fat tissue with reduced cost and culture times. In addition, the process is possible in clinical practices which would avoid chemical reagents.

CHAPTER 5

CONCLUSION

The buccal fat pad is a suitable intra-oral source of mesenchymal stem cells. The CD 271 surface marker could be used as a single marker for the sorting technique of ADSCs from buccal fat tissue. However, using co-expression markers was recommended for purifying the cells from the different origins. The CD271 positive ADSCs had potency of osteogenic differentiation similar to the PA cells. Therefore, they could be applied for bone tissue engineering.

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Appendix

Original Article

Characteristics of Adipose-derived Stem Cells Isolated from Buccal Fat Pads Using CD 271 Cell Sorting Technique

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Abstract

Buccal fat pad is a suitable intra-oral source of Adipose-Derived Stem Cells (ADSCs) for bone tissue engineering. CD271 is one of the most specific cell surface markers used to isolate mesenchymal stem cells from various tissues. However, there has not been a study done that has used the cell-sorting technique with this marker to isolate the ASC from buccal fat tissue. The aim of this study was to compare the characteristics of ADSCs isolated from intraoral buccal fat pads using CD 271+ magnetic-activated cell sorting (MACS) and plastic adherence (PA). Buccal fat tissue was harvested from ten patients who underwent orthognathic surgeries. ADSCs were isolated from the tissue using PA (Group A) and MACS; CD 271+ (Group B) and CD271- (Group C), (5 participants per group). The characteristics of the cells including colony forming unit fibroblast (CFU-F), immunophenotyping markers, and multi-differentiation into tri-lineages were comparatively assessed. Gingival fibroblast served as the negative control group. The results demonstrated that (CFU-F) formed in the Group B cells, but were not detected in the other groups. The cells of groups A-C expressed the mesenchymal stem cell including CD 73, 90 and 105. No statistical difference was detected among the groups. It was noted that CD 73 was detected at the highest levels followed by CD 105 and CD 90 respectively. The cells of the control group expressed those markers remarkably less than the experiment groups (significant differences were found in CD 73 and CD 105, $p<0.05$). In addition, the cells of all groups expressed hematopoietic stem cell markers including CD 14, 20, 34 and 45 at very low levels. The cells of groups A-C demonstrated adipogenic, chondrogenic and osteogenic differentiation when cultured in the inductive conditions. There was no significant difference of those properties among the groups. In conclusion, CD 271 is considered as a proper marker for sorting ADSCs from buccal fat tissue. However, it cannot be used as the sole marker. Although the ADSCs expressed CD 90 at the lowest levels, they still had osteogenic differentiation capacity. Therefore, they can be used as a stem cell source to repair bone defects.

Keywords: Buccal fat stem cells, Adipose-derived stem cells, Stromal vascular fraction, Mesenchymal stem cell, CD271, surface markers, cell sorting technique

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Introduction

Tissue engineering has become a popular alternative method in the field of reconstructive surgery. The bone tissue engineering triad includes scaffolds, cells, and signaling substances. A combination of scaffolds with bone forming cells as cell-scaffold constructs is a good strategy to enhance bone regeneration. Several studies[1-5] obtained better results in promoting new bone when the scaffolds were combined with several cell types, such as primary osteoblasts, mesenchymal stem cells (MSCs) from bone marrow (BM) (BM-MSCs), and dental pulp. Another source of MSCs is fat tissue from which adipose-derived stem cells (ADSCs) are obtained. ADSCs were found to express immunophenotyping markers similar to the BM-MSCs. Moreover, they can be differentiated toward various cell types, especially bone forming cells. Some studies[6-10] revealed that the buccal fat pads are suitable intra-oral sources of the ADSCs which provide a large amount of fat tissue that is easily harvested in routine intraoral surgical fields. Plastic adherence (PA) capacity of stem cells is commonly used to isolate the ADSCs from other cell types since non-adherent cells can be washed out after periods of culture. Although this technique is very simple and cheap, the amount of stem cells obtained is only about one cell per 105 of adherent cells.[11,12] A new method to purify stem cell populations is magnetic-activated cell sorting (MACS) which uses antibody-binding. The specific markers are conjugated to iron oxide microbeads that can retain the desired cells in the column containers in a magnetic field, whereas unlabeled cells are eluted.[13] Some studies[14-17] suggest using low-affinity nerve growth factor receptor (CD271,p75NTR) antibody as a positive selection marker of MSCs. The CD271+ cells obtain high purity of a MSC population that supports self-renewal capacity and multi-differentiation potential. However, there are no comparative results of characterization of the ADSCs isolated from the intraoral buccal fat pads using the conventional PA method or MACS. In this study, CD271 was used as the specific marker of the MSCs and the positive and negative cells to the marker were included for the investigation.

Materials and Methods

Patient enrollment

Ten volunteer patients were enrolled in the study. All patients underwent orthognathic surgeries to correct skeletal discrepancies at the Oral & Maxillofacial Surgery Clinic, Dental Hospital, Faculty of Dentistry, Prince of Songkla University. The protocol of this study was approved by the ethics committee of the Faculty of Dentistry, Prince of Songkla University (EC5909-38-P-LR). The inclusion criteria of the participants included ASA class I, age >20 years old, weight >50 kg and hematocrit ≥35 %. The excluded patients were those with systemic diseases including hereditary blood diseases, disorders of the blood and blood components, blood transmitted diseases, and diabetes.

Isolating ADSCs from fat tissue

The ADSCs of the participants were consecutively isolated using two different methods and divided into three groups. In Group A, five samples of cells were isolated from five patients using PA.[18] The other five patients were the sources of cells for Group B (five samples) and Group C (five samples). In Group B, the cells which were positive to CD 271 (CD271+) were isolated using MACS. In Group C, the cells which were negative to CD271 (CD271-) were isolated using MACS. Each participant underwent orthognathic surgery under general anesthesia. During the operations of Lefort I osteotomy of the maxilla or sagittal split osteotomy of the mandible, some parts of the fat pads were excised, then immediately placed into DMEM (Dulbecco's Modified Eagle Medium, Gibco, USA) and stored at 4°C until the isolation process. The fat tissue was washed several times with sterile phosphate buffered saline (PBS) to remove contaminating debris and red blood cells. The volumes were then measured using a 5 mL sterile disposable syringe. Afterwards, the tissues were minced into small

pieces and enzymatically digested using 0.75 % collagenase type I (Gibco, USA) in PBS at 37°C with gentle agitation for 60 min. The supernatants were collected, and then centrifuged at 400 g for 10 min to exclude the remaining adipocytes and lipid droplets. The cellular pellets were suspended in DMEM supplemented with 10 % fetal bovine serum (FBS) (Gibco, USA), and then filtered through a 100 µm filter (Corning, USA). In group A the cell suspension was plated onto 6-well culture plates (Corning, USA) and cultured in a humidified atmosphere with 5 % CO_2 at 37°C. In groups B and C, the cells were isolated using CD271 antibody for MACS (CD271 MicroBead Kit human, Miltenyi Biotec, Germany). In brief, the cell suspension was re-centrifuged at 300 g for 10 min and the cell pellets were resuspended in 60 µL of buffer (PBS with 0.5 % FBS, and 2 mM EDTA). The cell suspensions were incubated in 20 µL of FcR blocking reagent and 20 µL of CD271 MicroBeads for 15 min at 4°C. Afterwards, the cells were washed by adding 1-2 mL of buffer, and then centrifuged at 300 × g for 10 min. Re-suspended cell solutions were made by adding 500 µL of buffer, and then loaded onto a column which was placed in a MiniMACS separator (Miltenyi Biotec, Germany). The magnetically labeled CD271+ cells of group B were retained in the column, whereas the unlabeled CD 271- cells of group C were run through. The cells of Groups B and C were collected and cultured in DMEM supplemented with 10 % FBS in a humidified atmosphere with 5 % CO_2 at 37°C. The cells of all groups at passages 1-4 were used for the following experiments (five samples/ group/test).

Determining the Characteristics of the cells

Colony-forming unit fibroblast (CFU-F) assays

The cells of each group at passage 1 were plated at a density of 100 cells/well in a 6-well plate to define the numbers of CFU-F. Within 20 days, the cells were fixed with 4 % paraformaldehyde and stained with 0.1 % toluidine blue (Sigma, USA). The CFU-F was observed under a light microscope (Nikon, Japan). The numbers of colonies were counted if the aggregations were at least 50 cells or the colonies were >2 mm in diameters.[2] The cells of each patient were assessed in triplicate.

Flow cytometry analysis

The MSC immunophenotypes of the ADSCs were defined following the International Society for Cellular Therapy (ISCT) protocols.[19] The analysis was performed using a fluorochrome-conjugated monoclonal antibody cocktail in the MSC Phenotyping Kit human (Miltenyi Biotec, Germany). In brief, 5 x 10^5 cells from each group in passages 2 and 3 were incubated in the antibodies against the surface antigens CD73, CD90, and CD105 as the positive markers and CD14, CD20, CD34, and CD45 as the negative markers. At least 10,000 events were acquired for each sample using a fluorescent-activated cell sorting instrument (FACSCalibur, BD Biosciences) and the data were analyzed using CELLQUEST software (BD Biosciences).

Multi-differentiate potential of the ADSCs

Adipogenic differentiation

The cells at 1 x 10^4/well were cultured in adipogenic induction medium comprised of DMEM supplemented with 10 % FBS, 1 µM dexamethasone, 10 µg/mL insulin, 500 µM 3-isobutyl-1-methylxanthine and 200 µM indomethacin (Sigma, USA) for 21 days. The culture mediums were changed every two days. On day 21, the cells were fixed in 10 % formaldehyde for 1 h and stained with Oil Red O solution (20 mg/mL in isopropanol) (Sigma, USA) for 15 min. Lipid vacuoles were quantified by extracting with 100 % isopropanol for 10 min and reading with a microplate reader (Multiscan™Go, Thermo Fisher Scientific) at the absorbance of 540 nm.

Chondrogenic differentiation

The cells (5 x 10^5) were centrifuged at 600 g for 5 min to form cell pellets and re-suspended in 2 mL chrondogenic medium (StemPro Chondrogenesis Differentiation Kit, Gibco) in a 15-mL centrifugation tube (Corning, USA) for 21 days to induce chondrogenic differentiation.[20,21] The culture medium was changed every three days.[20] Determination of expression of

chondrogenic differentiation was performed after 21 days of culture using alcian blue staining.[20]

Osteogenic differentiation

The cells (1 x 10^4 cells/well) were cultured in osteogenic medium (DMEM supplemented with 10 % FBS, 5 mM beta-glycerophosphate, 100 nM dexamethasone, and 50 µg/mL ascorbic acid). Osteoblastic differentiation markers including alkaline phosphatase activity (ALP) and osteocalcin expression (OCN) at days 3, 7, 14, and 21 were assessed using ELISA (five samples/group/time point). On the days of the experiments, the cells were washed two times using PBS. After that, 200 µL of 1 % Triton X-100 in PBS was added into each well and then the cells were lysed by freezing and thawing in three cycles (30 min/cycle). The mixtures were transferred into microcentrifuge tubes and centrifuged at 2000 x g for 10 min. The supernatants were collected as cell lysis solutions and kept at -80°C for the analysis of total cellular protein content, ALP activity, and OCN expression. The quantification of total protein in the solutions were performed according to the manufacturer's instructions (Bio-Rad Protein Assay; Bio-Rad Laboratories, USA) based on the method of Bradford. Absorbance at 750 nm was read using the microplate reader. The ALP activities were measured according to the instructions using the commercial kit of Alkaline Phosphatase, AMP Buffer (HUMAN, Germany) according to the recommendation of the International Federation of Clinical Chemistry. The levels of activity were calculated per one milligram of the total cellular protein (U/L/mg protein). Quantification of OCN was performed according to the manufacturer instructions using the Osteocalcin ELISA kit (Biomedical Technologies Inc., USA). The solutions were read at 450 nm absorbance using the microplate reader and their concentrations were calculated with the serial diluted standard solution. The OCN levels were demonstrated as ng/mg protein.[22]

Statistical Analysis

The measured parameters were analyzed using statistics analysis software (SPSS, version 22.0, USA). One-way analysis of variance (ANOVA) and Tukey HSD were applied to compare the differences among the groups and time points. The level of statistical significance was set at $P<0.05$.

The average volume of buccal fat tissue harvested from the patients was 3.9 ± 2.6 mL.

CFU-F

CFU-Fs were detected only in Group B during 20 days of culture, whereas CFU-Fs were not detected in Groups A or C (Fig. 1).

Flow cytometry analysis

Expression of the MSC immunophenotypes of the ADSCs is demonstrated in Figure 2 and Table 1. Among the groups, the profiles of the positive markers of MSC were not statistically different. The cells of all groups expressed CD73 at the highest levels, followed by CD105 and CD90. Expression of the hematopoietic markers of all groups was less than 1 %. The fibroblasts expressed CD73 and 105 significantly less than those of Groups A and B ($P<0.05$).

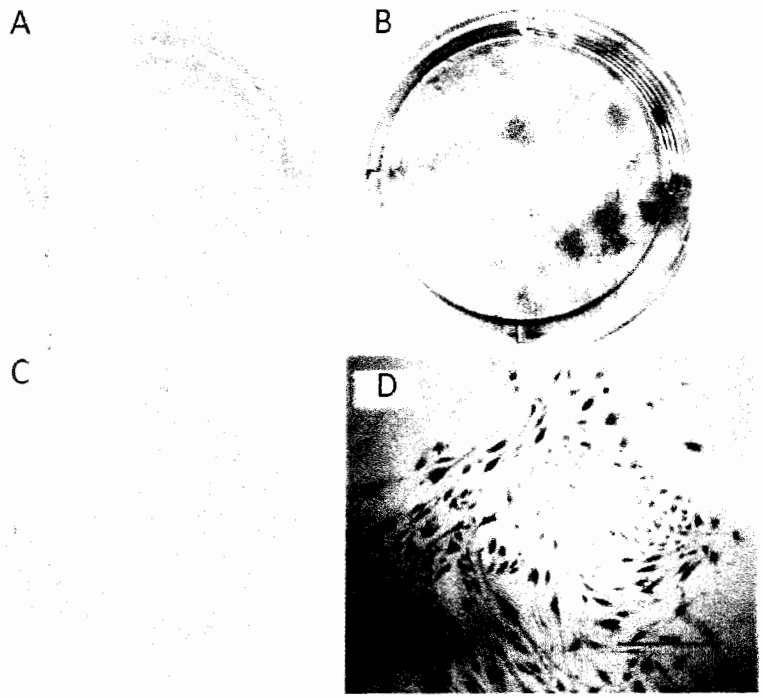

Figure 1 *Toluidine blue staining of CFU-F after 20 days of culture. A: Group A. B: Group B and C: Group C. D: The magnified image of Group B CFU-F.*

Table 1 *The percentages of immunophenotyping markers were demonstrated.*

CD Markers (%)	Groups	A (PA)	B (CD271+)	C (CD271-)	Gingival Fibroblasts (control)
MSCs markers	CD 90	54.5±27.4	48.7±16.7	58.1±12.6	56.4±9.3
	CD 105	78.7±12.1	60.6±9.7****	62.7±11.7*****	44.2±13.7$^{\#\#}$
	CD 73	88.8±5.3*	89.9±6.3**	86.7±13.5***	33.9±5.4$^{\#}$
Hematopoietic markers	CD 14, 20, 34, 45	0.71±0.4	0.72±0.4	0.99±0.1	0.2±0.17

*The percentages of CD73 of groups A-C were significantly higher than CD90 (*p=0.03, **p=0.002, ***p=0.006). The percentages of CD105 of groups B and C were significantly higher than CD90*

*(****p=0.018, *****p=0.02). The percentages of CD90, CD105 and CD73 of the control group were not significantly different. CD 73 and CD 105 of this group were significantly less than those of group A and B ($^{\#}$p =0.002, $^{\#\#}$p=0.007))*

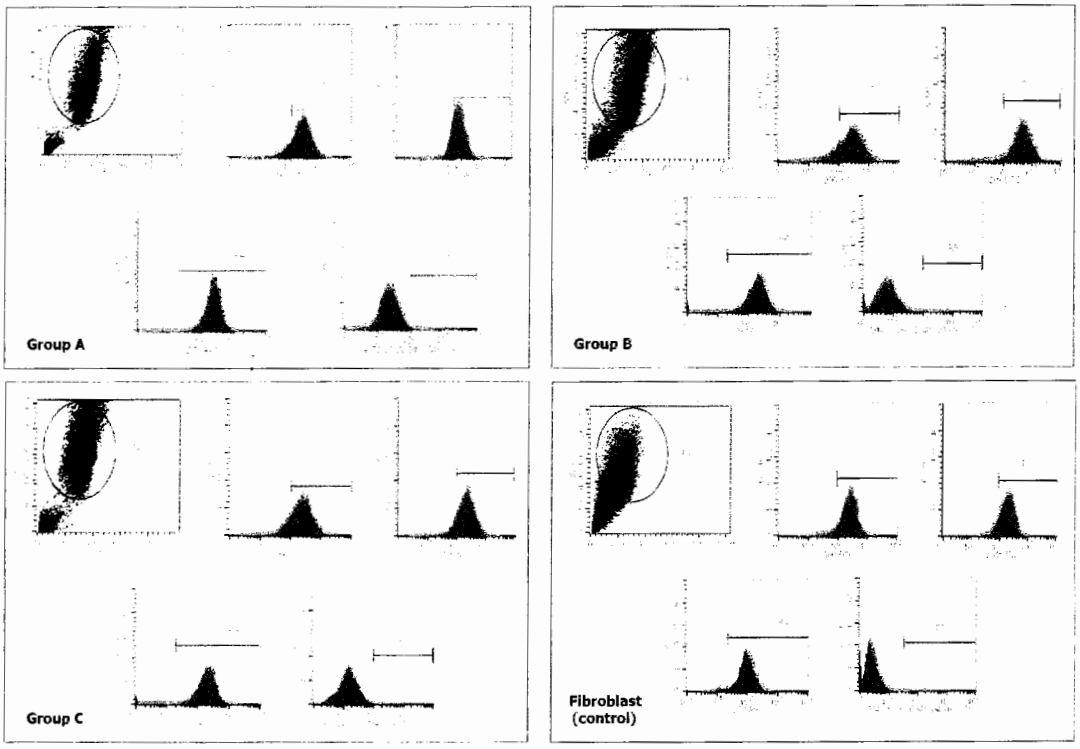

Figure 2 *The pictures of flow cytometry analysis show the profiles of the MSC markers and the hematopoietic markers of the experiment groups.*

Multi-differentiate potential of the ADSCs

Adipogenic differentiation

After 21 days of culture, lipid vacuoles were detected in red (Fig. 3A). The quantitative measurement of the extracted lipid vacuoles is demonstrated in Figure 3B. There was no statistical difference among the groups ($P<0.05$).

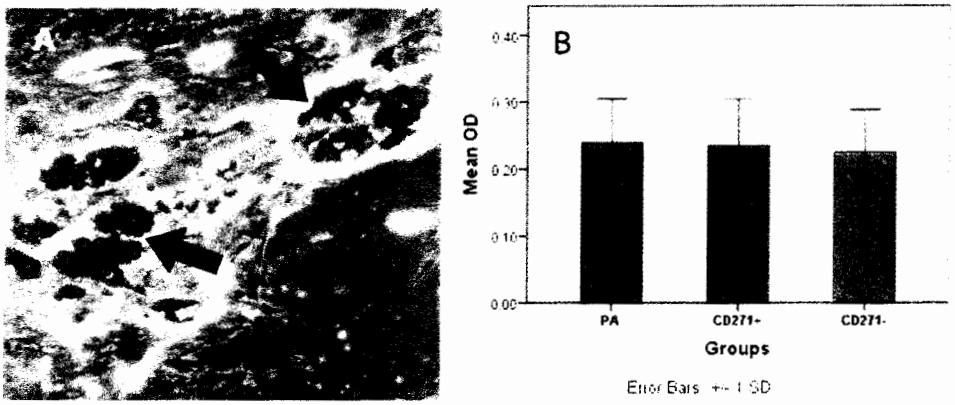

Figure 3 *(A) Oil Red O staining demonstrates the lipid vacuoles seen in red (arrows). (B) The bar graphs demonstrate the OD levels of the solubilized Oil Red O. No significant differences were detected among Groups A-C.*

Chondrogenic differentiation

After inductive culture, the cell pellets of Groups A-C could produce cartilaginous matrix (Fig. 4).

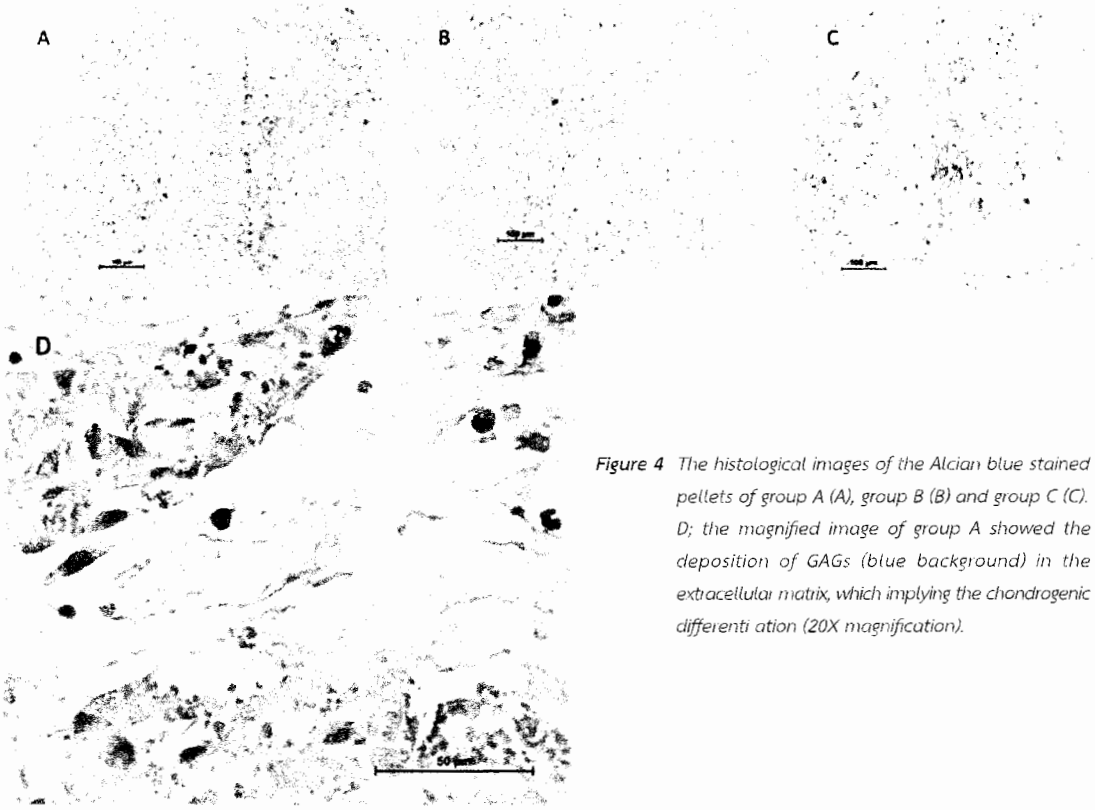

Figure 4 *The histological images of the Alcian blue stained pellets of group A (A), group B (B) and group C (C). D; the magnified image of group A showed the deposition of GAGs (blue background) in the extracellular matrix, which implying the chondrogenic differenti ation (20X magnification).*

Osteogenic differentiation

The ALP levels of Groups A-C are demonstrated in Figure 5. The levels of ALP of Groups A and B seemed to be stable during the first 14 days, and then they noticeably increased at day 21. On day 21, the ALP level of Group A was significantly greater than the other groups (P=0.000). In Group C, the highest ALP was detected at day 3 and then the levels decreased on the following days.

The OCN levels are shown in Figure 6. The levels of OCN in Groups A and B rapidly increased to reach the highest levels on day 7, and then decreased thereafter. The highest expression of OCN in Group C was detected at day 3, and then the levels rapidly decreased on the following days.

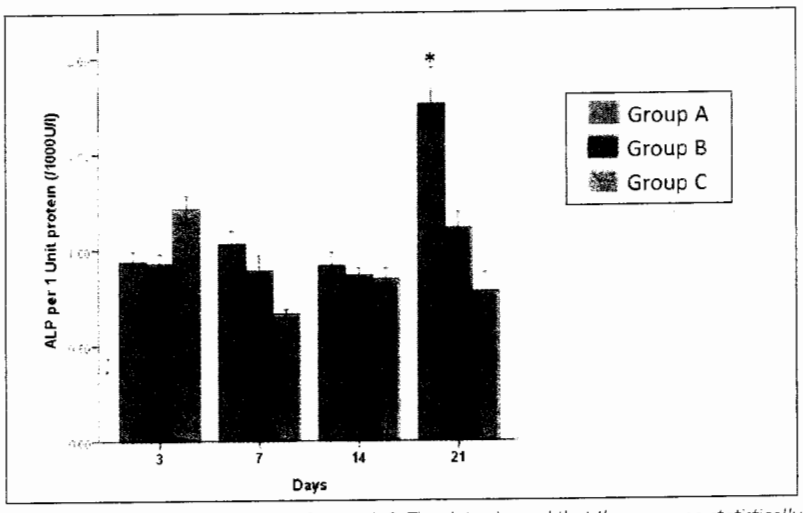

Figure 5 *The bar graphs demonstrate ALP activities of group A-C. The data showed that there was no statistically difference of the ALP levels among the groups on the first 14 days of culture. On day 21, the level of group A was significantly greater than the other groups (*p=0.001).*

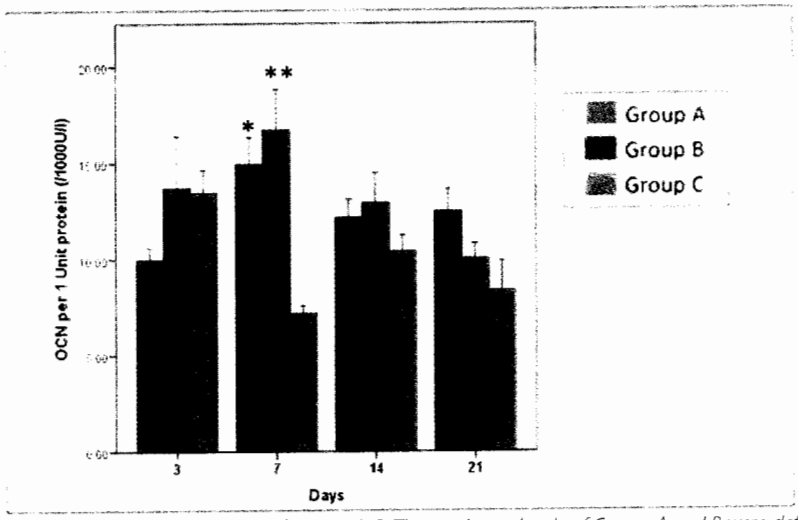

Figure 6 *Bar graphs demonstrate the OCN levels of Groups A-C. The maximum levels of Groups A and B were detected on day 7, whereas the maximum level was detected in Group C on day 3. The osteocalcin levels of Groups A and B were significantly higher than group C (*p=0.004, **p=0.001 respectively).*

Discussion

In the field of bone tissue engineering, adipose tissue is an alternative potential source of mesenchymal stem cells which have the ability to differentiate toward the lineage of osteoprogenitor cells.[6,23,24] Buccal fat pads are excellent intra-oral sources of adipose tissue that provide greater volumes of tissue compared with dental pulp and periodontal ligament. Moreover, the harvesting technique of the tissue is easily performed under local anesthesia which is less invasive compared with bone marrow sources. Plastic adherence is a conventional

technique used to isolate stem cells from tissue. The technique is routine and easy, but after enzymatic digestion, adipose tissue generates a pellet of stromal vascular fraction (SVF) which contains a heterogeneous cell population. At the cellular level, SVF is composed of mature adipocytes, fibroblasts, nerve cells, endothelial cells, immune cells, and preadipocytic cells.[25,26] Those cells usually have various protein and cytokine expressions and potency of differentiation.[25] Therefore, methods to purify them are still interesting. Cell sorting with specific surface markers to isolate the cells can obtain a more homogenous cell population. MACS would be the optimum method for identification of stem cells in clinical practices due to the fact that it can be done as a chairside procedure and the isolation processes can be finished within two hours. Moreover, it remarkably reduces cultivation time, and avoids contamination of the cell culture reagents, when compared with the conventional plastic adherence method. However, the amounts of stem cells from buccal fat tissue, retained in the column of MACS might be low and they should be further assessed. Several markers have been used to isolate MSCs from various sources. Nevertheless, a standard acceptable definition has not reached a consensus. The two types of CD surface markers of stem cells are sole markers and stemness markers. A sole marker is considered to be sufficient to identify stem cells from their in vivo environment, whereas stemness markers are used to identify subsets of cells with high CFU-Fs and trilineage potential. In principle, the sole markers are highly expressed, while the stemness markers may be moderately detected.[27] CD271 is considered to be one of the most specific markers to isolate MSCs from bone marrow, dental pulp, and adipose tissue.[14-16,28-32] A recent study[31] stated that CD271 is the best single marker to isolate dental pulp mesenchymal stem cells with the greatest differentiation potential. However, there has not been a study done which has used this marker to isolate ADSCs from buccal fat pads. Our study is the first to demonstrate the characteristics of the CD 271+ ADSCs isolated from buccal fat pads using the cell sorting method in terms of expression of MSC markers and the capacity to exhibit trilineage differentiation. Their properties were compared with those isolated with conventional plastic adherence and the gingival fibroblasts.

The results of CFU-F assay revealed that self-renewal capacity of the cells was detected only in the CD 271+ cells, whereas that property was not detected in the PA cells or in the CD 271- cells. These results corresponded with some previous studies.[14,15,29,33] Poloni, *et al.* compared numbers of CFU-Fs generated by human BMSCs isolated using Ficoll gradient and CD271+ mononuclear cells isolated using MACS. After 14 days of culture, the authors found higher numbers of CFU-Fs of the CD271+ cells compared with the unsorted BM-MSCs[15] Quirici, *et al.* determined the clonogenic potential in three different populations from human adipose tissue including PA, CD271+ cells, and CD34+ cells. At less than 10 weeks of culture, there were no detectable significant differences in the numbers of CFU-Fs among the groups. However, after 20 weeks, the number of CFU-Fs in the PA group nearly disappeared which was significantly less than in the CD34+ and 271+ groups.[29] Kuçi, *et al.* demonstrated that CFU-F activity was found only in the CD271+ cells, while there were no CFU-Fs detected in the CD271– cells.[14] Jarocha, *et al.*[33] compared the capacity for CFU-Fs among various methods of isolating MSCs from bone marrow including PA, RosetteSep-isolation, and CD105+ and CD271+ selection. The results showed that the CD271+ fraction had the highest number of CFU-F colonies compared with the other groups.[33] It is possible that the heterogeneous cells isolated using PA and the CD271- cells were possibly contaminated with hematopoietic cells, endothelial cells, erythrocytes, fibroblasts, lymphocytes, monocytes, and macrophages. The strong proliferation of cells can diminish the growth of progenitor cells.[34,35] Therefore, selective isolation using a specific marker can promote better self-renewal capacity.

Based on the minimum criteria of ISCT, the cells

that display the properties of PA, positive expression of CD90, CD105, CD73, and negative expression of hematopoietic markers, and multi-differentiation potency can be termed "MSC".[36] Our results demonstrated that the averages cell populations of PA, CD271+, and CD271- groups positively expressed CD90, CD105, and CD73 were less than 90 %, while the negatively expressed hematopoietic markers of CD14, CD20, CD34, and CD45 were less than 1 % without a significant difference among the groups. The positive cell numbers were less than the ISCT criteria to identify MSC, which purposes that the cell population should express the CD73, CD90 and CD105 ≥ 95 %.[36] However, our results corresponded to the consensus between ISCT and International Federation for Adipose Therapeutics and Science (IFATS)[35], which purposes that the ASC should be positive to CD13, CD29, CD44, CD73, CD90, and CD105 (>80 %) and negative to CD31, CD45, and CD235a (<2 %). Whereas, the SVF should express the primary markers of stromal cells including CD13, CD29, CD44, CD73, CD90 (>40 %), and CD34 (>20 %), but express the negative markers of CD31 (<20 %) and CD45 (<50 %).

Interestingly, the control group of fibroblasts could express the MSC markers, but the amounts were remarkably less than the experiment groups (significant differences were found in CD 73 and CD 105, $P<0.05$). In addition, they negatively expressed the hematopoietic markers less than the other groups. This character corresponded to previous studies,[27,37] which reported that the fibroblasts resembled many behaviors of MSCs such as cell morphology, self-renewing capacity, and cell surface protein expression, but they lacked multipotency.

It was noted that the CD271+ cells expressed CD73 at the highest levels of 89.9±6.3 % followed by CD105 at 60.6±9.7 % and CD90 at 48.7±16.7 %. Therefore, our results also demonstrated that the CD 271+ cells were co-expressed with CD105, 73, and 90, whereas they negatively expressed the hematopoietic stem cell markers. The result was similar to some previous studies which found that 49.6±1.7 % of CD271+ ADSCs co-expressed with CD90[17] and 99 % of CD 217+ cells co-expressed with CD90 and CD105[38], but contrasted to other studies, which found that only 10-20 % of CD271+ BMSCs co-expressed with CD90.[14,16] Several surface markers have been investigated as co-expression markers of the CD271+ cells. Some studies found that 82–85 % of the CD271+ cells from adipose tissue co-expressed with CD34.[29,38] Maria, *et al.*[39] reported the usefulness of using CD271 combined with CD45 to isolate fresh bone marrow MSCs. Mabuchi, *et al.*[40] suggested that a combination of markers using CD271, CD90, and CD106 for the isolation achieved the most potent and genetically stable MSC.

For differentiation of the cells, the results of our study demonstrated that the ADSCs could differentiate into three lineages including adipogenesis, chondrogenesis, and osteogenesis. Several previous studies investigated the correlations between the CD markers of the cells and their ability to differentiate. Some studies correspondingly demonstrated that MSCs, which are positive to the CD271, 73, and 105, have the potency of chondrogenic differentiation,[41-44] whereas those that are positive to CD90, have more potency for osteogenic differentiation in both *in vitro* and *in vivo*.[45-47] Arufe, *et al.*[41] investigated the differentiation of CD73+ and CD271+ synovial membrane cells and found that the CD271+ cells had higher potency of chondrogenic differentiation compared with CD73+ cells. Ruth, *et al.*[31] found that about 10.6 % of cultured dental pulp cells were positive for CD271 and they had promising odontogenic and chondrogenic potential. The CD105+ cells showed significantly greater chondrogenic potential *in vitro* even when cultured on tissue culture plastic, gel-embedded sheets[42] and biodegradable scaffolds.[43] Kavan, *et al.*[25] reported that CD90+ADSCs underwent improved osteogenic differentiation over CD90-, CD105+, and unsorted cells. *In vitro*, the authors found that co-selection of CD105low+/CD90high+ cells had more osteogenic phenotype compared with CD105low+/CD90low+ cells. Our results showed that although the amounts of the

CD 271+/90+ADSCs were remarkably less than those of the CD271+/73+ and 271+/105+cells, they could differentiate into osteoblastic lineage after culturing in the osteoblastic inductive conditions. Moreover, the levels of osteogenic differentiation markers of the CD271+cells were not statistically different to those of the PA cells. Therefore, it is presumed that the osteogenic differentiation capacity of the CD 271+ADSCs was similar to that of the PA ADSCs.

Various kinds of progenitor cells are present in the perivascular niche of adipose tissue, including tissue-resident mesoderm-derived cells, circulating bone marrow-derived cells, and neural crest (NC)-derived cells.[48] However, the developmental origin of ADSCs still remains unclear. Several literature reports hypothesize that some subsets of bone marrow, dental pulp, and adipose mesenchymal stem cells originate from the neural crest.[49-53] It is found that the neural crest-derived stem cells colonize earlier, but are largely replaced by non-NC derivatives. Therefore, the contribution of NC cells to either BM-MSCs or adipogenic progenitors sharply declines with age and a very small proportion of the NC-derived cells exist in adults.[54-56] Wrage *et al.*[57] reported that approximately 2 % of ADSCs are NC-derived and they do not contribute to neural differentiation under culture conditions. Cuevas-Diaz Duran, *et al.* and Quirici, *et al.*, reported that the amounts of CD271+ cells isolated from fresh human adipose tissue were approximately 2.89 % and 4.4 % respectively.[29,58] Correspondingly, Yoshihiro, *et al.*[58] demonstrated that a minor subpopulation of ADSCs was derived from NC cells and they exhibited an adipocyte-restricted differentiation potential, whereas chondrogenic potential was markedly attenuated. Another theory believes that the perivascular zone is the *in vivo* niche of mesenchymal stem cells which arise from a fibroblastic or pericytic origin. The cells are recognized as pericytes or perivascular cells which reside in the innermost layer of stromal cells contacting vessel endothelium.[27,58-60] CD146 is considered to be an early surface marker of MSCs derived from perivascular cells.[61] CD146+ perivascular cells can express general MSC surface antigens of CD73, CD90, and CD105, and they commonly negatively express CD31, CD34, and CD45.[25,62,63] On the other hand, CD146 is also highly expressed in MSCs, but not in dermal fibroblasts.[63] Feng-Juan, *et al.*[27] suggest that CD146 is another appropriate stem cell marker for universal detection of MSC populations from various tissues. The authors suggested that CD271+/CD146- cells are bone-lining cells, whereas, CD271+/146+ cells have perivascular localization. On the contrary, Yoshihiro, *et al.*[58] identified p75NTR-positive NC-derived cells along the vessels in the trunk fat tissue and found that almost none of them were positive to the pericyte markers. Therefore, the amounts of CD146 should be investigated as the co-expressed marker of CD271+ADSCs in future experiments.

Conclusion

The buccal fat pad is a suitable intra-oral source of mesenchymal stem cells. The CD271 surface marker seems not to be suitable to be used as the single marker for the sorting technique of ADSCs from buccal fat tissue.

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Best Oral Presentation - Second Place

(Graduate Student Track)

awarded to

Monsikan Phopetch

for the research entitled

"Characteristics of Adipose-Derived Stem Cells Isolated from Buccal Fat Pads Using CD271 Cell Sorting Technique"

the 16th National Scientific Conference of the Dental Faculty Consortium of Thailand

"Advancement in Dentistry"

on 18 - 20 July 2018

at BP Samila Beach Hotel, Songkhla, Thailand

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Award

Second Place in oral presentation for the research entitled "Characteristics of adipose-derived stem cells Isolated from buccal fat pads using CD 271 cell sorting technique" the 16 th National Scientific Conference of the Dental Faculty Consortium of Thailand "Advancement in Dentistry" on 18-20 July 2018 at BP Samila Beach Hotel, Songkhla, Thailand.

List of Publication and Proceeding

Phopetch M, Thuaksuban N, Nuntanaranont T, Leepong N, Vongvatcharanont S, Sretrirutchai S. Characteristics of adipose-derived stem cells Isolated from buccal fat pads using CD 271 cell sorting technique. *JDAT* 2018;68:36-49.

Phopetch M, Thuaksuban N, Nuntanaranont T, Leepong N, Vongvatcharanont S, Sretrirutchai S. Characteristics of adipose-derived stem cells Isolated from buccal fat pads using CD 271 cell sorting technique. The 16th National Scientific Conference of the Dental Faculty Consortium of Thailand. 18-20 July 2018.