Gingival Mesenchymal Stem Cells from Wistar Rat's Gingiva (*Rattus Novergicus*) – Isolation and Characterization *(In Vitro* Study)

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Abstract

Gingiva is emerging as a source of Mesenchymal Stem Cells. Gingival Mesenchymal Stem Cell has been isolated and characterized from the gingival connective tissue of wistar rat (*Rattus Novergicus*). Gingival Mesenchymal Stem Cell sources are rich, attainable and easy to isolate through minimal invasive procedure. Gingival Mesenchymal Stem Cells are ideal to accelerate bone regeneration. The aim of this study was to analyze Gingival Mesenchymal Stem Cells from Wistar Rats' gingiva (Rattus Novergicus) isolation and characterization by CD34, CD44, CD73, CD90, CD105 expression.

This study was descriptive observational with simple random sampling method. Gingival Mesenchymal Stem Cells were isolated from healthy, 200 gram, 1 month year old, male rat's (*Rattus Novergicus*) lower gingival tissue through gingivectomy procedure (n=4). Gingiva were minced into small fragments then cultured in 2 weeks. The culture was passaged every 3-5 days after cultured and plated. The isolated Gingival Mesenchymal Stem Cells in passage 5 were characterized by CD34, CD44, CD73, CD90, CD105 using Immunocytochemistry and flowcytometry examination.

Gingival Mesenchymal Stem Cells strongly expressed CD44+, CD73+, CD90+, CD105+ but did not express CD45- and CD34-. Gingival Mesenchymal Stem Cells' morphology was fibroblast-like, spindle-shaped, colony-forming abilities, and stick to the culture plate.

Gingiva is potential Stem Cell source. Gingival Mesenchymal Stem Cells has multipotency ability with proliferation and mesenchymal stem cells characteristic advantageous for tissue engineering and regenerative therapy.

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Introduction

The regenerative medicine and tissue engineering using mesenchymal stem cell have emerged in medicine nowadays.[1] Recently, many clinician have been interested to overcome the limitation in existing medical practice procedure by using mesenchymal stem cell.[2] Cells, biomaterials and biochemical factors were three fundamental factor of Tissue engineering. Mesenchymal Stem Cells (MSCs) have considerable regeneration ability that capable in tissue engineering.[3] The MSCs are multipotent stromal cells which have osteogenic, chondrogenic, and adipogenic ability.[4] MSC regarded as readily available regenerative cells that able mobilizing to the certain signal of various cell response in the body.[5] The MSCs have high proliferative growth capacity. The MSCs hold abundant favorable therapy for tissue

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engineering and regenerative medicine therapy clinically.[6]

Recently, the source for harvesting stem cells (SCs), progenitor/regenerative cells has gained lots of attention. MSCs have been isolated from various tissues such as bone marrow tissue, joint and muscle, adipose tissue, and blood.[4] The researchers have been explored the oral cavity to reserve cells with the prospective ability to various differentiated tissues.[1] Gingival Mesenchymal Stem/Progenitor Cells (GMSCs) was isolated from gingival connective tissue.[7]

Periodontium was the tooth supporting and investing tissue, comprising gingiva, alveolar bone, periodontal ligament, and cementum. The periodontal tissue develops functions as one unit.[8] The gingiva was a part of periodontal tissue. The gingiva characterized by it's the magnificent vigorous wound healing properties with a fast regeneration and reconstitution of tissue architecture following injury or excision without cicatrix.[1,9] Gingiva is easily accessible and is often resected during standard surgical procedures such as dental crown lengthening, gingivectomy and multiple periodontal surgeries with minimal patient discomfort.[10] Nowadays, the GMSCs with potential immunoregulator, modulate immunity and regenerative ability have been isolated and characterized from the gingival connective tissue. These cells, in contrast to other MSC sources, are abundant, easily obtainable and readily accessible through minimally invasive cell isolation techniques. Harvested gingival tissue is de-epithelized, and the lamina propria is separated and collected. The lamina propria of gingiva tissue is minced and used to isolate the MSCs.[8]

The isolation and characterization of MSCs can be done by condition. The MSCs has an adhere ability to the plate in standard culture conditions. MSCs have specific cell marker such as Homing Cell Adhesion Molecule (CD44), Extracellular Enzyme - Ecto-5'-Nucleotidase (CD73), Thy-1 (CD90) and Endoglin (CD105). However, the MSCs do not express Hematopoietic Stem Cells (HSCs) surface cell marker such as PTPRC (CD45).[4,11] The aim of this study was to analyze GMSCs from Wistar rats (*Rattus Novergicus*) isolation and characterization by CD34, CD44, CD73, CD90, CD105 expression.

Materials and methods

This study received an ethical clearance approval letter for animal subjects from the Faculty of Dental Medicine, Universitas Airlangga Surabaya, East Java, Indonesia Ethics Research Committee with number 289/HRECC.FODM/XII/2017. The research was conducted at an experimental laboratory within the Stem Cell Research and Development Centre, Universitas Airlangga, Surabaya, East Java, Indonesia.

The research was observational descriptive study incorporated the use of an animal model consisting of 4 samples determined using Lameshow's formula and selected randomly. The exploration of GMSCs involved the use of lower anterior gingiva from 1month year old Wistar male rats 200 gram. The immunofluorescence immunocytochemistry and flowcytometry was at passage 5 to examine and measured CD34, CD44, CD45, CD73, CD90, CD105 expression.

Gingival Mesenchymal Stem Cells – Isolation and Culture Procedure

GMSCs isolation and culture method was done based on research by Rantam et al.[12] Subsequently, all culture procedures should be conducted in a closed tissue culture using aseptic techniques. All samples were anesthetized using rodent's anesthesia before gingivectomy. Gingivectomy procedure was performed to produces sufficient gingival tissue to be cultured. Gingival tissue was attached to a 15ml Heparin tube (Sigma Aldrich®, USA) previously filled with 3 ml α-MEM. Each gingival tissue was transferred to a 15ml sterile tube with a blue cap and diluted with 1 x Phosphate Buffer Saline (PBS) (Sigma Aldrich®, USA) sterile to a total volume of 8 ml. Each tube was then rinsed twice with 5ml x PBS and the contents were combined with aspirate solution. In every case, the gingival tissue was placed in a Ficoll (Sigma Aldrich®, USA) temperature chamber in a separate 15 ml tube. Furthermore, each aspirate was coated with Ficoll before being centrifuged (Sorvall™ MX Series Floor Model Micro-Ultracentrifuge, Thermo Fisher, USA) at 1600 rpm for 15 minutes at room temperature. After centrifugation, collection was effected at the "buffy coat" location on the surface of Ficoll-PBS by means of a sterile Pasteur pipette and placed in a 15ml tube (Sigma Aldrich®, USA).

Each sample was diluted with 1x PBS to a total volume of 15ml, with the tube being turned 3-5 times as a means of achieving an even mix. At the next stage, centrifugation was undertaken for 10 minutes at a speed of 1600 rpm before the supernatant and cells suspended in 6ml of CCM were removed before heating. Having placed between $5cm^2$ and $10cm^2$ of cells on the plate, they were incubated at 37°C at 5% CO^2 moisture and allowed to attach to the cell for 18-24 hours. Approximately 24 hours later, the media and cells not attached were disposed of. 5ml 1x PBS was then added before being heated in the culture, shaken well and used to cover the surface area. It was then disposed of with 1x PBS prior to the washing process being repeated twice. Ten minutes later, 10ml of fresh CCM was added to the dish before it was returned to the incubator. The cells were incubated at 37°C and 5-10% CO^2 moisture with the culture being observed daily by means of an inverted microscope. The media was removed and the cells rinsed with 5ml or 10ml of 1x PBS prior to heating every three day. The PBS was subsequently discarded and the dish filled with 10ml of fresh CCM. This process was continued until the concentration of confluent cells reached between 60% and 80%. If the cell developed in the cell plant, the latter had to be balanced in the incubator at 5% CO^2 moisture and 37^o C for 48 hours before use.

Characterization GMSCs by immunofluorescence

GMSCs characterization using immunofluorescence was conducted based on research by Jin et al.[4] On examination, immunocytochemical cells that have been monolayer made single cell trypsin process. Trypsin was added and centrifuged at 1600 rpm for 5 minutes. The pellets were added to 1ml of αMEM growth medium (Sigma Aldrich®, USA). GMSCs were cultured in a chamber slide (BD Biosciences, USA) suspended and grown on a special glass of 20µl. The object glass was placed in a box containing wet paper and then incubated at 37^oC for one hour before the 4% paraformaldehyde (Sigma-Aldrich Co.) was used to fixed the cells, then permeabilized in PBS and 1% TritonX100 for 3 min. GMSCs were stained with anti-CD34, anti-CD44, anti-CD45, anti-CD73, anti-CD90, anti CD105 monoclonal antibodies (Sigma Aldrich®, USA) were added to each sample and then incubated at 37^oC for 45 minutes. The PBS washing and drying process was repeated. At the next immunofluorescence examination on glass object with 50% glycerin dropped above the glass object and viewed the results with a florescent microscope with 100x magnification (Automated Fluorescence Microscope, BX63, Olympus®, USA). When fluorescent the results are positive. If not fluorescent the results are negative.

Characterization GMSCs by Flowcytometry

The GMSC characterization using flowcytomery was done based on research by Du et al.[11] Surface markers for bulk-cultured GMSCs were quantified using flowcytometry. Trypsin/EDTA 0.05% resuspended in Hanks' Balanced Salt Solution (Sigma-Aldrich) supplemented with 5% FCS blocking buffer was used to detached cell after confluence. The immunophenotype was determined by flowcytometry 10^6 gingival cells (passage 5) were incubated with specific monoclonal antibodies (10 mg/mL), conjugated with Fluorescein Isothiocyanate (FITC), Phycoerythrin (PE) in 250ml PBS. The antibody anti-CD44, anti-CD45, anti-CD73, anti-CD90, anti CD105 primary monoclonal antibodies (Sigma Aldrich®, USA) was used. Cells then diluted in 4 ml PBS centrifugated and resuspended with 600ml phosphate buffered saline–formaldehyde 2% 1 hour on ice at dark room. The Isotype controls used were immunoglobulin G (IgG1) FITC and IgG1 PE monoclonal antibodies 45 minutes on ice in the dark room. Cells were fixed in fluorescence-activated cell sorting solution after washing. The analysis was performed with a flowcytometry (Epics-XL/MCL, Beckman Coulter, Fullerton, CA, USA).

Results

GMSCs from Wistar Rat's gingiva were isolated by adherence splitting and reaching 95% confluence. GMSCs initial cultures showed small rounded cells, spindle-shaped cells under inverted microscope. The cells homogeneously showed a fibroblast-like, spindle shape from the first until fifth passage (Figure 1). The GMSCs was expressed higher positive MSCs markers (Figure 2) than contrary HSCs (Figure 3). The CD105 expression has the highest mean among another positive MSC marker examined using

immunocytochemistry (Figure 4) and flowcytometry (Figure 5).

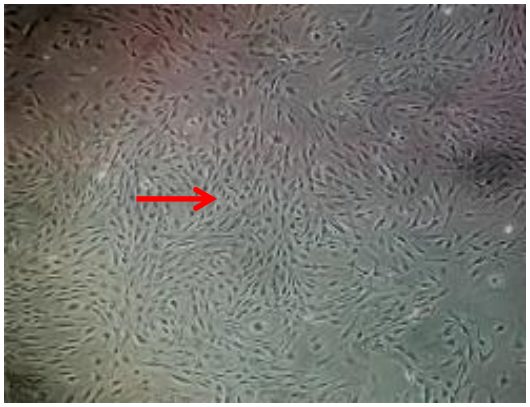


Figure 1. The morphology of Gingival Mesenchymal Stem Cells showed fibroblast-like morphology, colony-forming abilities, spindle-shaped, and adherence to plate.

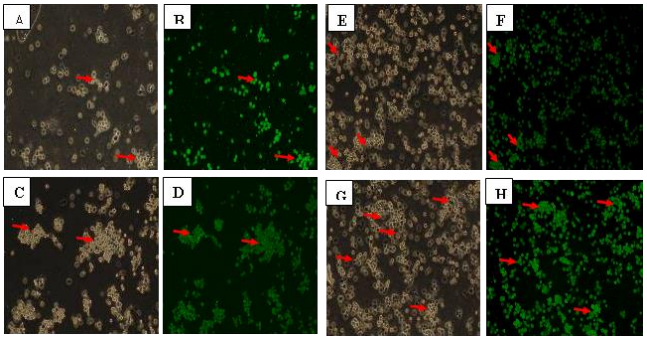


Figure 2. GMSCs expressed positive (+) MSCs surface cell markers (Red Arrow). A, B. CD44; C, D. CD73; E, F. CD90; G, H. CD105.

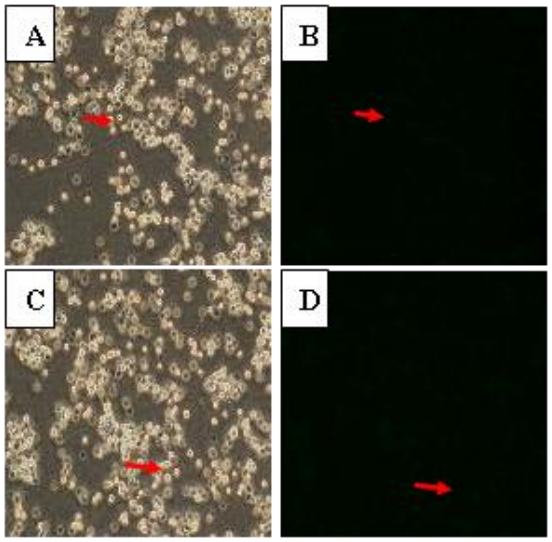


Figure 3. GMSCs expressed negative (-) HSCs surface cell markers (Red Arrow). A, B.

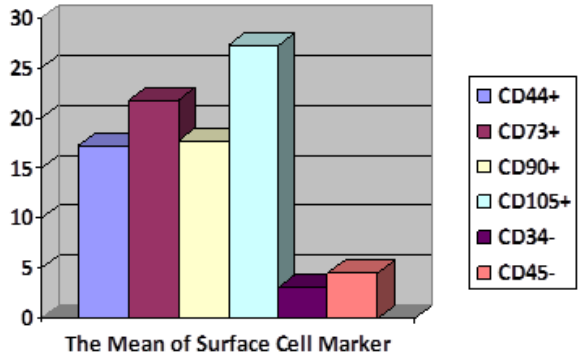
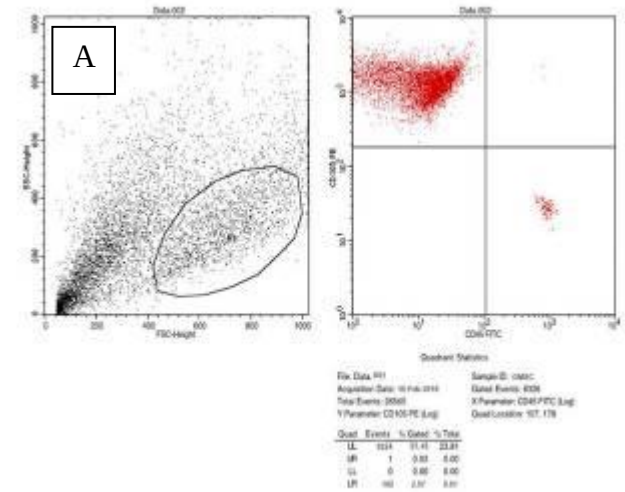


Figure 4. The mean of GMSCs expressed positive (+) MSCs surface cell markers CD44, CD73, CD90, CD105 and negative (-) HSCs surface cell markers CD34 and CD45.



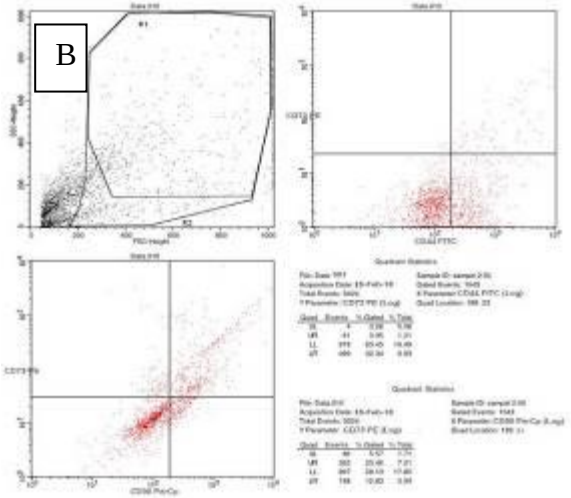


Figure 5. Flowcytometry result of GMSCs Characterization. A. GMSCs highly expressed CD105 but did not CD45; B. GMSCs expressed positive CD44, CD73, and CD90.

Discussion

MSCs can be isolated from adipose tissue and bone marrow but for a small harvest of this stem cell causes morbidity and pain.[13,14] Invasive procedures such as liposuction and surgery may be required to obtain bone marrow stem cells or fatty tissues stem cell. Thus, Umbilical Cord Mesenchymal Stem Cells origin can be only obtained at a certain stage.[4] Harvesting stem cells from oral cavity tissues easier than other tissue. The Oral cavity tissue could be a stem cell source with minimal invasive and it is painless only with local anesthesia procedure.[11,15] However, Dental pulp stem cells and periodontal ligament stem cells are easily accessible but number of teeth is limited.[7,16] In contrast, GMSCs can be isolated from minimally invasive procedures at any time in life. Nevertheless, GMSCs can be a future alternative cell for tissue engineering.[4]

Gingival tissue exhibits magnificent healing processes suggesting that GMSCs has a unique properties.[11] The firstly isolated a gingival tissue progenitor cell within gingival tissues named GMSCs.[17] Recent studies have also shown that GMSCs with the proper manipulation and application, GMSCs are essential in tissue engineering in dental medicine field.[18,19]

According to previous study, there are standard criteria was suggested for MSCs characterization (i) stick to culture plate in culture conditions; (ii) have osteogenic, chondrogenic, and adipogenic ability, (iii) specific marker expression (positive CD73, CD90 and CD105 expression; negative marker of HSCs).[4,11] GMSCs cultured cell morphology was showed fibroblast-spindle-shaped, colony-forming abilities, and stick to the culture plate in our study.

GMSCs have widely available population for therapeutic applications because of the accessibility of animal model or human gingival tissues. GMSCs contain subpopulations of various cells. Therefore, GMSCs should be purity to prevent fibroblast contamination is critically important.[1,11] The GMSCs phenotypic characterization has been extensively and widely documented.[20] Our study result, detected the positive MSCs marker of CD44, CD73, CD90, CD105 and negative expressed HSCs marker of CD34 and CD45 using immunofluorescence. This result was in accordance the International Society for Cellular Therapy position statement about MSCs marker profile.[21]

The most general cells sorting method by cell surface markers using flowcytometry. Flowcytometry detected the specific subpopulation enriched with certain surface makers.[8] GMSCs showed classical characteristics of multipotent postnatal stem/progenitor cells.[4] Our study showed that MSCs marker (CD44, CD73, CD90, CD105) expression and did not expressed HSCs marker (CD34, and CD45). The GMSCs' immunophenotypic profiles using flowyctometry and immunocytochemistry further verified our MSCs culture without HSCs contamination.[22]

Conclusions

Gingival tissues are promising future Stem Cell source. GMSCs have multipotency ability with high rate proliferation and MSCs characteristic that advantageous for regenerative medicine therapy through tissue engineering.

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