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Potency, Characteristic, and Differentiation of Iliac Crest Bone Marrow-Derived Mesenchymal Stem Cell of Systemic Lupus Erythematosus Patients Complicated with Avascular Necrosis of Femoral Head

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Abstract : Introduction. Mesenchymal stem cells (MSC) are very promising in the field of tissue engineering because it is multipotent, rapidly proliferates, with high ability to regenerate bone marrow. BM-MSC(Bone Marrow Mesenchymal Stem Cell) may be the treatment of choice for avascular necrosis (AVN) of femoral head that affects many systemic lupus erythematosus (SLE) patients in recent times. BM-MSC of SLE patients has phenotype, proliferation, and differentiation impairments. MSC therapy on femoral head AVN from autologous donors was reported to deliver good outcome and safety. This study aims to determine the potency, characteristics, and differentiation of BM-MSC in patients with SLE and their relation with age.
Methods. This is an in vitro study that examined four subjects of SLE patients in Cipto Mangunkusumo Hospital. BM-MSC of SLE patientswasisolated, expanded and differentiated. Pearson and Spearman correlation test were used as statistical analysis.
Results. The mean of confluent cell numbers was $7.44 \times 10^5 \pm 3.06 \times 10^5$cells/ml, mean of confluent time was 20.75 ± 4.99 days, median of adipogenic differentiation time was 17.5 days (range 14-21), osteogenic and chondrogenic differentiation time was 21 days. There were positive correlation between patient's age with confluence time ($p < 0.001$) and negative correlation with MSC confluence cell count ($p < 0.001$).
Conclusion. BM-MSC from iliac crest of SLE patients can be isolated, proliferated and differentiated. BM-MSC from SLE patients has longer confluence and differentiation time and lower confluence cell count.
Keywords: bone marrow derived mesenchymal stem cells, systemic lupus erythematosus, isolation, proliferation, differentiation.

Introduction

Mesenchymal stem cell (MSC) is a heterogeneous population group of fibroblast–like, multipotent, cells with the ability to regenerate itself and differentiate into specific mature cells[1].MSC is one of the stem cell population with a sizeable amount locatedin bone marrow tissue, adipose, umbilical cord, brain, liver, muscle, skin, tissue and embryo[1,2]. Because it is multipotent, rapidly proliferates, and has high regeneratingabilities, MSC is very promising in the field of tissue engineering[3].

Systemic lupus erythematosus (SLE) is a chronic inflammatory autoimmune disease which involvesa wide variety of organs such as musculoskeletal, kidneys, blood vessels, heart, nerves, and skin with diverse clinical manifestations[4]. Some studies suggest that the etiopathogenesis of SLEhas a role in the defect of bone marrow MSC's microenvironment[5]. In SLE, there are abnormalities of the body's immune system activity characterized by the presence of autoreactive B cells and T cells and the influence of an abnormal proliferation, migration, and differentiation of mesenchymal stem cells[6,7]. Several previous studies reported impaired phenotype, proliferation, differentiation, cytokine secretion, and immune modulation of MSCs in patients with SLE[4,8,9].

Decreasedmortality and increasedsurvival rate in patients with SLE in the last decade hasled to the increasing number of patients with SLE in old age[10]. This leads to an increase in the incidence of femoral head avascular necrosis (AVN)[11]. Femoral head AVN is found in 2.1% -30% of patients with SLE1[2]. There are some studies that suggest several factors, including age, can affect the amount of MSCthat can be isolated from bone marrow and the MSCs ability toproliferate and differentiate[13,14]. MSC cellular function in the elderly has decreased due to the decline of biological and mechanical responses due to a SLE dynamic actin cytoskeleton and there is an increased exposure to oxidative stress that support cell damage and macromoleculeaging[14]. Until now, no studies have reported the potential characteristics and differentiation ofiliac crest bone marrow MSCsin SLE patients suffering from femoral head AVNand its correlation with age.

Experimental

This is an observational in vitro study to analyzethe characteristics of MSCs in SLE patients who had femoral head AVN. The study was conducted from October 2014 to March 2015. Patients were included if they had SLE with femoral head AVN, had surgical intervention in CiptoMangunkusumoHosital (CMH), age above 18, screening test of hepatitis B, hepatitis C and HIV show negative result and willing to be the subject of the study. Patients were excluded if they have coexisting autoimmune disease. This study was approved by the Medical Research Ethics Committee of Faculty of Medicine Universitas Indonesia.

Iliac crest-bone marrow aspiration

The procedure is taken place in operating room under spinal or general anesthesia. After disinfected with 10% povidone iodine, an aspiration needle was inserted to iliac crest in a 45-degree angle. The hub was removed from the needle and 10 ml syringe containing 1000 IU/ml of heparine was attached to the needle. Thirty mL of iliac crest bone marrow were aspirated by rapidly pulling the plunger. Once the bone marrow aspiration was completed, the needle was removed, pressure was given to the wound, and it given appropriate dressing.

Isolation and culture

Phosphate buffer saline (PBS) was added into the syringe to dilute the bone marrow aspirate. Subsequently, PBS and bone marrow were adequately mixed and centrifuged in 2500 RPM for 15 minutes in 20-degree environment. The culture process was conducted in a level II biological safety cabinet. The pellet was then re-suspended with complete media filled with 10% Platelet Rich Plasma (PRP), 1% penicillin streptomycin, 1 % amphotericin B, heparine, glutamax and alpha minimum essential medium *(*α-MEM*)* and were divided into 75 ml flasks.

The cells were incubated for one week at a temperature of 37°C, 20%oxygen flow, and 5% CO_2.At the end of the first week, the media was removed carefully. The cultured cells were then washed several times with PBS, and replenished with fresh culture medium every 2 days. The culture flasks were observed daily to

evaluate the attachment of nucleated cells. The number of cells is then calculated using hemocytometer. The time needed to achieve 80%confluence was recorded in units of days starting from the first day of isolation.When the cultured cells have been filled (confluent), the cells were detached from the culture flasks using trypsin 0:25% -EDTA 1 mMol (Gibco, Grand Island, New York).

Characterization of cell expression

Characterization test is used to confirm the presence of MSCs by means of positive and negative immunoselection. A positive immunoselection confirms that the stem cells are not contaminated by hematopoietic cells.Markers of positive immunoselection are CD73, CD90, and CD105. Markers of negative immunoselections are CD34, CD45, CD19, CD11, and HLA-DR which are incorporated in a single package BD stemflow^{TM}kit(catalogue 562245).

Cell cultures were separated from the culture medium using trypsin, then fixed using 2% formaldehyde for 30 minutes. Then the cells were washed with flow cytometric buffer containing FCB, PBS 2%, and sodium azide of 0.05%.This was followed by incubation of the cells for 30 minutes using 20 uL containing FCB phycoeritrine (PE) conjugated with monoclonal antibodies CD105, CD73, CD90, CD34, CD45, CD19, CD11, and HLA-DR. Finally, the cells were analyzed using FACS calibur flow cytometer (Becton Dickson, San Jose, CA) and their background was stained with the PE conjugated with non-specific IgG.Flowcytometry results were presented by the percentage of expression.

Osteogenic, Chondrogenic, and Adipogenic Differentiation.

MSC was then differentiated into osteogenic, chondrogenic, and adipogenic cells. Differentiation is conducted by transferring the MSC into osteogenic medium consisting of complete medium (α-MEM, 10% PRP, 1% antibiotic/antimycotic), 50 μM ascorbate-2-phosphate, 10 mM β-glycerophosphate in a 24-well plate and incubated the temperature of 37°C, 20% O2, and 5% CO2.The culture medium used for the differentiation chondroblasts consisting of ascorbic acid, insulin, selenium acid, transferrin, sodium pyruvate, and transforming growth factor-beta 1 (TGF-b1).The culture medium used for the differentiation of adipocytes consisting of dexamethasone, indomethacin, 3-isobutyl-1-methylxanthine and insulin for 1-3 weeks. Every third day differentiation medium is replaced.

Confirmation of differentiation

Osteogenic, chondrogenic, and adipogenic differentiation were evaluated qualitatively by alizarin red staining, Alcian blue, and oil red O every week from week 1 to the differentiation. Osteogenic differentiation was characterized by alizarin red staining. Cells were incubated, then the medium is disposed and the cells are washed with PBS. The cells are further fixated using liquid alcohol (96%) for 30 minutes, then the liquid alcohol (96%) discharged. The next step is mixing 0.5 ml of alizarin red on each 24-plate well. The cell were then stored in a dark place for 15 minutes at room temperature followed by aspiration of alizarin red from the wells. Cells were thoroughly washed again with aquabidest. Evaluation of calcium deposits was observed macroscopically and microscopically. Osteogenic differentiation will show a brick red color from the calcium deposit within cells.

Chondrogenic differentiation is characterized by Alcian blue staining. As much as 0.5 ml of Alcian blue is mixed in each well and incubated in 37 ° C for 12-24 hours. The Alcian blue solution were aspirated from the wells, and the cell monolayer was washed again thoroughly with aquabidest. Evaluation of the deposit cartilage matrix was observed macroscopically and microscopically. A positive result will be obtained if the deposited cartilage matrix gives a blue color.

Adipogenic differentiation characterized by oil red O staining. Cells which were incubated were removed of their medium and washed with PBS. After slow aspiration of PBS, the cell monolayer was fixated with 0.5 ml of liquid buffered formalin (10%) for 60 minutes. The liquid buffered formalin (10%) is then discharged. 0.5 ml of 60% isopropanol is mixed, discharged, and the well of cell monolayer is left to dry. This is then followed by mixing 0.5 ml of oil red O in each well. The monolayer of cells that have been fixed were stained with oil red o solutionfor 10-15 minutes at room temperature and in a dark place. Following staining, the oil red o was aspirated and the cell monolayer washed again with aquabidest until clean. A positive result on the microscope showed vacuoles of fat cells that gave the red color. Microscopic

morphology will be observed using the inverted microscope Zeiss AxioCam Erc5s and images stored with AxioVision software Rel.4.8.lnk. Time of osteogenic, chondrogenic, and adipogenic differentiation were recorded in days.

The results were then analyzed using Statistical Program for Social Science version 17.0 (IBM). Data analysis of cell morphology and confirmation of differentiation results are to be expressed descriptively through images. Analysis of the correlation between age and total cell confluence and confluence time were conducted using the Pearson correlation test. The analysis of age in correlation with adipogenic differentiation time was conducted using the Spearman correlation test.

Results

Based on the method previously described, we obtained 4 SLE patients suffering from femoral head AVN and performed surgery on their pelvis in the central operating room of CMH. All subjects were female, with mean age 38.5 ± 16.36 years, the mean duration of the SLE was 82 ± 60.02 months, and the average duration of AVN was 41.75 ± 30.49 months. Characteristics of the subjects are shown in Table 1.

Tabel 1.Baseline Characteristics of Subjects

Subject	Sex	Age (years)	SLE duration (months)	AVN duration (months)	Diagnosis	Type of Surgery
R	Female	19	22	15	AVN of Left femoral head Ficat 1	Decompresionand bone grafting
N	Female	37	164	72	AVN of bilateral femoral head Ficat 4	LeftTotalHipReplacement
W	Female	39	82	64	AVN of bilateral femoral headFicat 4	Right Total HipReplacement
S	Female	59	60	16	AVN of bilateral femoral head Ficat 4	LeftTotalHipReplacement

Isolation and cell culture

In the first week of culture, mononuclear cells attached to plastic culture flask were discovered on all the subjects. This is one of the criteria for MSCs. In the second week of culture, the growth of cells with fibroblast-like morphology that is increasingly dense and homogeneous were seen, currently cell confluence reaches 40% - 50% in cultures of iliac crest bone marrow aspirates in all subjects. This is also one of the criteria for mesenchymal stem cells. In the third week,it can be seen that in all the iliac crest bone marrow aspirates, the culture reached 70-80% confluence. Analysis showed that the flowcytometryofall subjects gave positive results on CD105, CD90, and CD73 and negative results on the negative marker cocktail consisting of CD45, CD34, CD19, CD11, and HLA-DR. Osteogenic and chondrogenic differentiation on all subjects occurred on day 21. Adipogenic differentiation on the subject R and W occurs on the 14th day, while on the subject of the N and S occurred on day 21.

Spearman correlation test showed significant and strong positive correlation between age and confluence time ($r = 1.00$, $p < 0.001$), and a significant and strong negative correlation between age and the cell confluence count ($r = -1.00$, $p < 0.001$), There was no relationship between age and adipogenic differentiation time. Correlation between age, chondrogenic differentiation time and osteogenic differentiation time could not be performed as the data remained constant throughout the study.

Discussion

This study is an observational study in vitro. In this study as many as four people subject SLE patients suffering from femoral head AVN who underwent surgery on his hip has agreed to participate in the study and signed an informed consent, and then aspirated bone marrow from their iliac crest. Observation and evaluation were intended to assess the ability of proliferation and differentiation of MSCs in patients suffering from AVN

SLE femoral head.

All subjects in this study(4 subjects) were female, similar with previous studies with total subject numbers ranging from 4 to 22 SLE patients[5,6,7,8,9].SLE affects more women than men, with a ratio of 9: 1, because it is influenced by hormonal factors[4,13,16].The mechanism by which hormonal factors contribute to the pathogenesis of SLE remains unclear[17].However, hormonal therapy in SLE yielded disappointing results[13].The mean age of patients in this study is 31.67 ± 11 years in the range 19-59 years. The mean age in this study do not differ much with previous studies[5,6,8,9].SLE is most commonly found in women aged between 15-50 years[4,13].These range are categorized as the productive age, thus SLE has been causing great morbidity[17].

Isolation and culture of bone marrow mesenchymal stem cell

In this study, iliac crest bone marrow MSCs of subjects with SLE were isolated and cultured. After the 10^{th} passage, the MSC morphology cultured with α-MEM transformed into leaf-like, flat, and granulated phenotypical cells. α-MEM medium is better than DMEM in terms of cell proliferation. An α-MEMculture medium supplemented with 10% Platelet Rich Plasma (PRP) creates an optimal and appropriate medium for the isolation and culture of MSCs[18]. In the isolation and culture of BM-MSCs, many studies suggest that PRP is better than Fetal Bovine Serum (FBS) in terms of proliferation potential, immune modulation, and osteogenic, chondrogenic, and adipogenic differentiation. PRP is an option at this point because it does not cause immune reactions and physiological composition adequate. Currently, culturing MSCs with FBS is avoided because of the risk of immune reaction triggered by antigen xenograft FBS, disease transmission, and complex compositions with varying effectiveness[20].

We found that the morphological characteristics of the MSC were in the form of fibroblast-like cells attached to plastic culture flasks. Based literature, the morphological characters are characteristic of mesenchymal stem cells[21].A study by Sun et al reported cell morphology similar to our MSC from SLE normal controls[7].Isolation and culture of iliac crest bone marrow MSC SLE patients have been successfully carried out by some experts as Sun et al, Geng et al, Tang et al, Li et al, danGu et [5,7,6,8]. Sun et al stated MSC from SLE patients can be isolated and cultured but these MSC proliferate much longer than in a normal person[7].

In this study, the MSC confluence time had the median value of 19 days. This result was similar with previous study which showed that the confluence occurred within 10.5-26 days[7,21,22,23,24]. However the confluent cell counts are lower compared to other studies. In a study by Fiorentini et al, confluent cell counts were obtained with the 9×10^5 to 1.3×10^{6}[22].Mareschiet al studied the MSC expansion of bone marrow cultures in 10 normal subjects and had aconfluent cell count of 69×10^6 cells[23]. Bonab et al stated that the average number of confluent cells in their study at 17×10^5 cells / ml (range 5.4×10^5-39×10^5)[24]. A study of bone marrow MSCs in patients with SLE by Sun et al can obtain cell count of 20×10^6 cells in culture. This shows MSC SLE patients have a defect in some aspects, functional or number[7]. MSC proliferation defect in SLE remains an unclear mechanism, whether caused by immunosuppression therapy or pathology in SLE itself in the form of increased activity that the body's immune T cells, B cells, dendritic cells, cytokines, and NK cells are said to inhibit the proliferation of MSC and causes lysis. A lesser confluent cell count in SLE patients are caused by increased apoptosis of MSC in SLE. Apoptosis can occur through the intrinsic and extrinsic pathways. In the intrinsic pathway, it is caused by abnormalities in the mitochondria,translocation of Bax gene. While the extrinsic pathway is caused by TNF-alpha. In SLE, increased levels of TNF alpha will cause caspase activation which is a common mediator of apoptosis in human cells[8].This is also stated by Geng et al who stated that TNF alpha is recruited to the MSC area of inflammation in the body of patients with SLE. At normal speed, the MSC proliferation in vitro is influenced by several factors such as the following: source of the cells, the culture medium (serum FBS quality, concentrations of glucose, and glutamine), the condition of hypoxia, cell density, the size of the flask, plastic quality, and the addition of growth factors[6].

Value of markers CD105, CD73, and CD90 positive in this study is less than 95% and flowcytometry different characteristics in other studies in normal people that reached more than 95%. This is probably caused by subjects taking immunosuppressive drugs for the treatment of SLE. Expression of phenotypic markers indicate a lack of homogeneity population and less cultured MSCs due to impaired bone marrow MSC proliferation in patients with SLE. Several studies by ORCIANI et al and Latif et al stated MSC showed low expression of CD105. CD105 is a membrane glycoprotein complex part of the TGF beta receptor that plays a role in angiogenesis. Studies by Mark et al stated that the low expression of CD105 is caused by the culture

medium used in the serum free medium hMSC growth media. Cell surface markers is still debatable among experts, as Igura et al considered CD34 as a negative marker[25].

Osteogenic, chondrogenic, and adipogenic differentiation

In this study, all MSCs from every subject was able to differentiate into osteoblasts, chondroblasts, and adipocytes. Osteogenic, chondrogenic, and adipogenic differentiation were evaluated qualitatively i.e. by staining morphology. Osteogenic and chondrogenic differentiation time in all subjects were the same,21 days. Adipogenic differentiation in 2 subjects occurred on the 14th day whereas 2 subjects again occurred on day 21. Adipogenic differentiation had a median of14 days, similar with study conducted by Doan et al[21].Chondrogenic differentiation time in this study is longer than the Mardonez et al study which examined rabbits with results of 18 days differentiation time[26]. A study by Matsuoka etalobtained MSC osteogenic differentiation of bone marrow taken from the iliac crest of 3 normal subjects occurred on the 14th day[27]. Here we see that the bone marrow MSC of SLE patients have impaired differentiation. SLE occurs dysregulation of the BMP pathway that inhibits osteogenic and chondrogenic differentiation. This differentiation disorders are also caused by high levels of TNF alpha through phosphorylation of ERK ½[5].

Correlation between age's donor and confluence time and confluent cell count

This study shows that there was a significant correlation between age and confluence time, and also confluent cell count. Stolzing et al stated that the amount of bone marrow MSCs decrease with increasing age[28].As is known ,aging associated with the down-regulation of tissue regeneration capacity due to oxidation, deSLEi telomeres, and decreased ability to repair DNA and protein system changes. Just like the somatic cells, adult MSC were also exposed to stress during the life of the organism, causing a decrease in the function and the number of corresponding age. This resulted in the disruption of homeostasis, regeneration and tissue repair.

Meanwhile, the results of this study showed that there is no correlation between age at the time of MSC differentiation. This is due toa small number of subjects that are differentiate and evaluated by staining every week. These results are supported Siegel et al study on bone marrow MSC of 53 normal subjects who stated that the capacity of bone marrow MSCs differentiation is not affected by age[29].In this study, the morphological characteristics of MSC were fibroblast-like, homogeneous and attached to a plastic flask in confluent cell cultures; but the cultures had fewer cell confluent count and longer confluence. Bone marrow MSC of patients with SLE have the expression of surface antigen CD105, CD73, and CD90 are less than 95%. The functional characteristics of the MSCs in this has the ability to differentiate into osteoblasts, fat cells, chondroblasts, but require a longer differentiation time In this study it can be concluded that the iliac crest bone marrow MSCs of SLE patients can be isolated, cultured, and differentiated by showing morphological characteristics, immunobiology characteristics and functional characteristics that are not normal. For that further research is needed to look further and detailed morphological abnormalities onimmunobiology, and functional (differentiation) MSC SLE patient's bone marrow. The existence of osteoblasts, chondroblasts, adipocytes only proven and qualitatively through morphological and staining and do not do a more detailed examination of quantitative phenotype such as examination and immunohistochemical coloring.

Conclusion

The iliac crest bone marrow MSCs of SLE patients may be isolated, cultured, and differentiated into osteoblasts, chondroblasts, and adipocytes. The MSCs had a mean cell confluence number of $7.44 \times 10^5 \pm 3{:}06 \times 10^5$ cells / ml, with a mean confluence time of20.75 ± 4.99 days. The median time for adipogenic differentiation was 17.5 days, while both the osteogenic and chondrogenic differentiation time was 21 days. There is a significant correlation between the age of SLE patients and confluence time and total cell confluence ($p < 0.001$). There is no correlation between the ages of SLE patients and differentiation time.

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