Comparison of cultured mesenchymal stem cells derived from bone marrow or peripheral blood of rats

Achmad F. Kamal1, Diah Iskandriati2, Ismail H. Dilogo1, Nurjati C. Siregar3, Errol U. Hutagalung1, Achmad A. Yusuf4, Silmi Mariya2, Kurniadi Husodo1

INTRODUCTION
Many studies have reported that mesenchymal stem cells (MSC) can accelerate healing of bone fracture [1, 2], massive bone defect [3-7], and fracture non-union [8]. MSC can be stimulated to differentiate into desired cells, into mechanically and structurally appropriate tissue, and show excellent integration into surrounding tissues [9-11].

MSC can be isolated from various sources, such as bone marrow [12, 13], periosteum and peripheral blood. Although reported as a reliable source of MSC, bone marrow in fact contains only a little amount of MSC (0.1-5 per 10^6 cells from total nucleated cells in rat bone marrow). Therefore, a considerable amount of bone marrow aspirate is required [14-16]. In addition, it takes a considerably long time, about 3-4 weeks, until the stem cells cultured from bone marrow aspirate become confluent. Bone marrow aspiration also frequently causes trauma to the donor [15]. Therefore, other non-invasive methods of MSC isolation with the same or better potency compared to isolation from bone marrow need to be studied.

Isolation of MSC from peripheral blood is still a controversy [17, 18]. Previous studies show that not every isolation and culture of peripheral blood will able to produce MSC [19]. Drawbacks in isolation of MSC from peripheral blood include the limitations of the MSC to grow and proliferate in culture medium [19] and also difference in isolation methods and culture conditions [20, 21].

Isolation of MSC from peripheral blood covered the disadvantage found in isolation from bone marrow. Considerable amount of blood can be obtained from peripheral circulation and also the sampling technique is less traumatic than aspiration of bone marrow [19, 21]. In this study, we compared growth and potency of MSC cultured and isolated from bone marrow and those from peripheral blood of Sprague Dawley (SD) rats.

METHODS
Five male SD rats aged 8-12 weeks with average weight of 269 ± 15 g were prepared for harvesting of bone marrow and peripheral blood MSC. All procedures undertaken in this study have been approved by the Institutional Animal Care and Use Committee (IACUC) PT Bimana Indomedical Bogor (No R.03-11-IR) and ethical approval from Universitas Indonesia (No 131/PT02.FK/ETIK/2011).
Peripheral blood as a source of MSC was taken from inferior vena cava of SD rats. This protocol is a modification of existing protocols [22]. Rats were anesthetized by injection of 80 mg/kg ketamine (Ketamil®, Troy Laboratories, Glendenning NSW, Australia) and 10 mg/kg xylazine (Seton®), Laboratorios Calier, Barcelona, Spain), intraperitoneally. After disinfection with 10% povidone iodine and 70% alcohol in supine position, the rats underwent laparotomy to reach the inferior vena cava. Then, 6-6.5 ml of peripheral blood was obtained from each rat. Blood from each rat was put into a 20 ml sterile polypropylene-EDTA (ethylenediaminetetraacetic acid) tube, which was then brought to the cell culture laboratory, and centrifuged at 750g for 10 min.

Isolation of peripheral blood MSC

In biosafety cabinet, the plasma was removed, red and white blood cells were carefully extracted and put into a new polypropylene tube, with 1:1 RPMI (Roswell Park Memorial Institute medium) volume ratio, and then underwent re-suspension.

Suspension of red and white blood cells in RPMI was added to the Ficoll-Hypaque medium by overlay method, with a ratio of 1:1. Centrifugation was performed at 750g for 30 min at 20°C until the liquid was divided into several layers. The bottom layer was the group of erythrocytes and neutrophils, at the top there were Ficoll-Hypaque and plasma-platelet layer, and between the two layers there was a layer of thin white ring which was a population of mononuclear cells (buffy coat) [23]. Plasma layer was discarded carefully and then buffy coat layer was transferred into new tubes. Mononuclear cells were washed with RPMI medium without serum, and centrifuged at 750g for 10 min at 20°C. Later, the supernatant was removed.

Pellets were then resuspended with 10 ml RPMI, and centrifuged at 750g for 10 min, after which the supernatant was removed again. Cell pellet was mixed with 3 ml cell growth medium DMEM (Dulbecco’s Modified Eagle Medium; Gibco®, Life Technologies, Invitrogen™, Carlsbad, CA, USA) and then counted by using a hemocytometer.

Culture and expansion of peripheral blood MSC

Cells were grown in 6 wells of tissue culture plates with initial concentration of 10^7 cells on each well. Culture was incubated at 37°C with 5% CO2 concentration. Growth medium was replaced on day 7; and subsequently changed every 3 days. Observation was done by inverted microscopy (80x magnification) to evaluate the adhesion of the nucleated cells with fibroblast-like morphology to the plastic culture plate [23].

Harvest of bone marrow

After euthanasia procedures, disinfection was done with 10% povidone iodine and 70% alcohol from mid-body to the entire region of the right and left lower extremities which had been shaved previously. Incision was made around the proximal femur in the border of body-extremity and the anterolateral approach to the femur was done. Skin was sharply separated from muscles, pulled toward the foot and cut at the ankle region. Disarticulation of the hip and ankle joint was done, and the extremities of each rat were marked and put in RPMI transport medium. Tibial and femoral bones were separated aseptically at the knee joint in biosafety cabinet. Proximal tibial growth plate was cut together with the attached muscles, and the tibia was divided from fibula. All muscles and connective tissues in the femur were detached from the bone and the femoral condyle was cut.

Isolation and culture of bone marrow MSC

Bone marrow cells were taken using modified Dobson method by putting the bone in 25 ml polypropylene conical flask. The flasks were centrifuged at 750g for 30 min. After pellet was formed on the bottom of the tube, it was resuspended by adding 8 ml RPMI medium, then centrifuged at 750g for 10 min. Supernatant was removed, the pellet was added to 10 ml RPMI, and centrifuged at 750g for 10 min. Supernatant was removed again, the cell pellet was added to 3 ml of growth medium and counted by a hemocytometer. Cells were grown on 6 wells of tissue culture plates with concentration of 10^3 cells per well, and cells were incubated and evaluated with the same procedure as isolation and culture of peripheral blood [24].

Characterization of MSC

Characterization of MSC was done using reverse transcriptase-polymerase chain reaction (RT-PCR) and immunocytochemistry assay. RT-PCR was used to detect the expression of genes which encoded some of MSC surface proteins such as CD73, CD90, CD105, CD44, and STRO-1. Beside surface protein of MSC, the marker of hematopoietic stem cells such as CD34 (a marker of primitive hematopoietic progenitor cells and endothelial cells) and CD45 (a marker of pan-leukocytes) were also checked to ensure the isolated cells were not contaminated with hematopoietic stem cells. Immunocytochemistry assays were also performed to see the expression of MSC surface protein.

Statistical analysis

The statistical analysis was done by unpaired t-test and P < 0.05 was considered as statistically significant.

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RESULTS

The mean number of bone marrow nucleated cells isolated on day 0 was higher than those of peripheral blood (5.86 ± 2 x 10^7/ml vs 2.85 ± 0.7 x 10^7/ml; P = 0.016). The nucleated cells isolated from these two sources grew, underwent elongation, and finally became fibroblast-like shape. The colonies formed from the bone marrow nucleated cells proliferated more rapidly than the colonies formed from the peripheral blood. (Fig.1).

RT-PCR examination showed that cells with fibroblast-like morphology expressed genes which encode the surface protein of CD73, CD90, CD105, CD44, and STRO-1 as a MSC marker, and there were no gene expression of hematopoietic stem cells which encode surface proteins CD34 and CD45 (Fig.2). RT-PCR results were supported by immunofluorescent assay of CD73 and CD105 markers (Fig.3).

Only nucleated cells from bone marrow grew to 80% confluence within two weeks (day 14), and subculture could be performed on day 15 and day 28. On the contrary, nucleated cells derived from peripheral blood developed only few colonies of fibroblast like cells at the end of week 2 and week 4, almost the entire cells did not adhere to the surface of culture plates and finally died. There was significant difference in yield between bone marrow and peripheral blood nucleated cells on day 15 (3.59 ± 0.5 x 10^6/ml vs 0, P < 0.001) and day 28 (15.57 ± 5.4 x 10^6/ml vs 0, P < 0.001).

Table 1 provides summary of SD rats’ body weight, volume of peripheral blood, number of cells, and yields of culture on days 15 and 28 (bone marrow and peripheral blood nucleated cells).

Table 1. Cell count in the isolated and cultured mesenchymal stem cells from bone marrow and peripheral blood samples

<table>
<thead>
<tr>
<th>Rat</th>
<th>Body weight</th>
<th>Peripheral blood volume</th>
<th>Cell Count per ml</th>
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</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Bone marrow</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Day 0</td>
</tr>
<tr>
<td>1</td>
<td>284 g</td>
<td>6.5 ml</td>
<td>5.0 x 10^7</td>
</tr>
<tr>
<td>2</td>
<td>276 g</td>
<td>6 ml</td>
<td>6.7 x 10^7</td>
</tr>
<tr>
<td>3</td>
<td>254 g</td>
<td>6.5 ml</td>
<td>8.2 x 10^7</td>
</tr>
<tr>
<td>4</td>
<td>277 g</td>
<td>6.5 ml</td>
<td>6.7 x 10^7</td>
</tr>
<tr>
<td>5</td>
<td>254 g</td>
<td>6 ml</td>
<td>2.7 x 10^7</td>
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</table>

Figure 1. Colonies of cells with fibroblast-like morphology that were isolated and cultured from (A) peripheral blood, and (B) from bone marrow adhere to plastic culture plates.

Figure 2. RT-PCR assay showing positive expression of CD73, CD90, CD105, CD44, STRO-1 and negative for CD34 and CD45.
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DISCUSSION

Besides in the bone marrow, MCS can also be found in the periosteum, cartilage, muscle, synovium, adipose tissue, umbilical cord blood, blood vessel, and peripheral blood [25-28].

The defining characteristics of MSC are inconsistent among investigators. Many have their own methods to isolate and expand MSC [26-28], which show quite significant differences. Varied tissue sources and methodologies of cell preparation raised the question of whether the resulting cells are sufficiently similar to allow for a direct comparison. This was caused by the lack of universally accepted criteria to define MSC [16]. Therefore in 2005, The International Society of Cellular Therapy (ISCT) issued a consensus that MSC should have the following three criteria [16]. First, MSC must be plastic-adherent when maintained in standard culture conditions. Second, MSC must express CD105, CD73 and CD90, and lack expression of CD45, CD34, CD14, or CD11b, CD79a, or CD19 and HLA-DR surface molecules. Third, MSC could differentiate to osteoblasts, adipocytes and chondroblasts in vitro.

In order to prove that the cultured cells fulfill second ISCT criteria, we performed RT-PCR and immunocytochemistry assays. RT-PCR showed that cultured cells had positive gene for surface markers CD73, CD90, CD105 and STRO-1, and negative for CD34 and CD45. Those results were further supported by immunocytochemistry assay that showed positive expression of CD73 and CD105. It was the limitation of this study that we could only confirm two of three ISCT criteria. Considering their fibroblast-like morphology, plastic adherent properties, ability of cells to grow and proliferate on appropriate medium for mesenchymal stem cells, and the result of RT-PCR and immunocytochemistry, we concluded that those mononuclear cells with fibroblast-like morphology are very likely MSC.

The potential biological differences between progenitor cells from various tissues are influenced by the preparation and the origin of MSC so that the selection of cell sources for tissue engineering has practical implications [22, 25]. The capability of proliferation, differentiation and MSC surface antigen expression varies greatly depending on the sources [29]. The usage of peripheral blood as a source of MSC is still a controversy. From the previous study, it is known that not all of the isolation and culture from peripheral blood can produce MSC [19]. Difficulty in performing MSC isolation from peripheral blood is caused by limitations of peripheral blood MSC to grow and proliferate in culture medium [19], and by the difference in methods of isolation and culture conditions [20, 21].

Only few studies have successfully isolated peripheral blood MSC using the same conditions for the isolation of bone marrow MSC. However, this supports the existence of circulating population of MSC. Isolation of these cells are very difficult and tends to vary according to isolation and sorting methods as well as culture conditions [20, 21].
According to Rochefort et al [30], the presence of MSC in the peripheral blood depends on the model, age, treatment before isolation, and enrichment methods. Additionally, the population of rat’s peripheral blood MSC is very low in calm rats, but increased dramatically as the hypoxic stimulation was done.

By the different methods of isolation and culture, Kassis et al [19] reported the success in isolation of MSC from peripheral blood by administering the granulocyte-colony stimulating factor (G-CSF) for at least 5 days prior. Unfortunately, that isolation methods were ineffective and difficult to be repeated to get similar result [20, 21]. With different methods, Hu et al [20] isolated peripheral blood multipotent mesenchymal progenitors (PBMMC) with hematopoietic and mesenchymal potential.

In the present study, we found that MSC could be isolated from peripheral blood, although in smaller number compared to the MSC isolated from bone marrow. In addition, the potential for expansion of stem cells derived from peripheral blood was lower than those derived from bone marrow. Moreover, after 28 days, the MSC derived from peripheral blood eventually died. The most likely explanation is due to very limited population of peripheral blood MSC in rats, and the lack of stimulation such as G-CSF and hypoxia.

The knowledge of peripheral blood MSC is still very limited [31], of which the origin, homing site and their presence in the circulation is still in debates [30]. The presence of MSC in the peripheral blood circulation is alluded as a result of migration from bone marrow [31]. It has been reported previously that the presence of MSC in the peripheral blood is at a very low level [32].

In conclusion, while MSC can be isolated from peripheral blood, they are more enriched in bone marrow compared to peripheral blood. Bone marrow derived MSC grow more efficiently than those derived from peripheral blood.

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COMPETING INTERESTS
We hereby affirm that there is no conflict of interest for this research.

REFERENCES

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