

Proliferation and Biochemical Analyses of Osteoblast/Osteoclast Differentiation from Human Mononucleated Cells

(Analisis Proliferasi dan Biokimia Terhadap Sel Mononukleus Manusia yang dibezakan kepada Sel Osteoblas/Osteoklas)

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ABSTRACT

Stem cell is defined as the ability of the cell to proliferate themselves and differentiate into more than one type of cells. Human mononucleated cell (MN cell) is a suspension cell that was isolated from peripheral blood that was originated from monocyte-macrophage lineage or hematopoietic stem cells. The cells were cultured for 30 days in complete media (CM) which consist of Alpha Minimal Essential Medium (α MEM) with 2% (v/v) Penicillin-Streptomycin and 10% (v/v) Newborn Calf Serum (NBCS). The respective cells were differentiated at day 7 after in vitro proliferation in CM into osteoblastic cells by adding ascorbic acid and β -glycerophosphate. In addition, human recombinant Receptor Activator of Nuclear Factor- β Ligand (hrRANKL) and human recombinant Macrophage-Colony Stimulating Factor (hrM-CSF) were added to induce osteoclastic differentiation of MN cells. Cells that were cultured in CM served as a control and were subjected to the same approach as differentiated cells. The 30 days cultured cells in CM showed a significant increment ($p < 0.05$) of viable cells compared to day 0 ($n=3$). The specific activity of Alkaline Phosphatase (ALP) for osteoblast differentiation and Tartrate Resistant Acid Phosphatase (TRAP) for osteoclast differentiation were evaluated via biochemical assay until day 14 and day 10 for osteoblast and osteoclast sample, respectively. ALP and TRAP enzyme showed a significant increment ($p < 0.05$) after 14 and 10 days of differentiation compared to control cells. As a conclusion, human mononucleated cells are believed to have the potential to be defined as a multipotent stem cell based on their fulfillment of stem cell characteristics.

Keywords: Enzymological analysis; mononucleated; Osteoblast; Osteoclast

ABSTRAK

Sel stem ialah sel yang mempunyai keupayaan mengganda dan membeza kepada lebih daripada satu jenis sel. Sel mononukleus (MN) manusia merupakan sel terampai yang diasingkan daripada sel darah periferi; berasal daripada turunan monosit-makrofaj iaitu merupakan sel stem hematopoietik. Sel tersebut dikultur selama 30 hari di dalam media lengkap (CM) yang mengandungi 'Alpha Minimal Essential Medium' (α MEM), 2% (v/v) Penisilin-Streptomycin and 10% (v/v) 'Newborn Calf Serum' (NBCS). Sel mononukleus dibezakan pada hari ketujuh pengkulturan in vitro kepada sel osteoblas dengan menambahkan asid askorbik dan β -gliserofosfat. Bagi mengaruhkan pembezaan MN kepada sel osteoklas, 'Receptor Activator of Nuclear Factor- β Ligand' rekombinan manusia (hrRANKL) dan 'Macrophage-Colony Stimulating Factor' rekombinan manusia (hrM-CSF) ditambahkan ke dalam CM. Sel yang dikultur di dalam CM bertindak sebagai kawalan. Sel yang dikultur selama 30 hari di dalam CM menunjukkan peningkatan sel viabel yang signifikan ($p < 0.05$) berbanding hari 0 ($n=3$). Analisis biokimia dilakukan terhadap enzim Alkalin Fosfatase (ALP) bagi sel osteoblas sehingga hari 14 pembezaan dan enzim Asid Fosfatase Rintang Tartrat (TRAP) bagi sel osteoklas sehingga hari 10 pembezaan. Enzim ALP dan TRAP pada sel osteoblas dan sel osteoklas masing-masing menunjukkan peningkatan yang signifikan ($p < 0.05$) pada hari 14 dan 10 berbanding sel kawalan. Sebagai kesimpulan, sel MN manusia dipercayai mempunyai potensi untuk dikelaskan sebagai sel stem multipoten kerana memenuhi ciri-ciri sel stem yang telah disebutkan.

Kata kunci: Analisis enzimologi; mononukleus; Osteoblas; Osteoklas

INTRODUCTION

In complex organisms, mitosis of normal cells is usually accompanied by differentiation. Stem cells are the only cells which can self-renew and at the same time maintain the ability to differentiate into mature lineages. Stem cells are useful for many type of applications such as tissue engineering, cellular therapies and drug screening, due

to their extensive regeneration potential and functional multilineage differentiation capacity.

Other unique properties of stem cells for example, the mesenchymal stem cells, which produce the connective tissues of the body are transdifferentiated into other types of cells that are not from the same lineages (Winn 2005). A group of studies has demonstrated the ability of cultured

mesenchymal cells to differentiate into neural cell, skeletal cells, cardiomyocytes, endothelial cells and smooth muscle cells (Jiang et al. 2002).

Under normal conditions, bone homeostasis depends on the balance action of bone-forming osteoblasts and bone-resorbing osteoclasts. Such cultures should present proliferative and differentiation patterns representative of the *in vivo* osteoblast population, namely, an increased alkaline phosphatase (ALP) activity (Intan Zarina et al. 2008) and the ability to produce mineralized bone matrix (Beloti & Rossa 2005). Osteoclasts are of macrophage-monocyte origin and are giant multinucleated cells that degrade bone matrix. Current evidence suggests that osteoclast is responsible for bone erosion, based on studies that showed TRAP-positive, multinucleated cells at the pannus-bone interface areas of subchondral bone loss (Danks et al. 2002). Multinucleated osteoclast-like cells are characterized by osteoclast markers, such as TRAP, cathepsin K and calcitonin receptors (Gravallese et al. 2000).

Human mononucleated (MN) cells that were isolated from human peripheral blood originating from the monocyte-macrophage lineage can differentiate into all types of blood cells (Intan Zarina et al. 2010; Shahrul Hisham et al. 2005). This present study demonstrated a process known as transdifferentiation where the mononucleated cells were not only capable to differentiate into osteoclast cells but also can differentiate into osteoblast cells which originate from mesenchymal stem cells.

MATERIALS AND METHODS

ISOLATION OF HUMAN MONONUCLEATED CELLS

Approximately, 3 mL of human peripheral blood cells were drawn from a healthy donor. The blood was diluted with two volumes of Hank's Balanced Salt Solution (HBSS) (Sigma, USA) before being layered on 1.5 mL Ficoll Paque™ Plus (GE Healthcare, Sweden) per 1 mL of whole blood. The layer was centrifuged at 400 g for 20 min at room temperature (RT). Four distinct layers were produced which consist of red blood cell layer at the bottom, followed by Ficoll Paque™ Plus layer. The mononucleated (MN) cells were at the third grayish layer followed by a plasma layer. The plasma layer was carefully drawn out without disturbing the MN cell layer. After transferring the MN layer in another 15 mL tube, the cells were washed three times with Phosphate Buffered Saline (PBS) (Sigma, USA). The cells were dissolved in complete media (CM) containing Alpha Minimal Essential Medium (α MEM) (Fisher, USA), 2% (v/v) Penicillin-Streptomycin (PenStrep) (Gibco, US) and 10% (v/v) Newborn Calf Serum (NBCS) (Gibco, USA) in CO₂ chamber with 5% CO₂ at 37°C.

TRYPAN BLUE EXCLUSION ASSAY

The MN cells were culture in 24-well plate and fixed at 1×10^5 cell/mL. Data of viable cells were taken every day for 30 days starting at day 0 (day of cell's isolation)

by using trypan blue 0.4% (v/v) (Sigma, USA) solution with the ratio of 1 volume (trypan blue): 1 volume (cell's sample).

OSTEBOLAST (OB) AND OSTEOCLAST (OC) DIFFERENTIATION

After seven days of proliferation in CM, the MN cells were washed with PBS before cultured in for 14 days OB differentiation medium consisting of CM plus 50 ng/mL ascorbic acid and 10 nM β -glycerophosphate. The cells were fixed at 1×10^5 cell/mL. On the other hand, OC differentiation of MN cells was induced for 10 days by adding 50 ng/mL hrRANKL and 25 ng/mL hrM-CSF in the CM. The cells were also fixed at 1×10^5 cell/mL.

ALKALINE PHOSPHATASE (ALP) ASSAY

Cells were assayed at day 0, 3, 5, 7, 10 and 14 after being cultured in OB differentiation media. The cells were washed with PBS before 0.1 M NaHCO₃-Na₂CO₃ (pH 10.0), 10% (v/v) Triton X-100, 2 mM MgSO₄ and 6 mM p-nitrophenol inorganic phosphate (PNPP) were added and incubated at 37°C for 30 min. All chemical were from Sigma brand (USA). The p-nitrophenol liberated after 30 min of incubation at 37°C was converted into p-nitrophenylate by adding 1 M NaOH and the absorbance was immediately read at 405 nm wavelength. One unit ALP activity hydrolyzes 1 μ M PNPP per minute at 37°C.

TARTRATE RESISTANT ACID PHOSPHATASE (TRAP) ASSAY

Cells were assayed at day 0, 3, 5, 7 and 10 after culture in OC differentiation media. TRAP enzyme activity in osteoclast is extracellular enzyme and were assayed in cells extracts and in complete media using PNPP as a substrate in an incubation medium (1mL) containing the following: 10 mM PNPP, 0.1 M Na-Acetate (pH 5.8), 0.15 M KCl, 0.1% (v/v) Triton X-100, 10 mM Na-Tartrate, 1 mM ascorbic acid and 0.1 mM FeCl₃. All chemicals are from Sigma brand (USA). The mixture was incubated for one hour and the reaction was stopped by adding 0.3 M NaOH and immediately read at 405 nm wavelength. One unit TRAP activity hydrolyzes 1 μ M PNPP per minute at 37°C.

PROTEIN CONTENT ASSAY

A value of 0.01% (v/v) Triton X-100 was added to homogenize the cells. Bradford solution were added to the cells' lysate and incubated for 5 minutes before read at 595 nm wavelength for total protein content assay. Enzyme specific activity were determined by one unit (U) of enzyme activity over total protein content and were stated in percentage (%) to compare with control cells i.e. 100% activity. A standard curve of p-nitrophenylate and Bovine Serum Albumin (BSA) protein using Bradford solution with regression (R) 0.99 was prepared earlier.

STATISTICAL ANALYSIS

The data were expressed as mean \pm STD of at least three independent experiment ($n=3$). The data were analyzed using student paired t-test from SPSS ver15. Effects were considered statistically significant at $p < 0.05$.

RESULTS AND DISCUSSION

Viable cells were determined by using trypan blue exclusion assay during the proliferation assay. The cells were fixed at 1×10^5 cells/mL of viable cells and data of viable cells' proliferation were collected for up to 30 days (Figure 1). The cell's number was transformed into \log_{10} to give rise a linear graph. The viable cells were ~ 6300 times higher compared to viable cells at day 0. Statistical analysis using paired t-test showed a significant ($p < 0.05$) increment of viable cells at day 30 as compared to day 0. The division rate is approximately 0.42 ± 0.03 divisions per day ($n=3$). This result showed that the human MN cells have the ability to proliferate *in vitro* in CM containing α MEM, 10% (v/v) NBCS and 2% (v/v) PenStrep.

The MN cells that are isolated from bone marrow managed to survive after 30 days in culture media is considered as the most primitive stem cells (Morrison et al. 1997). The respective cells are capable to proliferate but still have the ability to differentiate into mature lineage, such as osteoblast and osteoclast cells.

To prove that the MN cells were successfully induced to become osteoblastic and osteoclastic cells, biochemical assays of ALP and TRAP enzymes were done. Osteogenic differentiation was accompanied by the increase level of known osteogenic markers, i.e. the increasing activity of cellular matrix ALP when differentiated into osteoblasts (Schilling et al. 2007). Ascorbic acid and β -glycerophosphate were added to induce differentiation of mononucleated stem cells into osteoblastic cells and also the survival of the cells (Intan Zarina et al. 2010; Shroder et al. 2000; Tumber et al 2000). Figure 2

shows that upon time, the MN cells were successfully induced to become osteoblastic cells, in correlation with a significant ($p < 0.05$) increment of the ALP enzyme specific activity. The highest of ALP specific activity is at day 14, i.e. 2500% higher as compared to control cells that is 100%.

On the other hand, the receptor activator of nuclear factor- β ligand (RANKL), a member of the tumor necrosis factor (TNF) family of cytokines, is a key molecule that involved in the differentiation of osteoclasts in the presense of macrophage colony-stimulating factor (M-CSF) (Arana-Chavez & Bradaschia-Correa 2009).

In this study, TRAP enzyme was used as a biochemical marker for osteoclast differentiation. The TRAP enzyme in the respective differentiation medium showed a significant increment ($p < 0.05$) of enzyme specific activity as compared to control cells (Figure 3). Control cells are human MN cells that were cultured in complete medium.

CONCLUSION

Human mononucleated cells that were isolated from peripheral blood do have the ability to proliferate *in vitro* and biochemically differentiate into osteoblast and osteoclast cells after induction of their specific inducer. As a conclusion, the respective cells manage to transdifferentiate and do have the potential to be defined as multipotent stem cells.

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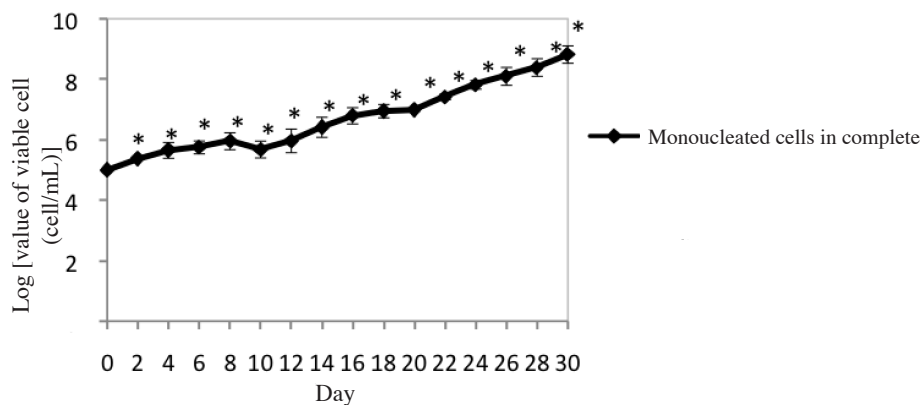


FIGURE 1. The graph showed the log value of viable cells (cells/mL) versus time (day). The MN cells were cultured in complete media consisting Alpha Minimal Essential Medium, 10% (v/v) Newborn Calf Serum and 2% (v/v) Penicillin-Streptomycin for 30 days ($n=3$). Trypan blue exclusion assay was used to count viable cells in the culture media. * indicated a significant increment ($p < 0.05$) as compare to day 0. The division rate was 0.42 ± 0.03 divisions per day

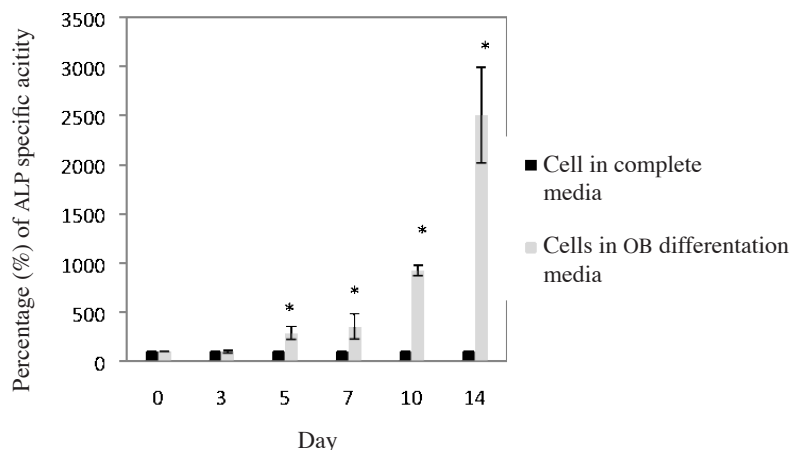


FIGURE 2. ALP assay for osteoblast differentiated cells. The ALP enzyme of MN cells cultured in complete media plus ascorbic acid and β -glycerophosphate were increased upon time when compared to control cells, i.e MN cells cultured in CM (n=3). The highest enzyme activity was at day 14 which was $2500 \pm 486\%$ higher as compared to control cells (100%). * indicate a significant ($p < 0.05$) increase of ALP enzyme compare to control cells using statistical paired t-test

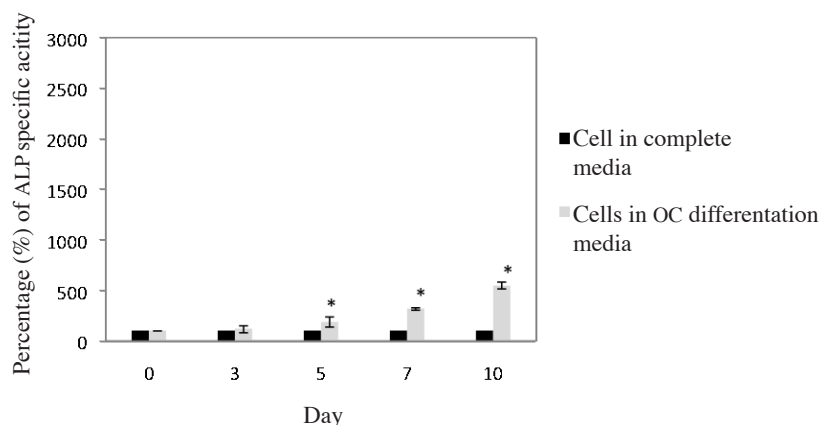


FIGURE 3. TRAP assay for osteoclast differentiated cells. The TRAP enzyme of MN cells cultured in complete media plus human recombinant hrRANKL and hrM-CSF were increased upon time when compared to control cells, i.e MN cells cultured in CM (n=3). The highest enzyme activity was at day 10 which was $550 \pm 34\%$ higher as compared to control cells (100%). * indicate a significant ($p < 0.05$) increment of TRAP enzyme compare to control cells using statistical paired t-test

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