

Potential Effects of Human Bone Marrow Non-Mesenchymal Mononuclear Cells on Neuronal Differentiation

Chareerut Phruksaniyom, Khwanthana Grataitong, Permphan Dharmasaroja, and Surapol Issaragrisil

Abstract—Bone marrow-derived stem cells have been widely studied as an alternative source of stem cells. Mesenchymal stem cells (MSCs) were mostly investigated and studies showed MSCs can promote neurogenesis. Little is known about the non-mesenchymal mononuclear cell fraction, which contains both hematopoietic and nonhematopoietic cells, including monocytes and endothelial progenitor cells. This study focused on unfractionated bone marrow mononuclear cells (BMMCs), which remained 72 h after MSCs were adhered to the culture plates. We showed that BMMC-conditioned medium promoted morphological changes of human SH-SY5Y neuroblastoma cells from an epithelial-like phenotype towards a neuron-like phenotype as indicated by an increase in neurite outgrowth, like those observed in retinoic acid (RA)-treated cells. The result could be explained by the effects of trophic factors released from BMMCs, as shown in the RT-PCR results that BMMCs expressed nerve growth factor (*NGF*), brain-derived neurotrophic factor (*BDNF*), and ciliary neurotrophic factor (*CNTF*). Similar results on the cell proliferation rate were also observed between RA-treated cells and cells cultured in BMMC-conditioned medium, suggesting that cells ceased proliferating and differentiated into a neuronal phenotype. Using real-time RT-PCR, a significantly increased expression of tyrosine hydroxylase (*TH*) mRNA in SH-SY5Y cells indicated that BMMC-conditioned medium induced catecholaminergic identities in differentiated SH-SY5Y cells.

Keywords—bone marrow, neuronal differentiation, neurite outgrowth, trophic factor, tyrosine hydroxylase

I. INTRODUCTION

TRANSPLANTATION of stem cells obtained from several sources has been proposed as a means for treating neurodegenerative diseases and injuries to the central and peripheral nervous system. Of these stem cells, bone marrow-derived stem cells (BMDCs) are of special interest because they are easily harvested, isolated, and purified. Several studies investigating a possible therapeutic role for these cells have used specific two subpopulations: the mesenchymal stromal cells (MSCs) and the mononuclear fraction (BMMCs; bone marrow mononuclear cells) [1]-[4]. MSCs have the

potential to differentiate into neurons, astrocytes, and endothelial cells in the brain [5], [6], making them the focus of much research. Some studies have suggested that a small proportion of BMDCs may differentiate into Schwann cells [7]. In addition to direct differentiation, BMDCs can promote neurogenesis and synapse formation in the injured brain [1] and can improve axonal regeneration and myelination of the injured peripheral nerve [4].

Human MSCs compose less than 0.01% of total bone marrow mononuclear cells [8]. Excluding MSCs, little is known about the role of the mononuclear fraction of the bone marrow in neurogenesis. Because BMMCs contain both hematopoietic and nonhematopoietic cells, including monocytes and endothelial progenitor cells, and the relative number of the hematopoietic cells is very low, it may be more appropriate to study unfractionated BMMCs. Cells of this fraction, including the trophic factors and cytokines that they may secrete, could have the potential to promote neurogenesis in the brain following injuries, including stroke, and the regeneration of peripheral nerves [4], [9], [10]. For example, nerve growth factor (NGF), which plays an important role neuronal plasticity, maturation, and survival, is produced in human monocytes under basal conditions [11]. Human monocytes, T cells, and B cells secrete brain-derived neurotrophic factor (BDNF), a member of the neurotrophin family that regulates the differentiation and survival of various neuronal populations [12]. Ciliary neurotrophic factor (CNTF), another factor involved in neurogenesis, is also expressed in monocytes, myeloid cells, lymphoblasts, T cells and B cells [13].

Several studies have indicated that human catecholaminergic neuroblastoma SH-SY5Y cells, following treatment with retinoic acid (RA), have the potential to develop neuron-like phenotypes, including neurite outgrowth and branches, and express tyrosine hydroxylase (TH) [14]-[16]. This study aimed to investigate the role of the non-mesenchymal mononuclear cell fraction of human bone marrow on neuronal differentiation of human SH-SY5Y neuroblastoma cells, with RA-treated cells as positive controls. We showed that BMMCs are a promising source for cell therapy in neurological diseases.

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II. MATERIALS AND METHODS

A. Maintenance of Bone Marrow Mononuclear Cells

Human bone marrow samples were aspirated from healthy donors. Informed consent was obtained prior to the procedure, and the protocol for bone marrow aspiration was approved by the Siriraj Ethics Committee of Siriraj Hospital. After isolation by Ficoll-Hypaque gradient centrifugation, the mononuclear cells were plated at a concentration of 1.5×10^5 cells/ml onto 75 cm² flasks containing low glucose-Dulbecco's Modified Eagle's Medium (LG-DMEM) supplemented with 10% fetal bovine serum (FBS; Lonza Group Ltd., Switzerland), 10 U/ml penicillin, and 100 µg/ml streptomycin (Sigma). Cells were incubated at 37°C, 5% CO₂, and 90% humidity. After 72 h, non-adherent cells were collected for culture in 1:1 mixture of Eagle's Minimum Essential Medium (MEM) and Nutrient Mixture Ham's F12 medium, supplemented with 10% FBS, 1 mM sodium pyruvate, 0.1 mM non-essential amino acids, 10 U/ml penicillin and 100 µg/ml streptomycin. After an additional 24 h, non-adherent cells were collected again and cultured in the medium described above in either the presence or absence of FBS. After 24 h, non-adherent cell conditioned medium (+FBS and -FBS) was collected and filtered using a 0.22-µm pore filter.

B. Culture of SH-SY5Y Cells in BMMC-Conditioned Medium

The neuroblastoma SH-SY5Y human cells were maintained in basal media consisting of a 1:1 mixture of MEM and Nutrient Mixture Ham's F12 medium supplemented with 1 mM sodium pyruvate and 0.1 mM non-essential amino acid, 1.5 g/L sodium bicarbonate, 100 U/ml penicillin, and 100 µg/ml streptomycin. Cells were seeded onto new plates containing BMMC-conditioned medium at an initial density of 1×10^4 cells/ml. As a control, cells were treated with 10 µM *all-trans* RA (Sigma) daily for 5 days to induce neuronal differentiation. Cells were visualized daily using a Nikon Eclipse TS100 inverted microscope equipped with a Nikon Coolpix 995 camera (Nikon Instruments Inc., Hollywood International Ltd., Thailand).

C. Measurement of Cell Proliferation

SH-SY5Y cells were seeded onto 96-well plates at a density of 5×10^3 cells/well in DMEM with 15% FBS (control) overnight. Media was then changed to one of three experimental conditions; medium without RA (control), medium with 10 µM RA, and BMMC-conditioned medium. Afterwards, the media was changed every two day. To quantitate the number of proliferating cells, a colorimetric [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)] assay was performed on days 2, 4, and 6. MTT (5 mg/ml) dissolved in Hank's balanced salt solution was added to each well. Plates were incubated for 4 h at 37 °C in a 5% CO₂ incubator. Supernatant was discarded, and precipitates were resuspended in 100 µl of DMSO. Absorbance at 570 nm with 665 nm as a reference was measured using a plate reader, and differences

between the two absorbances were analyzed. The experiments were performed in five replicates.

D. RT-PCR Analysis

The PARISTM kit (Ambion, Gene Systems Co. Ltd., Thailand) was used to extract total RNA from BMMCs according to the manufacturer's instructions. cDNA was synthesized from 0.25 µg of total RNA using the Masterscript RT-PCR system (5 PRIME, Prima Scientific Co. Ltd., Thailand) according to the manufacturer's instructions. After an initial denaturation step at 94 °C for 2 min, the actual cycling steps consisted of 28 cycles of a denaturation step at 94 °C for 15 s, an annealing step at 55 °C for 20 s, and the extension step at 68 °C for 30 s, followed by a final extension step at 72 °C for 10 min. The sense and antisense primers used to amplify *NGF*, *BDNF*, *CNTF*, and internal control beta-actin (*β-ACTIN*) in BMMCs were as follows: *NGF* (167 bp), 5'-TAAAAAGCGCGACTCCGTT-3' and 5'-ATTCGCCCTGTGGAAGATG-3'; *BDNF* (93 bp), 5'-ACTCTGGAGAGCGTGAATGG-3' and 5'-ATCCAACAGCTCTTCTATCACG-3'; *CNTF* (257 bp), 5'-ACCAGCAGGTGCATTTTACC-3' and 5'-GAAACGAAGGTCATGGATGG-3'; *β-ACTIN* (250 bp), 5'-CATGTACGTTGCTATCCAGGC-3' and 5'-CTCCTTAA TGTCACGCACGAT-3'. After PCR, 5 µl of the mixture was loaded onto a 1.5% agarose gel, electrophoresed, and stained with ethidium bromide to detect amplified products.

E. Real-time RT-PCR Analysis

The relative mRNA expression in SH-SY5Y cells of tyrosine hydroxylase (*TH*) and an endogenous housekeeping gene encoding for *β-ACTIN* as a reference were quantified using real-time PCR analysis with the ABI 7500 Real-time PCR system (Applied Biosystems, GenePlus Co. Ltd., Thailand). cDNA was synthesized from 0.18 µg of total RNA. Amplification of specific PCR products was detected using the KAPA SYBR[®] FAST qPCR Master Mix (Kapa Biosystems, Prima Scientific Co. Ltd., Thailand) according to the manufacturer's protocol. The primers used to amplify *TH* were as follows: 5'-GCCGTGCTAAACCTGCTCTT-3' and 5'-GTCTCAAACACCTTCACAGCTC-3' (77 bp). The primers for *β-ACTIN* are described above. Forward and reverse primers were used at a final concentration of 200 nM, and each assay was performed in triplicate. The cycling conditions were comprised of a polymerase activation step at 95 °C for 3 min, followed by 40 cycles of denaturation at 95 °C for 3 s and primer annealing/extension at 60 °C for 60 s. Data were analyzed using the SDS 7500 system software (v1.3.1, Applied Biosystems). PCR products were loaded onto a 4% agarose gel and electrophoresed to confirm primer specificity.

F. Statistical Analysis

Statistical analyses for cell proliferation studies were performed using a two-way Student's *t* test. Values plotted were the mean ± standard deviation computed for three samples per group. Values at a level of *p* < 0.05 were considered statistically significant.

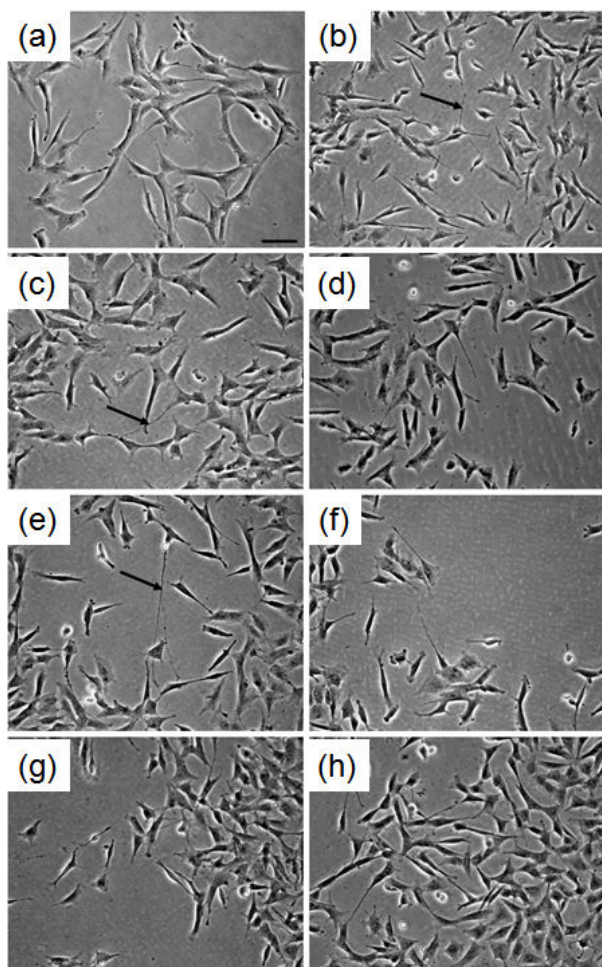


Fig. 1 BMMC-conditioned medium promotes neurite outgrowth in SH-SY5Y cells. Control cells were incubated with basal media for 4 days (a). Cells were treated with 10 μ M RA for 3 d and images were taken on d 4 (b). Cells were cultured in BMMC-conditioned medium for 4, 6, and 8 days with FBS (c, e, and g) or without FBS (d, f, and h). Arrows indicate neuritic extension. Scale bar = 50 μ m

III. RESULTS

A. BMMC-Conditioned Medium Promotes Neurite Outgrowth in SH-SY5Y Cells

SH-SY5Y cells were cultured in BMMC-conditioned media and neurite outgrowth was observed once a day for 8 days (Fig. 1). Cellular processes longer than twice the diameter of the cell body were considered neuritic processes and cells bearing such processes were considered neuron-like cells. Like RA-treated cells (Fig. 1b), SH-SY5Y cells, in the presence of BMMC-conditioned medium, exhibited morphological changes from an epithelial-like phenotype towards a neuron-like phenotype as indicated by an increase in neurite outgrowth (Fig. 1c-h). These processes could be observed as early as day 3, and cells showed a progressive increase in neurite outgrowth up to day 8. At day 5, small areas suggestive of a neurite network, as defined by extensions connecting

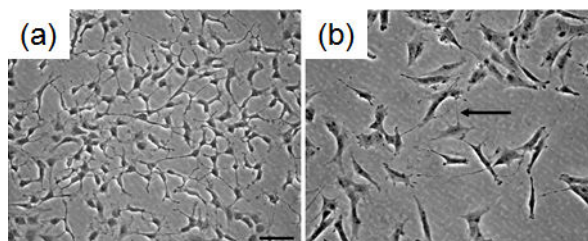


Fig. 2 BMMC-conditioned medium promotes formation of small areas of neuritic network. SH-SY5Y cells were treated with 10 μ M RA (a) or incubated in BMMC-conditioned medium (b) for 5 days. Arrow indicates a small area of neuritic network. Scale bar = 50 μ m

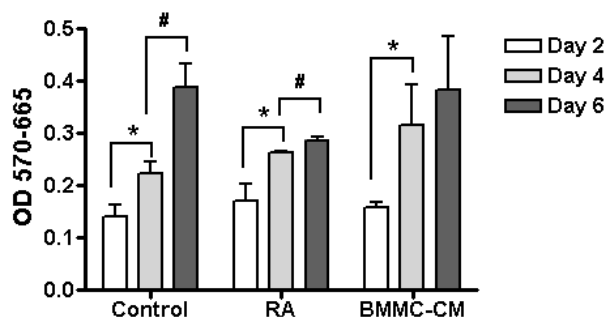


Fig. 3 The proliferation of SH-SY5Y cells cultured in control, RA-treated, and BMMC-conditioned medium. After seeding cells for 24 h, cultured medium was replaced with basal media (control), basal media with 10 μ M RA, or BMMC-conditioned medium (BMMC-CM). Cell proliferation was assessed using MTT assay. Graph represents the data (mean \pm SD) from five replicate wells. * and # represent $p < 0.05$ and $p < 0.005$, respectively.

neighboring cells, were observed in cells cultured in BMMC-conditioned medium (Fig. 2b). The network was not as complex as what was observed in RA-treated cells (Fig. 2a).

B. Effects of BMMC-Conditioned Medium on SH-SY5Y Cell Proliferation

To study the effects of BMMC-conditioned medium on the proliferation of SH-SY5Y cells, an MTT assay was performed to measure cell numbers (Fig. 3). MTT assay involves the use of mitochondrial activity of live cells to convert MTT to formazan, whose concentration can be measured spectrophotometrically. There was a statistically significant difference in cell number between day 2 and 4 in the control, RA-treated, and BMMC-conditioned medium-treated groups ($p < 0.05$). There was also a statistically significant change in cell number between day 4 and 6 in controls and the BMMC-conditioned medium-treated group ($p \leq 0.005$). Similar results on cell proliferation were obtained between RA-treated cells and cells cultured in BMMC-conditioned medium. Proliferation rate was estimated as a percentage of the OD570-665 changes from day 2 to day 4 and from day 4 to day 6 for each group. In agreement with previous studies that have shown that cells treated with 10 μ M RA for 5 days cease proliferating and differentiate into a neuronal phenotype [15], our results showed a decline in the cell proliferation rate from day 4 to day 6 for RA-treated cells and cells in BMMC-conditioned

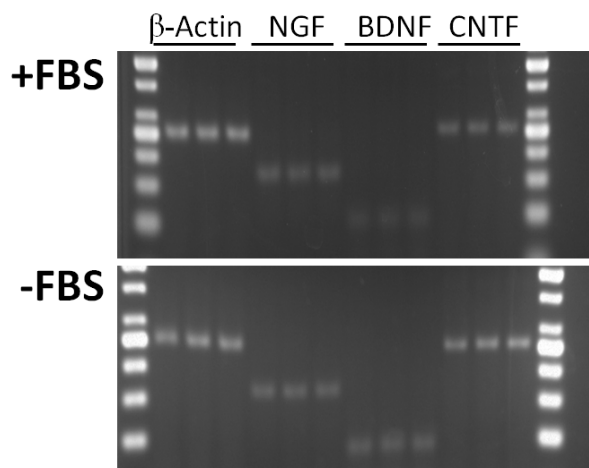


Fig. 4 RT-PCR analysis revealed expression of *NGF*, *BDNF*, and *CNTF* in BMMCs in both +FBS and -FBS conditions. Each gel image represents triplicate 5 μ l loads of the PCR products

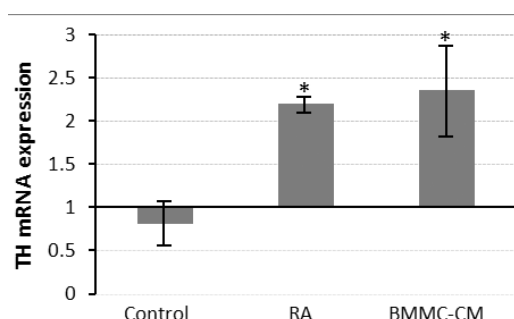


Fig. 5 Real-time RT-PCR analysis revealed that BMMC-conditioned medium induces *TH* mRNA expression in SH-SY5Y cells. Each experiment was performed in triplicates. Graph represents mean \pm SD. * represents $p < 0.05$ vs. control

medium. In controls, the proliferation rate remained high from day 4 to day 6, compared to that from day 2 to day 4.

C. Neuro-trophic Factors are Expressed in Populations of Human BMMCs

To determine whether BMMCs express trophic factors that might be involved in promoting neurite outgrowth of SH-SY5Y cells, *NGF*, *BDNF* and *CNTF* were selected as potential trophic factors. Using RT-PCR analysis, the results showed that populations of human BMMCs expressed these trophic factors (Fig. 4). To ensure that the expression of *NGF*, *BDNF*, and *CNTF* was not induced by factors within fetal bovine serum, we conducted serum-free experiments. The expression of *NGF*, *BDNF*, and *CNTF* was also observed in the serum-free condition.

D. BMMC-Conditioned Medium induces *TH* mRNA Expression in SH-SY5Y Cells

To determine whether SH-SY5Y cells morphologically differentiated in BMMC-conditioned media acquire the properties of catecholaminergic neurons, we investigated changes in *TH* mRNA expression as an indicator of functional differentiation. Three days after treatment with BMMC-

conditioned medium, real-time RT-PCR showed a significant increase in *TH* mRNA expression in SH-SY5Y cells ($p < 0.05$; Fig. 5). Similar results were observed in RA-treated cells ($p < 0.05$) as a positive control group.

IV. DISCUSSION

Previous studies investigating bone marrow-derived cells focused on specific subpopulations: either mesenchymal stromal cells or the mononuclear fraction. Here, we investigated the non-adherent BMMC fraction that remained after MSCs were adhered to culture plates for 72 h. Immediately after removal, BMMCs were cultured in specific media that differ from that used for MSC culture. After 24 h in this media, cells and conditioned media were collected for the experiments. Our cell separation method should exclude most of MSCs that might remain in the mononuclear fraction.

We demonstrate for the first time, a promoting effect of BMMC-conditioned medium on neurite outgrowth of SH-SY5Y human neuroblastoma cells. Our results suggest that BMMCs release cytokines or neurotrophic factors necessary for neuronal differentiation, and the amount of released factors is sufficient for the induction of neurite outgrowth in SH-SY5Y cells. Using RT-PCR, we show that cells in the mononuclear fraction express *NGF*, *BDNF*, and *CNTF*. Because gene expression is often regulated by both intrinsic genetic information and environmental signals, we conducted the serum-free experiments to confirm that the expression of *NGF*, *BDNF*, and *CNTF* was not influenced by components within fetal bovine serum; the expression of *NGF*, *BDNF*, and *CNTF* in BMMCs is most likely due to intrinsic factors.

The effects of BMMC-conditioned media on neuronal differentiation and proliferation on SH-SY5Y cells were similar to those of retinoic acid, which have been well documented. These include promotion of neurite outgrowth, reduction of proliferation rate, and slightly enhanced choline acetyltransferase activity [15]. RA can also induce responsiveness to BDNF in SH-SY5Y cells¹⁵. Expression and secretion of BDNF from cells, e.g. monocytes, in the mononuclear fraction may explain the similar effects of RA and BMMC-conditioned media. Similar effects of BMMCs have been demonstrated on the outgrowth of axons from peripheral nerves and the survival of neurons in dorsal root ganglia [4]. Bone marrow-derived stem cells constitutively synthesize and secrete *NGF*, *BDNF*, and *CNTF*. Multiple cell types, however, are present in the bone marrow mononuclear fraction. Primary CD34⁺ human HSCs express mRNA for a number of proteins, including receptors for trophic factors and other mediators involved in the development of neurons [17]. Most of the CD34⁺ cells are progenitors for myeloid and lymphoid lineages, which express some trophic factors, such as *CNTF*, that were also observed in our RT-PCR results [13]. Transplantation of bone marrow-derived CD34⁺ or CD133⁺ cells significantly reduces the motor deficits and infarct volume in stroke-induced rats, and both CD34⁺ and CD133⁺ cells have been proposed as a transplantable cell type for

stroke therapy [18], [19]. Previous evidence also supports our finding that the expression of trophic factors in BMMCs is likely intrinsic. There is a continuous influx of bone marrow stem cells into the ependymal and subependymal zones throughout life, and these may give rise to a variety of cell types of the central nervous system [20]. Studies have shown that the mobilization of hematopoietic stem/progenitor-enriched CD34+ cells from bone marrow to peripheral blood can be triggered by ischemic stroke or acute myocardial infarction [20]. Two hypotheses about the role of hematopoietic cells in promoting regeneration in the ischemic brain are as follows: 1) transdifferentiation of hematopoietic cells into neural lineages and 2) the effects of hematopoietic cytokines (stem cell factor [SCF] and granulocyte colony-stimulating factor [G-CSF]) that are released from the cells in response to brain injuries/stress. Here, we propose that growth or neurotrophic factors (NGF, BDNF, and CNTF) from hematopoietic cells/BMMCs may also contribute to neuro-regeneration. However, further studies are required to test this hypothesis.

We showed that BMMC-conditioned medium induced not only morphological but also functional differentiation of SH-SY5Y neuroblastoma cells into neuron-like cells. Increased expression of *TH* mRNA in differentiated SH-SY5Y cells could be explained by the effects of trophic factors released from BMMCs, and might indicate that differentiated cells have acquired catecholaminergic identities.

In conclusion, our results confirm previous studies showing that cells from the bone marrow mononuclear fraction express *NGF*, *BDNF*, and *CNTF*. Here, we show that the factors released from this fraction are sufficient to induce the neuronal differentiation of human neuroblastoma cells through either an individual or a synergistic effect. The bone marrow mononuclear fraction, which is easily obtained and manipulated without requirement of cell culture expansion, might have additional therapeutic potential and may potentially be an alternative source for cell therapy in diseases of the nervous system. Signals that regulate the expression of neuro-regulatory genes in BMMCs remain unknown. Further studies are required to quantify the contribution of trophic factors to BMMC-induced effects on neurite outgrowth and neuronal cell survival.

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