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The immunosuppressive capacity of human mesenchymal stromal cells derived from amnion and bone marrow

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ABSTRACT

Mesenchymal stromal cells derived from amnion (AM-MSCs) can be easily obtained in large quantity by less invasive method in comparison to bone marrow-derived MSCs (BM-MSCs). However, the biological and immunosuppressive properties of AM-MSCs are still poorly characterized. Previous studies demonstrated that BM-MSCs expressed indoleamine 2,3-dioxygenase (IDO) to suppress T-cell responses. This study was designed to address whether IDO contributes to the immunosuppressive function of AM-MSCs. MSCs isolated from amnion were cultured in complete medium similar to BM-MSCs. After culture, AM-MSCs exhibited spindle shape morphology and expressed MSC markers similar to that of BM-MSCs. In addition, AM-MSCs were able to differentiate into adipocytes and osteoblasts. Fascinatingly, AM-MSCs and BM-MSCs exhibited comparable degree of immunosuppressive effect when they were co-cultured with activated T-cells. In addition, IDO secreted by AM-MSCs was responsible for induction of immunosuppressive activities in the same manner as BM-MSCs. Taken together; the results of the present study demonstrate that while AM-MSCs and BM-MSCs show similar immunosuppressive effect, AM-MSCs may have additional advantage over the BM-MSCs in terms of availability. Therefore, AM-MSCs might be considered a potential source for therapeutic applications especially for treatment of immune related diseases.

1. Introduction

Mesenchymal stromal cells (MSCs) have been shown to have therapeutic potential for regenerative and anti-inflammatory therapy [1–4]. Recent study had demonstrated that bone marrow-derived MSCs (BM-MSCs) could suppress T-cell proliferation both in phytohemagglutinin (PHA) induced T-cell proliferation and mix lymphocyte models [5]. Although the immunomodulatory property of MSCs has been demonstrated by many experiments, the specific mechanisms are still controversial. Currently, several soluble factors either produced constitutively by MSCs or as a result of cross-talk with target immune cells have been attributed to immunomodulatory property of MSCs, including indoleamine 2,3-dioxygenase (IDO) [6], prostaglandin E2 (PGE_2) [7], interleukin 10 (IL-10) [8], nitric oxide (NO) [9], and hepatocyte growth factor (HGF) [10]. Interestingly, interferon γ (IFN-γ), proinflammatory cytokines secreted by activated T-cells, has been demonstrated to stimulate IDO, PGE_2, NO and HGF expressions [6].

MSCs were first recognized in bone marrow by Friedenstein *et al.* since 1976 [11]. Until now, the studies of MSCs both in preclinical and clinical studies have mainly focused on BM-MSCs. However, the procedure of bone marrow aspiration is invasive and the quality and quantity of BM-MSCs decreased with increasing age of donors [12]. These problems limit the clinical use of BM-MSCs. Currently; MSCs are isolated from various tissues, such as adipose tissue, gingiva, amnion, placenta and umbilical cord. Among these, amnion is one of the good candidate source of MSCs that exhibit a high degree of self-renewal capacity and multi-differentiation potential [13]. MSCs from amnion (AM-MSCs) have an abundant of collection source than that of BM-MSCs and can be collected with less ethical constraints.

Although the immunomodulatory property of BM-MSCs has been extensively characterized [14,15], the immunosuppressive behavior of AM-MSCs has not been studied. As BM-MSCs is the most well characterized source of the MSCs, this study aim to characterize and compare AM-MSCs in term of morphology, immunophenotype, differentiation potential, and immunosuppressive capacity to those of BM-MSCs. In addition, the mechanism underlying immunosuppressive effect of AM-MSCs is studied and compare to that of BM-MSCs. The results obtained from this study will help to illuminate the biological properties of AM-MSCs and the

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potentially for substituting BM-MSCs in several therapeutic applications, including the treatment of immune related diseases.

2. Methods

2.1. Cell isolation

This study was approved by the Human Ethics Committee of Thammasat University No. I. All subjects participated in the study after giving written informed consents. Bone marrow (n=5) obtained from healthy volunteer was carefully layered over Ficoll-Hypaque solution (GE Healthcare, Sweden) and centrifuged at 100xg (Hettich, Universal 320 K, USA) for 30 min at 20 °C. The amnion (n=5) obtained from pregnant woman after normal delivery was minced into small pieces and digested with 1.6 mg/ml collagenase XI (Sigma-Aldrich, USA) and 200 mg/ml deoxyribonuclease I (Sigma-Aldrich, USA). The cells from bone marrow and amnion were collected and cultured in complete medium [Dulbecco's Modified Eagle's Medium (GibcoBRL, USA.) containing 10% fetal bovine serum (FBS; BioWhittaker, USA), 2 mM L-glutamine (GibcoBRL, USA), 100 U/ml penicillin and 100 μg/ml streptomycin] at density of 1×10^5 cells/cm^2. After 72 h, non-adherent cells were removed and fresh medium was added. The culture medium was changed every 3–4 days. The cells were sub-cultured using 0.25% trypsin-EDTA (GibcoBRL, USA.) and replated at density of 1×10^4 cells/cm^2.

2.2. Immunophenotypical characterization

Primary culture from amnion and bone marrow (4×10^5 cells) were resuspended in 50 μl of phosphate buffer saline (PBS) and incubated with 10 μl of fluorescein isothiocyanate (FITC) or phycoerythrin (PE)-conjugated antibodies against CD34 (BD Bioscience, USA), CD45 (BD Bioscience, USA.), CD73 (BD Bioscience, USA.), CD90 (AbD Serotec, USA) or CD105 (AbD Serotec, USA.) for 30 min at 4 °C in the dark. After washing, the cells were fixed with 1% paraformaldehyde. The positive cells were then identified by comparison with isotype-match controls [FITC-conjugated mouse immunoglobulin G1 (IgG1) and PE-conjugated mouse immunoglobulin G2a (IgG2a)]. At least ten thousand labeled cells were acquired and analyzed using flow cytometry (FACScalibur™, Becton Dickinson, USA) and CellQuest® software (Becton Dickinson, USA).

2.3. Differentiation capacity assay

The adipogenic and osteogenic differentiation potential of cultured MSCs were studied according to manufacturer's protocol. For adipogenic differentiation, 3×10^4 MSCs from each source were cultured in adipogenic differentiation medium (Hyclone® Advance STEM™, USA) with complete change of medium every 3 days. After 3 weeks, cells were stained with Oil Red-O (Sigma-Aldrich, USA) and examined under inverted microscope. For osteogenic differentiation, 3×10^4 MSCs were cultured in osteogenic differentiation medium (Hyclone® AdvanceSTEM™, USA). The medium was replaced twice a week. After 21 days, cells were stained for alkaline phosphatase (ALP) activity and observed under inverted microscope. Cells maintained in completed medium served as controls.

2.4. Mixed lymphocyte reaction assay

Peripheral blood mononuclear cells (PB-MNCs) were prepared from 10 ml of heparinized blood of healthy volunteer using Ficoll-Hypaque solution. The stimulating PB-MNCs were treated with 10 μg/ml mitomycin C (Sigma-Aldrich, USA) for 2 h at 37 °C. Thereafter, responding PB-MNCs (2.5×10^4 cells) together with stimulating PB-MNCs (7.5×10^4 cells) were co-cultured in lymphocyte-cultured medium (RPMI 1640 medium supplemented with 10% FBS, 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin) in the presence or absence of MSCs (2×10^4 cells). PB-MNCs stimulated with 5 μg/ml phytohemagglutinin (PHA; Sigma-Aldrich, USA) were used as a positive control. The culture of PB-MNCs with MSCs was used as negative control. After 4 days, 100 μl of cell suspension from each well was transferred to the new 96-well plates and 10 μl of Cell Counting Kit-8 (Dojindo Laboratories, Japan) was added. After incubating at 37 °C for 4 h, the absorbance at 450 nm was measured with a microplate reader (BioTex, USA). The proliferative index was calculated according to the manufacturer's instruction:

Proliferative index=(O.D. sample−O.D. blank) / (O.D.control−O.D. blank)

To evaluation the effect of the number of MSCs on T-cell proliferation, PHA activated PB-MNCs (2×10^4 cells) were co-cultured with MSCs at various ratios, 20:1, 10:1, 5:1, 2:1, 1:1, 2:1, for 4 days. The culture of PHA activated PB-MNCs were used as controls. Cell proliferation was examined using Cell Counting Kit-8 and proliferative index was calculated as the above mention.

2.5. Gene expression analysis

MSCs (1×10^5 cells) were stimulated with PHA activated PB-MNCs (1×10^5 cells) or 10 ng/ml IFN-γ (Peprotech, USA). MSCs co-cultured with PB-MNCs (1×10^5 cells) were used as controls. After 72 h, total RNA was extracted from the cultured MSCs using PureLink™ RNA Mini Kit (Invitrogen, USA) and messenger RNA was reverse transcribed to cDNA using SuperScript® III First Strand Synthesis Kit (Invitrogen, USA). Quantitative real-time PCR samples were prepared using a SYBR® Green PCR Master Mix (Applied Biosystems, USA). Primer sequences of indoleamine 2,3-dioxygenase (*IDO*), cyclooxygenase 2 (*COX-2*) and inducible nitric oxide synthase (*iNOS*) were shown in Table 1. The amplification was performed on a Step one plus™ Real-Time PCR system (Applied Biosystems, USA) using 40 cycles of amplification (denaturation at 95 °C for 10 s, annealing at 60 °C for 10 s and extension at 72 °C for 20 s). Melting curves were assessed to ensure single products were quantified. The quantitation was based on normalizing the gene of interest to the invariant control gene (glyceraldehyde 3-phosphate dehydrogenase; *GAPDH*). The data were analyzed by comparative ΔΔCT method using StepOne™ Software version 2.2 and presented as the relative mRNA level.

Table 1
Primers and product size.

Gene	Forward primer	Reverse primer	Product size (bp)
IDO	5′-GGCAAAGGTCATGGAGATGT-3′	5′-TCCAGTTTGCCAAGACACAG-3′	127
COX-2	5′-GACTCCCTTGGGTGTCAAAG-3′	5′-AACTGATGCGTGAAGTGCTG-3′	147
iNOS	5′-CTCTATGTTTGCGGGGATGT-3′	5′-TTCTTCGCCTCGTAAGGAAA-3′	179
GAPDH	5′-GTCAACGGATTTGGTCGTATTG-3′	5′-CATGGGTGGAATCATATTGGAA-3′	193

2.6. Western blot analysis

MSCs (1×10^6 cells) were stimulated with PHA activated PB-MNCs (1×10^6 cells) or 10 ng/ml IFN-γ (Peprotech, USA). MSCs co-cultured with PB-MNCs (1×10^6 cells) were used as controls. After 72 h, the cells were resuspended in 200 μl of radioimmunoprecipitation assay (RIPA) buffer (Cell Signaling Technology, USA) contained 1 mM phenylmethylsulfonyl fluoride (PMSF; Sigma-Aldrich, USA). The protein concentrations were determined using Bradford protein assay (Bio-Rad, USA).

The protein was separated on 10% sodium dodecyl sulfate (SDS) polyacrylamide gel at 100 V for 90 min. Subsequently, the proteins were transferred to nitrocellulose membrane (Amersham Pharmacia, USA) by electroblotter at 100 V for 90 min. Then, the membranes were incubated with 5% nonfat dry milk for 1 h, followed with anti-IDO antibody (Sigma-Aldrich, USA.) at a dilution of 1:250 overnight at 4 °C. After washing, the membranes were then incubated with a horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (Sigma-Aldrich, USA) at a dilution of 1:2000 for 45 min at room temperature. The signals were visualized using chromogenic substrate detection system. After normalizing with actin, the densitometric value was expressed as the mean ± standard error of mean (SEM)

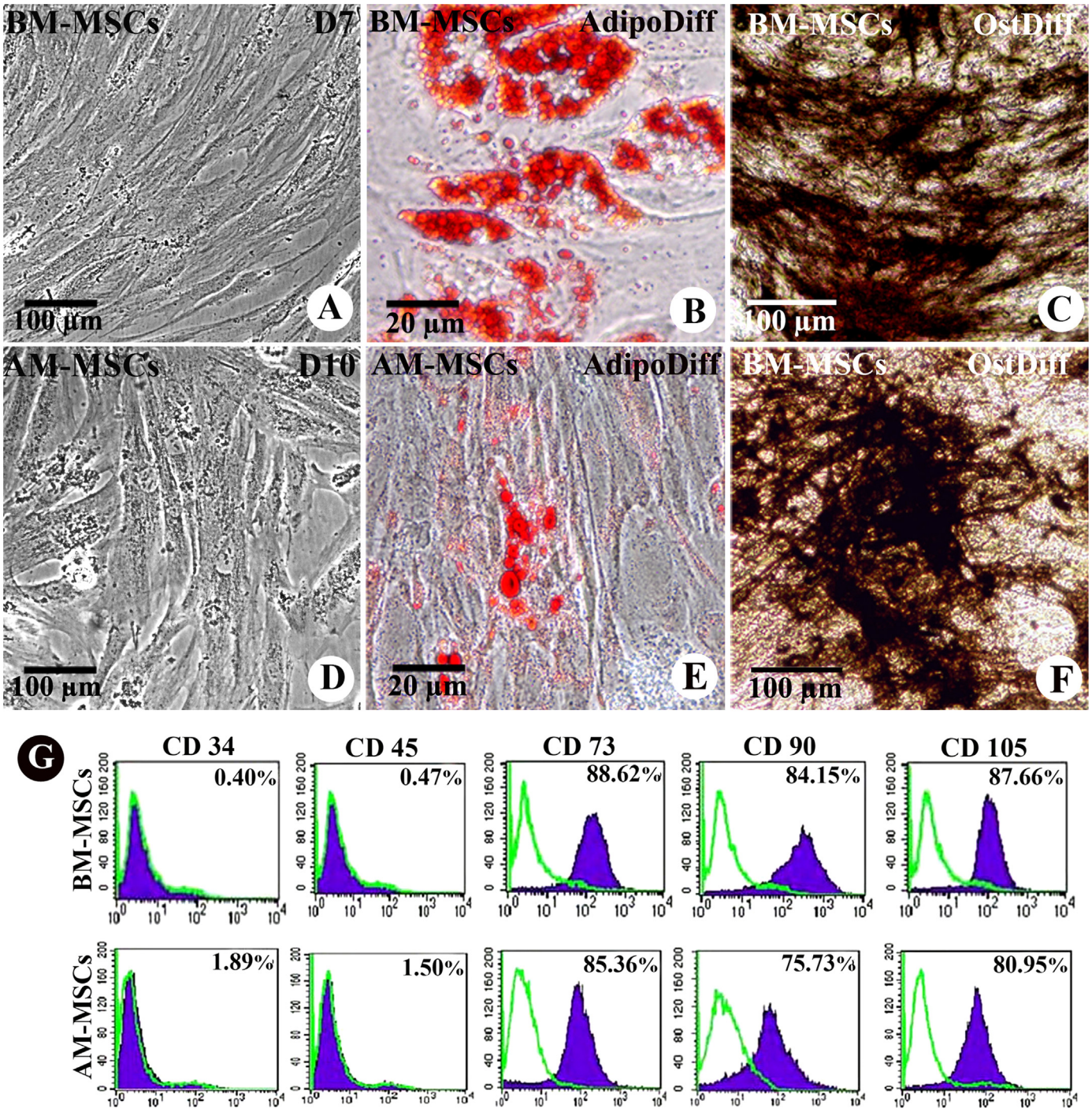

Fig. 1. The characteristic of cultured MSCs. (A) BM-MSCs at day 7. (B) Oil red-O staining of BM-MSCs at day 21. (C) ALP staining of BM-MSCs at day 14. (D) AM-MSCs at day 10. (E) Oil red-O staining of AM-MSCs at day 28. (F) ALP staining of AM-MSCs at day 28. (G) Flow cytometric analysis of surface marker expression. The data shown are representative of the three different experiments.

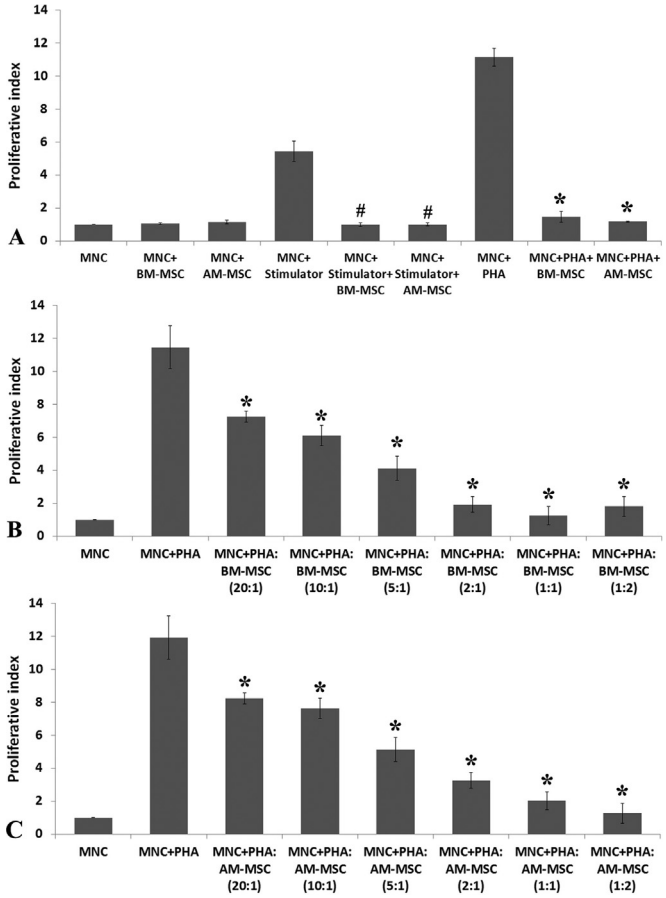

Fig. 2. Mean value of proliferative index of responder T cells co-cultured with stimulator T cells or PHA activated T cells in the presence or absence of MSCs. #, $^{*}p < 0.05$ significantly different compared to MNC+stimulator and MNC+PHA, respectively.

from three independent experiments.

2.7. Cytokine inhibition assays

To evaluate the involvement of IDO, COX-2 and iNOS in the suppressive effect of MSCs, PHA activated PB-MNCs (2×10^4 cells) were co-cultured with MSCs (2×10^4 cells) in the presented or absented of chemical antagonists for IDO [1-Methyl-L-Tryptophan (1-MT), 500 μM; Sigma-Aldrich, USA], COX-2 (Indomethacin, 20 μM; Sigma-Aldrich, USA), and iNOS [N-nitro-L-arginine methyl ester (L-NAME), 1 mM; Sigma-Aldrich, USA]. The culture of PHA-activated MNCs was used as the controls. After 4 days, cell proliferation was examined using Cell Counting Kit-8 and proliferative index was calculated as mention in the above section.

2.8. Statistical analysis

Data are presented as mean ± standard error of mean. The ANOVA test was used to assess the significance of differences between observed data. *p*-values of less than 0.05 was considered to be statistically significant.

3. Results

3.1. Morphology of MSCs

After plating, BM-MNCs exhibited a spherical shape and floated in the media. After 24 h, some cells attached on plastic surface of tissue culture flask and exhibited elongated shape. On day 7, the morphologically homogeneous population of spindle-shape cells was observed (Fig. 1A). These cells could multiply rapidly and reached 80% confluence within 10 days. AM-MSCs exhibited a spherical shape and floated in the media after initial plating. Some cells exhibited elongated shape after 48 h. On day 10, the homogeneous populations of elongated, spindle-shape cells were observed (Fig. 1D). After further cultured, these cells rapidly proliferated and reached 80% confluence within 14 days.

3.2. Immunophenotype of MSCs

AM-MSCs expressed high levels of typical MSCs markers; CD 73, CD90 and CD105, but did not express hematopoietic markers; CD34 and CD45, similar to that of BM-MSCs. However, the percentages of cells which expressed those markers were slightly difference from each MSC sources (Fig. 1G).

3.3. Differentiation potential of MSCs

After 3 weeks of adipogenic induction, AM-MSCs changed their morphology from spindle shaped cell to become large cells and shown accumulation of lipid vacuoles in their cytoplasm. These lipid droplets were positive for oil-red-O staining similar to that of differentiating BM-MSCs (Fig. 1B and E). For osteogenic induction, AM-MSCs formed large nodules and had appearance of refringent crystals in the cells after 3 weeks. Most of the cells were positive for alkaline phosphatase staining similar to that of BM-MSCs (Fig. 1C and F). The controls which cultured in complete DMEM medium without any differentiation stimuli maintained the fibroblast like morphology and did not positive for oil-red-O or alkaline phosphatase staining even after 3 weeks of culture.

3.4. Immunosuppressive effect of MSCs

The immunosuppressive effect of AM-MSCs and BM-MSCs were examined using mixed lymphocyte model. In allogeneic stimulation, the proliferative index was increased to 5.44 ± 0.63 in comparison with the control. Interestingly, the proliferative index of responder which was activated with PHA was increased to 11.14 ± 0.54 in comparison with control. After adding AM-MSCs, the proliferative indices of both allogeneic stimulation and PHA activated responder was significantly decreased ($p < 0.05$) similar to those of BM-MSCs (Fig. 2A).

The PHA-activated T cells co-cultured with MSCs at various ratios demonstrated that AM-MSCs and BM-MSCs could suppress T cell proliferation in dose dependent manner (Fig. 2B and C).

3.5. The expression of IDO, COX-2 and iNOS

To determine the expressions of cytokines involved in immunosuppression, MSCs were co-cultured with PHA-activated T cells and the expressions of *IDO, COX-2* and *iNOS* were examined using quantitative real time PCR. The results showed that the expression of *IDO* was significantly increased in both BM-MSCs and AM-MSCs in comparison with the control ($p < 0.05$, Fig. 3A). In addition, the expression of *COX-2* and *iNOS* was significantly increased, especially in BM-MSCs, after co-cultured with PHA activated T cells in comparison to control ($p < 0.05$, Fig. 3B and C).

To determine the factor secreted from activated T cells that could induce cytokine expression from MSCs, the experiment were performed by adding IFN-γ into the culture of MSCs and the expressions of *IDO, COX-2* and *iNOS* were examined using quantitative real time PCR. The result demonstrated that the level of *IDO* expression was significantly increased in both AM-MSCs and

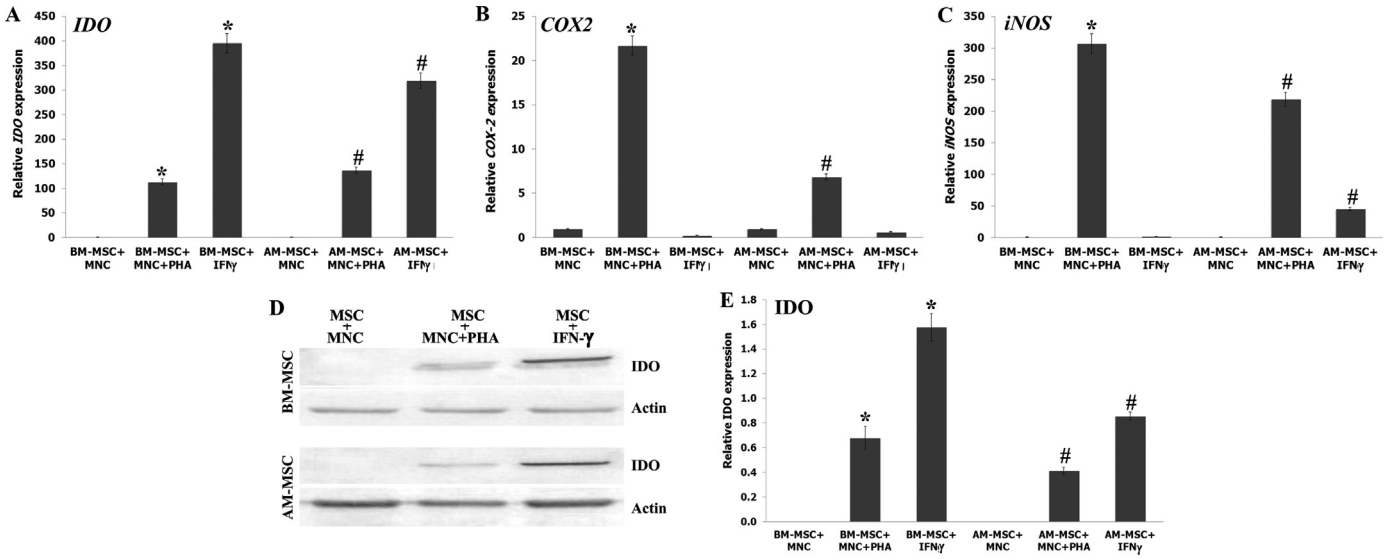

Fig. 3. (A–C) Relative gene expression in MSCs after cultured with PHA activated T cells or IFN-γ. (D, E) Western blot analysis of IDO expression in MSCs after cultured with PHA activated T cells or IFN-γ. $^{*,\#}p < 0.05$ significantly different compared to BM-MSCs + MNC and AM-MSC + MNC, respectively.

BM-MSCs in comparison with the control ($p < 0.05$, Fig. 3A). Interestingly, the expression of *IDO* in MSCs stimulated with IFN-γ was significantly higher than those of MSCs co-cultured with PHA-activated T cells. However, both AM-MSCs and BM-MSCs stimulated with IFN-γ did not express *COX-2*. Interestingly, AM-MSCs stimulated with IFN-γ could express *iNOS* but in lower level than that of MSCs co-cultured with PHA-activated T cells (Fig. 3B and C).

Moreover, the expression of protein, IDO, was significantly increased in both AM-MSCs and BM-MSCs after co-cultured with PHA-activated T cells or stimulated with IFN-γ in comparison with the control. Fascinatingly, the expression IDO in MSCs stimulated with IFN-γ was significantly higher than those of MSCs co-cultured with PHA-activated T cells. ($p < 0.05$, Fig. 3D and E).

3.6. Immunosuppressive effect of MSCs after cytokine inhibition

To determine the role of soluble factors, IDO, COX-2 and iNOS, in MSCs mediated T cell suppression; the inhibition assay was performed using chemical antagonists to those cytokines. The result demonstrated that the proliferation of PHA-activated T cells could reverse to the level of positive control (PB-MNCs + PHA) after co-cultured with BM-MSCs in the presence of 1-MT. The proliferative index of PHA-activated T cells co-cultured with AM-MSCs in the presented of 1-MT were higher than that of PHA-activated T cells co-cultured with AM-MSCs without 1-MT ($p < 0.05$, Fig. 4A). Interestingly, the proliferative index of these PHA-activated T cells did not reverse to the level of control (PB-MNCs + PHA). Indomethacin and L-NAME could also significantly reverse the proliferation of PHA-activated T cells which were co-cultured with BM-MSCs and AM-MSCs ($p < 0.05$, Fig. 4B and C), however, the proliferative index of these PHA-activated T cells did not reverse to the level of control.

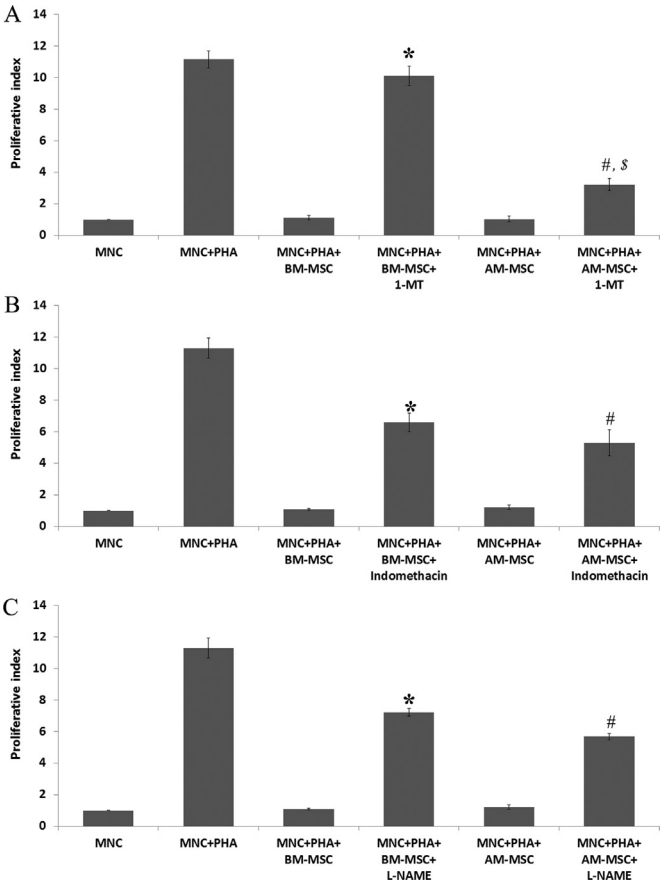

Fig. 4. Mean value of proliferative index of PHA activated T cells co-cultured with MSCs in the presence or absence of 1-MT (A), Indomethacin (B), and L-NAME (C). *, #, $ $p < 0.05$ significantly different compared to MNC + PHA + BM-MSCs, MNC + PHA + AM-MSC and MNC + PHA + BM-MSCs + 1MT, respectively.

4. Discussion

Human MSCs, mostly BM-MSCs, endow a great promise for application in regenerative medicine and immune related diseases [16]. However, the use of BM-MSCs has some limitations [12]. Therefore, the search for alternative sources of MSCs for therapeutic application is important. In this regard, the amnion represents an abundant source of MSCs that could be exploited for therapeutic application. In this study, AM-MSCs exhibited the unique stem cell-like properties similar to BM-MSCs including 1) *in vitro* proliferation as plastic-adherent cells with fibroblast-like morphology, 2) the expression of MSC immunophenotype, 3) the capacity to differentiate toward osteogenic and adipogenic lineages. The presented data demonstrate that AM-MSCs share the

minimum criteria for defining MSCs according to the International Society for Cellular Therapy [17].

Although MSCs from difference sources could share many similarities, however, some studies reported the differences at transcriptional and proteomic levels which possess different biological and functional characteristics [18,19]. Therefore, it is possible that differences in the regulation of immunosuppression may exist. Recent studies have shown that BM-MSCs can suppress the T cell proliferation induced by alloantigens or mitogens through an IDO dependent mechanism [20–22]. However, the immunosuppressive capacity of AM-MSCs and the specific mechanisms underlying AM-MSCs-mediated immunosuppression have been poorly studied. This study demonstrated that AM-MSCs could inhibit T-cell proliferation at the similar degree with BM-MSCs. Moreover, the number of MSCs has an effect to the immunosuppressive capacity of both BM-MSCs and AM-MSCs. Both of them could inhibit T cell proliferation in dose-dependent manner.

Recent studies have shown that the inhibition of T cell proliferation by BM-MSCs appeared to be mediated by both cell–cell interaction [23] and release of soluble factors such as IFN-γ [10,24]. The result from this study supports a crucial role of IFN-γ in inducing an immunosuppressive mechanism [10]. The addition of IFN-γ could significantly activate *IDO* expression in BM-MSCs and AM-MSCs. In addition, the protein expression exhibited a similar profiled to gene expression. Interesting, the expression of protein, IDO, in both AM-MSCs and BM-MSCs which stimulated with IFN-γ showed significantly higher than that of MSCs stimulated with activated T cells. It might be possible that the expression of IDO from both BM-MSCs and AM-MSCs is induced by IFN-γ released from activated T cells.

To determine whether protein, IDO, is exactly regulator for T cell suppression, inhibition assay using 1-MT were performed. The results demonstrated that the proliferative index of activated T cells co-cultured with BM-MSCs was significantly restored once IDO was inhibited. Interestingly, AM-MSCs exhibited a less response to 1-MT compared to BM-MSCs. Blocking IDO using 1-MT could not completely block immunosuppressive function of AM-MSCs. It might be possible that IDO partly contributed to immunosuppressive function of AM-MSCs which is different from BM-MSCs which use IDO as the main pathway for T cell suppression [25]. In addition to IDO, several cytokines and soluble molecules with immunomodulatory capacity, including PGE_2 and NO, have been implicated in the mechanism of immunosuppression by MSCs [9,26]. To determine whether PGE_2 produced by MSCs could account for the inhibition of T cell proliferation, the effect of blocking cyclooxygenase 2 (COX-2) was examined using indomethacin that blocks prostaglandin biosynthesis by inhibiting COX-2 [27]. The result showed that T-cell proliferation was significantly restored once PGE_2 production was prevented. Similar to PGE_2, blocking NO synthesis interfered with MSC-mediated immunosuppression. However, inhibition of these molecules does not result in a complete loss of the immunosuppressive activity of both AM-MSCs and BM-MSCs. Some discrepancies regarding the requirement of PGE_2 and NO on the mechanism of immunosuppression by MSCs exist [28,29]. However, overall results suggested that, although involved, PGE_2 and NO are not the main and crucial immunosuppressive mechanism.

Our finding demonstrates that cells isolated from amnion possess MSC characteristics. Remarkably, AM-MSCs displayed a similar degree of immunosuppression to that of BM-MSCs. Although immunosuppression of AM-MSCs rely on a highly complex mechanism with many factors involved, the key mechanism involved with the production of the tryptophan metabolizing enzyme, IDO, which is partly mediated by the stimulation of AM-MSCs with IFN-γ.

Acknowledgments

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Appendix A. Transparency document

Transparency document associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bbrep.2016.07.019.

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