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Original research article

Andrographolide Increases Cell Viability and Proliferation of Human Umbilical Cord-Derived Mesenchymal Stem Cells

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ABSTRACT

The decline in the number of bone marrow-derived human mesenchymal stem cells (BM-hMSCs) is one important factor that contributes to many degenerative diseases. BM-hMSCs have been extensively studied for research and clinical applications, but the invasiveness of the relevant procedure, and the fact that their number declines with age are of concern. Recently, human umbilical cord-derived MSCs (UC-hMSCs) have received more interest because they can be obtained easily by a non-invasive procedure. However, in vitro expansion is required for clinical use. This study aimed to search for new safe agents able to increase cell growth of UC-hMSCs. Therefore, we examined the proliferative effect of Andrographolide on UC-hMSCs. The characteristics of UC-hMSCs were verified before use. In this study, UC-hMSCs were cultured in a growth medium and treated with Andrographolide (0.01-50 μM) for 24-120 hours. At the indicated time point, cells were collected for evaluating cell viability and cell proliferation by MTT and BrdU assays, respectively. UC-hMSCs have shown characteristics of MSCs matching the criteria that can be used for further studies. After the UC-hMSCs were exposed to Andrographolide, at concentrations of 0.01-5 μM for 24-120 hours, it was found that Andrographolide at 0.01-10 μM had no toxicity to UC-hMSCs. Moreover, Andrographolide at 0.01-10 μM increased the proliferation of UC-hMSCs after treatment for 24-120 hours. In conclusion, this is the first report demonstrating that Andrographolide increases the expansion of UC-hMSCs in culture. These results suggest that Andrographolide may be a good candidate for further development as an alternative agent for increasing the number of MSC in therapeutic applications treating degenerative diseases.

Keywords: Andrographolide; Cell viability; Cell proliferation; Umbilical cord-derived mesenchymal stem cell

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1. Introduction

A decline in the number of bone marrow- derived mesenchymal stem cells (BM-hMSCs) has been shown to be an important factor contributing to many degenerative diseases such as osteoporosis, osteoarthritis, Alzheimer's, and Parkinson's diseases [1]. To date, MSCs have been isolated from many different tissues, including bone marrow [2], adipose [3], cartilage, muscle, as well as gestational tissues such as umbilical cord and placenta [4-5]. For in vitro expansion, the minimum criteria to define human MSC were suggested in 2006 by the Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy (ISCT) [6]. First, an MSC must be plasticadherent when maintained in standard culture conditions. Second, MSCs must express CD105, CD73, and CD90, and lack expression of CD45, CD34, CD14 or CD11b, CD79a or CD19, and HLA-DR surface molecules. Third, MSCs must be competent for in vitro differentiation into osteoblasts, chondroblasts, and adipocytes under standard culture conditions. Although bone marrow is the most frequently practiced location from which MSCs are harvested by standard operation procedure and with high success rate [7-8], cell harvesting requires an invasive procedure and the amount of BM is limited [9]. Therefore, in vitro cell expansion of BM-hMSCs has become a great restriction for their clinical use, resulting in alternative sources receiving more exploration and research. Umbilical cord-derived mesenchymal stem cells (UC-hMSCs), an alternative source for MSC isolation, can be acquired via non-invasive procedure and can be easily cultured, making them potentially superior candidates for use [10]. Although UC-hMSCs can be obtained in larger quantities than BM-MSCs, in vitro expansion is required for clinical research and application. This study aimed to explore new safe agents that are able to increase the cell growth of UC-hMSCs. Andrographolide is one popular compound that possesses several pharmacological activities including anti-inflammatory [11] and anti- oxidative properties [12]. To date, it has been wildly used clinically for treating fever, inflammation, diarrhea, and other infecti-ous diseases without any side effects [13]. Therefore, this study was interested in examining the effect of Andrographolide on *in vitro* UC-hMSC viability and cell proliferation.

2. Materials and Methods

2.1 Subject

This study was approved by the Human Ethics Committee of Thammasat University No.1 (Faculty of Medicine; MTU-EC-DS-6-146-61) in accordance with the Declaration of Helsinki, the Belmont Report, and ICH-GCP. The umbilical cord tissues were obtained from full-term pregnancies after labor and all donors gave written informed consent.

2.2 Isolation and culture of UC-hMSCs

The umbilical cord tissues were cut into small pieces and incubated with 0.25% (w/v) trypsin-EDTA (GIBCO™, Invitrogen Corporation, USA) for 30 minutes at 37°C. The pieces were then washed twice with PBS and cultured in DMEM+10% (v/v) FBS (GIBCO™, Invitrogen Corporation, USA) in a 25 cm^2 culture flask (Corning, USA). Cells were cultured at 37°C. For the removal of non-adherent cells, cultured media were changed every 3 days. The adherent cells were further cultured until colonies of fibroblast-like cells were obtained. For expansion, the cells were sub-cultured using 0.25% trypsin-EDTA. The morphology of hMSCs was observed and photographed under an inverted microscope (Nikon Eclipse Ts2R, Japan).

2.3 Immunophenotypical characterization of UC-hMSCs

The phenotype of the UC-MSCs was evaluated by flow cytometry

(FACScalibur™, Becton Dickinson, USA) and CellQuest® software (Becton Dickinson, USA). Native third to sixth passage UC-MSCs were trypsinized using 0.25% trypsin-EDTA and suspended in PBS. Cells were incubated with fluorochrome-labeled mouse anti-human monoclonal antibodies: anti-CD45-FITC (Bio Legend, USA), anti-CD34-PE (Biolegend, USA), anti-CD90-FITC (Bio Legend, USA), anti-CD73-PE (Bio Legend, USA), and anti-CD105-PE (BD Bioscience, USA) for 30 minutes at 4°C in the dark. After incubating with the antibodies, cell pellets were washed twice with PBS and fixed with 1% (w/v) paraformaldehyde in PBS.

2.4 Adipogenic and osteogenic and differentiation ability of UC-hMSCs

UC-MSCs (passages 3rd-6th) were used to evaluate their adipogenic and osteogenic differentiation potentials. Cells at a density of 1×104 cells/cm^2 were cultured in growth medium (10%FBS+DMEM). For adipogenic differentiation, after cells reached 70% confluence, the medium was replaced with adipogenic medium consisting of DMEM (high glucose) supplemented with 10% FBS, 0.5 mM isobutylmethylxanthine, 1 μM dexamethasone, 5 μg/ml insulin solution, and 100 μM indomethacin (Sigma-Aldrich; Merck KGaA). The adipogenic medium was changed twice weekly, and then after 28 days, the generation of lipid droplets was revealed by Oil Red O staining (Sigma-Aldrich; Merck KGaA). For osteogenic differentiation, after cells reached ~80% confluence, the medium was changed to the osteogenic differentiation medium, consisting of DMEM supplemented with 10% FBS, 100 nM dexamethasone (Sigma-Aldrich; Merck KGaA), 10 nM β-glycerophosphate (Sigma- Aldrich; Merck KGaA), and 50 μg/ml ascorbic acid (Sigma-Aldrich; Merck KGaA). Cells were cultured in the osteogenic differentiation medium for 21 days and the medium was changed every 3 days. Differentiated cells were analyzed by alizarin red staining and observed under an inverted microscope (Nikon Eclipse Ts2R, Japan).

2.5 Cell viability and proliferation assays

UC- hMSCs were seeded at 1×10^3 cells/well in 96-well plates (Costa, Corning, USA) containing 100 μl of 10% Fetal bovine serum (FBS) (GibcoBRL, USA) in growth medium. After 24 hours, cells were treated with 0.01-50 μM Andrographolide (Sigma-Aldrich, USA) for 24 - 120 hours. The cell viability was measured by colorimetric MTT assay. Cell viability (%) was calculated against untreated control cells. The IC50 values were calculated according to dose-dependent curves. Cell proliferation was measured using a bromodeoxyuridine (BrdU) cell proliferation assay kit (Sigma-Aldrich; Merck KGaA). The measurements were performed in triplicate.

2.6 Statistical analysis

Data are presented as the mean ± standard error of the mean (SEM). Group comparisons were made by one-way analysis of variance (ANOVA). Multiple comparesons between the groups were performed using the Student-Newman-Keuls method. A value of $p < 0.05$ was considered to indicate a statistically significant difference.

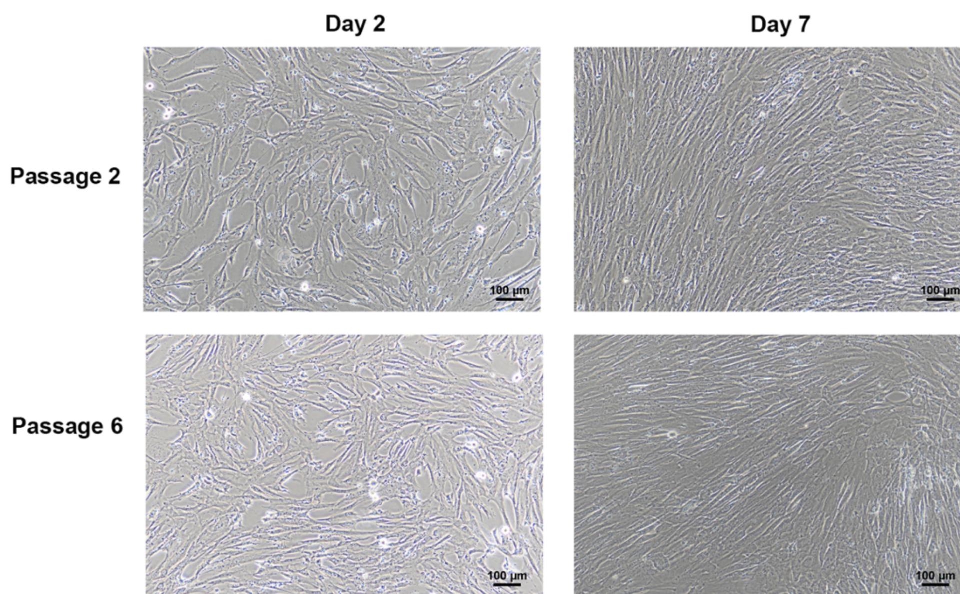

Fig. 1. The morphology of UC-MSCs.

3. Results and Discussion

3.1 Morphology of UC-hMSCs

UC-hMSCs have been considered as an excellent primitive source of noncontroversial stem cells [10]; as such, UC-hMSCs were used for this study. Firstly, cells were isolated and cultured in difference passage (passage 2 and passage 6). The UC-hMSCs exhibited fibroblast-like morphology in the growth medium which could attach, spread, and display a spindle-shaped morphology on the plastic surface of the tissue culture flasks (Fig. 1). The UC-hMSCs were successfully isolated and expanded.

3.2 Immunophenotype of UC-hMSCs

According to the standard definition of MSCs [6], the expression of MSC surface markers was assessed. As expected, the UC-hMSCs expressed typical hMSC surface markers including CD73, CD90, and CD105 but they did not express hematopoietic markers including CD34 and CD45 (Fig. 2).

3.3 Differentiation potential of UC-hMSCs

Under certain conditions, MSCs can be multipotent, meaning that they can differentiate into multiple different types of cells. After the UC-MSCs were cultured under adipogenic conditions for 4 weeks, the cells' shape changed from spindle to large and round, indicating the accumulation of lipid droplets in their cytoplasm (Fig. 3A). In contrast, lipid droplets were not found in the control cultured cells. Calcium deposits are an indication of successful in vitro bone formation, which can be specifically verified using alizarin red staining [14]. In this study, calcium depositions were detected in the osteogenic treated cells after 3 weeks (Fig. 3B), these deposits were not found in the control cells. These results suggest that UC-hMSCs have the potential to differentiate into adipocyte and osteoblast cells upon proper stimulation.

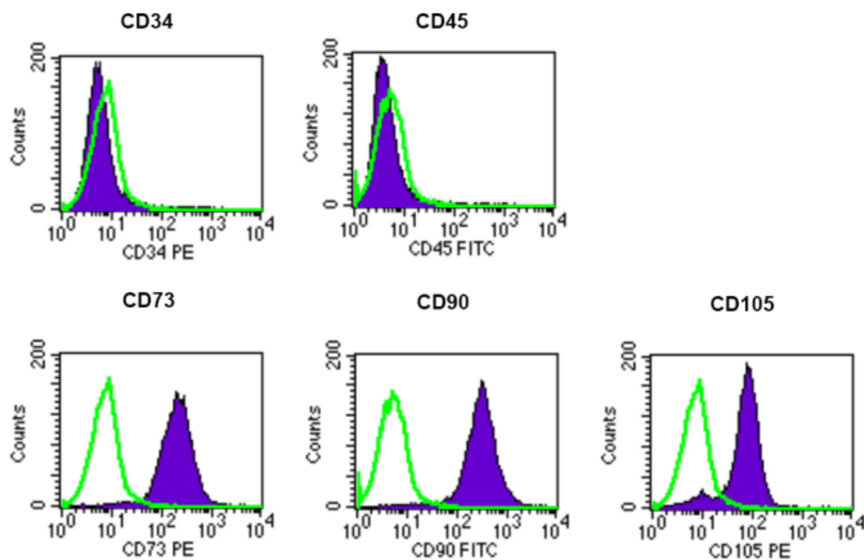

Fig. 2. The expressions of typical cell surface markers of mesenchymal stem cells.

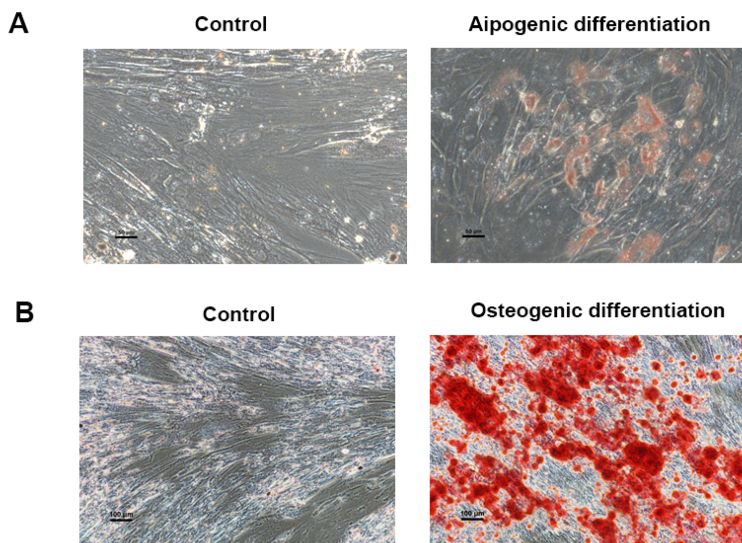

Fig. 3. Differentiation potential of UC-hMSCs. (A) Representative images of oil red O staining of UC-hMSCs cultured in adipogenic differentiation medium compared to growth control for 28 days. (B) Representative images of alizarin red staining of UC-MSCs cultured in osteogenic differentiation medium compared to growth control for 21 days. Scale bar, 100 µm.

Table 2. Effect of Andrographolide (AP) on UC-hMSCs cell viability. Data are expressed as percent cell survival compared to untreated control cells at each time point.

AP (μM)	Time (h)				
	24	48	72	96	120
Control	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00
0.01	121.64 ± 4.57**	112.47 ± 0.76*	115.94 ± 4.07*	109.78 ± 1.43	108.52 ± 2.04
0.1	130.98 ± 4.69**	117.57 ± 1.78**	123.67 ± 1.81**	113.19 ± 1.24*	117.13 ± 1.66*
1	132.78 ± 2.79**	122.58 ± 2.25**	128.41 ± 2.08**	125.32 ± 1.77**	134.50 ± 3.06**
2	133.00 ± 3.20**	123.38 ± 3.11**	138.05± 3.19**	122.70±2.38**	126.05 ± 1.91**
2.5	131.39 ± 3.55**	112.59 ± 1.33*	132.08 ± 3.79**	118.89 ± 3.48	123.12 ± 2.60**
5	116.01 ± 4.44**	112.79 ± 0.66*	124.03± 2.74**	108.06 ± 3.16	109.69 ± 0.85
10	101.26 ± 2.56	98.88 ± 0.83	109.18 ± 2.20	92.70 ± 1.08	89.53 ± 1.76**
20	83.86 ± 2.08**	77.36 ± 1.26**	75.51 ± 3.08**	68.25 ± 3.27**	58.47 ± 4.51**
50	49.89 ± 1.94**	31.84 ± 3.28**	43.65 ± 1.48**	26.64 ± 1.06**	16.53 ± 3.67**
IC 50 (μM)	50.56 ± 4.44	40.54 ± 1.92	37.28 ± 4.05	32.63 ± 3.57	24.90 ± 3.06

Each value is mean ± SEM. * $p<0.05$ and ** $p<0.01$ significant difference when compared to untreated control cells.

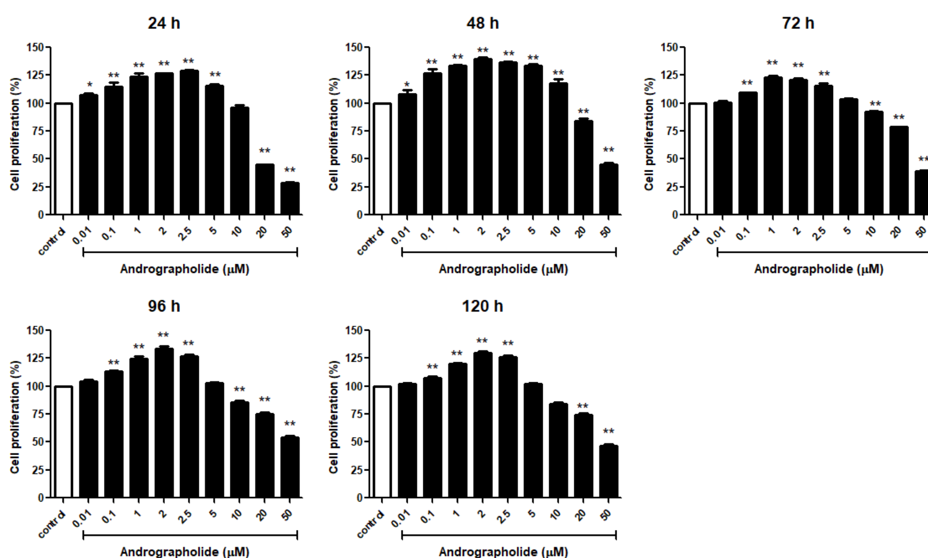

Fig. 4. Effect of Andographolide on UC-hMSC proliferation. Data are expressed as mean ± SEM from 3 independent experiments. *P<0.05, **P<0.01 significantly different from untreated control cells.

From MSCs characterization, the UC-hMSCs displayed a fibroblast- like morphology, typically positive for CD73, CD90, and CD105 and can develop in to adipocyte and osteoblast cells which meets the criteria of the International Society for Cellular Therapy. Therefore, UC- hMSCs were used for this study.

3.4 Effect of Andrographolide on UC-hMSC viability and proliferation

Table 1 shows cell viability of the UC-hMSCs treated with Andrographolide in growth medium compared to control group for 24-120 hours. The cell viability of UC-hMSCs treated with 0.1- 2.5 μM Andrographolide were significantly larger than the control group ($p<0.05$ and $p<0.01$). In contrast, cell viability of UC- hMSCs treated with 20- 50 μM Andrographolide were significantly lower than the control group ($p<0.05$ and $p<0.01$). The IC50 of Andrographolide on UC-hMSCs at 24 hours is more than 50 μM, and IC50 of Andrographolide on UC-hMSCs at 48, 72, 96 and 120 hours are 40.54 ± 1.92, 37.28 ± 4.05,

32.63 ± 3.57, and 24.90 ± 3.06, respectively. This difference also reflected an increase in cell proliferation which was confirmed by BrdU assay (Fig 4.). Cell proliferation of UC- hMSCs cultured in growth medium treated with 0.1- 5 μM Andrographolide was significantly increased after treatment for 24, 48, 72, 96, and 120 hours compared to the control group ($p < 0.05$ and $p < 0.01$). These results indicate that Andrographolide has a proliferative effect on UC-hMSCs.

Several studies have reported that the reduction of MSC growth and differentiation is related to excessive production of free radicals [15]. Increased levels of reactive oxygen radicals cause the increase of toxic pro-inflammatory cytokines that subsequently affect many cellular processes, including cell adhesion, migration, proliferation, and cellular senescence in MSCs [16-17]. In this study, it was demonstrated that Andrographolide increases cell viability and proliferation of UC-hMSCs. These effects may result from the anti-inflammatory and anti-oxidative properties of Andrographolide [11]. A previous study demonstrated that Andrographolide exerted an anti-inflammatory effect by inhibiting the activation of the NF-κB/MAPK signaling pathway and the induction of proinflammatory cytokines [18]. Moreover, Andrographolide downregulated free radical production to prevent neuronal cell degeneration [19] and also suppressed ROS-mediated NF- κB signaling [20]. However, the exact mechanisms involved in the cytoprotective and proliferative effects of Andrographolide on UC-hMSCs needs further investigation.

4. Conclusion

The current work is the first report demonstrating the proliferative effect of Andrographolide on UC-hMSCs in culture. These results suggest the potential opportunities of Andrographolide for further development as an alternative agent to increase the number of MSCs for use in therapeutic applications of degenerative disease treatment.

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