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ORIGINAL ARTICLE

Study the Proliferation Rate of Human Bone Marrow Stem Cells Post Induced by Osteogenesis Differentiation Medium and Human Adipose Stem Cells Conditioned Medium

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KEYWORDS

Human stem cells;
Proliferation;
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ABSTRACT: The human adipose stem cells (hADSCs) conditioned medium is easily prepared, transported, and stored. This medium is used in the treatment of diseases such as skin aging, wound, and scar repair, as well as nerve regeneration. In this research, the growth rate of human bone marrow stem cells (hBMSCs) under the effect of osteogenesis differentiation medium and the conditional medium was investigated separately. The third passage of hBMSCs was divided into three groups: 1- MED (cells cultured in α- MEM containing 10% fetal bovine serum), 2- OD (cells cultured in osteogenesis differentiation medium containing 7-10 M Dexamethason, 10 mM Beta-Glycerol-Phosphate, 50 ug ml^{-1} Ascorbic Acid bi-Phosphate, 10% FBS, 100 unit/ml Penicillin), and 3- CM (cells cultured in hADSCs conditioned medium). After 7 and 14 days, the proliferation rate was evaluated using the MTT method. The results showed that after 7 and 14 days of treatment, the rate of proliferation has increased considerably in both OD and CM groups versus the MED group, and the CM group versus the OD group. In addition, there has been an important increase in proliferation rate in both OD and CM groups compared to MED and in CM group compared to OD, 7 and 14 days post- treatment. Conditioned medium treatment effectively increased cell proliferation in hBMSCs, compared to osteogenic differentiation medium. This cell culture method provides the expansion of stem cells suitable for regenerative medicine.

INTRODUCTION

Self-renewal and also multilineage differentiation are important characters of mesenchymal stem cells (MSCs). MSCs can be separated from a various tissues, such as the umbilical cord, bone marrow, adipose tissue, etc [1].

MSCs can be differentiated into different types of mesodermal cells such as adipocytes, chondrocytes, osteoblasts, and skeletal muscle fibers, as well as non-mesodermal cells such as hepatocytes, pancreas, endocrine cells, neurons, myocardial and endothelial cells. MSCs are extensively applied in regenerative medicine and tissue engineering due to their self-renewal properties, generating various types of mature cells under specific conditions [2].

Bone marrow and fat tissue are the major sources of MSCs. Adipose tissue is a very alluring source of adult stem cells

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for use in further regenerative medicine applications because it is the main source of MSCs. The results of clinical trials did not support the efficacy of stem cell therapy, despite the immunomodulating qualities of adipose-derived stem cells (ASCs) and the secretion of a wide range of paracrine factors that promote tissue regeneration. [3].

Bone marrow stem cells (BMSCs) are isolated from different bones such as the ilium, femur, and tibia, but whether the proliferation and differentiation potential depend on the source of stem cells is still unknown. These cells can differentiate into different types of tissues like bone, cartilage, tendon, muscle, fat and nervous tissue to regenerate damaged tissue after transplantation [4].

In the body, there is a lot of adipose tissue [5] and the extracted cells from it are called ASCs [6]. Abdominal subcutaneous adipose tissue is the most common origin of ASCs [7]. These cells can secrete trophic factors in the culture medium which is called conditioned medium (CM) [8]. It was reported that rat BMSCs-CM increased the proliferation of MSCs and induced osteogenesis in injured rat calvaria. ASCs and BMSCs are similar in phenotype, surface markers expression, and differentiation, while ASCs have higher self-renewal capacity than BMSCs. The proliferation ratio of ASCs began to slow down in the earlier passage, perhaps due to collagenase digestion and spending more time from extraction to culture. The survival rate, differentiation capacity, and the number of stem cells in BMSCs decreased with increasing age [9].

In a study, mouse hepatocytes were treated with ASCs-CM produced under normoxia (N-CM) or hypoxia (H-CM) conditions, and after five days, the hepatocyte proliferation rate increased greatly [10]. In addition, ASCs-CM increased the proliferation rate of hair follicle papillary dermal cells and human keratinocytes [11].

In this study, the proliferation rate of hBMSCs induced by osteogenesis differentiation medium and hASCs - conditioned medium was assessed.

MATERIALS AND METHODS

Isolation and culture of hBMSCs

In this experimental study, human BMSCs were the kind gift of Dr.Junya Toguchida (Kyoto University, Kyoto, Japan) [12]. The cell samples were centrifuged at 1200 rpm for 5 minutes. After removing the supernatant, the cell pellets were cultured in α-MEM (α-minimal essential medium; Gibco/BRL; cat. No. 52100-0.3, USA) supplemented with 10% FBS (Gibco, cat. No.12203C, USA) and incubated [13]. The medium was changed every 3 days. The cells were passaged at 70 to 80% confluency [14]. In this research, the cells at passage three were used. Images were captured using a digital camera attached to a Nikon inverted phase contrast microscope (DXM 1200; Nikon Digital, Germany).

Isolation and Culture of hASCs

The subcutaneous adipose tissue samples were obtained from women (aged between 28 and 35) undergoing liposuction. Informed permission was obtained from the participants. This research has been confirmed under the ethical approval code of IR.DU.REC.1400.011. One gram of adipose tissue was digested mechanically by using a scalpel blade and then divided into small pieces under sterile conditions. Then, 0.2% collagenase enzyme (Invitrogen, USA) with the amount of 1.5 ml per gram of adipose tissue was used at 37℃ for 60 minutes. Collagenase was disabled in a similar volume of α-MEM medium enriched with 10% bovine fetal serum (FBS, Gibco, USA) and after centrifugation (1200 rpm, 5 min, and 37°C), the cells were incubated in α-MEM enriched with 10% FBS and 1% penicillin-streptomycin [15]. After 72 hours with replaced culture medium, the floating cells were removed and MSCs were adhered to the lowest level of the flask. The cells were passaged upon reaching 80% confluence. According to the method described in our previous study, isolated cells were characterized [16]. The third passage of cells was used to prepare the conditioned medium.

Preparation of hASCs-conditioned medium

The hASCs were washed with PBS three times at passage three, and cultured in medium without serum for 72 hours and allowed to secret neurotrophic factors. The conditioned medium was removed, centrifuged at 2000 rpm for 5 minutes, filtered through a 0.22 mm syringe filter, and then deposited at -80°C until use [17-20].

Experimental groups

At passage three, the hBMSCs were divided into three different groups: 1- MED: cultured cells in α-MEM with 10% FBS, 2- OD: cultured cells in osteogenesis differentiation medium containing 7-10 M Dexamethason, 10 mM Beta-Glycerol-Phosphate, 50 ug/ml Ascorbic Acid bi-Phosphate, 15% FBS, 100 unit/ml Penicillin), and 3- CM: cultured cells in hASCs-CM. The treatment duration was 7 and 14 days [21, 22].

Cell viability and proliferation rate assays by MTT assay

Cells at passage 3 were directly seeded on 96-well tissue culture plates at a density of 2×10^4 cells/well, and incubated in 200 µl α-MEM supplemented with 10% FBS. When the cells reached 70% confluency, cell proliferation was examined with MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl- 2H-tetrazolium bromide] assay (Sigma-Aldrich, Germany). Then, 0.005 g of MTT powder was dissolved in 1 ml of α-MEM. The supernatant was discarded and replaced in each well with 100 µl α-MEM supplemented with 10 µl MTT solution (5 mg ml^{-1}, Sigma, Germany) and incubated at 37°C for 4 h. After throw 85 µl of supernatant away, 50 µl of DMSO (Dimethylsulfoxide) was increased for 10- minute incubation. Finally, an ELIZA reader (BioTek) was used to determine the absorbance at 570 nm [23].

Statistical analysis

Data were statistically analyzed by one-way ANOVA test followed by Tukey using SPSS software version 16. To evaluate the significant differences between the experimental groups and LSD post-hoc tests were performed. The significant differences in studied cells on days 7 and 14 were evaluated using an Independent-sample T- test. The significance level adopted was $P < .05$ and each experiment was repeated 3 times.

RESULTS

Morphologies of in vitro cultured hBMSCs and hASCs

Primary cultured human BMSCs (A) and ASCs (B) were shown in Figure 1 and composed of heterogeneous colonies containing different cell morphologies (small round, spindle-shaped, or large flat cells). In higher passages, the cells gradually exhibited a fibroblast- like shape and displayed a more uniform morphology. At the third passage of BMSCs and hASCs, increased cell proliferation, polygonal cell morphology, and long uniform growth were shown in Figure 2 (A, B). hASCs with spindle-shaped and fibroblastic morphology were easily expanded up to passage 15.

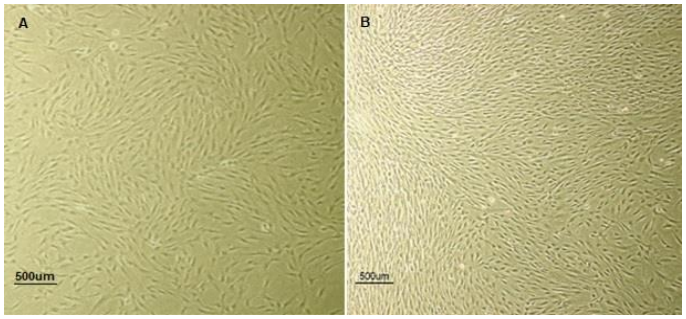

Figure 1. Morphologies of the first passage of human hASCs (A), and BMSCs (B).

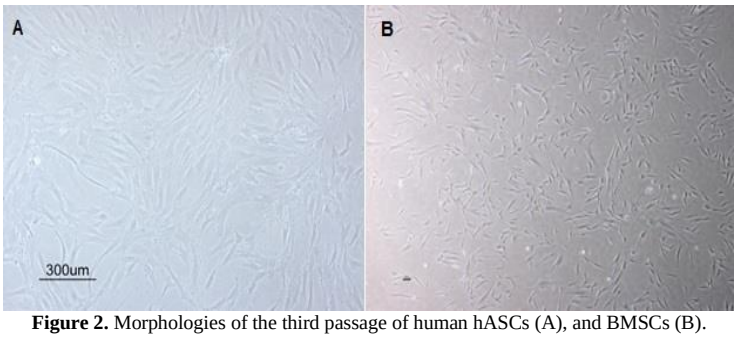

Figure 2. Morphologies of the third passage of human hASCs (A), and BMSCs (B).

The survival and proliferation rate of human BMSCs exposed to osteogenesis differentiation medium and the human ASCs conditioned medium

After 7 and 14 days of treatment, the proliferation rate of cultured hBMSCs in OD and CM groups was evaluated by the MTT method.

In Figure 2, there was a highly increased proliferation rate in both OD and CM groups versus the MED group and also, in the CM group versus OD group, 7 and 14 days post-treatment (P<0.05).

The cell viability and proliferation rate were assessed at day 7 compared to day 14. None of the test groups showed notable differences in cell proliferation and survival rate, at days 7 and 14 (P<0.05) (Figure 3).

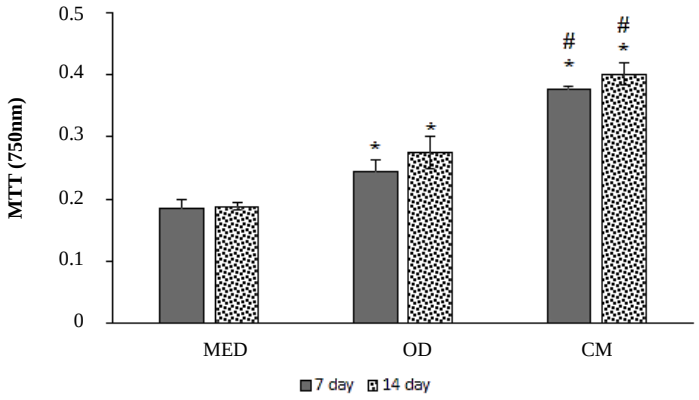

Figure 3. Study of hBMSCs proliferation rate in experimental groups after 7 and 14 days, * P < 0.05 versus MED group, # P < 0.05 versus OD group at the same day.

DISCUSSION

In recent decades, MSCs have received considerable attention in regenerative medicine and tissue engineering due to their differentiation ability, easy separation and culture growth, fast proliferation, and immunosuppressive impacts [24-26].

In this study, presence of osteogenesis differentiation medium and conditioned medium led to a significant increase in the proliferation rate of hBMSCs, and the CM group displayed a considerable increase compared to the OD group.

Although, dexamethasone, beta glycerophosphate, ascorbic acid, FBS, and antibiotics were used in the preparation of osteogenesis differentiation medium. Ascorbic acid (ASA) (an organic compound with a lactone structure) has been studied for a long period and has several physiological roles. ASA destroys reactive oxygen species caused by oxidative stress, has a great effect in cell division, and suppresses elderly [2]. It has been previously reported that

ASA supplementation causes the proliferation of MSCs [27]. In addition, many studies on cell proliferation stated that ASA plays a role in cell proliferation and differentiation [2].

In a study, it was mentioned that antioxidants such as ASA can stimulate the proliferation of MSCs while maintaining their differentiation ability without changing their phenotype [28]. However, the exact mechanism of ASA action is still unknown.

ASA has been found to increase the activity of hypoxia-inducible factor 1 alpha subunit (HIF1α) hydroxylase, suppresses the transcription of HIF1α and enhances its degradation, and leads to mitochondrial activity and cell proliferation [2]. The researchers recently demonstrated that dexamethasone (Dex) also stimulates the proliferation of osteoprogenitor cells [29].

The conditioned medium from adipose tissue- derived stem cells has some growth factors like fibroblast growth factor 2 (FGF2) and platelet-derived growth factor (PDGF) that induce cell proliferation [30] FGF2 belongs to the FGF family and is a heparin-binding protein that distributes between tissues and combines with cell surface proteoglycan [31].

In a study, it was reported that FGF2, the activity of Src/MEK1/2 (Src is a member of the Src kinase family) that binds to the cytoplasmic part of the cell membrane and is important in regulating the growth and differentiation of eukaryotic cells.

MEK1/2 is the downstream enzyme of Src and its activation by FGF2 is necessary for proliferation, and then it induces the phosphorylation of various signaling pathways such as Erk1/2, P38, and JNK and increases the proliferation of hASCs [31].

Recent research stated that FGF2 has a great effect on the proliferation and differentiation of hASCs by activating the Erk1/2 pathway [32]. It was demonstrated that FGF2 significantly increases the proliferation of synovial stem cells (SDSCs) [33].

Recent research has demonstrated that FGF supplementation improves the proliferation of hASCs [34].

It was reported that a mesenchymal- conditioned medium increases the in vitro proliferation and migration of alveolar epithelial cells by the JNK-P38 signaling pathway [35]. The increased proliferation of MSCs cultured with mesenchymal- conditioned medium was reported [9].

A study reported that ASCs-CM increases the proliferation of human follicular dermal papillary cells (HFDPCs) and human epithelial keratinocytes [11].

CONCLUSIONS

Mesenchymal stem cells have high potential for use in regenerative medicine. However, in laboratory conditions, the ability of these cells to proliferate and expand gradually decreases, while the differentiation and characteristics of stem cells are preserved, and this issue may limit their clinical applications.

In this study, osteogenesis differentiation medium and conditioned medium of human adipose stem cells were used as cell proliferation inducers in hBMSCs. Both the osteogenesis differentiation medium and conditioned medium increased the proliferation rate of hBMSCs, 7 and 14 days after treatment. In addition, the conditioned medium caused a remarkable increase in cell proliferation rate compared to the osteogenesis differentiation medium.

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Conflict of interests

The authors declare that there is no conflict of interest.

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