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The Cultivation of Bone Marrow Mesenchymal Stem Cells Derived from Patients with High Altitude Polycythemia

J. Zheng[1], N. Guo[1], Y. Xi[1], H-Q. Li[1], H. Zhang[1], L-H. Ji[2]✉, S. Cui[2], and Z-Q. Li[2]

[1] Department of Chemotherapy for tumor, Zhang Qiu Ren Min Hospital, Zhangqiu 250200, China
[2] Department of Hematology, Qinghai University Affiliated Hospital, Xining 810001, China

Corresponding author: Lin-Hua Ji, Department of Hematology, Qinghai University Affiliated Hospital, No. 29 Tongren Road, Xining 81000, China. Tel: +86 0971 6230877, Fax: +86 0971 6155740, Email: linhuajicn@163.com

Abstract

The aim of this study was to isolate and culture bone marrow mesenchymal stem cells (MSCs) from patients with high altitude polycythemia (HAPC) in order to provide a foundation for further exploration of their biological characteristics. MSCs were isolated and cultured from 10 HAPC patients and 10 healthy controls by using a density gradient centrifugation and an adherent screening method. The morphous of MSCs were observed under an inverted microscope, and its surface antigens were determined using flow cytometry. The growth of the MSCs was also detected to evaluate its proliferation. Bone marrow mononuclear cells were isolated from the bone marrow using a density gradient centrifugation, and they were cultured *in vitro*. The bone marrow MSCs were successfully isolated and cultured, which presented as fusiform and adherent cells. The MSCs in both groups expressed CD90,CD44,CD29,CD105, CD106, CD146, CD166,Stro-1 and CD13, but they did not express CD45, CD4,CD8,CD19,CD20,CD80,CD14,CD3,CD34 or HLA-DR ($P>0.05$). The bone marrow MSCs from HAPC patients had a higher proliferation than the bone marrow MSCs from the healthy controls ($P<0.01$). The bone marrow MSCs from HAPC patients can be effectively cultured *in vitro*.

Key words: High altitude polycythemia, cell culture, mesenchymal stem cells.

Introduction

High altitude polycythemia (HAPC) is a clinical syndrome that occurs in some individuals who live in a plateau area >2,500 m more than 3 years, yet they do not acclimatize to the low oxygen environment (1). Its main pathological features include severe hypoxemia and an excessive accumulation of red blood cells (RBCs), which is often accompanied by pulmonary hypertension. Extensive studies on the pathogenesis of HAPC have focused on its morphology, racial differences, cell apoptosis, the biological characteristics of the RBCs, and the hematopoietic regulation of the RBCs (2-5). The proliferation of erythroid cells in the bone marrow was found to contribute to the excessive accumulation of RBCs in those with HAPC. The excessive secretion of erythropoietin (EPO) is an important factor in the formation of erythrocytosis in those with HAPC; however, the mechanism for the excessive accumulation of RBCs in HAPC has not yet been clarified. Li et al. (6) reported that there are non-EPO-dependent mechanisms that exist in the process of erythropoiesis in HAPC.

Mesenchymal stem cells (MSCs) are important non-hematopoietic stem cells in the bone marrow microenvironment with multi-directional differentiation potentials, which are involved in the constitution of supporting a hematopoietic microenvironment (7). MSCs can differentiate into bone cells, cartilage cells, fat cells, tendon and muscle cells, nerve cells, and other cells (7). MSCs are important tools for treating immune disorders and in tissue repair due to their multi-potency, immunosuppressive properties, and production of cytokines or growth factors. MSC-mediated therapy is a fast growing field that has proven safe and effective in the treatment of various degenerative diseases and tissue injuries (8). They support hematopoiesis of hematopoietic stem cells and suppress the immune response in a dose-dependent manner (9). MSCs from aplastic anemia patients support *in vitro* homeostasis and *in vivo* repopulating function of CD34(+) cells while maintaining their immunosuppressive and anti-inflammatory properties (10). A study on the mechanism of MSCs supporting hematopoiesis provided current evidence, which demonstrated that the maintenance and regulation of hematopoiesis by bone marrow endosteal mesenchymal progenitors were dependent on hypoxia-inducible factors (HIFs) (11). When culture-expanded human bone marrow-derived (BM)-MSCs were stimulated with the parathyroid hormone (PTH), their ability to expand cocultured CD34(+) hematopoietic progenitor cells (HPCs) was enhanced. Furthermore, when PTH-treated BM-MSCs were subcutaneously implanted into non-obese/severe combined immunodeficiency mice, the induction of hematopoietic cells was enhanced (12).

MSCs have a strong ability to proliferate when cultured *in vitro*. MSCs can differentiate into marrow stroma cells to support hematopoiesis and are closely related to the homing of hematopoietic stem cells (HSCs). In addition, MSCs express a variety of hematopoietic cytokines, and they play very important roles in hematopoietic reconstruction (7).

However, few studies have focused on the MSCs of HAPC patients. Therefore, we isolated the MSCs from the bone marrow of HAPC patients and cultured them *in vitro*. Then we purified and amplified them in order to identify their phenotype and function, which may have

an important significance in studying the directional differentiation of MSCs in HAPC patients and in determining their roles during the onset of HAPC.

Materials and methods

Subjects

The bone marrow from 10 HAPC patients with a mean hemoglobin of 221±10 g/L were collected. All the patients were males aged 38±6 years, and they had lived in a plateau area with an altitude >2,500 m more than 3 years. Bone marrow was also obtained from the ribs of 10 patients with traumatic fractures and with normal hemograms and bone marrow pictures (control group). The subjects in the control group were all men aged 34±10 years. Sex and age showed no significant difference between the two groups (P>0.05). This study was conducted in accordance with the declaration of Helsinki. This study was conducted with approval from the Ethics Committee of Qinghai University Affiliated Hospital. Written informed consents were obtained from all participants.

Isolation and cultivation of the bone marrow mesenchymal stem cells

Under aseptic conditions, 5 mL of bone marrow, anticoagulated with 5×10^6 U/mL of heparin, were collected by paracentesis from the posterior superior iliac spine of 10 HAPC patients, and the bone marrow was diluted with an equal-volume of Dulbecco's modified Eagle's medium (DMEM) with low glucose (Gibco, Grand Island, NY, USA). The bone marrow from the control group were collected by washing the medullary cavity of the ribs from 10 patients with traumatic fractures using low glucose DMEM under aseptic conditions. Then, the diluted bone marrow was used to isolate the mononuclear cells using Ficoll separation liquid (density, 1.077 g/mL). After two washes with DMEM, the mononuclear cells were suspended in DMEM (Gibco), which was supplemented with 10% fetal bovine serum (FBS, Hyclone, Logan, UT, USA). The 5×10^6 cells in the DMEM supplemented with 10% FBS were seeded onto 25 cm^2 plastic culture flasks (Costar, Cambridge, MA, USA), and they were incubated in an incubator with 5% CO_2 with a saturated humidity at 37°C. Seventy-two hours later, the non-adherent cells were removed and changed with a new medium. The adherent cells were incubated again, and the medium was changed every 3 or 4 days. As it grew to 90% confluence, the cells were collected by digestion with 0.25% trypsin/EDTA (Gibco) at 37°C and were suspended in the DMEM containing 10% FBS. Then, the cells were subcultured at a ratio of 1:3 and were noted as P1. When the P1 cells grew to full confluence, they were subcultured for more passages at a ratio of 1:2. During the passage, the medium was changed every 3 days.

Morphological identification

The morphous and growth of the adherent cells were observed daily under an inverted microscope. The MSCs at P1, P3, and P6 were seeded onto Φ3.5 cm petri dishes with a glass slide inside, and they were incubated at 37°C with 5% CO_2 for 1 week, which encouraged the cells to grow on the glass slide. Then the glass slides were removed. After air-drying, the slides were stained with Switzerland-Giemsa and were observed under a light microscope.

Determination of surface markers

The cells were harvested by digestion with 0.25% trypsin and were washed twice with phosphate buffered saline (PBS). The 2×10^6 cells were stained with phycoerythrin- or fluorescein isothiocyanate-conjugated mouse anti-human monoclonal antibodies to determine the relevant surface markers, which included CD90,CD44,CD29,CD105,CD106,CD146,CD166,Stro-1,CD13,CD34,CD45,CD14,CD3,CD4,CD8,CD19,HLA-DR,CD80 and CD20 (BD Biosciences, San Jose, CA, USA). The reactions were stored in a dark at room temperature for 30 minutes. After two washes with PBS (centrifuged at 1000 r/min for 5 minutes each time), the cells were fixed with 1% paraformaldehyde and then were detected on an FACSCalibur flow cytometer (BD Biosciences). The data were analyzed using Cell Quest software (Beckton Dickinson Immunocytometry Systems, San Diego, CA, USA).

Identification of the proliferation

The bone marrow mononuclear cells in the HAPC and control groups were cultured and passaged from 5×10^6 cells. When the cells were subcultured to P3, P4, and P5 generations, the MSCs numbers were counted and compared between the HAPC and the control groups.

Statistical analysis

The measurement data shown were expressed as mean ± standard deviation, and the comparisons between the two groups were performed using the t test. The numeration data were expressed as percentages and were compared using the chi-square test. A P<0.05 was considered statistically significant. All the statistical analyses were performed using SPSS for Windows, version 11.5 (SPSS, Inc., Chicago, IL, USA).

Results

Morphology

Between 24 and 48 hours after the mononuclear cells were isolated from the bone marrow, they were cultured, and the cells were observed under an inverted microscope. A few cells adhered to the flask wall and had little cytoplasmic process. With the incubation time prolonged, the adherent cells began to split and grow. The cells presented fusiform fibroblast-like shapes with abundant cytoplasm, a centered nucleus, and obvious nucleolus. One week later, the cells were uniform in shape with extensive fusiform process, and they fused into a single layer. The adherent MSCs grew quickly, which took 10-14 days to grow to >90% confluence. Then the cells showed obvious directivity, such as parallel arrangement, spiral, mesh, and radial growth. The cells subcultured by trypsin digestion or the recovered cells were completely adherent in 24 hours with a shape similar to their primary cells. The cells with Switzerland-Giemsa staining presented with a fibroblast-like appearance, and the cytoplasm was stained as pale blue. Some of the cells contained vacuoles, and the nucleus

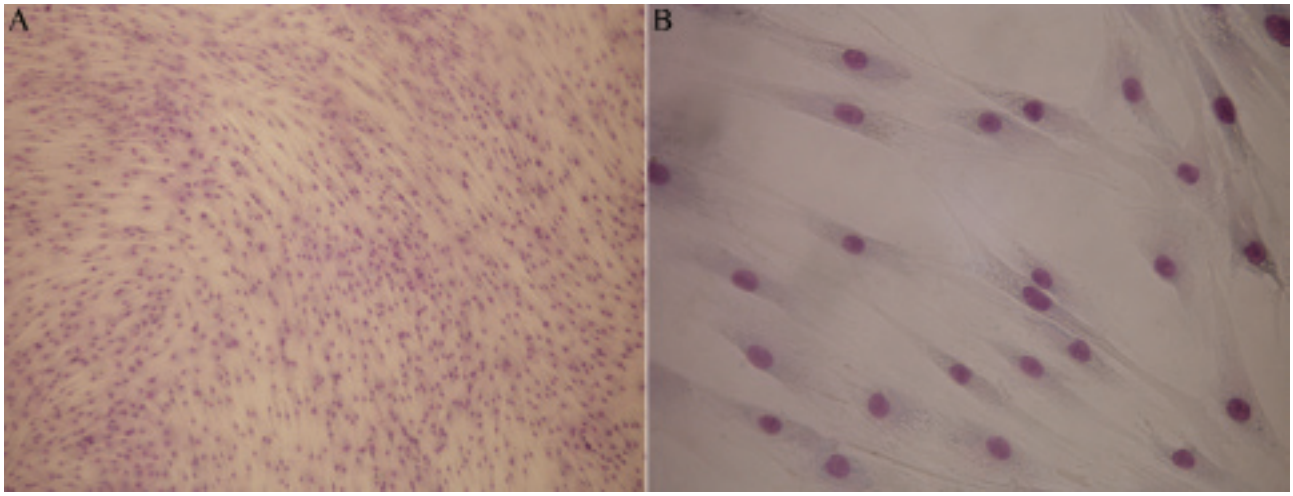

Figure 1. Primary culture of bone marrow MSCs isolated from HAPC patients (A, 40×; B, 400×).

Table 1. Cell count of bone marrow MSCs of the HAPC group and the control group amplified *in vitro*.

Group	Cases	Cell count of MSCs/ 5×10^6 mononuclear cells		
		P3($\times10^7$)	P4($\times10^7$)	P5($\times10^7$)
Control	10	1.35±0.5	3.75±1.2	8.22±1.9
HAPC	10	3.46±0.2	9.57±4.4	18.19±6.1
P value		<0.01	<0.01	<0.01

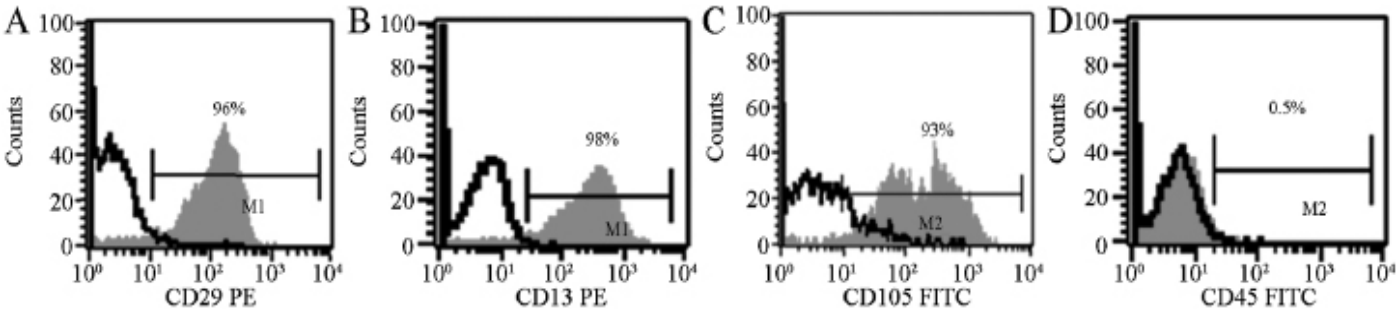

Figure 2. Phenotype of bone marrow MSCs isolated from HAPC patients determined by Flow cytometry.

was round or oval with several dark blue nucleoli and loose chromatin. The cells grew in parallel arrangement or a swirl (Figure 1). The bone marrow MSCs from the HAPC patients showed no different morphous compared to that of the healthy controls.

Surface markers

The typical antigen molecules of the hematopoietic cells, such as CD14,CD3,CD34,CD45,HLA-DR,CD4,CD8,CD19,CD20 and CD80 were not expressed by the bone marrow MSCs from the HAPC patients or the healthy controls. However, CD90, CD44, CD29, CD105,CD106,CD146,CD166,Stro-1 and CD13 were positive on the bone marrow MSCs (Figure 2). The cytokine expression profiles of the bone marrow MSCs showed no statistically significant difference between the two groups (P>0.05), indicating that the cell component was simple.

In vitro proliferation

The mononuclear cells isolated from bone marrows of the HAPC and control groups were counted, and 5×10^6 cells were used as the initial number for cultivation *in vitro*. As the cells were subcultured, the cell number at P3, P4, and P5 were compared between the HAPC and control groups. The cell count of the HAPC group was significantly higher than that of the control group, regardless of P3, P4, or P5 (P<0.01, Table 1).

Discussion

HAPC mainly presents as erythrocytosis and increased hemoglobin. The primary cause for HAPC is a lack of oxygen. MSCs are sensitive to the environment of ischemia and hypoxia, which can affect the physiological characteristics of the MSCs (13-16). The MSC is an important component of the hematopoietic microenvironment, which has become a hot research topic in the field of somatic stem cells in recent years, because of its characteristics of self-renewal and multi-potent differentiation as well as its function in supporting hematopoiesis and immune suppression (17-19). As a precursor cell of the marrow stroma cell, the MSC is an important component of the hematopoietic microenvironment in bone marrow. It not only supplies the mechanical support for the spatial location of HSCs in bone marrow, but it also secretes a variety of growth factors, such as the stromal cell-derived factor-1, stem

cell factor, platelet-3L, thrombopoietin, interleukin (IL)-7, granulocyte-macrophage colony-stimulating factor, IL-6, transforming growth factor-β, and IL-11, to support the hematopoietic function of bone marrow; this helps to maintain the undifferentiated state of the HSCs (20,21). Guarnerio et al. (11) found that murine endosteal mesenchymal progenitors express high levels of HIF-1α and HIF-2α, and by investigating the role of HIF factors in bone marrow mesenchymal progenitors, they proliferate preferentially in hypoxic conditions ex vivo. In addition, the extracellular matrix, such as the adhesion factor and integrin family produced by the MSC and its differentiated cells, can directly affect the HSCs and promote the adhesion and homing of HSCs (22). Therefore, the MSC is closely correlated with the self-renewal, proliferation, differentiation, and homing of HSCs.

MSCs constitute an essential component of the bone marrow hematopoietic microenvironment because of their immunomodulatory properties and their ability to support hematopoiesis. They have also been involved in the pathogenesis of several hematologic malignancies (10).

To ensure success in clinical trials as the MSCs field progresses, attention must be given to the optimization of the MSC culture (23). However, the percentage of MSCs in bone marrow is very low, accounting for about 0.001-0.01%, which decreases with the increase in age (24). Therefore, to isolate MSCs using the appropriate methods is the first step for future scientific research (25).

In this study, bone marrow MSCs from HAPC patients were isolated and cultured by density gradient centrifugation and the adherent screening method, providing a cell source for the MSC study of HAPC and for research on HAPC pathogenesis in the future. We successfully obtained MSCs from the bone marrow of patients with HAPC and amplified them. Compared with the bone marrow MSCs from the healthy controls, MSCs from the HAPC patients had no significant difference in the morphology and phenotype characteristics; however, they grew more actively.

Bone marrow MSCs were round when they were seeded onto the culture flasks. However, as time passed, the cells adhered to the flask wall and became fibroblast-like. They arranged into a single layer along the cell length axis and proliferated quickly. With the repeatedly passage, the cells grew in a similar morphology to their primary cells. Bone marrow MSCs showed strong proliferation ability during the passages, which is consistent with previous reports. In this study, we observed the cell counts in different passages and found that the amplification of bone marrow MSCs from HAPC patients were significantly higher than that from the healthy controls, indicating that MSCs from HAPC patients had a stronger ability for proliferation. *In vitro* cultivation of MSCs is under the condition of 20% O_2, which is significantly higher than the oxygen concentration *in vivo*. Oxygen concentration can influence the differentiation of MSCs into osteogenesis, cartilage, or fat, and the low oxygen can increase the migration mediated by the binding of the receptors and their specific ligands (26). It has been reported that moderate hypoxia is more suitable for the growth and survival of MSCs, and the colony-forming ability can also be increased significantly as well as the expression of the marker genes of the stem cells (27).

In conclusion, we successfully isolated, amplified, and cultured MSCs from the bone marrow of HAPC patients. The component of the cultured cells was simple and can be used in future experiments. We also observed the morphology, phenotype, and proliferation ability of bone marrow MSCs from HAPC patients, which may provide a foundation for further studies on the biological characteristics of MSCs, and it may supply important information for further research on HAPC.

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