Optimization of culture of mesenchymal stem cells: a comparison of conventional plate and microcarrier cultures

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Abstract

Objectives: There has been increasing interest in mesenchymal stem cells (MSCs) because of their potential use for regenerative therapy; however, there is no well-defined protocol for MSCs culture. This study compares techniques of conventional plate and microcarrier culturing of MSCs.

Methods and results: Here, different conditions for isolation and expansion of rat MSCs have been examined and it was found that plating density and plating time in primary culture played important roles for culture of these rat MSCs. When plated at $10^8/\text{cm}^2$ density for 72 h, in primary culture, recycling stem cells (RS cells) predominated, and characteristics of rat MSCs (including morphology, growth rate, phenotype and differentiation potentials) remained stable during expansion until passage 14. For subculture of the cells, it was found that their growth rate when incubated at 33 °C was higher than those incubated at 37 °C, and maximal increase was 10- and 6-fold respectively. When cultured using microcarriers, at a density of $1 \times 10^5/\text{mg}$ beads, growth kinetics, phenotype and differentiation potentials also remained constant for cells between passage 2nd and 14th; their maximal number increased 16-fold.

Conclusions: Compared to conventional plate culture, culture using gelatine porous microcarrier Cul-

tispher-S was superior for large-scale production of rat MSCs.

Introduction

In addition to haematopoietic stem cells, bone marrow contains a further type of stem cell-adherent, fibroblast-like and colony-forming, first described as mesenchymal stem cells (MSCs) by Friedenstein et al. more than 30 years ago (1). Due to their potential to differentiate into mesenchymal and non-mesenchymal lineages [into including cartilage (2), adipose tissue (2), muscle (3) and bone (4), as well as into neurones (5), endothelial cells (6) and hepatocytes (7)], they were defined to be multipotent stem cells, and play an increasingly important role in regenerative medicine. In addition, even though it has not been completely understood and there is lack of conclusive clinical evidence of it, some researchers have reported that allogeneic MSC transplantation obviated any need for immunosuppression (8,9) of a patient.

However, one of the problems limiting use of MSCs for regenerative therapies arises, as extended expansion leads to cell senescence and loss of multipotentiality. Recently, it has been reported that microcarriers in a suspension culture system, facilitate expansion of MSCs, this thus an environment is provided, that can easily be controlled and monitored (10,11). A further potential advantage of absorbable microcarriers is ability to avoid repeated trypsinization, which itself may increase apoptosis of cells.

This study described here was designed to determine optimized culture conditions of rat MSCs. First, we explored optimal conditions for conventional plate culture, including the method of isolating and expanding rat MSCs to build a plate-culture system. Secondly, we employed highly cross-linked gelatin Cultispher microcarriers that are enzymatically degradable, to investigate

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their influence on cell proliferation potential, phenotype characteristics and differentiation potential of rat MSCs.

Materials and methods

Isolation, plate culture and microcarrier culture

Eight-week-old male or female Wistar rats were bought from the Animal Centre of Chongqing University of Medical Sciences, and were used for isolation of MSCs from bone marrow. All procedures were approved by the Ethics Committee of Chongqing Medical University. Long and pelvic bones, dissected from euthanized rats, were crushed to release total bone marrow cells as described previously. Bone marrow was removed as quickly as possible, and soaked in DMEM F-12 (Gibco BRL, Gaithersburg, MD, USA), supplemented with 15% foetal bovine serum (FBS; Gibco BRL), cut into small pieces and dispersed by passage through a 16-gauge needle. Cells were then plated at $10^5/\text{cm}^2$, $10^6/\text{cm}^2$, $10^7/\text{cm}^2$ and $10^8/\text{cm}^2$ density, respectively.

Population of non-adherent cells was removed by changing medium after 24, 48, 72 and 96 h respectively. Adherent cells were then continuously cultured until 90% confluence was reached, around 1 week later. Cells were washed twice in PBS then isolated, by treating with 0.25% trypsin containing 0.01% EDTA (Sigma, St. Louis, MO, USA) for 6–7 min at 37 °C. Cells were diluted 1:2 or 1:3 at each passage and cultured on 100-mm plastic plates up to passage 14, when observations ended (some of these cells were stored at -130 °C for further study). Here, cells were incubated at 37 °C with 5% humidified CO_2 and medium was changed twice a week.

A quantity of 100 mg Cultispher-S gelatin porous microcarriers (40–77 μm in diameter, Percell Biolytica AB, Sweden, lot number 19122) was weighed, rehydrated in PBS (Ca^{2+} and Mg^{2+} free) for at least 1 h and sterilized by autoclaving, as recommended by the manufacturer (115 °C, 20 min). Then microcarriers were washed in PBS once and in culture medium twice, and soaked in culture medium overnight at 4 °C to maximize cell attachment; 1×10^5 and 1×10^7 MSCs at 2nd and 14th passages were added to 100 mg microcarrier, which was kept in 50-ml culture medium in 125-ml spinner flasks. Cultures were kept static until 24 h later, and then were agitated constantly at 107 g using an MSC stirrer (MSC-101L; Techne, Cambridge, England). Every 2 days, 50% supernatant was replaced by new medium after microcarriers were allowed to be settled down for 5–10 min. Cultures were incubated at 37 °C with 5% humidified CO_2 .

Morphology of the rat MSCs

Observed by inverted phase-contrast microscopy. Both plate-cultured cells and microcarrier-cultured cells were observed under an inverted phase-contrast microscope (type 1 X70; Olympus, Osaka, Japan) and photographs were taken with a digital camera coupled to the microscope, using HifuG5 software.

DAPI nuclear staining. Cells from primary culture and cells at 14th passage were plated in dishes (9.6 cm^2) $3 \times 10^4/\text{cm}^2$ density, and a drop of microcarrier with cells was taken and mounted on to one glass slide, every day, from the stirred culture system. Nuclei stained in 4, 6-diamidino-2-phenylindole (DAPI; Sigma), 5th day after plating or after drop of microcarrier was plated, and observed using a fluorescence microscope (Olympus). Images were taken using a digital camera matched with DP controller program.

Haematoxylin and eosin staining. Cells from primary culture and cells at 14th passage were plated on glass slides at $3 \times 10^4/\text{cm}^2$ density. They were then fixed in 4% formaldehyde when 80% confluence was reached and were stained with haematoxylin and eosin. Due to difficulty of securing them to glass slides, microcarrier-cultured cells were not stained with haematoxylin and eosin.

Electron microscopy. To study ultrastructure of the MSCs, 1×10^7 cells at passage 8th were harvested and centrifuged into small conglomerates then fixed in 3% glutaraldehyde. Samples were post-fixed in 0.5% osmium tetroxide, rinsed, dehydrated and embedded in araldite. Semi-thin sections (1.5 μm) were cut using a diamond knife and stained lightly with 1% toluidine blue. These were then embedded into araldite blocks and finally detached from slides by repeated freezing (liquid nitrogen) and thawing. Block with semi-thin section were further cut into ultra-thin (0.05 μm) sections using the diamond knife, stained with lead citrate, and examined under the transmission electron microscope (Type 600; Hitachi High-Technologies, Tokyo, Japan). Electron microscopy and image production was performed by the Electron Microscope Facility at Chongqing University of Medical Sciences.

Determination of growth kinetics of MSCs. Growth rates were tested at two different temperatures (33 and 37 °C). Cells at 2nd and 14th passages were plated in two 24-well plates at $10^5/\text{cm}^2$ density. Each day, three wells of cells were trypsinized and counted using a

haemocytometer, and numbers of cells were averaged.

For daily counting of microcarrier-cultured cells, triplicate samples (1 ml of each) of evenly mixed culture were collected from the spinner flask. Beads were rinsed three times in PBS and incubated in 1 ml trypsin-EDTA at 37 °C for 20 min, to dissolve microcarriers. Cells were then counted by using a haemocytometer, and numbers of cells were averaged each day.

To compare culture efficiency, equal quantities of cells (1×10^5) were cultured by plate and microcarriers respectively. Cells cultured in these two different ways were harvested on the 7th and 14th days, and total cell numbers were counted and averaged among three samples.

Phenotype of rat MSCs

To prepare samples for flow cytometry, plate cultured cells, 0.5×10^6 , from 2nd to 14th passages, were isolated by treatment with trypsin (0.25%) EDTA (0.01%), washed repeatedly in PBS, then re-suspended in PBS. They were then stained with fluorescein-coated antibodies (5 µl) against surface epitopes, including antibodies against CD29 (Coulter, Miami, FL, USA), CD44 (Immunotech, Marseilles, France), CD166, CD105 (Ansell, Bayport, MN, USA), SH3, SH4 (Osiris Therapeutics Inc., Baltimore, MD, USA), CD14, CD34 and CD45 (Becton Dickinson, San Jose, CA, USA). After being incubated at room temperature in the dark for 15 min, cells were washed in PBS, re-suspended in PBS, and analysed using a flow cytometer (FACs; Becton Dickinson). Cell preparation of equal quantities as above, were incubated with rabbit-anti-mouse monoclonal antibody (Becton Dickinson), and were used as negative controls to exclude possibility of non-specific fluorescence.

Stability of cell phenotype cultured with microcarriers was also tested by flow cytometry. Five millilitre microcarrier with MSCs was taken from spinner flasks and kept static for 5–10 min to remove supernatant. Then microcarriers with cells were washed twice in PBS, and dissolved using 0.3% trypsin with 0.02% EDTA (2 ml) for 10–20 min at 37 °C. Rinsing in PBS was repeated, then cells were re-suspended in PBS at 0.5×10^6 . Subsequent steps were followed as described above.

Differential potentials of the rat MSCs

To test multipotency, the rat MSCs from passage 2nd to 14th were taken, and differentiation into adipogenic, chondrogenic and osteogenic lineages was induced as described previously. Cells were tested by staining with specific agents, to indicated differentiation along the different lineages.

Adipogenic differentiation. Cells were plated at $4 \times 10^4/\text{cm}^2$ concentration and cultured in inductive or supportive medium for the adipogenic lineage. Supportive medium contained Dulbecco's modified Eagles medium-high glucose (DMEM-HG; Bio Whittaker, MD, USA), 0.05 U/ml penicillin, 0.05 µg/ml streptomycin (Invitrogen, Carlsbad, CA, USA), 10% FBS and 10 µg/ml insulin. The inductive medium consisted of components of supportive medium, with addition of 0.5 mM 1-methyl-3-isobutylxanthine, 0.2 µM indomethacin and 1.0 µM dexamethasone. Medium was replaced every 3 days for three cycles alternating between inductive and supportive medium up to the 21st day, then were stained with oil red O solution (Sigma) to certify existence of neutral lipids.

Chondrogenic differentiation. For induction of chondrogenic differentiation, 4×10^4 MSCs were cultured on micropellets in DMEM-HG with 0.05 U/ml penicillin, 0.05 µg/ml streptomycin, 1/100 insulin-transferrin-sodium selenite medium supplement (ITSS) (Sigma), 4 µg/ml linoleic acid, 1.25 mg/ml BSA, 0.17 mM ascorbate –2-phosphate, 0.1 µM dexamethasone, 1.2 mM Na pyruvate (Fluka AG, Buchs, SC, USA) and 0.3 mM proline (ICN Biomedicals, Costa Mesa, CA, USA). Chondrogenic differentiation was induced by adding 0.02 µg/ml TGF-β3 (Sigma) and medium was changed twice a week until the 21st day. Six samples were induced as above and harvested for quantitative RT-PCR of collagen I and collagen II respectively. A further sample was harvested on the 21st day and fixed in 10% formalin, embedded in paraffin wax, cut into sections and stained with alcian green for glucosaminoglycans.

Osteogenic differentiation. To induce osteogenic differentiation, MSCs were plated at $2 \times 10^4/\text{cm}^2$ density and cultured in DMEM-low glucose (LG) medium, with addition of 0.05 U/ml penicillin, 0.05 µg/ml streptomycin, 10% FBS, 0.05 mM ascorbic acid, 10 mM glycerophosphate and 0.2 µM dexamethasone. Medium was replaced twice a week until the 21st day. Six samples were induced and harvested for quantitative RT-PCR of osteocalcin and osteopontin respectively. A further sample was harvested by cytospinning, fixed in 10% formalin, embedded in paraffin wax, sectioned and stained for presence of minerals using the von Kossa method.

Quantitative RT-PCR was performed to certify chondrogenic and osteogenic differentiation of the MSCs on the 21st day after being induced. Total RNA was extracted from cell micropellets using RNeasy mini kit (Qiagen, Dusseldorf, Nordrhein-Westfalen, Germany), and from cells in monolayers using Trizol total RNA isolation kit (Invitrogen). Quantitative RT-

PCR was performed using QuantiTect SYBR Green RT-PCR kit (Qiagen, Dusseldorf, Nordrhein-Westfalen, Germany) and the Cepheid 1.2f Detection System. RT-PCR was performed with 25 µl total reaction volume, including 12.5 µl 2X QuantiTect SYBR Green RT-PCR master mix, 3 µl cDNA template and 0.2 µM each of target-specific primers designed to amplify the appropriate part of each gene. To quantify each target transcript, a standard curve was constructed using serial dilutions of standard plasmid (Invitrogen) with β -actin as reference housekeeping gene.

PCR reaction conditions for collagen I, collagen II, osteocalcin, osteopontin were performed as follows: initial cycle 94 °C, 5 min, followed by 35 cycles denaturation at 94 °C, 1 min, annealing at 60 °C, 1 min for collagen I and collagen II, and 56 °C, 1 min for osteocalcin and osteopontin, then a final cycle at 72 °C for 7 min, to allow completion of product synthesis. As negative control, containing all reaction components except for the template, was included in all experiments. Values of each gene (collagen I, collagen II, osteocalcin and osteopontin) were then normalized to β -actin.

Optimal oligonucleotide primers for real-time RT-PCR amplification of reverse-transcribed cDNA were selected with the aid of software program Oligo 4.0 (National Bioscience Inc., Plymouth, MN, USA). The following rat oligonucleotide primers were used:

ples of each gene were detected, and each sample was assessed at least twice for each gene of interest. All were analysed densitometrically, and expression of mRNA was conducted, after normalization for level of β -actin mRNA per sample. Data are expressed as mean $\pm$ SEM and mean values were compared using Student's *t*-test. *P*-value of <0.05 was considered statistically significant.

Results

Optimized conditions for primary culture of the rat MSCs

The variety of plating density and plating time was tested, and it was found that the two factors played important roles for establishment of rat MSC cultures. At $10^5/\text{cm}^2$ and $10^6/\text{cm}^2$ density, few cells attached to the plastic walls. At $10^7/\text{cm}^2$ density, cells attached walls and survived, but it was hard to become confluent. However, when plating at $10^8/\text{cm}^2$ density, specially when plating for 72 h, large numbers of cells adhered to the plastic surfaces; these presented as a population of small round cells. They grew rapidly and reached 90% confluence in roughly 1 week. All cells studied were taken from these.

Locus	Sense (5'–3')	Antisense (5'–3')
β -actin	CTATGACTT AGTTGCGTTACAC	GCATTACATAATTTACACGAAAGC
Collagen I	CGGGATCCCCGAGCAGACGGGAGTTTCACC	TCCCCCGGGGGAGAACTTACTGTCTTCTTGG
Collagen II	GCTCGCACCTGCAGAGACCTG	GTCCACACCGAATTCCTGCTCG
Osteocalcin	AAGTCCACACAGCAGCTTG	AGCCGAGCTGCCAGAGTTTG
Osteopontin	CCGATGAGGCTATCAAGGTC	ACTGCTCCAGGCTGTGTGTT

Statistical analysis

Unless otherwise stated, all experiments were performed at least three times. For quantitative RT-PCR, three sam-

Morphological characteristics of rat MSCs

Under the appropriate conditions described above, the primary culture was predominately of small, round and

Figure 1. RS cells in primary culture. (a) Primary culture of rat MSCs was dominated by RS cells, which were small, round and yellowish, showing no propensity for colony formation inverted contrast microscope, $\times 10$. (b) RS cells with multinuclei in different numbers and different shapes (H&E staining, $\times 40$).

Figure 2. Morphology of rat MSCs. (a) Subcultured rat MSCs grew to form colonies (inverted contrast microscopy, $\times 10$). (b) Subcultured rat MSCs were fibroblast-like, with a single oval nucleus and scattered granules within the cytoplasm (inverted contrast microscopy, $\times 20$). (c) Ultrastructure of rat MSCs showed that heterochromatin was mainly localized beneath nuclear membranes, and cytoplasm was rich in endoplasmic reticulum and Golgi apparatus (transmission electron microscopy, $\times 8000$).

Figure 3. Growth kinetics of rat MSCs. (a) Growth rate of cells incubated at 33 °C was higher than of cells incubated at 37 °C. (b) When cultured with microcarriers, a lag period was followed by exponential cell growth from day 7 to day 28 at $1 \times 10^5/\text{mg}$ density of beads, and few cells survived at density of $1 \times 10^3/\text{mg}$ beads. In images a–e, growth kinetics of microcarrier-cultured rat MSCs at higher density are shown.

yellowish cells when observed by inverted contrast microscopy, with no formation of colonies (Fig. 1a). This subpopulation was first observed on culture of human MSCs and cells were termed ‘recycling stem cells’ (RS cells). (12,13). RS cells with multinuclei in different numbers and in different shapes were observed after H&E staining (Fig. 1b).

In contrast to primary culture of rat MSCs, passaged rat MSCs grew gradually to form colonies (Fig. 2a). They remained morphologically homogeneous until the end of our observations (14th passage); they were characteristically spindle-like and fibroblastic in shape, with a solitary oval nucleus and scattered granules within the cytoplasm (Fig. 2b). Electron microscopy indicated that heterochromatin was mainly localized near nuclear membranes of the oval nucleus; Golgi apparatus and endoplasmic reticulum enriched the cytoplasm (Fig. 2c).

MSC growth kinetics

During expansion of plate-cultured MSCs, growth rate remained stable from passage 2nd to 14th. Cells were passaged roughly 7 days after being plated, when 80% confluence was reached. Comparison of population growth kinetics at different temperatures indicated that kinetics of cells incubated at 33 °C were of higher degree of those incubated at 37 °C. Plated at the same density, maximum numbers of cells increased in the region of 10 times at 33 °C, whereas increase was only around six times at 37 °C (Fig. 3a).

When cultured on microcarriers at density of $1 \times 10^5/\text{mg}$ per bead, growth kinetics also remained constant for cells between passages 2 and 14. A lag period of around 6 days was observed. This was followed by exponential cell growth from day 7 to day 28, of which maximal number increases was by 16 times. Few cells survived when cultured on microcarriers at $1 \times 10^3/\text{mg}$ density per bead (Fig. 3b).

Figure 4. Immunophenotypic profile of rat MSCs. Cultured rat MSCs were negative for CD14, CD34 and CD45, and positive for CD29, CD105, CD44, CD166, SH3 and SH4.

Phenotype of rat MSCs

Results of flow cytometry indicated that plate-cultured MSCs from passages 2 to 14, as well as microcarrier-cultured MSCs of passage 14, consisted of a phenotypically homogeneous cell population, which were negative for CD14, CD34 and CD45, while being

positive for CD29, CD44, CD166, CD105, SH3 and SH4 (Fig. 4). By use of specific surface antigens as previously reported for rat MSCs, it was certified that immunophenotypic profile of the cultured cells was similar to previously described MSCs, and remained stable when passaged on or when cultured on microcarriers.

Figure 5. Differentiation potentials of rat MSCs. Rat MSCs (a) were successfully induced into adipogenic (b), chondrogenic (c) and osteogenic lineages (d).

In vitro differentiation potentials of rat MSCs

The cultured rat MSCs (Fig. 5a) were successfully induced along adipogenic, chondrogenic and osteogenic lineages as described previously. When cultured in adipogenic induction medium, cells underwent morphological changes and derived vacuoles containing lipid (Fig. 5b). For cells cultured in chondrogenic induction medium, formation of glucosaminoglycans was confirmed by staining with alcian green (Fig. 5c). After being treated with osteogenic induction medium, mineralization was detected by staining using the von Kossa method (Fig. 5d). In addition, for cells detached from microcarriers, differentiation along adipogenic, chondrogenic and osteogenic lineages was successfully induced and confirmed.

Quantitative RT-PCR indicated that there was no expression of the above markers by untreated MSCs and remarkably elevated expression of collagen I and collagen II after being cultured in chondrogenic induction medium (Fig. 6a,b) and elevated expression of osteocalcin and osteopontin after culture in osteogenic induction

Figure 6. Differentiation potentials indicated by quantitative RT-PCR.

medium (Fig. 6c,d). Expression levels were similar between cells from different culture conditions, including cells from 2nd and 14th passages, which cultured both by plate and microcarrier.

Discussion

In this study, we report a new microcarrier-based way to optimize culture of MSCs. We found that the cells would grow normally when seeding at or higher than the density of $10^8/\text{cm}^2$. It has been reported that a hypoxic environment promotes expansion of stem cells through activation of hypoxia-inducible factors (14,15). Therefore, low oxygen could be involved through activation of hypoxia-inducible factors and expression pattern of these factors should be investigated further from our study. Different plating times were tested, including 24, 48, 72 and 96 h and we found that plating for 72 h was optimal for expansion of our MSCs.

By optimized culture conditions as described above, including method isolating MSCs, as well as plating density and plating time, cells with high homogeneity in morphology, phenotype and differentiation potentials were obtained. From passage 2 to passage 14, the cells revealed a fibroblast-like appearance and were characterized by an oval eccentric nucleus and scattered granules within the cytoplasm. Phenotypic characteristics of the cells were consistently positive for CD29, CD105, CD44, CD166, SH3 and SH4, and negative for haematopoietic proteins, CD14, CD34 and CD45. Multiple differentiation potentials were confirmed by positive staining of neural lipids, glucosaminoglycans and mineral after being induced along adipogenic, chondrogenic and osteogenic lineages. Chondrogenic and osteogenic differentiation was reconfirmed by markedly elevated expression of collagen I/collagen II and osteocalcin/osteopontin, tested by quantitative RT-PCR. All these data indicate that rat MSCs prepared by conventional procedures maintain all characteristics of stem cells and are capable of differentiating into different types of cells.

Cells cultured on plates exhibited high growth rates and phenotype remained stable during expansion. From passage 2 to passage 14, 80–90% confluence was usually reached in around 1 week after plating. We compared growth kinetics of passages 2 and 14 and found that growth kinetics of cells of passage 14 were almost the same as those of passage 2, which indicated that the cells did not lose their proliferation potential during expansion. Phenotypic characteristics of the cultured rat MSCs were also preserved during expansion. It has previously been reported that MSCs lose their adipogenic differential potential at passage 5 and lose chondrogenic

and osteogenic potentials at passage 8 (11). In this experiment, however, potentials of differentiation along adipogenic, chondrogenic and osteogenic lineages were not lost, at least up to passage 14.

We employed highly cross-linked gelatin CultiSpher microcarriers that are enzymatically degradable, to investigate their influence on proliferation potential, phenotypic characteristics and differentiation potential of rat MSCs. Results showed that growth kinetics of in microcarrier-culture system differed from those of the plate-cultured system. For conventional plate-culture, growth rates of cells varied at different temperatures, among which maximal increase was 10 times higher at 33 °C and 6 times at 37 °C. For plate-cultured systems at both temperatures, expansion of the cells started with a 1-day lag phase then entered exponential growth phase. For microcarrier culture system, the lag phase lasted for around a week and numbers of cells maximally increased by 16 times. Different seeding densities were tested and it was found that higher density ($1 \times 10^5/\text{mg}$ gelatin beads) was better than lower cell density ($1 \times 10^3/\text{mg}$ gelatin beads).

One key requirement for the culture system used to expand progenitor cells for subsequent transplantation, is that it should cause them to maintain their stem-cell characteristics and ability to differentiate into multiple lineages. Recent studies have indicated the preserved multipotency of rat MSCs when cultured with Cultispher-S microcarriers (11,16). Our results confirm their maintenance of multipotency into adipogenic, chondrogenic and osteogenic lineages, as well as phenotypic characteristics of rat MSCs, when cultured on microcarriers.

Conclusion

Our results have identified that plating density and time in primary culture played an important role in culture of rat MSCs, and the ideal condition is to plate at $10^8/\text{cm}^2$ for 72 h density. MSCs prepared by the conventional method maintained full potential to differentiate to all the types of cell. Thus, using Cultispher-S gelatin microcarriers was a promising culture system for rat MSCs.

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