Noninvasive estimation of cell cycle phase and proliferation rate of human mesenchymal stem cells by phase-shifting laser microscopy

Ayako Tokumitsu · Shigeyuki Wakitani ·
Mutsumi Takagi

Received: 12 May 2009 / Accepted: 24 June 2009 / Published online: 20 August 2009
© Springer Science+Business Media B.V. 2009

Abstract The simultaneous determination of the cell cycle phase of individual adherent mesenchymal stem cells (MSCs) using a fluorescence microscope after staining with 4',6-diamidine-2'-phenylindole dihydrochloride and bromodeoxyuridine and the laser phase shift by phase-shifting laser microscopy (PLM) revealed that the laser phase shift of cells in the G₂/M phase was markedly higher than that of cells in the G₀/G₁ phase. Even in the synchronous cultures to G₀/G₁ and G₂/M cell cycle phases, the laser phase shift of the cells in the G₂/M phase was markedly higher than that of the cells in the G₀/G₁ phase. The analysis of the cultures of MSCs from different donors with the addition of FGF2 at different concentrations revealed that there was a marked negative correlation between the average phase shift and mean generation time. In conclusion, it is possible to estimate noninvasively the proliferation activity of

MSCs population by measuring the phase shift using PLM.

Keywords Mesenchymal stem cell ·
Noninvasive · Cell cycle

Introduction

The quality of cells should be evaluated before transplantation from the viewpoints of quality and process controls because the populations of cells cultivated for transplantation in regenerative medicine are generally heterogeneous. Most transplantations require an autologous cell system and a small number of cells were cultivated in the autologous cell system. Thus, cells could not be discarded for quality evaluations, and the latter should be performed noninvasively. The important parameters concerning cell quality include growth activity and differentiation degree. We previously reported that the cells expressing a high level of aggrecan mRNA in the culture in which the differentiation of MSCs to chondrocytes occurred may be characterized on the basis of morphological parameters such as polygonal index and cell adhesion area (Takagi et al. 2008).

If the percentage of cells in the G₂/M cell cycle phase (mitotic period) could be determined by microscopy, the growth activity of the cell population could be estimated noninvasively. DNA condensation occurs and the cell morphology is round during the M

A. Tokumitsu · M. Takagi (✉)
Division of Biotechnology and Macromolecular
Chemistry, Graduate School of Engineering,
Hokkaido University, N13W8 Kita-ku,
Sapporo 060-8628, Japan
e-mail: takagi-m@eng.hokudai.ac.jp

S. Wakitani
Orthopedic Surgery, Osaka City University School
of Medicine, 1-4-3 Asahi-machi, Abeno-ku,
Osaka 545-8585, Japan

phase (Sanger and Sanger 1980). This suggested that the refractive index and height of the cells during the G₂/M phase were large. An atomic force microscope (AFM) can be used to observe the three-dimensional (3-D) morphology of adherent animal cells. However, AFM observation might be considered invasive for cells, because the 3-D observation of adherent animal cells using AFM requires a long time and the cells have to be fixed.

The refractive index of cell might be different from that of culture supernatant. So, there might be the phase difference between laser lights which passed through only the supernatant and cell, when laser light passed from the bottom to the top of culture. The phase difference (the laser phase shift) is a product of cell height and cell refractive index. The laser phase shift of adherent animal cells could be noninvasively determined using PLM (Endo et al. 2002; Takagi et al. 2007). The simultaneous determination of the cell cycle phase of individual adherent Chinese hamster ovary cells using a fluorescence microscope after staining with 4',6-diamidino-2'-phenylindole dihydrochloride (DAPI) and bromodeoxyuridine (BrdU) and the laser phase shift by phase-shifting laser microscopy revealed that the laser phase shift of the cells in the G₂/M phase was markedly higher than that of the cells in the G₀/G₁ and S phases (Ito and Takagi 2008).

MSCs in adult bone marrow have been shown to give rise to multiple mesodermal tissue types, including bone, cartilage, tendon, muscle, fat, and marrow stromal cells. MSCs are isolated from bone marrow aspirates and, because of their multilineage potential, present exciting opportunities for cell-based therapeutic applications. The morphology of MSCs is markedly different from that of CHO cells, i.e., fibroblast-like vs. epithelial-like. Moreover, the average doubling time of MSCs (approximately 50–130 h) is very long compared with that of CHO cells (approximately 30 h). Thus, it might be difficult to estimate the cell cycle phase of MSCs using PLM. Furthermore, it is not yet shown if the growth activity could be estimated through the cell cycle phase determined by PLM even for CHO cells.

In this study, the noninvasive estimation of the cell cycle phase and proliferation rate of human MSCs by using phase-shifting laser microscopy was investigated.

Materials and method

Cells

MSCs were isolated from bone marrow aspirate obtained by routine iliac crest aspiration from human donors (donor A, 30-year-old male; donor B, 19-year-old male; donor C, 67-year-old male), as previously reported (Takagi et al. 2003). All the subjects enrolled in this study, from whom primary human mesenchymal stem cells were isolated, gave their informed consent. This study was approved by our institutional committee on human research, as required by the study protocol. Briefly, bone marrow cells were plated on a dish (55 cm²; Corning, Tokyo, Japan) at a concentration of 6.0×10^5 nucleated cells/cm² using Dulbecco's modified Eagle's medium-low glucose (DMEM-LG; Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS; lot 1211852; Invitrogen), 2,500 U/L penicillin, and 2.5 mg/L streptomycin, and cultured at 37 °C in a humidified atmosphere containing 5% CO₂, during which the medium was changed on days 1, 2, 9 and 16. On day 19, the cells were detached using trypsin-EDTA (Sigma-Aldrich, St Louis, MO, USA), counted by the trypan blue dye-exclusion method, and subcultivated at an initial cell density of 1×10^4 cells/cm² (Takagi et al. 2003).

Medium

The growth medium was DMEM-LG (Gibco, NY, USA) supplemented with 10% (v/v) FBS (Gibco), 2,500 penicillin U/L, and 2.5 mg streptomycin/L. Human basic fibroblast growth factor (FGF2; Peprotech, London, England) was added for the cultivations shown in Fig. 4.

Cultivation

Human mesenchymal stem cells (hMSCs) were plated on a dish with grids (11.76 cm²; SARSTEDT, Nümbrecht, Germany) at a concentration of 0.15×10^4 cells/cm² using the growth medium and incubated at 37 °C under 5% CO₂ atmosphere. Cell density was determined by trypan blue staining after trypsinization.

Synchronous culture

hMSCs were cultured in the growth medium in a humidified incubator at 37 °C with 5% CO₂ until they were approximately 80% confluent. After the spent medium containing detached cells was removed and replaced with 1% FBS-supplemented growth medium, the cells were incubated for 24 h at 37 °C for synchronization to G₀/G₁ phase. Thereafter, the medium was replaced with 10% FBS-supplemented growth medium containing 0.2 mM hydroxyurea (Sigma–Aldrich) and incubated for 16 h. The cells were washed three times with serum-free medium and incubated for 12 h in 10% FBS-supplemented growth medium for synchronization to S phase. The old medium was removed and replaced with 10% FBS-supplemented growth medium containing 100 µg/mL nocodazole (Sigma–Aldrich) and the cells were incubated for approximately 24 h for synchronization to G₂/M phase. After the observation of round cells, the dishes were gently shaken and the cells at M phase were collected in the old medium (Lin and Arthur 2007).

Cell cycle analysis by flow cytometry

Cells (approximately 4×10^6 cells) harvested by trypsinization were stained with propidium iodide (PI) solution (1 mL, 0.25 mg/mL-phosphate buffered saline (PBS), Beckman-Coulter, Miami, USA) at 4 °C for 30 min and the cell cycle was analyzed using a flow cytometer (Martens et al. 1993).

Cell cycle analysis under fluorescence microscopy

A square on the bottom of the culture dish was marked and the presence of cells in this square was observed using an inverted phase-contrast microscope (CK-2, Olympus, Tokyo). The cells were incubated in a medium containing BrdU (0.01 mM, Roche, Basel, Switzerland) for 30 min at 37 °C under 5% CO₂ atmosphere and washed with PBS for 5 min. The cells were fixed with paraformaldehyde (4%/PBS) at room temperature for 10 min. The cells in the square were then observed under PLM and an inverted phase-contrast microscope. The cells were incubated with Triton X-100 (0.2%/PBS) at room temperature for 5 min and washed with PBS for

5 min. The cells were incubated with bovine serum albumin (BSA, 1%/PBS) for 30 min and washed with PBS for 5 min. The cells were incubated with HCl (4 M) for 10 min and washed three times with PBS for 5 min. The cells were incubated with mouse anti-BrdU monoclonal IgG antibody (DAKO, Glostrup, Denmark) for 1 h and washed three times with PBS for 5 min. The cells were incubated with rabbit anti-mouse IgG-tetramethylrhodamine isomer R (TRITC) polyclonal antibody (DAKO) for 30 min in the dark and washed three times with PBS for 5 min and once with pure water for 1 min. The cells were incubated with DAPI (1 mg/mL, Roche) for 5 min in the dark and washed with pure water. The cells in the square were observed using a fluorescence microscope (IX70, Olympus, Tokyo, Japan) under a constant exposure condition. The cell fluorescence intensities for BrdU and DAPI were calculated using Photoshop (Adobe, San Jose, CA). The cell cycle phase of each cell was analyzed using the two-dimensional plot of fluorescence intensities of BrdU and DAPI (Fig. 1; David et al. 2002).

Cell morphology analysis under PLM

The cells attached to the bottom surface of the culture dish with grids were observed using PLM (FK Optical Laboratory, Saitama, Japan). Two neighboring fields of view with and without cells were selected as sample and reference fields, respectively, and the laser phase shift ($\Delta\phi$) was determined for all the pixels in the sample field under PLM.

Statistical analysis

Statistical analysis was performed using *t*-test, and the significance level was set at 0.01 or 0.05.

Results

Estimation of cell cycle phase of MSCs by staining

MSCs (donor B) were cultivated in the growth medium without FGF2 for 72 h without synchronization. After the laser phase shifts of the cells were analyzed using PLM, their cell cycle was analyzed using a fluorescence microscope after staining

Fig. 1 Single-cell analysis of cell cycle phase under microscope. The cell cycle phase of adherent MSCs stained with anti-BrdU antibody and DAPI was determined using a fluorescence microscopy image (**a** bright field; **b** BrdU; **c** DAPI). Scale bar indicates 200 μm. The numbers of cells

having fluorescence intensities in the respective range (**d** BrdU; **e** DAPI) are shown (e.g., 13.1–17.0; $13.1 \leq \text{DAPI}$ and $\text{DAPI} \leq 17.0$). By employing the threshold of fluorescence intensities for BrdU (8) and DAPI (45), the 151 cells were classified into each cell cycle phase (**f**)

with BrdU and DAPI and by flow cytometry. The percentage of cells in each cell cycle phase estimated by fluorescence staining was compared with that obtained by conventional flow cytometry. The numbers of cells having fluorescence intensities in the respective ranges are shown (e.g., 13.1–17.0; $13.1 \leq \text{DAPI}$ and $\text{DAPI} \leq 17.0$) in Fig. 1d and e, and the distributions of fluorescence intensities were analyzed. In the histograms of BrdU and DAPI fluorescence intensities (Fig. 1d, e), the number of cells belonging to the ranges higher than 8.1 for BrdU fluorescence intensity was apparently lower than those belonging to the lower ranges (2.1–8.0; Fig. 1d). More cells belonged to the ranges lower than 45 for DAPI fluorescence intensity than to the other ranges of higher intensity (45.1–73.0; Fig. 1e). Thus, the fluorescence intensity upper thresholds for BrdU and DAPI were determined to be 8 and 45, respectively. The percentage of G₀/G₁ cells was generally higher

than those of S and G₂/M cells and this might be the reason behind those distributions.

Then, the cell cycles were determined. The percentages of cells in each cell cycle phase (G₀/G₁, 66.0%; S, 23.3%; G₂/M, 10.7%) were determined using these thresholds and compared with those determined by flow cytometry (G₀/G₁, 74.5%; S, 16.1%; G₂/M, 9.4%). There was no marked difference in the percentages determined using a fluorescence microscope and a flow cytometer. Thus, the cell cycle phase determined using a fluorescence microscope was considered accurate.

Relationships between the phase shift of MSCs determined by PLM and the cell cycle

To investigate the relationships between the phase shift of the cells and the cell cycle, MSCs (donor B) were cultured in the growth medium without FGF2

Fig. 2 Effect of cell cycle on laser phase shift. By employing the threshold values for fluorescence intensities and classifying 72 cells into their respective cell cycle phase, the laser phase shift ($\Delta\phi$) in each cell cycle phase was determined (average $\pm$ SD, * $p < 0.01$, ** $p < 0.05$)

for 72 h and observed under a fluorescence microscope after treatment with BrdU and DAPI. Then, the laser phase shifts of these cells were analyzed using PLM ($n = 72$). The cell cycle phases (G_0/G_1 , 66.6%; S, 16.7%; G_2/M , 16.7%) were determined using the thresholds, which were determined in the same manner as that in Fig. 1. The laser phase shifts of G_2/M and S cells were markedly ($p < 0.01$) larger than that of G_0/G_1 cells (Fig. 2).

Relationships between the phase shift and the cell cycle of synchronized MSCs

After the percentage of cells in the G_2/M phase was increased by synchronization, the relationship between the phase shift and the cell cycle of MSCs was confirmed. MSCs synchronized in G_0/G_1 phase, which arise in the course of G_2/M synchronization, were also examined. Besides these synchronized cells, asynchronous cells were stained simultaneously and used for the determination of the thresholds of fluorescence intensity. The percentages of cells in G_0/G_1 , S and G_2/M cell cycle phases after synchronization to G_0/G_1 phase were 90.0% (54 cells), 0% (0 cells) and 10.0% (6 cells), respectively (Fig. 3a), and their phase shift was analyzed (Fig. 3c). The percentages of cells in G_0/G_1 , S and G_2/M cell cycle phases after synchronization to G_2/M phase were 37.5% (24 cells), 26.6% (17 cells) and 35.9% (23

cells), respectively (Fig. 3b), and their phase shift was analyzed (Fig. 3d). These results showed that the phase shift of G_2/M cells was markedly larger than that of G_0/G_1 cells in the synchronous culture to G_0/G_1 . The phase shift of G_2/M cells was also markedly larger than that of G_0/G_1 cells in the synchronous culture to G_2/M .

Relationship between the average phase shift and proliferation rate

To examine the relationship between the average phase shift and proliferation rate, MSCs (from donors A, B and C) were cultured simultaneously in the growth medium without FGF2, and the cell density was measured at 0, 36 and 83 h. Moreover, MSCs (from donor B) were cultured simultaneously in the growth medium with FGF2 at various concentrations (0, 2 and 20 ng/mL), and the cell density was measured at 0, 29 and 65 h. Mean generation times were calculated using these cell densities. The phase shifts of approximately 80 cells were determined at 36 and 29 h in these cultures. The mean generation times of MSCs obtained from donors A, B and C were 55, 107 and 65 h, respectively. The mean generation times of MSCs obtained from donor B in the presence of FGF2 at 0, 2 and 20 ng/mL were 130, 69 and 55 h, respectively. A negative correlation was observed between the average phase shift and mean generation times (Fig. 4), and the correlation coefficient was 0.71.

Discussion

In asynchronous culture (Fig. 2), the phase shift of G_2/M cells was markedly larger than that of G_0/G_1 cells, while the number of investigated cells in the G_2/M phase (12 cells) was less than that of the G_0/G_1 phase (48 cells). Thus, to obtain more reliable data, the percentage of cells in the G_2/M phase was increased by synchronization to the G_2/M phase, and it was also shown that the phase shift of G_2/M cells was markedly larger than that of G_0/G_1 cells. The reason for the larger phase shift of MSCs in G_2/M phase may be DNA aggregation and the large height of the cells.

While the phase shifts of cells in the G_0/G_1 phase were almost constant [2.70 (Fig. 2), 2.63 (Fig. 3c) and

Fig. 3 Analysis of cell cycle phase and laser phase shift after synchronization to G_0/G_1 and G_2/M . After synchronizations to G_0/G_1 (a, c) and G_2/M (b, d), the 60 and 64 cells were classified into each cell cycle phase by employing the threshold of fluorescence intensities for BrdU (13 in a, 11 in b) and DAPI (29 in a, 33 in b). The laser phase shift ($\Delta\phi$) in each cell cycle phase was determined (c, d). (average $\pm$ SD, * $p < 0.01$, ** $p < 0.05$)

Fig. 4 Relationship between mean generation time and the average phase shift. Mean generation times of MSCs from donor B cultivated in the presence of FGF2 (open circles) and MSCs from donors A, B and C (closed circles) were plotted against their average phase shift ($\Delta\phi$). The correlation coefficient was 0.71

2.90 (Fig. 3d)], there was fluctuation in the phase shift of the G_2/M cells [3.69 (Fig. 2), 4.43 (Fig. 3c) and 5.64 (Fig. 3d)]. The addition of nocodazole might inhibit cell division in synchronous culture to G_2/M phase (Fig. 3d) and increase the percentage of cells with aggregated DNA. This may be the reason for high phase shift of the G_2/M cells in Fig. 3d. Nevertheless, the cells in the G_2/M phase may be distinguished by PLM. On the other hand, the phase shift of the G_2/M cells was markedly larger than that of the G_0/G_1 cells even in the culture synchronized to G_0/G_1 , in which the percentage of cells in the G_2/M phase was very low (10%). This suggested that the sensitivity of the method to distinguish G_2/M cells was not low.

The phase shift of G_2/M MSCs was markedly larger than that of G_0/G_1 MSCs. It agreed with our previous result for CHO cells (Ito and Takagi 2008). However, the average phase differences for G_2/M and G_0/G_1 CHO cells were 2.0 and 3.1, respectively,

which were apparently lower than those for MSCs as mentioned above. The reason for this difference is not clear.

The proliferation rate of MSCs from donor B was the lowest among the MSCs from the three donors; donor B was the youngest among the three donors. The reason for this is unclear. The proliferation rate increased with increasing FGF2 concentration. This result agreed with our previous result (Takagi et al. 2003). In the cell population growing actively, the percentage of cells in the G₂/M phase may be large. Our results showing that the phase shift of the G₂/M cells was higher than that of the cells in other phases suggested that the average phase shift of the actively growing cell population with a high percentage of G₂/M cells may be larger. This agreed with our result, that is, the higher average phase shift was observed in the faster growing cell population as shown in Fig. 4.

The average phase shifts of cells in the G₀/G₁ and G₂/M phases in Figs. 2 and 3 were 2.74 and 4.59, respectively. Consequently, if the percentage of G₂/M cells increases from 16.7% (asynchronous culture, Fig. 1) to 35.9% (synchronous culture to G₂/M, Fig. 3b), the average phase shift may increase from 2.5 to 3.1. Thus, the increase in the proliferation rate could cause the increase in the average phase shift from 2.5 to 3.1 as shown in Fig. 4. In conclusion, it might be possible to estimate noninvasively the proliferation activity of an MSC population by measuring the phase shift using PLM.

References

- David AB, Anthony SA, Tiffany B, Jennifer LM, Heidi R, Linette O, Anna AD, Mary CH (2002) Cyclin G₂ associates with protein phosphatase 2A catalytic and regulatory B' subunits in active complexes and induces nuclear aberrations and a G₁/S phase cell cycle arrest. *J Biol Chem* 277:27449–27467
- Endo J, Chen J, Kobayashi D, Wada Y, Fujita H (2002) Transmission laser microscope using the phase-shifting technique and its application to measurement of optical waveguides. *Appl Opt* 41:1308–1314
- Ito S, Takagi M (2008) Correlation between cell cycle phase of adherent Chinese hamster ovary cells and laser phase shift determined by phase-shifting laser microscopy. *Biotechnol Lett* 31:39–42
- Lin W, Arthur G (2007) Phospholipids are synthesized in the G₂/M phase of the cell cycle. *Int J Biochem Cell Biol* 39:597–605
- Martens DE, Gooijer CD, Velden CA, Beuvery EC, Tramper J (1993) Effect of dilution rate on growth, productivity, cell cycle and size, and shear sensitivity of a hybridoma cell in a continuous culture. *Biotechnol Bioeng* 41:429–439
- Sanger JW, Sanger JM (1980) Surface and shape during cell division. *Cell Tissue Res* 209:177–186
- Takagi M, Nakamura T, Matsuda C, Hattori T, Wakitani S, Yoshida T (2003) In vitro proliferation of human bone marrow mesenchymal stem cells employing donor serum and basic fibroblast growth factor. *Cytotechnology* 43: 89–96
- Takagi M, Kitabayashi T, Ito S, Fujiwara M, Tokuda A (2007) Noninvasive measurement of three-dimensional morphology of adhered animal cells employing phase-shifting laser microscope. *J Biomed Opt* 12:054010
- Takagi M, Kitabayashi T, Koizumi S, Hirose H, Kondo S, Fujiwara M, Ueno K, Misawa H, Hosokawa Y, Masuhara H, Wakitani S (2008) Correlation between cell morphology and aggrecan gene expression level during differentiation from mesenchymal stem cells to chondrocytes. *Biotechnol Lett* 30:1189–1195