Effect of the distance between adherent mesenchymal stem cell and the focus of irradiation of femtosecond laser on cell replication capacity

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Abstract The effect of femtosecond laser irradiation on adherent mesenchymal stem cell was investigated with the aim to develop a novel noninvasive cell purification system. A single mesenchymal stem cell was irradiated with a femtosecond laser on the center of the cell using several energy values and the cell lost its replication capacity with one time irradiation with an energy of 2.0 μJ . Besides at a neighbor point in the major axis, when irradiated in the minor axis at a distance shorter than 10 μm , the cell stopped its replication capacity. The accumulation effect of cell damage caused by multiple laser shots at a neighbor point in the minor axis was correlated with the critical

distance at which the cell lost its replication capacity. Finally, a novel equation of laser cell damage as a function of laser pulse energy and laser shot number is proposed.

Keywords Mesenchymal stem cell · Irradiation · Femtosecond laser · Noninvasive · Purification · Regenerative medicine

Introduction

Human mesenchymal stem cells (hMSCs) offer the possibility of obtaining many kinds of tissue such as bone, tendon, and cartilage and can be isolated from adult bone marrow by aspiration (Darwin 1997). However, heterogeneity has been reported during the isolation, proliferation and differentiation (Yokoyama et al. 2008). The quality of the cells, in terms of heterogeneity in the culture, should be estimated before transplantation and it should be performed noninvasively.

There have been reported methods that include cell morphological analysis in two and three dimensions for the estimation of cell quality, in terms of heterogeneity. Two-dimensional analysis employed a conventional inverted microscope for determining whether the differentiation of hMSCs into chondrocytes had occurred (Takagi et al. 2008). Three-dimensional analysis employed a phase-shifting laser

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microscope (PLM) to estimate the cell cycle, mean generation time and tumorigenesis (Ito and Takagi 2009; Tokumitsu et al. 2009, 2010).

A heterogeneous cell population should be purified into a homogeneous cell population from the viewpoint of quality control for cell medicine. This issue might be especially critical when the heterogeneous cell population contains malignant cancer cells. The cell purification should be performed noninvasively (Uchugonova et al. 2008).

The conventional methods of cell purification, including flow cytometry and magnetic separation, involve extremely invasive procedures such as detachment of adhesive cells into suspension and addition of antibodies. Although the conventional method requires a specific antibody conjugated with a fluorescence or magnetic particle, there might not be a 100% specific antigen. Moreover, the conventional method can be performed only once during the long-time cell processing because cells need to be in suspension and stained (Lindstrom and Andersson-Svahn 2010). Consequently, it is virtually impossible to obtain 100% purity by the conventional invasive method.

When a femtosecond laser with a low intensity (i.e., 1 nJ) and high repetition rate (i.e., 80 MHz) is directly focused on a cell through an objective lens, the cell could be knocked out by photo thermal and photochemical interactions (Uchugonova et al. 2008). Alternatively, when a femtosecond laser with a high intensity (i.e., 1 μ J) and a low repetition rate (i.e., 1 kHz) is focused into a cell culture medium through an objective lens, a stress wave due to a shockwave and a cavitation bubble are generated, propagating a wave packet. The impulsive force due to the stress wave is localized in the vicinity of the laser focal point (a few tens of μ m) and has been used to detach a single animal cell from a cell culture (Takagi et al. 2008). The impulsive force is considered to knock out the cell, not only at the laser focal point but also in the vicinity of the laser focal point by mechanical interactions.

Each unnecessary cell needs to be respectively irradiated and many irradiation procedures might be required to remove many unnecessary cells, while the conventional method can remove many unnecessary cells at the same time. So, the novel noninvasive cell purification system using a femtosecond-laser-induced impulsive force in this study might be appropriate to

perform with a small number of cells in an early stage of cultivation and to remove a small number of cancer cells appearing during cultivation.

We aimed to propose a novel noninvasive cell purification system using a femtosecond-laser-induced impulsive force, in which the laser is focused on or in the vicinity of an unnecessary cell such as a cancer cell. To establish this system, irradiation conditions such as the intensity of irradiation for the killing of an unnecessary cell should be determined. Moreover, the minimum distance between an unnecessary cell and a necessary cell, required for the survival of the necessary cell, should be determined.

In this study, the energy value necessary to prevent the replication of an hMSC was determined when a femtosecond laser was focused on its center. Then, the critical distance between the irradiation point and a neighboring necessary cell for the survival of the latter was determined. An equation to correlate the critical distance to the power and number of irradiation is suggested.

Materials and methods

Cells

Human bone marrow mesenchymal stem cells (hMSCs) were used. The cells were isolated from bone marrow aspirate obtained by routine iliac crest aspiration from a human donor (19-year-old male). All subjects enrolled in this study gave their informed consent, which was approved by our institutional committee on human research, as required by the study protocol.

The isolated cells were plated on dishes (55 cm²; Corning, Tokyo) at a concentration of 0.15×10^4 cells/cm² using Dulbecco's modified Eagle's medium–low glucose (DMEM-LG; Gibco, Carlsbad, CA, USA) supplemented with 10% FBS (Gibco), 2,500 U/l penicillin, and 2.5 mg/l streptomycin, and cultured at 37 °C in a humidified atmosphere containing 5% CO₂.

Single cell culture

A single cell culture was obtained by limited dilution. Cells were detached using trypsin–EDTA from the dish and plated onto 96-well plates (Sumilon, Tokyo) at a concentration of approximately 12.5 cells/cm²

using the medium supplemented with 2 ng/ml FGF2 (Peprotech, Rocky Hill, NJ, USA) and cultured at 37 °C in a humidified atmosphere containing 5% CO₂. The presence of a well containing only one adherent cell was confirmed on day 1 and laser was irradiated on that well on day 2.

Irradiation with femtosecond laser

Femtosecond laser pulses were produced by a regeneratively amplified Ti:Sapphire laser system (Spit Fire, Spectra-Physics, 800 nm, 50 fs), which was then led to a microscope and focused into the samples with a 20X/0.4 NA objective lens (LWD CA20 PL, Olympus). The repetition rate of the pulse train was tune to be 200 Hz and the amount of shots was controlled with a mechanical shutter (Fig. 1).

Irradiation was focused on the center or the neighbor point on the major or minor axis of the single fibroblast-like cell (Fig. 2) and cultured for one more week at 37 °C in a humidified atmosphere containing 5% CO₂. The height of the focus was the same as that of upper surface of the bottom of well and

the average depth of the cell was approximately 1.5 µm (Takagi et al. 2007). Then, it was determined using an optical microscope whether there were plural cells in a well. Each irradiation condition was performed at least three times and the same result was confirmed.

Results

Influence of laser energy hitting a cell on cell replication

Single-shot laser irradiation was performed in the center of the cell with different energy values, and cell replication was confirmed to determine the amount of energy necessary to stop cell proliferation. For an amount of energy of 1 µJ, the cell maintained its replication capacity, but for an amount of energy higher than 2 µJ, the replication capacity was lost (Table 1), indicating disappearance of the cell replication. The energy value of 2 µJ was employed for further experiments.

Fig. 1 Experimental conditions: **a** Experimental timeline, **b** Experimental setup for irradiation of cells

Fig. 2 Typical morphology of mesenchymal stem cell. Minor and major axes are clearly indicated

Table 1 Influence of laser energy hitting the center of cell on cell replication

Energy (μJ)	1	2	3	4	6	8
Replication	+	–	–	–	–	–

Table 2 Influence of the distance between cell surface and laser hit point in the major axis on cell replication

Distance (μm)	0	5	10	15	20	30
Replication	–	+	+	+	+	+

Influence of distance in the major axis between laser focal point and cell surface on cell replication

A single-hit shot of 2 μJ at a neighbor point in the major axis of a single cell was performed to determine the critical distance at which cell proliferation was stopped.

When the outer surface of the cell was irradiated, it lost its replication capacity. On the other hand, the irradiation at points 5, 10, 15, 20 and 30 μm away from the membrane resulted in replication (Table 2).

Consequently, no loss of cell replication capacity was observed when the stress wave was exposed in the direction of the major axis.

Influence of distance in the minor axis between laser focal point and cell surface on cell replication

Single-shot irradiation at a predetermined distance from the cell in the minor axis with a 2 μJ laser was

Fig. 3 Influence of laser irradiation on cell replication capacity. Closed circles indicate that the cell lost its replication capacity and open circles indicate that the cell maintained its replication capacity

performed to determine the critical distance at which cell proliferation was stopped.

When the cell was irradiated at a point 5 μm away from the membrane, it lost its replication capacity. On the other hand, the irradiation at points 10, 15, 20 and 30 μm away from the membrane resulted in replication (Fig. 3).

Consequently, a distance larger than 10 μm between the laser focal point and cell surface in the minor axis was necessary to maintain the cell replication capacity.

Influence of laser shot number in the minor axis on cell replication

Multishot irradiation (2–16 times) of 2 μJ pulses at a neighbor point in the minor axis of the cell was performed to determine the influence of laser hit number on cell replication. Two, four, eight and sixteen shots at the points between 10 and 15, 30 and 35, 35 and 40 and 50 and 55 μm, respectively, resulted in the loss of replication capacity (Fig. 3). Consequently, the necessary distance between the laser focal point and cell surface in the minor axis for the maintenance of the cell replication capacity increased with the laser shot number.

Discussion

The mean generation time in the pre-culture was approximately 110 h. So, the replication capacity was considered to be lost, when plural cells were not found

at day 9. However, it is not clear whether the irradiated cell loses the replication capacity eternally.

The femtosecond laser pulse is partially absorbed at the laser focal point with multiphoton absorption. Possible pathways of the absorbed energy are plasma radiation, local heating in the focal volume, and mechanical effects such as shock wave emission and cavitation bubble generation, whose mechanics are explained in terms of optical breakdown (Vogel et al. 1999).

The loss of replication capacity when the laser pulses hit the center of a cell could be explained by laser ablation of the cytoskeleton and nucleus due to the local heating, mechanical effects, and multiphoton absorption (Shen et al. 2005).

The loss of replication capacity when the laser hits a point away from the cell surface could be explained by the impulsive force due to the stress wave generated by the laser irradiation (Vogel et al. 1999; Doukas et al. 1991).

The local stress generated by the impulsive force decreased proportionally to $1/r^2$, where r is the distance from the laser focal point, because the stress wave approximately propagates spherically (Doukas et al. 1991). When the cell was hit in the minor axis, a larger amount of surface was exposed to the stress wave than when it was hit in the major axis. This could explain why the influences of laser irradiation at the point 5 μm away from cell surface were different between the major and minor axes.

The threshold of the stress generation was the pulse energy between 1 and 2 μJ . Although the stress generation fluctuates at the vicinity of the threshold in the case of nanosecond and picosecond laser irradiation, such fluctuation is negligible even at the threshold because of highly effective multiphoton absorption. Actually, a stable generation of the stress wave was observed at 2 μJ . Consequently, since the impulse generated at the pulse energy higher than 2 μJ is stable and strong enough to eliminate the replication capacity, the experiment has a good reproducibility.

The critical distance (R_c) is defined as average between the maximum distance at which cell proliferation was stopped, and the minimum distance at which the cell could maintain its replication capacity. In the case of single-shot irradiation in the minor axis of the cell, the maximum distance at which the cell proliferation was stopped was 5 μm , and the minimum distance at which the cell could maintain its

replication capacity was 10 μm , thus, the critical distance was 7.5 μm . In the case of two, four, eight and sixteen hits, the critical distances were 12.5, 32.5, 37.5, and 52.5 μm , respectively.

It has been shown that irradiation with a femtosecond laser causes oxidative stress (a) evoking the production of ROS, (b) resulting in membrane barrier dysfunction, (c) inducing structural deformation and fragmentation of the nuclei as well as DNA strand breaks, (d) leading to cell death by apoptosis (Tirlapur et al. 2001). It is predictable that the longer the irradiation time, the larger the damage to the cell, and this is shown by the increase in the critical distance with the increase in the laser hit time.

A function for Laser Cell Damage (LCD) was defined in terms of the pressure of the shock wave (P) and number of irradiations (n). The dependence of LCD on the number of hits was assumed to be an exponent function with the number of hits being the base and an unknown exponent (θ).

$$\text{LCD} = k \times P \times n^\theta, \quad (1)$$

where k and θ are constants, and P and n are the pressure of the shock wave and the laser shot number, respectively.

The pressure of the shock wave is given in terms of the force at the laser focal point, F , and the distance, R , between the laser focal point and the membrane of the cell as

$$P = \frac{F}{4 \times \pi \times R^2} \quad (2a)$$

Therefore, the LCD is rewritten as

$$\text{LCD} = k \times \frac{F}{4 \times \pi \times R^2} \times n^\theta \quad (2b)$$

LCD was assumed to be greater than 1 when the cell lost its replication capacity, equal to 0 when the cell maintained its replication capacity and equal to 1 for the critical distance.

In the case of the critical distance, that is, when LCD is 1, Eq. 2b was converted to a logarithmic scale:

$$\log_{10}(R_c) = \left(\frac{\theta}{2} \times \log_{10}(n) \right) + \left(\frac{1}{2} \times \log_{10} \left(\frac{k \times F}{4 \times \pi} \right) \right) \quad (3)$$

The experimental data shown in Fig. 3 were plotted in Fig. 4 and the value of θ was calculated on

Fig. 4 Critical distance for multishot irradiation in logarithmic scale

Fig. 5 Force determined by atomic force microscopy on the focal point

the basis of the regression line obtained using Eq. 3.

$$\log_{10}(R_c) = 0.72 \times \log_{10}(n) + 0.92 \quad (4)$$

By comparing Eqs. 3 and 4, θ was found to be 1.44, indicating nonlinearity in the accumulation of cell damage caused by femtosecond laser pulses.

Furthermore, we estimated F under the present experimental conditions using our original force measurement system by atomic force microscopy (AFM) and the results are shown in Fig. 5 (Hosokawa et al. 2004). The force at the laser focal point, F , which is indicated as a unit of impulse (Ns), was increased linearly with laser pulse energy I as indicated in Eq. 5:

$$F = 18.5 \times I - 22 \quad (5)$$

From Eqs. 1 to 5, we summarized the critical distance for cell damage as a function of laser pulse energy and laser shot number as:

$$R_c = \sqrt{(86 \times I - 102) \times n^{1.44}} \quad (6)$$

Conclusions

The minimum laser energy required to stop the replication of MSC irradiated with a femtosecond laser was estimated. The influence of the distance between the laser focal point and cell surface on the replication capacity was also studied. Moreover, the cumulative effect of cell damage caused by multiple laser shots was correlated with the critical distance at which the cell lost its replication capacity. Finally, a novel equation for LCD as a function of laser pulse energy and laser shot number was proposed. This equation may be useful for estimating the optimum parameters for further applications, while the parameter values in the equation may vary depending on the pulse width, NA of lens, and so on. A novel noninvasive cell purification system using the irradiation of femto second laser may contribute a noninvasive quality control of cell processing for transplantation in future.

References

- Darwin JP (1997) Marrow stromal cell as stem cells for nonhematopoietic tissues. *Science* 276:71–74
- Doukas AG, Zweig AD, Frisoli JK, Birngruber R, Deutsch TF (1991) Non-invasive determination of shock wave pressure generated by optical breakdown. *Appl Phys B* 53:237–245
- Hosokawa Y, Takabayashi H, Miura S, Shukunami C, Hiraki Y, Masuhara H (2004) Nondestructive isolation of single cultured animal cells by femtosecond laser-induced shockwave. *Appl Phys A* 79:795–798
- Ito S, Takagi M (2009) 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
- Lindstrom S, Andersson-Svahn H (2010) Overview of single-cell analyses: microdevices and applications. *Lab Chip* 10:3363–3372
- Shen N, Datta D, Schaffer C, LeDuc P, Ingber D, Mazur E (2005) Ablation of cytoskeletal and mitochondria in live cells using a femtosecond laser nanoscissor. *Mech Chem Biosyst* 2:17–25
- Takagi M, Kitabayashi T, Ito S, Fujiwara M, Tokuda A (2007) Noninvasive measurement of three-dimensional morphology of adhered Chinese hamster ovary cells employing phase-shifting laser microscope. *J Biomed Opt* 12:September/October, 54010-1-5
- 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
- Tirlapur U, König K, Peuckert C, Krieg R, Halbhuber K (2001) Femtosecond near-infrared laser pulses elicit generation of reactive oxygen species in mammalian cells leading to apoptosis-like death cell. *Exp Cell Res* 263:88–97
- Tokumitsu A, Wakitani S, Takagi M (2009) Noninvasive estimation of cell cycle phase and proliferation rate of human mesenchymal stem cells by phase-shifting laser microscopy. *Cytotechnology* 59:161–167
- Tokumitsu A, Wakitani S, Takagi M (2010) Noninvasive discrimination of human normal cells and malignant tumor cells by phase-shifting laser microscopy. *J Biosci Bioeng* 109:499–503
- Uchugonova A, Isemann A, Gorjup E, Tempea G, Bückle R, Watanabe W, König K (2008) Optical knock out of stem cells with extremely ultrashort femtosecond laser pulses. *J Biophot* 1:463–469
- Vogel A, Noack J, Nahen K, Theisen D, Busch S, Parltitz U, Hammer DX, Noojin GD, Rockwell BA, Birngruber R (1999) Energy balance of optical breakdown in water at nanosecond to femtosecond time scales. *Appl Phys B* 68:271–280
- Yokoyama M, Miwa H, Maeda S, Wakitani S, Takagi M (2008) Influence of fetal calf serum on differentiation of mesenchymal stem cells during expansion. *J Biosci Bioeng* 106:46–50