Protocol of mesenchymal stem cell inoculation to nonwoven fabric scaffold

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Abstract To obtain a large number of human mesenchymal stem cell (hMSCs) for allograft, non-woven fabrics (NWF) were used as a cell culture scaffold. NWF are three-dimensional fiber aggregates formed by heat bonding and have a high surface area for cell adhesion and elongation. Inoculation hMSC was done to a center of NWF disc (diameter, 15.1 mm; depth, 0.1 mm). A cell suspension inoculum had a volume of 10 μL , which was close to the void volume of the disc, and resulted in a high initial (24 h) cell adhesion efficiency. Use of green fluorescent protein expressing rat MSCs and fluorescence microscopy revealed that adding an additional 10 μL of medium at 0–2 h after the cell inoculation made the initial horizontal distribution of cells in the NWF disc more uniform. Addition of 10 μL of the medium after 1 and 2 h of hMSC inoculation (0.15×10^3 cells/ cm^2 NWF-

fiber) markedly increased the final cell density (21 days) from 2.48 to 7.45×10^3 cells/ cm^2 NWF-fiber and fold increase in cell density by 16–48-fold. In conclusion, the addition of an additional medium after inoculation made the initial cells distribution in NWF more uniform, which might result in higher final cell density.

Keywords Mesenchymal stem cell · Nonwoven fabric · Scaffold · GFP · Polyester

Introduction

Human mesenchymal stem cells (hMSCs) are an attractive candidate for cell-based therapies of disorders such as cartilage impairments (Sato et al. 2013), graft-versus-host disease (GVHD) (Yamahara et al. 2014), and brain infarct (Chua et al. 2010), because they can be harvested by a minimally invasive procedure. These therapies with an allograft system might require a large number of hMSCs in a single lot, and thus large-scale growth cultivation. For such cultivation of adhesion-dependent mammalian cells, microcarriers are used in bioreactors to provide a large surface area per unit volume of bioreactors (Takagi et al. 1994; Nienow 2006; Schop et al. 2008).

However, in mass culture using microcarriers whose surface is curved, it was inferred that hMSCs

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can hardly adhere to the microcarriers and the cells tend to peel off owing to shear stress caused by agitation. Therefore, NWF was investigated as a culture carrier of hMSCs, because NWF has three-dimensional fiber aggregates formed by heat bonding, which is different from woven fabrics and knitted fabrics. NWF has a long surface in the longitudinal direction of a fiber and a cell can elongate on that long flat surface. Moreover, NWF has sufficient space for a cell to crosslink between fibers. Indeed, a bone marrow stromal cell line (SR-4987) attached to a polyester NWF disc could grow and spread well on NWF and a fibroblast-like cell shape was observed by scanning electron microscopy (Takagi et al. 1999; Tomimori et al. 2000). Thus, NWF was thought as a candidate for excellent porous carries. However, there are few reports on methods of inoculating cells to NWF. Therefore, we investigated the methods of inoculating cells to NWF to obtain high adhesion efficiency and fold increase in the density of hMSCs.

Materials and methods

Isolation and cultivation of hMSCs

hMSCs were isolated from bone marrow aspirates obtained by routine iliac crest aspiration from human donors (donor A, 76-year-old female and donor B, 65-year-old female) as reported previously (Sato et al. 2013). The donors gave their informed consent in this study, which was approved by our institutional committee on human research, as required by the study protocol.

The isolated cells, whose population doubling level (PDL) was defined to be zero, were inoculated at a concentration of 0.15×10^4 cells/cm² on a dish (55 cm²; Corning, NY, USA) containing Dulbecco's modified Eagle's medium–low glucose (DMEM-LG; Invitrogen, Carlsbad, CA, USA) supplemented with 10% FCS (Invitrogen), 2500 U/l penicillin, and 2.5 mg/l streptomycin (Sigma-Aldrich, St. Louis, MO, USA). The cells were cultivated at 37 °C in a humidified atmosphere containing 5% CO₂. When the cells density reached near confluence, they were detached using trypsin–EDTA (Sigma) and inoculated onto dishes and NWF (polyester, Y-15050, Asahi Chemical Co., Tokyo, Japan) as described below.

Adipose-derived rat mesenchymal stem cells (rGFP-MSC) were obtained from adipose tissue isolated from green fluorescent protein (GFP) transgenic Lewis rats (weight, 300–360 g). All the rats were treated humanely in accordance with the Principles of Laboratory Animal Care (formulated by the National Institutes of Health, Publication No. 56-23, received 1985), and the research protocol (No. 13034) was approved by the ethics committee of the National Cerebral and Cardiovascular Center Research Institute, Japan. Briefly, approximately 5 g of fat tissue was obtained from the subcutaneous fatty layer of rats under 2% inhaled isoflurane anesthetic at 0.3 ml/min airflow and was digested using 0.1% collagenase-type I solution (Wako Pure Chemical, Osaka, Japan) at 37 °C for 1 h with gentle agitation. After filtering the digest through a 100-μm-pore nylon mesh (BD Biosciences, NJ, USA) the filtrate was centrifuged at 1300 rpm for 4 min, and the resulting cell pellet was collected. The cell pellet was re suspended in the medium. The cell suspension was then filtered through a 70-μm-pore nylon mesh (BD Biosciences) before the cells were plated on a dish (100 mm; Asahi Glass Co., Tokyo, Japan) with the medium and was cultured in an atmosphere of 5% CO₂ at 37 °C. When the cells in culture were nearly confluent, they were harvested and sub cultivated at $3\text{--}6 \times 10^3$ cells/cm².

NWF

A disc (diameter, 15.1 mm; surface area of fibers; 12.24 cm²) of NWF (Fig. 1 Asahi Kasei, Y-15050) was used for the culture of hMSCs. The void volume in the NWF disc was calculated using Eq. (1), where V_{NWF} is the apparent volume of the NFW disc, and V_{fiber} is the volume of fibers in a disc calculated using Eq. (2). The volume of all fibers (V_{fiber}) in one disc was calculated using Eq. (3) as the ratio of the weight of a NWF disc (W_{fiber}) to the density of fibers (ρ_{fiber}). R_{NWF} and H_{NWF} are the radius and thickness of the NWF disc, respectively.

$$\text{Void volume } (\mu\text{L}) = V_{\text{NWF}}(\mu\text{L}) - V_{\text{fiber}}(\mu\text{L}) \quad (1)$$

Fig. 1 Whole image of culture system and SEM image of NWF. A NWF disc was put in a dish, and cell suspension will be put in the center of disc. The volume of disc was V_{NWF} . Many fibers were contained in a disc, and the volume of all fiber in one disc was V_{fiber}

$$\begin{aligned} V_{NWF}(\mu\text{L}) &= R_{NWF}^2 \times \pi \times H_{NWF} \\ &= 0.76(\text{cm})^2 \times 3.14 \times 0.01(\text{cm}) = 18(\mu\text{L}) \end{aligned} \quad (2)$$

$$\begin{aligned} V_{fiber}(\mu\text{L}) &= W_{fiber} / \rho_{fiber} \\ &= 9(\text{mg}) / 1.4(\text{g}/\text{cm}^3) = 6.43(\mu\text{L}) \end{aligned} \quad (3)$$

$$\begin{aligned} \text{Void volume}(\mu\text{L}) &= V_{NWF} - V_{fiber} \\ &= 18(\mu\text{L}) - 6.43(\mu\text{L}) = 11.57(\mu\text{L}) \end{aligned} \quad (4)$$

The surface area of fibers in a disc was calculated using Eq. (5).

$$\begin{aligned} \text{Surface area of fiber in a disc}(\text{cm}^2) &= 2V_{fiber} / R_{fiber} \\ &= 2 \times 6.43(\mu\text{L}) / 10.5(\mu\text{m}) \\ &= 12.24(\text{cm}^2) \end{aligned} \quad (5)$$

Cultivation on NWF

A NWF disc was placed in a 24-well plate (MS-8024R, Sumitomo Bakelite Co. Ltd., Japan) for suspension culture. A cell suspension of a predetermined volume was inoculated at the center of the NWF disc and incubated at 37 °C in 5% CO₂ atmosphere. Eight hours after inoculation, 1 ml of medium was added into the well, and the culture was incubated for predetermined times. The medium was changed with a fresh one every 7 days. In some experiments, 10 μl of

only medium was added besides the inoculation to the center of disc 0–2 h after inoculation.

Cell number analysis

The cell number in the NWF disc was determined by nucleus staining (Sanford et al. 1950) method. Briefly, after the culture supernatant was removed from well, a NWF disc was transferred to a 1.5 ml microtube. The NWF disc was mixed with 0.5 ml nuclei staining solution (500 μL, crystal violet, 1 g/l; citrate, 21 g/l), and incubated for 3 days at 25 °C. Then, the mixture was vigorously mixed, and the concentration of stained nucleus was counted using hemocytometer.

Fluorescence microscopy observation

In order to visualize cells in the NWF disc, GFP-MSCs were inoculated in the NWF disc, and observed under a fluorescence microscope (OLYMPUS IX 70, Tokyo). Specific three areas containing the center(A) and surrounding two areas (B, C) on a NWF disc were fixed and their photos were taken with time. The cells number (fluorescence dot) in each photo were counted.

Scanning electron microscopy (SEM) observation

Cells in a NWF disc were fixed with 2.5% glutaraldehyde (Wako, Osaka). After dehydration in sequentially increasing ethanol solution concentration from 50 to 100%, the samples were washed by isopentyl

acetate, and finally dried in a critical point dryer (HITACHI, HCP-2). After coating with platinum, the samples were observed by SEM (JEOL, JSM-6510LA)

Results

Effect of volume of cell suspension for inoculation on cell adhesion efficiency

To improve the adhesion efficiency of hMSCs to the NWF disc, the effect of the volume of cell suspension for inoculation on cell adhesion efficiency was investigated with references to the void volume (11.57 μl) of a NWF disc. A NWF disc in a well of a 24-well plate was inoculated with 10–320 μl of cell suspension (donor A, $4.7\text{--}15 \times 10^4$ cells/ml), to obtain an inoculum cell density of 1.23×10^3 cells/cm² NWF-fiber and incubated for 24 h. Thereafter, the cells adhering to the NWF disc and those suspended in the culture medium cells were respectively counted, and adhesion efficiency was calculated. Figure 2 shows the numbers of adherent cells on the NWF disc and cells suspended in the culture medium 24 h after inoculation, respectively.

With increasing volume of inoculum cell suspension, the number of suspended cells increased and that

Fig. 2 Effect of inoculum volume on adherent cell number. Cells (donor A, 1.5×10^4 cells) in inoculum at various volumes were inoculated to a NWF disc in a well and incubated for 24 h. The number of adherent cells on the NWF disc (open bars) and suspended cells (closed bars) were counted. The data shows the average of three wells

of adherent cells on the NWF disc decreased (Fig. 2). At the smallest volume of inoculum cell suspension (10 μl), the adhesion efficiency reached the highest at 81%. In the subsequent experiments, the volume of the inoculum cell suspension was set at 10 μl .

Effect of inoculum density on fold increase in density of hMSC in cultivation

To investigate the effect of inoculum density on fold-increase of hMSCs in cultivation, hMSCs (donor A) were inoculated to a NWF disc at inoculum cell densities of 0.15, 0.31, 0.61, 1.22, 2.45, and 4.9×10^3 cells/cm² NWF-fiber. Adherent cell density was determined by the nuclei staining method.

Even when the inoculum density was decreased from 4.9×10^3 to 2.45 and 1.22×10^3 cells/cm² NWF-fiber, the final cell density was almost the same (about 3.3×10^3 cells/cm² NWF-fiber). However, when the inoculation density was decreased to 0.61, 0.31, and 0.15×10^3 cells/cm² NWF-fiber, the final cell densities decreased to about 2.43, 1.93, and 1.18×10^3 cells/cm² NWF-fiber, respectively. When the inoculum density was 0.15×10^3 cells/cm² NWF-fiber, the highest fold increase in cell density (about 7.7 times) was obtained (Fig. 3).

Fig. 3 Effect of inoculum cell density on fold-increase of MSC in culture. hMSCs (donor A) were inoculated to a NWF disc in a well at inoculum densities of 0.15 (open circles), 0.31 (open squares), 0.61 (open triangles), 1.22 (open inverted triangles), 2.45 (closed circles) and 4.9×10^3 cells/cm² NWF-fiber, respectively. Cell density is shown on the y-axis. (n = 3, average $\pm$ S.D.)

Effect of medium addition on cell distribution in NWF disc

The effect of medium addition (10 μ l) after inoculation on the distribution of cells in NWF disc was investigated, in which a 10 μ l suspension of rGFP-MSC was initially inoculated at the center of a NWF disc (0.61 $\times$ 10³ cells/cm² NWF-fiber) in a well. Ten microliters of the medium was added to the center of the NWF immediately after inoculation (2–3 min later) or 1 h later. Three fields of view (A, B, and C) in the NWF disc [Fig. 4(1)] were observed under a fluorescence microscope [Fig. 4(2–1), (2–2), (2–3)].

The cell number in a field of view and the standard deviation (SD) among the three fields were calculated [Fig. 4(3–1), (3–2), (3–3)].

In the case of without medium addition, the distribution of cells in the three fields (A, B, and C) did not change even after 1 h [Fig. 4(2–1)]. On the other hand, the addition of the medium immediately after inoculation or 1 h later, markedly decreased the cell number at A after the addition [Fig. 4(2–2), (2–3)]. Although the SD did not change from 0 to 1 h without the additional medium [Fig. 4(3–1)], adding of the medium at 0 or 1 h decreased the SD after the addition [Fig. 4(3–2), (3–3)].

Fig. 4 Effect of medium addition on cell distribution in a NWF disc. Immediately (0 h) or 1 h after inoculation of 10 μ l cell suspension to the center of NWF disc, 10 μ l of the additional medium was added to the same place. Specific three areas containing the center (A) and surrounding two areas (B, C) on a NWF disc (1) were fixed and their photos were taken with time. (2–1) were photos of A, B, and C at 0 h and 1 h without adding

an additional medium. (2–2) were photos of A, B, and C at 0 h and 1 h, the additional medium was added at 0 h (0 + h). (2–3) were photos of A, B, and C at 0 h and 1 h, the additional medium was added at 1 h (1 + h). The cells numbers (fluorescence dot) in each photo and the standard deviation (SD) of cells numbers among the three fields are shown (3–1), (3–1), (3–3)

Effect of medium addition on the final cell density

To confirm the effect of additional medium addition on the final cell density, 10 μl of hMSC suspension (donor B, 0.61×10^3 cells/cm² NWF-fiber) was inoculated at the center of the NWF disc (Fig. 5). After 1 h, 10 μl of the medium was added to the same place and incubated as described above. Besides, cells were inoculated at 1.22×10^3 and 0.61×10^3 cells/cm² NWF-fiber without medium addition and cultured for 21 days. Cell density was determined (Fig. 5). Data are presented as average $\pm$ SD ($n = 3$). Two-tailed Student's *T* test was carried out for statistical analysis of the data. Values of $p < 0.05$ were considered to be statistically significant.

The cell density stopped increasing on day 14 under all conditions. Decreasing the inoculum density from 1.22×10^3 (circles) to 0.61×10^3 cells/cm² NWF-fiber (triangles) without medium addition resulted in the decrease in the final cell density from 3.34×10^3 to 2.98×10^3 cells/cm² NWF-fiber. On the other hand, 10 μl of medium addition at 1 h after inoculation (squares) increased the final cell density from 2.98×10^3 to 4.25×10^3 cells/cm² NWF-fiber.

Effect of time of medium addition on growth of hMSCs

The effect of additional medium addition timing in the culture with a lower inoculum density (0.15×10^3

Fig. 5 Effect of additional medium addition on growth of MSCs. MSCs (donor B) were inoculated to a NWF disc at cell densities of 0.61 (squares, triangles) and 1.22 (circles) cells/cm² NWF-fiber. The medium (10 μl) was added after 1 h (squares). ($n = 3$, average $\pm$ SD, * $p < 0.05$)

cells/cm² NWF-fiber) than those in Fig. 5 was investigated (Fig. 6). Ten microliters of hMSC suspension (donor B) was inoculated to a NWF disc at a cell density of 0.15×10^3 cells/cm² NWF-fiber. After 0, 1, or 2 h, 10 μl of the medium was added to the center of the NWF disc. Besides, hMSCs were inoculated at concentrations of 1.22×10^3 and 0.15×10^3 cells/cm² NWF-fiber without medium addition. All wells were incubated for 28 days. Cell density was determined (Fig. 6). Data are presented as average $\pm$ SD ($n = 3$). Two-tailed Student's *T* test was carried out for statistical analysis of the data. Values of $p < 0.05$ were considered to be statistically significant.

Decreasing the inoculum density from 1.22×10^3 (open circles) to 0.15×10^3 cells/cm² NWF-fiber (open squares) without medium addition resulted in the decrease in the final cell density from 5.11×10^3 to 2.48×10^3 cells/cm² NWF-fiber. On the other hand, additional medium addition after 0 (open triangles), 1 (open inverted triangles), and 2 h (closed triangles), increased the final cell density to 6.03, 7.45, and 4.65×10^3 cells/cm² NWF-fiber, respectively.

The highest fold increase was 48-fold in the culture with an inoculum density of 0.15×10^3 cells/cm² NWF-fiber and medium addition at 1 h. The NWF disc was observed by SEM at the end of culture. Cell adhesion was observed not only at the center but also in the periphery part (Fig. 7a). Besides the cells

Fig. 6 Effect of additional medium addition timing on growth of hMSCs. hMSCs (donor B) were inoculated to a NWF disc at densities of 0.15 (open triangles, closed triangles, open squares, open inverted triangles) and 1.22 (open circles) cells/cm² NWF-fiber. The medium of 10 μl was added after 0 h (open triangles), 1 h (open inverted triangles), and 2 h (closed triangles), respectively (* $p < 0.05$)

Fig. 7 SEM images of hMSCs grown on NWF for 21 days. The inoculum cell density was 0.15×10^3 cells/cm² NWF-fiber and 10 μ L of the medium was added 1 h after inoculation

adhering along with a fiber, some cells were cross linking between multiple fibers (Fig. 7b).

Discussion

The inoculum cell suspension volume of 10 μ L, which is close to the void volume of the NWF disc gave the maximum adhesion efficiency, and a large volume of inoculum cell suspension increased the number of floating cells. When the volume of the inoculum cell suspension is more than 10 μ L, it was supposed that the excess cell suspension overflowed from the NWF disc and the cells in the excess suspension could not adhere to the NWF disc. Therefore, in the subsequent experiments, the inoculum volume of 10 μ L was used.

Even the decrease in the inoculum cell density from 4.9 to 2.45, 1.22×10^3 cells/cm² NWF-fiber resulted in almost the same final cell density (about 3.3×10^3 cells/cm² NWF-fiber). Therefore, 3.3×10^3 cells/cm² NWF-fiber is considered to be the confluence density or contact inhibition density (Fig. 3). On the other hand, when the inoculum cell density was decreased to 0.61, 0.31, and 0.15×10^3 cells/cm² NWF-fiber, the final cell density decreased to 2.43, 1.94, and 1.18×10^3 cells/cm² NWF-fiber (Fig. 3), respectively. We supposed that the non-uniform distribution of cells in the NWF disc was the reason for the lower final cell density. In other words, even if cells locally reach the confluence density, the whole NWF disc might not reach confluence density. This speculation was confirmed in another experiment using rGFP-MSCs.

rGFP-MSCs were inoculated to the center of the NWF disc. Immediately or 1 h after inoculation, 10 μ L of the medium was added to the same place in the disc. This medium addition resulted in the decreases in the cell number at the center of the NWF disc [Fig. 4(1)A] and decrease the SD of the cell number among the three fields of view (Fig. 4A–C). These results suggest that rGFP-MSCs moved from the center (A) to the periphery (B and C) following the addition of the medium (10 μ L) and making the distribution of cells on the disc tended to be uniform.

Therefore, the effect of medium addition on the final cell density and the fold increase in cell density during culture were investigated using hMSCs. When cells were inoculated at a density of 0.61×10^3 cells/cm² NWF-fiber, 10 μ L medium addition 1 h after inoculation increased the final cell density from 2.98 to 4.25×10^3 cells/cm² NWF-fiber (Fig. 5). It was suggested that the distribution of cells in the NWF disc became more uniform after the medium addition, thus, the final density of cells increased.

The inoculum cell density was further decreased to 0.15×10^3 cells/cm² NWF-fiber, and the effect of the timing of the medium addition was examined (Fig. 6). When the medium was added 0, 1, and 2 h after the inoculations, the cells stopped to increase and reached the confluence density approximately on day 21 independently from the medium addition time. Among conditions, the addition of the medium 1 h after the inoculation provided the highest fold increase of 48. The reason why the addition 1 h after the inoculation resulted in the highest fold increase was unclear.

Consequently, decreasing the volume of inoculum cell suspension close to the void volume (about 10 μ L)

and a small amount (10 µL) of the medium added immediately after inoculation might improve cell distribution in the NWF disc and result in the higher density of grown cells and the higher fold increase in the density of cells.

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