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Cardiomyogenic induction of human mesenchymal stem cells by altered Rho family GTPase expression on dendrimer-immobilized surface with D-glucose display

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ABSTRACT

The commitment of stem cells to different lineages is regulated by many cues in the intercellular signals from the microenvironment system. In the present study, we found that alterations in Rho family GTPase activities derived from cytoskeletal formation can lead to guidance of cardiomyogenic differentiation of human mesenchymal stem cells (hMSCs) during *in vitro* culture. To regulate the cytoskeletal formation of hMSCs, we employed a dendrimer-immobilized substrate that displayed D-glucose. With an increase in the dendrimer generation number, the cells exhibited active migration, accompanied by cell morphological changes of stretching and contracting. Fluorescence microscopy for F-actin, vinculin and glucose transporter1 (GLUT1) clarified the localization of integrin-mediated and GLUT-mediated anchoring, introducing the idea that the morphological changes of the cells were responsive to variations in the generation number of the dendrimer with D-glucose display. On the 5th-generation dendrimer surface, in particular, the cells exhibited RhoA down-regulation and Rac1 up-regulation during the culture, associated with alterations in the cellular morphology and migratory behaviors. It was found that cell aggregation was promoted on this surface, supporting the notion that an increase in N-cadherin-mediated cell–cell contacts and Wnt signaling regulate hMSC differentiation into cardiomyocyte-like cells.

1. Introduction

Bone marrow-derived mesenchymal stem cells (MSCs) are a population of self-renewing, multipotent cells that have significant clinical potential for cellular therapies and tissue engineering [1]. These cells are able to differentiate into several committed phenotypes including osteogenic, chondrogenic, adipogenic, cardiomyogenic, and neurogenic lineages when stimulated under appropriate conditions [1,2]. However, one of the limitations of using MSCs for clinical applications is their low differentiation efficiency, because the differentiation pathways of MSCs are highly regulated by their niches, including both intrinsic and extrinsic signals [1–3].

Many strategies have been proposed to control MSC differentiation during *in vitro* culture [3–10]. A trend is to employ a target signaling pathway by means of signaling molecule-derived exogenous stimulation [5]. There are some candidate signals that direct MSCs to differentiate into specific cell lineages, based on physical or chemical factors as well as mechanical stimulators. An alternative strategy is to adopt endogenous stimulation by regulating the cell morphology in relation to the stem cell differentiation [6]. It has been demonstrated that the cell morphology regulates the switch in lineage commitment by modulating endogenous Rho family GTPase activities [6–10]. McBeath et al. [7] reported that the differentiation of human MSCs (hMSCs) is regulated by the cell morphology, based on observation that hMSCs allowed to adhere, flatten and spread underwent osteogenesis, while unspread and round cells became adipocytes. Furthermore, they reported that changes in the cell morphology activated signal transduction pathways involving the main elements of the Rho family GTPases and their downstream signaling. These findings suggest that extracellular signals can be locally integrated by the endogenous Rho family GTPases into a desired output to coordinate the dynamic cytoskeletal rearrangements that are necessary for directing stem cell fates.

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In previous studies, we have introduced our approaches to design of culture surface using a dendrimer substrate. A polyamidoamine dendrimer with a semispheric architecture was immobilized on a culture surface and glucose molecules as peripheral ligands were bound to the terminal amine moiety in dendrimers with different generation numbers [11–14]. The changes in cell morphology were appreciable on the dendrimer surface with D-glucose display, and were particularly, dependent on the generation number of the dendrimer [13]. It was also demonstrated that alterations of the generation number of the dendrimer with D-glucose display could promote the cell morphological changes, accompanied by dynamic cytoskeletal formation in the cells on the culture surface. Based on a series of studies, we propose a concept that the D-glucose-displaying dendrimer substrate can induce desmin expression relating to hMSC differentiation in the absence of any aqueous differentiation-inducing factors [14]. Within these contexts, the present study employed various dendrimer surfaces with D-glucose display as a tool for regulating MSC morphology to clarify the correlations of Rho family GTPase activities derived from cytoskeletal organization with MSC differentiation. Moreover, based on examination of cell differentiation, possible mechanisms by which these proteins function in intercellular signal transduction via cell–cell contacts are discussed from the aspect of cardiomyogenic lineage-related differentiation.

2. Materials and methods

2.1. Cells and culture conditions

Cryopreserved hMSCs derived from bone marrow (Lot no. 6F4085; Lonza Walkersville Inc., Walkersville, MD, USA) were thawed according to the supplier's instructions. Routine subcultures of hMSCs were conducted in 75-cm^2 flask (Corning Costar, Cambridge, MA, USA) using hMSC growth medium (Lonza) at 37 °C in a humidified atmosphere containing 5% CO_2. After reaching 70% confluence on the culture surface, the cells were detached by enzymatic treatment with a 0.1% trypsin/0.02% EDTA solution (Sigma–Aldrich, MO, USA). The cells that had experienced less than five passages were used in subsequent experiments.

For all experiments, hMSCs were expanded for specified days in Dulbecco's modified Eagle's medium (DMEM, Sigma–Aldrich) supplemented with 10% fetal bovine serum (Invitrogen, CA, USA) and antibiotics. The seeding density was fixed at a viable cell concentration of 5.0×10^3 cells/cm^2. On culture day 3, the spent medium was exchanged for fresh medium. The number of viable cells was estimated by the trypan blue exclusion test via direct counting of suspended cells in a hemacytometer.

2.2. Surface preparation

The conventional PS surface of a square 8-well plate (surface area; 9.6 cm^2, Nunc, Roskilde, Denmark) was used as a plain or starter material. Three types of dendrimer-immobilized surfaces with D-glucose display, i.e., first-generation (G1), third-generation (G3) and fifth-generation (G5) surfaces, were prepared by changing the generation number of synthesized dendrimers through four-step reactions conducted under sterile conditions. In step 1, the hydroxyl groups were displayed on the plain surface by pouring 50 μmol/ml potassium *tert*-butoxide into the wells, followed by incubation for 1 h at ambient temperature. The wells were then washed three times with sterilized water. In step 2, an aqueous solution of 360 μmol/ml glutaraldehyde was introduced into the wells. The wells were allowed to stand for 1 h, followed by washing with a large amount of sterile water. The wells were then treated with 360 μmol/ml tris(2-aminoethyl) amine solution (pH 9.0, adjusted with 0.1 mmol/ml NaOH) for 1 h to produce a dendron structure and then rinsed with sterile water. In step 3, D-glucose was displayed as a terminal ligand by keeping 0.1 mmol/ml D-glucose solution in the wells for 2 h. In step 4, a sodium borohydride solution (0.5 μmol/ml) was poured into the wells and left to stand for 24 h. Subsequently, the wells were washed with sterile water to yield the G1 surface with D-glucose display. To prepare the G3 and G5 surfaces, step 2 was repeated until the prescribed generation numbers of dendrimer were achieved. Thereafter, D-glucose liganding was carried out through steps 3 and 4, in which the applied concentration of D-glucose was changed to 1.6 μmol/ml.

2.3. Immunofluorescence staining

For the visualization of cytoskeletal elements, GLUT and Rho family GTPases, the cells were fixed with 4% paraformaldehyde in phosphate buffer (Wako Pure Chemical Industries, Tokyo, Japan) for 10 min at room temperature and rinsed with phosphate-buffered saline (PBS, Sigma–Aldrich), followed by soaking in PBS with 0.25% Triton X-100 for 4 min. After masking of non-specific proteins by incubation in Block Ace (Dainippon Sumitomo Pharma Co., Ltd. Osaka, Japan) for 15 min at ambient temperature, the cells were treated with a primary antibody at 4 °C overnight. Specifically, the cells were incubated with anti-vinculin (Chemicon International, CA, USA), anti-GLUT1 (Abcam, Cambridge, MA, UK), anti-RhoA (Abcam) and anti-Rac1 (Abcam) primary antibodies that were adequately diluted in PBS containing 10% Block Ace. The cells were washed with Tris-buffered saline (TBS) followed by immunolabeling with Alexa Fluor 488-conjugated goat anti-rabbit or anti-mouse IgG (Molecular Probes, OR, USA) for 1 h. Fluorescein-rhodamine-phalloidin and TOPRO-3 (Molecular Probes) were used to stain F-actin and nuclei, respectively. Images were obtained using a confocal laser scanning microscope (CLSM, model FV-300; Olympus, Tokyo) through 60× and 100× objective lenses.

To investigate cell–cell contacts, the cells were fixed with 2% paraformaldehyde in PBS for 10 min at room temperature and rinsed with PBS. After masking of non-specific proteins with Block Ace as described above, the cells were incubated with an anti-N-cadherin primary antibody (Santa Cruz Biotechnology, CA, USA) for 1 h at room temperature. The cells were washed with TBS and incubated with Alexa Fluor 568-conjugated goat anti-rabbit IgG (Molecular Probes) for 1 h. Alexa Fluor 488-phalloidin (Molecular Probes) was used to stain F-actin. The preparations were mounted and examined using the CLSM under fixed conditions for image capturing.

To analyze hMSC differentiation into muscle cell lineages, the cells were fixed with paraformaldehyde as described above, and permeabilized by treatment with 0.25% Triton X-100 in PBS for 4 min. After masking of non-specific proteins with Block Ace as mentioned above, the cells were incubated with mouse primary antibodies against desmin (Abcam), cTnT (Abcam) or fast skeletal MHC (Sigma–Aldrich) at 4 °C overnight. The cells were washed with TBS and incubated with Alexa Fluor 488-conjugated anti-rabbit or anti-mouse IgG for 1 h. The specimens were mounted and examined using the CLSM.

To examine Wnt signaling, the cultured cells were fixed with paraformaldehyde and premeablized with Triton X-100 as described above. After masking of non-specific proteins with Block Ace as described above, the cells were treated with anti-Wnt7a (Santa Cruz Biotechnology) and anti-Wnt11 primary antibodies (Santa Cruz Biotechnology) at 4 °C overnight. The cells were washed with TBS followed by incubation with Alexa Fluor 488-conjugated goat anti-donkey IgG (Molecular Probes) for 1 h. The specimens were examined using the CLSM.

3. Results

3.1. Cytoskeletal organization and glucose transporter1 (GLUT1) localization

Time-lapse observations of hMSCs were conducted to clarify the dynamics of the cell behaviors on polystyrene (PS) surface (Video S1 in the Supporting Information) as well as three types of dendrimer surfaces with D-glucose-display, i.e., first (G1), third (G3) and fifth (G5) generation numbers of dendrimer (Videos S2, S3 and S4, and Fig. S1). After inoculation, the cells in all the cultures started to adhere to the surfaces and put out cell extensions exhibiting dynamic protrusions with filopodia and lamellipodia. In particular, the cells on the G5 surface showed a round shape with temporal stretching. In addition, three-dimensional cell aggregate formation was found to occur spontaneously on the G5 surface through cell division as well as coalescence of migrating cells. As shown in Fig. 1, single-cell observation on day 3 revealed that most of the cells on the G5 surface were round, while those on the PS, G1 and G3 surfaces showed stretched morphologies. Moreover, on day 7, the cells on the G5 surface formed loosely attached aggregates, while those on the PS, G1 and G3 surfaces formed flatter and confluent cell monolayers attached firmly to the surfaces.

To confirm the cytoskeletal formation associated with integrin- and GLUT-mediated binding of cells to the surfaces, the cells cultured on the PS, G1 and G5 surfaces for 3 days were evaluated by confocal laser microscopy. As shown in Fig. 2A, many stress fibers appeared in both the cytoplasm and cell periphery in the cells on the PS surface and some stress fibers stretching across the cytoplasm were developed. Moreover, vinculin was detected in the central and peripheral regions of the cells on the PS surface, especially at the ends of the stress fibers with intensive staining in the longitudinal direction in a scattered manner. In contrast, the cells on the dendrimer surfaces exhibited extended lamellipodia