**Changes in human mesenchymal stem cell behaviors on
dendrimer-immobilized surfaces due to mediation of fibronectin
adsorption and assembly**

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Footnote: Much of this work forms the basis of the Ph.D. dissertation of Yuuki Ogawa.

Abstract

Dynamic changes of morphologies in human mesenchymal stem cells (hMSCs) were investigated on dendrimer surfaces with different capacities for fibronectin adsorption by changing the polymeric generation numbers of first (G1), third (G3), and fifth (G5) generations. The amount of adsorbed fibronectin on dendrimer surfaces increased with the generation number. Time-lapse observations revealed that cells on the G1 surface maintained their shape with formation of fibronectin fibrils in the bodies, introducing to the stabilization of focal adhesion with enriched stress fibers. Cells on the G3 surface showed partial contraction with degradation of fibril structures in the trailing edge. Cells on the G5 surface changed the shape by active extension and strong contracting without stabilization of focal adhesion through the formation of fibronectin aggregates and immature stress fibers. In addition, the paxillin which is a focal adhesion protein at lamellipodia was phosphorylated, leading to active lamellipodium protrusions. These results indicate that the amount and structure of fibronectin affects dynamic hMSC behaviors through the formation of cytoskeletons and focal adhesions.

[Key words: dendrimer-immobilized surface, human mesenchymal stem cells, cell behaviors, fibronectin adsorption on surface, fibronectin assembly]

INTRODUCTION

In the culture of anchorage-dependent cells, designing the culture surface is one of the most important strategies to regulate cellular functions such as migration, growth, and differentiation (1,2). The adhesion of cells onto a culture surface involves interactions between integrins of the cells and the extracellular matrix (ECM) adsorbed on the surface (3). Fibronectin is an abundant and ubiquitous ECM component that supports cell adhesion by displaying integrin-binding domains (4). On a fibronectin-coated surface, morphological variation of the cells occurs based on the density of binding sites which can be regulated by the coating concentration of fibronectin (5). As an alternative method, Keselowsky et al. (6) reported that the charge of the culture surface by displaying functional groups modulates the amount of fibronectin adsorption and its orientation to display integrin-binding domains. Surface chemistry can also be manipulated to affect cellular functions through regulation of fibronectin adsorption and assembly (6–8). Rico et al. (8) reported that the formation of fibronectin fibrils is influenced by surface chemistry, leading to morphological changes of the cells.

Interactions between integrins and fibronectin on the surface form focal contact, leading to the polymerization of fibronectin through the conformation changes (4,9,10). Fibronectin polymerization promotes stabilization of cell–matrix adhesions that are responsible for the morphological changes of cells with F-actin fiber formation (11,12). Therefore, morphological changes are considered to be due to both the density and stability of cell–substrate adhesions.

In previous studies, culture surfaces that immobilize polyamidoamine dendrimers have been proposed as a tool to regulate cell morphologies by modification of surface roughness, electrostatic charge, and ligand density, depending on the generation number (13–16). In addition, morphological variations of human mesenchymal stem cells (hMSCs) on dendrimer surfaces with different generation numbers induce directed differentiation of hMSCs toward a muscle cell fate commitment (15,16). In the present study, we investigated the possible mechanisms of dynamic cell behaviors in terms of focal adhesion and cytoskeletal formations of hMSCs on dendrimer surfaces with various

generation numbers by considering the mediation of fibronectin adsorption and assembly.

MATERIALS AND METHODS

Cells and culture conditions Bone marrow-derived hMSCs (Lot no. 0000183402; Lonza, Walkersville, MD, USA) were grown on tissue culture-treated polystyrene (PS) surfaces (Corning Costar, Cambridge, MA, USA) in MSC growth medium (Lonza) at 37°C in a humidified atmosphere containing 5% CO₂ as routine subcultures. At 70% confluence on the culture surface, the cells were passaged by enzymatic treatment with a 0.1% trypsin/0.02% EDTA solution (Sigma-Aldrich, St. Louis, MO, USA). Cells that underwent less than five passages were used in experiments.

For all experiments, hMSCs were expanded for the indicated number of days in Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich) supplemented with 10% fetal bovine serum (FBS) and antibiotics (Life Technologies, Carlsbad, CA, USA) at a depth of 4 mm in the culture vessels. The seeding density was 5.0×10^3 cells/cm², and medium changes were performed every 3 days.

Preparation of dendrimer-immobilized surfaces The PS surface of a square 8-well plate (Nunc, Roskilde, Denmark) and 35 mm culture dish (Ibidi GmbH, Martinsried, Germany) were used as starting materials as described previously (16). In brief, the hydroxyl groups were displayed on the starting material by addition of potassium *tert*-butoxide. Next, dendron structures were established by reactions of glutaraldehyde and tris (2-aminoethyl) amine solutions, which were repeated for the suitable generation numbers. A D-glucose solution was kept in the vessels and a sodium borohydride solution was added to the vessels without removal of the D-glucose solution. Subsequently, the dishes were washed with sterile water before use of the dendrimer-immobilized surfaces.

Western blotting Protein collection and western blotting were carried out as described in previous report (13). After incubation with culture medium, the culture surfaces were rinsed with phosphate-buffered saline (PBS; Sigma-Aldrich) twice and adsorbed protein was dissolved in lysis buffer (CellLytic M; Sigma-Aldrich) for 15 min at room temperature. After harvesting the protein samples by scraping and centrifugation at 4°C, the supernatant was collected. The incubated medium

1 was reacted with 20% trichloroacetic acid for 30 min at 4°C. After centrifugation, the precipitates
2 were washed with acetone twice and then dissolved in the lysis buffer for 15 min at room
3 temperature. After centrifugation at 4°C, the supernatant was collected. The protein samples were
4 mixed with Laemmli buffer and then heated at 95°C for 10 min. Proteins were separated by
5 SDS-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride nylon
6 membranes (Hybond-P; GE Healthcare, Buckinghamshire, UK). After masking the non-specific
7 proteins (ECL Blocking Agent; GE Healthcare) at room temperature for 2 h, the membrane was
8 reacted with a primary antibody against fibronectin (Santa Cruz Biotechnology, Dallas, TX, USA) at
9 4°C overnight. The membrane was washed with PBS containing 0.1% Polyoxyethylene (20) sorbitan
10 monolaurate (PBS-T), followed by the incubation with a peroxidase-linked secondary antibody (GE
11 Healthcare) at room temperature for 1 h. The membranes were washed with PBS-T and reacted with
12 detection reagent (ECL Detection Reagent; GE Healthcare) for 1 min before exposure to X-ray film
13 (Fujifilm, Tokyo, Japan).

14 **Immunofluorescence staining** Cells were fixed with 4% paraformaldehyde in PBS for 10
15 min at room temperature and then rinsed with PBS twice. The cells were then incubated in PBS with
16 0.5% polyoxyethylene (10) octylphenyl ether for 10 min. After masking the non-specific proteins
17 (Block Ace; Dainippon Sumitomo Pharma Co., Ltd., Osaka, Japan) for 90 min at room temperature,
18 the cells were incubated with primary antibodies against fibronectin, paxillin (Millipore, Billerica,
19 MA, USA), and phosphorylated-paxillin (Cell Signaling Technology Inc., Danvers, MA, USA) at
20 4 °C overnight. The cells were washed with Tris-buffered saline (TBS; Dako, Glostrup, Denmark)
21 and reacted with secondary antibodies (Alexa Flour 488-conjugated anti-mouse IgG or Alexa Flour
22 594-conjugated anti-rabbit IgG; Life Technologies) for 1 h at room temperature. Nuclei and F-actin
23 were stained with 4',6-diamidino-2-phenylindole and fluorescence-phalloidin (Life Technologies),
24 respectively. Images were obtained using a confocal laser scanning microscope (FV-1000; Olympus,
25 Tokyo, Japan) through a 60× objective lens.

Time-lapse fluorescence observations The cells were transfected with actin-RFP by baculovirus (CellLight actin-RFP; Life Technologies) according to the commercially available protocol. Then, the cells were cultured in DMEM with 10% FBS containing 5 µg/ml fluorescence-labeled fibronectin (Cytoskeleton, Denver, CO, USA). Images were obtained every 5 min using a time-lapse confocal laser scanning microscope (FV-10i; Olympus) through a 60× objective lens.

Quantitative RT-PCR RNA isolation and quantitative RT-PCR assays were carried out as described previously (13). Total RNA was extracted from the cells (RNeasy Mini Kit; Qiagen, Hilden, Germany) according to the commercially available protocol. Reverse transcription was conducted (SuperScript II Reverse Transcriptase kit; Takara Bio Inc., Shiga, Japan). Quantitative PCR assays were carried out (SYBR Premix EX Taq; Takara Bio Inc. and 7300 Real-Time PCR System; Applied Biosystems, Foster City, CA, USA). Specific primers were membrane type 1-matrix metalloproteinase (MT1-MMP), forward 5'-CCATTGGGCATCCAGAAGAGAGC-3'; reverse 5'-GGATACCCAATGCCCATTTGGCCA-3' and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), forward 5'-CAACGGATTTGGTCGTATTGG-3'; reverse 5'-GCCATGGGTGGAATCATATTG-3' according to previous reports (13,17). The relative quantitation of target mRNA expression was estimated by the cycle threshold value (C_t). Relative mRNA expression was normalized to the GAPDH expression to obtain the ΔC_t value and calculated by $2^{-\Delta C_t}$.

RESULTS

Adsorption of fibronectin on dendrimer surfaces Western blotting was carried out using samples of the proteins adsorbed on G1, G3, and G5 surfaces and the unadsorbed proteins in the supernatant after 1 day of incubation with 10% FBS/DMEM. As shown in Fig. 1, the amount of adsorbed fibronectin on the surfaces increased with the generation number of the dendrimer surface, while a decrease in the amount of fibronectin occurred in the medium.

Cytoskeletal formation and fibronectin assembly

Cytoskeletal formation and fibronectin assembly were observed by immunostaining of F-actin and fibronectin in hMSCs on the G1, G3, and G5 surfaces at days 1 and 3. As shown in Fig. 2, the spindle-shaped cells on all surfaces showed abundant F-actin filaments at day 1. Fibronectin was detected on all surfaces but was concentrated in the interfaces between cells and surfaces (Fig. S1). Fibril and small structures of fibronectin were found on the surfaces (Fig. 2), indicating fibronectin fibrils (10^1 – 10^3 μm in length) and fibronectin aggregates (10^0 – 10^1 μm in diameter), respectively (18). In particular, fibril structures were not detected in the cell edge (Fig. 2A1, B1, and C1), and similarly to the formation of stress fibers, much more fibril structures were observed of the longitudinal direction in cell bodies on the G1 and G3 surfaces, compared with those on the G5 surface (Fig. 2A–C). At day 3, on all surfaces, little fibronectin fibril was detected in the cell edge. In addition, the spindle-shaped cells on the G1 surface maintained the formation of stress fibers, and the fibronectin fibrillogenesis was promoted in the bodies, compared with those at day 1 (Fig. 2D). On the G3 surface, stress fibers and fibril structures were localized in half side of the bodies along the major axis (Fig. 2E2), although little fibronectin fibril was detected in the other one (see the arrow in Fig. 2E). In contrast, on the G5 surface, both the round- and spindle-shaped cells showed many lamellipodia with less stress fibers, compared with those on the other surfaces. And the fibronectin formed small compact structures in the cell bodies (Fig. 2F and G).

Actin-RFP expressing hMSCs were observed with fluorescent labeled-fibronectin on the G1, G3, and G5 surfaces at day 3. On the G1 surface, spindle-shaped cells showed lamellipodia with little fibril structures in the leading edge and stress fibers with fibrill structures in the bodies. With extension of the cells, fibronectin fibrillogenesis was seen in the bodies along the direction of migration. Then, the cells contracted their trailing edge with shortening of stress fibers and fibronectin fibrils (Movie S1). On the G3 surface, spindle-shaped cells showed lamellipodium formation with little fibronectin fibril in the leading and trailing edges as well as stress fibers with

fibronectin fibrils in the bodies. Upon extension of the cells, it was observed that lamellipodium protrusions with fibronectin fibrillogenesis in the front-side of the bodies and partial contraction of the cells through release of trailing edge with disruption of fibronectin fibrils (Movie S2 and Fig. S1). Furthermore, on the G5 surface, round-shaped cells showed lamellipodia with little fibril structures in whole bodies. The cells extended the shape to bipolar directions with active lamellipodium protrusions. Upon the extension of the cells, immature stress fibers and small structures of fibronectin were formed in whole bodies. Then, the cells contracted with sudden release of the trailing edge. This temporal stretching was repeated with increasing the amount of small structures of fibronectin on the cells (Movie S3 and Fig. S2).

Enzymatic activity for matrix degradation The mRNA expression of MT1-MMP, which is one of the enzymes for fibronectin degradation, was examined in hMSCs cultured on G1, G3, and G5 surfaces at days 1 and 3. As shown in Fig. 3, relative mRNA expression of MT1-MMP in hMSCs on the G5 surface was significantly higher than that on G1 and G3 surfaces at day 1. In addition, at day 3, the expression of MT1-MMP on the G1 surface was lower than that on the G3 surface, while the expression in hMSCs on the G5 surface at day 3 was 2-fold higher than that at day 1.

Formation and phosphorylation of paxillin Immunostainings of F-actin, paxillin, and phosphorylated-paxillin were conducted in hMSCs cultured on the G1, G3, and G5 surfaces at day 3. As shown in Fig. 4, paxillin spots were distributed at terminal of stress fibers at edge and bodies of the cells on the G1 surface (Fig. 4A). With increasing generation numbers of the dendrimer surface, the distribution of paxillin spots was more intensive at cell edge, while being less in the bodies, compared to those on the G1 surface. In particular, on the G5 surface, almost all paxillin had localized in the lamellipodia in the edge in both round- and spindle-shaped cells (Fig. 4C and D). Furthermore, as shown in Fig. 5A–C, expression of phosphorylated-paxillin was not observed in hMSCs cultured on G1 or G3 surfaces, although phosphorylated-paxillin spots were detected in

1 the edge of cells on the G5 surface.

2 **DISCUSSION**

3 The present study describes dynamic changes in behaviors of hMSCs on dendrimer surfaces
4 with various generation numbers due to the mediation of fibronectin adsorption and assembly. These
5 behaviors consist of the formation of attachments between integrins of extended membranes and
6 adsorbed fibronectin on the surface, stabilization of adhesion in the cell body, and contraction of the
7 cell rear with release of attachments from the substrate (19). Time-lapse observation (Movie S1)
8 revealed that cells on the G1 surface migrated with maintenance of their spindle-shape through
9 extension in the leading edge, stabilization of focal adhesion, and contraction in the trailing edge
10 with promotion of fibrillogenesis and stress fiber formation in the bodies (Fig. 6Aa and Ab), as well
11 as with fibronectin aggregation and release of focal adhesion in the trailing edge (Fig. 6Ab and Ac),
12 similarly to those on the conventional PS surface. It has been documented that fibronectin
13 fibrillogenesis stimulates cell contraction associated with shortening of stress fibers through a
14 Rho-dependent mechanism (20–22). In our previous report (16), cells on the G1 surface exhibited the
15 higher activity of RhoA than those on the G3 and G5 surfaces. These evidences suggested that
16 fibronectin fibrillogenesis stimulates the RhoA activity to facilitate the stress fiber formation, causing
17 the migration with spindle-shape. On the G3 surface, the dynamic cell behavior (Movie S2) exhibited
18 that the cells made the extension in the leading edge, low stabilization of focal adhesion, and
19 partially strong contraction with promotion of fibronectin fibrillogenesis and stress fiber formation
20 (Fig. 6Ad and Ae), as well as with degradation of fibril structures in the trailing edge and stress fiber
21 disruption (Fig. 6Ae and Af). On the G5 surface (Movie S3), the cells changed the shape by active
22 extension and strong contracting without stabilization of focal adhesion through the formation of
23 fibronectin aggregates and immature stress fibers (Fig. 6Ag and Ah). In addition, the cells showed
24 active extension to bipolar directions, causing sudden release of the trailing edge with collection of
25 fibronectin aggregates (Fig. 6Ah and Ai), which was considered to contribute to restarting the cell

1 extension because of the adsorption of fibronectin from the surrounding components in the medium.
2 Thus, the changes in fibronectin assembly and cytoskeletal formation were responsible for the
3 dynamic cell behaviors on the dendrimer surfaces.

4 The possible processes for fibronectin adsorption and assembly associated with the formation of
5 focal adhesions and actin cytoskeletons at cell–surface interfaces were considered as follow (Fig. 6B).
6 The adsorbed fibronectin with a compact conformation is unfolded to display the N-terminal
7 fibronectin–fibronectin binding domain, leading to the formation of fibronectin fibrils (4). It has been
8 documented that fibronectin fibrillogenesis induces the formation of fibrillar adhesion, which is
9 essential for stress fiber formation through constructing focal adhesions (11,21). In the present study,
10 immunostainings indicated that cells on the G1 surface had stable focal adhesion at the terminal of
11 stress fibers in the edge and bodies, accompanied by the formation of fibronectin fibrils (Fig. 6Ab,
12 B1, and B2). In contrast, on the G5 surface, localization of focal adhesion was detected in
13 lamellipodia with little formation of stress fibers and fibronectin fibrils compared with those on the
14 G1 surface (Figs. 2 and 4). Similar phenomena of fibronectin assembly have been reported by
15 Faucheux et al. (7) in cultures of human skin fibroblasts. The inhibition of fibronectin fibrillogenesis
16 occurred on a positively charged surface by showing a larger amount of fibronectin adsorption
17 compared with that on a negatively charged surface. In this study, compared with the other surfaces,
18 the amount of adsorbed fibronectin from the culture medium was larger on the G5 surface (Fig. 1).
19 On a larger amount of adsorbed fibronectin, it has been documented that the turnover of focal
20 adhesions is promoted with active cell migration through paxillin phosphorylation (23,24). Paxillin
21 phosphorylation enhances the turnover of focal adhesion with lamellipodium protrusions, whereas
22 non-phosphorylated paxillin leads to the formation of fibrillar adhesions (25). In the present study,
23 phosphorylated-paxillin was detected in cells, although less phosphorylated-paxillin was found in
24 cells on the G1 and G3 surfaces (Fig. 5). These suggested that the active extension on the G5 surface
25 occurred due to high turnover of paxillin through high adsorption of fibronectin and its promotion of

paxillin phosphorylation (Fig. 6B3). Furthermore, the inhibition of fibronectin fibrillogenesis occurred in cells on the G5 surface (Fig. 2), which is considered to be related to matrix degradation mediated by specific enzymes such as MMPs (26-28). In particular, MT1-MMP plays a role in the degradation of fibronectin fibrils to promote cell motility (28), and the presence of fibronectin made up-regulation of expressions for MT1-MMP (29). These evidences supported our current results of higher expression of MT1-MMP in cells on the G5 surface, which was made by higher adsorption of fibronectin (Figs. 1 and 3), suggesting that the unstable adhesions of cells on the G5 surface appeared through no formation of fibronectin fibrils and focal adhesions in cell bodies by degradation of unfolding fibronectin (Fig. 6B4). These results suggested that a dendrimer surface with high cationic properties was sufficient for fibronectin adsorption and the inhibition of fibronectin fibrillogenesis through MMP activation, causing the active extension and adhesion turnover with formation of immature stress fibers on the G5 surface. Cells on the G3 surface with middle level of fibronectin adsorption exhibited the transitional behaviors, suggesting that the partial degradation of fibronectin fibrils by MMPs caused the extension and stable adhesion with fibronectin fibrillogenesis and stress fiber formation in the leading edge, and partial unstable adhesion and contraction with fibronectin aggregation in the trailing edge (Fig. 6Af, B1, B2, and B4).

In conclusion, the present study indicates that fibronectin adsorption and assembly by the cells changes dynamic cell behaviors. Moreover, we found that adsorption of fibronectin induces the extension of cells, and that fibronectin assembly affected by MMPs plays important roles in the stabilization of focal adhesions associated with cytoskeletal formations. These results suggest that control of the cell–surface interface in fibronectin adsorption and assembly represents a versatile approach to manipulate specific cellular responses to biomaterials for tissue engineering applications.

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FIGURE LEGENDS

FIG. 1. Western blotting of fibronectin adsorbed on the culture surfaces and remaining in the culture medium on the G1, G3, and G5 surfaces after 1 day of incubation.

FIG. 2. Immunostainings of F-actin (red), fibronectin (green), and nuclei (blue) in hMSCs cultured on the G1 (A and D), G3 (B and E), and G5 (C, F, and G) surfaces at days 1 and 3. Panels A1–G2 show enlargements of boxed areas in panels A–G, respectively. Scale bars: 50 μm (A–G) and 10 μm (A1–G2).

FIG. 3. Quantitative RT-PCR analysis of MT1-MMP expression in hMSCs cultured on the G1 (dark bar), G3 (gray bar), and G5 (blank bar) surfaces at days 1 and 3. Data represent average values with the standard deviation determined from at least triplicate independent experiments. Data were analyzed using the Tukey-Kramer method. Statistical significances among the data sets were considered at $*p < 0.01$ and $**p < 0.05$.

FIG. 4. Immunostainings of F-actin (red), paxillin (green), and nuclei (blue) on the G1 (A), G3 (B), and G5 (C and D) surfaces at day 3. Panels A1–D2 show enlargements of boxed areas in panels A–D, respectively. Scale bars: 50 μm (A–D) and 10 μm (A1–D2).

FIG. 5. Immunostainings of paxillin (green), phosphorylated-paxillin (red), and nuclei (blue) on the G1 (A), G3 (B), and G5 (C) surfaces at day 3. Panel C1 shows an enlargement of the boxed area in panel C. Scale bars: 50 μm (A–C) and 10 μm (C1).

FIG. 6. Schematic illustrations of dynamic changes in behaviors of hMSCs associated with formation of focal adhesion and cytoskeleton due to the adsorption and assembly of fibronectin (A) on the G1 surface (Aa–Ac), the G3 surface (Ad–Af), and the G5 surface (Ag–Ai), and cell–surface interfaces in adsorption and assembly of fibronectin associated with the formation of focal adhesions and actin cytoskeletons (B) for cell extension (B1), stable adhesion (B2), active extension (B3), and unstable adhesion (B4).

Fig. 1

Fig. 2

Fig. 3

Fig. 4

Fig. 5

Fig. 6