Morphological Changes of Human Mesenchymal Stem Cells on a Large Area of Nanopatterned Surface

Jaeyeon Lee, Kyuback Lee, Kyung Sun, and Yongdoo Park*

1Department of Biomedical Engineering, Biomedical Science of Brain Korea 21, Medical College, Korea University, Seoul, Korea
2Korea Artificial Organ Center, Korea University, Seoul, Korea
3Department of Biomedical Engineering, Medical College, Korea University, Seoul, Korea
4Department of Interdisciplinary Bio/Micro System Technology, College of Engineering, Korea University, Anam-dong 5-1, Seoul 136-713, Korea
5Department of Biomedical Engineering, College of Health Science, Korea University, Sna-1, Jeongneung 3-dong, Seoul 136-703, Korea
6Department of Thoracic and Cardiovascular Surgery, Medical College, Korea University, Seoul, Korea

(Received January 09, 2013/Accepted January 31, 2013)

Cells receive various physical and chemical signals from their tissue’s microenvironment. In this study, human mesenchymal stem cells were cultured on variously sized nanostructures and their cellular responses were evaluated. Various nanotopological structures were fabricated on polystyrene polymer surfaces using anodized aluminum oxide (AAO) with different nanopost diameters (70, 200, 400 nm) as nanoimprinting templates. Before cell culture experiments, nanopatterns were coated with collagen types I and IV. Human mesenchymal stem cells were cultured on the various nanotopological structures for 24 h. Cell viability in the nanopatterned surfaces was evaluated by a live/dead assay which showed that various nanostructures do not affect cellular viability. Cellular viability in all the samples was more than 95%. Cell proliferation assays showed that cells cultured on 70 nm nanostructures pre-coated with collagen type IV increased proliferation rates three folds compared to those of control, 200 nm, or 400 nm surfaces. Additionally, the cell area showed significant differences between samples cultured on 70 nm nanopatterned surfaces compared to other conditions. However, Nanopatterned surfaces pre-coated with collagen type I showed no significant differences between samples cultured on a 70 nm nanopatterned surface compared to the other samples. This result reflects that cells showed differential reaction depending on surface coated ECM proteins as well as nanostructures. The effect of pre-coated ECM proteins is one of the major determinants that guides cellular reactions and nanopatterned surfaces could be used to further guide stem cell differentiation and proliferation.

Key words: extracellular matrix, collagen, nanotopography, hMSC, cell area, proliferation

Introduction

Cells are surrounded by an extracellular matrix (ECM) within tissues that interacts with the cells to elicit various cellular activities such as proliferation and differentiation. ECM is largely comprised of proteins and its physical structure is based on nanosized structures. The geometries of these nanotopologies in three-dimensional tissues are pits, protrusions and fibers ranging from 6- to 200 nm in size. For example, nanoprotuberances and other raised topological features are found in many ECMs and nanopore structures are found in the basement membrane of the cornea, the aortic heart valve, and in vascular systems. The myocardium has grooved nanotopological ECM structures while collagen has various nanofibril structures ranging from 50 to 100 nm.

The effects of nanotopology on cells were first studied by Curtis, and Brunette who observed that surfaces patterned with nanostructures can control cellular morphology and behavior. The development of nanofabrication technologies has improved the study of cellular behavior on nanostructures and is one of the leading areas in nanobiology research. Nanostructures such as nanostrips, nanopits, and nanogrooves can be fabricated by new technologies, such as chemical vapor deposition, polymer phase separation, colloidal lithography, photolithography, and electron beam lithography (EBL).

A leading subject area in research involving nanostructure cell interactions is the morphological change of cells on nanostructures through cytoskeletal modulation. Dalby et al. found that human epithelial cells cultured on nanogrooved structures molded with 70 nm ridges and 400 nm pitches changed their...
shape from round to elongated. Cells, such as fibroblasts, cultured on randomized nanoislands recognized the nanostructures and filopodia and lamellipodia were formed on the isotropic nano geometries. Yim et al. and Kim et al. found that nanostructures also modulate cell proliferation and motility. Cells cultured on nanograting structures with a 350 nm line width, a 700 nm pitch, and a 350 nm depth resulted in reduced proliferation activity. The polarization of the microtubule organization center (MTOCs) shifted along with the axis of nanostructures also modulate cell proliferation and motility. Cells cultured on nanograting structures with a 350 nm line width, a 700 nm pitch, and a 350 nm depth resulted in reduced proliferation activity. The polarization of the microtubule organization center (MTOCs) shifted along with the axis of nanostructures.

Cell conduction activity in cardiomyocytes increased in nanopatterned PEG hydrogel surfaces. Nanotechnology has also been shown to change the gene expression profiles of cells. Gasiorowski et al. cultured human umbilical vein endothelial cells on 400 nm grooved surfaces and found that genes for differentiation were upregulated but genes for proliferation and cyclins were downregulated. Dalby et al. also showed that human mesenchymal cells (MSCs) cultured on nanopit arrays up to 28 days showed overexpression of osteoblast specific genes.

Cells also receive signals from the microenvironments of the ECM. The composition of ECM plays a key role on cellular behavior such as proliferation, migration, and differentiation. Therefore, changing ECM composition in culture dishes or in three-dimensional matrices could modulate certain cellular functions. Surfaces coated with fibronectin or laminin elicit neural cell migration as a guidance cue. Immobilization of RGD peptides also induces cells to spread on these coated surfaces. ECM composition is important given that its components can control cell behavior. The geometry and patterns of ECM are other important factors. The combination of ECM components and nanoscaled topologies will elicit a new era for controlling cells in two- and three-dimensional conditions.

In this paper, large-scale nanopost arrays were fabricated with nanoimprinting technologies. Cellular behavior on these nanopatterns was evaluated with ECM proteins including collagen types I and IV. Cellular behavior is closely related to surface ECM proteins and nanocolumn size. Especially, We demonstrate how the combination of geometry and ECM proteins affect the proliferation and area of human mesenchimal stem cell.

Materials and Methods

Nanostructure fabrication
The nanopatterns on PS were obtained by imprinting AAO stamps on ST313120 PS plates (Goodfellow, UK) using the NANOISISTM610 nanoimprinting device (Nano & Device, Korea). The ST313120 PS plate was used as received. AAO fabricated by anodizing pure aluminum in oxalic acid (AAO-O45) had pores of 45 nm in diameter and 110 nm in spacing. AAOs fabricated by anodizing pure aluminum in phosphoric acid (AAO-P250, AAO-P410) had pores of 250- and 410 nm in diameter and 500 nm in spacing.

Scanning electron microscopy (SEM)
Samples were fixed in 4% paraformaldehyde, in PBS for 1h and rinsed three times with PBS. The specimens were dehydrated with ethanol and sputter-coated with gold for SEM observation.

Cell culture
Human MSCs were seeded on the untreated and nanopatterned surfaces. Before seeding, nanopatterned surfaces were coated with collagen type I (collagen solution, StemCell Technologies) and collagen type IV (Mouse Collagen Type IV, BD Biosciences). Cells were seeded at a density of 5 × 10^3 cells per sample in 200 µl of complete medium. Cells were cultured in DMEM with 10% (v/v) FBS and 1% (v/v) antibiotics, containing penicillin and streptomycin. Cells were incubated at 37°C with 5% CO₂ for 24h.

Live/dead assay
For the viability test, cells were treated with Live/Dead assay reagents (LIVE/DEAD Viability/Cytotoxicity Assay Kit, Molecular Probe) and incubated for 40min. Live cells (green fluorescence) and dead cells (red fluorescence) were observed under a fluorescence microscope. The viability of human MSCs on the nanopatterned surface was determined by dividing the number of live cells by the number of total cells counted.

Cell proliferation assay
The proliferation of human MSCs on the nanopatterned surface was evaluated by the Cell Counting Kit-8 (CCK-8) assay (Dojindo Laboratories, Kumamoto, Japan). CCK-8 is a colorimetric assay that is based on the amount of mitochondrial dehydrogenase in viable cells. For the assay, human MSCs were rinsed twice with PBS and CCK-8 solution was applied. CCK-8-treated cells on the nanopatterned surface were incubated at 37°C under 5% CO₂ for 4h. The optical density was measured at a reference wavelength of 450 nm using an ELISA reader.

Cell adhesion area analysis
After 24h of culture, cellular morphology on the nanopatterned surface was documented by photomicrography. The cell area was measured from the cellular images using image analysis tools. The percentage(%) of cell area was presented as the ratio of single cell area versus total image area.

Immunostaining of Actin Filament
Cells on the nanopatterned surface were fixed with 4% paraformaldehyde in PBS for 1h, followed by three PBS washes. The membrane was permeated by incubating in 0.1% Triton X-100 for 5min, followed by three PBS washes. Cells
Morphological Changes of Human Mesenchymal Stem Cells on a Large Area of Nanopatterned Surface

were then blocked with 3% BSA in PBS for 30min, followed by three PBS washes. The sample was incubated with actin staining reagents (Alexa 594 phalloidin, Molecular Probes; Eugene, OR, USA) for 30min, and viewed by a fluorescence microscope.

Electrophoresis for the detection of proteins and glycoproteins

Statistical Analysis
All the data are reported as mean ± deviation (SD). All the analyses were performed with OriginPro 7.5 (OriginLab; Northampton, MA). The comparisons between the groups were carried out using a student t-test. Results of \( p < 0.01 \) were considered significant (differences of \( p < 0.05 \) denoted by* and \( p < 0.01 \) denoted by**).

Results and discussion

Preparation of nanopatterned surfaces
ECM consists of micro- and nanosized topologies and in tissue this modulates cellular behavior.24 Nanofabrication technologies have been developed for creating biomimetic environments by controlling sizes from 1- to 500 nm scales. In this study, nanopatterned surfaces were fabricated on polystyrene polymer surfaces using AAO with different pore sizes as nanoimprinting templates to mimic in vivo ECM topology. Figure 1 shows the SEM images of the resulting nanostructure substrates with various pore sizes. The sizes of the nanopits were 70 nm, 200 nm, and 400 nm (Figure 1). The space between nanopost was 110 nm, 500 nm, and 500 nm, respectively. The patterned surface was treated with EO gas to remove residual elements toxic to the cells.

Cellular viability cultured on nanopatterned surfaces
Cell viability was influenced by ECM geometry and components such as collagen.25 In this study, nanopatterned surfaces were coated with collagen types I and IV to facilitate cell adhesion (Figure 2). Collagen types I and IV have domains that function as ligands that can specifically bind integrins on cell membranes, and thus can affect cell attachment, spread, and viability.26 To investigate cellular viability, human MSCs were cultured on various nanopatterned surfaces coated with either collagen type for 24h and after seeding, viability was evaluated. We observed that the viability of human MSCs on nanopatterned surfaces (70-, 200-, and 400 nm) was higher than 95%. These results were similar to cells cultured on a flat surface (Figure 3). The viability of human MSCs is not affected by nanopatterned surfaces or collagen type.

Cell proliferation on collagen types I- or IV-coated nanopatterned surfaces
The proliferation of human MSCs on nanopatterned surfaces was measured with a CCK-8 assay 24h after seeding. Human MSCs cultured on nanopatterned surfaces with different nano-sizes showed different proliferations (Figure 3). Cells cultured on 70 nm sized nanostructures post coated with collagen type IV increased proliferation three-fold over the control, 200 nm, and 400 nm surfaces when the nanopatterned surface was precoated with collagen type IV (Figure 3(A)). However, there was no significant differences among samples coated with collagen type I (Figure 3(B)). The major difference of 70 nm nanopost to 200 and 400 nm post is the spacing. The spacing of 70 nm post sample is 110 nm whereas other samples spacing

Figure 1. SEM (scanning electron microscopy) images of nanopatterned surfaces fabricated using AAO. (a) 70 nm, (b) 200 nm and (c) 400 nm.

Figure 2. Synthetic scheme of nanopatterned surfaces with collagen types I and IV.
is 50 nm. The size of basic unit in 70 nm sample is 180 nm, which is 2.5 times smaller than that of 200 nm sample and 4 times smaller than that of 400 nm samples. Therefore, cells could have much more contact points on the nano post in 70 nm samples compared to 200 and 400 nm samples. However, this effect is not shown in the samples treated with collagen type I because there was no significant differences among samples. It is still not quite clear that coating ECM proteins on the surface is the major determinants for cell proliferation compared to nano structures. However, it is clear that surface protein could be the one of the major determinants in stem cell behavior such as migration and proliferation.

**Cell morphology and area**

Cell area also indicated significant increases in cell spreading in cells on a 70 nm patterned surface compared to cells cultured on the other surfaces pre-coated with collagen type IV (Figures 4(A)). This is correlated to the surface density of nano post of 70 nm sample. However, when the nanopatterned surface was pre-coated with collagen type I, the area of human MSCs on the nanopatterned surface showed no significant differences (Figures 4(B)). These results showed the similar patterns to cell proliferation assay. As Thagar et al. indicated in their micropattern study, the decrease in MSC proliferation could be due to the decrease in cell spreading and change in the mechanical force exerted on the cell. Micro- and
Morphological Changes of Human Mesenchymal Stem Cells on a Large Area of Nanopatterned Surface

Vol. 17, No. 1

nanotopologies affect cell proliferation and is expected to be closely connect with cell area. In order to confirm the area of human MSCs on nanopatterned surfaces precoated with collagen type IV, the actin filaments of human MSCs were stained. Actin filaments of human MSCs tended to be more spaciously spread on 70 nm-sized nanopatterns than other samples. Changing cell behavior by changing nanopatterns has been reported to be associated with the regulation of focal adhesion and signaling. However, the processes that mediate the cellular reactions to nanoscaled surface structures are not well understood.

Conclusion

In this study, we evaluated human MSC behavior on nanopatterned surfaces in combination with collagen types I and IV. Nanopatterned surfaces were fabricated with nanoimprinting with different column sizes (70-, 200- and 400 nm) and human MSCs were cultured on different nanopatterned surfaces adsorbed with different ECM proteins. Cellular activity was the highest in 70 nm patterns coated with collagen type IV. Based on these results, we concluded that adsorbed proteins on the nanocolumns are a major determinant that controls cellular behavior. The study of conformational changes of ECM proteins on nanostructures will be an important future topic of study for the nanobiology field.

Acknowledgements

This study was supported by the Health 21 R&D Project the Ministry of Health & Welfare of the Korean government (Contract grant number A120313).

Reference


