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Impact of selective fibronectin nanoconfinement on human dental pulp stem cells

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ABSTRACT

In this study, it was aimed to investigate the combinatory effect of biophysical and biochemical factors on human dental pulp stem cells' (hDPSCs) behavior. For this purpose, well-defined nanotopography of nanowells with two different pitch size of 109 nm and 341 nm were prepared on polyhydroxymethylsiloxane (PHMS) by using colloidal particles nanofabrication. The nanopatterned PHMS surfaces (PHMS/109 and PHMS/341) were subsequently used for fibronectin (Fn) adsorption. With this approach, nanotopographical details were combined with biochemical signals from Fn. Depending upon the size of cavities created by the nanowells, Fn molecules followed a site-selective adsorption. While they adsorbed both inside and outside the nanowells of PHMS/341, they preferred to adsorb outside the cavities of PHMS/109 surfaces. Human dental pulp stem cells were cultured on nanopatterned PHMS with or without Fn adsorption in the presence and absence of serum. Scanning electron microscopy and fluorescence microscopy analyses showed the interaction of cells was dependent on nanotopography size especially in serum-free medium. Furthermore, hDPSCs' morphology and cytoskeletal organization changed in correlation with preferential Fn adsorption. On Fn adsorbed PHMS/109 surfaces, cells displayed stretched bundles whereas, they showed extensive spreading and followed the Fn adsorbed sites inside the cavities of PHMS/341 surfaces. The observed effects are interpreted in terms of the preferential exposure of different Fn epitopes occurring on PHMS/109 and PHMS/341 as a consequence of the different hydrophilic/hydrophobic adsorbing surface.

1. Introduction

Stem cells are considered to be the most versatile and promising cell source in tissue regeneration [1,2]. Critical properties like differentiation into specific lineages and decreased immunogenity intensified the need to control the stem cell activity [3]. As a class of adult stem cells, human dental pulp stem cells (hDPSCs), identified within the dental pulp of third molars, show multi-lineage differentiation capacity including, adipogenic, osteogenic, chondrogenic and neuronal lineages [4–6]. These easily accessible stem cells have been identified to be bone-cell-like mechano-responsive in vitro which shall make them attractive especially for bone tissue engineering [4].

Both the biochemical and biophysical factors of the microenvironment have been reported to regulate the stem cell fate [1–3,7–9]. Among biophysical factors, surface topography with precise control, has been evidenced to influence stem cell behavior. In particular, the biomimetic, cell-modulating cues introduced by nano-scaled topography have been shown to influence cell shape, which is a potent regulator in cell growth and differentiation through a cytoskeletal-mediated mechanism [3,10–14]. The change in elongation and alignment of human mesenchymal stem cells (hMSCs) and human embryonic stem cells (hESCs) cultured on nanopatterned surfaces induced the differentiation into osteogenic and neuronal lineages [15,16]. Human MSCs were reported to switch their decision between adipogenic and osteogenic lineages by regulating their cytoskeletal network depending on the various

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densities of the nanotopographical cues [17]. Meanwhile, the biochemical factors contributing to the stem cell environment have been considered important in directing stem cell fate related to surface composition. Particular emphasis was based on the bioactive molecules of extracellular matrix (ECM) proteins available to interact with cell surface receptors to regulate cell adhesion and differentiation [18]. Fibronectin (Fn) is one of the major proteins present in the ECM promoting cell adhesion and spreading [19,20]. Role of Fn in inducing osteogenic differentiation of hMSCs has been reported on Fn treated tissue culture plates [18]. Furthermore, Fn was found to promote the differentiation of neural crest progenitors into smooth muscle cell phenotype [21].

Development of functional surfaces providing the combined effect of nanotopographical stimulation and bioactive surface composition is significant for manipulation of stem cell fate. However, few studies have been reported on this combinatory effect [22,23].

In this study, Fn adsorbed nanostructured surfaces were prepared by using a very simple and versatile method [24]. The technique uses nano sized colloidal particles to produce nano arrays on surfaces which enable selective protein adsorption [25]. Polystyrene particles were used to create hydrophilic nanowell arrays with different pitch size on hydrophobic polyhydroxymethylsiloxane (PHMS) surfaces. Further, Fn was selectively adsorbed on nanostructured PHMS. Human DPSCs were used to investigate the stem cell behavior on Fn nanopatterned PHMS surfaces. The distribution and preferential adsorption and/or specific epitope exposure of Fn on nanostructured surfaces were observed by atomic force microscopy (AFM). Cell culture studies were performed both in serum-containing and serum-free media with or without Fn adsorption on nanostructured surfaces. While the studies without Fn adsorption enabled us to evaluate the effect of nanotopography size on hDPSCs behavior, serum-free conditions allowed investigation of specific hDPSC–Fn interaction. The selective cell attachment and behavior of hDPSCs with respect to availability of the adsorbed Fn molecules on the material surface influenced by nanotopography were determined by scanning electron microscopy (SEM), immunofluorescence staining of cell cytoskeleton and focal adhesion points.

2. Materials and methods

2.1. Materials

Polyhydroxymethylsiloxane (PHMS, Accuglas-T-12B) was purchased from Honeywell (USA). Monopolished monocrystalline ⟨1 0 0⟩ silicon wafers and gold covered quartz sensors were obtained from Siegert (Germany) and QSense AB (Sweden), respectively. Aqueous solutions of colloidal carboxylated-polystyrene nanoparticles (PS-COO^-) with 109 and 341 nm diameters were supplied from Polyscience (Warrington, USA). Fibronectin (Fn, MW: ~466 kDa, $16.0 \times 11.0 \times 2.0\,nm^3$, IP: 5.4) [26] was purchased as lyophilized powder (Sigma, Germany) and solubilized in ultrapure (UP) water.

2.2. Preparation and characterization of homogeneous PHMS surfaces

Homogeneous PHMS surfaces with different surface properties were prepared as reported, previously [27]. Briefly, thin films of PHMS were spin-coated on either silicon wafers or gold covered quartz sensors at 3000 rpm for 60 s (P6700, Specialty Coating Systems, USA) to prepare untreated hydrophobic PHMS (PHMS-UT) surfaces (static water contact angle > 90°). The deposited polymer films were then modified by exposure to UV-O_3 for 15 min at atmospheric pressure in a Jelight Instruments (USA) apparatus (λ_{exc} of 185 and 254 nm) to obtain high surface energy substrates with hydrophilic character (static water contact angle < 10°). After UV-O_3 treatment the samples were washed several times with UP water and blow-dried under nitrogen gas to obtain treated hydrophilic PHMS (PHMS-T) surfaces. After modification, due to extensive polymer surface oxidation the original siloxane matrix was converted into [SiO_x] phases, containing a high percentage of Si—OH surface groups.

2.3. Preparation and characterization of nanostructured PHMS surfaces

Nanostructured surfaces with arrays of hydrophilic nanowell with different dimensions were prepared by combining simple spin coating and nanoparticle removal techniques [28]. Briefly, 50 μL suspension (5%, w/v in UP) of PS-COO^- nanoparticles (either 109 nm or 341 nm) was spin coated on UV-O_3 treated PHMS (PHMS-T) thin films to produce close-packed monolayer particles. Afterwards, 100 μL of PHMS was deposited on each substrate and spin-coated to obtain a hydrophobic PHMS thin film with low surface free energy. Subsequently, the PS-COO^- nanoparticles were removed from the surface by sonication in chloroform for 5 min. A schematic presentation of the procedure is presented in Fig. 1a.

The morphology of nanopatterned PHMS surfaces was characterized by using Atomic Force Microscopy (AFM, Nanoscope IIIA, Veeco Instr., USA). Images were acquired at room temperature, by using p-doped silicon tips operated in tapping mode with a nominal force constant of 40 N/m and a resonance frequency of 300 kHz.

2.4. Protein adsorption studies

The protein adsorption studies were performed at two stages. At the first stage either PHMS-UT or PHMS-T homogeneous surfaces (without nanowells) were used to investigate Fn adsorption characteristics by quartz crystal microbalance-dissipation (QCM-D) instrument (E1 from Q-Sense AB, Sweden). For this purpose, Fn was dissolved in phosphate buffer saline (PBS, pH: 7.4) at a concentration of 2.5×10^{-6} M. The QCM-D sensor covered by PHMS- either PHMS-T or PHMS-UT-was used as substrates. The measurements were performed inside a measurement chamber and the temperature was kept constant at 37 °C. After a continuous flow of PBS inside the chamber, the substrates were left to interact with Fn solution for 30 min of incubation time. The total mass of the adsorbed protein on the QCM-D sensor is calculated by using the Sauerbrey relation: $\Delta f = -C_f \Delta m$, where Δf is the measured frequency change (Hz), Δm is the area mass density of the film (ng/cm^2) and C_f represents the QCM sensitivity (≈0.171 Hz cm^2/ng at 5 MHz) [29,30].

At the second stage, the specific protein adsorption on nanopatterned PHMS surfaces was visualized by AFM. For this purpose, PHMS/109 and PHMS/341 surfaces were used as substrates (in 1 cm × 1 cm dimensions). Fn solution of 200 μL (dissolved in UP water at a concentration of 2.5×10^{-6} M) was placed on the surfaces and incubated for 30 min at room temperature. After several washing steps with UP water, the protein adsorption on nanopatterned surfaces was analyzed by AFM. Images were acquired at room temperature. All the measurements have been performed by using p-doped silicon tips operated in tapping mode with a nominal force constant of 40 N/m and a resonance frequency of 300 kHz.

2.5. Cell culture studies

Cell culture studies were carried out with human dental pulp stem cells (hDPSCs) isolated from third molars of healthy female individuals (aged 16–25). Briefly, the pulp tissue was pulled out with excavator, cut into pieces and digested in 0.075% (w/v) collagenase type I (Biochrom AG, Germany) in Ca^{2+}, Mg^{2+} free PBS for 1 h