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Influence of nanostructural environment and fluid flow on osteoblast-like cell behavior: A model for cell-mechanics studies

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ABSTRACT

Introducing nanoroughness on various biomaterials has been shown to profoundly effect cell-material interactions. Similarly, physical forces act on a diverse array of cells and tissues. Particularly in bone, the tissue experiences compressive or tensile forces resulting in fluid shear stress. The current study aimed to develop an experimental setup for bone cell behavior, combining a nanometrically grooved substrate (200 nm wide, 50 nm deep) mimicking the collagen fibrils of the extracellular matrix, with mechanical stimulation by pulsatile fluid flow (PFF). MC3T3-E1 osteoblast-like cells were assessed for morphology, expression of genes involved in cell attachment and osteoblastogenesis and nitric oxide (NO) release. The results showed that both nanotexture and PFF did affect cellular morphology. Cells aligned on nanotexture substrate in a direction parallel to the groove orientation. PFF at a magnitude of 0.7 Pa was sufficient to induce alignment of cells on a smooth surface in a direction perpendicular to the applied flow. When environmental cues texture and flow were interacting, PFF of 1.4 Pa applied parallel to the nanogrooves initiated significant cellular realignment. PFF increased NO synthesis 15-fold in cells attached to both smooth and nanotextured substrates. Increased collagen and alkaline phosphatase mRNA expression was observed on the nanotextured substrate, but not on the smooth substrate. Furthermore, vinculin and bone sialoprotein were up-regulated after 1 h of PFF stimulation. In conclusion, the data show that interstitial fluid forces and structural cues mimicking extracellular matrix contribute to the final bone cell morphology and behavior, which might have potential application in tissue engineering.

1. Introduction

Cellular behavior is a complex phenomenon, which depends on multiple factors acting simultaneously [1]. A major challenge in cell biology and biomaterials research remains the understanding of such complexity. Current experimental designs for testing cellular behavior on biomaterials usually choose single-parameter approaches, which are atypical for cells within tissues [2]. Bone tissue, in particular, contains cells that experience structural cues from the extracellular matrix (ECM), together with compressive or tensile forces due to mechanical loading and shear stress resulting from fluid flow [3–5]. Since there is a correlation between tissue geometry and normal tissue function [6], these forces contribute to the final bone tissue structure, which eventually leads to normal tissue function.

In this respect, atomic force microscopy (AFM) analysis on the morphology of the lacuna–canalicular system in cortical bone has revealed the presence of structural cues in the form of ridges and grooves, with a periodicity of 125 nm, formed by collagen fibrils. These ridges and grooves are especially visible in the non-mineralized small channels called canaliculi [7]. Also, dense collagen fibrils organized in parallel or random direction with a periodicity of 67 nm [8,9] are present on the exterior surface of trabecular bone. Such structural cues are sources of roughness that affects cellular morphology and behavior, and thus the overall tissue structure. Indeed, mimicking such structural components on biomaterials by introducing surface nanoroughness has been shown to have a profound effect on bone cell morphology and differentiation [10,11]. One important aspect of cell behavior in such organized structural environment has not yet been investigated, i.e., the influence of fluid-applied forces.

The fluid flow hypothesis proposes that, when bone is mechanically loaded, an interstitial fluid flow is created through the

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canaliculi [12]. When the interstitial fluid is squeezed through the channels, a shear stress is produced, acting on the surface of the osteocyte cell membrane. Theoretical extrapolation predicts that strain-induced flow in bone elicits shear stresses up to 3 Pa at 20–30 Hz [13], while real-time measurements of the shear stress at the osteocyte membrane approximate 5 Pa [14]. Thus, morphological shape adaptation of cells within bone tissue during the bone remodeling seems to occur not only as a result of structural contact guidance through the collagen fibrils, but also as a result of the cells sensing the shear stress arising from the mechanical-loading-induced interstitial fluid flow. Similarly to substrate texture, it has been shown in vitro that fluid flow can trigger cell alignment [15] and modulate gene expression of proteins involved in proliferation and differentiation processes through a variety of cell-signaling pathways involving β-catenin/wnt, integrins and the ERK5 pathway, growth factor synthesis, nitric oxide (NO) synthesis and integrin-cytoskeletal interactions [3,16,17].

One recent study combining specific substrate nanoislands and oscillatory fluid flow has already shown enhanced mechanosensitivity when such dual stimulation for non-osteoblastic cells is used [18]. Thus, to further understand cellular behavior on biomaterial surfaces, the aim in the current study was to develop an experimental setup combining a nanogrooved substrate with PFF and study osteoblast-like behavior. The hypothesis was that the predetermined parallel cell alignment arising from the nanogrooved substrate will be affected by fluid flow. Moreover, it was hypothesized that both parameters can act together and influence the final cellular morphology. Thus, the effects of such different experimental conditions were compared on cell behavior in terms of morphology, NO production and genes involved in cell attachment, ECM formation and cell differentiation.

2. Materials and methods

2.1. Preparation of nanotextured substrates

Nanotextured groove and ridge templates were made on Prime quality 4″ silicon wafers using laser interference lithography [10]. A setup was used based on the Lloyd's interferometer, where a regular pattern was produced by interference of an incident laser beam and a mirror reflected beam [19]. The period of the interference pattern, and thus of the grating recorded in the resist layer on the substrate, is given by the equation $P = \lambda/(2\sin\theta)$, where the period ($P$) is determined by the wavelength (λ) of the beam source and the angle (θ) at which two coherent beams are interfering. With a 266 nm light source, nanogrooved patterns 200 nm wide and 50 nm deep were produced [20]. A tri-layer positive resist system was spin-coated on a silicon wafer consisting of a 13-nm-thick bottom antireflective coating DUV46 (Brewer Science, Derby, UK), a 140-nm-thick positive for polystyrene (PS) replication photosensitive polyvinyl-based resist PEK500 (Sumitomo Chemical, Tokyo, Japan), and a 5 nm top antireflective coating (Aquatar-6A, AZ Electronics, Wiesbaden, Germany) (Fig. 1). After illumination and development of the resist layer, the grating was transferred to the substrate by a reactive ion etching (RIE) process using a Plasmatherm 790 system (Unaxis, Utrecht, The Netherlands). An optimized method of RIE using parameters giving anisotropic etch profiles in nanoscale was used. Sulfur hexafluoride/oxygen ($SF6:O_2$) plasma chemistry gave well-defined structures transferred into the silicon substrate. Using this setup, highly regular patterns were produced. In this study, the wafers were not used directly, but served as templates to produce PS replicas for cell culture materials. Wafers with a planar (smooth) surface were used as controls. Thus, using the method of solvent casting, 0.5 g PS was dissolved in 3 ml chloroform, cast onto the silicon wafer and followed by overnight chloroform evaporation. The next day, the PS replicas were detached from the wafers in a water bath, cut in 6×3 cm substrates to fit in the fluid flow chamber. Before cell seeding, all experimental substrates received a plasma/radiofrequency glow-discharge (RFGD; Harrick, Ithaca, NY, USA) treatment for 5 min in argon gas at 10^{-1} bar for sterilization. The plasma treatment results in bombardment of the PS surface with argon ions, which involves alteration of the surface composition by bond breaking of the phenyl rings, inducing radical formation, but does not produce functionality or incorporation of new species on the surface. Thus, the treatment leads to an increase in surface hydrophilicity and subsequent protein and cell attachment [21].

2.2. Surface analysis with AFM

The groove dimensions of the nanopatterned PS replicas were routinely verified by AFM. A multimode AFM (Nanoscope IIIa, Veeco, Santa Barbara, CA, USA) with NanoScope Analysis software (version 1.20, Veeco) was used to confirm the surface topography of the nanopatterned replicas. Tapping in ambient air was performed with high aspect ratio NW-AR5T-NHCR cantilevers (NanoWorld AG, Wetzlar, Germany) with average nominal spring constants of 30 N m^{-1} [22]. Height images of each nanopattern were captured in ambient air at 50% humidity at a tapping frequency of ~250 kHz. The analyzed field was scanned at a rate of 0.8 Hz with 512 scanning lines.

2.3. Cell culture

The mouse MC3T3-E1 osteoblast cell line (ATCC#CRL-2593) was maintained in alpha Minimal Essential Medium without ascorbic acid (α-MEM; Gibco BRL, Life Technologies, Breda, The Netherlands) supplemented with 10% fetal bovine serum (FBS; Gibco) and 0.5 mg ml^{-1} gentamicin (Gibco). For all experiments, 1×10^6 cells were defrosted from passage 23, and seeded in cell culture flasks (T-75). When the cell monolayer became confluent, cells were detached using trypsin/EDTA (0.25% w/v trypsin/0.02% EDTA) (Sigma–Aldrich, Zwijndrecht, The Netherlands), seeded on nanotextured and smooth PS substrates at a density of 1×10^4 cells cm^{-2} and placed in a Petri dish containing osteogenic medium (α-MEM, 10% FBS, 10 mM sodium β-glycerophosphate, 10^{-8} M dexamethasone, 50 mg ml^{-1} ascorbic acid and 50 mg ml^{-1} gentamycin; all from Gibco). After overnight incubation to allow cell initial attachment, the substrates containing the cells were placed in the fluid flow chamber and subjected to different fluid flow regimes, as described below.

2.4. Pulsating fluid flow

A parallel-plate flow chamber and a recirculating flow circuit were used to introduce pulsating laminar fluid flow over the cells [23]. The flow chamber was fully characterized for the pulsating fluid flow (PFF) regimes that arise in such a experimental setup in a previous study [24]. Briefly, the circuit included a variable-speed roller pump, CO_2 influx and a reservoir with culture medium. PFF was generated by pumping medium through two parallel plates, divided by a space maintainer, as shown on Fig. 2. A flow rate was chosen using the formula for shear stress $t = 6Q\mu/bh^2$, where t is wall shear stress (Pa), Q is the flow rate ($cm^3\ s^{-1}$), μ is the viscosity (dynes $s^{-1}\ cm^{-2}$), h is the channel height (cm), and b is the channel width (cm). The present setup used $\mu = 0.0078$ dynes $s^{-1}\ cm^{-2}$, $b = 2.4$ cm, $h = 300$ μm. First, cell morphology on the smooth surface and cell alignment on the grooved samples were assessed in the absence of fluid flow. Subsequently, fluid flow was applied, and the time of flow was increased from 1 to 5 h to observe changes in cell alignment on the smooth surface.