

Enhanced proliferation of osteoblast cells on mechanically deformed elastic membranes

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ABSTRACT

This paper investigates the effect of transmission of forces on bone cells attached to a deformable membrane. A silastic membrane was functionalized and coated with ECM protein, fibronectin, FN. MC3T3-E1 osteoblast-like cells were cultured on the functionalized FN-coated membrane after which cellular attachment and proliferation were evaluated. An immediate cellular attachment and proliferation on the functionalized surfaces were observed. Upon the application of a dynamic equibiaxial strain of 2% magnitude at 1Hz frequency for 2 hours, this resulted in a slightly elevated phalloidin fluorescence which suggested that there had been a reorganization of cell cytoskeleton. It looks like the engineered surfaces transduced applied mechanical forces directly to the adherent osteoblast-like cells resulting in the enhanced cellular attachment and proliferation.

Keywords: elastic membrane, mechanical, deformation, osteoblast cells, attachment, proliferation.

1 INTRODUCTION

Bioactive substrates are capable of enhancing adhesion and proliferation of osteoblast cells. The reactivity of such surfaces and their interfacial adhesion to bone cells are attributed to the nature and type of surface modifications as well as the functionalized layers attached to the bioactive materials. Bone cells transduce changes in the mechanical environment through adhesion molecules that link them to the ECM. This paper investigates the effect of mechanical stimulus and substrate surface characteristics on osteoblast attachment and proliferation. This publication is a fundamental study in the process of mechanotransduction in bone cells that are seeded on modified bioactive substrates.

Minimal strains are reported to occur in bone cells as a result of the application of mechanical stresses under physiological conditions [1]. While some studies have suggested, that strain rate correlated with bone formation [6], others had suggested that mechanical forces were transmitted to cells through the ECM [2, 3]. In a recently published data, it was suggested that the ECM-cell surface

receptors, called the integrins, acted as mechanoreceptors [4]. The authors, in a study that focused on the mechanosensitivity of human bone derived cells, demonstrated that response of human osteoblast-like cells depends on the frequency and cycle number of the proliferative cells [5]. The authors further concluded that those two effects were interrelated. In a more recent study [6], some investigators reported that short periods of applied physiological mechanical stresses induced immediate early gene expression and growth in MC3T3-E1 osteoblasts cells.

Studies in mechano-stimulation of bone cells have often used systems that included cell culture with controlled delivery of a mechanical input like hydrostatic pressure, fluid shear stress, and a substrate strain [7]. The apparatuses that were devised in the laboratory for such studies included a variety of complex systems that featured mechanical inputs of varied degrees of precision and homogeneity although early efforts in cell culture mechanostimulus were of a nonquantitative nature.

Techniques for mechanical stimulation of cells that were used in the past, included an application of hydrostatic pressure, axial compression, longitudinal mechanical straining, out-of-plane systems, and fluid shear stress systems. Special purpose systems were also developed to study the combined effect of fluid stress and substrate deformation concurrently [8]. Authors, in a most recent study, however, confirmed that although mechanical stress was essential for the survival of cells and the maintenance of tissues, the mechanisms of cellular response to mechanical stress were not fully elucidated due to the diversity of mechanical stresses and mechanosensors [9].

In this study, we designed a system that enhanced the attachment and proliferation of bone cells to a deformable membrane. The objectives of this study were to anchor osteoblast cells to a substrate that was chemically functionalized and then modified with protein molecules; and to ascertain whether the combined effect of substrate modifications and applied equibiaxial mechanical strain could cause a change in the cytoskeletal architecture of the adherent bone cells.

2 MATERIALS AND METHODS

The silicon membrane on which the cells were seeded was first functionalized by exposure to ultra violet (UVO) radiation. They were then characterized using the established surface characterizations tools such as contact angle goniometry, atomic force microscopy (AFM), and Rutherford backscattering spectroscopy (RBS). MC3T3-E1 osteoblast-like cells were then seeded onto the biomimetic surface, after which the evaluation of the cellular function was done to determine changes in the cytoskeletal organization of the adherent cells.

2.1 Characterization of Silicone membranes

Silicon membranes of 0.005 inches thickness and 2.5 inches cross-sectional diameter, were functionalized, coated with FN, and then cultured in cells, prior to the application of mechanical strain. They were exposed to 10 to 30 min UVO radiation, in order to functionalize and oxidize the surfaces. This resulted in the formation of active hydroxyl (-OH) groups on the surfaces of the membranes. These surfaces were, therefore made hydrophilic by exposure to UVO radiation.

The functionalized surfaces were characterized by means of contact angle goniometry, using the sessile drop method. Roughness analysis of the silicone surfaces were performed using AFM techniques under ambient conditions. The mean surface roughness, denoted by Ra (in nm) of both the nontreated and functionalized surfaces were measured.

Rutherford backscattering spectrometric analysis was done on the surfaces to quantitatively determine the elemental composition and the depth profile of the Si, O and C elements on the sample surfaces.

2.2 Adsorption of FN and cell culture

Both the functionalized and the nontreated silicone membranes were incubated in a 2.5 µg/mL concentration of FN at 37°C for 1 h to attain a monolayer FN surface coverage.

MC3T3-E1 osteoblast like cells that were maintained in 22 mL DMEM medium, were passaged once a week and fed every other day with DMEM. The cells were cultured at 37°C in a humidified atmosphere containing 5% CO₂.

2.3 Cellular response to Mechanical Stimulus

The system that was designed and characterized to enable to apply mechanical strain to a cell culture. It was made up of an a device capable of deforming a compliant substrate as well as generating mechanical strains that could be reproduced. This system, thus, mimicked conditions that prevailed *in vivo*.

A cell stretching device that incorporated the four-point bedding system was fabricated at the Department of

Anatomy and Cell Biology, School of Dental Medicine, the University of Pennsylvania. This device, provided a sinusoidal waveform and an equibiaxial stress of 0.2 to 2.0 Hz for 2 h under an atmosphere of 100% humidity comparable to physiological conditions. It was capable of deforming a compliant substrate in order to generate a reproducible mechanical strain. A load application of 1000 Pa produced strain regimes of up to 2500 µε in all four directions. The cells were seeded at concentrations of 100,000 cells/cm² for 24 h, after which the membranes were subjected to a uniform equibiaxial strain. The experiments were terminated after 2 h and the cellular responses were evaluated. In the control experiment, the cells were seeded on membranes prepared, as previously described but not stretched.

Actin filaments were visualized by treatment with phalloidin after which cells were analysed with a confocal microscope. In order to quantify the cells, a plane of maximum fluorescence was determined by means of a photomultiplier tube voltage.

The data obtained were replicated. Besides, the data was analyzed using one way ANOVA where appropriate, with Scheffe's test at level 0.05. Cell incubation times were also statistically analysed.

3 RESULTS

The procedures for treating the silicone membranes were optimized in order to maintain their elastic properties. Initial observations that were made indicated that there were very minimal alterations to the physical properties of the membrane, that is, there was no evidence of surface deformations after the elastic membranes were subjected to equibiaxial strain.

Data also showed a steady decrease in surface wettability of the membranes. There was up to 20° reduction in the surface wettability after the membranes were subjected to 10 min of UVO radiation. Longer period of exposure times, however, did not further reduce the hydrophobic nature of the membranes.

RBS analysis of surfaces showed similarities in their surface profiles, while AFM imagery showed a steady increase in the surface roughness at increasing UVO treatments (Table 1). Similarly the surface density of FN coatings on the functionalized surfaces indicated a nanoscale monolayer coverage of the proteins of up to 150 ng/cm². There was generally a significant decrease in roughness on all three surfaces after adsorbing 2.5 µg/mL FN (Fig. 1).

The cytoskeletal organization of the MC3T3-E1 cells that were evaluated after the application of the dynamic equibiaxial strain displayed a significant level of increased actin fluorescence, especially at the cell periphery (Fig. 2). The fluorescence intensity was however, generally lower than the level displayed on stretched cells.

4 TABLES AND ILLUSTRATIONS

UVO radiation Time (min)	UVO + 0 ug/mL FN Ra (nm)	UVO + 0 ug/mL FN RMS (nm)	UVO + 2.5 ug/mL FN RMS (nm)
0	1.2	1.4	0.9
10	1.9	2.4	1.5
20	2.8	3.6	2.3
30	4.7	5.9	3.7

Table 1: The RMS surface roughness values obtained by AFM measurements on non – UVO/UVO – activated silicone membrane surfaces with and without adsorbed 2.5 ug/mL FN molecules..

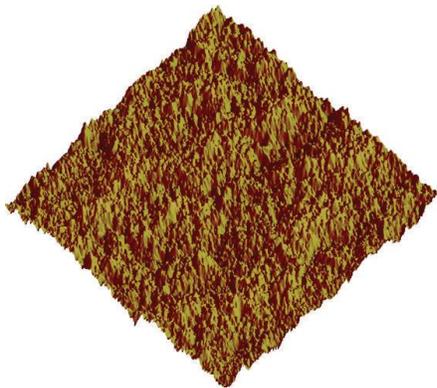
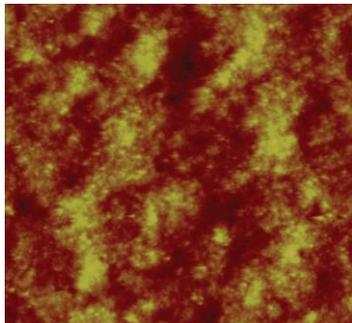


Figure 1: Representative AFM images of a 30 min UVO-activated Si membrane surface. Roughness analysis on a 10µm scan size of the images indicated that the Surface roughness (Ra) on these surfaces was 4.7nm; while the RMS roughness value was 5.9 nm [two different orientations of the same sample].

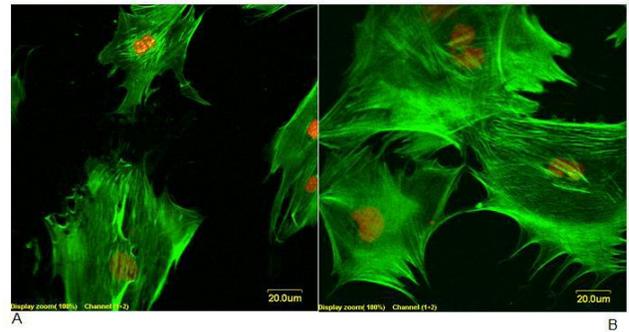


Figure 2: Phalloidin-stained actin filaments of osteoblasts grown on silicone membranes. MC3T3-E1 osteoblast cells were seeded on FN-coated silicone membrane and subjected to equibiaxial strain for 2 h. Cells were treated with rhodamine-labeled phalloidin (1:100) and then visualized by confocal microscopy. (A) Cells on: *non-stretched* UVO-activated FN-coated silicone membrane; (B) Cells on: *stretched*, 30 min UVO-activated FN-coated silicone membrane. Note the slightly elevated fluorescence of the actin filaments in the stretched sample.

5 DISCUSSION

The application of biophysical forces, according to the Frost mechanostatic theory, translates into cellular response. That is, the cellular organisms tend to adapt to their mechanical environment.

The functionalized silicone-biomolecule substrate engineered in this study promoted cellular attachment and proliferation, which was in conformity with data that has been reported in previous studies [10]. After subjecting the osteoblast-like cells to the dynamic equibiaxial strain regimes via the functionalized FN-coated silicone membranes, there were noticeable changes in their cytoskeletal architecture with minimum cell damage. The data we obtained further indicated that modified elastic membrane surfaces transduced mechanical stimuli onto osteoblast-like cells, through integrin receptors that are present in FN.

We have therefor engineered a system which employs functionalization techniques to activate surfaces of elastic membranes, like silicone, to achieve the linkages of various biomolecules, proteins and cells. Furthermore, the adherence and proliferation of osteoblast-like bone cells were seen to be more enhanced on strained, functionalized Si-RGD (Fn molecules) surfaces. As a result of the elastic characteristics of the functionalized Si surfaces, further mechanisms whereby bone cellular functions could be physiologically influenced through the applications of mechano-physical strain regimes, can be further explored.

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