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Relative influence of substrate stiffness on osteogenic potential of human adipose-derived mesenchymal stem cells

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Introduction

Mesenchymal stem cells (MSCs) are multipotent adult stem cells with a high capacity for self-renewal. So far, MSCs have been isolated from several human tissues, e.g. bone marrow, adipose tissue, umbilical cord matrix, tendon, lung and periosteum. Among all types of MSCs adipose-derived cells provide many advantages for tissue engineering and in particular, for bone formation, due to their abundance in supply, readily and safely accessibility and immense osteogenic potential. Although many researchers' efforts to understand the mechanisms of osteogenic differentiation, what the factors driving the differentiation of MSCs into osteoblasts are, still remains largely unknown. It is now appreciated that not only the soluble factors but also the physical properties of the scaffold, such as roughness, micro- and nano-topography, surface energy, and porosity influence stem cell commitment. In the recent years substrate stiffness is recognized as a 'passive' mechanical cue that tends to be more selective than soluble factors in regulation of stem cell lineage specification. Therefore, in this study we investigated the effect of substrate stiffness on osteogenic potential of human adipose-derived mesenchymal stem cells (AD-MSCs) using flexible fibronectin-coated polydimethylsiloxane (PDMS) materials. Varying the amount of the crosslinking agent we prepared PDMS substrates with different Shore hardness and have characterized their surface properties and efficiency of stem cell adhesion, proliferation, fibronectin matrix secretion and osteogenic potential.

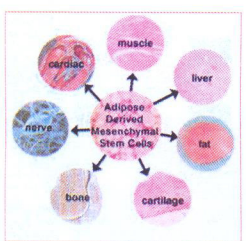


Fig.1 Mulipotential differentiation of adipose derived mesenchymal stem cells /prolotherapy.com/

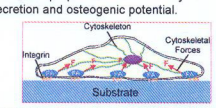


Fig.2 Mechanotransduction- the process by which cells convert mechanical stimuli into a chemical response /Breuls et al. The Open Orthopaedics Journal, 2008, 2, 103-109/

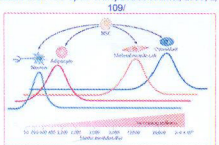


Fig.3 Role of the substrate stiffness in differentiation of mesenchymal stem cells /Halder et al. Nature Reviews Molecular Cell Biology, 2012, 13, 591-600/

Experiments

Synthesis of polydimethylsiloxane elastomers

Polydimethylsiloxane materials were prepared with three different Shore hardness (40, 55 and 70) using two-component siloxane rubber (KCC Corporation, Korea). The two compounds, prepolymer and crosslinker, were mixed in an extruder in different ratio in order to achieve materials with different hardness. After their polymerization the extruded elastomeric sheets were vulcanized at 180° C for 15 minutes. For cell culture experiments the samples were cut and sterilized in an autoclave.

Physico-chemical characterization of materials

Surface nanotopography of the samples was observed with an atomic force microscope (AFM, Easyscan 2, Nanosurf, Switzerland).

Water contact angle of the samples was measured by the sessile drop method under ambient conditions. Drop's profile was recorded by the drop analyzer (Easy Drop FM40, Kruss, Germany).

The elastomers' surface hardness was evaluated by the Depth Sensing Indentation method. The microindentation tests were performed by Dynamic Ultramicrodurometer (DUH -211 S, Shimadzu, Japan).

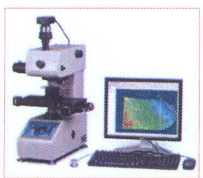


Fig.4 Microhardness testing system

Cell experiments

Cell culture

Human AD-MSCs were cultured in DMEM (Invitrogen, USA), supplemented with 10% fetal bovine serum (Invitrogen, USA) at 37° C and 5% CO_2 in a humidified incubator. For the experiments cells were seeded at an initial concentration of $2x10^4$ cells/ml on the elastomers, pre-coated with 50 µg/ml fibronectin.

Cell adhesion

Initial adhesion of stem cells to the PDMS materials was characterized in respect to their overall morphology, development of actin cytoskeleton and focal adhesion complex after 2 hours of incubation in serum-free medium. Formation of focal adhesion complexes in fixed and permeabilized cells was investigated by immunostaining of vinculin. Actin cytoskeleton was visualized with phalloidin. Micrographs were taken by Axio Observer Z1, Zeiss, Germany.

Cell proliferation

Proliferation of human AD-MSCs was assessed at the 1st, 3rd and 7th day of incubation of the cells on PDMS materials using Cell Counting Kit (CCK-8, Sigma-Aldrich, Germany). The amount of formazan produced is directly proportional to the number of living cells.

Fibronectin matrix secretion and organization

After 24 hours of incubation of human ADMS cells on PDMS elastomers cells were fixed, permeabilized and stained with primary polyclonal anti-fibronectin antibody (Abcam, USA) followed by secondary anti-rabbit IgG antibody (Invitrogen, USA). Cell nuclei were stained with Hoechst (Invitrogen, USA).

Osteogenic differentiation

Osteogenic potential of stem cells was determined by Alizarin Red S staining for detection of calcium deposition in the extracellular matrix after 21 days of cell culture on the elastomers in osteogenic medium. Calcium forms an Alizarin Red S-calcium complex in a chelation process, and the end product is orange-red and can be measured spectrophotometrically at λ=550nm.

Results

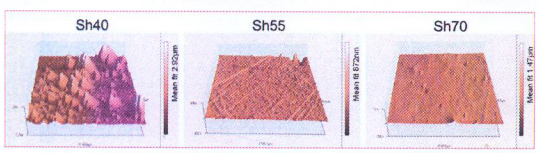


Fig. 5 AFM topographic images of PDMS elastomers with different hardness

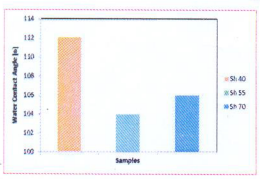


Fig. 6 The mean water contact angle values of PDMS elastomers with different hardness

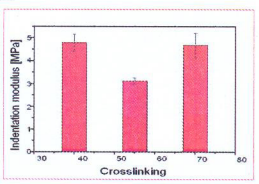


Fig. 7 Indentation elastic modulus of the PDMS elastomers as a function of the crosslinking

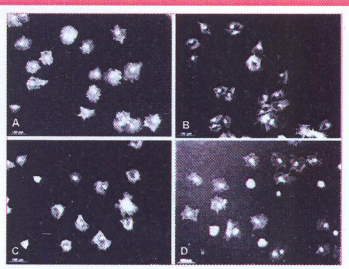


Fig. 8. Adhesion of hADMSCs cultured for 2 hours on fibronectin-coated PDMS elastomers with different hardness: A) Cover glass, B)Sh40, C) Sh56 and D) Sh70; Bar 100 µm

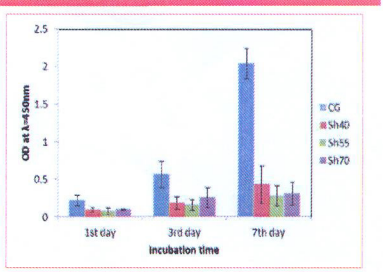


Fig. 9 Proliferation of hADMSCs on PDMS elastomers, determined using CCK-8 assay

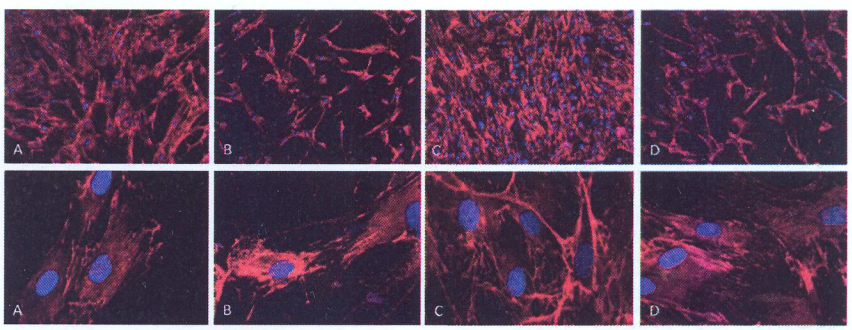
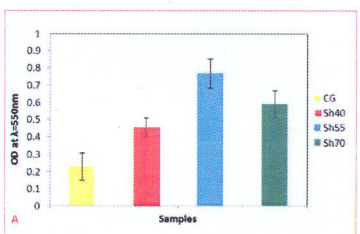


Fig. 10 Secretion and organization of fibronectin matrix by hADMSCs cultured for 24 hours on PDMS elastomers with different hardness: A) Cover glass, B)Sh40, C) Sh55 and D) Sh70; cell images upper panel magified x10, lower panel magified x63.



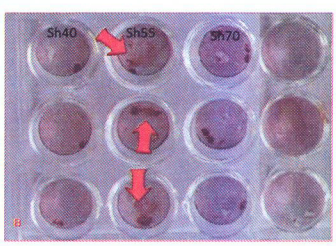


Fig. 11 Calcium deposits of hADMSCs cultured for 21 days in osteogenic medium on PDMS elastomers with different hardness, determined A) Spectrophotometrically, B) Macroscopically

Summary

Surface characterization of the materials

PDMS materials with different Shore hardness were characterized in respect to their surface topography, wettability and surface elastic modulus.

AFM images showed that materials surface became smoother with increasing the Shore hardness, respectively the amount of the crosslinker.

Water contact angle (WCA) measurement revealed that the PDMS elastomers were highly hidrophobic with WCA varying between 104° and 112°. When the amount of crosslinker, respectively the hardness, was increased the materials' hydrophobicity decreased. The least hydrophobic was PDMS with Sh55 with WCA 104°.

Indentation elastic modulus measurement demonstrated that the surface of the elastomer with 55 Shore hardness was the softest.

Cell behaviour

Cell adhesion studies showed that on Sh55 material the cells were homogeneously distributed, well spread with well developed actin stress fibers and focal adhesion contacts. On Sh40 the cells were smaller, tended to make aggregates and actin fibers were observed mainly at the cell periphery. On Sh70 the cells tended to round up and actin was less organized.

Proliferation ability of human ADMSCs after one week incubation on PDMS was significantly suppressed compared to the control. Among all elastomers cell growth was highest on PDMS with Shore hardness 40. Results from staining of FN matrix and calcium deposits showed that on Sh55 the cells synthesized, organized and mineralized the matrix in the greatest degree.

Conclusions

Our results demonstrated that material's stiffness had an influence on the osteogenic potential of human AD-MSCs suggesting that biomaterials with different stiffness can be used to control the differentiation of MSCs into osteoblasts. We found that among PDMS materials with three different hardness the material with intermediate hardness (Sh55) possessed the greatest osteogenic potential because it was shown to accelerate matrix deposition and mineralization in human AD-MSCs. Varying the amount of the crosslinker, however, altered not only bulk properties, e.g. the hardness, but also the surface properties including surface topography, wettability as well as surface elastic modulus. Therefore, the effect of substratum stiffness on stem cell behaviour is very complicated and it is dependant on material' surface properties that should be taken in account in the developing of biomaterials for tissue engineering application.

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