Materials Science and Engineering C 31 (2011) 357–362

Contents lists available at ScienceDirect

Materials Science and Engineering C

journal homepage: www.elsevier.com/locate/msec

Differentiation of human mesenchymal stem cells on nano- and micro-grain size titania

A.J. Dulgar-Tulloch [a,c,*], R. Bizios [b,c,1], R.W. Siegel [a,c]

[a] Department of Materials Science and Engineering, Rensselaer Polytechnic Institute, Troy, NY 12180, United States
[b] Department of Biomedical Engineering, Rensselaer Polytechnic Institute, Troy, NY 12180, United States
[c] Rensselaer Nanotechnology Center, Rensselaer Polytechnic Institute, Troy, NY 12180, United States

ARTICLE INFO

Article history:
Received 19 May 2010
Received in revised form 1 September 2010
Accepted 16 October 2010
Available online 21 October 2010

Keywords:
Mesenchymal stem cells
Stem cell differentiation
Titania
Nanophase
Material chemistry
Topography

ABSTRACT

The current study investigated the osteogenic differentiation of human mesenchymal stem cells cultured on titania surfaces with grain sizes ranging from 50 to 1500 nm in either control or osteogenic medium. Characterization of osteogenic differentiation included quantification of the osteopontin and alkaline phosphatase expression by the cells, as well as of the content of calcium in the extracellular matrix. Mesenchymal stem cell differentiation was not observed on any of the grain sizes tested without dexamethasone and osteogenic-stimulating chemical agents (specifically, ascorbic acid and beta-glycerolphosphate) in the culture medium. Little-to-no mesenchymal stem cell differentiation was detected on the 50 nm substrates under osteogenic media. In contrast, osteogenic differentiation occurred earlier, and to greater extent, on the 200 nm grain size titania, compared to results obtained on either the 50 or 1500 nm grain sizes, or the glass (reference) surfaces, under osteogenic media. These results demonstrated that biomaterial substrate topography, such as ceramic grain size, affects mesenchymal stem cell differentiation in a size-dependent but, non-linear, manner.

1. Introduction

Adult mesenchymal stem cells (MSCs) are spindle-shaped, anchorage-dependent, multipotent cells that reside in bone marrow, adipose, and other tissues and give rise to such lineages as adipocytes, skeletal and smooth muscle myocytes, chondrocytes, fibroblasts, and osteoblasts [1,2]. Due to their continued presence in adults and their lineage plasticity, MSCs are the focus of considerable research for tissue engineering and regenerative medicine applications. To date, moderate success has already been achieved using MSCs for repairing critical-size defects in bone [3–5], cartilage [6,7], and tendons [8].

In order to realize the full potential of human mesenchymal stem cells (HMSCs) in tissue engineering, however, improved methodologies to isolate, expand, and maintain HMSCs *in vitro*, as well as to direct their differentiation both *in vitro* and *in vivo* using chemical and physical stimuli, must be developed. Toward this purpose, the ability of substrate topography to mediate functions of anchorage-dependent cells such as HMSCs is of particular interest. Previous studies have demonstrated that material topography (specifically, ceramic surface features in the nanophase regime) can enhance the adhesion and proliferation of osteoblasts [9–11] and fibroblasts [11,12], which are mesenchymal stem-cell-derived lineages. Similar trends of enhanced adhesion and proliferation were reported for HMSCs cultured on nanophase ceramics [11], but to date the effect of substrate surface topography on mesenchymal stem cell differentiation has not been systematically investigated.

As such, the current study hypothesized that nanoscale topography of material substrates may mediate HMSC differentiation and investigated the effect of ceramic grain size on the osteogenic differentiation of HMSCs cultured on titania surfaces of 50, 200, and 1500 nm grain sizes under select cell culture conditions.

2. Materials and methods

2.1. Materials

Titania powder from Nanophase Technologies Corporation was consolidated into cylindrical substrates (12.5 mm in diameter and 2 mm in height) via uniaxial loading and sintered for 2 h each at 600 °C, 850 °C, and 1100 °C to produce substrate surfaces with grain sizes of 50 nm, 200 nm, and 1500 nm, respectively. After sintering, all substrates were cleaned sequentially by sonication in acetone, 95% ethanol/5% water, and deionized water. Borosilicate glass coverslips (Fisher Scientific; 12 mm diameter) were used as a reference substrate because glass is a well-characterized material surface that has been used for cell culture and the study of various cell functions. In addition, because glass is an amorphous material, the potential of

* Corresponding author. Now at: Biosciences, GE Global Research Center, 1 Research Circle, K1-5B28A, Niskayuna, NY 12309, United States. Tel.: +1 518 288 8550; fax: +1 518 387 7765.
E-mail address: dulgar@research.ge.com (A.J. Dulgar-Tulloch).
[1] Now at: Department of Biomedical Engineering, The University of Texas at San Antonio, San Antonio, TX 78249, United States.

0928-4931/$ – see front matter © 2010 Elsevier B.V. All rights reserved.
doi:10.1016/j.msec.2010.10.010

grain size effects in the reference substrate is eliminated. These coverslips were etched in NaOH (1 N) for 1 h, cleaned with acetone and ethanol, and used as a reference surface for the select cell interactions and functions of interest to the present study. All substrates were sterilized in a steam autoclave, dried at 120 °C for 8 h, and stored under sterile conditions until use in cell experiments.

2.2. Titania surface characterization

Titania surfaces were polished using standard techniques and thermally etched for 30 min at their respective sintering temperatures. The resulting substrates were coated with 15 nm of carbon (Denton Vacuum BTT-IV) and visualized under field emission scanning electron microscopy using a FEG SEM 6330F (JEOL).

2.3. Human adult mesenchymal stem cell (HMSC) culture

HMSCs from the bone marrow of adult subjects were obtained commercially (Cambrex) and used without further characterization. These cells were cultured in HMSC-maintenance medium (Cambrex), maintained under standard cell culture conditions (that is, a humidified 95% CO_2/5%O_2, 37 °C environment) and used at passages 3–5.

2.4. HMSC differentiation

HMSCs (6000 cells/cm^2) were seeded on the titania substrates and allowed to adhere under standard cell culture conditions for 24 h. At that time, the supernatant medium was replaced with one of the following two options: either (i) "control medium," that is, HMSC-maintenance medium (Cambrex) supplemented with 10 mM β-glycerolphosphate and 50 μg/ml ascorbic acid, which provided all necessary growth factors for HMSCs, but did not include chemical compounds that forced the cells to differentiate; or (ii) "osteogenic medium", that is, control medium supplemented with a proprietary quantity (Cambrex) of dexamethasone, to induce osteogenic differentiation of HMSCs. The respective supernatants were replaced with fresh medium every 2–3 days. The HMSCs were cultured under the aforementioned conditions for 4, 7, 14, 21, and 28 consecutive days. These specific prescribed time points were used throughout the present study unless noted otherwise.

2.5. Evaluation of adipogenesis, osteogenesis and chondrogenesis

HMSCs were stained for adipogenesis with Oil Red O according to standard techniques after 7, 14, 21, and 28 days of culture. Briefly, HMSCs were fixed in 10% buffered formalin for 15 min. Oil Red O (Sigma-Aldrich) in isopropanol was then diluted in deionized water and added to the fixed cells for 5 min. Counter staining was performed using Harris hematoxylin for 1 min and the resulting samples were visualized under light microscopy. This procedure, which results in lipids staining red and cell nuclei staining blue, was used to detect the presence of fat vacuoles in cells differentiating along the adipogenic pathway.

HMSCs were stained for osteogenesis and chondrogenesis using immunohistochemistry after 7, 14, 21, and 28 days of culture. Briefly, HMSCs were gently washed with phosphate buffered saline (PBS), fixed with 10% buffered formalin for 10 min, permeabilized with 1% Triton-X 100 for 15 min, blocked with 8% bovine serum albumin (BSA) in PBS for 1 h, washed again with PBS, and incubated with primary antibody (either AB758 goat anti-human type I collagen or AB761 rabbit anti-human type II collagen; Chemicon International) in 1% BSA at room temperature for 1 h. These preparations were then washed in PBS, incubated with the appropriate secondary antibody (either Ap106F rabbit anti-goat IgG fluorescence or AP132R goat anti-human IgG rhodamine; Chemicon International) in 1% BSA at room temperature for 1 h, washed in PBS, and either visualized immediately, or stained with DAPI and then visualized, using fluorescent (E600; Nikon) and/or confocal fluorescent (LSM 510; Confluent) microscopy with UV excitation and DAPI, fluorescence, and rhodamine emission filters.

2.6. CyQuant cell quantification

At the prescribed time points cell number was quantified by mixing 100 μl cell lysate (prepared using cells from each substrate tested) with 100 μl of CyQuant GR dye (CyQuant; Molecular Probes) and maintaining at room temperature in the dark for 5 min. The results of fluorescence reading of each sample (excitation of 480 nm, emission of 520 nm) were expressed as cell number by comparison to a standard curve prepared from a serial dilution of one million HMSCs and run in parallel with the experimental samples.

2.7. Osteopontin expression

At the prescribed time points the supernatant medium from each sample of interest in the present study was supplemented with 10 μl of protease inhibitor (Complete Mini, EDTA-free; Roche Diagnostics) and frozen at −70 °C until samples from all time intervals were collected.

After thawing, the osteopontin content in these samples was quantified using TiterZyme EIA (Assay Designs, Inc.) following the manufacturer's instructions. For each analysis, optical density was converted to concentration of osteopontin (ng/ml) by comparison to a standard curve of known osteopontin concentrations run in parallel with the experimental samples. Total values of osteopontin concentration per sample were obtained by normalizing against the respective total cell number, as determined by using the aforementioned CyQuant cell quantification technique.

2.8. Alkaline phosphatase activity

At the prescribed time points cells adherent on the titania substrates were gently washed with PBS and frozen at −70 °C. When all pertinent samples had been collected, they were thawed and lysed using CyQuant lysis buffer (Molecular Probes) for 15 min. Each lysate was collected and developed with p-nitrophenyl phosphate (1:1 v/v) at 37 °C for 45 min. Light absorbance of these samples was determined spectrophotometrically at 405 nm, converted to units of alkaline phosphatase (using a standard curve obtained from a range of known alkaline phosphatase concentrations and run in parallel with the experimental samples), and normalized against their respective cell numbers determined by the aforementioned CyQuant cell quantification technique.

2.9. Calcium content

Accumulation of calcium in the extracellular matrix was quantified at 14, 21, and 28 days of HMSC culture. Briefly, after removal of the supernatant medium and cell lysis, the respective matrices were washed with PBS and the calcium present was dissolved via exposure to 0.5 M HCL at 4 °C for 12 h. The resulting calcium-rich solution was centrifuged (at 1700×g for 10 min). The calcium content (in respective 10 μl aliquots) was quantified using a commercially available kit (Sigma-Aldrich). Baseline levels of calcium present in each type media (without cells) used in the present study were determined at the aforementioned time points and were subtracted from the respective values obtained in the presence of HMSCs.

2.10. Data analysis

All experiments were run in duplicate and repeated at three separate times. The resulting numerical data were averaged and