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Effects of pseudowollastonite ($CaSiO_3$) bioceramic on *in vitro* activity of human mesenchymal stem cells

Nianli Zhang [a], James A. Molenda [b], John H. Fournelle [c], William L. Murphy [a,b,d,e], Nita Sahai [a,c,*]

[a] Materials Science Program, University of Wisconsin, Madison, WI, USA
[b] Department of Biomedical Engineering, University of Wisconsin, Madison, WI, USA
[c] Department of Geoscience, 1215 W. Dayton St., University of Wisconsin, Madison, WI, USA
[d] Department of Orthopedics and Rehabilitation, University of Wisconsin, Madison, WI, USA
[e] Department of Pharmacology, University of Wisconsin, Madison, WI, USA

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This work is dedicated to the memory of Prof. Michel Anseau, University of Mons-Hainaut, Belgium.

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ABSTRACT

We report the effects of two pseudowollastonite (β-$CaSiO_3$) substrates on the attachment, viability, proliferation and osteogenic differentiation of human mesenchymal stem cells (hMSCs), and provide detailed mechanistic links of surface texture, soluble factors and culture media to cell activities. Cell attachment and viability were lower for psWf (fine-grained, roughness 0.74 μm) than for psWc (coarse-grained, roughness 1.25 μm) surface, and were ascribed to the greater specific area of the finer psWf particles resulting in higher release rate of Si, which is cytotoxic at high levels. Interestingly, proliferation was greater on psWf. Osteogenic differentiation occurred on both surfaces, indicated by calcium phosphate bone nodule formation and by osteocalcin, osteopontin and core-binding factor alpha-1 gene expression. Gene levels were lower on psWf than on psWc at day 8 in growth medium, explained by differences in Ca and/or Si concentrations between the two surfaces. Similar gene expression on both surfaces at day 16 in both growth and osteogenic induction media was attributed to pro-osteogenic effects of Ca and P at specific concentrations and complementary Ca and P levels on the two surfaces. In summary, soluble factors from substrates may be more important for osteogenic differentiation in growth medium than small surface roughness variations within a factor of 2. Optimum concentration ranges exist for individual soluble factors to balance cell toxicity/growth versus osteogenic differentiation, and soluble factors together have complex, cooperative or opposing, effects on a given cell activity.

1. Introduction

Human mesenchymal stem cells (hMSCs) have generated much attention in tissue engineering because of their high ability to proliferate and multilineage differentiation potential [1–3]. After isolation from patient bone marrow, hMSCs may be cultured and differentiated to osteoblastic lineage on implants prior to implantation. The cells may then promote bonding to the living bone tissue with the implant within a relatively short period. The effective use of hMSCs requires an osteoinductive scaffold that can promote differentiation of the hMSCs down the osteoblastic lineage. The current challenge of this strategy is to create the optimum conditions for promoting hMSC differentiation on implants, which requires a detailed understanding of the cellular and molecular bases of the reactions at the biomaterial–tissue interface [4–8].

The osteoconductive properties of silicate glasses, ceramics, and glass–ceramic composites have long been established, and the osteoinductive properties of silicate bioglasses are beginning to be explored [9–16]. The chemical effects responsible for the osteoconductive properties of silicate Bioglass® are well-established and can be broadly generalized to other silicate biomaterials [9–12,17,18]. The first five steps involve surface dissolution and precipitation. The reaction starts with ion exchange of Na^+ and Ca^{2+} ions of the glass with H^+ ions from body fluids, leaving behind a silica-rich, leached layer. The release of Ca ions increases the local super-saturation of apatite, so that a calcium phosphate (CaP) layer is precipitated on the leached silica layer. The CaP layer then crystallizes into carbonated hydroxyapatite by incorporating CO_3^{2-} and OH^-. The newly formed carbonated hydroxyapatite layer bonds to

* Corresponding author. Materials Science Program, University of Wisconsin, Madison, WI, USA.
E-mail address: sahai@geology.wisc.edu (N. Sahai).

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the existing bone. The effects of soluble Si released from Bioglass® on osteoblastic cell proliferation and differentiation have been explored [13–16]. While the chemical reactions are well-established, few studies, if any, have examined surface texture effects of silicate bioceramics on cell behavior.

We have examined the effects of fine- and coarse-grained surfaces of pseudowollastonite (β-$CaSiO_3$, a high-temperature polymorph of calcium silicate) on the rate of release of soluble factors and their effects on adhesion, viability, proliferation, and differentiation of human mesenchymal stem cells (hMSCs). We chose pseudowollastonite because it is osteoconductive, so the chemical reactions at its surface are well-established in the literature [19–25], and because the active surface site that promotes growth of an epitaxial layer of hydroxyapatite has been identified by *ab initio* molecular orbital calculations [18,24,25]. A few studies previously examined the effects of soluble factors released from pseudowollastonite on osteoblastic cell activities [19,20,26], but we believe that the present work is the first to examine whether pseudowollastonite can induce osteoblastic differentiation of hMSCs. Furthermore, we provide detailed mechanistic linkage of cell activities to surface texture differences that resulted in differences in release of soluble factors and the surface precipitates formed.

2. Materials and methods

2.1. Pellet synthesis

In order to form pseudowollastonite pellets (12 mm diameter × 1 mm thick) of coarse-grained (psWc) and fine-grained (psWf) textures, we pressed 0.4 g of $CaSiO_3$ ceramic powder of different particle sizes (4.5 and 1 μm, respectively) in a hardened steel die, followed by heating at 1300 °C for 5 h and 900 °C for 10 h, respectively, at a heating rate of 5 °C min^{-1} starting from room temperature.

2.2. Pellet characterization

The crystallinity and purity of the psW phase was confirmed by X-Ray Diffraction (XRD) using a Scintag Pad V diffractometer with Cu K_α for both kinds of pellets. Pellet surfaces were characterized both before and after cell culture in terms of their roughness and chemical and mineralogical composition. Before cell culture, surfaces were washed with anhydrous ethanol.

Surface roughness (R_a), the average vertical deviation of the surface profile from the mean line, was determined by white light interferometer (Zygo New View) over an area of 2.81 × 2.10 mm of the pellets.

The surface of pellets was imaged with a Hitachi S-3400 Variable Pressure Scanning Electron Microscope (SEM) at an accelerating voltage of 15 KeV, using one of the following detectors, depending upon the vacuum level: secondary electron detector, environmental secondary electron detector, or backscatter electron detector. Samples were carbon-coated, although variable pressure (30 Pa) was utilized to fully eliminate charging. In order to prepare surfaces after cell culture, cells were lysed and carefully flushed away using 0.2% Triton-X 100 solution, dried under ambient conditions, and carbon-coated. The surface morphology, composition change, and identity of phases precipitated were determined by SEM combined with Energy Dispersive Spectrometry (EDS) and Electron Back-Scattered Diffraction (EBSD). The tilt-view images were obtained with the sample holder at a 75° angle and imaged via the environmental secondary electron detector at 20 mm working distance.

To prepare TEM sample, fine particles were scratched off from mineral pellets after cell culture experiment. The powder was then dispersed on the carbon-coated grids using anhydrous ethanol.

2.3. Cell culture

The 24-well culture plates were pre-coated with 1% BSA (Bovine Serum Albumin, overnight at 4 °C) to prevent cell attachment and growth on the culture plate surfaces. All pellets before cell seeding were first washed with anhydrous ethanol and then sterilized overnight at a temperature of 180 °C. The pellets were placed in the culture plates (one pellet per well) at the desired cell densities specified below for cell growth, proliferation and cell differentiation. Human mesenchymal stem cells (hMSCs, Cambrex®) at passage 5 were seeded on the psW surfaces. Two kinds of cell culture media were used: (a) growth medium (GM), made of 10.08 g L^{-1} Alpha Modification of Eagle's Medium (AMEM), 1% penicillin/streptomycin as antibiotics and 10% Fetal Bovine Serum (FBS); and (b) osteogenic induction medium (IM), made of GM + 50 μg mL^{-1} 2-phosphate ascorbic acid + 100 nM dexamethasone + 10 mM glycerol 2-phosphate. After seeding, the cells were incubated for 24 h in GM and then cultured in GM or IM for the following cell growth and differentiation experiment up to 30 days. All cultures were maintained at 37 °C in a humidified incubator with 5% CO_2. Media were changed every 2 days. The "day 0" time point in the following figures means 24 h after cell seeding. Pellets in GM and IM without cells served as controls (psWcB, psWfB). All the quantitative assays were carried out in quadruplicate.

2.4. Si, Ca and P analyses in culture media

Cell culture media were collected every other day. After media collection, solutions were diluted 10 times and acidified by high purity HNO_3 until pH dropped below 2. The samples were then stored at 4 °C until analysis. The Si, Ca and P concentrations were measured using Inductively Coupled Plasma-Optical Emission Spectroscopy (ICP-OES, Varian Vista-MPX). The error bars shown in the figures below represent the standard deviations of four replicates.

2.5. Cell adhesion, morphology, and viability

Pellet surfaces from cell culture at days 0, 12, and 28 were washed twice with Phosphate Buffered Saline (PBS). Cells on the pellet surfaces were then incubated in 2 μM calcein AM (calcein acetoxymethyl ester) and 4 μM ethidium homodimer (EthD-1) solution (LIVE/DEAD® Viability/Cytotoxicity Kit for Mammalian Cells, Invitrogen) at room temperature for 40 min. This assay identifies esterase activity in live cells via green fluorescence emission from calcein AM and nuclear permeability in dead cells via red fluorescence emission from EthD-1 [27]. Cell adhesion, morphology, and viability were investigated by epifluorescence microscopy.

2.6. Cell growth

Cells were cultured in osteogenic induction medium and seeded on mineral pellet surfaces at a low seeding density of 10,000 cells/cm^2 in quadruplicate. For total DNA assay, cells were harvested using lysis buffer containing 0.2% Triton-X 100, at days 2, 8, and 12, and the resulting solutions were centrifuged and assayed using CyQuANT® Assay Kit (Invitrogen) based on standard procedures. Total cell number was determined at days 0, 2, 8, 12, 20, and 28 by adding CellTiter Blue® reagent (Promega) into cell culture wells and incubating for 4 h. Aliquots (100 μL) of culture medium were then transferred to 96-well plates for determining the concentrations by fluorescence absorbance at a wavelength of 560 nm/590 nm.

2.7. Osteoblastic cell differentiation

In order to compare osteoblastic cell differentiation on the two psW substrates, we measured activity of the enzyme, alkaline phosphatase (ALP), and gene expression of osteopontin (OPN), osteocalcin (OCN), and the osteoblast-specific transcription factor, core-binding factor alpha-1 (Cbfα-1) or Runx2, compared to the housekeeping gene, β-actin, using Reverse Transcription-Polymerase Chain Reaction (RT-PCR).

For ALP analysis, cells on the substrates were lysed with 0.2% Triton-X 100. Aliquots (20 μL) of the cell lysates were transferred into a 384-well plate. The concentration of ALP was analyzed by ANASpec SensoLyte™ FDP kit (Anaspec®) using standard procedures. Total DNA contents were also measured by CyQuANT® Assay (Invitrogen®) as described above. Cell differentiation was expressed as μg ALP per ng DNA after subtraction of values obtained for controls.

We also attempted to measure the OCN concentration in cell culture media over time, but cross-reaction of both the Mid-Tact Human Osteocalcin EIA Kit (Cat #: BT-480, Biomedical Technologies Inc.) and the human osteocalcin Instant ELISA kit (Cat #: BMS2020INST, Bender MedSystems™) with OCN from the 10% FBS added to the culture media resulted in background levels higher than levels produced by the osteoblastic cells. The OCN analyses were, therefore, abandoned.

For RT-PCR, the cells were seeded on psW surfaces at a high density of 30,000 cells cm^{-2}. Experiments were carried out in quadruplicate, at days 8 and 16, using both growth and osteogenic induction media. At the end of each culture period, total RNA was extracted using RNeasy® Mini kit (Qiagen) according to the manufacturer's instructions, and total RNA concentrations were determined by light absorbance at 260 nm wavelength. For each sample, 50 ng of RNA was reverse transcribed into complementary DNA (cDNA) with Illustra™ Ready-to-Go RT-PCR beads according to the manufacturer's instructions, and the cDNA samples were then amplified. Specifically, 0.5 μg of random hexanucleotide primer, $pd(N)_6$, was added into the RNA template to synthesize the first-strand cDNA at a temperature of 42 °C for 20 min, followed by denaturing at 95 °C for 5 min. After first-strand cDNA synthesis, gene-specific primers (Table 1) were added into the solutions. The mixture was then subjected to the thermal cycling processes.

The thermal profile consisted of an initial denaturation step at 95 °C for 5 min, 35 thermal cycles, and an elongation step at 72 °C for 7 min. Each of the 35 thermal cycles consisted of a denaturing step at 95 °C for 30 s, an annealing step at 55 °C for 45 s, and an elongation step at 72 °C for 1 min.

Agarose gel was prepared by melting 2.25 g of agarose in 150 mL water in a microwave and then pouring the solution into a prepared gel tray. When the gel