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A screening approach reveals the influence of mineral coating morphology on human mesenchymal stem cell differentiation

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Abstract

“Biomimetic” inorganic coating on biomaterials has been an active area of research with the intention of providing bioactive surfaces that can regulate cell behavior. Previous studies have demonstrated that human mesenchymal stem cell (hMSC) behavior is differentially regulated by physical and chemical properties of inorganic mineral coatings, indicating that modulation of mineral properties has potential importance in regulating hMSC behavior. However, the lack of an efficient experimental context in which to study stem cell behavior on inorganic substrates has made it difficult to systematically study the effects of specific mineral coating parameters on hMSC behavior. In this study, we developed an efficient experimental platform to screen for the effects of mineral coating morphology on hMSC expansion and differentiation. hMSC expansion on mineral coatings was regulated by micro-scale morphology of mineral coatings, with greater expansion on small granule-like coatings when compared to plate-like or net-like coatings. In contrast, hMSC osteogenic differentiation was inversely correlated with cell expansion on mineral coating, indicating that mineral coating morphology was a key parameter regulating hMSC differentiation. The effect of mineral coating morphology on hMSC behavior suggests the utility of this inorganic screening platform to identify optimal coatings for medical devices and bone tissue engineering applications.

Keywords

Inorganic coatings; hMSC differentiation; Micro-morphology; Medical devices

1 Introduction

Inorganic, calcium phosphate (CaP)-based mineral coatings have been applied to various biomaterials due to their ability to improve physicochemical and biological properties of implanted medical devices. Nucleation and growth of “bone-like” or “biomimetic” minerals on biomaterials have resulted in bioactive surfaces, which can promote osteoconductivity and osteointegration in orthopedic implants [1-3]. Furthermore, a series of studies have demonstrated that intrinsic mineral coating properties, including solubility, chemical composition and surface morphology are important factors for regulating diverse cell behaviors [4-8]. The broad range of variable inorganic coating parameters, coupled with the

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diverse set of cell behaviors that can be affected, indicate a need for efficient, clinically relevant platforms to screen for the effects of mineral coatings on cell behavior.

Human mesenchymal stem cell (hMSC) behavior on mineral surfaces has been of considerable interest, as these cells are often present during musculoskeletal tissue healing and may be a useful component in bone tissue engineering schemes [9, 10]. The physical and chemical properties of CaP minerals have been implicated in regulating hMSC behavior, including cell adhesion, proliferation, and differentiation [11-13]. For example, changes in CaP mineral morphology have been shown to influence hMSC lineage commitment in previous studies [14, 15]. However, previous studies have not systematically studied the effects of CaP mineral morphology on hMSC proliferation and differentiation. As compared with generation of a large library of polymers using combinatorial approach to screen the effect of polymer properties on cell behaviors, this limitation is due in part to the lack of an efficient experimental context in which to study stem cell behavior on inorganic substrates [16]. We hypothesized that same type of screening concept could be applied to inorganic materials.

Here we developed an efficient experimental platform to study the influence of inorganic CaP mineral coatings on hMSC expansion and osteogenic differentiation. Mineral coatings were formed using modified simulated body fluids (mSBFs), which have been used to simulate in vivo conditions of ionic components of blood plasma for formation of CaP-based minerals [17]. Systematic modification of ion concentrations in mSBF solutions resulted in coatings with variable physical and chemical properties, including surface morphology. In view of the potential clinical utility of these coatings in future studies, we formed mineral coatings on one of the most commonly used bioresorbable polymers in clinical applications, poly(lactide-co-glycolide) (PLG) [18]. Our results indicated that hMSC proliferation and differentiation were dependent on the micro-scale morphology of mineral coatings. Interestingly, we also found that the extent of osteogenic hMSC differentiation was greater on mineral coatings that promoted lower rates of stem cell expansion. In addition, pro-osteogenic media supplements clearly stimulated differentiation of hMSCs on coatings that promoted high stem cell expansion, but did not significantly influence differentiation on coatings that promoted low stem cell expansion. These results suggest that enhanced throughput mineral coatings may provide a library of biologically relevant mineral properties, which can potentially be used to design mineral coatings for medical devices and bone tissue engineering applications.

2 Materials and methods

2.1 Fabrication of mineral coatings

To generate polymer coatings on polypropylene 96-well plates, 1g/1mL of poly (lactide-co-glycolide) (PLG, lactide:glycolide (85:15), Mw = 50000-70000) dissolved in acetone were transferred in each well and dried in chemical hood. PLG-coated wells incubated in 0.5 M NaOH solution were hydrolyzed for 30 min to present COOH and OH groups on the surface. For mineral formation on hydrolyzed PLG surfaces, a set of supersaturated mineral solutions were prepared by dissolving NaCl, KCl, MgCl₂, MgSO₄, NaHCO₃, CaCl₂, KH₂PO₄, and MES (Fisher Scientific) in DI water with varying Ca²⁺ and PO₄³⁻ ion concentrations and pH of each solutions was adjusted with NaOH and HCl solutions (these solutions are referred to as “modified simulated body fluid” (mSBF) and each solution is named as A – B (A: [Ca²⁺]; B: [PO₄³⁻]) (Table 1). Precursor mineral coatings were formed in mineral solution containing 5 mM Ca²⁺ and 2 mM PO₄³⁻ ions than that in human blood plasma (this condition is referred to as a “5 – 2”) after 3 d incubation at 37°C with refreshing media every 12 h. Precursor mineral coatings were further incubated in various mineral

solutions for 4 d at 37°C. Resulting mineral coatings were rinsed in DI water then dried for characterizing mineral properties or used immediately for cell culture.

2.2 Analysis of mineral properties

For imaging surface morphology of mineral coatings, samples were coated with gold and analyzed by scanning electron microscopy (SEM) (Carl Zeiss SMT). Phase of mineral coatings was analyzed by X-ray diffractometry (XRD) (Bruker AXS) under Cu K radiation. The surface roughness of mineral coating was analyzed by optical surface profiler (Zygo Corporation).

2.3 Proliferation and differentiation of hMSC on mineral coatings

Before seeding cells on mineral coatings, human mesenchymal stem cells (hMSCs) were grown on flask at 37 °C and 5% CO₂ in Minimum Essential Medium (MEM) (Mediatech) with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (P/S) until cells were 70% confluent. 2.4×10^4 cells/cm² of hMSCs were seeded on the mineral coatings after collected by treatment with 0.05% trypsin-EDTA and incubated in MEM at 37 °C and 5% CO₂ for 1 d. After 1 day of incubation, cells were rinsed with phosphate buffer saline (PBS) and refreshed with cell growth medium (MEM, 10% FBS, 1% P/S) and growth medium with osteogenic supplements (OS: 50 µM ascorbic acid-2-phosphate, 10 mM glycerophosphate, 100 nM dexamethasone). At the time of analysis, cells on mineral coatings were washed with PBS and lysed using 200 µL of solution containing 0.2% Triton X-100. For cell proliferation analysis, supernatant after cell lysis was assayed using CyQUANT cell proliferation assay kit (Invitrogen). 50 µL of cell lysate were mixed with working solution and the fluorescence measured at 528 nm was converted to amount of total DNA using standard curves prepared with known amount of DNA in solution. Alkaline phosphatase (ALP) activity was analyzed using SensoLyte FDP ALP assay kit (AnaSpec). In brief, 50 µL of cell lysate were mixed with FDP reaction mixture and fluorescence at 528 nm was converted to ALP concentration using standard curves relating fluorescence intensity to ALP concentration.

3 Results and discussion

3.1 Control of mineral morphology with modified simulated body fluids

We developed enhanced throughput mineral coatings to study the effects of mineral properties on hMSCs (Fig. 1A). In this study, mineral coatings on PLG-coated 96-well plates provide enhanced screening efficiency as compared with previous studies that used mineral coatings formed on centimeter-scale substrates or tissue engineering scaffolds. However, each study is not practical to systematically vary the broad ranges of parameters that can influence mineral properties. We hypothesized that systematically varied ion concentrations in mSBF solutions would provide control over mineral coating morphology on PLG surfaces. To test this hypothesis, we modulated Ca²⁺ and PO₄³⁻ ion concentrations in mSBF solutions and characterized mineral coating properties (Fig. 1B). Mineral coatings grown on PLG surfaces formed layers with different micro-scale structures. The small granule-like morphology formed in relatively low Ca²⁺ and PO₄³⁻ ion concentrations was transformed into plate-like and net-like morphologies with increasing Ca²⁺ and PO₄³⁻ ion concentrations. Previous study of the interdependent relationship between mineral properties and simulated body fluid (SBF) solution conditions has indicated that the ion concentration can influence the mineral phase [19]. However, in this study mineral coatings with different micro-morphology were each shown by X-ray diffraction analysis to be composed of a poorly crystalline hydroxyapatite-like mineral, which is a mineral phase similar to bone composition and structure (Fig. 1B, inset) [20]. It should be noted that the morphology of native bone, composed of organic and inorganic materials, is different depending on

hierarchical levels of structure [21]. Even if mineral coatings formed in 5 – 2 condition are more similar to native bone, which are composed of nanometer-scale plate-like mineral structure, when compared to other mineral coatings in terms of morphology, it is useful to screen various mineral morphologies that alter hMSC behaviors for optimizing mineral properties for specific biological or biomedical purpose.

3. 2 hMSC expansion on mineral coatings

Differences in micro-morphology of mineral coatings influenced hMSC expansion in both growth medium (GM) and growth medium with osteogenic supplements (GM-OS) (Fig. 2A and 2B). Cell expansion was greater when the cells were grown on mineral coatings with small granule-like morphology when compared to mineral coatings with plate-like and net-like morphology (Fig. 2A). After 8 days of cell culture on mineral coatings, no significant difference in cell density was observed among mineral coatings with small granule-like morphology. However, cell density was significantly reduced on mineral coatings with plate-like and net-like morphology. In addition, the cell density on small granule-like mineral coatings was further increased from 8-16 days, while no significant difference in cell density was observed among plate-like and net-like mineral coatings from 8-16 days. Previous studies demonstrated that several physical properties resulted from mineral morphology change, including porosity, roughness, and crystallinity, can be parameters to influence cell proliferation [6, 8, 13, 14]. In this study, the combinational effect of surface characteristics may be attributed to difference in hMSC expansion on mineral coatings. For example, surface roughness of mineral coatings with plate-like morphology is relatively rough when compared with mineral coatings with other morphologies (Fig. 1B, inset).

Small granule-like mineral coatings supported reduced cell density in OS medium when compared to growth medium, while plate-like and net-like mineral coatings did not show significant differences in cell expansion in growth versus OS media (Fig. 2B). This result indicated that the effect of OS on cell expansion was dependent on mineral surface morphology. In addition, high cell density on small granule-like mineral coatings relative to that on other morphologies in growth and OS media indicated that hMSC proliferation was strongly correlated with mineral surface morphology.

3.3 hMSC differentiation on mineral coatings

Mineral coating morphology also influenced ALP activity, a hallmark of osteogenic hMSC differentiation (Fig. 2C). Cells on small granule-like mineral coatings showed lower ALP activity relative to cells on plate-like or net-like mineral coatings after 8 days in growth medium. A similar influence of mineral morphology on ALP activity was observed when cells were grown in OS medium. The stimulatory influence of OS on ALP activity was evident when hMSCs were grown on small granule-like mineral coatings, while there was no significant influence of OS on ALP activity of hMSCs on plate-like or net-like mineral coatings. These results indicated that ALP activity was significantly influenced by mineral morphology, both in the presence and absence of OS. This result is consistent with previous studies, which have demonstrated that control of surface topography can induce differentiation of hMSC in growth medium [22-24]. It is possible that the observed influence of mineral morphology may be related to cell shape, as previous studies have shown that surface topography influences hMSC shape and intracellular structure, which in turn regulates hMSC fate [25-27]. Further studies will be required to delineate the mechanism for mineral coating effects on hMSC differentiation.

Several previous studies of cell density effects on MSC differentiation have demonstrated that ALP activity was higher at low cell density than at high cell density [26, 28]. Therefore, we further investigated the influence of the hMSC expansion rate, and associated hMSC

density, on ALP activity. The ALP activity was inversely correlated with cell expansion, and the data followed a power law (Fig. 2D). Therefore, at a low rate of cell expansion ALP activity was higher than that at a high rate of cell expansion, in both growth and OS media. In addition, hMSCs in OS media exhibited higher ALP activity at each cell expansion rate compared to hMSCs grown in growth medium, as demonstrated by a shift in the slope of the ALP/cell expansion dependence. Therefore, cells with a low cell expansion rate show higher ALP activity than those with a high cell expansion rate, whether the expansion rate is influenced by surface morphology or medium conditions.

4 Concluding remarks

We developed enhanced throughput mineral coatings to study the effects of inorganic coating properties, particularly mineral morphology, on hMSC differentiation. Bone-like hydroxyapatite mineral coatings formed layers on PLG surfaces, and different micro-scale morphologies were produced by systematically changing $[Ca^{2+}]$ and $[PO_4^{3-}]$ in mSBF solutions. hMSC expansion on mineral coatings was correlated with mineral surface morphology, with higher levels of cell expansion on small granule-like coatings when compared to plate-like or net-like coatings. In contrast, hMSC osteogenic differentiation was inversely correlated with cell expansion on mineral coatings in growth medium or OS medium, indicating that mineral coating morphology is a key parameter regulating hMSC differentiation. These results suggest the potential application of inorganic screening in design of mineral properties for medical devices and bone tissue engineering scaffolds. For example, in some medical device applications, there is a need to optimize surface coatings for rapid engraftment and efficient osteogenic differentiation of autologous stem cells (e.g. hMSCs). A combinatorial approach that can screen for the effects of mineral properties such as the approach described here could be ideal for this purpose.

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Abbreviations

hMSC	Human mesenchymal stem cell
CaP	Calcium phosphate
SBF	Simulated body fluid
mSBF	Modified simulated body fluid
PLG	poly(lactide-co-glycolide)
SEM	Scanning electron microscopy
XRD	X-ray diffractometry
MEM	-Minimum Essential Medium
FBS	Fetal bovine serum
P/S	Penicillin/streptomycin
PBS	Phosphate buffer saline
GM	Growth medium
GM-OS	Growth medium with osteogenic supplements

OS	Osteogenic supplements
ALP	Alkaline phosphatase

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Figure 1.

(A) Schematic of enhanced throughput mineral coatings for screening of hMSC expansion and differentiation. (B) Mineral coatings formed on pre-hydrolyzed PLG after 7 days of incubation in solutions containing various Ca²⁺ and PO₄³⁻ ion concentrations. Ca²⁺ and PO₄³⁻ concentrations are shown as a ratio relative to the ion concentrations in SBF solution, which are 2.5 mM and 1.0 mM, respectively. mSBF solutions are named as A – B (A: [Ca²⁺]; B: [PO₄³⁻]). SEM images of the mineral coating morphology change from small granule-like morphology to plate-like and net-like morphology with increasing [Ca²⁺] and [PO₄³⁻]. Scale bar = 40 μm. Inset: XRD spectra of mineral coatings, each showing two dominant peaks at 26° and 31° (•), which are consistent with hydroxyapatite mineral. Surface roughness was measured using an optical surface profiler. Data represents mean ± SD (*n* = 6).

Figure 2. hMSC expansion and differentiation on mineral coatings. (A) hMSC expansion on mineral coatings after 8 and 16 days in growth medium, as measured by total DNA quantification. (B) hMSC expansion and (C) ALP activity on mineral coatings after 8 days in growth (GM) or OS medium (GM-OS). (D) Correlation between ALP activity and cell expansion ratio. ALP activity of hMSC after 16 days of culture (normalized to total DNA) was plotted versus cell expansion ratio (total DNA at 16 days divided by total DNA at 8 days). Statistical significance between hMSC behaviors on the same mineral coatings (denoted by %, Student's t-test) or on different mineral coatings respect to 5 – 2 (denoted by *, #, Dunnett t-test) at different time points (A) or in medium conditions (B and C) was set at $p < 0.05$. Data represents mean $\pm$ SD ($n = 4$).

Table 1

Ion concentrations of the mSBF solutions

	Ca ²⁺ [mM]	PO ₄ ³⁻ [mM]	pH
SBF	2.5	1	7.4
	5	2	6.8
	5	2.5	6.6
	5	3.3	6.5
	5	5	6.2
	8.8	3.5	6.1
	8.8	4.4	5.9
mSBF	8.8	5.8	5.8
	8.8	8.8	5.7
	12.5	5	5.8
	12.5	6.3	5.7
	12.5	8.3	5.5
	12.5	12.5	5.3