Acta Biomaterialia 6 (2010) 591–597

Contents lists available at ScienceDirect

Acta Biomaterialia

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

Adhesion of osteoblast-like cells on nanostructured hydroxyapatite

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ARTICLE INFO

Article history:
Received 27 April 2009
Received in revised form 27 July 2009
Accepted 30 July 2009
Available online 3 August 2009

Keywords:
Hydroxyapatite
Osteoblast
Cell adhesion
Cell proliferation
Nanotopography

ABSTRACT

The response of osteoblast-like cells seeded on hydroxyapatite (HAp) substrates consisting of nanosized crystals was investigated. Various types of HAp nanocrystals, such as nanofibers, nanoneedles and nanosheets, were selectively prepared as substrate through the hydrolysis of a solid precursor crystal of $CaHPO_4$ in alkaline solutions by varying the pH and ion concentrations. Although all the substrates were macroscopically flat and smooth, the nanoscale topography influenced cell activity, including the adhesion, proliferation, elongation and formation of actin stress fibers. The presence of fine nanoneedles and nanofibers on the surface restricted the cellular activities, while the cells steadily proliferated on a nanoscopically smooth surface of large grains and on a substrate consisting of wide nanosheets. These results suggest that the adhesion and subsequent responses of osteoblast-like cells were affected by the contact domain size between the cell and the substrate. Isolated small domains of the nanostructured HAp limited focal adhesion formation in the cells associated with the formation of stress fibers. Stable adhesion with contact domains larger than 100 nm in width was suggested to be required for cell survival. On the other hand, insufficient adhesion on the fine nanoneedles was found to lead to apoptosis.

1. Introduction

The interactions between cells and solid surfaces are crucial to many biological phenomena for a wide variety of biomaterials. Since cells are inherently sensitive to their surroundings, the performance of biomaterials strongly depends on their initial interaction with a biological environment. Thus, the surface properties of biomaterials are associated with cell adhesion and subsequent various cell behaviors, such as proliferation, migration, cytoskeletal arrangement, differentiation and apoptosis [1]. In particular, a large number of studies on cell adhesion to various substrate surfaces have been conducted. Cell adhesion and its performance have been reported to depend on the characteristics of substrates, including the chemical composition [2,3], surface charge [4], water wettability [5–7], roughness [8–12] and size of the cytophilic area [13]. Understanding the mechanisms whereby cells sense and respond to chemical, physical and biological signals from material surfaces will facilitate the development of novel biomaterials for the control of cell behavior.

Conventionally, artificial biomaterials have been designed to match the properties of host natural tissues in real organs at the macroscopic level of the cell–matrix interaction [14]. In recent years, several systematic studies using nanoscopically regulated substrates have been reported. The nanoscale spacing and density of adhesive islands containing arginine–glycine–aspartic acid (RGD) peptide sequences can modulate various cell functions [15–19]. Self-assembled layers of vertically oriented TiO_2 nanotubes critically influence the adhesion, spreading, growth and differentiation of mesenchymal stem cells [20]. Nanofeatures on anisotropic patterned substrates regulate cell elongation and alignment with nanoscale grooves and ridges [21–24]. An increase in nanostructural depth restricts proliferation on needle-like nanoposts and enhances cell elongation with alignment on blade-like nanograte [25].

In the field of biomaterials for substitution and reorganization of hard tissue, calcium phosphate ceramics are quite important because natural hard tissues are primarily composed of hydroxyapatite (HAp). In consequence, the nanoscale topography of calcium phosphate ceramics determines the cellular performance of mesenchymal stem cells and osteoblast cells. Osteoblast proliferation was reported to be enhanced on nanophase HAp in comparison with borosilicate glass, nanophase alumina and nanophase titania [26]. However, studies of cell responses to nanotopography for HAp are not mature due to the technological limitations of controlling the surface structure of the material. Recently, we succeeded in the selective preparation of nanoscale needles, fibers and sheets of HAp through the hydrolysis of a solid precursor crystal of dicalcium phosphate (DCP, $CaHPO_4$) in alkali solutions by varying the pH and the ionic concentrations [27]. In this study, we investigated the influence of the nanoscale topography of HAp on cell adhesion using disk-shaped HAp substrates with various nanostructures prepared from the DCP precursor. Cell activities, including the adhesion, proliferation, elongation and formation of actin stress

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doi:10.1016/j.actbio.2009.07.037

fibers, were studied with osteoblast-like cells seeded on the nanostructured substrates.

2. Materials and methods

2.1. Preparation and characterization of HAp substrates

Nanoscale HAp was synthesized from a precursor crystal of DCP as per our previous report. Commercial dicalcium phosphate dihydrate (DCPD, $CaHPO_4{\cdot}2H_2O$; Junsei Chemical, 98.0%) powder was immersed in a 2.0 M NH_4Cl solution. After being stirred for 20 min, the precipitate was filtered and dried in air at 60 °C for 24 h, and DCP powder was then obtained by dehydration of DCPD. Culture substrates were prepared by compaction of 0.15 g of the resultant DCP powder into disk-shaped pellets with a diameter of 13 mm. Ten pellets of DCP were then immersed in 150 ml of alkaline solutions to hydrolyze DCP into HAp. The nanostructure of the HAp substrates was regulated by varying the condition of the alkaline solution. The pH was adjusted and kept at a specific value in the range 9.0–13.0 by the addition of NH_4OH or NaOH during the hydrolysis reaction at 70 °C for 24 h. An aqueous solution containing 3.0 M $(NH_4)_2HPO_4$ at pH 9.0 with NH_4OH was used for the HAp nanoflakes. The hydrolysis of DCP at a lower pH was carried out with urea to obtain larger HAp crystals. A 0.5 M urea solution containing DCP pellets was adjusted to pH 3.0 with addition of HNO_3 and then heated to 85 °C. The temperature was kept at 85 °C for 72 h, and the pH of the solution increased to 7.8 owing to the NH_3 formed by the decomposition of urea. As a reference material with a smooth surface, commercial HAp powder (Junsei Chemical, 96%) was compacted into a disk-shaped pellet with a diameter of 13 mm and sintered at 1200 °C in air for 24 h. The sintered HAp pellets were soaked in a modified simulated body fluid (SBF; $[Na^+] = 145.0$, $[K^+] = 5.0$, $[Ca^{2+}] = 0.7$, $[Cl^-] = 141.1$, $[HPO_4{}^{2-}] = 9.6$ mmol l^{-1}) [28] at 38 °C for 24 h to achieve a nanostructure on the flat surface. All the HAp pellets were soaked in purified water at 65 °C for 15 h and then dried in air at 65 °C for 9 h to remove the reaction solution; this washing process was repeated 3–7 times.

The morphology of substrates consisting of nanocrystals was characterized by field-emission scanning electron microscopy (FESEM) using a Hitachi S-4700 microscope. X-ray diffraction (XRD) patterns were recorded on a Bruker D8 Advance diffractometer with CuK_α radiation.

2.2. Cell culture and characterization

Rat bone-marrow-derived mesenchymal stem cells (RMSCs) as a precursor of osteoblast-like cells were purchased from DS Pharma Biomedical. Adherent osteoblast-like cells differentiated from RMSCs with dexamethasone supplementation were cultured in Eagle's MEM supplemented with 2.0 mM L-glutamine, 41.7 mM sodium hydrogen carbonate and 10% fetal bovine serum at 37 °C in a humidified 5% CO_2 atmosphere. The resultant cells were washed twice with PBS(−) (Dulbecco's phosphate-buffered saline without magnesium and calcium, Takara Bio) and removed from the flask by treatment with 0.05% trypsin–EDTA (Gibco).

Before the cell experiments, the substrates were soaked for 24 h in Eagle's MEM (Nissui Pharmaceutical, containing kanamycin and phenol red) supplemented with 2.0 mM L-glutamine, 41.7 mM sodium hydrogen carbonate and 10% fetal bovine serum at 37 °C in a humidified 5% CO_2 atmosphere to equalize the initial condition of the HAp surface. For a cell adhesion assay, HAp pellets as a substrate were individually placed on the bottom of each well of Nunclon™Δ (Nunc) 4-well multidishes. A Teflon tube (diameter = 10 mm) was then put on each HAp pellet to restrict the culture area and to prevent cell growth on the plastic area of the bottom of the culture dish. Osteoblast-like cells suspended with 500 μl of the culture medium were seeded on the substrates in the wells at a cell density of 10^4 cells $well^{-1}$. The cells were cultured at 37 °C in a humidified 5% CO_2 atmosphere for 1–4 days.

The number of adherent cells was determined using a CellTiter 96® non-radioactive cell proliferation assay (Promega). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide tetrazolium salt (MTT, yellow) is converted into a water-insoluble formazan salt (purple) by the metabolic activity of viable cells based on the principle of the mitochondrial conversion. Cells were cultured for 24 h or 4 days and then washed twice with PBS. We added 30 μl of dye solution and 270 μl of PBS to each well and incubated them for 4 h at 37 °C. During the 4 h incubation, viable cells converted the tetrazolium component of the dye solution into a formazan product. Three hundred microliters of the solubilization solution/stop mix was then added to each well to dissolve the formazan product, and the absorbance at 570 nm was recorded using a Jasco V-560 absorption spectrometer. The cell number was determined using a standard curve of the absorbance vs. numbers of cells seeded with graduated concentrations in the wells.

The alkaline phosphatase (ALP) activity was evaluated by the quantitative measurement of *p*-nitrophenol formed from the enzymatic hydrolysis of *p*-nitrophenylphosphate (pNPP). Cells were cultured for 24 h or 4 days and then washed twice with PBS. 300 μl of the pNPP solution (Wako Pure Chemical) was then added to the washed cells. After incubation for 60 min at 37 °C, 300 μl of

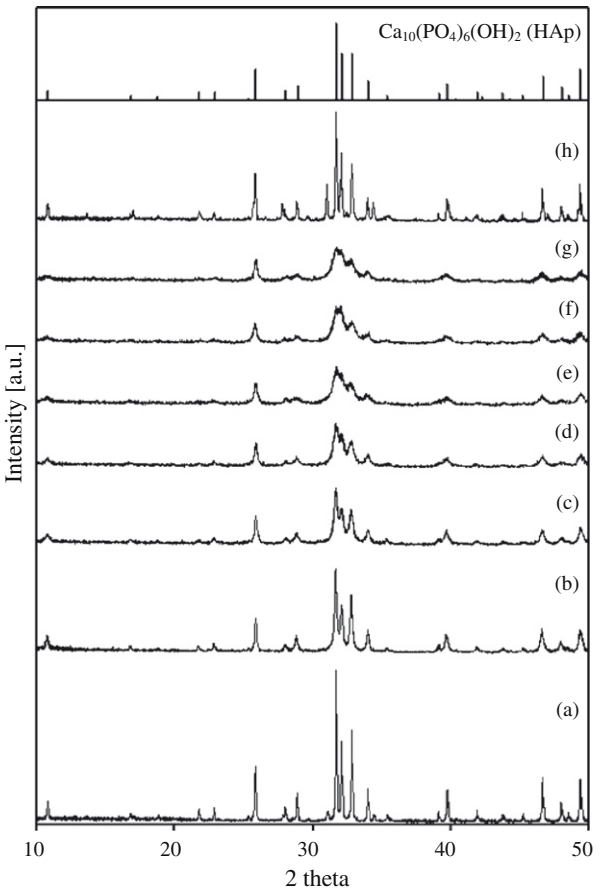

Fig. 1. XRD patterns of: (a) dense surface obtained by sintering commercial HAp at 1200 °C; (b) submicron fibers obtained by hydrolysis of DCP at pH 3 with urea; (c) nanofibers obtained by hydrolysis of DCP at pH 9; (d) nanofibers obtained by hydrolysis of DCP at pH 10; (e) nanoneedles obtained by hydrolysis of DCP at pH 11; (f) nanoneedles obtained by hydrolysis of DCP at pH 13; (g) nanoflakes obtained by hydrolysis of DCP in 3 M $(NH_4)_2HPO_4$ solution at pH 9; (h) wide nanosheets obtained by soaking dense surface in SBF.

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