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Surface free energy predominates in cell adhesion to hydroxyapatite through wettability

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ABSTRACT

The initial adhesion of cells to biomaterials is critical in the regulation of subsequent cell behaviors. The purpose of this study was to investigate a mechanism through which the surface wettability of biomaterials can be improved and determine the effects of biomaterial surface characteristics on cellular behaviors. We investigated the surface characteristics of various types of hydroxyapatite after sintering in different atmospheres and examined the effects of various surface characteristics on cell adhesion to study cell-biomaterial interactions. Sintering atmosphere affects the polarization capacity of hydroxyapatite by changing hydroxide ion content and grain size. Compared with hydroxyapatite sintered in air, hydroxyapatite sintered in saturated water vapor had a higher polarization capacity that increased surface free energy and improved wettability, which in turn accelerated cell adhesion. We determined the optimal conditions of hydroxyapatite polarization for the improvement of surface wettability and acceleration of cell adhesion.

1. Introduction

Numerous studies of the interfaces between biomaterials and living tissues have been carried out to control osteogenic cells and improve osteoconductivity, which is the formation of new bone near implanted biomaterials. Osteoconduction progresses in six stages at the interfaces between implanted biomaterials and injured bone tissues: (1) serum adsorption, (2) cell recruitment, (3) adhesion, migration, and proliferation of osteogenic cells, (4) osteoblast differentiation and osteoid production, (5) matrix calcification, and (6) bone remodeling [1]. Further improvements in osteoconductivity require an understanding of the processes that take place at the interfaces between biomaterials and living tissues.

The initial adhesion of cells to biomaterials is key in the regulation of the subsequent proliferation, differentiation, and formation of extracellular matrix after cell spread and migration. In general, cell adhesion is primarily affected by two properties—namely, the surface characteristics of biomaterials and stimulation from outside the cells. Surface characteristics, including the topography, functional groups, and wettability, affect cell adhesion [1–5]. The surface characteristics of ceramic biomaterials are reportedly determined by surface topography [6], roughness, crystallinity [2–4], grain size [7], constituent elements at the surface, and the incorporation of ions [5,8,9]. Stimulation from outside cells, which includes electrical stimulation such as capacitive coupling, inductive coupling, and combined electromagnetic fields, also affects the adhesion of osteoblast-like cells [10].

Understanding of the interactions between osteogenic cells and biomaterials may help clarify physiologic cellular reactions at the biomaterial-cell interface. The mechanism of cell adhesion to biomaterials varies according to substratum type. For instance, hydroxyapatite (HA) and titanium surfaces are superior for initial adhesion of human osteoblast-like cells [11] and gene expression at the early phase of adhesion as well as at later phases of proliferation and differentiation [4]. The signal transduction pathways involved in the adhesion of osteoblasts to HA and titanium have been confirmed by the subsequent expression $\alpha_v\beta_1$ integrins [11]. Cell shapes on substrata depend on integrin-mediated cytoskeletal and signal transduction molecules such as actin filaments [12] and, during cell-substratum adhesion, are critical to subsequent cell behaviors such as proliferation and differentiation [8,13]. We focused on the shapes of attached cells indicated by actin structure to study osteogenic cell adhesion, using osteocyte-like cells for cell adhesion assays because of their clearly branched shape during spread.

We recently demonstrated that compared with conventional HA, HA with charged surfaces induced by polarization treatment has enhanced osteoconductivity [14], early-stage protein adsorption after implantation in vivo [15], and initial adhesion [16] and migration [17] of osteoblast-like cells in vitro. Among the critical factors for enhancing cellular behaviors through electrical polarization is the improvement of surface wettability [18], which results from increases in the free energy of solid surfaces [19]. Other studies have reported similar effects on osteoblast-like cells [20] when wettability is improved via electrical

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polarization [21] and electrical stimulation of electro-sprayed surface charges of HA.

Although enhancements during the initial stages of osteoconduction have been reported, the mechanisms of polarization-induced effects on cellular behavior have not been elucidated completely. Accordingly, we are intrigued by the surface characteristics of polarized ceramic biomaterials, which might be useful for clarifying the interactions between these materials and living cells. Several questions about the mechanism of surface wettability improvement and the effects of surface characteristics on cellular behaviors remain under discussion. To resolve these questions, we combined approaches from material and biological perspectives in this study to clarify the interactions of cells and biomaterials. We prepared various HA surfaces via sintering in different atmospheres to study the mechanism of surface wettability improvement. In addition, we investigated the interactions between cells and ceramic biomaterials by observing the effects of various surface characteristics on osteogenic cell adhesion. In the present study, we used osteocytes for the cell evaluations because they are the most abundant cells in bone tissue and induce osteoclast differentiation from osteoclast precursors through the expression of differentiation factors [22], which means that they are involved in bone metabolism and osteoconduction process.

2. Materials and methods

2.1. Preparation of HA specimens

HA powder was synthesized from analytical-grade calcium hydroxide and phosphoric acid with the wet method. The HA powder was calcined at 850 °C for 2 h and pressed in a mold at 200 MPa. Some HA compacts were sintered in a saturated water vapor atmosphere at 1250 °C for 2 h to suppress dehydration (hereafter referred to as wHA) [23,24]. Other HA compacts were sintered in air at 1250 °C for 2 h (hereafter referred to as aHA).

The obtained dense wHA and aHA were characterized with X-ray diffraction (XRD), Fourier transform infrared spectroscopy (FTIR), and scanning electron microscopy (SEM). XRD measurements of wHA and aHA were performed for phase analysis at room temperature (RT) with Cu Kα radiation at 40 kV and 40 mA on a Philips PW 1700 diffraction spectrometer equipped with a graphite monochromator. FTIR spectra of the powdered wHA and aHA were recorded in the range of 400–4000 cm^{-1} (FTIR spectrophotometer, JASCO 4100) after dispersion in KBr. The surfaces of wHA and aHA were observed with a scanning electron microscope (HITACHI, S-3400NX).

For the comparison of sintering atmosphere (wHA and aHA), the dense HA specimens were electrically polarized with a pair of platinum electrodes at 400 °C in direct-current (dc) electric fields of 5 kV/cm for 1 h in air according to our previously described protocol [24]. The negatively charged aHA and wHA were designated aHA-N and wHA-N, respectively, and the positively charged aHA and wHA were designated aHA-P and wHA-P, respectively.

To compare the effects of polarization temperature on wHA, we electrically polarized dense wHA specimens with a pair of platinum electrodes at 200, 300, 400, 500, and 600 °C in dc electric fields of 5 kV/cm for 1 h in air. The negatively charged wHA surfaces were designated wHA (200)-N, wHA (300)-N, wHA (400)-N, wHA (500)-N, and wHA (600)-N, respectively, and the positively charged wHA surfaces were designated wHA (200)-P, wHA (300)-P, wHA (400)-P, wHA (500)-P, and wHA (600)-P, respectively. wHA specimens treated thermally at 200, 300, 400, 500, and 600 °C without polarization were designated wHA (200), wHA (300), wHA (400), wHA (500), and wHA (600), respectively.

Polarization of the HA specimens was verified with thermally stimulated depolarization current (TSDC) measurements. These measurements were carried out in air from RT to 800 °C at a heating rate of 5.0 °C/min according to our previously described method [24]. The depolarization current was measured with a Hewlett-Packard 4140B pA meter. The values of the polarization charge (Q_p) were calculated from the TSDC spectra according to the following equation:

$$Q_p = \frac{1}{\beta}\int J(T)dT$$

where J(T) is the measured dissipation current density at temperature T, and β is the heating rate.

2.2. Surface characterization of HA specimens

The surface roughness of HA specimens was analyzed with a laser microscope (Keyence, VK8500). A total of 15 measurements were performed on each specimen to obtain an average.

Each polarized HA specimen was subjected to attenuated total reflectance FTIR spectroscopy (ATR-FTIR) at five points with a Perkin-Elmer spectrum BX spotlight spectrophotometer with a diamond ATR attachment. Before measurement, samples were conditioned in a standard atmosphere of 65 ± 2% relative humidity and 20 ± 2 °C for 48 h and then held in a desiccator to maintain the same atmosphere as that of the FTIR equipment. Scanning was conducted from 4000 to 400 cm^{-1}, with 64 scans averaged for each spectrum. The ratios of peak intensity of OH^- (3570/3571 cm^{-1}) and PO_4^{3-} (1040/1015 cm^{-1}) in the ATR-FTIR spectra were used to estimate hydroxide ion content. The rations of OH/PO_4 in the ATR-FTIR spectra were compared relatively before and after the electrical polarization.

The zeta potential analysis in deionized water was performed with a zeta potential analyzer (Otsuka Denshi, ELSZ-1000Z) equipped with a holder for bulk samples. Contact angle measurements were performed on the HA specimens with distilled and deionized water (Kyowa Interface Science, DropMaster DM-500).

For the calculation of the surface free energy of the HA specimens, contact angle measurements were performed with the two-liquid-phase method. The contact angles of water on the HA specimens were measured in hydrocarbon oils such as hexane (18.4 $mJ \cdot m^{-2}$), heptane (20.1 $mJ \cdot m^{-2}$), octane (21.7 $mJ \cdot m^{-2}$), decane (23.8 $mJ \cdot m^{-2}$), and hexadecane (27.5 $mJ \cdot m^{-2}$). Surface free energy was calculated according to Jouany's equation:

$$\gamma_W - \gamma_H + \gamma_{HW}\cos\theta = 2\sqrt{\gamma_S^d}\left(\sqrt{\gamma_W^d} - \sqrt{\gamma_H}\right) + I_{SW}^p$$

where subscripts W, H, and S refer to water, hydrocarbon, and solid, respectively; γ_S^d and γ_W^d refer to dispersion components; and I_{SW}^p refers to nondispersive interaction between the solid and water as expressed by:

$$I_{sw}^p = 2\sqrt{\gamma_s^p \times \gamma_w^p}$$

where γ_s^p and γ_w^p refer to polar (nondispersive) components.

According to Fowkes, work of adhesion (W) between the solid and water is divided into two interaction components, which are dispersive and nondispersive interactions [25]:

$$W_{sw} = I_{sw}^d + I_{sw}^p$$

$$W_{sw} = 2\sqrt{\gamma_s^d \times \gamma_w^d} + 2\sqrt{\gamma_s^p \times \gamma_w^p}$$

where dispersive component of water (γ_w^d) is 21.8; and polar component of water (γ_w^p) is 51.0 [25].

The geometric mean expression for I_{sw}^d is after Fowkes, and that for I_{sw}^p is an extended one.