Improvement of Osteoblast Adhesion Through Polarization of Plasma-Sprayed Hydroxyapatite Coatings on Metal

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Abstract

Hydroxyapatite coatings (HACs) have long been applied to orthopedic and dental implants made of titanium and its alloys because of their high biocompatibility and osteoconductivity. We have recently demonstrated that the charged surfaces on HAC induced by polarization enhance mineral deposition in simulated body fluid and osteoconductive capabilities in vivo. The present study evaluates the effects of the electrical polarization of HAC on surface characteristics and osteoblast adhesion. It was found that electrical polarization has no effect on surface roughness and crystallinity. Morphological observations and quantitative analyses of adhered osteoblasts on HACs revealed that the cell areas positively stained for actin, which indicates the degree of cell spreading, were distinctly larger on negatively and positively charged HAC than that on uncharged HAC.

Keywords: Hydroxyapatite coating, Polarization, Osteoblast adhesion

1. Introduction

In clinical applications, hydroxyapatite (HA) is coated onto metal substrates, such as titanium (Ti), Ti alloys, and stainless steel (SUS 316), to offset its mechanical weakness. Various methods of fabricating HA coatings (HACs) have been developed for dental and orthopedic implants, such as plasma spraying, radio-frequency (RF) magnetron sputtering, dip coating, electrochemical deposition, pulsed-laser deposition, and electrophoresis deposition methods. HACs have long been applied to orthopedic and dental implants made of metals because of their high biocompatibility and osteoconductivity that is the formation of new bone in the vicinity of the implanted biomaterials. The osteoconductivity of HACs has been found to be inferior to those of autografts and thus HAC is unsuitable for filling the wide clearance between implants and bone tissues with newly formed bone due to a slow osteoconduction process. Osteoconduction is considered to progress in six stages at the interface between the implanted biomaterials and injured hard tissues: (1) serum adsorption; (2) recruitment of various cell types; (3) attachment, motility, and proliferation of various cell types; (4) osteoblast differentiation and osteoid production; (5) matrix calcification; and (6) bone remodeling [1,2]. The adhesion of osteoblasts to the biomaterials is crucial in the regulation of the subsequent differentiation and the formation of the extracellular matrix following spreading and motility [3]. The present study thus focuses on the analysis of osteoblast behavior near biomaterials.

The mechanism of cell adhesion on biomaterials varies according to the type of substrate. Human osteoblast-like cells initially attach and spread more quickly on HA than on titanium [3]. HA and titanium surfaces, furthermore, influence gene expression at an early phase of adhesion as well as at the later phases of proliferation and differentiation [4]. These behaviors of osteoblasts can be attributed to the differences in surface characteristics, which affect the signal transduction pathways. The signal transduction pathways involved in the adhesion of osteoblasts on HA and titanium were confirmed by the subsequent expression of α5β1 integrins [3].

The adhesion of osteoblasts primarily depends on the surface characteristics of the biomaterials involved and stimulation from outside the cells. The surface characteristics are affected by surface roughness, surface crystallinity [4-6], constituent elements at the surface, and the incorporation of ions such as carbonate or fluorine [7,8]. Electron-induced surface energy modifications such as surface photovoltage
spectroscopy can be used to improve surface characteristics [9]. Stimulation from outside the cells includes electrical stimulation such as capacitive coupling, inductive coupling, and combined electromagnetic fields, which affect osteoblast attachment, adhesion, and motility [9]. Cell shapes on substrates depend on the integrin-mediated cytoskeletal and signal transduction molecules, such as actin filaments and vinculin [10,11], and are important during cell-substrate adhesion for subsequent cell behaviors such as proliferation and differentiation [8,12]. Therefore, the present study focuses on adhered cell shapes, as indicated by the actin structure, to study osteoblast adhesion.

We have recently demonstrated that the charged surfaces on dense HA induced by polarization [13-17] enhance osteoconductive capabilities in vivo [18,19] and that the charged surfaces on HAC induced by polarization enhance mineral deposition in simulated body fluid (SBF) [20] and osteoconductive capabilities in vivo [21]. Additionally, polarized HA affects both hard and soft tissues. It enhanced the blood vessel regeneration of a vascularity injured model [22] and epidermal recovery from full-thickness skin wounds in vivo [23]. Polarization treatment is thus considered to affect cell behaviors. The initial adhesion and motility of osteoblast-like cells on polarized HA were accelerated in vitro [24]. Although the polarization treatment enhanced new bone formation in the vicinity of the polarized HA in vivo, the mechanisms of the effects induced by the polarization treatment on osteoblast behavior were not completely identified.

In a previous study, β-tricalcium phosphate (β-TCP) as a starting material was coated onto Ti by the plasma-spraying method and transformed into the HA phase through hydrothermal treatment [20]. The different coating method was tried to intend to a wide range of applications. The HA was directly coated onto SUS 316 using the gas-tunnel-type plasma-spraying method. The electrical properties of HA ceramics were highly sensitive to the crystal structures and microstructures. In particular, the electrical polarization of HA was appreciably influenced by the crystal structure in the vicinity of protons and the grain boundary in the microstructure. The present study characterizes HAC with or without thermal treatment and the effects of electrical polarization of HAC on osteoblast adhesion.

2. Materials and methods

2.1 Sample preparation

HA powder as starting powder for the plasma spraying was synthesized from the analytical-grade reagents calcium hydroxide and phosphoric acid by the wet method [16]. The metal substrate was stainless steel (SUS-316) blocks (1 cm × 1 cm × 3 mm). The HAC of the metal substrates were prepared using the gas-tunnel-type plasma-spraying method developed by Arata et al. [25]. The thickness of the HAC layers was ca. 30 μm.

Surface characterization was performed to investigate the differences before and after heat treatment. Surfaces of HAC and heated HAC (H-HAC) specimens were observed by scanning electron microscopy (SEM; Hitachi S-2400, Japan). HAC and H-HAC specimens were characterized by X-ray diffraction (XRD). XRD measurements were performed for phase analysis at room temperature (RT) with CuKα radiation at 40 kV and 40 mA on a diffraction spectrometer (Philips PW1700, Netherlands) equipped with a graphite monochromator.

The HA-coated SUS 316 blocks were electrically polarized with a pair of platinum electrodes at 300 °C in a DC electric field of 30 V for 1 h in air according to our previous work (Fig. 1) [16,20]. The temperature for electrical polarization was decided to provide the appearance of the maximum point in thermally stimulated depolarization current (TSDC) spectrum less than 600 °C. The electrically polarized HAC are either negatively charged (N-HAC) or positively charged (P-HAC). The surface of HAC heated at 300 °C was denoted as H-HAC.

Polarization of the HA specimens was verified by TSDC measurement. The TSDC measurements were carried out according to our previous study [20,21] in air from RT to 600 °C at a heating rate of 5.0 °C/min. 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 using:

\[
Q_p = \frac{1}{\beta} \int J(T) \, dT
\]

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

2.2 Osteoblast culture

Osteoblast cells (MC3T3-E1 cell line) obtained from the RIKEN Cell Bank (Tsukuba, Japan) were used for the osteoblast adhesion assay. This cell line is widely used in the biomaterials field. It was easy to observe the morphology of each cell adhered on the HAC specimens. The cells were maintained in α-modified minimum essential medium (α-MEM), supplemented with 10% fetal bovine serum (FBS), 50 units/ml penicillin, and 50 μg/ml streptomycin in a humidified atmosphere of 5% CO₂ in air at 37 °C. After

![Figure 1. Schematic illustration of experimental procedure. HAC specimens were electrically polarized in a DC field of 30 V with a pair of platinum electrodes in air at 300 °C for 1 h. The electrically polarized HAC was either negatively charged (N-HAC) or positively charged (P-HAC). The surface of HAC heated at 300 °C was denoted as H-HAC.](image-url)
reaching 70% confluency, the cells were detached by treatment with 0.25% trypsin and then seeded into culture plates at a density of 0.5 x 10^4 cells/cm² in α-MEM containing 10% FBS, 50 units/ml penicillin, and 50 µg/ml streptomycin. The medium was changed every 3-4 days.

2.3 Osteoblast adhesion assay

The HAC specimens were sterilized with 70% ethanol and immersed in the cell culture medium for 30 min. The cells were seeded into HAC specimens at a density of 0.5 x 10^4 cells/ml in α-MEM containing 10% FBS, 50 units/ml penicillin, and 50 µg/ml streptomycin. After 1 h and 3 h, the cells on the HAC samples were fixed with 4% paraformaldehyde and permeabilized with 0.1% Triton X-100 in phosphate-buffered saline (PBS). The cells were stained with rhodamine phalloidin and Hoechst. Fluorescent signals were observed using a fluorescence microscope (Olympus IX71, Japan). The cell areas positively stained for actin were measured using MetaMorph® software. The measurement was performed for a minimum of 50 cells on each surface.

Accurate quantification in three HAC samples was achieved using three independent experiments. The differences were analyzed using one-way or two-way analysis of variance (ANOVA). Student’s t-test (paired or unpaired) was used to ascertain the differences between the two groups. The statistical significance was defined as p < 0.05.

3. Results and discussion

The XRD patterns of HAC and H-HAC were highly consistent with the published data of HA (ICDD No. 9-432), indicating that the HAC surfaces consisted of a single phase of hexagonal HA before and after heating at 300 °C (Fig. 2). SEM images of the surfaces of HAC and H-HAC are shown in Fig. 3. The surfaces were partially covered with spherical crystals approximately 30 µm in diameter and molten grains under the spherical crystals were observed. The surface characteristics of H-HAC revealed that heating at 300 °C had no effect on surface crystallinity, crystal phase, and morphology.

As shown in Fig. 4, the TSDC curve of electrically polarized H-HAC increased at ca. 400 °C, reached a shoulder point at ca. 600 °C, and then gradually increased. The shoulder of the TSDC curve indicates depolarized current being released from the electrically polarized H-HAC specimen. The stored charges were calculated from the TSDC spectra at 52 µC·cm⁻¹. The maximum current density and the stored charges calculated from TSDC spectra of the HAC were higher than those of dense HA [16]. The value of the stored charges of HAC was approximately 3.5 times higher than that for dense HA. It was previously suggested that the polarization and depolarization can be attributed to the migration of the protons of OH⁻ in apatite columnar channels [16]. According to a study on the complex impedance of HA [16], protons migrate through both the grains and grain boundary. The resistances of the grain boundaries were higher than those of the grains, indicating that the grain boundaries were obstacles to proton transfer.

Because the number of protons was proportional to volume, HAC should have a much smaller number than that of HA. However, the value of the stored charges of HAC was much higher than that of HA. It was suggested that the defects of calcium ions stimulate proton migration and act as possible trap sites of protons [13]. Moreover, the OH⁻ ions in HAC were unstable due to the heat history during plasma spraying, such as rapid cooling after heating at a high temperature. Consequently, the protons easily migrated.
The longer migration distance of the protons is considered to be another reason for the high stored charges of HAC. It was reported that the grain boundaries are obstacles for proton migration during the polarization of dense HA [13]. The observed average grain size of HA was approximately 1 μm [20]. Some HA grains had obvious boundaries and the average grain sizes were approximately 30 μm in diameter (Fig. 3). Most of the HA grains were molten because of the high-temperature plasma flame, and the grain boundary was indistinct. Therefore, the higher value of the stored charges of HAC can be attributed to the complicated structures of HAC.

Figure 5 shows actin and nuclei labeling of the cells after seeding onto the HAC specimens for 1 h and 3 h. During cultivation 1 h after cell seeding, the cells that adhered on H-HAC showed a round or spherical configuration. The cells that adhered on N-HAC and P-HAc showed a spindle- or fan-like shape after 1 h of cultivation. During subsequent incubation after 3 h of cell seeding, the cells that adhered on H-HAC were spread out and showed a slightly spindle-like or rectangular shape. An accumulation of actin filaments in the periphery of lozenge-shaped cells was observed for both N-HAC and P-HAC. In addition, bundles of actin fibers forming stress fibers appeared in the cells on N-HAC and P-HAC as the attached cells spread. Well-defined stress fibers showing a regular arrangement with particular polarities were found in some cells grown on N-HAC and P-HAC. In some cells on N-HA and P-HA, the actin filaments were mostly distributed near the edge of pseudopodia-like structures and formed weak bundles of stress fibers.

![Figure 5. Morphology of adhered osteoblasts on HAC, N-HAC, and P-HAC. Fluorescence images with actin and nuclei staining of the cells cultured on the HAC specimens for 1 h and 3 h. Scale bar = 50 μm.](image)

To quantify the difference in the degree of cell spreading between the three HAC specimen types, the cell areas positively stained for actin were measured (Fig. 6). The cell areas increased from 1 h to 3 h, which means that the cells spread and elongated on the HAC specimens. The cell areas were significantly larger on N-HAC and P-HAC compared to that on H-HAC 1 h and 3 h after seeding. The area of the cells cultured on the polarized HAC was approximately 1.2 times and 1.4 times larger than that on H-HAC 1 h and 3 h after seeding, respectively. However, no significant differences in cell area were observed between N-HAC and P-HAC. It would be useful to further investigate whether the polarization affects the proliferation and differentiation of osteoblasts, which are subsequent events in the osteoconduction process.

![Figure 6. Cell areas positively stained for actin for the three HAC specimens. The cell area was significantly larger on N-HAC and P-HAC compared to that on HAC at 1 h (*p < 0.005 compared with HAC) and 3 h (*p < 0.001 compared with HAC) after seeding.](image)

4. Conclusion

The surface characteristics of HAC specimens reveal that electrical polarization has no effect on surface roughness and crystallinity. Morphological observations and quantitative analyses of adhered osteoblasts on HAC specimens revealed that the cell areas positively stained for actin, which indicates the degree of cell spreading, were distinctly larger on N-HAC and P-HAC than that on H-HAC.

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References

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