The cell attachment and morphology of neonatal rat calvarial osteoblasts on the surface of Ti-6Al-4V and plasma-sprayed HA coating: Effect of surface roughness and serum contents

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The biocompatibility of material plays an important role in the bone–implant interface for the prosthetic implant fixation. The biocompatibility of implants is associated with the chemical composition, surface topography, surface energy and surface roughness of biomaterials. The effects of two factors, surface roughness and serum contents, on osteoblast behavior at the surface of Ti-6Al-4V and plasma sprayed HA coating were investigated in the experiment. The osteoblasts derived from neonatal rat calvarial were cultured in Dulbecco's modified Eagle medium (DMEM) with fetal bovine serum (FBS) on the surface of polished Ti-6Al-4V (Ti-p), grit-blasted Ti-6Al-4V (Ti-b), polished HA coating (HAC-p), and as-sprayed HA coating (HAC). Under culture medium containing 4% FBS, the level of cell attachment to the polished surface is significantly higher than the rough surface of the same experimental materials during all culture periods. Increasing the contents of FBS up to 10%, the difference of osteoblast attachment is not found between Ti-p and Ti-b. Under 4% serum condition, the cell morphology attached to smooth surfaces (Ti-p and HAC-p) is spread faster and are more flattened than the one to rough surface of the same experimental materials by SEM. After 24 h culture, the corroded cracks are easily observed at the surface of polished HA coatings, and the cell morphology on HAC-p coatings are elongated and less flattened compared with Ti-p. The result is consistent with statistical difference of cell attachment between Ti-p and HAC-p under 4% serum condition.

Introduction

In the application of biomaterial in orthopaedic surgery, bone cell response and biocompatibility of material play important roles in the bone-implant interface for long term survivors of the prosthetic implant fixation. The effect of the biocompatibility on bone apposition and bone formation is associated with the bone cell response induced by biomaterials. Current studies use the model of cell culture to investigate the biological response of bone implant interface during early phase [1, 2]. The factors of chemical composition, surface topography, surface energy and surface roughness of biomaterials would affect the bone cell response of implants, and further influence the biocompatibility in clinical use.

Some researchers investigate the influence of surface roughness on bone integration of implants [3–6]. *In vivo*, rough surfaces were found to produce better bone fixation than smooth surfaces [3–6], suggesting that this surface property might have a direct effect on the attachment of osteoblasts and their subsequent proliferation and differentiation. However, in the study of Larsson *et al*. [7], a reduction of surface roughness of titanium had no influence on the amount of bone after one year in rabbit cortical bone, and a high degree of bone contact and bone formation is achieved after one year titanium implants which are modified with respect to different surface roughness. Recently, some studies investigate the surface roughness of implants which affect bone cell response examined with cell culture [8–13]. Most of these results suggest that cell proliferation, differentiation, protein synthesis and matrix production are affected by surface roughness and composition *in vitro*.

In orthopaedic surgery, hydroxyapatite (HA) has the same chemical and crystallographic structure as the

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apatite of living bone, and can bond physicochemically with bone and promote bone growth onto its surface [14–17]. HA is therefore considered as an excellent bone substitute [18]. Among metals, increasingly widespread use of porous coating devices manufactured from titanium or its alloys is due to their relatively excellent corrosion resistance and favorable biocompatibility, compared to stainless steel and cobalt chromium alloys [19, 20]. The combination of the mechanical properties of titanium and the biocompatibility of HA might provide an implant device with good fixation to the surrounding bone. Clinical applications of HA-coated implants are reported to promote the formation of normal bone at its surface [21–23]. Revealing excellent biocompatibility and mechanical properties, plasma-sprayed bioactive HA-coated Ti-alloy implants have raised much interest as an approach to achieve reliable implant to bone fixation.

Most *in vivo* studies reported that HA-coated Ti-6Al-4V implant is more osseointegrate and osteoconductive than uncoated ones [15, 24]. However, Carlsson *et al.* [25] have found that there were no statistically significant differences in bone apposition seen between the commercially pure titanium and hydroxyapatite-coated implant after three- and six-month implantation in arthritic human knees. In some studies, the cell growth and biological function of bioactive HA is superior to bioinert Ti-6Al-4V [26]. But, the study of Puleo *et al.* [27] reports that less percentage of cells attached on HA surface than titanium surface, and the doubling time of osteoblast growth on HA surface is more than on titanium at early cultured stage (four days). The confused results express that the HA is not absolutely superior through the stage of bone attachment and bone formation. In order to distinguish the influence of biomaterial on cell response, the stage from bone attached to bone formation need further investigation.

In this study, four kinds of specimens, grit-blasted Ti-6Al-4V (Ti-b), polished Ti-6Al-4V(Ti-p), plasma-sprayed HA coatings (HAC), and polished HA coatings (HAC-p), were carefully prepared. The properties of Ti-6Al-4V and HA coatings were well identified to prevent the deviation during the preparation of specimens. The purpose of this investigation was to evaluate the influence of Ti-6Al-4V and HA coatings with different surface roughness on neonatal rat calvarial osteoblast adhesion and morphologies during the early phase ($<$ two days). Besides, the different contents of serum in cell culture medium were also adopted to investigate the cell attachment on the Ti-6Al-4V and HA coatings with different surface roughness.

Materials and methods

Preparation of specimens

The 12.7 mm $\varnothing \times$ 2.0 mm disk plate used in this study was a surgical grade Ti-6Al-4V alloy (ASTM F136-92). The high-purity feed stock HA powder was adopted in the plasma-sprayed process, and the spraying parameter is listed in Table I. For smoother surface of HA coatings, specimens are ground by SiC papers and finally polished by 1.0 μm Al_2O_3 slurry. In the group of the Ti-6Al-4V discs, the rough surface of Ti-6Al-4V was obtained by Al_2O_3 grit-blasted, and the smooth surface of the Ti-6Al-4V specimens was prepared by the following procedure: ground by SiC and finally polished by 1.0 μm Al_2O_3 slurry. All the specimens were subjected to sonication five times in de-ionized water and once in absolute alcohol and packed in double-sealed autoclaving bags and steam sterilized at 121 °C for 30 min and dried at 121 °C for 15 min.

TABLE I Plasma spraying parameter employed for preparing the HA coatings

Parameter	
Primary gas, flow rate (1 min^{-1})	Ar, 41
Secondary gas, flow rate (1 min^{-1})	H_2, 8
Powder carrier gas, flow rate (1 min^{-1})	Ar, 3.2
Powder feed rate (g min^{-1})	20
Power (kW)	40.2
Stand-off distance (cm)	7.5
Surface speed (cm min^{-1})	2400
Transverse speed (cm min^{-1})	60

Plasma spraying was performed with a Plasma-Technik system (M-1100 C).

Characterization of specimens

After surface and autoclaving treatment, the morphology of specimens was observed by scanning electron microscopy (SEM) (Hitachi S-2500). The surface roughness of the specimens was quantified using a surface profilometer (Kosaka Laboratory Ltd., Surfcorder SE-30H). The phase composition of HA coatings with or without polished treatment were analyzed by X-ray diffractometer (XRD, Rigaku D/Max III. V) with a scan speed of 4° min^{-1}, using Cukα radiation (30 kV, 20 mA).

Shallower surface chemical analyses by X-ray photoelectron spectroscopy (XPS) were carried out for Ti-6Al-4V subjected to surface and autoclaving treatments. All analyses were carried out using VG Scientific ESCALAB 210 (analyzed area: 0.5 mm in diameter), operated at 12 kV and 20 mA in a pressure less than 10^{-8} mbar, using Mg Kα radiation. Measurements of binding energy in the range 0–1000 eV were made at a ''take-off'' angle of 45° with respect to the sample surface. High-resolution scans of Ti, Al, V, and O peaks were performed on selected specimens.

Cell culture

Osteoblasts were isolated by sequential trypsin-collagenase digestion on calvaria of neonatal ($<$ four days old) Sprague-Dawley rats [28]. In short, the calvaria were excised under aseptic conditions and kept in ice cold phosphate buffered saline (PBS). The fibrous layers of the periostea were mechanically removed. The calvaria were then incubated with Dulbecco's modified Eagle medium (DMEM) for 2 × 10 min at 37 °C and rinsed 2 × 5 min with PBS. To diminish fibroblastic contamination and cell debris we preincubated the calvaria for 20 min with enzyme solution (18 000 units/ml collagenase I, 0.125% trypsin, 0.004 M EDTA, and 0.02% DNase I at 37 °C) and discarded the supernatant. After continuous enzyme treatment (6 × 20 min) the third and fourth supernatants were centrifuged (10 min at 1000 r.p.m.; 250 g). The pellets were resuspended in

DMEM containing 10% fetal bovine serum (FBS) and maintained in a humidified, 5% CO_2/balance air incubator at 37 °C. The phenotype and function of the osteoblasts were characterized by the presence of alkaline phosphatase and deposition of calcium phosphate mineral *in vitro* [28]. Subcultured cells were used for experiments after two or three passages.

The sterilized disks were placed in 24-well culture plates. These tissue culture plates coated with 12% poly-HEMA (hydroxyethylmethacrylate) held the biomaterial samples to ensure that the osteoblasts would grow on the biomaterials only and not the tissue culture wells [29]. For experiments, the osteoblast cells were seeded on the disks at a density of 75 000 cells/ml and flooded with growth medium supplemented with 4% FBS, 50 μg/ml ascorbic acid and 10 mM β-glycerophosphate. The culture medium was changed every two days during culture.

Cell number

The cell numbers on Ti-b, Ti-p, HAC, and HAC-p, were determined after 3, 6 and 24 h of osteoblasts seeding. The two different contents (4% and 10%) of FBS were adopted to evaluate the effect of culture medium on cell number responses at the early phase. At the designated times (2×10 min), the osteoblasts released from the biomaterial surface by addition of 0.05% trypsin contain 1 mM EDTA in PBS. A second trypsinization was performed to ensure that any remaining cells had been removed from the surface. SEM examination of disks used in preliminary studies showed that following two trypsinizations, all cells and matrix were removed from the surfaces of specimens (data not shown). Cell suspensions from both trypsinization were combined and centrifuged at 250 g for 10 min. The supernatant was decanted, and the cell pellet was reconstituted with DMEM for measurement with hemocytometer. Cell viability was determined by trypan blue exclusion, then counted the cell number with a hemocytometer.

Cell morphology

Under culture medium containing 4% FBS, specimens (Ti-b, Ti-p, HAC, and HAC-p) were prepared for SEM after 3-, 6-, 24- and 48 h cultures. The medium was pipetted out from the dishes, and the plates rinsed several times with cacodylate buffer (pH 7.2), and fixed for 2 h with 2.5% glutaraldehyde in 0.1 M cacodylate buffered at pH 7.2; with post-fixation in 1% OsO_4 in buffer for 1 h; treatment with 1% tannic acid in buffer for 1 h; dehydration in an ascending alcohol series; and immersion in HMDS (hemaethyldisilazane) for 10 min in lieu of critical point drying [30]. Finally, after sputter coating with gold, the specimens were examined using SEM at an accelerating voltage of 25 kV.

Statistical analysis

Each data point represents the mean ± standard deviation of five individual cultures. The analysis of one-way variance (ANOVA) was used to evaluate the significance differences between cell growth on different kinds of materials. Differences were considered significant at $p \leq 0.05$.

Results

Characterization of specimens

Surface morphology

The SEM observation on the surfaces of Ti-p, Ti-b, HAC-p, and HAC is shown in Fig. 1(a)–(d). After plasma

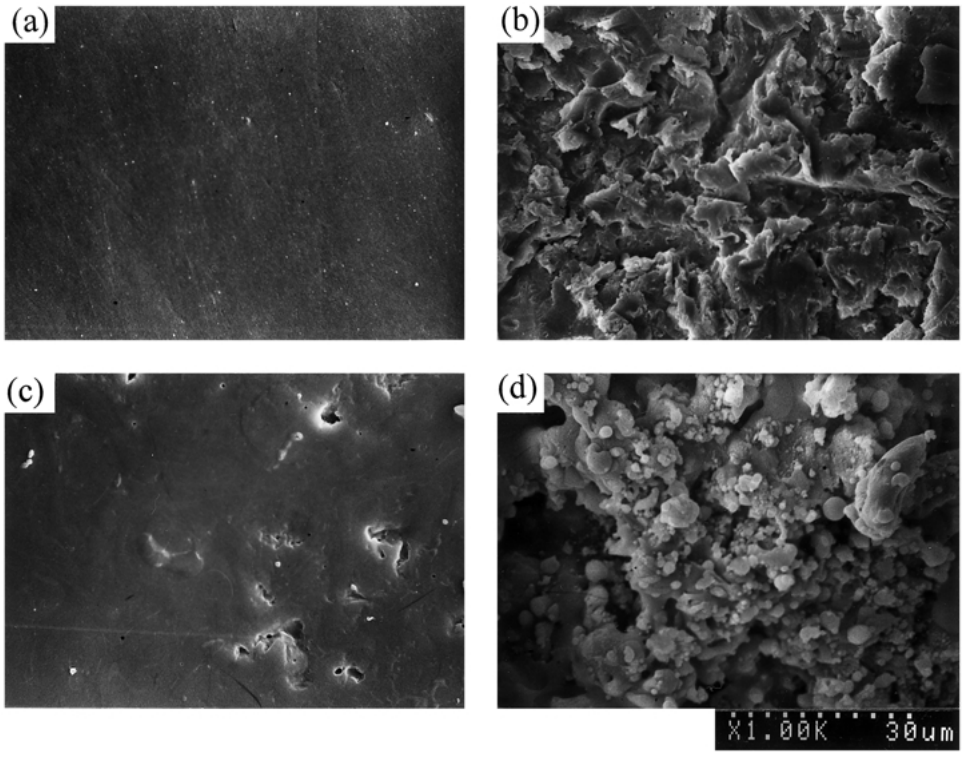

Figure 1 The surface morphologies of specimens: (a) Ti-p, (b) Ti-b, (c) HAC-p, and (d) HAC.

TABLE II Average surface roughness (μm) of Ti-6Al-4V and HA coating (HA) with different surface treatment

Materials	Surface treatment	Roughness
Ti-6Al-4V	Polished	0.63 ± 0.11
	Grit-blasted	4.64 ± 0.38
HA coating	Polished	0.67 ± 0.13
	As-sprayed	10.37 ± 1.35

The value is presented by mean ± standard deviation.

spraying, HA is a complete molten matrix with accumulated splats, and the surface morphology has craggy shapes of peaks and valleys. In contrast, after polishing, HA coatings have a smoother surface with pores found by the SEM observation. In comparison with Ti-p, the pores found in the surface of HA-p could be the original closed pores in the inner HA coatings, and the open pores were observed after removal of outer surface of HA coatings during the polishing process. After grit-blasted treatment, surface morphology of Ti-b is characterized as irregular with rough topography in comparison with the smooth surface of Ti-p.

Surface roughness

The surface roughness of specimens is shown in Table II. After polishing, the average surface roughness of Ti-p and HAC-p is significantly lower than Ti-b and HAC, and there is no significant difference found between Ti-p and HAC-p. As shown in Table II, HAC is the roughest among all groups of specimens and statistically rougher than Ti-b.

XRD analysis

By XRD analysis, the phase composition of plasma-sprayed HA coatings consist of HA phase and several impurity phases. These impurity phases include α-$Ca_3(PO_4)_2$ (α-TCP), β-$Ca_3(PO_4)_2$ (β-TCP), $Ca_4P_2O_9$, and CaO. After polishing, the phase composition of HAC-p is consistent with HAC specimens. The relative contents of phase composition of HAC and HAC-p are shown in Table III. The results indicate that the polishing treatment has not changed the phase composition and contents in HA coatings.

XPS analysis

After autoclaving treatment, the broad-range XPS spectra from the nano-surface of two different surface treatments for Ti-6A1-4V are shown in Fig. 2. The scans for the two surface treatments are very similar, with no indication of the presence of V, while the presence of V on the alloy sample can be detected by the V 2p3 peak at 515 eV on a more sensitive scale. A summary of the surface chemical composition by XPS analyses is given in Table IV. As shown in Table IV, the results of XPS analysis indicate no apparent difference in nano-surface composition for Ti-p with respect to Ti-b. High-resolution Ti 2p spectra are examined to probe the changes in the nature of the oxide layers as a function of surface treatment. The predominant oxide phase is TiO_2, and the various suboxide phases, Ti^{2+},Ti^{3+}, and metallic Ti are also seen in the spectra of Ti-b and Ti-p. The thickness of the oxide layer is estimated by comparing peak intensity of the total Ti 2p3 oxide component to the Ti 2p3 metallic component. Using the formula devised by Lausmaa et al. [31] with an electron mean free path of 1.7 nm, the surface oxide thickness for Ti-p and Ti-b after autoclaving treatment are 2.25 nm and 2.34 nm, respectively. In our previous study [32], the oxide thickness of Ti-6Al-4V is significantly decided by the factors of passivation treatment and autoclaving procedure. In this study, we adopt the same passivation treatment and autoclaving procedure for Ti-p and Ti-b. Therefore, Ti-p and Ti-b have a similar the surface oxide thickness after the same passivated and autoclaving treatment. In high-resolution scans of O 1s spectra, basic and acidic OH groups are formed in the oxide surface of Ti-p and Ti-b by assuming chemisorption, and their

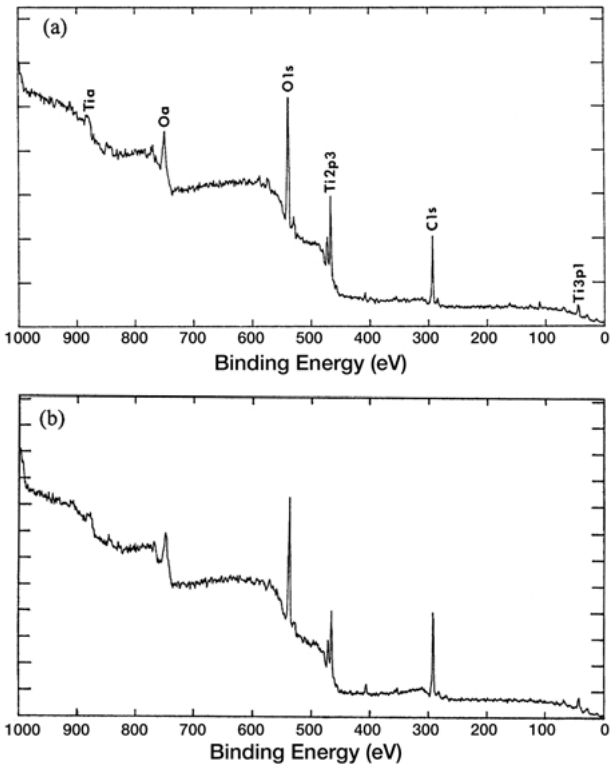

Figure 2 Broad-range XPS spectra of Ti-6Al-4V with autoclaving: (a) polished and (b) grit-blasted.

TABLE III The concentrations (in wt %) of the impurity phase of the as-sprayed and polished HA coatings

Impurity phase	As-sprayed	Polished
α-TCP	16.38%	16.38%
β-TCP	12.77%	12.75%
$Ca_4P_2O_9$	15.23%	15.12%
CaO	5.99%	6.03%

Data obtained by the internal standard method.

TABLE IV Surface chemical composition (wt %) by XPS analyses for Ti-6Al-4V specimens with autoclaving treatment

Surface treatment	Ti	Al	V
Polished	82.79	13.96	3.25
Grit-blasted	81.26	14.33	3.41

TABLE V High resolution XPS surface chemical analyses (at %) of oxygen spectra for Ti-6Al-4V specimens with autoclaving treatment

Surface treatment	Ti-OH basic	OH acidic	Ols
Polished	10.76	21.66	67.58
Grit-blasted	11.09	20.98	67.93

chemical nature arises from the way they bond, either to one or two titanium ions. Table V summarizes the results of oxygen spectra for the two different specimens with autoclaving treatment. As shown in Table V, the contents of basic and acidic groups are similar between Ti-p and Ti-b.

Cell attachment

After 3-, 6-, and 24 h culture in medium containing 4% FBS, the cell number of osteoblasts attached on the different specimens (Ti-p, Ti-b, HAC-p and HAC) were counted. Under culture medium with 4% FBS (Fig. 3 and Table VI), the level of cell attachment to the surface of Ti-p is significantly higher than Ti-b and HAC during all culture periods. However, the statistical difference is found between Ti-p and HAC-p only after 24 h culture. The osteoblast attachment to the smooth surface of HAC-p is also statistically higher than the rough surface of HAC in all culture periods. During all experimental periods, the cell attachment on the surface of HAC-p is higher than Ti-b, while the statistical differences are not found in cell attachment for HAC-p and Ti-b. Between HAC and Ti-b specimens, the higher osteoblast adhesion is found at the surface of Ti-b than HAC, and the significant differences are found in all experiment periods.

Under 10% FBS, the graph of the osteoblast attachment versus time for four kinds of specimens is illustrated in Fig. 4. Increasing the content of FBS from 4% to 10% in medium, the higher level of osteoblast attachment is found on the surface of specimens under 10% serum, and significant differences are found between the same specimens cultured with two different contents of FBS in all experimental periods. Under

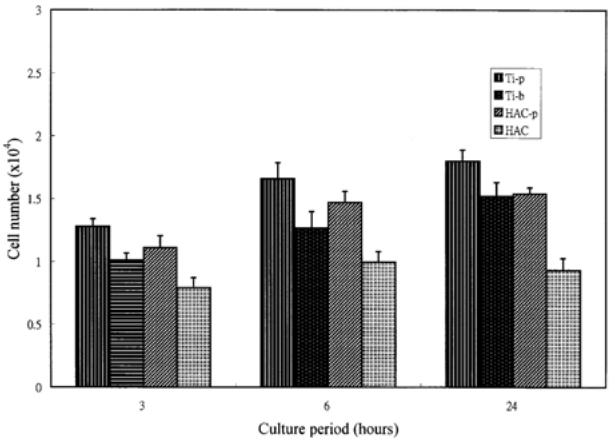

Figure 3 Under 4% serum condition, the growth of osteoblasts on different specimens. Values are the mean ± standard deviation ($n = 5$).

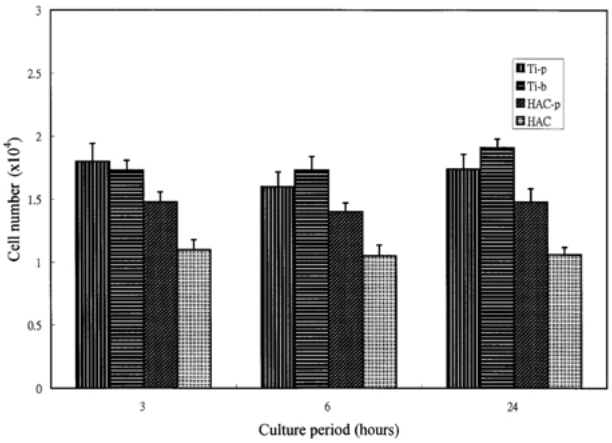

Figure 4 Under 10% serum condition, the growth of osteoblasts on different specimens. Values are the mean ± standard deviation ($n = 5$).

culture medium with 10% FBS, the osteoblast attachment could be divided into three levels based on three groups of specimens, (1) Ti-p and Ti-b, (2) HAC-p, and (3) HAC. During all culture periods (Table VII), the level of osteoblast attachment to the surface of Ti-b and Ti-p is significantly higher than HAC-p and HAC, and there is no statistical difference between Ti-p and Ti-b. Between HAC-p and HAC, the significantly higher level of osteoblast attachment on HAC-p is also found than the one to HAC during all culture periods.

Cell morphology

In contrast to 10% serum condition, there are apparent differences found among four kinds of specimens in 4% FBS condition. Therefore, in this study, the osteoblast morphology is investigated under medium containing 4% FBS to evaluate the influence of surface roughness.

After 3 h of culture, the osteoblast morphology on the surface of specimens is observed by SEM (Fig. 5). On the smooth surface of Ti-p, the cell morphology demonstrates rough texture, and the thin rim of cytoplasm has spread out from the cell body whose nucleus is clearly observed. The osteoblast morphology on HAC-p surface is similar to the one on Ti-p, however the more elongated and less flattened morphology is observed on HAC-p. By the observation of SEM, the attached cells demonstrate spherical shape and protruded surface on the rougher surface of Ti-b and HAC.

After 6 h of culture, Fig. 6(a)–(d) represent the micrographs of osteoblasts on all four specimens, respectively. On the surface of Ti-p, the cells spread from the center, and the filopodia extending along the substrate surface at the base of the cells with fibers observed in the extracellular matrix. The osteoblast morphology on HAC-p surface is similar to the one on Ti-p. However, the more elongated and less flattened morphology is observed on HAC-p. In comparison with the smoother surfaces of HAC-p and Ti-p, the multiple filopodia extend and the cells appear polygonal on the rougher surface of Ti-b and HAC.

After 24 h of culture, the osteoblast morphology on the surface of specimens is shown in Fig. 7. The cytoplasm is

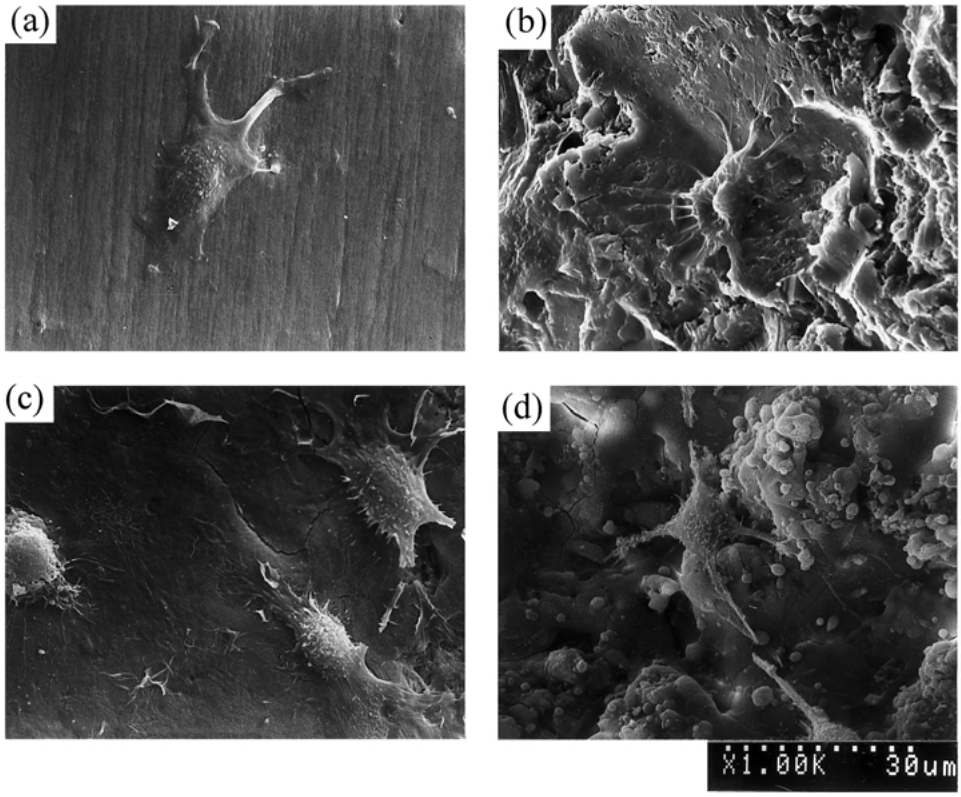

Figure 5 Under 4% serum condition, scanning electron micrographs of osteoblasts cultured for 3 h on (a) Ti-p, (b) Ti-b, (c) HAC-p, and (d) HAC.

almost completely spread, and shows a flattened morphology with almost no dorsal ruffles and filopodia on the surface of Ti-p. The osteoblast morphology on the surface of HAC-p is similar to Ti-p, while the more elongated and less flattened morphology is still observed on HAC-p compared to the one on Ti-p. Notice the presence of corroded surface is observed easily by the existence of cracks in the surface of HAC-p. The corroded surface is possibly induced by the dissolution of soluble phases, α-TCP, β–TCP and $Ca_4P_2O_9$, in HA coatings. The rapid dissolution of soluble phases will decrease the mechanical strength and prompt the formation of cracks. Besides, the original pores found at the surface of Ti-p are easily attacked by medium. At 24 h culture, the cell morphology on the rough surface of Ti-b and HAC are similar to 6 h.

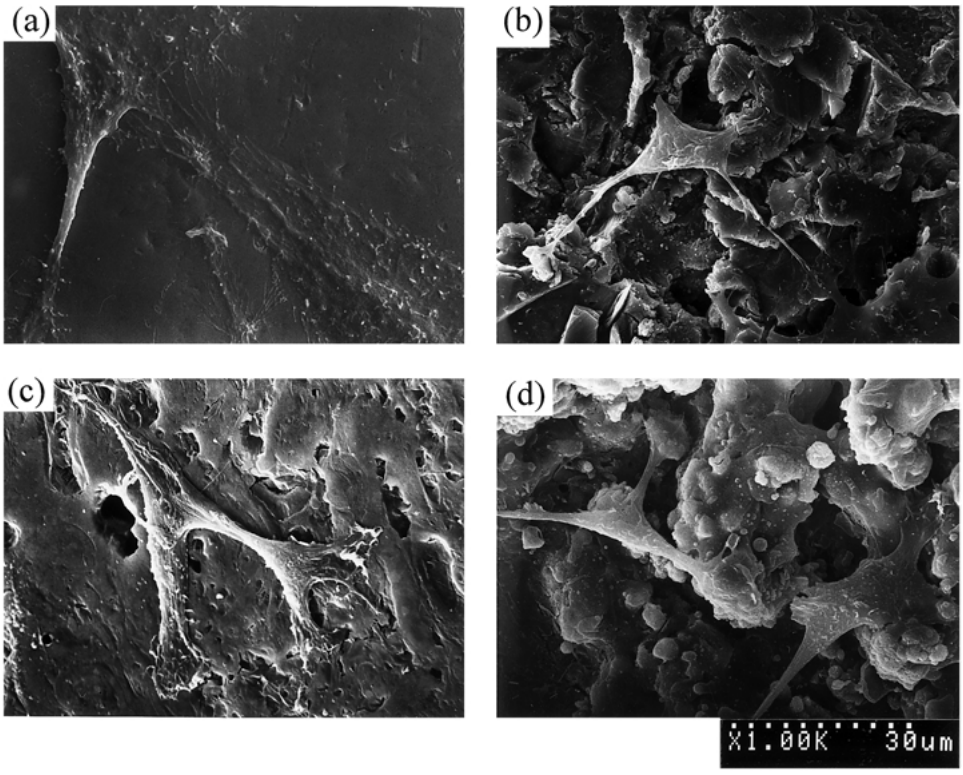

Figure 6 Under 4% serum condition, scanning electron micrographs of osteoblasts cultured for 6 h on (a) Ti-p, (b) Ti-b, (c) HAC-p, and (d) HAC.

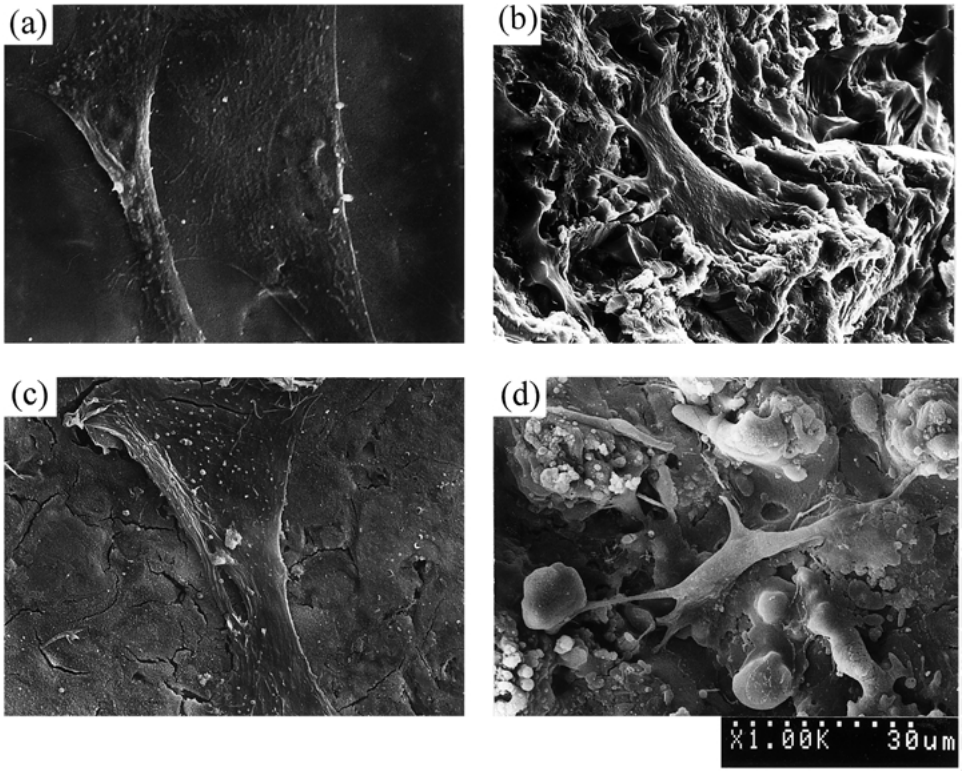

Figure 7 Under 4% serum condition, scanning electron micrographs of osteoblasts cultured for 24 h on (a) Ti-p, (b) Ti-b, (c) HAC-p, and (d) HAC. After 24 h culture, the presence of corroded surface is observed by the existence of crack in the surface of HAC-p.

Fig. 8(a)–(d) represent randomly taken SEM osteoblast on Ti-p, Ti-b, HAC-p, and HAC after 48 h culture. On the surface of Ti-p, the osteoblast morphology at 48 h is similar to 24 h, and 48 h exhibits a more confluent and a fibrous network between the cells. The osteoblast morphology on the surface of HAC-p is similar to Ti-p at 48 h. The edge of the spreading cytoplasm at the rougher surface of Ti-b and HAC is not readily distinguishable, and the cells on Ti-b and HAC have spread incompletely and the cell structure is more compact. They exhibit more cuboidal cell morphology, and appear more elongated and less flattened. Some cells span the macropores apparently by first extending long filopodia across the pores.

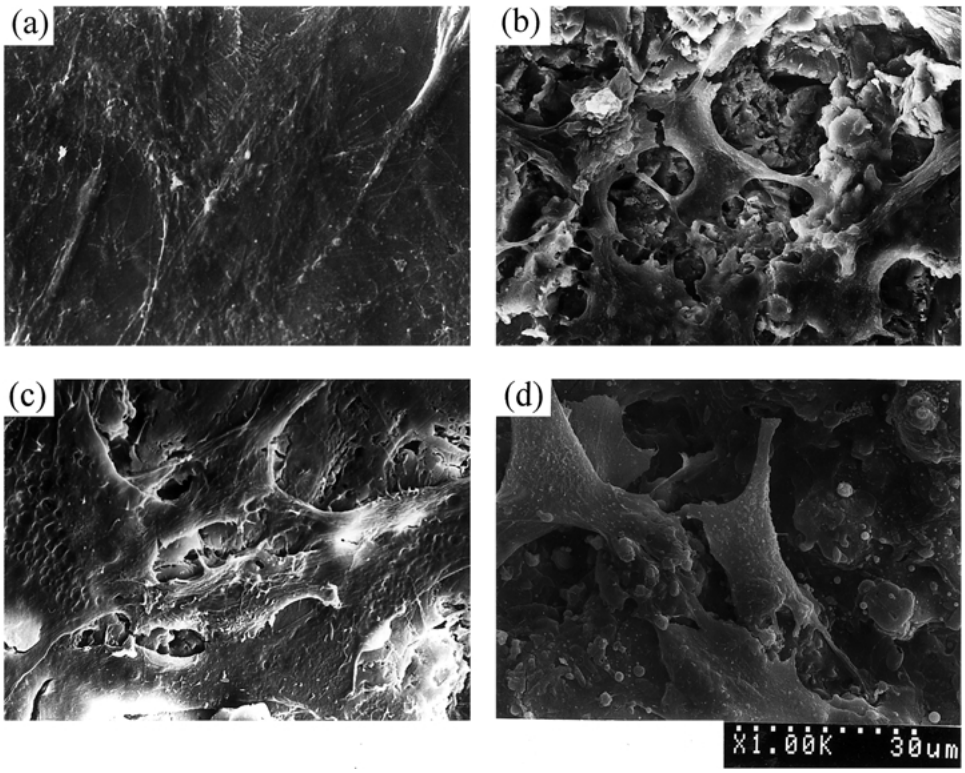

Figure 8 Under 4% serum condition, scanning electron micrographs of osteoblasts cultured for 48 h on (a) Ti-p, (b) Ti-b, (c) HAC-p, and (d) HAC.

TABLE VI Under 4% serum condition, the results of statistical comparison applied to the data of cell attachment to the surface of specimens

	Ti-p	Ti-b	HAC-p	HAC
3 hours				
Ti-p	—	S	NS	S
Ti-b		—	NS	S
HAC-p			—	S
HAC				—
6 hours				
Ti-p	—	S	NS	S
Ti-b		—	NS	S
HAC-p			—	S
HAC				—
24 hours				
Ti-p	—	S	S	S
Ti-b		—	NS	S
HAC-p			—	S
HAC				—

S, significant difference ($p \leq 0.05$); NS, not significant difference; Ti-p, polished Ti-6Al-4V; Ti-b, grit-blasted Ti-6Al-4V; HAC-p, polished HA coating; HAC, as sprayed HA coating.

TABLE VII Under 10% serum condition, the results of statistical comparison applied to the data of cell attachment to the surface of specimens

	Ti-p	Ti-b	HAC-p	HAC
3 hours				
Ti-p	—	NS	S	S
Ti-b		—	S	S
HAC-p			—	S
HAC				—
6 hours				
Ti-p	—	NS	S	S
Ti-b		—	S	S
HAC-p			—	S
HAC				—
24 hours				
Ti-p	—	NS	S	S
Ti-b		—	S	S
HAC-p			—	S
HAC				—

S, significant difference ($p \leq 0.05$); NS, not significant difference; Ti-p, polished Ti-6Al-4V; Ti-b, grit-blasted Ti-6Al-4V; HAC-p, polished HA coating; HAC, as sprayed HA coating.

Discussion

In this study, we adopt the polishing treatment to produce two different roughness of HA coatings. In our previous studies [33, 34], different roughness levels of the surface could be manipulated by different plasma-sprayed parameters. By XRD analysis, we found, moreover, different phase contents in HA coatings could be changed by different plasma sprayed parameters. For example, higher enthalpy parameter will obtain rougher surface with higher impurity phases in HA coatings, which would present the higher dissolution rate and more loosened structure in serum added simulated body fluid [33, 34]. *In vivo*, the higher contents of impurity phase in HA coating would easily induce dissociated particles from coatings and cause adverse biological responses in comparison with lower contents of impurities phase in HA coatings [24, 25].

For Ti-6Al-4V, two kinds of surface treatment were used to obtain different surface roughness. There is a possibility that we might obtain two different surface properties with different treatment, polishing or grit-blasting. In clinical use, the reasons for the apparent success of titanium or its alloy implants have been attributed to the existence of a thin, stable passivating oxide layer of TiO_2. The reasons for the apparent success of titanium and its alloy implants have been attributed to the existence of a thin, stable passivating oxide layer of TiO_2. The oxide layer of TiO_2 is known to have at least two types of hydroxyl groups: acidic OH with oxygen doubly coordinated to titanium, and basic Ti–OH groups singly coordinated. The presence of basic and acidic OH groups is derived from the hydrated titanium oxide exposed to ambient moisture. The acidic hydroxyl groups tend to act as cation exchange sites, while the basic hrdroxyl groups may act as anion exchange sites [35–41]. Healy and Ducheyne [42] have suggested that titanium is not in direct contact with the biological milieu; rather there is a gradual transition from the bulk metal: near-stoichiometric oxide, Ca and P substituted hydrated oxide, adsorbed lipoprotein and glycolipids, proteoglycans, collagen filaments and bundles to cells. Albrektsson *et al*. [20] and Kasemo [36] mentioned that the biocompatibility of titanium implants is associated with the surface titanium oxide, not with the titanium metal. After autoclaving treatment, the properties of titanium oxide on the surface of polished Ti-6Al-4V are similar to grit-blasted Ti-6Al-4V by XPS analysis. For example, the similar nano-surface composition, oxide thickness and contents of amophertic OH groups are observed between two different surface treatment specimens (Tables IV and V). In this study, we tried to compare the behavior of osteoblasts at the Ti-p and Ti-b with the same surface and nano-surface properties except for the surface roughness, and the different results of cell culture of Ti-p and Ti-b could be possibly attributed to the factor of surface roughness.

Under 4% FBS condition, the levels of cell attachment on the smooth surface are significantly higher than those on the rough surface of the same experimental materials. The result is consistent with the cell morphology. By the observation of SEM, the osteoblasts on the smoother surface exhibit a broadly spread and flattened morphology compared to cuboidal morphology on the rougher surface. In the studies of other researchers, they concluded that more flattened and well spread cells would show higher proliferate rates than those round spherical cells. For example, Folkman and Moscona [43] and Archer *et al*. [44] mentioned that one of the main regulators of proliferate rate in anchorage dependent cells is shape. In their studies, the cells, attached to materials with less spread, will show lower proliferate rates than those on the material with better spreading. Similar results were also observed by Hunter *et al*. [45]. They found that the surface of biomaterials with the greatest number and area of adhesion plaque spread well and flattened whilst those materials with the least number of adhesion plaque were more rounded and less spread.

Increasing FBS contents up to 10%, no significant

difference of cell attachment was found between Ti-p and Ti-b during all culture periods. The result indicates that by increasing FBS content, it is possible to diminish the factor of Ti-6A1-4V surface roughness. As a general rule, cells do not directly bind to material surfaces. According to the results of Eriksson *et al*. [46], cells associated with the titanium surface were almost never seen directly apposed to the implant but were attached to the surface via fibrin strands. After plating, the serum conditioned the reaction of material surface to provide better environment for cell attachment and spreading. In the study of Schneider *et al*. [47] and Bagambisa *et al*. [48], some cell adhesion proteins in serum enhanced cell attachment and spreading. However, the precise composition of the material surface adsorbed protein is not clear because the surfaces are different from the corresponding bulk of the material. For thermodynamic reasons they contain unsaturated bonds which lead to the formation of the surface reactive layers and adsorbed contamination layers [49]. In the study of Buser *et al*. [50], they evaluated the shear strength of titanium implants with different surface treatments (sandblasted and acid-etched surface), and the results indicated that the interface strength of titanium implants is significantly influenced by their surface characteristics. In this study, by careful preparation, the phase composition and content of HA and surface properties of Ti-6Al-4V were unchanged after surface treatment. Therefore, we could compare the behavior of osteoblasts on HA coatings and Ti-6Al-4V influenced by the factor of surface roughness and serum contents. The higher serum content (10% FBS) diminished the factor of surface roughness on the osteoblasts attachment at Ti-6A1-4V, but a similar result was not found between HAC-p and HAC. As shown in Table II, the average roughness of HAC-p and HAC are 10.37 μm and 0.67 μm, respectively. The deviation of roughness between HAC-p and HAC is significantly higher than 0.63 μm for Ti-p and 4.64 μm for Ti-b. It is possible that the significantly different levels of surface roughness between HAC-p and HAC would reduce the influence of serum contents.

Of the two different experimental materials, Ti-6Al-4V with polishing treatment provided more optimal surface properties for osteoblast attachment than HA coating with the same polishing treatment. As shown in Fig. 7(c), the surface of HAC-p was attacked by surrounding medium, and cracks are also easily found after 24 h culture. In our previous study, the corroded surface of HA coatings are well investigated in simulated body fluid containing serum [34]. The results show that a bonding degradation of approximately 25–33% of the original strength was measured after HA coatings had been immersed in SBF, and that predominantly depended on the period of immersion. The surface morphologies of HA coatings have dissolved in the SBF, and it is suggested that the interlamellar structure of the HAC was weakened and, therefore, the bonding strength degraded. In this study, the loosened structure of HAC-p can not provide optimal surface for osteoblasts attachment compared to Ti-p. Because it is impossible to produce 100% denser HA coatings by plasma-sprayed technique, the porosity in HAC-p is possibly another reason for the behavior of osteoblasts.

In this study, titanium alloy has superior osteoblast adhesion compared to HA coatings during 2-day culture. The result is consistent with the studies of Puleo *et al*. [27] and Santis *et al*. [13]. It is possible that the titanium alloy is more inert and stable than HA coatings. After soaking in simulated body fluid with or without serum content [33, 34], some impurity phases (TCP, $Ca_4P_2O_9$) in HA coatings would significantly dissolve in the early phase, and the behavior of dissolution possibly decreases the mechanical properties. After soaking in simulated body fluid [51], the interface reaction between titanium alloy and the surrounding environment is insignificant, and the new growth of oxide film formed at the titanium alloy surface would further protect the substrate from the aggressive medium. The stable surface of titanium alloy compared to HA coating possibly provides better conditions for cell attachment in this study. Although the significant differences in bone apposition between the commercially pure titanium and HA coated implant are not found in arthritic human knees [5], some *in vivo* studies have found that the HA-coated Ti-6Al-4V implant is more osseointegrate and osteoconductive than uncoated implants [15, 24]. *In vivo* and *in vitro*, bone formation is a serial process, and the cell behavior is modified with serial changes of environment. Although the Ti-6Al-4V provides better surface for osteoblast adhesion during the early phase, the effect of biomaterials on later frames of osteoblasts differentiation, matrix production, and calcification is as important as cell attachment and plays a major role in long term implant success. However, in order to comprehend the effects of biomaterials and to obtain a complete interpretation of these events, further investigation of a series experiment at different stages is required.

Conclusions

This work was carried out to investigate the effect of surface roughness, serum contents, and experimental materials on osteoblast attachment and spreading. By careful preparation, the phase composition and content of HA and surface properties of Ti-6Al-4V were unchanged after surface treatment. Under 4% FBS condition, the cell attachment at the smoother surface was significantly higher than rougher surface of same experimental materials. Increasing the serum content up to 10%, there was no statistical difference found between polished and grit-blasted Ti-6Al-4V. By SEM, the osteoblasts were early spreading and flattened at the smoother surface under 4% FBS, while the cells showed later adhesive, elongated and less flattened morphology at the rougher surface. During early periods (up to 48 h), Ti-6Al-4V provided more optimal surface properties for osteoblasts attachment than HA coating after the polishing treatment, and this could be attributed to the looser structure of HA coatings after immersion in culture medium.

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