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Journal of the European Ceramic Society 32 (2012) 2647–2652

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Response of osteoblast-like MG63 cells to TiO_2 layer prepared by micro-arc oxidation and electric polarization

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Available online 22 March 2012

Abstract

Micro-arc oxidation (MAO) is one of the useful surface modifications of titanium implants to improve bioactivity. Also, electric polarization treatment enhances bioactivity of calcium phosphate. The aim of this study was to evaluate the effect of the combination of two surface modifications, micro-arc oxidation (MAO) with electric polarization, on the behavior of osteoblast-like osteosarcoma MG63 cells. MAO-treated materials had a surface geometry that was favored by MG63 cells as determined using scanning electron microscopy and X-ray diffraction; additionally, electric polarization induced surface electric fields, which were measured using thermally stimulated depolarization currents. The results of assays to study cell–material interactions suggest that these two approaches could regulate cell attachment, spreading, proliferation, and differentiation without the addition of other reagents. This new surface modification processes produce materials with a good surface geometry, generate surface electric fields and enhance the osteopromotive ability of osteoblasts.

Keywords: Electric polarization; TiO_2; Interfaces; Biomedical applications

1. Introduction

Titanium is more widely used as a biomaterial for dental and orthopedic implants than to other metallic implant materials because of its surface oxide layer, which allows favorable interactions between tissues and implants. To enhance the biocompatibility, a number of physical and chemical TiO_2 coating methods have been proposed to achieve a fast and strong fixation to bone at implant surfaces[1] and modified implants are in widespread clinical use. Among them, micro-arc oxidation (MAO), an electrochemical procedure for modifying Ti surfaces, has attracted a great deal of attention for the surface modification of Ti implants.[2] This process is well suited for the modification of a number of metal substrates with complex geometries, produces an effective chemical barrier against the release of metal ions from the substrate, and can enhance the corrosion resistance of titanium alloys. Moreover, the MAO-TiO_2 coating is porous and firmly adheres to the substrate, properties that are beneficial to the biological performance of implants. Another advantage of the MAO process is the possibility of incorporating Ca and P ions into the surface coating by controlling the composition and concentration of the electrolyte during processing. Recent studies on the biological response to Ti implants have demonstrated that the MAO process is one of the best methods for modifying implant surfaces.[3,4] Although this MAO-TiO_2 coating is an osteoconductive implant material, it takes a few months to heal after operation using available modified implants. There are much earlier osseointegrated implants especially for old ages. Because our purpose in this study is to shorten the duration of treatment, osteopromotive or osteoinductive ability is also required.

Several strategies have been intensively studied to promote bone formation, including the incorporation bone morphogenetic proteins, osteoinductive agents, and enamel matrix derivatives, as osteopromotive agent.[5,6] Previous studies have shown that electric polarization treatment can be utilized to induce surface electric fields, which induce osteopromotive ability in biomaterials such as hydroxyapatite (HAp), HAp-coated titanium, and β-tricalcium phosphate composite in vivo[7,8] and in vitro; this treatment affects the interfaces between charged

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doi:10.1016/j.jeurceramsoc.2012.03.002

surfaces and biomolecules or such surfaces and cultured cells.[9–12] The electric fields which enhance biological processes in living cells could exert osteopromotive ability in the absence of growth factors and drugs. Therefore, we combined MAO-TiO_2 with electric polarization to fabricate new surface-modified Ti substrates that could enhance bone formation in this study.

After sustaining injury to bone, osteogenesis is initiated to repair bones. Osteoblasts are cells found in bone that are responsible for osteogenesis. When osteocytes and/or osteoblasts themselves detect various physical and chemical signals for bone formation, they recruit osteoblasts. Pre-osteoblasts which differentiate directly from mesenchymal cells, and the inactive osteoblasts that line bone surfaces (bone-lining cells) proliferate and differentiate into osteoblasts and secrete minerals to form the bone matrix. If a biomaterial implant were to be entirely englafted by, proliferating and, differentiating osteoblasts, early healing by accelerated bone bonding would be expected because new bone would not only form around damaged bone but also around the implant.

It is reported that the initial attachment of osteoblasts to materials requires the materials to have a geometry and chemical composition that allows the adsorption of biomolecules.[13,14] It is also well known that the degree of cell adhesion affects subsequent signal transduction and therefore linking the ability of the adhering cells to proliferate and differentiate.[15] The interactions between cells and bone-related biomaterials have been investigated in vitro using various osteoblast cell lines, including MG63 cells. MG63 cells are derived from an osteoblast-like cell line from a human sarcoma. Despite being a tumor cell line, MG63 cells exhibit many traits characteristic of bone-forming cells. The cells showed an increased alkaline phosphatase activity following differentiation inducing reagent administration.[16] To study the osteopromotive ability of the polarized MAO surfaces compared to non-polarized MAO ones, we estimated the cytocompatibility by observing the adhesion, spreading, proliferation, and differentiation of MG63 cells. We investigated the initial attachment characteristics by counting nuclei stained with DAPI (4′,6-diamidino-2-phenylindole) and assessed spreading by actin cytoskeletal staining followed by fluorescence microscopy, proliferation by an MTT (3-(4,5-dimethyithiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay, and differentiation by an alkaline phosphatase (ALP) assay.

2. Materials and methods

2.1. Sample preparation

Titanium discs (ϕ10 mm × 2 mm) were fabricated using commercially pure titanium (grade-2) and were successively ground using 320# and 600# waterproof abrasive paper and then ultrasonically cleaned in acetone, ethanol and distilled water. The titanium discs were subjected to MAO treatment in an aqueous electrolyte containing 0.15 M calcium acetate monohydrate ($(CH_3COO)_2Ca{\cdot}H_2O$, Wako, Japan) and 0.02 M calcium glycerophosphate ($C_3H_7CaO_6P$, Wako, Japan) at 400 V for 5 min by applying a d.c. field to the samples. MAO processing was carried out in a water-cooled bath, and a stainless steel plate was used as the cathode. After MAO treatment, the samples were rinsed with distilled water and dried in an oven at 40 °C for 24 h.

2.2. Characterizations

The surface morphologies of the coatings before and after polarization were observed by scanning electron microscopy (SEM; Hitachi S-3400NX, Tokyo, Japan). The phase compositions of the sample surfaces were analyzed by X-ray diffraction (XRD; PW1700, PANalytical, Tokyo, Japan) over a 2θ range between 10° and 70° using Cu Kα radiation at 40 kV and 10 mA.

2.3. Electric polarization and TSDC measurement

In accordance with the electric polarization process reported in previous work,[8,17] the MAO-TiO_2 coated samples were sandwiched between a pair of platinum (Pt) plate electrodes, electrically polarized in a d.c. field of 5 kV cm^{-1} in air at 400 °C for 1 h, and then cooled to room temperature under the applied dc voltage. The TiO_2 coating in contact with the anode was labeled the negative surface (N-surface), and the TiO_2 coating placed in contact with the cathode was labeled the positive surface (P-surface). A non-polarized specimen was heated in air at 400 °C for 1 h under no field and labeled the 0-surface.

The thermally stimulated depolarization current (TSDC) of the polarized samples was measured using a picoampere meter (Keithley Inc., OH, USA). Each polarized sample was sandwiched between Pt electrodes and heated to 570 °C in an electric furnace equipped with a stainless-steel shielded sample chamber at a heating rate of 5 °C min^{-1}. The stored charge was calculated by integration of the current density on the measurement as previously reported.[17]

2.4. Cell culture

Human osteoblast-like cells (MG63) were purchased from the American Type Culture Collections (ATCC, Manassas, VA) and were grown in DMEM supplemented with 10% fetal bovine serum and penicillin/streptomycin at 37 °C in an atmosphere of 5% CO_2 in air.

2.5. Cell attachment and adhesion assay

The attachment of cells was evaluated by counting the number of cells attached to the surface. Cell spreading was assessed by measuring the cell-covered area, as determined by actin cytoskeleton staining. MG63 cells were seeded at a density of 1×10^3 cells cm^{-2} for 30 and 120 min on the surfaces of samples. After washing, cells were fixed in 4% paraformaldehyde and permeabilized with 0.3% Triton X-100 in PBS. Cells were stained with 14 μM rhodamine-labeled phalloidin, which binds specifically and with high affinity to the polymerized form of actin (F-actin) (Cytoskeleton, CO, USA). DNA was stained with DAPI (Dojindo, Kumamoto, Japan). Imaging of the samples was performed using an Olympus IX71 fluorescence microscope and DP71 digital camera (Olympus, Tokyo, Japan). The surface

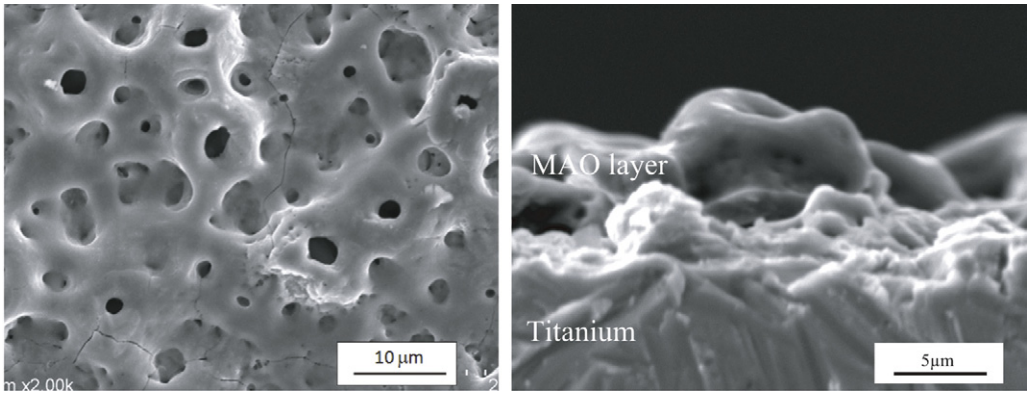

Fig. 1. Surface morphology of titanium subjected to micro-arc oxidation, as imaged using SEM. (A) Top view and (B) cross-sectional view.

areas covered by the cells were quantified using MetaMorph software (Molecular Devices, CA, USA).

2.6. Cell proliferation assay

Cell viability was quantitatively analyzed by using an MTT assay which is widely used for measuring cytotoxity and proliferation. After seeding for 24 or 72 h, the specimens were incubated with an MTT solution (5 mg ml^{-1}, Dojindo, Japan) at 37 °C for 3 h. The formazan product, which was obtained by the reduction of MTT in the mitochondria of viable cells, was dissolved in dimethyl sulfoxide for 10 min and the absorbance was measured at 570 nm using a microplate reader (Bio-Rad, CA, USA).

2.7. Cell differentiation assay

ALP activity in osteoblasts was determined by histochemical staining, using a kit in accordance with the protocol provided by the manufacture (Wako, Osaka, Japan). ALP activity is an early marker of osteoblast differentiation. The staining areas were estimated as described in Section 2.5.

2.8. Statistical analysis

The number of cells and the staining areas measured in 5 separate 40-fold magnification viewing fields on each of 3–4 independent surfaces in Sections 2.5 and 2.7. All values are presented as means ± SD, and a statistical software (ANOVA4 on the Web, Japan) was used for statistical analysis. A one-way analysis of variance (ANOVA) was used following multiple comparisons with Ryan's method to assess the data, and $P < 0.05$ was considered to indicate statistical significance.

3. Results

3.1. Surface characteristics of the MAO-TiO_2 coatings

The surface morphology of the MAO-TiO_2 coating is shown in Fig. 1. The top view (Fig. 1A) showed that a porous layer was formed on the surface of the Ti substrate after MAO treatment, with pore sizes of about a few μm or less distributed homogeneously. The average thickness of the surface layer was 5 μm with connecting holes. The complicated structures must be produced by local breakdown with small sparks and generation of the subsequent oxygen gas.

The XRD patterns of the polarized and non-polarized MAO-TiO_2 coatings are shown in Fig. 2. The MAO-TiO_2 coating consisted of rutile and anatase. Despite the existence of Ca and P in the electrolyte, no Ca- or P- containing phases were detected by XRD.[2] There were no obvious changes in morphology or phase composition after electric polarization.

3.2. TSDC spectrum and stored electric charge of the polarized MAO-TiO_2 coatings

The behavior of a polarized material can be estimated by TSDC measurements. TSDC analysis is a widely used experimental technique for the investigation of polarized material qualities such as stored charges (Q), activation energy for the depolarization processes and relaxation times. Ionic polarization inside a material causes the generation of TSDC when the material's temperature is increased. Representative TSDC spectra of the non-polarized and polarized MAO-TiO_2 samples are shown in Fig. 3. The TSDC curve began to increase at ca. 150 °C, reached its maximum at ca. 500 °C, and then gradually decreased. Because the non-polarized MAO-TiO_2 has Q after

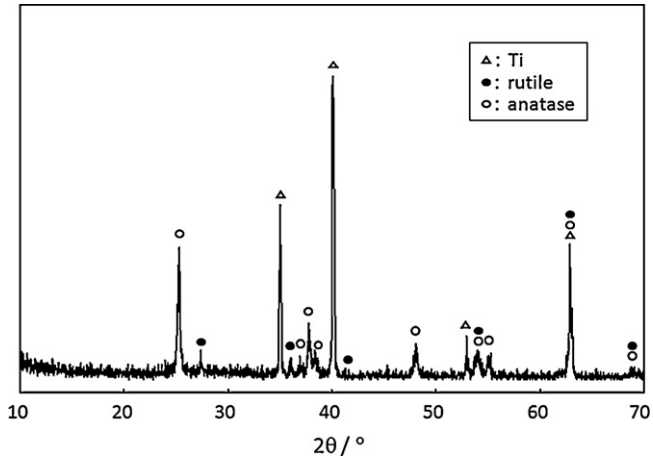

Fig. 2. XRD spectra of MAO-TiO_2 coating.

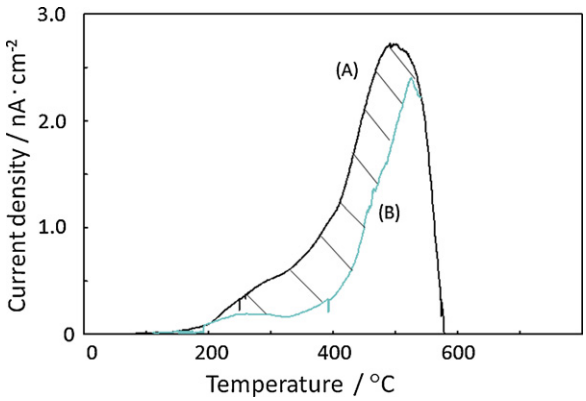

Fig. 3. Thermally stimulated depolarization current (TSDC) spectra of (A) polarized and (B) non-polarized MAO-TiO_2 coatings. The shaded portions indicate the stored charges of the polarized specimen.

heat treatment, the fraction of Q in the non-polarized samples was subtracted from the total Q of polarized one to estimate Q in the polarized specimen. The shaded portions in Fig. 3 is Q of polarized specimens that were 2.7 $\mu C\,cm^{-2}$. This result confirms that MAO-TiO_2 coatings can be polarized under the utilized experimental conditions.

3.3. Attachment and spreading behavior of MG63 cells on the polarized MAO-TiO_2 coatings

The initial osteoblast attachment to three specimens is depicted in Fig. 4A. The diffused actin meshwork is indicated in red across the entire cell image. Actin was generally distributed, with circumferential banding near the edge of the cell and a cytoplasmic meshwork. Most of cells seeded on each MAO-TiO_2 showed normal behavior: rounding after 30 min and spreading after 120 min (Fig. 5). The formation of lamellipodia was observed at the edge of the cells on the N-surface, indicated by arrows in Fig. 5. Polymerized actin at the periphery of cells formed focal adhesion and was found at 30 and 120 min. More cells had attached to polarized MAO-TiO_2 than to the non-polarized surface at 30 min (Fig. 4B). At 120 min, there was no statistical differences in the number of attached cells (ca. 1×10^3 cells) between non-polarized and polarized specimens; thus, almost all seeded cells attached to all surfaces. This result indicates that all surfaces exhibited little cytotoxity. For all surfaces, the individual attached cells were spread over a greater area after 120 min of contact than after 30 min. The cells had spread out more on polarized surfaces after 30 min than on 0-surfaces. After 120 min, the total actin-stained areas of cells spread on the N-surface were significantly greater than the areas on the other surfaces, suggesting that the N-surface favored cell adhesion (Fig. 4C).

3.4. Proliferation of MG63 cells on the polarized MAO-TiO_2 coatings

The proliferation of cells was estimated by an MTT assay 1 day and 3 days after seeding (Fig. 6). At 1 day after seeding, statistically more osteoblasts were present on the N-surface than

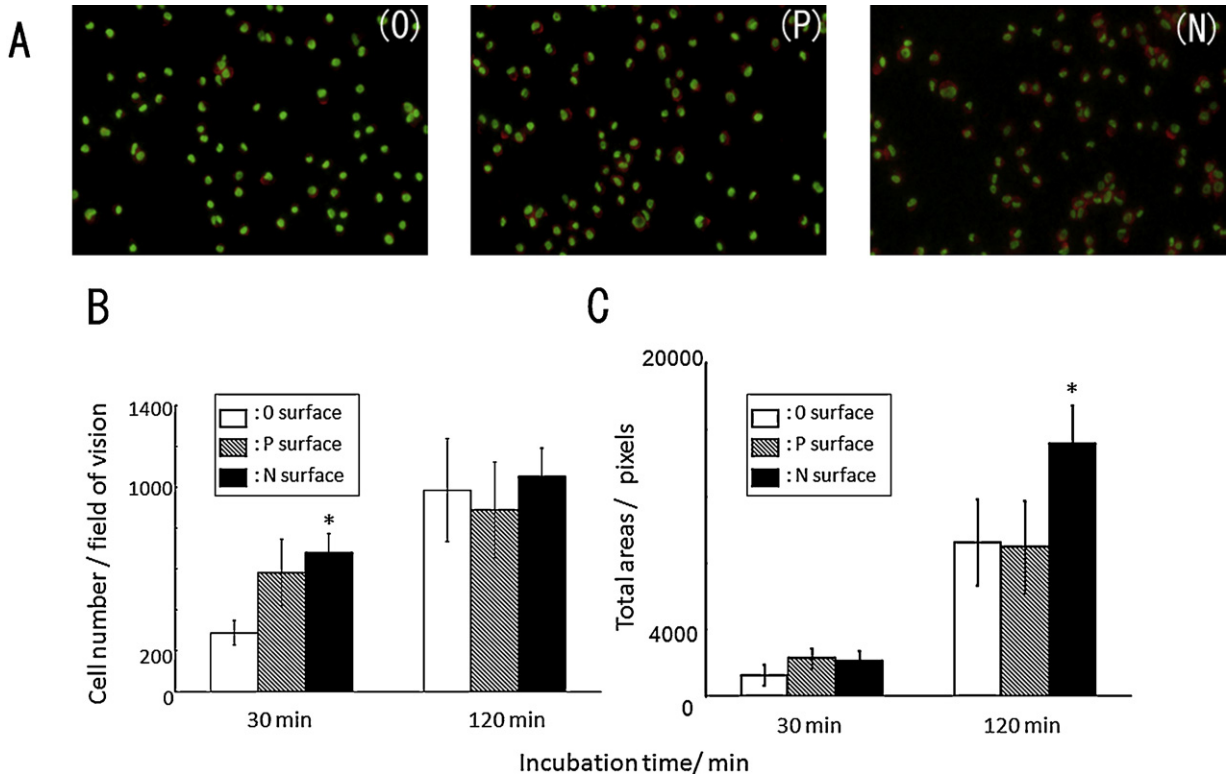

Fig. 4. (A) Representative images of cells stained with rhodamine-conjugated phalloidin and DAPI on all surfaces, (B) cell number (as determined by DAPI staining) after 30 min and 120 min and (C) actin-stained (phalloidin staining) areas after 30 min and 120 min, $^*P < 0.05$, compared with 0-surface. Results are the mean standard deviation ((B) $n = 3$, (C) $n = 4$).

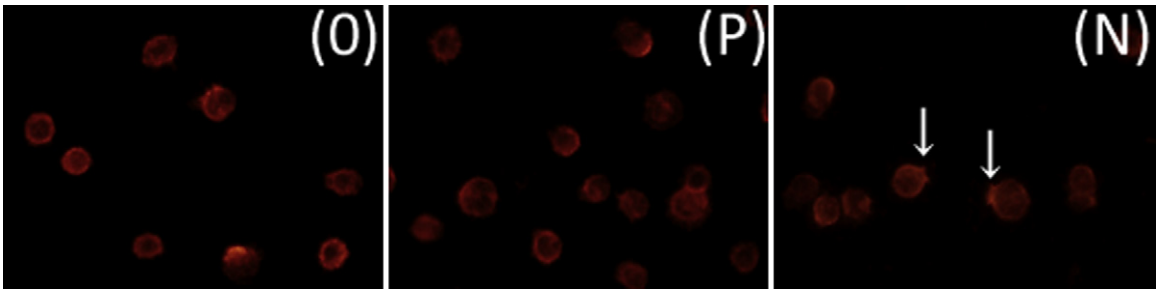

Fig. 5. High-magnification images of cells at 120 min after seeding on all surfaces. The formation of lamellipodia was indicated by arrows.

the other surfaces, although after 3 days, there was no significant difference among the materials. It was observed that the cell layers were confluent on all surfaces after 3 days. These results suggest that surface coverage by cells was faster on the N-surface.

3.5. Differentiation of MG63 cells on the polarized MAO-TiO_2 coatings

The differentiation of cells was observed 11 days after seeding, by using ALP staining (Fig. 7A). ALP stained areas (brown color) were observed in the cells on all surfaces in the absence of differentiation-inducing reagents and the stained areas on the N-surface were more than the areas on the other surfaces. The cells on the N-surface were more differentiated than those on the other surfaces (Fig. 7B).

4. Discussion

This study demonstrated that the early behavior of osteoblast cells upon interacting with the polarized MAO-TiO_2. The

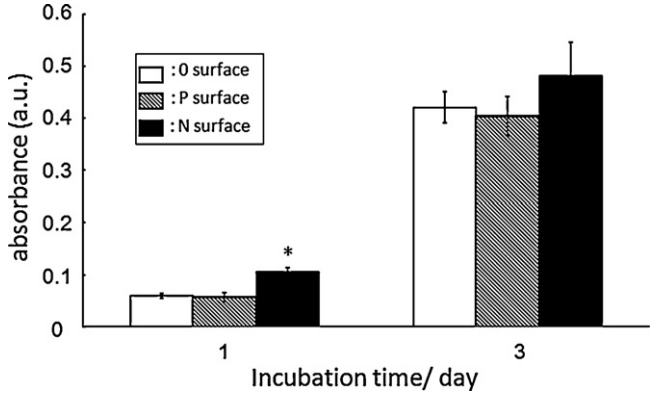

Fig. 6. MTT assay at 1 day and 3 days after seeding, $*P < 0.05$, compared with 0-surface. Results are the mean standard deviation ($n = 4$).

MAO-TiO_2 surface exhibits good topography favored by MG63 cells [16,18] and we showed that the cells more favored the surface roughness with surface electrical fields produced by electric polarization. It was previously reported that the electrical fields reduced the water contact angle of the polarized surfaces, which

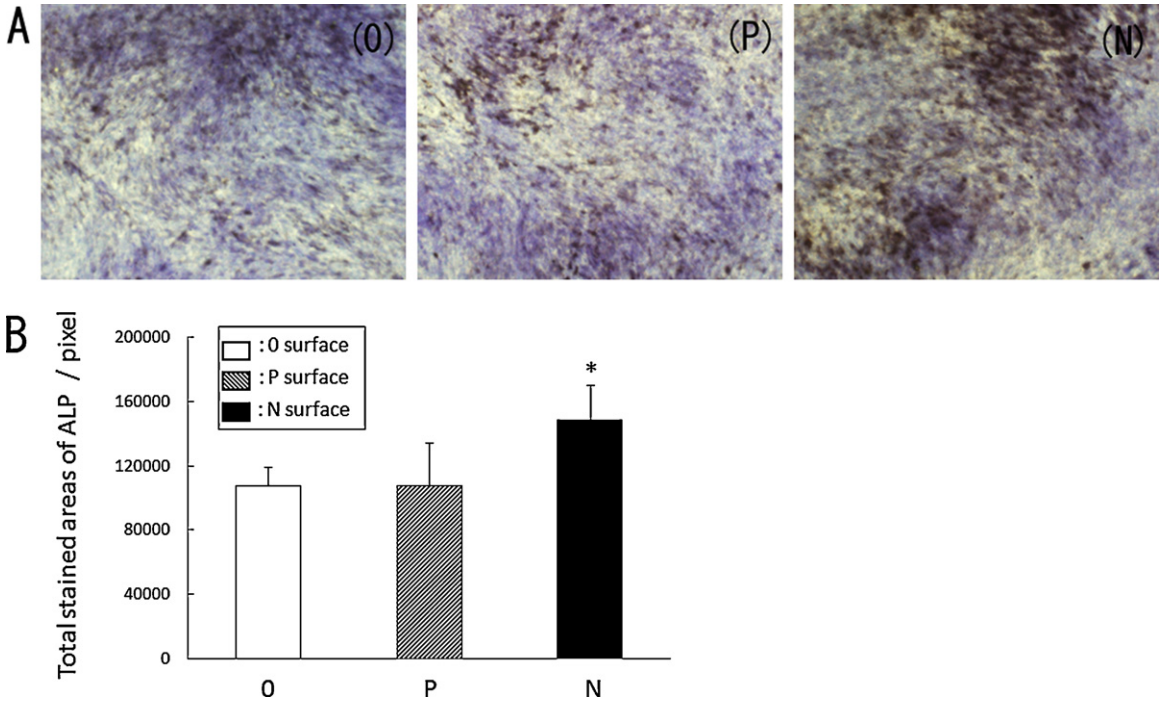

Fig. 7. (A) Representative images of cells stained with ALP on all surfaces and (B) ALP-stained areas at 11 days after seeding, $*P < 0.05$, compared with 0-surface. Results are the mean standard deviation ($n = 4$).

may a manifestation of the electrowetting effect.[19–21] The electrowetting effect can be defined as a change in the solid–liquid contact angle due to an electric field applied on the solid and the liquid. The electrostatic forces induced by the electric polarization increase the surface energy of the TiO_2 surfaces, which makes it easy for cells to adhere to the surfaces. Because there were similar quantities of stored electric charges on the N-surface and the P-surface, the reduction in the contact angle was found on both surfaces.[19–21] We concluded that the surface electrical fields could be used to increase the initial attachment of MG63 cells on the polarized MAO surfaces with respect to the MAO surface.

The attachment of cells to substrates is the initial process in cell–surface interactions. Initial attachment is an important event because it leads to the production of extracellular matrix proteins and cytoskeletal proteins, and is involved in signal transduction and thus regulates gene expression. Contacts formed between cells and the surface influenced the ensuing cellular response and spreading on substrates. Diminished cell adhesion is used as an indicator of toxicity. Cell spreading is an essential function of a cell that has adhered to surface and precedes cell proliferation, which finally results in a cell-covered surface. In this study, the spreading, proliferation, and differentiation of MG63 cells were occurred faster on the N-surface of MAO-TiO_2 than on other surfaces. It is assumed that biomolecules that are important for early cell behaviors, such as calcium ions and growth factors, were recruited to the N-surface because of its overall negative charges.[22,23]

The results of this study indicate that the combination of MAO and the electric polarization process made Ti implants osteopromotive. Each MAO surfaces were observed to be osteoconductive and thus well suited as scaffolds for MG63 cells. Moreover, the N-surface of MAO was shown to enhance the differentiation of MG63 cells without the addition of other reagents. Further in vivo testing of implants is needed to verify the surface-induced formation of new bone.

5. Conclusion

In this study, we found that the surface modification of Ti using the combination of MAO and electric polarization could regulate cell adhesion, spreading, proliferation, and differentiation without the addition of any other reagents. The initial cellular attachment could be accelerated by a reduction in the interfacial surface tension between the cells and the polarized surfaces. Serial cell behaviors after attachment enhanced on the N-surface of MAO. These findings indicated that the polarized MAO-TiO_2 is cytocompatible and has osteopromotive ability.

Acknowledgment

This work was supported by Grants-in-Aid for Scientific Research (C)(23500540) from JSPS.

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