Effect of strontium ions on calcification of preosteoblasts cultured on porous calcium- and phosphate-containing titanium oxide layers formed by micro-arc oxidation

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Strontium (Sr) ions were added to calcium- and phosphate-containing porous titanium oxide layers formed by micro-arc oxidation (MAO) of titanium (Ti) substrates to improve their osseointegration. An MC3T3-E1 preosteoblast was used to evaluate the effect of the incorporated Sr species on cell calcification. Similar surface microporous morphologies of the oxide layers were observed for all specimens produced by MAO, while the contents of the incorporated Sr ions increased with increasing Sr concentrations in MAO electrolytes. The calcium- and phosphate-containing porous layers promoted the cell alkaline phosphatase (ALP) activity, while cell calcification was promoted by the Sr addition. In particular, the ALP activity significantly increased after 10 days of culture, and larger areas of calcified deposits were observed for the specimens treated with MAO electrolytes containing 0.15 mol L⁻¹ of Sr species. The effect of Sr addition on the calcification of the MAO-treated Ti oxide layers was established in this study.

Keywords: Strontium, Micro-arc oxidation, Titanium, Osteogenic differentiation and calcification, MC3T3-E1

INTRODUCTION

Titanium (Ti) and its alloys have been used in dentistry as implant materials because of their good mechanical properties, high corrosion resistance, and low cytotoxicity. However, their poor bone fusion properties limit their clinical applications¹. Various techniques have been explored to modify the surfaces of Ti and its alloys to improve their osseointegration, including plasma spraying, sol-gel method, and electrochemical deposition²⁻¹². Among these modification methods, micro-arc oxidation (MAO) has attracted special attention since it is a relatively convenient and effective technique for depositing oxide coatings on the surfaces of nonferrous metals⁹⁻¹². More importantly, by using this technique, various desired elements can be introduced into the coated layers with porous structures¹¹, such as calcium (Ca), phosphorus (P), magnesium (Mg) and strontium (Sr), which are trace elements in the human body playing a significant role in bone formation.

Sr is considered a bioactive element that improves implant osseointegration. As was previously reported, Sr ions have been shown to enhance bone collagen synthesis and decrease bone resorption by inhibiting osteoclast resorbing activity¹³⁻²⁰. Therefore, it is expected that a local release of Sr ions from implant surfaces would directly improve implant osseointegration.

Since our previous studies have shown that MAO is a promising method to produce porous and firmly adherent bone formation concerned elements contained oxide deposition layers on metal substrates¹¹, it is

easy to optimize the Sr amounts introduced into the oxide deposition layers by altering MAO electrolyte compositions. Moreover, except for the first several days, a long-term stable release of metallic ions from the oxide deposition layers can be achieved by using the microporous structures formed during the MAO process⁸.

In this study, Ti surfaces were modified by MAO using electrolytes with different Sr concentrations to promote calcification of preosteoblasts. Morphologies and elemental compositions of the surfaces as well as cross-sections of the Ti specimens obtained with and without MAO were characterized by scanning electron microscopy (SEM) and energy dispersive X-ray spectroscopy (EDS). A mouse preosteoblast MC3T3-E1 was utilized to study the calcification effect of Sr contents in the MAO-deposited layers on the Ti substrates *in vitro*. Osteogenic differentiation and calcified depositions of the MC3T3-E1 cells cultured on all specimens were evaluated using alkaline phosphatase (ALP) activity assay and alizarin red s staining, respectively.

MATERIALS AND METHODS

Ti substrate preparation

Commercially pure Ti discs (grade 2, 8 mmφ×2.0 mm in thickness, Rare Metallic, Tokyo, Japan) were used in this study. Before the MAO treatment, Ti discs were mechanically polished with up to #800-grid SiC abrasive paper and then ultrasonically rinsed in acetone and ethanol. The obtained specimens (not subjected to MAO) were named “BMAO” and stored in an auto-drying desiccator until further use.

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Received Jan 22, 2016; Accepted Mar 24, 2016

doi:10.4012/dmj.2016-032 JOI JST.JSTAGE/dmj/2016-032

Table 1 Compositions of the electrolytes used for the MAO treatment of the Ti surfaces (M)

Specimen name	Calcium acetate monohydrate	Strontium acetate hemihydrate	Calcium glycerophosphate
Sr0M	0.15	0	0.10
Sr0.05M	0.10	0.05	0.10
Sr0.10M	0.05	0.10	0.10
Sr0.15M	0	0.15	0.10

MAO treatment

As reported in our previous work¹⁰⁻¹², Ti substrates (BMAO) were fixed in a polytetrafluoroethylene holder, which was used as an anode, while a stainless steel cylindrical container served as the cathode. The electrolytes used for the MAO procedure were composed of calcium acetate monohydrate (Wako Pure Chemical Industries, Osaka, Japan), strontium acetate hemihydrate (Wako Pure Chemical Industries), and calcium glycerophosphate (Wako Pure Chemical Industries) (see Table 1). Both electrodes were connected to a DC power supply (PL-650-0.1, Matsusada Precision), while the treatment was performed at a positive voltage of 420 V and constant current of 12 mA for 10 min. After the MAO treatment, all specimens were fully washed with distilled water and dried. All surface characterizations were performed for the MAO-treated areas.

Surface characterization

Images of the specimens produced with and without MAO were obtained using an Olympus SZX12 microscope (Olympus, Tokyo, Japan) at the same magnification, while their detailed surface morphology and compositions were investigated by scanning electron microscopy and energy dispersive X-ray spectroscopy (SEM/EDS, S-3400N, Hitachi High-Technologies, Tokyo, Japan).

Cell cultures

A mouse preosteoblast cell line (MC3T3-E1, RIKEN Bioresource Center, Ibaraki, Japan) was purchased and stored in a cell growth medium consisting of an alpha modification of the eagle's minimum essential medium (α -MEM, GIBCO, CA, USA) supplemented with 10% fetal bovine serum (FBS, GIBCO, NY, USA) and an antibiotic-antimycotic (100 U mL⁻¹ of penicillin, 100 mg mL⁻¹ of streptomycin, and 0.25 mg mL⁻¹ of amphotericin B; GIBCO).

Before *in vitro* testing, all specimens were sterilized in 70 vol.% ethanol for 20 min and thoroughly rinsed with deionized water. Then the cells were seeded onto the sterilized specimens with an approximate initial density of 5,000 cells cm⁻². All the cell-containing specimens were incubated at 37°C in fully humidified atmosphere containing 5% CO₂ in air. When the cells reached 100% confluence on the specimens, the osteogenic differentiation was induced, and this day was counted as 0 day. The growth medium was changed to a differentiation induction medium, where 2 mM of

β -glycerophosphate (Calbiochem, Darmstadt, Germany) and 50 mg mL⁻¹ of L-ascorbic acid (Wako Pure Chemical Industries) were supplemented to the cell growth medium. This differentiation medium was changed every 3 days.

Cell proliferation evaluation

In order to evaluate the cell proliferation on the Ti specimens obtained with and without MAO, the number of attached cells was counted on each specimen using a cell counting kit (CCK-8, Dojindo Laboratories, Kumamoto, Japan) after 6, 10, and 14 days. The specimens were washed with a phosphate-buffered saline (PBS) buffer solution to remove any unattached cells, while the attached cells were harvested from each specimen and then assayed to obtain the cell number according to the instructions for the CCK-8 kit. Sample absorbance was measured at a wavelength of 450 nm using a microplate reader (ChroMate Microplate Reader, Awareness Technology, Palm City, FL, USA).

Osteogenic differentiation evaluation

The ALP activity is very important for evaluating the osteogenic differentiation of cells²¹. After 6, 10, and 14 days of culture, the MC3T3-E1 cells cultured on all specimens were washed twice with ice-cold PBS and then distributed by sonication (UD100, Tomy Seiko, Japan) on ice. The ALP activity of the cells was determined by LabAssayTM ALP (Wako Pure Chemical Industries) according to the manufacturer's instructions, while their absorbance was measured at a wavelength of 405 nm using a ChroMate Microplate Reader (Awareness Technology).

Calcification evaluation

Calcification of the MC3T3-E1 cultures on the Ti specimens obtained with and without MAO was visually observed in the form of calcified deposits revealed by alizarin red s staining. After 14, 21, and 28 days of culture, the MC3T3-E1 cells cultured on all specimens were washed with ice-cold PBS and fixed with 4% formaldehyde. All specimens were stained with a 1% alizarin red s solution (adjusted to pH=4.2 with ammonium hydroxide) at room temperature and then rinsed with ultra-pure water. After being fully dried, all specimens were photographed using a stereomicroscope (Olympus SZX12). In addition, the stained calcified areas were calculated as [(stained area/total dish area)×100]%.

using an image analyzing software (ImageJ, National Institutes of Health, Bethesda, MD, USA). The details of this procedure were described elsewhere²²⁾.

Statistical analysis

All data were presented as mean±standard deviation of the mean. The obtained results were compared by one-way ANOVA followed by a Student-Newman-Keuls (SNK) test for multiple comparisons using SPSS Statistics 22 (SPSS, Chicago, IL, USA). Results with p values < 0.05 were deemed statistically significant.

RESULTS

Specimen characterization

The stereomicroscopic images of the specimens produced with and without MAO (Fig. 1) show that the microporous deposition coatings on the Ti specimens obtained by MAO are located in circular regions with diameters of 7 mm and characterized by a similar color regardless of the Sr concentrations in electrolytes. The areas outside the circular regions (Fig. 1b) may be a result of the leakage during treatment.

No significant difference in the surface microporous morphology were observed for the MAO-treated Ti specimens—even at different Sr concentrations in electrolytes (Figs. 2a–e). The results of the EDS analysis are presented in Fig. 2f. The obtained fractions of various elements indicate that with increasing the concentration of Sr in the electrolyte, its fraction on the surface of the microporous Ti oxide layer increases, while that of Ca decreases. Moreover, the observed changes in Sr electrolyte concentrations do not have a significant effect on the percentage concentrations of P on the surfaces of the microporous oxide layers.

The cross-sectional images of the specimens (Fig. 3) reveal that the MAO-deposited coating layers with an approximate thickness of 10 μ m are integrated with the Ti substrates. The EDS mapping images indicate that the Ti atoms originate from the substrate, the O atoms come from the oxide, and the C atoms originate from surface contamination; Ti, Ca, P, and Sr elements were also detected in the oxide layers. Due to the rapid growth of microporous deposition during the MAO treatment, the electrolyte elements were incorporated into the oxide layers on the Ti substrates. More importantly, all elements (including Sr, Ti, Ca, P, and O) were homogeneously distributed in these Sr-containing microporous oxide layers, while increasing Sr electrolyte concentrations results in an increase in the EDS intensities of Sr.

Cell proliferation

The proliferation of the MC3T3-E1 cells cultured on all specimens was examined through the attached cell number on each specimen for the 6, 10, and 14-days cultures (Fig. 4). The number of the attached cells on all specimens increases with culture time. After 6 days of culture, the highest cell number was obtained for BMAO, whereas after 10 days culture, there was no

Fig. 1 Stereomicroscopic images of the specimens obtained with and without MAO.

Images of the specimens (a) before the MAO treatment (BMAO) and after the MAO treatment with the Sr concentrations of (b) 0 M (Sr0M), (c) 0.05 M (Sr0.05M), (d) 0.10 M (Sr0.10M), and (e) 0.15 M (Sr0.15M). The diameters of all specimens are 8 mm ϕ , and the diameters of the MAO-treated areas are about 7 mm ϕ .

significant difference in the attached cell numbers for all specimens. After 14 days of culture, a larger number of the attached cells were obtained for BMAO than for the MAO-treated specimens.

Cell differentiation

To evaluate the cellular osteogenic differentiation at different Sr concentrations in the oxide layers obtained by MAO, the intracellular ALP activity levels of the MC3T3-E1 cells cultured on all specimens were assayed after 6, 10, and 14 days of culture. The ALP activity levels increased for all specimens from the 6 to 14-days culture. After 6 days of culture, no significant differences were observed for the cells cultured on different specimens. However, higher levels of the ALP activity were detected for the cells cultured on the Sr0.15M specimen compared to the other specimens after 10 days

Fig. 2 SEM images and surface elemental compositions (% atomic concentration) of all specimens obtained by SEM/EDS. SEM images of (a) BMAO, (b) Sr0M, (c) Sr0.05M, (d) Sr0.10M, (e) Sr0.15M, and (f) surface elemental compositions determined by EDS.

of culture. After 14 days of culture, the cells cultured on the MAO-treated Ti specimens exhibited higher ALP activity levels compared to BMAO.

Cell calcification

Alizarin red s staining was used to present visible results of calcification for the MC3T3-E1 cells on all specimens after 21 and 28 days of culture. The calcified deposits observed for all specimens were stained with a crimson color (black color in the grayscale photos, Fig. 5a). However, compared with the calcified deposits on BMAO, the MAO-treated specimens were almost fully stained with the crimson color. The quantified results obtained by the ImageJ software show that a higher percentage of the calcified area was obtained

for the MC3T3-E1 cells cultured on the Sr-containing microporous oxide coatings after 21 days of culture. Notably, the cells cultured on the Sr0.15M and Sr0.1M specimens produced the highest percentage of the calcified area among all the specimens after 28 days of culture.

DISCUSSION

In this study, different concentrations of Sr-containing microporous oxide layers were fabricated on the Ti specimens by MAO. Compared with other surface modification methods, the MAO treatment procedure employed in this study was a simple and easy way of producing Ti oxide surface coating layers containing

Fig. 3 Cross-sectional images of the specimens acquired by SEM and elemental maps of the specimens with multiple elements obtained by EDS.

specific target metal elements with the microporous structure. The microporous structures deposited on substrate surfaces by MAO can be controlled by varying operating voltage and current values^{11,13}; for example, a higher voltage leads to a larger pore diameter. In this study, the consistent conditions of the MAO treatment were used, except for the Ca and Sr concentrations. Therefore, similar surface microporous oxide layers

were obtained for the Ti substrates even when the Sr concentration was varied (Figs. 2b–e). Because of the same MAO treatment time, similar thicknesses of the microporous oxide layers were observed for all specimens after the MAO treatment (Fig. 3).

Using the MAO technique, different Sr concentrations were obtained on/in the microporous oxide layers of the Ti specimens; more importantly, all

Fig. 4 Proliferation of the MC3T3-E1 cells on all specimens. The attached cell numbers were counted using a cell counting kit-8. All values are presented as mean±standard deviation ($n \geq 3$). The obtained data were compared by one-way ANOVA followed by a Student-Newman-Keuls test for multiple comparisons. * $p < 0.05$; n.s. means non-significant.

Fig. 6 ALP activity levels of the MC3T3-E1 cells on all specimens for the 6, 10, and 14-days cultures. All values are presented as mean±standard deviation ($n \geq 3$). The obtained data were compared by one-way ANOVA followed by a Student-Newman-Keuls test for multiple comparisons. * $p < 0.05$; n.s. means non-significant.

the elements (including Sr, Ca, and P) were uniformly distributed on/in these layers (Fig. 3). In addition, increasing Sr concentrations in electrolytes resulted in larger Sr contents on/in the microporous oxide layers (Figs. 2f and 3). Certainly, the surface microporous morphology and the thickness of the surface oxide layers can be easily controlled by changing the conditions of

Fig. 5 Calcification evaluation of the MC3T3-E1 cells on all specimens for the 21 and 28-days cultures by alizarin red s staining.

(a) Grayscale photos of calcified deposits produced by the MC3T3-E1 cells on all specimens for the 21 and 28-days cultures after alizarin red s staining; (b) proportions of the calcified area to the total area for each specimen. All values are presented as mean±standard deviation ($n = 3$). The obtained data were compared by one-way ANOVA followed by a Student-Newman-Keuls test for multiple comparisons. * $p < 0.05$; n.s. means non-significant.

the MAO treatment. However, the exact concentrations of Sr on/in these microporous oxide layers obtained by MAO are difficult to determine.

As the Ti specimens were surface-modified with the Sr-containing microporous oxide layers, an MC3T3-E1 preosteoblast was used to evaluate their potential effects on cell proliferation, differentiation, and calcification behaviors.

For the cell proliferation, no significant differences were observed for the specimens obtained by MAO (Fig. 4) indicating that adding different Sr concentrations to electrolytes (between 0 and 0.15 M) produces no significant effect on the cell proliferation. However, the MAO treatment procedure may itself have an effect on cell proliferation, as the number of the cells attached to the specimens obtained by MAO was less than that obtained for BMAO (Fig. 4). This phenomenon may be explained by a larger number of metal ions released from the microporous oxide layers, which may induce a higher cellular stress and influence the cellular signaling

related to cell proliferation.

On the other hand, the incorporation of Sr, Ca, and P on/in the microporous oxide layers by the MAO treatment promotes cell osteogenic differentiation. Higher levels of the ALP activity were observed for the MC3T3-E1 cells on the microporous oxide layers after 14 days of culture (Fig. 6), which indicates larger osteogenic differentiation capacity. In particular, after 10 days of culture, a higher level of the ALP activity was observed for the cells cultured on the Sr0.15M specimen. Therefore, the incorporation of Sr should be beneficial for cell osteogenic differentiation—especially, for the MAO-treated specimens with electrolytes containing 0.15 M of Sr.

As the Sr-containing microporous oxide layers produced by MAO promote cell osteogenic differentiation, they may have an effect on the consequent cell calcification of preosteoblasts. The results obtained by the alizarin red s staining revealed that almost the whole MAO-treated areas were stained with a crimson color (Fig. 5a) indicating that the microporous oxide layers improved the hard tissue compatibility of the Ti substrates. This phenomenon was also confirmed by the ALP activity results obtained after 14 days of culture. More importantly, increasing Sr concentrations in electrolytes results in an increase in the incorporated Sr content on/in the microporous oxide layers and larger surface areas of the calcified deposits (Fig. 5b). Thus, increasing concentrations of the incorporated Sr on/in the microporous oxide layers improves osseointegration of the Ti substrates. In addition, similarly good osseointegration was observed for the Sr0.10M and Sr0.15M specimens.

The concentration of Sr incorporated on/in the microporous oxide layers on the Ti substrates is an important parameter for obtaining good osseointegration metal properties. It was reported that treatments with 0.1 mM of Sr^{2+} promoted differentiation of osteoblast precursors into mature osteoblasts and increased the bone nodule number²⁰, which was also used in a clinical treatment; while a 0.12 mM solution of Sr ranelate was used to treat blood active concentrations in postmenopausal osteoporotic patients¹⁸. However, excessively high concentrations of Sr (up to 0.4 mM) delayed the effect on the osteoblastic differentiation with delayed expression on Cbfa1 and osteonectin, and the inhibitory effect on the bone sialoprotein expression²⁰. In this study, MAO electrolytes with higher Sr concentrations were prepared. Compared with other surface modification methods, a better intake of target metal ions as well as stable and continuous ion release can be achieved through the MAO treatment. During the first several days of the cell attachment, higher Sr concentrations may be released from the surface of microporous deposition coatings; however, in the following several months, stable and lower Sr concentrations can be obtained from the inside of the coating layers through their microporous structure. Our data also exhibit improved osteogenic differentiation and calcification characteristics obtained for the MC3T3-E1

cells on the Sr0.15M specimen.

The results of this work show that adding Ca and P through MAO is beneficial for cell osteogenic differentiation, which is enhanced by incorporating Sr on/in microporous oxide layers. Moreover, these Sr-containing microporous oxide layers also promote cell calcification, which is important for boosting metal implant osseointegration.

CONCLUSION

In this study, Sr was added to the microporous Ti oxide layers containing Ca and phosphates species formed by MAO on the Ti substrates to improve their osseointegration. Increasing Sr concentrations in electrolytes produces no effect on the microporous structure of the oxide layers deposited on the Ti specimens, while the concentration of the incorporated Sr ions was increased. In addition, the incorporated Ca, phosphates, and Sr species were homogeneously distributed on/in these microporous oxide layers. Although the MAO-treated oxide layers on the Ti substrates produce a small inhibitory effect on cell proliferation (compared with that observed for the BMAO specimen), they also promote cell osteogenic differentiation. The calcified deposits stained by alizarin red s show that the Sr-containing microporous oxide layers promote calcification of MC3T3-E1 cells (especially on the MAO-treated Ti specimens) using electrolytes with Sr concentrations of 0.1 and 0.15 M. The obtained results suggest that the specimens treated with MAO electrolytes containing 0.15 mol L⁻¹ of Sr can be beneficial for cell osteogenic differentiation and calcification. The effect of Sr addition on the calcification of the MAO-treated Ti substrates was clearly revealed. Our study provides an effective surface modification strategy for achieving better implant osseointegration of metallic biomaterials.

ACKNOWLEDGMENTS

This work was partially supported by a Grant-in-Aid for Scientific Research (Kiban A: No. 22240059) from the Ministry of Education, Culture, Sports, Science, and Technology of Japan.

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