MG63 Cell Attachments on the Titanium Disks after Micro-Arc Oxidation

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타이타늄 임플랜트의 양극산화 표면처리에 따른 세포부착 특성에 관한 연구

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이번 연구의 목적은 순수 타이타늄의 표면을 양극산화법으로 표면처리하여 표면의 특성변화를 연구하고, 이에 따른 세포부착 특성의 차이를 연구하는 것이다.

원반 모양의 타이타늄을 전해질용액에서 300V - 550V의 전압을 주어 양극산화 하고 표면특성을 관찰한 결과, 전압이 높아짐에 따라 표면의 분화구 크기가 커지는 양상을 보였으며 아울러 표면 거칠기도 증가되었다.

세포 부착 실험결과 전압이 증가함에 따라 세포부착 및 증식세포수는 감소하였다.

300V 이상의 양극산화 전압은 표면의 거칠기는 증가시키지만 세포증식은 오히려 억제되는 것이 관찰되었다.

주용어 ; 세포부착, 양극산화, 전압, 타이타늄 임플랜트

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I. INTRODUCTION

Pure titanium (Ti) and titanium alloys are frequently used as dental and orthopedic implant materials because of their excellent mechanical strength, chemical stability, and biocompatibility[1]. The biocompatibility of titanium is closely related to the properties of the surface oxide layer, in terms of its structure, morphology and composition. Various physical and chemical treatments of the Ti surface have been proposed with a view to obtaining the most biocompatible implant surface. Among the techniques, which have been found to be beneficial to the biological performance of the implants, are increasing the surface roughness, the oxidation of Ti to form a TiO2 layer on the surface, and the incorporation of Ca or P ions into the surface layer, and the validity of these results has been confirmed by several different researchers[2,3].

Recently, an electrochemical procedure for modifying the Ti surface was proposed, which has since attracted much attention. By applying a positive voltage to a Ti specimen immersed in an electrolyte, anodic oxidation (or anodizing) of Ti occurs to form a TiO_2 layer on the surface. When the applied voltage is increased to a certain point, a micro-arc occurs as a result of the dielectric breakdown of the TiO_2 layer. At the moment that the dielectric breakdown occurs, Ti ions in the implant and OH ions in the electrolyte move in opposite directions very quickly to form TiO_2 again. This process is generally referred to as micro-arc oxidation (MAO) or plasma electrolysis[4,5]. The newly formed TiO_2 layer is both porous and firmly adhered to the substrate, which is beneficial for the biological performance of the implants. Another advantage of this MAO process is the possibility of incorporating Ca and P ions into the surface layer, by controlling the composition and concentration of the electrolyte[5,6]. The incorporated Ca and P ions were even precipitated into hydroxyapatite crystals by a hydrothermal treatment[7,8]. Recent studies on the biological response of Ti implants demonstrated that the MAO process constitutes one of the best methods of modifying the implant surface[9-11]. However, further research is necessary for the complete characterization of the oxide layer and also for the identification of the optimum conditions for the MAO process.

In vitro cell studies showed that cell proliferation level, and cell morphology and arrangement varied with surface roughness of the discs[12].

In this study, we formed TiO2 layers with different thicknesses and roughnesses on the Ti surface, by controlling the applied voltage used in the MAO process. The phase, composition and morphology of

the oxide layer were monitored with respect to the applied voltage. The biological properties of the layers were evaluated by in-vitro tests, in terms of the proliferation and differentiation of certain cell lines

Ⅱ. MATERIALS AND METHODS

1. Micro-arc Oxidation (MAO)

Commercially available pure Ti (CP-Ti, Grade 2, Ka-Hee Metal Industry Co., Seoul, Korea), machined into disks with dimensions of 12 mm (diameter) × 1 mm (thickness), was used as the substrate. These disks were ground using 400-grit SiC sandpaper and cleaned ultrasonically in acetone, ethanol and de-ionized water. Micro-arc oxidation (MAO) of the specimen was carried out in an aqueous electrolyte, by applying a pulsed DC field to the specimen. The frequency and duty of the pulsed DC power were 660 Hz and 10 %, respectively. The electrolyte was prepared by dissolving 0.15 mol calcium acetate monohydrate $\{Ca(CH_3COO)_2{\cdot}H_2O\}$ and 0.02 mol calcium glycerophosphate ($CaC_3H_7O_6P$) in de-ionized water [10]. To obtain oxide layers with different degrees of roughness and thickness, a wide range of DC fields (300-550 V) were applied to the specimens, with each treatment lasting 3 min. All of the MAO processing was carried out in a water-cooled bath made of stainless steel, and a stainless steel plate (100 × 60 × 1 mm) was used as the counter electrode. The titanium disks which were anodized at 6 different voltage were examined.

2. Biological properties

The biological properties of the specimens were evaluated by in-vitro cell tests. For the in-vitro tests, the MG63 and human osteosarcoma (HOS) cell lines were used to characterize the proliferation and differentiation behaviors of the cells, respectively. The pre-incubated cell lines were plated onto specimens with a cell density of 1.5 × 104 cells/cm^2 for the MG63 cells and 5 × 103 cells/cm^2 for the HOS cells, and then cultured in a humidified incubator with 5% CO_2 at 37℃. Dulbecco's modified Eagle's medium (DMEM, Life Technologies, Inc., USA) supplemented with 10% fetal bovine serum (FBS, Life Technologies, Inc., USA) was used as the culturing medium.

The proliferation behavior was determined by counting the number of cells after culturing them for 7 days. The cells were detached from the specimens with 0.05% trypsin-EDTA and counted using a hemocytometer (Superior Co., Germany). The differentiation behavior was estimated by measuring the alkaline phosphatase (ALP) activity of the HOS cells after culturing them for 10 days [20]. The cell layers were washed with Hank's balanced salt solution (HBSS) and detached using trypsin-EDTA solution. After centrifugation at 1200 rpm for 7 min, the cell pellets were washed once with PBS and resuspended by vortexing them in 200 ㎕ of 0.1 % Triton X-100. The pellets were disrupted by 4 cycles of successive freezing and thawing. After centrifugation, the cell lysates were assayed colorimetrically in order to measure their ALP activity using p-nitrophenyl phosphate as the substrate (Sigma Kit, as described fully in Procedure No. 104). The reaction lasted for 60 min at 37℃, and was then stopped by quenching on ice. The quantity of p-nitrophenol produced was measured at 410 nm using a spectrophotometer (Shimadzu, Japan). The morphology of the proliferated cells was observed by means of SEM after fixation with 2.5 % glutaraldehyde, dehydration with graded ethanols (70, 90 and 100 %), and critical point drying using CO_2. Each set of tests was performed in triplicate, and the data was normalized by taking the surface area into consideration.

Ⅲ. RESULTS

1. Morphology of oxide layer

Before the oxidation treatment, only the machining

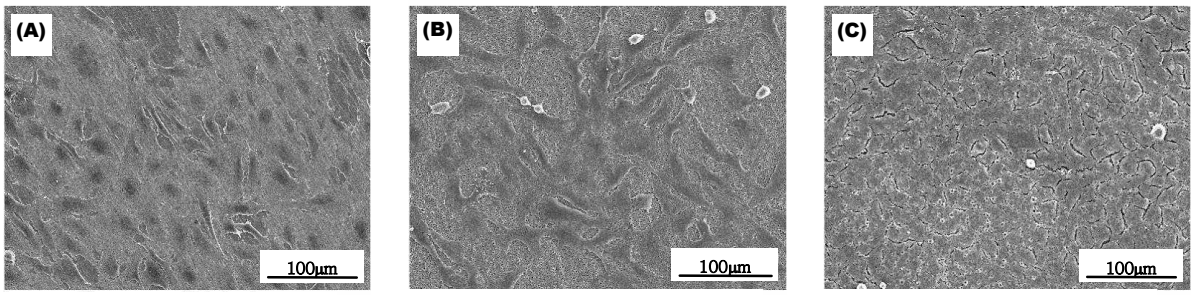

Fig. 1. SEM morphology of MG63 cells after culturing for 3 days on (A) pure Ti, and micro-arc oxidation treated Ti at (B) 300 V and (C) 550 V.

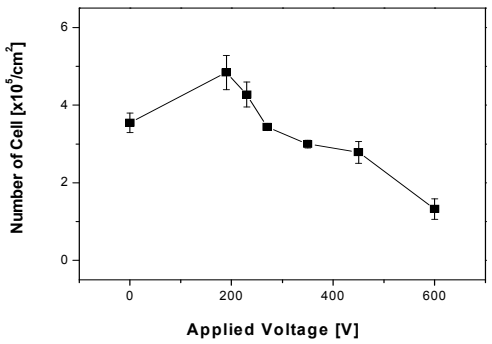

Fig. 2. Number of MG63 cells after proliferation for 7 days. Each set of tests was performed in triplicate and error bars stand for ± 1 standard deviations.

grooves were observed on the surface. When a pulsed DC field was applied, a porous oxide layer began to be formed. When the field was lower than 300 V, this layer was very thin and uniform without any porosity. As the voltage increased to 300 V, the whole layer became porous and the layer became uniformly porous. The resulting layer was actually composed of small craters with holes at the center. When the voltage was increased to 400 V, the size of these craters became so large that they were connected together, and the presence of tiny cracks was observed. When the voltage attained 500 V, the size of the holes, as well as that of the craters, became much larger, and this trend continued as the applied voltage was further increased up to 550 V.

2. Biocompatibility

The morphologies of the MG63 cells grown on the specimens for 3 days are shown in Fig. 1. On pure Ti, the cells were in close contact with the specimen and spread out uniformly over the surface, as shown in Fig. 1 (A). The morphologies of the cells grown on the MAO treated specimens were not much different when the applied voltage was relatively low, as illustrated in Fig. 1 (B). However, when the applied voltage was excessively high (> 500 V), the number of cells decreased, as shown in Fig. 1 (C).

Table 1. summary of oxide roughness of the four different types of c.p. titanium implants

Oxide characteristics	Group I	Group II	Group III	Group IV
Anodic forming voltage	300V	400V	500V	550V
Morphology	porous	porous	porous	porous
Roughness (Ra)	0.4§	0.9§	1.7§	2.2§

The number of cells was counted after culturing them for 7 days. In all cases, the cells proliferated at least 10 times compared to the originally plated cells. When the Ti was MAO treated at 300 V, the proliferation rate was the highest. As the voltage used for the oxidation process increased, the number of cells decreased steadily, as shown in Fig. 2.

Ⅳ. DISCUSSION

The purpose of this study was to improve the biocompatibility of Ti implants by modifying the composition and morphology of the implant surface. Micro-arc oxidation (MAO) is a simple, controllable, and cost-effective method of forming a porous TiO_2 layer on the implant surface. Moreover, MAO provides an ideal method of producing an oxide layer on the surface of implants which have a complex shape. The properties of the oxide layer, such as its thickness, microstructure, roughness, and concentrations of Ca and P are easily controllable by adjusting the voltage, current, processing time, and the concentration of the electrolyte during the MAO process .

The microstructural change of the oxide layer was found to be closely related to the voltage used for the MAO treatment. Increasing the voltage resulted in an increase in both the roughness and pore size, as well as in the thickness of the oxide layer (Table 1). These microstructural evolutions are attributed to the dielectric breakdown of the oxide layers. During the MAO process, as the TiO_2 layer becomes thicker, micro-arc discharges occur on the local area of the substrate, that break down the surface dielectric layer to form micro pores. At the same time, the oxide layer becomes thicker due to the increased extent of the electrochemical reaction. As the oxide layer becomes thicker, the resistance of the oxide layer increases and a higher potential energy is required to break down the dielectric layer. As a result of this series of reactions, the pore size and the roughness of the oxide layer increase rapidly.

The changes in chemical composition and roughness of the Ti surface played crucial roles in the biocompatibility of the implant. The proliferation rate was highest when the specimen was oxidized at the relatively low voltage of 300 V, and it decreased steadily with increasing voltage (Fig. 2). Even though there was some variation depending on the applied voltage, the number of cells increased more than 10 times compared to the originally plated cells. These proliferation results simply indicate that all of the specimens offered a biologically favorable environment. Our result shows that the roughness strongly affect the cell response. Especially, cell proliferation at higher voltages was decreased, which is deemed to be closely related to the increase in surface roughness of the oxide layer.

Ⅴ. CONCLUSION

The MAO treatment of Ti, by bringing about positive physical and chemical changes to the Ti surface, changed in vitro cell proliferation of MG63.

Increasing the MAO voltage increased the thickness and roughness of the oxide layer. As a result of these changes the cell proliferation rate decreased.

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