Microarray-based expression analysis of human osteoblast-like cell response to anodized titanium surface

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Abstract
An anodized surface significantly enhanced the adhesion of human osteoblast-like MG-63 cells to titanium. Using cDNA microarray analysis, five genes were differentially expressed while the rest remained unaltered. The results demonstrated that the anodized surface enhances cellular adhesion without significantly affecting the pattern of gene expression.

Introduction
Titanium implants are increasingly used in dentistry and orthopedics due to their excellent biocompatibility and mechanical properties. In air at room temperature, the surface of titanium is covered by an oxide layer which is 1.5–10 nm in thickness (Brånemark et al., 1977). The oxide layer has a low electrical conductivity, good thermodynamical stability and a low tendency of ion formation in aqueous environments which may be the reason for the excellent biocompatibility of titanium implants. Moreover, in the studies of implants retrieved from patients, successfully osseointegrated implants have an increase oxide thickness up to 200 nm but failed implants show no changes.

The method used in these experiments for preparing the porous oxide surface was the electrochemical anodic oxidation of titanium. In anodic oxidation (anodization), the ionic current and the processes which occurs at the electrode lead to the growth of an oxide film (anodic oxide). Due to their electrical potential, anions and water molecules are attracted to the anode where they are absorbed. This electrical potential also sets up an electrical field in the pre-existing surface oxide layer. Ti ions from the metal-oxide interface can then migrate by field enhancement to the oxide-electrolyte interface. There will be charge transfer and dissociation of the water molecules and/or the absorbed anions takes place. The dissociation of the water molecules results in the production of atomic oxygen or $O^{2-}$ ions, and oxidation of the Ti. The growth of the oxide proceeds as long as the electrical field strength in the oxide is high enough to allow ionic transport through it. Because the oxide layer of TiO₂ has a high resistance, the oxide growth stops at a thickness which causes the potential over the oxide to drop, and at which point ionic current can no longer flow through the oxide or electrolyte. By means of anodization, a rough and porous oxide layer can be formed. The chemical composition, crystallinity, roughness, and topography of the implant surface are changed after the anodizing procedure (Sul et al., 2001).

The response of cells and tissues to implants is affected by not only the chemical properties of the implant surface but also by the surface topography or its roughness. The purpose of this study was to evaluate the effect produced by the anodization of the titanium surface on cell cultures by cell adhesion test, and to perform a large-scale analysis of gene expression using cDNA microarray. The surface characteristics of
the anodized implants were analyzed in three categories (the surface morphology, the surface roughness, and the crystal structure of TiO2), and an investigation was made to determine the effect of surface characteristics on the cell adhesion and phenotypic expression.

Methods

Titanium preparation

The Ti disks used for the cell culture were fabricated from grade 2 commercially pure Ti. The disks were prepared to be 1 mm thick and 25 mm diam. Prior to use, all disks were washed in acetone, then with a solution of 2% (v/v) NH4F/2% (v/v) HF/10% (v/v) HNO3 at 55 °C for 30 s, and soaking in 2% (v/v) HF/10% (v/v) HNO3 at room temperature for 30 s. The pretreated disks were further processed, as described below, to produce surface of varying roughness.

- Group 1: Machined surface, used as control.
- Group 2: Anodized using a constant voltage, 270 V.

The disks were anodized using pulse power (650 Hz). The electrolyte solution contained 0.15 M calcium acetate and 0.02 M calcium glycerophosphate. All procedures were performed at room temperature, and the total time for the anodization of one disk was 3 min. Each disk was attached at the anode and stainless steel was used as the cathode.

Surface analysis

The surface characteristics of the machined and anodized titanium disks were analyzed in three categories (the surface morphology, the surface roughness, and the crystal structure of TiO2). The morphologies of the surface of the implants were observed with a scanning electron microscope (JSM-840A, JEOL, Japan).

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Cell cultures and RNA extraction

MG-63 cells were obtained from the Korean Cell Line Bank (KCLB21417). For all experiments, cells were cultured on disks placed in 100 ml dishes. The control groups consisted of cells cultured on the machined surfaces. The experimental groups were composed of cells cultured on the anodized titanium surfaces under 270 V. Cells were plated at 10^7 ml^-1 in Dulbecco’s modified Eagel’s medium (DMEM) containing 10% (v/v) fetal bovine serum (FBS) and 1% (w/v) antibiotics and cultured at 37 °C in 5% (v/v) CO2 for 24 h on each sample. Total RNA extraction was performed with Qiagen mini kit (Qiagen, Chatsworth, CA).

Cell adhesion assay

After 12, 24 and 48 h, cell adherence on both the machined and anodized (under 270 V) Ti disks was determined. After cell culture, cells were washed in PBS and fixed with 10% (v/v) formalin for 15 min. Cells were stained overnight with 1 ml of 1% (w/v) Crystal Violet. After staining, the disks containing cell culture were washed three times with distilled water. Cells were released from the disk surface by the addition of 1% (w/v) SDS and mixed for 5 min. Lysed cells were transferred onto a 96-well plate and the cell adhesion was quantified by measuring the absorbance of colored solutions in a spectrophotometer at 570 nm.

Microarray analysis

We used a cDNA microarray GCK 5.0 K human chip (Genocheck, Korea) and monitored the expression of 5049 genes. The samples (10 µg total RNA per condition) were processed according to the manufacturer’s recommendations. Genepix software was used to analyze the data. The gene expression of cells incubated on the machined titanium surfaces was chosen as the reference. For analysis, the difference in gene expression is based on the experimental finding that either upregulation or down-regulation by more than 2.5-fold was considered as significant.

Results and discussion

The surface oxide films on the titanium were obtained by anodic oxidation. Figure 1 shows representative scanning electron micrographs of the anodized and machined surfaces. The anodic surfaces were observed to be porous and average pore size was 0.79 ± 0.27 µm. The roughness of machined Ti surface was 0.54 ± 0.16 µm and the roughness of anodized one was 0.88 ± 0.13 µm (Figure 2). The pores of the anodized oxide layer results from micro
Fig. 1. Representative scanning electron micrographs of (A) machined and (B) anodized surface. (Marker bar = 1 µm.)

Fig. 2. Surface roughness of machined and anodized titanium (\( ^* p < 0.05 \)).

arcing which occurs on the surface of titanium anode. These results suggest that the exposure of the titanium surface to anodic oxidation result in the formation of a rough, thick and porous crystalline oxide layer.

Next, we examined the effects of surface treatment on the cell adhesion. As shown in Figure 3, the human osteoblast-like MG-63 cells plated on anodized surface attached and spread better typically containing filopodia- and lamellipodia-like extensions compared to the control machined surface. These results indicate that the anodized surface improves the adhesion and spreading of the MG-63 cells and may enhance the stability of initial implant. However, the mechanism by which anodization induces the enhancement of osteoblast adhesion is not clear at this point.

After 24 h incubation of the MG-63 cells, we evaluated the modulation of gene expression by the surface treatment. Cells on the anodized surface had 2 up-regulated genes and 3 down-regulated genes in

Fig. 3. Effect of cell adhesion on machined and anodized surface. (A) Representative scanning electron micrographs and of machined and anodized surface. (Marker bar = 100 µm.) (B) MG-63 cell adhesion on anodized titanium is significantly (\( ^* p < 0.05 \)) increased compared with machined titanium. Serum-starved MG-63 cells were cultured on titanium plates. Results are given as absorbance (O.D.) values of Crystal Violet-stained cells and represent the mean ± SD.
Table 1. Gene expressions between the machined surface (control) and anodized surface (experimental).

<table>
<thead>
<tr>
<th>Up/down</th>
<th>Gene name</th>
<th>Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Up</td>
<td>Heat shock protein (HSP-90α)</td>
<td>3.16</td>
</tr>
<tr>
<td></td>
<td>Mitochondrion: 8904–9205</td>
<td>2.41</td>
</tr>
<tr>
<td>Down</td>
<td>Attractin</td>
<td>0.28</td>
</tr>
<tr>
<td></td>
<td>Retinol dehydrogenase 1</td>
<td>0.39</td>
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<tr>
<td></td>
<td>Proteoglycan link protein</td>
<td>0.4</td>
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In conclusion, our findings suggest that the surface modification of the titanium with anodic oxidation enhances cellular adhesion with minor change in the gene expression of osteoblast cells. Thus, the enhanced cell adhesion produced by anodic oxidation might result in increased bone growth, and contribute to the achievement of a tight fixation within a shorter period of time after surgery.

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References


