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Effects of the nanotopographic surface structure of commercially pure titanium following anodization–hydrothermal treatment on gene expression and adhesion in gingival epithelial cells ☆

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ABSTRACT

The long-term stability and maintenance of endosseous implants with anodized–hydrothermally treated commercially pure titanium surfaces and a nanotopographic structure (SA-treated c.p.Ti) depend on the barrier function provided by the interface between the transmucosal portion of the implant surface and the peri-implant epithelium. This study investigated the effects of extracellular and intracellular gene expression in adherent gingival epithelial cells cultured for 1–7 days on SA-treated c.p.Ti implant surfaces compared to anodic oxide (AO) c.p.Ti and c.p.Ti disks. Scanning electron microscopy (SEM) showed filopodium-like extensions bound closely to the nanotopographic structure of SA-treated c.p.Ti at day 7 of culture. Gene expressions of focal adhesion kinase, integrin-α6β4, and laminin-5 (α3, β3, γ2) were significantly higher on SA-treated c.p.Ti than on c.p.Ti or AO c.p.Ti after 7 days ($P < 0.05$). Our results confirmed that gingival epithelial cells adhere to SA-treated c.p.Ti as the transmucosal portion of an implant, and that this interaction markedly improves expression of focal adhesion molecules and enhances the epithelial cell phenotype. The cellular gene expression responses driving extracellular and intracellular molecular interactions thus play an important role in maintenance at the interface between SA-treated c.p.Ti implant surfaces and the gingival epithelial cells.

1. Introduction

An endosseous implant system usually includes a mucosa-penetrating component [1–3] to facilitate close contact between the implant surface and the oral epithelium [4]. Attachment of the surrounding epithelial tissue to the transmucosal portion of an endosseous implant surface is mediated by hemidesmosomes and the internal basal lamina [4–7]. Formation of a normal peri-implant epithelium with intact internal basal lamina is therefore an important first transmucosal barrier against complex confounding factors including oral bacteria and their components [4–7]. The peri-implant epithelial integrity also blocks apical migration of the junctional epithelium and prevents loss of crestal bone [4,6–8]. The effectiveness of this soft-tissue biological seal around the transmucosal portion of an endosseous implant depends on the topographic and physicochemical properties of the endosseous implant surface, which basically serves as a three-dimensional interface with the tissue [9]. Such properties potentially impact on cellular attachment, spreading, proliferation, and differentiation [4,9,10]. Indeed, preclinical and clinical studies suggested that the transmucosal portion of commercially pure titanium (c.p.Ti) endosseous implants could regulate the peri-implant oral epithelial and connective tissue cell behaviors by virtue of these specific implant surface properties [10–12].

Discharge anodic oxidation followed by hydrothermal treatment (spark-discharged anodic oxidation (SA) treatment) is used successfully to coat c.p.Ti implants (SA-treated c.p.Ti) with a highly crystalline, thin hydroxyapatite (HA) layer anodic titanium oxide film to provide a suitable nanotopographic structure for clinical oral implants [13–29]. The HA crystals remain stable and do not impact on living tissue [15,16,23,25,26]. However, a thorough molecular understanding of possible interactions between the transmucosal portion of the implant surface and the host defense mechanisms is still needed, particularly regarding the specific and nonspecific immune responses to oral bacteria and their components [19]. In this context, our previous investigations demonstrated that the SA-treated c.p.Ti surface provides good tissue biocompatibility and stimulates little discernible response from lymphocytes and macrophages [1,19,22,30]. Moreover, our recent *in vitro* data clearly demonstrated that murine fibroblast-like cells adhere successfully to the SA-treated c.p.Ti implant surface and start to express focal adhesion kinase (FAK) over a 1- to 3-day culture period [28], as well as producing a greater initial epithelial cellular response after 1 to 3 days in culture than an untreated c.p.Ti implant, and showed

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potentially greater capacity to induce the activation of integrin-α6β4 in the epithelial cells to interact with laminin-5 [29]. These findings suggested that the topographic and physicochemical properties of SA-treated c.p.Ti surface structures enhance the very early cellular responses of gingival epithelial cells to the transmucosal portion of the SA-treated c.p.Ti implant. Therefore, it is very important to investigate the specific extracellular and intracellular gene expression activated to drive such responses in the adherent cells on SA-treated c.p.Ti.

We hypothesized that the thin HA layer and anodic titanium oxide film with a nanotopographic structure on SA-treated c.p.Ti implants could regulate and enhance integrin-mediated epithelial cell adhesion and the associated gene expression to better function as a transmucosal barrier. In this study, we therefore examined these cellular-implant surface properties using adherent gingival epithelial cells, and how specific cellular mechanisms could be related to the surface topography and physicochemical properties of SA-treated c.p.Ti implants by examining cell morphology and proliferation, as well as the gene expressions of FAK, the integrin-α6β4 adhesion molecule, and laminin-5 (α3, β3, γ2) in immortalized murine gingival epithelial (GE1) cells over a 7-day culture period on SA-treated c.p.Ti surfaces.

2. Materials and methods

2.1. Sample preparation and properties

Commercially pure titanium (c.p.Ti; Ti > 99.8%, JIS-Grade 2, Shinko Wire Company, Ltd., Osaka, Japan) disks (diameter, 15 mm; thickness, 1.5 mm) were machined (0.29 ± 0.01 μm Ra) and used in this study [27]. The c.p.Ti disks were cleaned by ultrasound, washed with distilled water, dried in a dessicator, and then anodized at 350 V in an electrolytic solution containing 0.01 M β-glycerophosphate disodium salt pentahydrate and 0.15 M calcium acetate monohydrate dissolved in distilled water. Anodic oxidation was conducted at a current density of 50 mA/cm^2 using a regulated DC power supply (419A-630; Metronix, Japan) to produce the AO c.p.Ti disks (0.73 ± 0.04 μm Ra) [27]. The AO c.p.Ti disks were then washed with distilled water, dried, and then hydrothermally heated using high-pressure steam at 300 °C for 2 h in an autoclave (volume 1.3 l; Nitto-kouatsu, Japan), resulting in the precipitation of HA crystals onto the disk surface (0.75 ± 0.03 μm Ra; SA-treated c.p.Ti) [27]. The surface morphology of the AO c.p.Ti and SA-treated c.p.Ti disks was confirmed by scanning electron microscopy (SEM; JSM-7001F, JEOL Ltd., Tokyo, Japan), which showed an anodic titanium oxide film with a porous microstructure containing numerous craters and micropores of 1–3 μm in diameter [13–15,27]. The precipitated HA crystals had a typical single hexagonal columnar shape and very high crystallinity [13–16,21,23,27], and were observed over approximately 60% of the anodic titanium oxide film containing calcium and phosphate (AOFCP) surface [13–15,27]. The AOFCP surface on AO c.p.Ti disks was slightly smoother with less nanoscale topography (0.83 ± 0.03 nm; AO c.p.Ti) than the AOFCP surface underlying the HA crystals on SA-treated c.p.Ti disks (2.75 ± 0.10 nm; SA-treated c.p.Ti) [27]. The HA crystals formed by AO and hydrothermal treatment of c.p.Ti were characterized using X-ray photoelectron spectroscopy (XPS) and X-ray diffraction (XRD), which determined the Ca/P ratio to be 1.48 [14,21]. Prior to cell culture, all disks were sterilized by autoclaving at 121 °C for 20 min followed by exposure to ultraviolet light in a sterile tissue culture hood for 72 h.

2.2. Cell culture

The GE1 cell line was obtained from the RIKEN BioResource Center Cell Bank (RCB1709, Tsukuba, Japan) [31]. The cells were seeded at a density of 1.5 × 10^5 cells/cm^2 in tissue culture plastic dishes (Falcon, Becton Dickinson, NJ, USA), containing SFM101 medium (Nissui, Tokyo, Japan) supplemented with 1% fetal bovine serum (Gibco-BRL, Rockville, MD, USA) and 10 ng/ml of mouse epidermal growth factor (Sigma-Aldrich Japan, Tokyo, Japan) in a fully humidified 95% air, 5% CO_2 atmosphere at 33 °C. Cells (1.5 × 10^4 cells/disk) were seeded onto c.p.Ti, AO c.p.Ti, and SA-treated c.p.Ti disks in 12-well plates (Falcon) and incubated for 7 days for the analysis of cell morphology and proliferation. Cells (1.5 × 10^5 cells/disk) were also seeded onto c.p.Ti, AO c.p.Ti, and SA-treated c.p.Ti disks in 24-well plates (Falcon) to determine the expression levels of FAK mRNA after 1, 3, and 7 days in culture, and integrin-α6β4 and laminin-5 (α3, β3, γ2) mRNA after 7 days in culture.

2.3. Analysis of cell morphology

GE1 cells incubated on c.p.Ti, AO c.p.Ti, or SA-treated c.p.Ti disks for 7 days were washed twice in phosphate-buffered saline (PBS; Nissui Pharmaceutical Company, Ltd., Tokyo, Japan) to remove nonattached cells, and then twice with 0.1 M sodium-cacodylate buffer pH 7.4, at 37 °C for 5 min. The cells on all disks were fixed for 3 h at 4 °C according to Karnovsky's method (4% paraformaldehyde, 3% glutaraldehyde, 0.1 M sodium-cacodylate buffer pH 7.4) and postfixed in 4% osmium tetroxide. After fixation, the cells were dehydrated using a graded-ethanol series of solutions, followed by critical point drying. Finally, the disks were coated with platinum by ion sputtering (Hitachi E-1030, Tokyo, Japan). GE1 cell adhesion and morphology on all disk types were examined using SEM (JSM-7001F, JEOL Ltd., Tokyo, Japan). Experiments were performed with five disks each of c.p.Ti, AO c.p.Ti, and SA-treated c.p.Ti.

2.4. Analysis of cell proliferation

Cell proliferation was assayed by a colorimetric method based on the reduction of water-soluble tetrazolium salts, using the WST-1 reagent (Roche Diagnostics, Basel, Switzerland) according to the manufacturer's instructions [29]. GE1 cells were seeded onto c.p.Ti, AO c.p.Ti, and SA-treated c.p.Ti disks and cultured for 7 days. Briefly, WST-1 reagent was added to the cell culture incubated for 1 h at 33 °C, after which the absorbance was measured at 450 nm with a Gene Spec III spectrophotometer (Hitachi). The level of cell proliferation is shown as a fold increase, relative to day 1. Experiments were performed in triplicate with five disks each of c.p.Ti, AO c.p.Ti, and SA-treated c.p.Ti.

2.5. RT-PCR evaluation of FAK, integrin-α6β4, and laminin-5 (α3, β3, γ2) gene expression

Total RNA was isolated from the GE1 cells using TRIzol reagent (Invitrogen Co., Ltd., CA, USA) based on the single-step method. First-strand cDNA was synthesized from the total RNA using the PrimeScript RT Master Mix (TaKaRa Shuzo Co., Ltd., Shiga, Japan), and then real-time quantitative RT-PCR (qRT-PCR) was performed using a Thermal Cycler Dice Real Time System (TP800, TaKaRa) with SYBR Premix Ex Taq II (TaKaRa) and specific oligonucleotide primers. The primer was used as template for PCR with primers specific for FAK (TaKaRa), integrin-α6 (TaKaRa), integrin-β4 (TaKaRa), laminin-α3 (TaKaRa), laminin-β3 (TaKaRa), or laminin-γ2 (TaKaRa) (Table 1). The FAK, integrin-α6β4, and laminin-5 (α3, β3, γ2) mRNA levels for cells on c.p.Ti, AO c.p.Ti, or SA-treated c.p.Ti disks were normalized to those obtained for GAPDH mRNA. Experiments were performed in triplicate with five disks each of c.p.Ti, AO c.p.Ti, and SA-treated c.p.Ti.

2.6. Statistical analysis

The cell proliferation and qRT-PCR data are expressed as mean values ± SD. Statistical analysis was performed by one-way analysis of variance (ANOVA), followed by Bonferroni *post-hoc* analysis of multiple groups to determine significance. A value of $P < 0.05$ was considered significant. Statistical analyses were performed on a personal computer using the statistical software package SPSS, version 15.0 for Windows (SPSS Japan, Tokyo, Japan).