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Interaction of progenitor bone cells with different surface modifications of titanium implant

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ABSTRACT

Changes in the physical and chemical properties of Ti surfaces can be attributed to cell performance, which improves surface biocompatibility. The cell proliferation, mineralization ability, and gene expression of progenitor bone cells (D1 cell) were compared on five different Ti surfaces, namely, mechanical grinding (M), electrochemical modification through potentiostatic anodization (ECH), sandblasting and acid etching (SLA), sandblasting, hydrogen peroxide treatment, and heating (SAOH), and sandblasting, alkali heating, and etching (SMART). SAOH treatment produced the most hydrophilic surface, whereas SLA produced the most hydrophobic surface. Cell activity indicated that SLA and SMART produced significantly rougher surfaces and promoted D1 cell attachment within 1 day of culturing, whereas SAOH treatment produced moderate roughness (Ra = 1.26 μm) and accelerated the D1 cell proliferation up to 7 days after culturing. The ECH surface significantly promoted alkaline phosphatase (ALP) expression and osteocalcin (OCN) secretion in the D1 cells compared with the other surface groups. The ECH and SMART-treated Ti surfaces resulted in maximum ALP and OCN expressions during the D1 cell culture. SLA, SAOH, and SMART substrate surfaces were rougher and exhibited better cell metabolic responses during the early stage of cell attachment, proliferation, and morphologic expressions within 1 day of D1 cell culture. The D1 cells cultured on the ECH and SMART substrates exhibited higher differentiation, and higher ALP and OCN expressions after 10 days of culture. Thus, the ECH and SMART treatments promote better ability of cell mineralization in vitro, which demonstrate their great potential for clinical use.

1. Introduction

Ti implants have significant functions in orthopedics, bone reconstruction, and dentistry because of their biocompatibility, corrosion resistance, low weight, and exceptional mechanical properties [1]. Ti is widely used in manufacturing oral implants. However, if Ti surface is not treated properly, the implant material might cause biological interactions that lead to infections and gum inflammation [2,3]. The consequences are dangerous and possibly lead to increased treatment risks, costs, and medical resource consumption [4,5]. Aside from biocompatibility, implants should adapt and respond according to the biological environment after implantation [6]. Ti surface should be significantly modified to improve their physical and chemical properties for promoting faster osseointegration with greater efficiency. The modified Ti surfaces should enhance the attachment, proliferation, and differentiation of progenitor bone cells while the implant is in contact with the surrounding tissues to accelerate bone attachment and provide anchor strength to the mandibular and maxillary alveolar bones [7–10].

Several commercialized products have been derived using different physical surface modification methods, such as machine grinding (M) and sand/grit blasting with micrometer-rough and nanometer-rough Ti surfaces (e.g., in laser ablated method), which should be performed while fabricating a system for increased utilization [3,11]. Other important factors for Ti surfaces include increasing hydrophilicity and preventing the stress-shielding effects of stress cycles, as well as load and temperature changes [12–17]. Therefore, this study focused on improving and evaluating different plate Ti surface modifications. The methods used in this study were the application of micro-rough Ti surfaces via Al_2O_3 sandblasting and acid etching (SLA), heat treatment and alkali treatment (SMART), hydrogen peroxide and heat treatment (SAOH), and potentiostatic anodization in sulfate electrolytes through constant electric current supply (ECH) [4,18–21]. The products with these surface modifications are clinically used commercial products. For example, the products with roughened surfaces obtained by M and sand/grit blasting are SLA from Straumann AG, TiOblast™ and OssoSpeed™ from Astra Tech, and Anker from Alliance. The other products with roughened surfaces were obtained through a combination of SLA, heat, alkali, hydrogen peroxide, and potentiostatic anodization, such as OSSEOTITE and Nano Tite™ from Biomet 3i, whereas RBM and

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Bio Tite-H are from DIO SM dental implants. These commercialized products with various Ti surfaces were developed to generate roughened surfaces to guarantee stable mechanical tissue–implant interfaces and to reduce recovery time. The degree of osseointegration in the early stages of wound healing (1 month to 3 months) is critical for determining the mechanical stability of the implant. The recovery time using Ti oral implants without surface modifications is generally ~12 weeks after implantation. With surface modifications, the recovery time can be reduced to 6 weeks to 8 weeks, demonstrating the clinical advantage of shorter healing period [20,21].

Pure Ti implants are commercially used in current teeth reconstruction procedures, but their safety must first be considered in vitro using cells before proceeding with animal studies and clinical tests. This safety precaution helps adjust and evaluate surface modification techniques, as well as analyzing the efficacy of the techniques in vivo. Evaluating the effects of different surface modifications through in vitro tests contributes to the optimization of results. Despite the existence of various surface modifications, no specific standard has been established to determine the best method. The factors involved in various types of surface modifications and the combinations of surface modification protocols affect the interaction of implants with tissues. Therefore, this study evaluated the optimization of multiple surface modifications by examining the effects of these treatments on Ti specimen in vitro. Alkaline phosphatase (ALP) is a membrane-bound enzyme in osteoblasts. ALP enhances osteogenesis and mineralization; thus, this enzyme is an early marker for osteoprogenitor bone cells (D1) that can be measured via cell counting and staining. Osteocalcin (OCN) is a specific marker for the bone maturation of osteoblasts. OCN is also studied in D1 cells as a late-stage marker for cell differentiation [8,22]. Therefore, the effects of the surface modifications on cell affinity, proliferation, and ALP production were also analyzed.

2. Materials and methods

2.1. Substrate surface modifications

Commercially pure Ti (c.p. Ti grade IV) was cast into 14.8 mm-diameter, 2 mm-thick circular disks. The substrates were successively smoothed with 400-, 800-, and 1200-grit sandpaper. The samples were then ultrasonically washed for 30 min in acetone. The same procedure was repeated with isopropanol and again with deionized water. Finally, the disks were dried at 40 °C in an incubator set. The samples comprised the control group, labeled as machined surface (M). Further modifications involved additional sample groups, namely, SLA, SAOH, and SMART. In SLA treatment, the samples were sandblasted for 30 s using an air compressor with 2 kg/cm^2 to 3 kg/cm^2 of powder (with Al_2O_3 particles with a mean size of 200 μm blasted over a distance of ~75 mm). Ultrasonic cleaning was performed to dry the disks, after which they were immersed in HCl (37%, Panreac, Barcelona, Spain), H_2SO_4 (95% to 98%, Panreac, Barcelona, Spain), and deionized water at 1:1:100. Subsequently, temperature etching was performed at 100 °C for 30 min. SAOH disks were processed in sulfuric acid (0.1 M, H_2SO_4) and hydrogen peroxide (8.8 M, H_2O_2) for ~20 min. The disks were successively heat-treated at 100 °C for 30 min [19]. SMART disks were immersed in NaOH (5.0 M) and etched at 100 °C for 1 h. The disks were cleaned with deionized water, dried, and immersed for 30 min in hydrochloric acid (0.1 M, HCl) for etching. The samples were cleaned, dried, and heat-treated again at 100 °C for 1 h in an oven. For the ECH treatment, the samples were processed for potentiostatic or galvanostatic anodization of Ti in a neutral solution with 0.1 M sulfate at a constant direct current density of ~200 A/m^2. Two Pt plates were used as cathodes on both sides of Ti anode. The samples were electrochemically prepared. Surface oxides increased to the anodic forming voltage of 125 V, which is essential for surface chemical modification. The anodization resulted in a thickening of Ti oxide layer to several micrometers. After cleaning and drying, the samples were also heat-treated at 100 °C for 1 h in an oven. All samples were cleaned and then autoclaved (121 °C/1.2 atm) for sterilization [4,19].

2.2. Substrate surface analysis

The surface wettability of the substrates was tested in triplicate using a contact angle meter (CAM-100, Creating Nano Technologies, Inc., Taiwan). The central line average surface roughness of Ra was measured using a roughness tester (SJ-301 Mitutoyo, Ltd., Japan). The topographies of different groups were analyzed under a scanning electron microscopy (SEM) system (Hitachi S-3000N, Hitachi, Tokyo, Japan). The samples were sequentially dehydrated in graded ethanol before they were coated with gold for SEM analysis. The substrate surface structures, cell morphology after cell attachment, and repopulation were examined.

2.3. Short-term cell attachment and long-term cell proliferation tests

The bone marrow cells from mesenchymal stem cells (osteoprogenitor cells, D1) that were cloned from BALB/c mice were purchased from the American Type Culture Collection. The D1 cells were maintained in Dulbecco's modified Eagle's medium. The cells were supplemented with 10% fetal bovine serum in 37 °C incubators with 5% CO_2. The cells were used before the eighth passage. The substrate disks were placed into 48-well plates, after which 50 μL of 5 × 10^3 cells was dripped onto the substrates for culturing. The cells were incubated for 1 h, 1 day, and 4 days. At different periods, the substrates were washed carefully with phosphate buffered saline (PBS) and were fixed with glutaraldehyde. The substrates were then gold-plated and screened using SEM to determine the interval required for cell attachment and proliferation.

The substrates were placed in 48-well plates, after which 50 μL of 1 × 10^5 D1 cells was again dripped onto the disks for incubation for 4 h to allow cell attachment. Additional media were applied to the plates. The cells were incubated for 1, 4, 7, 10, and 14 days and were washed twice with PBS at each time point. Up to 500 μL of a medium with alamarBlue® solution from an alamarBlue® cell viability assay kit (AbD Serotec, US) was added into each well. The cells were then incubated for 4 h at 37 °C. The reaction medium was spectrophotometrically measured at 570 and 600 nm using an enzyme-linked immunosorbent assay (ELISA) microplate reader (UVM-340; ASYS Hitech GmbH, Eugendorf, Austria). The cell numbers were determined from a plot of absorbance (OD values) versus the respective D1 cells after adjustment using the alamarBlue® assay. Each experiment was performed five times ($n = 5$).

2.4. ALP quantification and staining

ALP production on the surfaces of different sample groups was determined using *p*-nitrophenyl phosphate (pNPP) tablets (Sigma, USA). The pNPP and Tris-buffered saline tablets were placed in 20 mL deionized water and mixed. Testing was performed simultaneously with the same intervals as in the cell proliferation tests. At the end of each interval, Ti substrates were washed twice with PBS, after which 500 μL of the prepared solution was added into each well. This solution was subsequently incubated for 30 min. ALP activity was determined through absorbance measurements using ELISA reader at 405 nm.

ALP staining was performed using tartrate-resistant acid phosphatase and ALP double-stain kit (Takara Bio Inc., Shiga, Japan) in accordance with the manufacturer's instructions. As in the previous experiment, 50 μL of 1 × 10^5 D1 cells was dripped onto the disks during incubation and cell attachment for 4 h and medium was subsequently added. The cells were incubated for 14 days, after which the cells were washed twice with PBS and fixed with citrate buffer (pH 5.4) containing 60% acetone and 10% methanol. After the surface cells were fixed and washed with distilled water, the substrate solution was added to