Physicochemical properties of anodized-hydrothermally treated titanium with a nanotopographic surface structure promote osteogenic differentiation in dental pulp stem cells

Atsushi Aoyagi, Masaki Hata, Ryohei Matsukawa, Yuka Imanishi, Jun Takebe
Department of Removable Prosthodontics, School of Dentistry, Aichi Gakuin University, Nagoya, Japan

Abstract

Purpose: Implants made of anodized-hydrothermally treated commercially pure titanium with a nanotopographic surface structure (SA-treated c.p.Ti) may advantageously promote contact osteogenesis during the early stages of healing. We hypothesized that utilizing SA-treated c.p.Ti with dental pulp stem cells (DPSCs) might improve osteoconduction during the process of osseointegration. This in vitro study investigated the effect of initial adhesion of DPSCs to SA-treated c.p.Ti compared with conventional c.p.Ti and anodic oxide (AO) c.p.Ti.

Methods: DPSCs were obtained from the mandibular incisors of Sprague-Dawley rats and cultured without osteogenic induction medium on c.p.Ti, AO c.p.Ti, and SA-treated c.p.Ti disks for up to 14 days. The morphology, proliferation, and differentiation of DPSCs were assessed by scanning electron microscopy, an MTT assay, and Alizarin Red S staining, respectively. A real-time quantitative polymerase chain reaction was used to quantify the mRNA expression of osteocalcin, osteopontin, and bone sialoprotein.

Results: On all disks, the DPSCs appeared flattened with the formation of extensions over time. The filopodium-like extensions were closely bound to the SA-treated c.p.Ti surface. The proliferation of DPSCs was not significantly different among the c.p.Ti treatments. However, DPSCs on SA-treated c.p.Ti showed the greatest mRNA levels of osteopontin, osteocalcin, and bone sialoprotein, as well as increased Alizarin Red S staining.

Conclusions: The results of the present in vitro study demonstrate that the surface properties of SA-treated c.p.Ti disks enhance osteogenic differentiation of DPSCs and may facilitate mineralized matrix formation on SA-treated c.p.Ti implant surfaces, which can enhance early bone regeneration.

Keywords: Dental implants, Titanium, Discharge anodic oxidation and hydrothermal treatment, Nanotopography, Dental pulp stem cells

1. Introduction

In general, the goal of prosthetic rehabilitation is to improve patient quality of life. Early bone formation can improve implant stability under clinical conditions [1–3]. The surface topography and physicochemical properties of endosseous implants are important factors that promote successful bone formation during the process of osseointegration [4–6]. Commercially pure titanium (c.p.Ti) is well documented as a successful implant surface in dental clinics [7–9]. C.p.Ti implant surface topography can be modified down to the nanoscale to promote bone formation at the implant-bone interface [5,10,11]. Although surface topography at the microscale may play an important role in osteoconductivity for osseous wound healing, the so-called “nanotopography” of c.p.Ti, may influence surface-specific biological responses such as cell activity [6,12–14].

Previous studies have shown that discharge anodic oxidation followed by hydrothermal treatment (spark-discharged anodic oxidation (SA)) coats c.p.Ti implants (SA-treated c.p.Ti) with a highly crystalline, thin hydroxyapatite (HA) layer of an anodic titanium oxide film that provides a suitable nanotopographic structure for clinical oral implants [15–32]. Previous in vivo and in vitro studies confirmed that SA treatment induced a coating that promoted good bone conduction, early bone matrix mineralization, and high adhesive strength when paired with a c.p.Ti substratum [20,22,24,26,27,29]. Moreover, the topography, including HA crystals and anodic titanium oxide film, provides a porous microstructure to the SA-treated c.p.Ti surface, which effectively induced mineralized accretion in simulated body fluid and beagle jaw bone body fluid models [20,28]. This suggests that the chemical and physical properties of SA-treated c.p.Ti surfaces enhance extracellular bone matrix mineralization at the implant-bone tissue interface during the early stages of wound healing [20,24,26–28].

Dental pulp stem cells (DPSCs) are a type of mesenchymal stem cells that can differentiate into adipocytes, osteoblasts, and odontoblasts [33,34]. DPSCs, which can be easily obtained during orthodontic treatment by third molar or premolar extraction, retain their differentiation potential even after extended cryopreservation [35,36]. Furthermore, DPSCs exhibit greater osteogenic differentiation capacities than that of bone marrow mesenchymal stem cells (BMSCs) [37]. Because of these qualities, DPSCs are a promising cell source for bone regeneration. It has been reported that DPSCs with titanium materials show effective osteoblastic differentiation and bone formation [38,39], and the use of dental pulp-derived mesenchymal stem cells with simultaneous dental implant placement is effective in creating newly formed bone [40]. Therapeutic strategies that utilize DPSCs can promote bone formation on SA-treated c.p.Ti implants at earlier stages than current treatments. Therefore, we plan to inject DPSCs when placing the implant in areas with bone defect.
We hypothesized that utilizing SA-treated c.p.Ti surfaces with DPSCs would improve the success rate of osteoconduction during the process of extracellular bone matrix mineralization in wound healing. The current study examined cellular-implant surface properties using adherent DPSCs. Cell morphology and proliferation were observed to examine how specific cellular mechanisms are related to the surface topography and physicochemical properties of SA-treated c.p.Ti surfaces compared with c.p.Ti and anodic oxide (AO) c.p.Ti surfaces. Osteogenic differentiation was quantified at the level of the bone matrix by observing osteocalcin, osteopontin, and bone sialoprotein expression in DPSCs after a 3-day and 5-day culture period on these different surfaces. Alizarin Red S staining was measured after 7 and 14 days of culture to quantify the mineralized matrix. The results of this study suggest that DPSCs paired with SA-treated c.p.Ti present an advantageous strategy to promote early osseointegration.

2. Materials and methods

2.1. Sample preparation

C.p.Ti (> 99.8% titanium, JIS-Grade 2; Takacoh Company, Ltd., Aichi, Japan) (diameter = 15 mm, thickness = 1.5 mm, machine prepared surface roughness = 0.29 ± 0.01 μm Ra) were initially used. They were 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/cm² using a regulated DC power supply (419A-630; Metronix, Tokyo, Japan) to produce AO c.p.Ti disks (0.73 ± 0.04 μm Ra) [29]. SA treatment was completed by washing with distilled water, drying, and then hydrothermally heating the AO c.p.Ti disks using high-pressure steam at 300 ℃ for 2 h in an autoclave (1.3 L; Nitto Kouatsu Co., Ltd., Tsukuba, Japan). This resulted in HA crystal precipitation on the disk surface (0.83 ± 0.03 μm Ra) [15,28,29,32]. The surface morphologies of the AO c.p.Ti and SA-treated c.p.Ti disks were confirmed by scanning electron microscopy (SEM; XM177007-0007; JEOL, Ltd., Tokyo, Japan) after coating with carbon by chemical vapor deposition. The AO c.p.Ti surface was coated with an anodic titanium oxide film characterized by a porous microstructure with numerous craters and micropores with a diameter of 1-3 μm [15–18]. Conversely, the HA crystal layer of the anodic titanium oxide film containing calcium and phosphoric acid (AOFCP) were observed on the AO film of SA-treated c.p.Ti, characterized by a typical single hexagonal columnar shape, highly crystallized [15–18,23,24,27,29], and covering more than 60% of the AOFCP on SA-treated c.p.Ti [15–17]. The overall film had a lamellar structure with a 1 μm-thick HA layer over the AOFCP layer (4.5 μm) with a total thickness of 5.5 μm [16]. The AOFCP surface nanoscale topography on the SA-treated c.p.Ti, and SA-treated c.p.Ti disks. The adhesion and morphology of DPSCs on all disk types were analyzed using SEM. Experiments were performed in duplicate (n = 6 for c.p.Ti, AO c.p.Ti, and SA-treated c.p.Ti disks).

2.2. DPSCs isolation and cell culture

DPSCs were isolated from 6-week-old male Sprague-Dawley rats (Chubu Kagaku Co., Ltd., Aichi, Japan) and cultured. The lower incisors were surgically extracted, and the dental pulp was immediately collected, minced, and degraded with phosphate-buffered saline (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) containing 0.1% collagenase and 0.25% trypsin. DPSCs were seeded at a density of 1.5 × 10⁴ cells/cm² in plastic tissue culture dishes (Falcon; Becton Dickinson and Company, Franklin Lakes, NJ, USA) containing alpha-minimum essential medium (α-MM) (Eagle Alpha Modification; Gibco Laboratories Inc., Grand Island, NY, USA) with 20% fetal bovine serum (FBS; Gibco) under a humidified atmosphere of 5% CO₂/95% air at 37 ℃. DPSCs (1.5 × 10⁴ cells/disk) were seeded onto c.p.Ti, AO c.p.Ti, and SA-treated c.p.Ti disks in 24-well plates (Falcon) and incubated for 3, 5, or 7 days to prepare the conditioned media without the osteogenic induction medium. Following this, all disks with attached cultured cells were washed twice in phosphate-buffered saline to remove non-attached cells. Then, the cells were fixed for more than 6 h at room temperature with 2% glutaraldehyde and for more than 2 h at 4 ℃ with 1% osmium tetroxide. After fixation, the cells were dehydrated using a series of graded ethanol solutions, followed by critical point drying. Finally, the disks were coated with carbon by chemical vapor deposition. The adhesion and morphology of DPSCs on all disk types were analyzed using SEM. Experiments were performed in duplicate (n = 6 for c.p.Ti, AO c.p.Ti, and SA-treated c.p.Ti disks).

2.5. DPSCs proliferation analysis

DPSCs were cultured in α-MM with 20% FBS under a humidified atmosphere of 5% CO₂/95% air at 37 ℃. DPSCs (1.5 × 10⁴ cells/disk) were seeded onto c.p.Ti, AO c.p.Ti, and SA-treated c.p.Ti disks in 24-well plates (Falcon) and incubated for 3, 5, or 7 days to prepare the conditioned media without the osteogenic induction medium with the plastic tissue culture used as a control. The cells were cultured, and cell proliferation was measured on day 3 and day 5 using the MTT assay (Cayman Chemical Company, Ann Arbor, MI, USA). The MTT reagent was added on each disk, and the plates were incubated for 3 h. Formazan crystals were dissolved, and absorbance was measured at 570 nm using a multimode microplate reader (SPARK 10M; Tecan Japan Co., Ltd., Kanagawa, Japan). Experiments were performed in duplicate (n = 6 for c.p.Ti, AO c.p.Ti, and SA-treated c.p.Ti disks).

2.6. Real-time quantitative PCR (qRT-PCR) analysis of osteogenic differentiation at the level of bone matrix protein gene expression

DPSCs were maintained in α-MM with 20% FBS under a humidified atmosphere of 5% CO₂/95% air at 37 ℃. DPSCs (1.5 × 10⁴ cells/disk) were seeded onto c.p.Ti, AO c.p.Ti, and SA-treated c.p.Ti disks to prepare the conditioned media without the osteogenic induction medium with the plastic tissue culture used as a control. DPSCs were cultured and analyzed on day 3 and day 5. Total RNA was isolated from DPSCs using TRIzol (Thermo Fisher Scientific, Waltham, MA, USA). cDNA was synthesized from total RNA using ReverTra Ace reverse
transcriptase (Toyobo Co., Ltd., Osaka, Japan). qRT-PCR was performed using the StepOneTM and StepOnePlusTM real-time PCR systems (Thermo Fisher Scientific) with osteocalcin, osteopontin, and bone sialoprotein primers specific for DPSCs. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a control. The relative quantity was calculated using the ΔΔCt method [44]. Experiments were performed in duplicate (n = 6 for c.p.Ti, AO c.p.Ti, and SA-treated c.p.Ti disks).

2.7. Examining the mineralized matrix

DPSCs were maintained in α-MEM with 20% FBS under a humidified atmosphere of 5% CO₂/95% air at 37 °C. DPSCs (1.5 × 10⁶ cells/disk) were seeded onto c.p.Ti and SA-treated c.p.Ti disks to prepare the conditioned media without the osteogenic induction medium, with the plastic tissue culture used as a control. After 7 or 14 days of culture, the cells were fixed with 10% formaldehyde for 30 min at room temperature and then stained with Alizarin Red S (Iwai Chemicals, Co., Ltd., Tokyo, Japan) for 30 min. The calcium content was detected using a colorimetric method. Two percent formic acid (Iwai Chemicals) was added to each well, and the plate was incubated at room temperature after shaking for 10 min. The solution was transferred to a new tube, and the Alizarin Red S concentration was determined by measuring the absorbance at 405 nm [45]. Experiments were performed in duplicate (n = 12 for c.p.Ti and SA-treated c.p.Ti disks).

2.8. Statistical analysis

The cell proliferation, PCR, and Alizarin Red S staining data are expressed as mean ± standard error. One-way analysis of variance followed by Bonferroni post-hoc analysis was used to compare multiple groups. A probability (P)-value < 0.05 or < 0.01 was considered significant. Statistical analyses were performed on a personal computer using SPSS software for Windows, version 15.0 (SPSS Japan, Tokyo, Japan).

3. Results

3.1. Identification of DPSCs

Phase-contrast microscopy revealed that DPSCs had a typical spindle-shaped morphology on a plastic dish (Fig. 1A). The cultured DPSCs were identified by their surface markers using FACS. Mesenchymal stem cells were positive for the common stem cell markers CD29, CD49d, and CD90 but negative for the hematopoietic stem cell markers CD34 and CD45 (Fig. 1B).

3.2. DPSCs differentiated into adipocytes and osteoblasts

After 21 days of culture in adipogenic differentiation and osteogenic induction medium, DPSCs differentiated into lipid-containing adipogenic cells, as indicated by positive staining with Oil Red O and FABP-4. DPSCs also differentiated into osteoblasts, as indicated by positive staining with ALP and osteocalcin (Fig. 1C).

3.3. Cell morphology analysis

The SEM morphological evaluation showed that DPSCs adhered to the c.p.Ti surfaces for 3 days and appeared to be flattened with extensions and numerous microvilli (Fig. 2A). Adhesion of the cell cytoplasm to the c.p.Ti disk surface was confirmed under higher magnification (Fig. 2B). Analysis of DPSCs on the AO c.p.Ti disks showed that the cytoplasm was thinly spread and adhered tightly to the disk surface (Fig. 2C), which was confirmed under higher magnification (Fig. 2D). The DPSCs on the SA-treated c.p.Ti were thinly spread with a flattened cytoplasm. The HA crystals on the anodic titanium oxide film contacted the filopodium-like processes from the cells (Fig. 2E). This close connection between the cell processes and the SA-treated c.p.Ti disk surface was confirmed under higher magnification, which also revealed the nanoscale topography (Fig. 2F).

After 5 days of culture, DPSCs were bounded to the c.p.Ti surfaces and had flattened and elongated bodies with extensions (Fig. 3A). High-magnification micrographs of the c.p.Ti surface (Fig. 3B) confirmed that the cytoplasm adhered to the disk surface. DPSCs bound tightly to the AO c.p.Ti disks with flattened bodies and projections extending into the cytoplasm (Fig. 3C). High-magnification micrographs (Fig. 3D) confirmed the tightly adhered cytoplasm and revealed filopodium-like processes that extended towards and adhered to the anodic titanium oxide film surface. DPSCs were bound tightly to the SA-treated c.p.Ti surface and were also spread with a flattened cytoplasm. The HA crystals and the anodic titanium oxide film appeared to be in close contact with the cells and were surrounded by cytoplasm and filopodium-like processes (Fig. 3E). High-magnification micrographs (Fig. 3F) confirmed that the cytoplasm and extending filopodium-like processes adhered closely to the anodic titanium oxide film at the level of nanoscale topography.

After 7 days of culture, DPSCs were tightly bound to and spread across all disk surfaces (Fig. 4A-F). DPSCs on the c.p.Ti disks had flattened bodies with extensions (Fig. 4A). Higher magnification confirmed the close cellular connection to the disk surface (Fig. 4B). DPSCs on the AO c.p.Ti disks also showed a flattened body with an extending cytoplasm (Fig. 4C).
High-magnification micrographs (Fig. 4D) confirmed that these cells had filopodium-like processes that extended to the anodic titanium oxide film on the disk surface. DPSCs on SA-treated c.p.Ti disks were extensively spread with a flattened cytoplasm on the disk surface. The HA crystals on the anodic titanium oxide film were in close contact with the filopodium-like processes from the cells (Fig. 4E). Higher magnification of the nanoscale topography confirmed a closer connection between the cell processes and the SA-treated c.p.Ti disk surface, compared with the cells on the AO c.p.Ti disks (Fig. 3D). Moreover, the apical parts of the cells were spread across the nanoscale topography and migrated into the micropores on the SA-treated c.p.Ti disks (Fig. 4F).

3.4. Proliferation of DPSCs

The proliferation of DPSCs was quantified using the MTT assay. As shown in Figure 5, the number of DPSCs was almost equal on c.p.Ti and SA-treated c.p.Ti disks on days 3 and 5. Following 3 days of culture, no significant differences were observed between the c.p.Ti, AO c.p.Ti, SA-treated c.p.Ti, and control surfaces. Following 5 days of culture, the AO c.p.Ti and SA-treated c.p.Ti disks had significantly more DPSCs than the c.p.Ti disks. However, there were noticeable differences between the AO c.p.Ti and SA-treated c.p.Ti disks.

The cell proliferation rate did not increase during day 3 to day 5 of culture on the c.p.Ti disks but increased significantly on the AO c.p.Ti and SA-treated c.p.Ti disks.

3.5. qRT-PCR analysis of bone matrix protein gene expression

Osteocalcin, osteopontin, and bone sialoprotein mRNA expression levels of DPSCs adhered to the treated disks were analyzed after day 3 and day 5 of culture (Fig. 6A-F).

3.5.1. Osteocalcin

After 3 days of culture, osteocalcin mRNA expression increased significantly on SA-treated c.p.Ti disks compared with c.p.Ti disks and the control surfaces but not on the AO c.p.Ti disks. There were no significant differences between the c.p.Ti disks, AO c.p.Ti disks, and control surfaces (Fig. 6A).

Following 5 days of culture, osteocalcin mRNA expression increased significantly on SA-treated c.p.Ti disks compared with c.p.Ti disks, AO c.p.Ti disks, and control surfaces. There was no significant difference between the c.p.Ti disks, AO c.p.Ti disks, and control surfaces (Fig. 6B).

3.5.2. Osteopontin

After 3 and 5 days of culture, osteopontin mRNA expression increased significantly on SA-treated c.p.Ti disks compared with c.p.Ti disks, AO c.p.Ti disks, and control surfaces. There was no significant difference between the c.p.Ti disks, AO c.p.Ti disks, and control surfaces (Fig. 6C, D).

3.5.3. Bone sialoprotein

After 3 and 5 days of culture, bone sialoprotein mRNA expression increased significantly on SA-treated c.p.Ti disks compared with c.p.Ti disks, AO c.p.Ti disks, and control surfaces. There was no significant difference between the c.p.Ti disks, AO c.p.Ti disks, and control surfaces (Fig. 6E, F).

3.6. Examination of the mineralized matrix

Alizarin Red S staining was performed after 7 and 14 days of culture (Fig. 7A, B) to assess the specific calcium-binding sites of the mineralized matrix.
matrix. Representative images of calcified matrix deposition are shown in Fig. 7A. At both time points, DPSCs showed marked mineralization on SA-treated c.p.Ti disks compared with that on the c.p.Ti disks and control surfaces (Fig. 7A). Quantification of Alizarin Red S staining showed that the SA-treated c.p.Ti disks had significantly greater mineralized matrices compared with the c.p.Ti disks and control surfaces at both time points (Fig. 7B). Moreover, the SA-treated c.p.Ti surfaces exhibited a significantly greater mineralized matrix on day 14 compared to day 7 (Fig. 7B).

4. Discussion

This is the first report characterizing the effect of SA treatment on the differentiation of DPSCs adhered to c.p.Ti disks along the osteoblastic lineage. The main finding was that SA treatment enhanced osteogenic differentiation of DPSCs on c.p.Ti disks without an osteogenic induction medium. This phenomenon was associated with increased mRNA expression of the bone matrix proteins osteocalcin, osteopontin, and bone sialoprotein. Furthermore, a significantly greater mineralized matrix was formed on the SA-treated c.p.Ti disks in DPSCs that were cultured for 7 or 14 days. These in vitro results support our hypothesis that the early cellular responses of DPSCs interact with the physicochemical properties of SA-treated c.p.Ti surfaces at the nanotopographic level, which may be crucial for successful osteoconductance during the process of matrix mineralization in osseous wound healing. Therefore, these findings suggest that using SA-treated c.p.Ti surfaces with DPSCs may be an advantageous strategy to promote bone regeneration during the early stage of osseointegration in areas with bone defects.

Using DPSCs is advantageous over alternative methods. Our previous study used human BM MSCs that were required to be drawn from the iliac crest of adult donors [46], a highly invasive and painful collection method. Conversely, obtaining DPSCs from teeth requires a less invasive method, that is, extraction, and the cells retain their differentiation potential even after long periods of cryopreservation [41,47]. DPSCs are also isolated from extracted teeth at a young age for orthodontic treatment because DPSCs are not affected by systemic diseases that may induce cellular dysfunction, such as hypertension, diabetes, and arteriosclerosis. DPSCs also secrete various factors, including angiogenic and anti-inflammatory factors that promote vascularization at transplant sites [47,48]. An in vitro study comparing the expression patterns of osteogenic differentiation markers showed that DPSCs produce more mineralization than BM MSCs after osteogenic differentiation [37], indicating their enhanced potential for osseointegration. Autologous DPSCs are an effective cell source that contributes to the success rate of implant treatment to replace lost teeth [49].

Osseointegration represents the processes of osteoinduction, osteoconduction, and osteogenesis [50]. Recruiting mesenchymal stem cells into osteoblasts is regarded as a central step in osseointegration. The surface topography and physicochemical properties of endosseous implants may positively influence bone formation during the early stages of wound healing [51]. However, long-term stability and retention are dependent on the remodeling of the jawbone, with initial stabilization of the implant strongly influenced by the early stages of osseous wound healing that begin at the time of implantation [14]. The current theory describes that the primary mechanical stability at the implant-bone interface is provided by the implant design (shape, diameter, and length). Moreover, biological (secondary) stability is provided by the implant surface topography at the micro- or nano-level, and the chemical and hydrophilic properties support the newly formed bone during early wound healing [1]. Our recent data indicated that macrophages are a source of the osteogenic growth factors BMP-2 and BMP-6 [25,52,53]. Moreover, we demonstrated that macrophage adhesion to SA-treated c.p.Ti surfaces and surface-specific expression of osteogenic growth factors play key roles in the processes of osseointegration [25,52,53]. We speculate that cells, such as macrophages, but not undifferentiated adherent osteoprogenitor cells that are present on SA-treated c.p.Ti implants contribute to early osteoinductive signals during early wound healing [22,25,28,32,52,53]. SA-treated c.p.Ti surfaces promote early osteoconduction that is compatible with new bone tissue [24]. The present study indicates that utilizing SA-treated c.p.Ti surfaces with DPSCs may promote the early formation of the mineralized matrix due to cell differentiation on the endosseous implant surface.

Interactions of DPSCs with the SA-treated c.p.Ti surface are dependent on the surface topography and physicochemical properties. The current study found that the proliferation rate of DPSCs on SA-treated c.p.Ti disks after 3 and 5 days of culture was associated with filopodium-like cellular extensions that were tightly bound to the nanotopographic structure surface and HA crystals on the SA-treated c.p.Ti disks. Furthermore, mRNA expression of the non-collagenous extracellular matrix proteins osteocalcin, osteopontin, and bone sialoprotein was significantly greater on SA-treated c.p.Ti disks compared with that on the c.p.Ti disks and control surfaces at both time points (Fig. 7B). Moreover, the SA-treated c.p.Ti surfaces exhibited a significantly greater mineralized matrix on day 14 compared to day 7 (Fig. 7B).

Fig. 4. SEM morphological evaluation of the adherence of DPSCs following 7 d of culture. Representative SEM images of cellular adhesion on c.p.Ti (A, B), AO c.p.Ti (C, D), and SA-treated c.p.Ti (E, F) disks. Higher magnification micrographs of c.p.Ti (B, enlargement of the frame shown in A), AO c.p.Ti (D, enlargement of the frame shown in C), and SA-treated c.p.Ti disks (F, enlargement of the frame shown in E). Magnification was × 1,500 for A, C, and E; and × 10,000 for B, D, and F. White arrows indicate the interface between cells and the disk surfaces.
On day 3 of culture, mRNA expression levels of bone sialoprotein and osteopontin were higher than those of osteocalcin since the stage of bone formation is characterized by matrix mineralization. On day 5 of culture, mineral accumulation is thought to occur. Therefore, the mRNA expression level of osteocalcin was higher than that of bone sialoprotein and osteopontin. Osteopontin expressed prior to bone mineralization is regulated by binding to HA and enhances osteoblast and osteoblast adhesion. Osteocalcin is an abundant calcium-binding protein in the bone extracellular matrix that may contribute to bone resorption or remodeling. Osteocalcin expression reflects a cellular commitment to the osteoblastic lineage and is a useful molecular marker for osteoblast differentiation [54]. Osteopontin has defined roles in cell attachment and differentiation [55]. Finally, bone sialoprotein, a mineralized tissue-specific and adhesive extracellular matrix protein, is produced by committed osteoblastic cells. It is a key indicator of adherent cell differentiation and osteogenesis [56].

The present study analyzed osteogenic differentiation after 7 or 14 days of DPSC culture without osteogenic induction medium. Unfortunately, it was impossible to analyze mRNA expression by qRT-PCR because the formation of the mineralized matrix was promoted. Therefore, osteogenic differentiation was analyzed by Alizarin Red S staining. SA-treatment significantly increased the mineralized matrix of DPSCs on c.p.Ti disks after 7 and 14 days of culture. Alizarin Red S staining was unable to detect the formation of a mineralized matrix on the AO c.p.Ti disks. A previous study reported that the energy of the endosseous implant material surface could affect the latter stages of bone matrix mineralization and bone formation by influencing initial cell adhesion to the material and cell differentiation at the implant-cell interface [57]. The AO c.p.Ti and SA-treated c.p.Ti disks had different properties that may have influenced this process [26,28,29]. Therefore, we propose that DPSCs can increase bone matrix proteins and mineralized matrix expression on SA-treated c.p.Ti surfaces. These findings provide a molecular characterization of the osteogenic differentiation of DPSCs on SA-treated c.p.Ti disks, which may contribute to tissue engineering of endosseous implant materials for clinical implantation. We are also considering the application of this method to titanium mesh for the repair of large bone defects or the reconstruction of lumbar burst fractures [58–60].

In addition, we speculate that the observed increase in osteopontin and bone sialoprotein mRNA expression may stimulate osteogenic differentiation of DPSCs and mineral formation in the extracellular matrix via integrin-mediated signaling pathways. Regarding this phenomenon, we previously demonstrated that the organization of cytoskeletal actin filaments and focal adhesion kinase protein is dependent on the topography and physicochemical properties of SA-treated c.p.Ti surfaces [22,30,32]. However, further studies are needed to elucidate the mechanism underlying signal transduction among DPSCs on SA-treated c.p.Ti surfaces.

Our previous and present studies demonstrated that after hydrothermal treatment of AO c.p.Ti, the AO surface with the HA crystals had a nanotopographic structure, which enhanced or induced osteogenic differentiation and formation of bone matrix mineralization [17-24,25-29]. Initially, calcium and phosphorous seemed to be ionically contained in the anodic titanium oxide film on the AO c.p.Ti surface and then virtually lost from this film after hydrothermal treatment. The AOFCP surface nanotopographic structure, which enhanced or induced osteogenic differentiation and formation of bone matrix mineralization [17-24,25-29]. Initially, calcium and phosphorous seemed to be ionically contained in the anodic titanium oxide film on the AO c.p.Ti surface and then virtually lost from this film after hydrothermal treatment. The AOFCP surface nanotopographic structure with the HA crystals on the SA-treated c.p.Ti surface is formed as a result of Ca$^{2+}$ and PO$_4^{3-}$ leaching from the AOFCP surface and precipitated onto the AOFCP surface again [22,28,29]. It has been reported that calcium-phosphorous biomaterials are capable of adsorbing cell adhesive proteins [61,62], and our previous report suggested that mesenchymal stem cells actin filaments on SA-treated c.p.Ti surfaces were accumulated by focal adhesion via integrin, and regulation of cellular differentiation and growth may be mediated by the molecules involved in focal adhesion formation [22,24,26,27]. Therefore, we consider that the AOFCP surface nanotopographic structure with the HA crystals on the SA-treated c.p.Ti had a strong adsorptive property for serum proteins and then
mediated the specific adhesion of DPSCs. Recent studies have demonstrated that the contact angle measurements of serum albumin and Hank’s balanced salt solution (HBSS) indicated that SA-treated c.p.Ti with a nanotopographic surface structure became superhydrophilic and exhibited marked improvement in wettability compared with c.p.Ti and AO c.p.Ti surfaces [28,29]. We also demonstrated that the surface free energy levels exhibited marked improvement in SA-treated c.p.Ti with a nanotopographic surface structure compared with c.p.Ti and AO c.p.Ti surfaces [28,29]. Specifically, serum protein and surface free energy could play an important role in initial cell contact and adhesion to the interface surface [53,61,62]. Moreover, we previously demonstrated that the AOFCP surface nanotopographic structure with the HA crystals on the SA-treated c.p.Ti was completely covered with a deposited layer containing calcium phosphate after immersion in HBSS, while the deposition of calcium phosphate on the AO c.p.Ti surface was not detected. This indicates that the biomineralization of the calcium phosphate layer on the adsorption of calcium and phosphorous ions is a function of the electrostatic interaction, including surface energy or polarity of the AOFCP surface nanotopographic structure with the HA crystals on the SA-treated c.p.Ti [22-29]. It was demonstrated that calcium and phosphorous concentrations affect the osteogenic differentiation of human DPSCs [63]. In addition, adenosine triphosphate (ATP) enhances osteogenic differentiation of rat DPSCs, which involves a calcium ion-dependent mechanism via the PLC-IP3 pathway [64].

Thus, the nanotopographic structure of SA-treated c.p.Ti surface exhibited marked improvement in wettability of the superhydrophilic surface with higher levels of surface free energy and higher affinity, number of hydrogen bonds, and polarization, compared with c.p.Ti and AO c.p.Ti surfaces. These are important factors in determining biological responses [17,18,20-32]. Therefore, the biological interactions of these physicochemical properties of the AOFCP surface nanotopographic and nanotopographic structures with the HA crystals on the SA-treated c.p.Ti surface serve as important regulators of DPSC phenotypes with osteogenic differentiation ability.

The present in vitro study characterized the early stage adhesion of DPSCs to SA-treated c.p.Ti and demonstrated enhanced osteogenic differentiation of DPSCs, likely due to SA-treated c.p.Ti surface properties. These results suggest that the DPSCs strategy paired with an SA-treated c.p.Ti implant surface may promote successful osteoconduction during osseointegration via an enhanced mineralized matrix. Therefore, utilizing SA-treated c.p.Ti endosseous implants with DPSCs may promote early bone regeneration in implant therapy. In future studies, we plan to validate the efficacy of creating bone tissue by observing the bone-implant interface and in vivo tracking of cells.

5. Conclusion

In summary, the results of the present in vitro study indicate that SA-treated c.p.Ti disks produced a greater cellular response than c.p.Ti or AO c.p.Ti disks with a greater capacity to induce activation of DPSCs without an osteogenic induction medium. The nanotopographic structure of the thin HA layer and anodic titanium oxide film on the SA-treated c.p.Ti implant surface markedly improved the adhesion and spreading of DPSCs, bone matrix protein mRNA expression, and mineralized matrix formation. These in vitro early cellular responses support our hypothesis that utilizing SA-treated c.p.Ti surfaces with DPSCs could facilitate successful osteoconduction during the process of matrix mineralization. Therefore, utilizing DPSCs with SA-treated c.p.Ti may be an advantageous strategy to promote bone regeneration during the early stage of osseointegration. Nonetheless, further in vivo studies are needed to confirm whether SA-treated c.p.Ti surfaces enhance the biological response to an endosseous implant.

Funding

This work was supported by a grant from JSPS KAKENHI (grant no. JP16K11616, JP20K10082) to Jun Takebe.
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