Longitudinal Observation of Thin Hydroxyapatite Layers Formed on Anodic Oxide Titanium Implants after Hydrothermal Treatment in a Rat Maxilla Model

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Clinical significance
Highly crystalline thin hydroxyapatite (HA) layers deposited on commercially pure titanium (cpTi) surfaces by discharge anodic oxidation and hydrothermal treatment (SA treatment) enhance the value of cpTi as endosseous implants. This study indicated that the thin HA layer remained stable during the process of bone formation in rat maxilla.

Abstract
Purpose: Highly crystalline thin hydroxyapatite (HA) layers deposited on the surface of commercially pure titanium (cpTi) by discharge anodic oxidation and hydrothermal treatment (SA treatment) enhance the value of cpTi as endosseous implants in clinical dentistry. In particular, the SA treatment affects the osteoconductive properties of cpTi. Determining whether this HA layer retains its chemical stability during osseous wound healing is crucial for its use in implants. In this study, we characterized the morphological, chemical, and structural features of HA layers on SA-treated cpTi implants in rat maxilla.

Materials and methods: SA-treated cpTi implants (diameter: 1.0 mm, length: 2.0 mm) were placed in the maxilla of 8-week-old Wistar rats. After 14, 21, and 28 days, the maxilla were harvested and the implant surfaces were analyzed by scanning electron microscopy (SEM), electron probe microanalysis (EPMA), and x-ray photoelectron spectroscopy (XPS).

Results: SEM analysis revealed precipitated HA crystals on the implant surface; the crystals had a typical single hexagonal columnar shape and they were highly crystalline. Implantation up to 28 days changed neither the morphology nor the crystalline features of the thin HA layer. EPMA revealed an even distribution of P and Ca in the HA layers before and after implantation in the maxilla, while XPS indicated no change in the binding energies of P and Ca in the HA crystals.

Conclusion: The thin HA layer formed on the SA-treated cpTi implants remained stable during the process of jaw bone formation.

Key words: discharge anodic oxidation, hydrothermal treatment, hydroxyapatite crystal, endosseous titanium implant, animal studies

Introduction
The successful integration of endosseous implants relies on bone formation at the implant site. The surface topography of these implants is important for the osseointegration process, and modifying the surface of endosseous implants may enhance the progress of bone formation.1,2 Commercially pure titanium (cpTi) is widely used in endosseous implants due to its mechanical strength, stability, and compatibility with bone tissue.3 As proposed, changing the surface topography or properties of cpTi promoted the extent of bone formation at the implant-bone interface.4,5 In particular, a hydroxyapatite (HA) coating of cpTi led to the direct chemical bonding of the implant surface with the bone soon after implantation due to the superior osteoconductive properties of HA-coated cpTi as compared to uncoated cpTi.6,9

The HA-coating of cpTi for endosseous implants is achieved by first forming an anodic oxide film containing calcium and phosphate (AOFCP) on the outermost cpTi surface by anodization in an aqueous electrolyte solution containing β-glycerophosphate (β-GP) and calcium acetate monohydrate (CA) dissolved in distilled water. HA crystals are then precipitated on the AOFCP by hydrothermal treatment using high-pressure steam in an autoclave at 300°C (SA treatment).10-13 The high adhesive strength of HA, measured at 38-40 MPa, is believed to result from the AOFCP existing as an intermediate layer between the HA layer and the...
cpTi substrate.\textsuperscript{11,12} Studies have confirmed that an SA-treated cpTi surface promotes the early production and mineralization of the bone matrix,\textsuperscript{14,15} as well as early bone formation. SA-treated cpTi surfaces are also biologically compatible with bone tissue,\textsuperscript{16} and have no adverse effect on immune cells.\textsuperscript{17,18} These data suggest that the chemical or physical state of the SA-treated cpTi surface influences the phenotypic expression of cells involved in bone formation. We have also shown previously that the thin HA layer formed on SA-treated cpTi increases in stability during the process of bone matrix mineralization in the in vitro osteoblastic culture model.\textsuperscript{19} However, it remains unknown whether the HA layer formed by SA treatment maintains long-term chemical stability during bone formation after implantation in jaws.

We hypothesized that a thin HA layer with high crystallinity deposited on cpTi matrices following SA treatment might be crucial for successful osteoconduction and chemical stability during early osseous wound healing and for bone formation in the jaw. Therefore, in this study, we investigated the morphological, chemical, and structural features of a thin HA layer on SA-treated cpTi implants during the process of bone formation in rat maxilla.

Materials and methods
SA-treated cpTi implants
Custom-made, tapered, commercially pure titanium implants (cpTi; Ti purity > 99.8%, Shinko Wire Company Ltd, Osaka, Japan) (diameter: 1.0 mm, length: 2.0 mm, taper: 5°) were anodized at 350 V in an electrolytic solution containing 0.01 mol/L of β-glycerophosphate disodium salt pentahydrate (β-GP) and 0.15 mol/L of calcium acetate monohydrate (CA) dissolved in distilled water. Anodic oxidation was conducted at a current density of 50 mA/cm\textsuperscript{2} using a regulated DC power supply (419A-630; Metronix, Japan). After anodic oxidation, the cpTi implants were washed with distilled water and dried. They were then hydrothermally heated using high-pressure steam at 300°C for 2 h in an autoclave, resulting in the precipitation of HA crystals on the implant surface (SA treatment)\textsuperscript{11,15} (Fig. 1). Prior to implantation, the SA-treated cpTi implants were sterilized by dry heat at 180°C for 45 min. The observation of the HA crystals by scanning electron microscopy (SEM) (S-4700, Hitachi, Tokyo, Japan) showed that approximately 60% of the surface of the AOFSCP was covered with HA crystals.\textsuperscript{10-12} The precipitated HA crystals had a typical single hexagonal columnar shape and they were highly crystalline.\textsuperscript{10,11,13,19} This film had a lamellar structure: a thin HA layer of 1-μm thickness was formed on an AOFSCP layer of 4.5-μm thickness, for a total film thickness of 5.5 μm.\textsuperscript{15} All SA-treated cpTi implants were exposed to ultraviolet light in a sterile tissue culture hood for 72 h.

Surgical placement
Twenty-one 8-week-old male Wistar rats (170–190 g; Japan Clea Co., Ltd., Tokyo, Japan) were housed during experiments at two per cage with free access to food and water. The animals were anesthetized by an intraperitoneal injection of pentobarbital sodium 50 mg/mL (Nembutal Sodium Solution, Abbott Laboratories, North Chicago, IL). The front part of the first molar tooth at the maxilla implantation sites was then thoroughly disinfected with 10% iodine. The maxilla surface was carefully exposed by the exfoliation of the mucous membrane, and after the dissection of the periosteum, trans cortical holes were drilled at 3-mm intervals using a slow-speed (500 rpm) dental hand-piece equipped with a round burr (diameter: 0.8 mm), followed by a taper fissure burr (diameter: 1.0 mm, thickness: 2.0 mm) to reach the bone marrow. Profuse irrigation with physiological saline was maintained throughout the drilling process. The SA-treated cpTi implants were inserted into each of the surgically prepared holes by tapping with a mallet. After washing the implant site with physiological saline, the skin flap was closed with 4-0 absorbable surgical sutures. After implantation, the animals were housed with free access to water and food. No antibiotics were administered.
SA-treated cpTi implants were placed in the rat maxilla for 14, 21, 28 days.

- Animals: 8-week-old male Wistar rats
- Place: Front, the upper first molars on both side
- Anesthesia: General anesthesia by pentobarbital sodium intraperitoneal injection

SA-treated cpTi implants were extracted from circumferential bone.

The specimen was fixed according to Karnovsky’s method and removed the SA-treated cpTi implant.

Analysis

(Fig. 2). The study protocol was approved by the Iwate Medical University Animal Research Committee (#D-17-066, June 21, 2005).

**Specimen preparation**

At 14, 21, and 28 days following surgery, animals were euthanized by anesthetic overdose, and the SA-treated cpTi implants were retrieved by dissection from the maxilla bone. The implants and the surrounding maxillary bone pieces were washed twice in phosphate-buffered saline (PBS) and then twice with 0.1 M sodium cacodylate buffer (pH 7.4, 37°C) for 5 min each. The specimens were fixed for 3 h at 4°C according to Karnovsky’s method (4% paraformaldehyde, 3% glutaraldehyde, and 0.1 M sodium cacodylate buffer, pH 7.4), washed twice in PBS, and then dehydrated through a graded ethanol series, followed by critical-point drying. The bone surrounding the SA-treated cpTi implants was removed carefully by tweezers, and then parts of the bone tissue including the mineralized bone, extracellular matrix, and collagen fibers were removed by mechanical force. Finally, SA-treated cpTi implants that were to be examined by scanning electron microscopy (SEM, S-4700, Hitachi, Tokyo, Japan) and electron probe microanalysis (EPMA, JXA-8900L, JEOL, Tokyo, Japan) were coated by ion sputtering (E-1030, Hitachi, Tokyo, Japan). The SA-treated cpTi implants were examined by SEM, EPMA, and X-ray photoelectron spectroscopy (XPS, AXIS-His, Kratos, Manchester, UK), as described below (Fig. 2).

**SEM of HA crystal morphology**

The HA crystals on the implant surfaces were examined by SEM (original magnification 1500x, 5000x) before and after placement in the rat maxilla for 14, 21, and 28 days.

**EPMA of elemental distribution**

The distributions of phosphorus (P) and calcium (Ca) in the thin HA layer on the surface of the SA-treated cpTi implants were monitored using EPMA at an acceleration voltage of 15 kV and specimen current of $3 \times 10^{-8}$ A before and after placement in the rat maxilla for 14, 21, and 28 days.

**XPS analysis of HA crystals**

The elemental and chemical composition of the crystals comprising the thin HA layer on the implants was determined by XPS before and after placement in the rat maxilla for 14, 21, and 28 days. The chamber was evacuated to a minimum pressure of $1 \times 10^{-8}$ Pa. A magnesium electrode (Al-K$_{12}$ radiation at 1486.6 eV) at 15 kV and 3 mA was used as the X-ray source. The XPS take-off angle was set at 45°. XPS wide scans of the P2p, C1s, Ca2p, Ti2p, and O1s regions were conducted to quantify the atomic compositions. Narrow XPS scans of the P2p, Ca2p$_{3/2}$, and Ca2p$_{1/2}$ regions were also conducted.

**Statistical analysis**

For EPMA and XPS experiments, seven SA-treated cpTi implants were analyzed at five points per implant. The average values were compared by one-way analysis of variance (ANOVA), and the data are expressed as mean values ± standard deviation (SD).

**Results**

**SEM of HA crystal morphology**

The SEM analysis of the bone matrix mineralization process visualized the bone-tissue and crystal morphology of the HA layer on the surface of SA-treated cpTi implants (Figures 3a, b). Needle-like crystals covered the implant surface before placement in the rat maxilla; these crystals had a single hexagonal columnar shape that is typical of HA. Thin, early bone trabeculae appeared in the rat maxilla 14 days after implantation. Under these forming trabeculae, and on the surface of the SA-treated cpTi implant, was a layer of collagen fibers, numerous accretions, and mineralized bone tissue. After 21 days, the trabeculae were
filled with new bone, and a bone-implant interface was apparent, formed by the fusion of globular calcified accretions on the thin HA layer. After 28 days, a mature bone was observed apposing much of the implant surface. An interface, formed by the fusion of globular calcified accretions, was still apparent. Moreover, the needle-like crystals were no longer visible due to the presence of accretions. Low-magnification SEM imaging showed a layer of calcified-like microspheres and multilayered mature bone trabeculae on the HA layer that was markedly thicker than it was before placement in the rat maxilla. SEM imaging at high magnification revealed no change to the HA-layer crystal morphology during bone matrix mineralization and bone tissue formation in the maxilla.

**EPMA analysis of elemental distribution**

The EPMA results quantified the presence of P (Fig. 4a) and Ca (Fig. 4b) on the SA-treated cpTi implant surface before and after being placed in the maxilla.

The EPMA showed an even distribution of P and Ca across the HA layer before being placed in the rat maxilla (9.87 ± 1.20 vs. 34.38 ± 1.19), as well as after implantation for 14 (9.41 ± 0.95 vs. 34.63 ± 1.46), 21 (9.58 ± 1.40 vs. 34.62 ± 1.04), and 28 (9.73 ± 1.58 vs. 34.41 ± 1.57) days. No significant differences were found among the P or Ca densities before and after placement in the rat maxilla for 14, 21, and 28 days (ANOVA: F = 0.230, P = 0.875 for P; F = 0.101, P = 0.959 for Ca).

**XPS analysis of HA crystals**

Figure 5 shows the XPS wide-scan spectra of the implant surfaces after placement in the rat maxilla for 14, 21, and 28 days as compared to that before placement in the rat maxilla. Phosphorus, carbon, calcium, titanium, and oxygen were detected on all sample surfaces. P2p, C1s, Ca2p, Ti2p, and O1s conformed to the wide-scan spectra of the thin HA layer. According to the XPS narrow-scan spectra, before placement in the rat maxilla, the respective binding energies of P2p and Ca2p were as follows: 133.46 ± 0.21 eV and 347.40 ± 0.25 eV, respectively (Fig. 5a, b). After placement in the rat maxilla, the respective binding energies of P2p and Ca2p were as follows: 133.36 ± 0.16 eV and 347.32 ± 0.14 eV after 14 days; 133.41 ± 0.21 eV and 347.31 ± 0.14 eV after 21 days; and 133.39 ± 0.18 eV and 347.29 ± 0.15 eV after 28 days. No significant differences were found among the P2p or Ca2p binding energies before and after placement in the rat maxilla for 14, 21, and 28 days (ANOVA: F = 0.480, P = 0.698 for P2p; F = 0.768, P = 0.520 for Ca2p).
Successful osseointegration requires the careful characterization of the thin HA layer that forms on the cpTi substrates by anodization and hydrothermal treatment. This study sought to characterize the morphological, chemical, and structural features of this layer during the process of bone formation in rat maxilla. In particular, our study evaluated the highly crystalline HA layers that were proposed to have a key role in osteoconduction during the initial stages of osseous wound healing. Our results indicated that the thin HA layer on the surface of SA-treated cpTi implants is likely to remain stable during the process of bone matrix mineralization and bone formation in the jaw.

Enhancing bone formation by altering the responses of bone tissue is the current goal of therapy using endosseous implants. The surface topography and physicochemical properties of the implants are important factors for successful bone formation during early wound healing and blood clotting. Previous works have demonstrated that the chemical modification of a topographically enhanced surface maintained the surface topography and associated advantages for contact osseointegration.1-4,21,22

The titanium implant surface contains a TiO$_2$ layer that contributes to its high compatibility with bone tissue, which is important for osseointegration. Moreover, the surface topography, micro-morphology, and physicochemical properties of the endosseous implant surface also affect cell proliferation and differentiation, as well as the production of extracellular matrix (ECM) proteins and mineralized bone.23,24 The cell-to-implant surface strength may be related to the ECM development and interaction on implant surfaces.23 In a previous in vitro study, the SA-treated cpTi surface provided a suitable surface area for the attachment of ECM proteins and cells.14,15,18 This was possibly attributable to the SA treatment enhancing the hydrophilic nature of the cpTi surface,15,18 in turn inducing changes in the surface physicochemistry due to environmental interactions and surface oxidation states. The HA crystals observed by SEM in the present study were a typical shape. In addition, the surrounding mineralized bone tissue and multilayered mature bone trabeculae were in direct contact with the HA layer, thus increasing osteoconductivity with the implant surface. These morphological observations were strikingly similar to those in previous studies of rat bone marrow stromal cells19 and dog mandibles.16 A comparison of the SA-treated cpTi implants with HA coatings on implants formed by plasma-spraying revealed excoriation and bioresorption of the plasma-sprayed HA, but not that formed by SA treatment. The plasma-sprayed HA also exhibited reduced bone-tissue compatibility and adhesive strength.12 These findings suggested the precipitation of numerous HA crystals on the surface of SA-treated cpTi implants, rendering the implant surface more stable in the bone tissue following implantation. Our EPMA data collected during bone matrix mineralization and bone formation in rat maxilla also indicated an even distribution of P and Ca density in the HA layer on the implant surfaces at all times after implantation, supporting both the present SEM observations and those made in a previous study.19 The hydrothermal-treated AOFCP surface induced the formation of a porous TiO$_2$ matrix and numerous HA microcrystals that

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**Fig. 6** XPS narrow-scan spectra of the SA-treated cpTi implants before and after placement in the rat maxilla for 14, 21, and 28 days. a. Ca2p$_{3/2}$ and Ca2p$_{1/2}$ spectra, b. P2p spectra.
markedly improved the surface wettability. These surface properties are potentially crucial for osteoconduction at the surface of SA-treated cpTi implants, and for inducing changes in the osteoblastic shape and gene expression profiles consistent with the process of osseointegration. 15,18

When an SA-treated cpTi implant is placed in bone tissue, both chemical and biological reactions occur at the bone/implant interface. While the long-term stability of an SA-treated cpTi implant is gained through the remodeling of bone, the initial stabilization probably depends on the early stages of bone wound healing, specifically at the time of implantation. During the initial stages of wound healing, a fibrin clot is formed at the endosseous implant and in hematopoietic cells, including peripheral monocytes that give rise to macrophages, populate the bone/implant interface. 18,26-29 The HA layer on the implant surfaces makes contact with a variety of ions, blood-cell proteins, serum proteins, bone morphogenetic proteins, and noncollagenous bone proteins that are present in the maxillary tissue fluid. The adsorption of these proteins is a function of the HA-layer surface energy. Furthermore, the physicochemical state of the surface may affect the adsorption of the ions and proteins that support cell attachment. 25 The fusion of globular calcified accretions on the HA surface crystals was apparent after implantation in the rat maxilla for 21 and 28 days. Despite this observation, XPS analysis indicated no significant difference in the binding energies of P and Ca in the HA crystals before and after placement for any duration tested. These phenomena may reflect the high crystallinity of the HA crystals precipitated by the SA treatment, thereby resulting in no overall change in the binding energies of the elemental constituents. This is despite contributing factors such as interactions between the HA layer on the surface of the SA-treated cpTi implant and ECM proteins, bone matrix mineralization, globular calcified accretions, or mineralized bone tissue. 30,31

Our present characterization of the thin HA layer formed by the anodic oxidation and hydrothermal treatment of cpTi indicated a highly crystalline HA providing enhanced osteoconductive properties to the endosseous implant surface. In addition, the HA layer remained stable during the process of bone matrix mineralization and bone formation in the jaw.

**Conclusion**

The thin HA layer formed on the surface of SA-treated cpTi implants by SA treatment remained stable during the process of bone matrix mineralization and bone formation in jaws. Furthermore, the highly crystalline HA produced by the SA treatment may play a key role in osteoconduction during the process of osseointegration.

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