Effect of carbon coating on the biocompatibility of titanium — in vitro cytotoxicity evaluation using human bone marrow cells —

Osamu Yamamoto*,❖ and Masayuki Fukuda**

The in vitro biocompatibility of a novel carbon-coated titanium implant (CTi), fabricated by heating a poly(vinyl) alcohol (PVA)-coated titanium disk at 700 °C in argon gas, was investigated. The obtained CTi possessed a surface layer of anatase-TiO$_2$ covered by amorphous carbon about 20 nm thick. In order to establish the effectiveness of the CTi as one of the implant materials, in vitro tests using human bone marrow-derived mesenchymal cells (hBMCs) were performed to check cytotoxicity, by examining cell proliferation, cell differentiation and mineralization capability. After 10 days of culture a higher degree of cell viability was observed on the surface of CTi. On the other hand, hBMCs cultured in direct contact with CTi continued to show alkaline phosphatase activity (ALP) and showed mineralization similar to the control cultures. These results indicated that the titanium coated with carbon possessed better biological response than that without carbon, which was demonstrated by the higher proliferation rates of osteoblasts, higher osteo differentiation and higher mineralization ability.

KEYWORDS: Titanium, Pyrolytic carbon, Coating, Biocompatibility, Biomaterials

1. Introduction

The titanium-based implants have been applied as clinical materials, which contribute to the improvement of quality of life (QOL) for the patients. The main aim of the current biomaterials available for dental and orthopedic applications is to induce rapid healing as well as controlled and guided bone growth around the implant leading to improved osseointegration. A strong interfacial layer with high mineralization potential able to assure the biomechanical stability of the implant is desired as well. The complicated osseointegration process involves numerous factors in which the key role is connected by chemistry on the surface of implant. The surface modification of implant by forming a bioactive layer affects the enhancement of the cell adhesion and proliferation. It results in the increase of the osseointegration.

Numerous surface treatment modalities such as chemical treatment (acid and alkali treatment), electrochemical treatment (anodic oxidation), sol-gel, chemical vapor deposition (CVD), thermal oxidation, etc. have been employed to improve the bonding strength between interfacial bone and implant. The thermal oxidation treatment is a low cost and simple process that can be readily applied to modify the external surface of metals without changing the core or the shape of the implants. Furthermore, the highly crystalline oxide layer (principally based on rutile-TiO$_2$) generated by thermal oxidation have lead to enhanced bone formation around dental implants after functional loading. On the other hand, the carbon materials, carbon nanotube and graphite-like amorphous carbon, have been studied on the cytotoxicity including the in vitro evaluation of cell proliferation, cell differentiation and mineralization, which indicated a good biocompatibility. The characteristic of the carbon suitable for the living body is extremely attractive. Therefore, we tried to coat the carbon film on the surface of titanium implant.

In our current investigation, a newly implant material based on titanium as a substrate has been fabricated by means of a especial thermal oxidation treatment, and the analyses of the chemical surface situation and the biomimetic characteristic on the fabricated material have been reported. The surface of the material has been modified, the top 20 nm consists of amorphous carbon layer, subsequently the upper 140 nm consists of anatase-TiO$_2$ layer. That is, the fabricated material is a carbon-coated titanium implant (CTi).

The aim of this study is to build upon our previous work. Given that osteoblasts are responsible for producing the mineralized extracellular matrix of bone, the assessment of the osteoblasts

❖ Corresponding Author, E-mail: yamamoto@cges.akita-u.ac.jp

* Center for Geo-Environmental Science, Graduate School of Engineering and Resource Science, Akita University : 1-1 Tegata Gakuen-machi, Akita 010-8502, Japan

** Division of Dentistry and Oral Surgery, Akita University Hospital : 1-1-1 Honda, Akita 010-8543, Japan

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response when the cells are in direct contact with the biomaterial is necessary. Therefore, we have focused in evaluating in vitro cell proliferation, osteogenic differentiation and mineralization capability of human bone marrow-derived mesenchymal cells (hBMCS) cultured directly on the surface of CTi.

2. Experimental

2.1 Fabrication of CTi disk

Commercial high purity titanium disk (Ti disk) with the size of \( \varnothing 10 \text{ mm and } 0.5 \text{ mm thick (Ti} > 99.9 \text{ %, Nilaco Co.)} \) was used as a starting substrate. Fig.1 illustrates the fabrication procedure of CTi. In a few words, the surface of the Ti disk was cleaned ultrasonically in pure acetone, ethanol and distilled water for 20 min followed by immersion into a 20 wt. % aqueous solution of poly (vinyl alcohol) (PVA#2000, average molecular weight \( M = 88 \text{ kg/mol, 72} \sim 82 \text{ mol} \text{ %, hydrolyzed, Kanto Chemical Co. Inc.)} \). The PVA-coated Ti disk was allowed to dry on a glass plate at 70 °C for 24 h. Subsequently, the disk was thermally treated at 700 °C for 1 h in an argon (purity : 99.999 %) atmosphere. The flow rate of argon gas was 50 cm³/min. Before use the disk was placed in sterile watch glass and was dry-heat sterilized at 180 °C for 2 h. CTi disk was thus obtained and was used in evaluating in vitro cell proliferation, osteogenic differentiation and mineralization capability. As-cleaned Ti disk was used in order to compare with CTi disk as well.

2.2 Surface characterization

The surface of the CTi and the cleaned Ti disks was examined before the analysis using a field-emission scanning electron microscopy (FE-SEM; HITACHI S-4500). The phases and the crystal structure of the surface in the fabricated disk were identified by X-ray diffraction (TF-XRD; RINT 2000, Rigaku Co.) using Cu Kα radiation at 40 kV and 20 mA power. X-ray photoelectron spectroscopy (XPS) analyses were conducted using Perkin-Elmer ESCA 5600 X-ray photoelectron spectrometer. X rays were produced by a monochromatic Al Kα source of 1486.6 eV. The thickness of the carbon film formed on the surface of CTi disk was measured by a 3D laser microscope (VK-9700G, KEYENCE).

2.3 In vitro biocompatibility assays

2.3.1 Cell culture

Healthy human bone marrow-derived mesenchymal cells (hBMCS) were isolated from bone marrow aspirates, after informed consent, from a newborn baby donor via density gradient centrifugation\(^{18}\). Tissue was used with the approval of Akita University Research Ethics Committee. The cells were expanded in minimum essential medium Eagle (α-MEM) medium (Sigma, M8042) supplemented with 10 % fetal bovine serum (FBS; CELLect GOLD, ICN Biomedicals Inc.) and 1 % penicillin/streptomycin (Gibco, Invitrogen Co.) at 37 °C in a humidified atmosphere of 5 % CO₂ in air and subcultured every 7 days. Medium was completely renewed every third day. Passages 4 ~ 9 were used in experiments. In all the experiments cells grown on the polystyrene cell culture dish (Costar, Cambridge, MA) in the absence of disks were used as a control.

2.3.2 Cell viability : MTT assay

Viability of the hBMCS was determined by means of MTT assay. Cells were seeded onto dry-heated sterilized Ti and CTi disks that perfectly fitted the bottom of the wells of a 48-well cell culture dish. Cells were seeded onto the disks at a density of 5 × 10³ cells per well and incubated for 1, 4, 7, and 10 days. Changes in the number of the viable cells on the substrates were quantitatively assessed by MTT-based colorimetric assay\(^{19}\). Thirty microliters of tetrazolium salt solution, 3-[4,5-dimethyl-2-thiazolyl]-2,5-diphenyltetrazolium bromide (MTT) (Dojindo Laboratories) (600 mg/l in cell culture medium), was added to each well. After incubation for 4 h at 37 °C, MTT solution in the medium was removed. The incorporated formazan crystals in the cells were solubilized with 300 μl of dimethylsulfoxide. The absorbance of each well was then read at 570 nm using a 96-well microplate reader (Multiskan JX; THERMO Electron Co.) equipped with Ascent version 2.6 microplate analysis software (Labsystems, Waltham, MA, USA). A standard curve of known viable cells numbers was used to calculate viable cell numbers for the different time points.

2.3.3 Alkaline Phosphatase Activity (ALP)

hBMCS were put on a 48-well cell culture dishes at a density of 5 × 10³ cells per well and cultured for 2, 5, 7, 14, 21 and 28 days. The medium was replaced on days 5, 8, 12, 15, 19, 22 and 26. After each culture time, the cell layers were washed twice with 300 μl of PBS. Substrate, p-nitrophenylphosphate disodium (Wako Pure Chemical Industries LabAssay™ ALP, 1 tablet) was dissolved in substrate buffer (0.1 mol/l Carbonate buffer, pH 9.8, 2.0 mmol/l MgCl₂) (5.0 ml), and 250 μl of this solution was added to each 48-well plate directly onto the monolayer. The plates were incubated at 37 °C for 20 min. The reaction was stopped by adding 200 μl of 0.05 mol/l NaOH. The amount of released p-nitrophenol (pNP) was measured at 405 nm in a 96-well microplate reader. Enzyme activity was calculated from calibration curves and was expressed.
as concentration of pNP in 20 min/10^6 cells. A minimum of two samples was assayed at each time point and values were normalized by the number of cells at each time point determined by the crystal violet assay20.

2.3.4 Mineralization assay

Mineralization was induced in confluent monolayers after 7 days of culture in ordinary medium. The cells were plated at a concentration of 5 × 10^3 cells per well in 48-well cell culture dishes. The primary ordinary medium was replenished after 4 days. After 7 days, the cells were provided with a secondary osteogenic medium consisting of α-MEM supplemented with 15 % fetal bovine serum (FBS; CELLect GOLD, ICN Biomedicals Inc.) and 1 % penicillin/streptomycin (Gibco, Invitrogen Co.), 10 mmol/l β-glycerophosphate (Sigma-Aldrich), 0.28 mmol/l ascorbic acid 2-phosphate (Wako Pure Chemical) and 100 mmol/l dexamethasone (Sigma-Aldrich) [18]. The osteogenic medium was renewed twice a week and the cultures were continued up to 28 days. Materials samples incubated in the absence of cells were used as a negative control.

At the end of the culture period, the monolayers in 48-well dishes were washed with PBS solution, and then were fixed for 15 min with 300 µl of 10 % cold neutral phosphate buffered formalin solution. The monolayers were then rinsed 3 times with distilled water prior to the addition of 300 µl of 40 mmol/l Alizarin red S (pH 4.1) solution. The plates were incubated at room temperature for 10 min and then washed well with distilled water. Nodules were visualized by using an inverted microscope for the control samples and a metallographic microscope for the metallic samples. The stain was solubilized for 30 min at room temperature with 0.5 mol/l HCl-5 % sodium dodecyl sulphate (SDS). Then, 100 µl of solubilized stain was moved to 96-well cell culture dish and the absorbance was measured at 405 nm using a microplate reader.

3. Results and Discussion

3.1 Characterization of the surface of CTi

FE-SEM micrographs of the surface of Ti and the CTi disks are shown in Fig.2, together with photographs of those disks. By heat-treating the PVA-coated Ti disk at 700°C, the color of Ti disk changed from silver to black. The surface morphology of the Ti disk was similar to the CTi disk, and the wounds at the production of original disk were observed in both disks. The thickness of the carbon film coated on the disk was found to be about 20 nm from the measurement of a 3D laser microscope.

Fig.3 shows the TF-XRD pattern of the fabricated CTi disk. The diffraction peaks corresponding to Ti and anatase-TiO₂ were detected on the surface of CTi disk. None of the diffraction peaks were detected for carbon, such as graphite. Thus, it can be inferred that the carbon residues generated due to the thermal decomposition of the PVA are amorphous in nature.

The XPS spectrum of the CTi disk is shown in Fig.4, with the elements Ti, O, C and Na being detected on the surface. As a trace element, Na was present on the surface of CTi disk but was the absence on the surface of Ti disk. The Na presence as a residual element in the CTi was presumed to be due to the origin from the sodium poly-alginate used in the polymerization of PVA with the hydrolysis of the homopolymer/co-polymer. A well-defined Ti 2p doublet with the typical interval of about 6 eV between the 2p_1/2 and 2p_3/2 signals was found, suggesting that the binding energy of about 459 eV for Ti 2p_3/2 is comparable with TiO₂ [21]. The C 1s signal appeared around 285 eV. According to several reports [22], [23], the C 1s signal around 285 eV is anticipated to arise from the residual presence of graphitic carbon or hybridized sp³ carbon.

3.2 In vitro biocompatibility evaluation

3.2.1 Cell viability : MTT assay

Fig.5 shows the results of the MTT proliferation assay. The
The number of metabolically active cells on the material surfaces was measured over different periods (1, 3, 5 and 10 days). The increase of the absorption can be judged as the enhancement of the osteoblasts proliferation. It was observed that although the osteoblasts proliferation resulted to be smaller on the surface of Ti and CTi disk, after 10 days, osteoblasts cultured on CTi disk showed significantly higher cell numbers than Ti disk.

Surface modification on Ti can alter the surface topography and chemistry, which directly affect the biological reaction to implants, that is, the interaction among implants and biological environments. In this work, the carbon layer with 20 nm thick was formed on the surface of Ti disk. The formation of the carbon layer seems to be suitable for implant applications, because none of the cytotoxicity effect to hBMCs was found in the carbon layer formed on the surface of Ti disk.

3.2.2 Alkaline Phosphatase Activity (ALP)

To examine the cell differentiation, the ALP was measured as a marker of osteoblasts differentiation. The expression of ALP is needed before matrix mineralization. It provides localized enrichment of inorganic phosphate, one of the components of the mineral phase of bone. Fig.6 shows the results of the ALP tests performed on the Ti and CTi disks. In this study, the ALP was expressed as the relation to the cell number, eliminating the effect of proliferation in this parameter. The cells cultured on the plastic cell culture dish (control) exhibited a maximum in the ALP activity after 7 days in culture. In the Ti and CTi disks, this maximum was observed at 5 days of culture and then significantly decreased on 7 days. In comparison with the control and the Ti disk, the fabricated CTi disk showed higher ALP activity during the first 5 days of culture. At 14 days, cells cultured on the CTi disk showed higher ALP activities than the Ti disk and the obtained values were very similar to the ALP activity of the control culture. ALP plays a crucial role in the initiation of matrix mineralization, and after that expression of this enzyme is down-regulated in the early stages of the mineralization.

This fact can explain the decline in the ALP activity observed during 7 and 14 days of culture.

3.2.3 Mineralization assay

In culture, hBMCs have the ability to form bone-like structures for this reason bone marrow cell cultures are recommended to screen biomaterials on their osteoinductive capacity. Bone nodules formation was observed by using Alizarin red staining. Alizarin red is a calcium-sensitive dye used to identify tissues highly rich in hydroxyapatite. Osteoblast cells developed red-stained calcified nodules after 28 days of culture in the presence of osteogenic medium. It was observed that the density and distribution of the calcified nodules varied according to the material surface. On the CTi disk, circular-shaped nodules were evenly distributed across the metallic surface in very similar feature to the control cultures. On the other hand, on the Ti disk the nodules were found in some areas and not in the totality of the surface.

Fig.7 shows the extent of calcium deposition of the Ti and CTi disks, after incubation in osteogenic cell culture medium for 28 days. The amount of calcium deposition was determined quantitatively by dissolving the nodules in a diluted chloridric acid solution and the absorbance was read at 405 nm. The results demonstrated that...
the CTi disk exhibited significantly elevated levels of mineral nodule formation, being similar to the control group. Compared with the Ti disk, CTi disk was found to promote the mineralization in the human osteoblasts culture. To confirm that the in vitro mineralization observed represents bone formation, the chemical composition of the mineral nodules formed on the surface of CTi disk was examined by EDX elemental analysis. The Ca/P ratio of the nodules was 1.32 close to the value of the Ca/P of the rabbit bone used in the in vivo experiments (1.58).

As mentioned above, specifically, the cells were able to proliferate, synthesize cellular matrix, express the enzyme alkaline phosphatase, which is related to bone differentiation and show mineralization toward calcium-phosphate material.

Compared with the Ti disk and culture dish, increased ALP activity was found on the CTi disk, indicating that the osteoblast differentiation process was not inhibited due to material toxic effects. On the contrary, CTi disk may contribute to osteogenic differentiation. The elevated ALP activity levels of the CTi disk indicate that higher differentiated number of cells was present and this results correlates well with the mineralization capability. The increased ALP activity found on the CTi disk resulted into more mineralized bone-like nodules on carbon-coated titanium, and the biocompatibility of titanium was improved.

4. Conclusions

The formation of carbon layer on titanium was performed to increase the cellular biocompatibility of titanium, and in vitro cytotoxicity of carbon-coated titanium was evaluated by using human bone marrow cells. According to the biological tests, the carbon layer of the external surface of titanium resulted to be non-cytotoxic. When human osteoblasts were cultured directly on carbon-coated titanium, cell proliferation and high cell numbers were obtained in carbon-coated titanium. The results showed high ALP activity and the presence of mineralized bone-like nodules on carbon-coated titanium, suggesting that the titanium coated with carbon did not impair the differentiation and mineralization process of the osteoblast cells. The carbon-coated titanium can be concluded to be a promising biomaterial capable to accelerate the bone formation and improve the osseointegration in cortical bone implants.