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Evaluation of Osteoblast-Like Cell Viability and Differentiation on the Gly-Arg-Gly-Asp-Ser Peptide Immobilized Titanium Dioxide Nanotube via Chemical Grafting

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This study examined the effect of the immobilization of the Gly-Arg-Gly-Asp-Ser (GRGDS) peptide on titanium dioxide (TiO_2) nanotube via chemical grafting on osteoblast-like cell (MG-63) viability and differentiation. The specimens were divided into two groups; TiO_2 nanotubes and GRGDS-immobilized TiO_2 nanotubes. The surface characteristics of GRGDS-immobilized TiO_2 nanotubes were observed by using X-ray photoelectron spectroscopy (XPS) and a field emission scanning electron microscope (FE-SEM). The morphology of cells on specimens was observed by FE-SEM after 2 hr and 24 hr. The level of cell viability was investigated via a tetrazolium (XTT) assay after 2 and 4 days. Alkaline phosphatase (ALP) activity was evaluated to measure the cell differentiation after 4 and 7 days. The presence of nitrogen up-regulation or C═O carbons confirmed that TiO_2 nanotubes were immobilized with GRGDS peptides. Cell adhesion was enhanced on the GRGDS-immobilized TiO_2 nanotubes compared to TiO_2 nanotubes. Furthermore, significantly increased cell spreading and proliferation were observed with the cells grown on GRGDS-immobilized TiO_2 nanotubes ($P < .05$). However, there was no significant difference in ALP activity between GRGDS-immobilized TiO_2 nanotubes and TiO_2 nanotubes. These results suggest that the GRGDS-immobilized TiO_2 nanotubes might be effective in improving the osseointegration of dental implants.

Keywords: Gly-Arg-Gly-Asp-Ser (GRGDS) Peptide, Titanium Dioxide Nanotube, Chemical Grafting.

1. INTRODUCTION

Even though titanium has shown favorable biocompatibility and proper mechanical characteristics, titanium implant surfaces react with oxygen in the air to form titanium oxide.[1]

In order to complement this limitation, "anodic oxidation" can be used to form a thin, rough, and porous oxide layer on titanium electrochemically.[2]

On the other hand, surface modification with bioactive molecules such as collagen, bone morphogenetic protein, transforming growth factor, and Arg-Gly-Asp (RGD) peptide have been suggested as alternatives to anodic-oxidation. The RGD peptide is a major sequence of amino acids of integrin, which aid in the attachment of cell and extra-cellular matrix proteins and promote cell adhesion when fixed to a surface.[3,4] Moreover, grafting of Gly-Arg-Gly-Asp-Ser (GRGDS) peptide was reported to be effective in osteoblast adhesion, proliferation, and differentiation.[5,6]

The objective of this study is to evaluate the effect of the immobilization of the GRGDS peptide on titanium dioxide (TiO_2) nanotube via chemical grafting on osteoblast-like cell viability and differentiation.

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2. EXPERIMENTAL DETAILS

2.1. Fabrication of Specimens

Commercially pure titanium discs (ASTM Grade II, Kobe Steel, Japan), 10 mm in diameter and 3 mm in thickness, were used. For the anodization process, the platinum was connected to an anode, and the specimen was connected to a cathode. Then, 20 V of power were applied a using DC power supply (Fine Power F-3005; SG EMD, Anyang, Korea) to 1 M H_2SO_4 and a 1.0 wt% HF-containing solution for 10 min.[7] Consequently, nanotubes of 100 nm size was created. Before the cell assay, all specimens were sterilized with ethylene oxide gas.

2.2. Immobilization of GRGDS Peptide

For the immobilization of GRGDS peptide on TiO_2 nanotubes, aminosilane linker was used.[1,8,9] For silanization, 2 ml of (3-aminopropyl)-triethoxysilane (APTES, Aldrich, China) with 38 ml anhydrous hexane was used to immerse TiO_2 nanotubes at room temperature for 2 hr (while stirring). After the reaction was finished, the specimens were washed twice with anhydrous hexane, ethanol, and deionized water. Then, the nanotubes were immersed in 36.3 ml of a *N*,*N*-dimethyl-formamide (DMF, Sigma, Germany) solution with 275 mg *N*-succinimidyl-3-maleimidoproprionate (Aldrich, Japan) for 1 hr at room temperature. After rinsing with DMF and deionized water, each specimen was immersed in 0.2 mg/ml GRGDS (Anaspec Inc., USA) dissolved in deionized water for 2 hr at room temperature. When the reaction was finished, the specimens were rinsed with deionized water sevearl times and dried for 24 hr. The specimens were divided into two groups in accordance with GRGDS peptide application;

Group 1: TiO_2 nanotubes (control), and
Group 2: GRGDS-TiO_2 nanotubes.

2.3. Surface Characterization

Surface property evaluation following peptide coating was observed using a field emission scanning electron microscope (FE-SEM, JSM-7500F, JEOL, Japan), and X-ray photoemission spectroscopy (XPS, MultiLab 2000, Thermo electron corporation, England).

2.4. Biological Activity

Human osteosarcoma (MG-63) cells were cultured with Dulbecco's modified Eagle's medium (DMEM, Gibco BRL, USA) containing 10% fetal bovine serum and 1% penicillin/streptomycin at 37 °C in a humidified atmosphere of 5% CO_2.

The morphology of the cells were observed using FE-SEM after fixation at 2 and 24 hr. After removing the culture medium from the specimens, they were rinsed with phosphate buffered saline (PBS). For fixation, they were placed at room temperature in 0.1 M PBS with 2.5% glutaraldehyde for 2 hr. Then, they were rinsed twice for 10 min each with PBS. For dehydration, they were immersed once in each of the following concentrations of ethanol: 40, 50, 60, 70, 80, 90% for 15 min, and three times in 100% ethanol for 10 min. After the fixation, the specimens were dried at 37 °C overnight. The cell attachment and morphology were observed using FE-SEM.

Cell proliferation was examined using a cell viability assay kit (EZ-Cytox, Daeil Lab Service Co., Korea). MG-63 cells were seeded at a density of 1×10^4 cell/well in 24-well plate, and the proliferation was calculated after 2 and 4 days of culture. The EZ-Cytox solution (40 μl) was added to each cell culture well, and the mixtures were incubated for 1 hr at 37 °C, in 5% CO_2. After that, 100 μl mixtures were measured using an ELISA reader (VERSA max, USA) at 450 nm.

Cell differentiation was evaluated an using alkalinephosphatase (ALP) activity assay (Biovision Research, USA). The ALP activity assay process was performed according to the manufacturer's instructions. MG-63 cells were plated at 1×10^4 cell/well in a 24-well plate, and measured after 4 and 7 days of culture. ALP activity was normalized with the protein concentration.

2.5. Statistics

All data were analyzed using independent *t*-tests ($p < 0.05$) with SPSS 20 software (SPSS Inc., USA).

3. RESULTS AND DISCUSSION

The TiO_2 nanotubes and the immobilization of GRGDS peptide on TiO_2 nanotubes were examined by FE-SEM (Fig. 1). The diameter of the nanotubes formed was about 100 nm (Fig. 1(a)). After GRGDS peptide immobilization, the multi-porous nanotubes showed a little clogging, but no difference in image (Fig. 1(b)).

XPS was used to observe the elemental content ratio of each specimen. Only the peptide-coated group with the chemical immobilization method demonstrated the elemental Si and S. With the presence of the Si element, we found that the silane compound remained stable. The carbon 1*s* spectrum contained the evidence of C–C peaks (~284.8 eV), C–O peaks (~ 286.3 eV), C═O, and N–C═O peaks (~288.3 eV), possibly from the GRGDS-TiO_2 nanotubes (Fig. 2). The GRGDS peptide

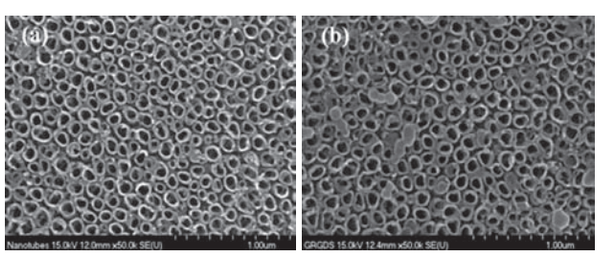

Figure 1. FE-SEM images of (×50, 000) (a) TiO_2 nanotubes, (b) GRGDS-TiO_2 nanotubes.

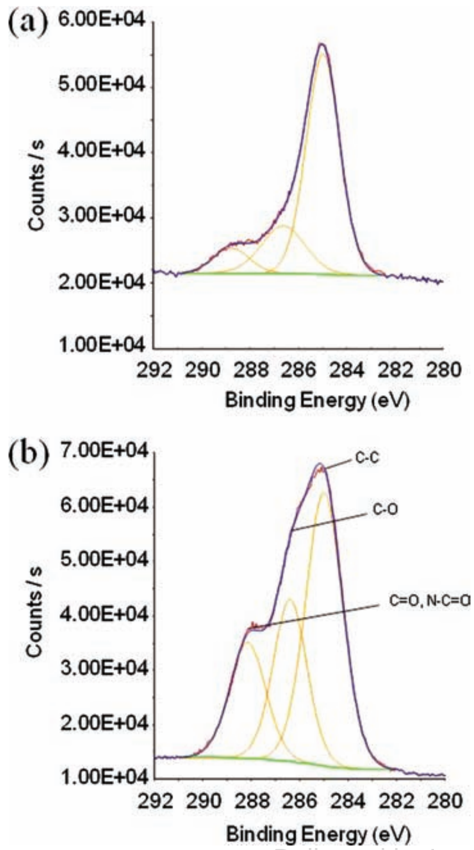

Figure 2. XPS C1*s* spectrum of (a) TiO_2 nanotube, (b) GRGDS-TiO_2 nanotube.

coating on TiO_2 nanotubes surface was confirmed by the increase of nitrogen and detection of C═O carbons. Similar results had been previously demonstrated by other experiments.[10–12]

After 2 hr, the cell morphology demonstrated was circular (Figs. 3(a) and (b)). After 24 hr, osteoblast-like cells cultured on the TiO_2 nanotubes surfaces showed polygonal morphology (Fig. 3(c)). However, the osteoblast-like cells

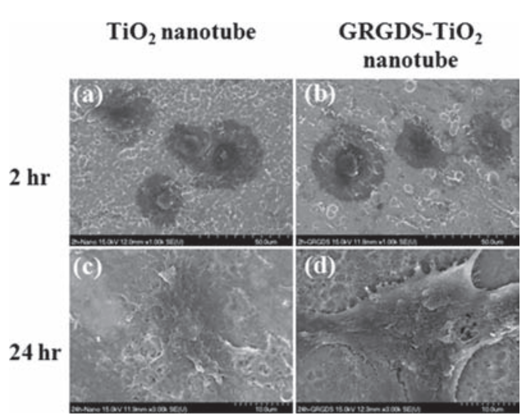

Figure 3. SEM images of osteoblast-like cells cultured after 2 hr (a), (b) (×1000) and 24 hr (c), (d) (×3000) on different surfaces.

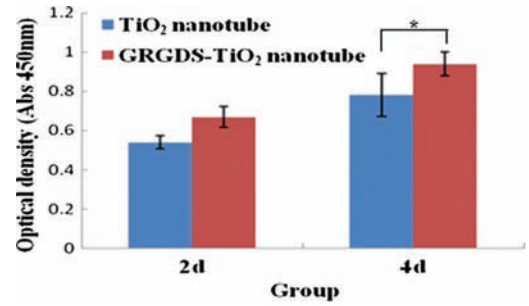

Figure 4. Cell proliferation of osteoblast-like cell on different titanium surfaces.
Note: *Significant difference ($p < .05$).

cultured on the GRGDS-TiO_2 nanotubes surfaces exhibited more firmness and filopodia (Fig. 3(d)).

The cell proliferation assay was carried out after 2 and 4 days of cell culture. There was no significant difference between two groups after 2 days. However, after 4 days, the cell viability of the GRGDS-TiO_2 nanotubes group was significantly higher than that of the TiO_2 nanotubes group (Fig. 4). Lee et al., reported that the GRGDS grafted chitosan cell adhesion has no significant difference after 7 days.[6] However, this study showed a significant difference after 4 days, which may suggest that GRGDS produces a positive effect on cell proliferation in an early phase. Figure 5 shows the ALP activity after 4 and 7 days of cell culture in each group. Even though the ALP activity of the GRGDS-TiO_2 nanotubes group was higher than that of the TiO_2 nanotubes group, there was no significant difference between the groups after 4 or 7 days.

Kim et al. reported that ALP activity was increased in RGD peptide-coated titanium sample after 14 days.[13] In this study, ALP activity was evaluated after 4 and 7 days, which is shorter than the previous study. Furthermore, Kilpadi et al. reported that RGD-containing peptide increased ALP activity in a late phase.[14] Therefore, in order to overcome the limitations of this study, further studies are required to evaluate the effect of the immobilization of the GRGDS peptide on TiO_2 nanotubes via chemical grafting on osteoblast-like cell differentiation during the late phase.

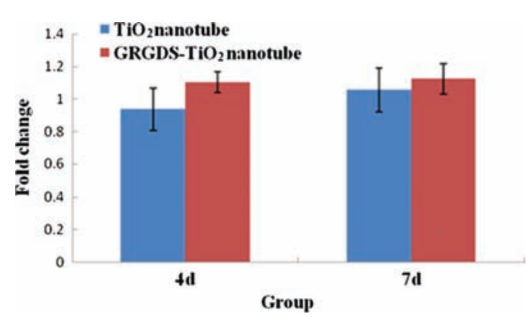

Figure 5. ALP activity of osteoblast-like cell on different titanium surfaces.

4. CONCLUSION

GRGDS-immobilized TiO_2 nanotubes might be effective in improving the osseointegration of dental implants.

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