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Hydrocarbon Deposition Attenuates Osteoblast Activity on Titanium

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APPENDIX

MATERIALS & METHODS

Titanium Samples

Commercially pure grade 2 titanium disks were mirror-polished to obtain identical surface topography (8 mm φ × 1.0 mm thickness; Rare Metallic Co. Ltd., Tokyo, Japan). Then, titanium samples were ultrasonically rinsed in acetone for 30 min and rinsed in double-distilled water for 10 min. These samples were kept in an auto-dry desiccator. The carbon concentration on the titanium surface was experimentally regulated. First, the titanium surfaces were varnished with machine oil, and the titanium disks were then soaked in acetone for 3 different durations (60, 15, and 5 min) to control the C/Ti ratio, which is the ratio of the integrated intensity of the C1s electron peak to that of the Ti2p electron peak. Finally, 3 different models were prepared for this study, with C/Ti ratios of 0.3, 0.7, and 1.0. These C/Ti values were confirmed by x-ray photoelectron spectroscopy (XPS: JEOL Ltd., Tokyo, Japan) analysis. C/Ti values were calculated from the integral values of the output waveform with the use of Casa XPS software (Casa XPS; Casa Software Ltd., Teignmouth, UK). The surface wettability of each sample was evaluated according to the contact angle of 10 μL of H_2O by means of an automatic contact angle measuring device (DM-301; Kyowa Interface Science, Saitama, Japan).

Cytotoxicity Test (cell viability test)

The specific components of the machine oil used in this study were unknown, so we tested cellular viability for the osteoblasts on the oil-varnished glass plates compared with the cells on the polystyrene plates to evaluate whether cytotoxic molecules were contained in the machine oil. Both the attached cells on the substrates and the floating cells in the culture medium were collected for the measurement after 24 and 72 hr of culture. The quantification of viable cells was performed with a cell-counting reagent and water-soluble tetrazolium salt, WST-8 (Dojindo Molecular Technologies, Inc., Rockville, MD, USA) which is reduced by dehydrogenase activities in cells to give a formazan dye.

Protein Adsorption Assay

Bovine serum albumin (Thermo Scientific, Rockford, IL, USA) was used as the model protein; a 200-μL quantity of protein solution (1 mg/mL protein/saline) was pipetted onto and spread over each titanium disk. After 3 hr of incubation at 37°C, non-adherent protein was removed, and the initial entire solution was mixed with working reagent (Thermo Scientific) at 37°C for 60 min. The amount of protein was measured with a microplate reader (MICROTEC Co. Ltd., Chiba, Japan) at an absorbance of 562 nm.

Osteoblastic Cell Culture

MC3T3-E1 cells (RIKEN BioResouce Center, Ibaraki, Japan) were grown in α-modified medium (Wako Pure Chemical Industries, Ltd., Osaka, Japan) supplemented with 10% fetal bovine serum (JRH Bioscience, Inc., Shawnee Mission, KS, USA) and 100 $U \cdot mL^{-1}$ penicillin-streptomycin. The cells were incubated at 37°C in a fully humidified atmosphere of 95% air and 5% CO_2. Sample titanium specimens were placed on non-treated polystyrene dishes (AGC TECHNO GLASS Co. Ltd., Tokyo, Japan). For induction of bone differentiation, the cells were seeded on the titanium specimens and cultured to 80% confluence in the culture medium, which was α-MEM supplemented with 10% fetal bovine serum (FBS), 2 $mmol \cdot L^{-1}$ β-glycerophosphate (Wako Pure Chemical Industries), and 50 $\mu g \cdot mL^{-1}$ L-ascorbic acid (Wako). The medium was changed every 3 days.

Cell Attachment Assay

We evaluated the initial attachment of osteoblastic cells by measuring the quantity of cells attached to titanium substrates after 6 and 24 hr of incubation. The quantification was performed by

colorimetry with water-soluble tetrazolium salt, WST-8, which is reduced by dehydrogenase activities in cells to give a formazan dye. The cultures were incubated at 37°C for 2 hr with 10 µL of WST-8 reagent for 100 µL of culture medium. The amount of formazan product was measured in a microplate reader at 450 nm (Microtec). Further, after 6 hr, the cultured cells were stained with calcein for observation by fluorescent microscopy (IX71; Olympus Corporation, Tokyo, Japan) to confirm the cell density results. After 6 hr of incubation, the medium was removed and stained with calcein (Dojindo). The cells were photographed by fluorescent microscopy (Olympus).

Cell Morphology and Morphometry

A fluorescent microscope (Olympus) was used to examine the cell morphology and cytoskeletal arrangement of osteoblasts seeded onto the titanium samples. After 12 hr of culture, osteoblastic cells were fixed in 10% formalin and stained for actin with rhodamine-conjugated phalloidin (Cytoskeleton, Inc., Denver, CO, USA). Cell area, perimeter, and Feret's diameter were quantitatively assessed with an image analyzer (ImageJ, NIH, Bethesda, MD, USA).

Vinculin Expression Analysis

The expression and localization of vinculin were analyzed microscopically. In the above-mentioned fluorescent microscopic analysis, cultures were also immunochemically stained with a mouse anti-vinculin monoclonal antibody (Cytoskeleton), followed by a fluorescein isothiocyanate-conjugated anti-mouse secondary antibody (Cytoskeleton). Vinculin expression was quantified in terms of pixel-based density by means of an image analyzer (ImageJ).

Alkaline Phosphatase (ALP) Activity

The ALP activity of cultured osteoblasts was examined at day 10 by means of a colorimetry-based assay. The cultures were rinsed with double-distilled water, supplemented with 250 µL *p*-nitrophenyl phosphate (Wako Pure Chemical Industries), and then incubated at 37°C for 15 min. The ALP activity was evaluated as the amount of nitrophenol released through the enzymatic reaction and measured at 405 nm in a microplate reader (Microtec).

Mineralization Assay

The mineralization capability of cultured osteoblastic cells was examined by colorimetry-based quantification of calcium deposition at day 20. Cultures were washed with phosphate buffered saline (PBS) and incubated overnight in 200 µL of 0.5 M HCl with gentle shaking. The solution was mixed with *o*-cresolphthalein complexone in an alkaline medium (Cayman Chemical Company, Ann Arbor, MI, USA) to produce a calcium-cresolphthalein complexone complex. The color intensity was measured at 575 nm in a microplate reader (Microtec).

Statistical Analysis

Five samples were used in each analysis (n = 5). This study used one-way analysis of variance (ANOVA) and Tukey's test with significance defined as $p < .05$ to examine differences in variables among the surfaces with different C/Ti ratios.