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Bioadaptive Nanorod Topography of Titanium Surface to Control Cell Behaviors and Osteogenic Differentiation of Preosteoblast Cells

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Titanium (Ti) nanorods fabricated using selective corrosion of Ti substrate by anodic technology show better biocompatibility with pre-osteoblast cells. The current study investigated the response of the murine pre-osteoblast cell MC3T3-E1 on Ti nanorod topography and untreated Ti surfaces by means of examination of the morphology and osteogenic differentiation responsible for the pre-osteoblast reaction. The morphology of MC3T3-E1 cells was observed using scanning electron microscopy, and alkaline phosphatase (ALP) activity was measured using a colorimetric assay after incubation for 7, 14, and 21 days. The expression of three osteogenic differentiation markers including ALP, osteocalcin (OCN), and collagen type 1A1 (COL1A1) and two transcription factors including runt related transcription factor 2 (Runx2) and osterix (Osx) at different time points was detected using real-time polymerase chain reaction analysis in both groups. Osx was used to confirm the protein level. The results showed that Ti nanorod surfaces provided prolonged higher levels of ALP activity compared with unmodified Ti surface on the 14th and 21st days. Gene expression analysis of ALP, OCN, and COL1A1 showed significant upregulation with modified nanorod topography after incubation for 14 and 21 days. Osteogenic transcription factors of Runx2 and Osx exhibited changes consistent with the osteogenic differentiation markers, and this may contribute to the persistently active differentiation of MC3T3-E1 cells in the Ti nanorod group. These results demonstrated that the current nanostructured surface may be considered bioadaptive topography to control cellular behaviors and osteoblast differentiation. The *in vivo* performance and applicability are further required to investigate osseointegration between implant and host bone in the early stages for prevention of aseptic implant loosening.

1. Introduction

Orthopedic and dental implants, which have greatly improved the function and quality of life of affected patients, are becoming increasingly popular due to the rapidly aging population[1]. However, aseptic loosening resulting from poor osseointegration is one of main causes of implant failure, making re-treatment inevitable[2]. Since most patients undergoing orthopedic or dental implant treatment suffered from age-related, postmenopausal, or other forms of secondary osteoporosis (due to systemic diseases or pharmacological), the potential for proliferation and differentiation of pre-osteoblast cells is relatively limited. Thus, there is a clinical need to ameliorate the bone–implant interfaces to stimulate pre-osteoblast cell differentiation and facilitate better osseointegration. Surface modifications such as sandblasting and acid etching are often utilized on titanium (Ti) implant surfaces to increase their biological performance by improving wettability, cell-implant adhesion and attachment, cell proliferation, and osseointegration[3–5].

The emergence of nanotechnology has demonstrated the extraordinary efficacy of nanoscale structures in improving the activity and fate of biological systems[6]. Nanoscale surface modifications of Ti-based metal implants have greatly improved osseointegration

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between the material surface and host tissue because bone itself has a structural hierarchy at the first level in the nanometer range[7–9]. Among various nanostructures studied, titanium nanotube arrays have shown promise for clinical application because of controllable dimensions, mechanical/chemical stability, good biocompatibility, and improved bone-forming functionality on implant by self-ordering anodization on a Ti surface[10–12]. Ti nanorods, another novel one dimensional nanostructure fabricated by selective corrosion of Ti substrate using electrochemical anodization technique, have generated increasing interest in recent years because of simple and easy-going anodization method. Because of the monolithic structure between the nanorod and underlying Ti substrate, the nanorod film could integrate strongly with the Ti substrate and avoid the peeling-off behavior commonly observed in TiO_2 nanotubes[13]. In a previous study[14–16], Ti nanorods of different lengths were fabricated by applying a constant current of 200 mA under different anodization conditions. Lin et al.[17] compared the murine mesenchymal stem cell response to nanorods with different lengths, and concluded that Ti nanorods of 100-nm length showed better biocompatibility in promoting MSCs adhesion and elicited higher level of alkaline phosphatase (ALP) activity, suggesting greater bone-forming ability than controlling Ti and Ti nanorods of 30 nm and 120 nm. Thus in the current study, we investigate pre-osteoblast cell response to Ti nanorods of 100-nm length in long-term culture and attempt to understand the gene expression underlying this response.

2. Experimental

2.1. Preparation of Ti foil and Ti nanorods

The surface treatment of Ti foils and fabrication of nanorods was carried out by Professor Ning and has been described in other literature[13,14]. Briefly, Ti foils (1 mm thick, 3 cm × 3 cm; conforming to the standard ASTM F67-2002 for biomedical application; obtained from Baoji Qichen New Material Technology Co. Ltd) were degreased by sonicating in acetone and ethanol, treated with 1:1 (v/v) HF and HNO_3 solution, rinsed with deionized water, and air dried. Electrochemical anodization was conducted in a two-electrode configuration where the Ti and Cu foils served as the working and counter electrodes, respectively. A mixture of 1.45 wt% NH_4F and 1.93 wt% $H_2C_2O_4$ was used as the electrolyte. Prior to electrochemical treatment, the Ti foil was placed in the electrolyte for 10 min. Anodization was performed by applying a constant current of 200 mA for 80–125 min at room temperature via a DC power supply. The resulting specimens were then washed with deionized water.

2.2. Surface characterization

Field emission scanning electron microscopy (FESEM, Nova Nano SEM 430, Germany) was used to characterize the morphology of the untreated Ti surface and the Ti nanorod surface.

2.3. MC3T3-E1 cell culture and amplification

Murine bone pre-osteoblast MC3T3-E1, purchased from ATCC, was cultured in low-glucose DMEM containing 10% FBS in an incubator at 37 °C with 5% CO_2 under saturated humidity conditions. The culture medium was replaced routinely. Cells were passaged according to 1:6 after reaching 90% confluence. Osteogenic differentiation of MC3T3-E1 was induced using an osteogenic medium (culturing medium supplemented with 10 mM Na-β-glycerophosphate (Sigma), 10^{-8} M dexamethasone (Sigma), and 50 μg/mL ascorbic acid (Sigma)). Square samples of control Ti and Ti nanorods (10 mm in length and 1 mm in thickness) were placed at the bottom of each well of a 24-well plate, and 500 μL cell suspensions were seeded at a density of 1×10^4 cells/mL.

2.4. Morphological observation

The untreated control Ti plate and Ti nanorod plates were placed at the bottom of the 24-well plate; cells at a density of 1×10^4 cells/mL were seeded and cultured for 24 h; and the plates were then removed and fixed with 3% glutaraldehyde for 6 h. After rinsing with PBS and dehydrating sequentially in a series of ethanol (50%, 70%, 85%, and 100%), the cells were observed by SEM.

2.5. Immunofluorescence staining of cytoskeletal actin

MC3T3-E1 cells were seeded at a concentration of 2×10^4/mL on the control Ti and Ti nanorod surfaces. After incubating for 24 h, the cells were fixed in 4% paraformaldehyde for 30 min at 4 °C. Thereafter, the cells were washed and permeabilized with 0.1% Triton X-100 for 10 min. They were then washed and incubated for 1 h at room temperature in a blocking solution (1% BSA/1× PBS). TRITC-conjugated phalloidin (Sigma) was added after washing the cells, incubated for 1 h at room temperature, and then washed off from the cells using PBS three times. DAPI (Sigma) was added and incubated for 30 min at 37 °C. The cells were washed three times with PBS, transferred to glass slides, visualized, and photographed using a green (actin) and blue (DAPI) filter by fluorescence (Olympus 1X2-ILL 100).

2.6. Detection of alkaline phosphatase activity

One milliliter of cell suspension was seeded in the 24-well plates at a density of 1×10^4 cells/mL, and the samples were transferred to new plates after 7, 14, and 21 days of culture in the osteogenic medium. The cells were washed three times with PBS and lysed in 0.2 vol.% Triton X-100 for 12 h at 4 °C. ALP activities were determined using a colorimetric assay with an ALP reagent containing *p*-nitrophenyl phosphate (Amresco) as the substrate. The absorbance of the *p*-nitrophenol formed was measured at a wavelength of 405 nm. The intracellular total protein content was determined using the BCA protein assay kit (Pierce). OD values of ALP activity were finally normalized to the total protein content with corresponding OD values.

2.7. Real-time polymerase chain reaction analysis

MC3T3-E1 cells cultured on the control Ti and Ti nanorods on days 7, 14, and 21 were digested and collected. Total RNA was extracted from these cells using TRIZOL (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Reverse transcription was performed at 42 °C for 50 min using the SuperScript First-Strand Synthesis Kit (TOYOBO; Biotech Co. Ltd., Shanghai, China). ALP, runt related transcription factor 2 (Runx2), osteocalcin (OCN), collagen type 1A1 (COL1A1), osterix (Osx), and GAPDH gene expression was quantified by real-time polymerase chain reaction (PCR) using the ABI 7500 Sequence Detection System. Amplification was conducted for all genes using SYBR Green Master Mix (Applied Biosystems, Foster City, CA). Primer sequences were designed with Primer5 (Table 1). The PCR conditions were as follows: 94 °C for 30 s (initial step); 39 cycles at 94 °C for 5 s and the appropriate annealing temperature for 30 s; and extension in the last cycle for 5 s. Cycle threshold (CT) values were defined as the cycle in which fluorescence intensity reached the geometric phase of amplification. Samples were performed in triplicate, and CT values were averaged. Values for individual genes were then normalized to the value of the housekeeping gene GAPDH, allowing calculation of fold