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Surface modification of titanium plate enhanced fibronectin-mediated adhesion and proliferation of MG-63 cells

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Abstract: An understanding of osteoblast adhesion and proliferation on biomaterials is crucial to optimizing the surfaces of artificial implants used in clinical practice. Polished, anodic oxidation (AO) and micro-arc oxidation (MAO) treated titanium (Ti) plates were used as model surfaces to study the adhesion of MG-63 cells. Cells were monitored for 0.5 and 4 h; faster adhesion and spreading of MG-63 cells were observed on the AO and MAO modified samples. Stimulated secretion of fibronectin (FN) influenced the adhesion rates. In addition, AO and MAO modified surfaces promoted cell proliferation through apparent up-regulation of FN and integrin α_5 transcription via outside-in signaling. This strongly suggests that FN secretion by osteoblasts plays an essential role in enhanced cell adhesion, spreading and proliferation on these modified Ti surfaces.
Key words: titanium; surface modification; Outside-in signaling; fibronectin; integrin

1 Introduction

Titanium is an ideal choice for the long-term replacement of hard tissue because of its excellent mechanical properties, corrosion resistance and biocompatibility. Enhancement of titanium bioactivity via surface modification has been reported for particular clinical applications [1,2]. Frequently used methods are anodic oxidation (AO) and micro-arc oxidation (MAO) [3,4]. Anodic oxidation produces vertically aligned TiO_2 nanotubes on a titanium substrate, and micro-arc oxidation can provide titanium with a microporous calcium phosphate coating. Both modifications have been well tested in vivo and in vitro [5−7], and have exhibited benefits for osteoblast growth and a potential for future clinical application.

For the successful clinical use of biomaterial implants, cell/surface interactions are considered to be a key determinant [8]. Research is focused on establishing the relevant surface characteristics of biomaterials, such as chemical composition [9,10] and topography [11,12], to unravel the processes whereby biomaterials affect cell behavior. However, because of the complex and varied surface properties of biomaterials, and the application of different cell culture conditions, it is difficult to arrive at a general conclusion on how biomaterials regulate cell behavior [13]. Our mechanistic understanding of cell alteration on biomaterial surfaces remains incomplete [14]. The relationship between osteoblast behavior and titanium surface modification is the focus of study here, in order to provide further insight into cell/biomaterial interface. Moreover, the system has clinical relevance for hard tissue replacement.

Outside-in signaling is one of the most important processes mediating the interaction of cells and biomaterials. The cell, typically, uses integrins to transduce information from the extracellular matrix (ECM) to the cell interior [13,15], but such outside-in signaling also mediates the response to biomaterial surfaces [16,17]. This signaling exerts significant influence on cell adhesion and proliferation, and if we can fully understand this signaling function, we can also better control cell behavior through improved design biomaterials. An outside-in signaling study of the interaction of osteoblasts with AO or MAO modified

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titanium implants has not yet been reported.

The specific objective of this study is to understand the mechanism of outside-in signaling at osteoblasts cultured on AO or MAO modified titanium. Polished, AO and MAO treated Ti plates were used as the biomaterial models, and MG-63 cells, a human osteosarcoma cell line were used as the osteoblast model. Protein expression and gene transcription of fibronectin (FN) and collagen I (COLI), respectively, were observed by Western blot and RT-PCR assay. RT-PCR assay was also employed to examine the FN and COLI-related integrin transcription.

2 Experimental

2.1 Biomaterial preparation

Commercially available titanium plates (>99.9% purity, ASTM GR.1, Baoji INT Titanium Material Co., Ltd., China) of 2 mm×10 mm×10 mm were chemically polished and used for AO or MAO treatment. Scratches or blemishes on the plates were first removed using 400-grits SiC polishing paper, and chemical polishing was then carried out by using acid etch solution comprising HNO_3 (65%−68% in mass fraction) and HF (40%) in volume ratio of 1:1. The samples were then ultrasonically cleaned using double distilled water (ddH_2O) and ethanol (99.7%), respectively. Finally, the samples were dried at room temperature.

AO treatment of the titanium plates was performed in electrolyte solution containing NaF (0.138 mol/L) and H_3PO_4 (0.5 mol/L) according to a reported procedure [18]. A constant voltage of 10 V was also applied to the titanium plates for 20 min at 40 °C. All prepared samples were then ultrasonically cleaned with ddH_2O and dried at room temperature.

MAO treatment of plates (prepared by Shanghai Institute of Ceramics, Chinese Academy of Sciences) was carried out in electrolyte solution comprising $Ca(CH_3COO)_2$ (0.1 mol/L) and $Na_2C_3H_5(OH)_2PO_4$ (0.1 mol/L) at 10 °C [19,20]. During this treatment, an AC voltage was applied for 4 min at 800 Hz and current density of 0.15 A/cm^2. The MAO treated samples were cleaned by the same method as for AO treatment.

2.2 Surface analysis

The surface morphology of the specimens was observed using a field emission scanning electron microscope (FESEM, JSM6700F, JEOL, Japan).

2.3 Cell culture

A human osteoblast-like MG-63 cell line (purchased from the Center of Cell Resource, Shanghai Institutes for Biological Science, China) was used to evaluate the biological response to the samples. Cells were cultured in RPMI 1640 medium supplemented with 10% (v/v) fetal bovine serum and 1% (v/v) antibiotic (100 U/mL penicillin, and 100 g/mL streptomycin) at 37 °C in a humidified incubator (BB15, Thermo Scientific, USA) with an atmosphere of 5% CO_2. All reagents were from Thermo Scientific, USA.

2.4 Cell morphology

Titanium samples were collected and washed twice with PBS prior to their use for MG-63 cell culture. Culture periods of 0.5 h and 4 h were used. For imaging, samples were pre-fixed with 3% glutaraldehyde for 30 min, and then dehydration gradients of ethanol and of hexamethyldisilazane were applied. The dehydrated specimens were observed by a field emission scanning electron microscope (FESEM, JSM6700F, JEOL, Japan).

2.5 AlamarBlue assay

AlamarBlue (Invitrogen, USA) assay of cell viability was carried out according to the protocol described previously [9].

2.6 Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was extracted from MG-63 cells which had been cultured on polished Ti, AO and MAO treated Ti for either 4 h or 5 d using TRIzol reagent (Invitrogen, USA). The RNA was precipitated with isopropanol, washed with 75% ethanol and dissolved in RNase-free water.

Following DNase I (Fermentas, USA) treatment, the extracted RNA was used to produce cDNA using a reverse transcription kit (Fermentas, USA). PCR reactions were undertaken using a thermal cycle system (2720 Thermal Cycler, Applied Biosystems, USA) with the following programs: a denaturation step for 2 min at 94 °C followed by a sequence of 25−30 cycles at 94 °C for 30 s, 55−57 °C for 30 s and 72 °C for 60 s. The PCR products were detected using a gel image analysis system (2600R GIS, Tanon, China). Typically, five repeats were performed for every sample. The primers used in this study are listed in Table 1.

2.7 Western blotting assay

MG-63 cells were collected after they had been incubated with samples for 4 h and 5 d, respectively, and were washed once with PBS and once with washing buffer (10 mmol/L tris (pH 8.0), 150 mmol/L NaCl, 1 mmol/L EDTA (pH 8.0)). The cells were then lysed in 200 μL of lysis buffer which contained 20 mmol/L tris-HCl (pH 8.0), 30 mmol/L $MgCl_2$, 2 mmol/L ethyleneglycol tetraacetic acid (EGTA), 10% glycerol, 1% CHAPS, 1.2 μL of 10% β-mercaptoethanol and 100 mmol/L phenylmethanesulfonyl fluoride (PMSF).