Available online at www.sciencedirect.com
ScienceDirect
Acta Biomaterialia 4 (2008) 1985–1995
Acta BIOMATERIALIA
www.elsevier.com/locate/actabiomat

Osteoblast response to biomimetically altered titanium surfaces

J. Barbara Nebe [a,*], Lenka Müller [b], Frank Lüthen [a], Andrea Ewald [c], Claudia Bergemann [a], Egle Conforto [d], Frank A. Müller [b,e]

[a] University of Rostock, Department of Internal Medicine, Biomedical Research Centre, Cell Biology, Schillingallee 69, Biomedicum, D-18057 Rostock, Germany
[b] University of Erlangen-Nürnberg, Department of Materials Science – Biomaterials, Henkestr. 91, 91052 Erlangen, Germany
[c] University of Würzburg, Department for Functional Materials in Medicine and Dentistry, Pleicherwall 2, D-97070 Würzburg, Germany
[d] University of La Rochelle, Centre Commun d'Analyses, Allee de l'ocean 5, F-17071 La Rochelle Cedex 9, France
[e] Friedrich-Schiller-University of Jena, Institute of Materials Science and Technology (IMT), Loebdergraben 32, D-07743 Jena, Germany

Received 27 September 2007; received in revised form 16 April 2008; accepted 9 May 2008
Available online 11 June 2008

Abstract

Bioinert titanium (Ti) materials are generally encapsulated by fibrous tissue after implantation into the living body. To improve the bone-bonding ability of Ti implants, we activated commercially pure titanium (cpTi) by a simple chemical pre-treatment in HCl and NaOH. Subsequently, we exposed the treated samples to simulated body fluid (SBF) for 2 (TiCT) and 14 days (TiHCA), respectively, to mimic the early stages of bone bonding and to investigate the in vitro response of osteoblasts on thus altered biomimetic surfaces. Sample surfaces were characterized by scanning electron microscopy, energy-dispersive X-ray analysis, cross-sectional transmission electron microscopy analyses, Fourier transform infrared and Raman spectroscopy. It was shown that the efflorescence consisting of sodium titanate that is present on pre-treated cpTi surfaces transformed to calcium titanate after 2 days in SBF. After 14 days in SBF a homogeneous biomimetic apatite layer precipitated. Human osteoblasts (MG-63) revealed a well spread morphology on both functionalized Ti surfaces. On TiCT, the gene expression of the differentiation proteins alkaline phosphatase (ALP) and bone sialo protein was increased after 2 days. On both TiCT and TiHCA, the collagen I and ALP expression on the protein level was enhanced at 7 and 14 days. The TiCT and the TiHCA surfaces reveal the tendency to increase the differentiated cell function of MG-63 osteoblasts. Thus, chemical pre-treatment of titanium seems to be a promising method to generate osteoconductive surfaces.

Keywords: Titanium; Simulated body fluid; Biomimetic apatite; Osteoblast adhesion; Gene expression

1. Introduction

Bone-interfacing implants require a bioactive surface with osteoconductive properties to avoid the formation of fibrous tissue. Several surface modifications, including physico-chemical [1], morphologic [2] and biochemical ones [3], have been investigated to overcome the problems of bone integration by creating osteoconductive as well as osteoinductive surfaces. It is known that a limited number of bioactive ceramics can bond to living bone without the formation of fibrous tissue. These materials create a hydroxycarbonated apatite (HCA; $Ca_{10-x}(HPO_4)_{x-y}(CO_3)_y(PO_4)_{6-x}(OH)_{2-x}$) layer on their surface after implantation [4,5]. HCA layers can be reproduced on the surface of bioactive ceramics even in an acellular protein-free simulated body fluid (SBF) with ion concentrations nearly equal to the inorganic part of human blood plasma [6]. Thus, in vitro HCA formation is believed to be the main requirement for bone-bonding ability of materials according to our understanding of bioactive behaviour [7,8]. In recent years various methods have been developed to prepare SBF solutions for in vitro bioactivity tests [9–13].

Titanium and its alloys are materials that are widely used for bone implants under biomechanical loading conditions due to their excellent mechanical properties and

* Corresponding author. Tel.: +49 381 494 7771; fax: +49 381 494 7778.
E-mail address: barbara.nebe@med.uni-rostock.de (J.B. Nebe).

1742-7061/$ - see front matter © 2008 Acta Materialia Inc. Published by Elsevier Ltd. All rights reserved.
doi:10.1016/j.actbio.2008.05.028

biocompatibility. Nevertheless, bioinert titanium materials are generally encapsulated by fibrous tissue after implantation into the living body [14,15]. Chemical methods to provide titanium with bioactive surface characteristics include soaking in NaOH followed by heat treatment [16] or acid etching in HCl and subsequent NaOH treatment [17]. Both methods induce the growth of a bioactive, nanostructured sodium titanate layer on the surface of the titanium substrate. This altered surface acts as a site for the subsequent in vitro nucleation of calcium phosphates from SBF [13,18], mimicking the earliest in vivo surface reaction stages after implantation (up to 2 days), where the crystallization of HCA is described as occurring as a result of chemical reactions without cellular activity [19].

In our study, we investigated the cellular response of osteoblasts in vitro to biomimetically altered bioactive titanium surfaces after 2 and 14 days of mineralization in SBF, respectively, to mimic the early stages of bone bonding.

Sample surfaces were characterized by scanning electron microscopy (SEM), energy-dispersive X-ray analysis (EDX), cross-sectional transmission electron microscopy (TEM) analyses, and Fourier transform infrared (FTIR) and Raman spectroscopy. Cell behavior on the titanium samples, such as proliferation, gene expression, protein synthesis and the organization of adhesion structures (actin cytoskeleton, integrins), was investigated by flow cytometry, real time reverse transcriptase-polymerase chain reaction (RT-PCR), Western blot and confocal microscopy, respectively.

2. Materials and methods

2.1. Biomimetically altered titanium surfaces

Commercially pure titanium (cpTi) squares (Timet GmbH, Germany), with an edge length of 25 mm and a thickness of 1 mm, were ultrasonically cleaned in isopropyl alcohol and etched in 37 wt.% HCl under an argon atmosphere for 2 h at 50 °C. Subsequently, the specimens were soaked in a 10 mol l^{-1} NaOH aqueous solution at 60 °C for 24 h, washed with distilled water and dried at 100 °C [17]. All samples were freely suspended in SBF under static conditions at 37 ± 0.4 °C in a volume of model solution roughly corresponding to a surface to volume ratio $S/V = 0.05\ cm^{-1}$. SBF10 was prepared by mixing concentrated solutions of KCl, NaCl, $NaHCO_3$, $MgSO_4{\cdot}7H_2O$, $CaCl_2$ and KH_2PO_4 into double-distilled water, buffered with *tris*-hydroxymethyl aminomethane and HCl to pH 7.4 at 37 °C according to a procedure reported earlier [13]. Sodium azide (NaN_3) was added to the solution to inhibit the growth of bacteria. In order to mimic the early in vivo stage after implantation, 40 chemically treated Ti samples were soaked in SBF for 48 h (labelled as TiCT). In a second group (labelled as TiHCA), 40 samples were coated with biomimetic HCA by soaking the chemically treated samples in SBF for 14 days.

2.2. Characterization of the sample surface

Changes in the Ti surface after chemical treatment and soaking in SBF were characterized on gold sputtered samples by SEM (Quanta 200, Fei, Netherlands) and EDX (INCA x-sight, Oxford Analytical Instruments Ltd., UK). Cross-sectional TEM analyses were carried out on a Philips EM-430 microscope, operated at 300 kV, and a JEOL 2010 microscope, operated at 200 kV. For this purpose, two parts of a sample that were cut perpendicular to the surface, were joined with epoxy glue, surface against surface. Subsequently slices were cut, mechanically polished and thinned to perforation by ion milling at a low angle in order to obtain the largest possible thin area, from the substrate up to the surface. Raman spectra were collected in the range from 4000 to 400 cm^{-1} using a dispersive Raman spectrometer (Nicolet Almega XR, Thermo, USA) equipped with a 532 nm laser. FTIR spectra were measured in transmission using the KBr technique in the range from 4000 to 400 cm^{-1} at a resolution of 4 cm^{-1} (Impact 420, Nicolet Instruments, USA). Approximately 1 mg of the coatings formed on the chemically treated titanium during soaking in SBF solutions for 2 weeks was removed from the substrate, mixed with 300 mg of dry KBr powder and ground using an agate mortar and pestle. The resulting mixture was pressed into transparent pellets with a diameter of 13 mm applying a force of 10^5 N (Perkin-Elmer Hydraulic Press, Germany). Crystalline phases were determined by X-ray diffraction (XRD) analysis using Cu $K\alpha$ radiation at a scan rate of 1 min^{-1} over a 2θ range of 10–70° (D 500, Siemens, Germany).

2.3. Cell culture of MG-63 osteoblastic cells

TiCT and TiHCA samples were placed into Petri dishes (Greiner, Ø 60 mm). As reference material for cell experiments, cpTi was polished (Ti pol) with SiC wet grinding paper (grit P4000, $R_a = 0.19 = \mu m$) [20] and placed into 6-well plates (Greiner bio-one, Frickenhausen, Germany). As controls, collagen I (COL; rat tail, 20 μg cm^{-2}) (TEBU, Frankfurt/Main, Germany) coated cover glass and tissue culture plastic (TCPS) were used.

Throughout the experiments, human MG-63 osteoblast-like cells (osteosarcoma cell line, ATCC, LGC Promochem, Wesel, Germany) were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal calf serum (FCS, PAA gold) and 1% gentamicin (Ratiopharm GmbH, Ulm, Germany) at 37 °C and in a 5% CO_2 atmosphere. Cells were seeded with a density of 3×10^5 cells $specimen^{-1}$ on gamma-sterilized (Gamma-Service GmbH, Radeberg, Germany) TiCT and TiHCA samples, as well as on polished titanium sterilized with 70% ethanol.

2.4. Cell morphology visualized by SEM

Cells were grown on the specimens for 48 h, fixed with 4% glutaraldehyde (1 h), postfixed with 0.5% OsO_4, dehy-

ID	Title	Pages
1772	Osteoblast response to biomimetically altered titanium surfaces	11