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The osteogenic differentiation of human osteoprogenitor cells on Anodic-Plasma-Chemical treated Ti6Al7Nb

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ABSTRACT

Biological integration of an implant to surrounding bone is an important event for its clinical success and is driven by numerous factors, including the attraction of bone forming cells. The implant's surface properties influence the initial cell response at the cell/material interface, ultimately affecting the rate and quality of new tissue formation and the stability of the implant. As a consequence, various surface treatments have been developed to increase the clinical performance of titanium-based implants. Among them, the Anodic Plasma-Chemical (APC) technique allows for the combined chemical and morphological modification of titanium surfaces in a single process step. In the present study, we compared the potential of APC surface treatment of high-strength titanium alloys with vacuum plasma spray treatment and yellow gold anodization in supporting osteogenic differentiation of two different osteoprogenitor cell types. Both human fetal osteoblast cell line (hFOB1.19) and human mesenchymal stromal cells showed extensive cell spreading, faster cell growth and differentiation on APC surfaces compared to vacuum plasma spray treated and yellow gold anodized surfaces. Our findings showed that APC titanium-based surfaces provided an effective substrate for osteoprogenitor cells adhesion, proliferation and differentiation.

1. Introduction

The clinical success of an implant is determined by its stability. Primary stability is defined as the absence of mobility in the bone bed upon insertion of the implant and depends on the quality and quantity of bone, the type of implant and the surgical technique. Secondary stability is the increase in stability as a result of bone formation and remodeling at the implant-bone interface and is influenced by the implant surface and the healing time [1].

Osteointegration is defined as the direct structural and functional connection between living bone and the surface of a load-bearing artificial implant and results from a complex and multi-steps process [2], involving osteogenic cells and their precursors. *In vivo*, cells from the surroundings (most of them undifferentiated and from the mesenchymal lineage) migrate and attach to the implant, where they will start to differentiate toward an osteoblastic cell type, and secrete their own extra-cellular matrix that will later mineralize [3]. *In vitro*, cells settle down on the surface of the material, attach, and then integrin-dependent adhesion occurs; if appropriate conditions are met, cells can further differentiate and mineralize. For anchorage-dependent cells (such as mesenchymal stromal cells) attachment and adhesion phases are crucial both *in vitro* and *in vivo* as these regulate cellular subsequent behavior (cell growth, differentiation, matrix synthesis, etc) and *in fine* the implant osteointegration.

Human bone marrow stromal cells (hBMSC) are the predominant cells that will colonize the implant *in vivo* and represent a relevant cell type for *in vitro* models of osteointegration. However, hBMSC constitute a very heterogeneous cell population; therefore, a cell-line might be preferred for the initial *in vitro* assessment of implants. Recently, Harris et al. have developed the osteoprogenitor cell line hFOB1.19, which is characterized by a temperature-induced proliferation (at 34 °C) or differentiation (at 39 °C) [4]. The assessment of the *in vitro* cell response to a surface in terms of adhesion, growth and differentiation will give indications on the implant secondary stability *in vivo*.

Titanium and its alloys are well established biomaterials, successfully used for the fabrication of hard-tissue implants, particularly in load-bearing applications because of their excellent mechanical strength and good biocompatibility with bone tissue [5,6]. However, due to the rather passive properties of titanium materials [7] and subsequent lack of osteointegration, implant loosening is still an unsolved problem associated with titanium

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internal fixation. Therefore, increasing implants bio-reactivity will promote cell/material interactions and improve osteointegration of the implants. Among the various surface modifications and deposition techniques developed to improve the tissue-biomaterial interface [8], electrochemical anodization techniques - as voltage deposition, anodic spark deposition or the newly developed anodic plasma chemical ("APC") treatment - are particularly efficient on titanium-based implants. However, a coating could offer a real advantage in terms of early stability only if it promotes precursor cells differentiation toward an osteoblastic phenotype.

In the present study, cell adhesion, growth and differentiation of hFOB1.19 and hBMSC were investigated on gold-anodized ("Anodized"), vacuum plasma-sprayed titanium ("VPS") and APC treated titanium alloys. The hFOB1.19 cell line was chosen as they constitute a well established cellular model showing osteoblastic differentiation features when the right conditions are met. In parallel, we studied the behaviour of hBMSC as they represent a more clinically relevant cell type.

2. Material and Methods

2.1. Surface treated Ti6Al7Nb discs

Ti6Al7Nb discs (30 mm in diameter, 2 mm thick; Synthes GmbH, Switzerland) treated by Vacuum Plasma Spray (VPS) of chemically pure titanium, anodic oxidation of titanium (Anodized) type "yellow gold" according to AMS 2487 or anodic plasma chemical (APC) treatment [9] were used for this study.

The microstructure of the surface treated Ti6Al7Nb discs was characterized by scanning electron microscopy (SEM) (mod. EVO MA 25, Zeiss, Oberkochen, Germany) at 5 keV with a secondary electrons detector. Samples were sputter-coated with a 10 nm layer of gold prior to observation. The surface roughness was measured by image analysis with MeX version 5.1 software (Alicona Imaging GmbH, Grambach, Austria) of SEM micrographs of samples surface. Because of differences between surfaces, the area used for image analysis was 50x70 μm^2 for APC and Anodized, and 2000x2600 μm^2 for VPS-treated surfaces. Coating thickness was measured by image analysis of SEM micrographs of transversal sections of the samples. For the APC treated surfaces, Raman and XPS analysis were used to characterize the calcium phosphate present in the coating. Micro-Raman spectra were recorded with an Invia Raman microscope (Renishaw, Wotton-under-Edge, Gloucestershire, UK) with a diode laser (wavelength = 782 nm) as excitation source, with an integration time of 30 s and a resolution of 0.8 cm^{-1}. X-ray photoelectron spectroscopy (XPS) was performed on an Axis Nova instrument (Kratos Analytical Ldt, UK) using a monochromatic AlK_α radiation source, operated at an electron take-off angle of 90° relative to the surface plane. Survey spectra were collected over a range of 0-1200 cm^{-1} and detailed spectra in 800-1000 cm^{-1}. All peaks were referred to the C1s band at 285.0 eV. The three surfaces have been prepared under strict cleanliness standards and were gamma sterilized and sterile packaged for cell culture experiments.

2.2. hFOB1.19 amplification

The human fetal osteoblast cell line (hFOB1.19, ATCC CRL-11372) was purchased from LGC Promochem (Molsheim, France). This cell line is transfected with a gene coding for a temperature sensitive mutant of SV40 T-antigen (tsA58) and a gentamycin resistance coding gene, enabling continuous proliferation at 34 °C and cell quiescence at 39 °C, leading to differentiation when the right culture conditions are met [4]. hFOB1.19 were amplified in Dulbecco's modified Eagle/Ham's F12 (DMEM/F12) medium, containing 25 mM L-glutamine, 15 mM HEPES (Invitrogen AG, Switzerland), 10% charcoal stripped fetal calf serum (sFCS, Invitrogen AG, Switzerland), 0.3 mg/ml Geneticine 418 (G418, Sigma-Aldrich, Switzerland), 1.2 g/l $NaHCO_3$ and 0.5 mM sodium-pyruvate (Sigma-Aldrich, Switzerland). Cells were placed in a humidified atmosphere incubator at 34 °C, 5% CO_2, 95% air until 85-90% confluence was reached.

2.3. hBMSC isolation and culture

Human bone marrow (BM) samples were obtained after informed consent of patients, according to Insel Spital Bern (Switzerland) ethical commission's guideline. After homogenization, BM aspirates were diluted 1:4 with Iscove's modified Dulbecco's medium (IMDM) containing 5% (v/v) fetal bovine serum (FBS, Invitrogen AG, Switzerland). After cell separation on a Ficoll gradient, mononucleated cells of the interphase were collected and seeded at densities of 8-10 x 10^6 monocytes per 150 cm^2 T-flask in IMDM containing 10% FBS, 1% nonessential amino acids and 100 U/ml penicilline/streptomycine (PenStrep). After 5 days, fresh medium containing 5 ng/mL basic-fibroblast-growth-factor (bFGF) was added [10-12]. Medium was changed every 3 days and cells were subcultured 1:3. Cells of passage 3 were used in this study.

2.4. Cell seeding and differentiation on the different surfaces

Prior to cell seeding, the three types of materials were transferred into 6 wells cell culture plates, rinsed in DMEM/F12 medium alone and then left to dry overnight in a sterile hood.

On the next day, 70 000 cells in 250 μL of DMEM/F12 medium containing 10% FBS were seeded on each of the 7 cm^2 material and transferred in a cell culture incubator set up at 39 °C, 5% CO_2. After 2 h, 4 ml of differentiation medium (DMEM/F12, 10% sFBS, 0.1 mM ascorbic acid, 10^{-8} M menadione, 8 x 10^{-8} M VitD3, 10 mM β-glycerophosphate, 0.5 mM sodium-pyruvate and 0.3 mg/ml G418 - all from Sigma-Aldrich, Switzerland) were added to the materials seeded with hFOB1.19s, while classical osteogenic medium (IMDM, 10% FBS, 0.1 mM ascorbic acid, 10^{-8} M dexamethasone, 10 mM β-glycerophosphate, and PenStrep) was added in the case of hBMSC. Cells were incubated for different periods of time prior to further analysis.

2.5. Cell morphology

2.5.1. Cell fixation

All procedures were carried out at room temperature (20 to 22 °C) and 0.1 M Piperazine-N-N′-bis-(2-ethane sulphonic acid) (PIPES) (Aldrich, Switzerland) was used as buffer. After rinsing in PIPES (pH 7.4) samples were fixed in 2.5% glutaraldehyde in PIPES (pH 7.4) for 5 min and rinsed three times for 2 min in PIPES (pH 7.4). Additional contrasting of cells was accomplished by staining the cells with 1% osmium tetroxide (Simec Trade AG, Zofingen, Switzerland) in PIPES (pH 6.8) for 1 h. Cells on experimental substrates were dehydrated through an ethanol/water series (50, 60, 70, 80, 90, 96 and 100%). Samples were critical point dried (CPD) with a Polaron E3100 (Quorum Technologies, East Sussex, UK).

2.5.2. Scanning electron microscopy (SEM)

Samples were mounted on aluminum stubs and sputter-coated with a 12 nm layer of gold/palladium 80:20 (MED020, Bal-Tec, Balzers, Liechtenstein). Samples were observed at 5 kV accelerating voltage and 40 μA emission current in a field emission scanning electron microscope (FESEM S-4700, Hitachi, Japan) with a secondary electron detector and Quartz PCI digital imaging system.

2.6. Cell growtlh

Cell growth was determined as described by Labarca et al.[13]. Briefly, DNA was quantified by measuring the binding of Hoechst 33258 (Polysciences Inc, 09460) to the DNA helix after BMSC overnight digestion at 56 °C in a proteinase K solution (0.5 mg/mL in 3.36 mg/mL disodium-EDTA-PBS). After appropriate dilution of the samples in Dulbecco's phosphate buffered saline (DPBS) containing 0.1% (v/v) H33258, the bound fluorescence was measured using a PE HTS 7000 Bio Assay Reader at 360 nm excitation and 465 nm emission wavelength.

2.7. Quantification of alkaline phosphatase activity

Alkaline phosphatase (ALP) activity was measured according to the Sigma Technical Bulletin Procedure No.104. Briefly, the cell layers were extracted by incubation of 500 μL of 0.1%Triton-X in 10 mM Tris-HCl (pH 7.4) for 2h at 4 °C. After 15 min incubation at 37 °C with p-nitrophenyl phosphate as substrate (Sigma Kit No.104), p-nitrophenol production was measured on a Perkin Elmer Bio Assay Reader HTS 7000.

2.8. Alizarin red staining

After rinsing in ice-cold PBS, cell layers were fixed in 70% ethanol overnight at 4 °C. Following washing steps in MilliQ water, 2 ml of a 40 mM Alizarin Red solution (ARS) were added to each well and incubated for 1 h at room temperature. ARS was then removed and materials were intensively washed in MilliQ water, then in PBS until all non-specific staining was removed. After air drying, samples were imaged with a digital camera under day light.

2.9. Gene expression analysis

2.9.1. RNA Isolation and Reverse Transcription

Total RNA was extracted using TRI-Reagent® (MRC Inc. TR-118) according to the manufacturer's instructions. Reverse transcription was performed on 1 μg of total RNA sample, using TaqMan® reverse transcription reagents (Applied Biosystems, Foster City, Calif.) with random hexamer primers.

2.9.2. Real-time Polymerase Chain Reaction (PCR)

PCR was performed on a 7500 Real Time PCR System (Applied Biosystems). Genes of interest were detected using specific oligonucleotide primers and TaqMan® probes (Microsynth, Switzerland) or Assays on Demand (Applied Biosystems, Foster City, CA) as specified in Table 2. PCR conditions were set at 95 °C for 10 min, followed