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Biophysical characterization of nanostructured TiO_2 as a good substrate for hBM-MSC adhesion, growth and differentiation

L. Petecchia, C. Usai, M. Vassalli, P. Gavazzo*

Institute of Biophysics, National Research Council, Via De Marini 6, 16149 Genova, Italy

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ABSTRACT

Mesenchymal stem cells from human bone marrow (hBM-MSC) are widely utilized for clinical applications involving bone healing. In this context, their use has been often optimized in association to variously designed titanium substrates, being this material of great use in orthopaedic implants.

According to recent findings, the ability of hBM-MSC to differentiate towards a specific lineage is not only driven by biochemical signals, but physical stimuli, such as rigidity or roughness of the substrate, can also support a commitment towards osteogenic differentiation. Moreover, the presence of features with defined dimensional scales, in particular nanometer-size, also proved to elicit specific biological effects. Here we evaluated the effectiveness of a nano-patterned titanium surface in sustaining hBM-MSC adhesion, growth and differentiation by means of a panel of biophysical tools: morphometry, electrophysiology, intracellular calcium measurements and immunocytochemistry. The results substantiate the idea that this micro-textured titanium dioxide is a good surface for growth and differentiation of hBM-MSC and it exhibits a stimulating action mainly in the initial period of differentiation. Moreover, the basal concentration of free cytosolic Calcium $[Ca^{2+}]_i$ is confirmed to be a good hallmark of the hBM-MSC maturation stage. The study could provide relevant hints to help improving the biocompatibility and osteointegration potential of clinical titanium implants.

1. Introduction

Stromal stem cells from human bone marrow (hBM-MSC) were first identified as “osteogenic stem cells” being a second stem cell type, different from hematopoietic [19]; later they were qualified as “mesenchymal stem cells” [5] and then “skeletal stem cells” [2] to highlight their remarkable potential in healing large bone defects and bone regeneration [1,40]. Due to their multipotency, hBM-MSCs can divide or become committed from the modulation of extrinsic and intrinsic factors to differentiate through discrete pathways into chondrocytes, adipocytes or, most important to implantology, into osteoblasts. Interestingly, it has been shown that hBM-MSC, among the various populations of adult stem cells, are the most susceptible to in vitro osteogenic differentiation [8]. For these reasons, many aspects of hMSC biology have been studied in deep and, in search of putative hallmarks of definite physio/pathological conditions to be exploited, specific features have been monitored and highlighted. We recently characterized hBM-MSC osteo-differentiation focusing our analysis on Ca-related aspects of cell metabolism, finding that cytosolic free Ca^{2+} concentration can be regarded as a reliable marker of the osteogenic stage. Furthermore, we proved that cell exposure to a pulsed electromagnetic field acts synergistically with hBM-MSC differentiation induced by osteogenic culture medium, thus offering elements to outline a successful protocol for in vivo bone disease treatment and tissue regeneration [37].

A challenging issue of hBM-MSC osteodifferentiation is that this process can be mediated by mechanisms controlled by micrometer and sub-micrometer features of the growth surface. In this context a seminal paper was published by Dalby and colleagues [14] proving that the adhesion and differentiation of hBM-MSC can be stimulated by specific disordered nanotopographies suggesting that disorder may be an effective strategy within a physiological and regenerative framework. Since then, many evidences unequivocally demonstrated that flat untreated titanium shows poor bone cell adhesion and scarce hBM-MSC differentiation, while a considerable improvement is determined by the insertion of nano- features on the TiO_2 surface in order to form a nanoscale network resembling the extracellular matrix (ECM) [12,15,39,10]. As a matter of fact, using different culture substrates with tunable elasticity, it was shown that, without changing the biochemical stimulus, hBM-MSC were forced to differentiate toward different lineages depending only on the elasticity, proving that cells usually growing in a soft native microenvironment branch in soft

* Corresponding author.
E-mail address: gavazzo@ge.ibf.cnr.it (P. Gavazzo).

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matrices and vice-versa [17].

Titanium and, especially, titanium dioxide are widely employed in implantable devices because of their high biocompatibility, good mechanical properties, and good osteo-integration potential even in animal models [3,47].

It has been shown that, tuning the size of titanium oxide nanotubes, it is possible to modulate the adhesion of hBM-MSC to the substrate and, eventually, to induce a lineage commitment toward osteogenesis. Noticeably, this effect was achieved using only geometric cues, but in the absence of any inducing agent in the medium [31]. Another study has shown that the microtexture of a hydrophilic titanium surface can modulate Bone Morphogenetic Protein (BMP) signaling in hBM-MSCs and induce more robust bone formation than the mere addition of exogenous BMP [23,34,35]. Interestingly, a complex cross-talk between BMP, the "Wingless-related integration site" (Wnt) signaling and integrins exists in hBM-MSCs and it is involved in the modulation of the differentiation toward the osteoblastic phenotype. Altogether, there is a bunch of evidence for the role of the microtexture of the substrate in the differentiation potential, but the related molecular details are still to be clarified.

More specifically, titanium dioxide (TiO_2), due to its chemical stability, has been produced and utilized in multiple designs such as nanowires, nanotubes, nanofibers and nanoparticles. Indeed it has been shown that, beside the traditional protocols, chondrogenic or osteogenic differentiation of hBM-MSCs growing onto vertically-aligned BMP-2-coated nanotubes are alternatively triggered depending on the size of the applied nanotubes [36].

From the material point of view, another interesting property of TiO_2 is the ability to be immobilized and spread as a thin layer onto other materials, such as glass [7], obtaining full transparency to the visible light. Accordingly, it can be used as growth substrate [44] even in experiments requiring the optical access to cells, such as patch clamp and immunocytochemistry.

The use of these nanostructured materials opens up novel perspectives for biomedical applications and tissue regeneration, providing access to a deep understanding of the influence of the microenvironment in the destiny of stem cells.

In this context the aim of the present work was to investigate hBM-MSCs simply proliferating on a newly designed nanostructured titanium dioxide surface or on glass coverslips, or stimulated to osteo-differentiate by the addition of osteo-inductive agents in the culture medium. Several aspects of cell physiology have been evaluated and compared. Overall the results suggest an active role of nano-patterned TiO_2 in favouring the ongoing differentiation process, thus highlighting the biocompatibility of the surface and confirming the reliability of this material for biomedical applications.

2. Materials and methods

2.1. Cell cultures

hBM-MSCs were grown at 37 °C in a 5% CO_2 humidified atmosphere, and maintained in low-glucose DMEM (Dulbecco's modified Eagle's) supplemented with 10% FBS, 1% glutamine, 50 μg/ml penicillin-streptomycin and amphotericin B (Lonza Group Ltd.) (proliferative medium, PM) for proliferation or in Osteogenic Differentiating Medium (OM), i.e. α-MEM (Minimum Essential Medium) with 10% FBS, antibiotics and a mixture containing 100 nM dexamethasone, 5 mM β-glycerophosphate disodium and 50 mg/ml ascorbic acid (Sigma-Aldrich, S. Louis, MO, USA) for osteogenic differentiation. OM Treatment lasted up to 27 days and the medium was changed every 3 days. The study was conducted in accordance with the Institution Review Board of Fondazione IRCCS Policlinico San Matteo and the University of Pavia (2011).

2.2. TiO_2 substrate

TiO_2 slides used in our experiments were bought from Tethis spa (Milan Italy). These substrates are based on a 20 mm diameter glass coverslip (0.17 mm thickness) on top of which a thin film of nanostructured titanium was deposited under vacuum conditions, using a supersonic seeded beam of titanium oxyde clusters produced by a microplasma cluster source [6]. The resulting surface features a 30 nm thick coating with a clustered morphology (average roughness of 4–5 nm and average surface slope of about 8°) which are optimized to mimic the granularity and porosity of the extracellular matrix (ECM) environment [7], while keeping a disordered organization which is suggested to provide a good cue for stem cells differentiation [14]. These substrates have been found to have a good degree of biocompatibility [42] and maintain the transparency of the glass which is mandatory to perform experiments requiring optical access (as electrophysiology or high resolution fluorescence microscopy). Moreover, in order to foster an efficient bio-integration of the surface, mediated by an initial phase of protein adhesion, the surface was plasma-treated to provide a contact angle lower than 100°, comparable with the one observed in bare glass (see patent PCT/EP2010/050820).

2.3. Cell viability

The cell viability of hBM-MSC growing on glass or on TiO_2 coverslips evaluated by means of resazurin assays was previously reported [43]. The results indicated that after 27 days of incubation the number of cells was quite similar with no significant differences correlated to the substrate. Thus the TiO_2 coating used in this work doesn't exert cytotoxic effect on the cells and doesn't alter proliferation rate.

2.4. Electrophysiology

Single-cell current recording was performed by Patch-clamp technique as previously described [37]. Cells were seeded on 20 mm diameter glass- or TiO2- coated coverslips and grown to reach subconfluence. Then cells were detached from the substrate and resuspended in Standard Solution containing (in mM): 150 NaCl, 5.4 KCl, 2.0 $CaCl_2$ 1.0 $MgCl_2$, 10 HEPES, 10 glucose (pH adjusted to 7.4 with NaOH). During current recording cells were continuously perfused by gravity flow (10 ml/min) as described [20,38] with the standard solution described above. Conversely voltage activated Ca^{2+} currents were measured maintaining cells in (mM) 108 $BaCl_2$ and 10 HEPES, (pH 7.4). Recording pipette were backfilled with a solution containing (in mM): 8 NaCl, 40 KCl, 100 Aspartic Acid, 100 KOH, 2 $CaCl_2$, 5 EGTA and 4 adenosintriphosphate (ATP). Only cells with no evident contact with other cells were patched. Bay-K 8644 was dissolved in Ethanol at a stock concentration 1 mg/ml. GEPULSE software was used for current acquisition and ANA [http://users.ge.ibf.cnr.it/pusch/programs-mik.htm] for current analysis. Further analysis was performed using Sigma Plot (SPSS Science, Chicago IL, USA).

2.5. Intracellular calcium measurements

hBM-MSCs grown on 20 mm diameter coverslips were loaded with Fura-2 AM in the presence of Pluronic F-127 (Sigma-Aldrich GmbH) for 45 min at 37 °C. Measurements were performed at room temperature in a Standard solution containing (in mM): 150 NaCl; 5 KCl; 2 $MgCl_2$; 10 glucose; 10 HEPES; 2 $CaCl_2$; pH was adjusted to 7.3 with NaOH. Fluorescence ratio calculation and calibration and Data analysis were performed as previously described [50].

2.6. Digital Holography

Digital Holography (DH) is an interferometric imaging technique

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