In vitro osteoinduction of human mesenchymal stem cells in biomimetic surface modified titanium alloy implants

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Interaction between cells and implant surface is crucial for clinical success. This interaction and the associated surface treatment are essential for achieving a fast osseointegration process. Several studies of different topographical or chemical surface modifications have been proposed previously in literature. The Biomimetic Advanced Surface (BAS) topography is a combination of a shot blasting and anodizing procedure. Macroroughness, microporosity of titanium oxide and Calcium/Phosphate ion deposition is obtained. Human mesenchymal stem cells (hMSCs) response *in vitro* to this treatment has been evaluated. The results obtained show an improved adhesion capacity and a higher proliferation rate when hMSCs are cultured on treated surfaces. This biomimetic modification of the titanium surface induces the expression of osteoblastic differentiation markers (RUNX2 and Osteopontin) in the absence of any externally provided differentiation factor. As a main conclusion, our biomimetic surface modification could lead to a substantial improvement in osteoinduction in titanium alloy implants.

Keywords: Osteoinduction, Biomimetic surface, Titanium implant, Human mesenchymal stem cells, Combined surface treatment

INTRODUCTION

Titanium implants are widely used in orthopaedics and dentistry because of their good biocompatibility, resistance to corrosion and mechanical properties^{1,2}. One essential aspect in the performance of an implant is the mechanical and biological behaviour of its interface with bone. A stable and sufficiently stiff and resistant interface between the biomaterial surface and the surrounding tissue is a vital prerequisite both for immediate implant loading and for the long-term success of such implants. This phenomenon has been extensively analyzed in the literature^{3–6}. The interface is usually achieved by the biological process (partially regulated by mechanical and material properties) known as osseointegration of the implant, which is obtaining an intimate connection of the implant within the bone by means of an appropriate and sufficient growth of new bone on the surface of the implant⁷. Achieving a good osseointegration depends on physiological variables such as bone quantity and quality, on the particular properties of the implant material, on the biocompatibility of the implant, and on the material and implant surface properties^{8,9}.

Various surface modifications of titanium implants that greatly improve the osseointegration process have been described in the literature. One of the most widely used processes is introducing different chemical and/or biochemical substances on the surface. Such

modifications of the atomic composition of the surface have led to significant improvements in the cell response on the surface material both *in vitro* and *in vivo*^{10–13}. Impregnation or coating with hydroxyapatite^{14,15} or calcium phosphate^{16,17} have been proposed as biomimetic approaches. However, despite the success achieved through surface chemistry modifications in the early host-implant integration, there is a general consensus that the modulation of surface texturing is also an important and desirable variable, regardless of the surface chemistry approach followed in endosseous implants^{18,19}. The benefits of topographical surface modifications have been previously described *in vitro* and *in vivo*^{20–23}. *In vitro* studies have shown that surface topography affects different cellular functions such as cell adhesion, proliferation, differentiation and local factor production²⁴, but that it also favours appropriate implant-cell interactions on the bone-implant interface^{25–27}. For example, implant biocompatibility largely depends on the particular effect of the implant (especially its surface) on cells. In particular, osteoblastic cells tend to adhere quickly on rough surfaces^{28,29}. Also, cell differentiation on rough surfaces is conditioned by parameters such as cell morphology, extracellular matrix production, the specific activity of alkaline phosphatase, and osteocalcin production²⁹. The interaction between cells and the extracellular matrix (ECM) is another critical factor for regulating cell functions such as cell shape, migration, proliferation, and survival.

However, despite the widely accepted crucial role

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of the initial interaction between cells and implant surface in achieving a faster initial bone formation and therefore in the final clinical success, little is known about the specific molecular mechanisms involved in the surface chemistry-dependent protein adhesion and its interaction with mechanical variables such as roughness, surface topography, friction, stiffness and strength^{30–32}.

The use of human mesenchymal stem cells (hMSCs) in promoting rapid osseointegration of implants has been proved to be highly relevant in terms of their potential to integrate with and promote functional restoration of bone²⁷. Due to their strategic location, MSCs have been described as one of the most important players in bone fracture healing and implant incorporation, even more important than pre-existing osteoblast. Implant collocation induces MSCs recruitment into the damaged zone being those cells the main responsible cells of the bone healing and bone formation after becoming osteoprogenitor cells³³. It is also well known that bone marrow mesenchymal stem cells have the ability to differentiate into various cell types including osteogenic lineage³⁴.

Therefore, the present study was designed to evaluate the cellular response *in vitro* of human bone marrow derived MSCs cells (BMH cells) on biomimetic modified titanium surfaces. The main purpose of this study was to compare the adhesion, proliferation and osteoblastic differentiation potential of hMSCs culture that have been analyzed in an *In vitro* system that offers a tandem model to *in vivo* applications.

MATERIALS AND METHODS

Titanium disc fabrication

Grade 5 ELI Ti (Ti6Al4V) titanium discs supplied by Avinent Implant System (Barcelona, Spain) were used. The machined and polished discs of 10 mm diameter and 2 mm thick were divided in two groups: non-treated polished discs and discs treated with BAS (Biomimetic Advanced Surface). The BAS surface treatment was obtained by a combination of two processes. First, a shot blasting procedure was carried out using aluminium oxide as a blast media with a particle size between 212 and 300 µm for 30–40 s at 3–5 bars. Afterwards, the discs were anodized for 40–90 s by connecting them to a Direct Current power supply and using an electrolyte solution rich in Ca and P results in a ratio 2/1 of a Ca and P. The BAS surface topography is characterized by the presence of macroroughness and microporosity in the titanium oxide with a calcium and phosphorus deposit. The non-treated discs were prepared through initial polishing with silicon carbide paper and then with aluminium oxide with a particle size of 1 µm and 0.05 µm. The titanium discs were washed in an ultrasound bath to remove traces of aluminum oxide particles that may remain during treatment and autoclaved before being used in cell cultures. Standard tissue culture plastic was used as control.

Titanium disc characterization

Sample topography and roughness were measured optically with a white light interference microscope (NewView 7100, ZygoLot, Darmstadt, Germany). Two different objective lenses (×10, ×50) were used to characterize the sample surface. The microporosity of the samples was examined by scanning electron microscopy (Inspect F, FEI, Hillsboro, Oregon, USA) at an accelerating voltage of 20 kV. The chemical characterization was carried out with X-ray photoelectron spectroscopy (XPS) (AXIS Ultra DLD, KRATOS, UK) as described by Boyd *et al.*³⁵.

Cell culture

hMSCs from human bone-marrow (Lonza, Basel, Switzerland) were maintained as subconfluent monolayers in alpha-Modified Eagle's Medium (Sigma, Steinheim, Germany) supplemented with 10% (v/v) fetal bovine serum, 100 U/mL penicillin and 100 mg/mL streptomycin (Sigma) and 5 µg/mL FGF-2 (SIGMA) at 37°C. The cells were harvested using trypsin-EDTA (Sigma) and seeded on control, treated and non-treated samples in 24-well plates for differentiation, proliferation and adhesion tests. The medium was changed twice a week. For all the experiments, 10% FBS normal medium was used without any differentiation factors. All the experiments were conducted using cells up to passage 5.

Cell morphology observation

The sample surfaces were also examined by scanning electron microscopy after cell seeding (Inspect F, FEI). After 1 day in culture, the seeded discs were fixed with 2% glutaraldehyde. Following fixing, the titanium discs were washed in phosphate buffered saline (PBS). The samples were then dehydrated through the ascending ethanol series, dried to critical point, gold coated (10–15 nm) and viewed through a scanning electron microscope (SEM) as previously described.

Cell adhesion assay

Cell attachment on each substrate was evaluated by recording the number of cells attached after incubation: 2×10^5 cells/sample were seeded on the materials and incubated for 6, 12 and 24 h. The specimens were then washed with PBS to eliminate unattached cells. The adherent cells were removed from the substrate by incubation twice with trypsin 0.25% in EDTA (Sigma). The resulting cell suspension was counted with a Neubauer chamber. Three measurements on each group were performed per experiment. The results were expressed as the number of total adherent cells per disc.

Proliferation assay

The proliferation of hMSCs cells cultured on the discs was evaluated by measuring the cell number present on the material after 7, 14, 21 and 28 days of culture. The previously used cell concentration induced contact inhibition due to lack of space after 28 days in culture (data not shown). An optimal concentration of 50,000

Fig. 1 Titanium disc characterization: SEM images (5000×) of non-treated (a) and treated discs (b); 3D roughness measured optically (×10) with a white light interference microscope. The 3D model and the roughness parameters of the untreated samples (c) and the biomimetic treated surface (d); Chemical analysis of the titanium discs. Treated samples are represented in green and control samples represented in red. The specific areas of calcium (e) and phosphorus (f) are shown.

Fig. 2 The graphs show cell adhesion measured with an initial cell concentration of 200,000 cells/disc. A normal plastic culture surface was used as control (data not shown). Data are presented as mean $\pm$ S. E. of at least three independent experiments. Differences were analysed by one-way analysis of variance, with $p < 0.05$ being considered statistically significant (*).

cells per disc was used for long culture periods. Once the culture period was over, the cells were detached by trypsinization and counted with a Neubauer chamber. Three measurements on each group were performed per experiment.

Gene expression by real time-polymerase chain reaction

Semi-quantitative RT-PCR was used to assess the gene expression level of several well known osteogenic markers in hMSCs cells. The hMSCs cells were cultured on titanium discs (5×10^5 cells/mL) over a time period of 7, 14, 21 and 28 days. Plastic tissue culture was used as control. At these times intervals, the cells were trypsinised twice and RNA extracted using a Trizol reagent (Invitrogen, Life Technologies Ltd, Paisley, UK). The first-strand synthesis of single-strand cDNA from RNA for use as a PCR template was carried out with a SuperScript™ First-Strand cDNA Synthesis Kit (Invitrogen). Expression of osteogenesis-related genes including runt-related transcription factor-2 (RUNX-2) and Osteopontin (OPN) were quantified using Real

Fig. 3 SEM images of cells on non-treated (a, b) and treated (c, d) titanium surface at different magnifications after 24 h in culture. Pictures at 700 $\times$ magnification of the control (a) and the biomimetic treated samples(c); Pictures at 5000 $\times$ magnification of non-treated and treated samples, respectively (b) and (d).

time-PCR (Applied Biosystem, Real time-PCR detection system) TaqMan Assay on demand expression Assays (GAPDH Assay ID: Hs00266705_g1; OPN: SPP1 Assay ID: Hs00959010_m1; RUNX-2: RPL32 Assay ID: Hs00851655_g1). All the samples were analyzed in triplicate. The comparative cycle threshold-value method was used to calculate the relative quality of RUNX-2, OPN, and GAPDH. The relative expression levels for each gene of interest were normalized with the expression of the housekeeping gene GAPDH and with the expression of the genes of interest in the control sample.

Statistical analyses

All cell culture experiments were carried out in triplicate at least three times. Analysis of variance (ANOVA) and pairwise multiple comparison tests (t student test) were used to determine differences within each group (SPSS software package). A statistical significance was obtained for $p < 0.05$.

RESULTS

Surface characterization

The optical roughness measurements and the scanning electron microscopy pictures of the different surfaces show that the BAS surface samples have macro and microscopic roughness due to the impact of aluminium oxide particles during the shot blasting process, and pores with a diameter of 1–2 μm uniformly distributed throughout the surface (Figs. 1a and b). Polished and treated samples showed a R_a mean value of $0.276 \mu\text{m} \pm 0.08 \mu\text{m}$ and $2.8 \mu\text{m} \pm 0.36 \mu\text{m}$ respectively. Significant differences ($p < 0.05$) in 3D roughness were observed between both samples (Figs. 1c and d). Chemical

analysis showed the presence of oxygen, calcium, phosphorous and sodium on the surface of the treated samples, elements introduced on the surface during the anodization process (Figs. 1e and f).

Cell adhesion

The effects of treated and non-treated superficial Ti discs on enhancing human MSC adhesion were evaluated by cellular counting. A cellular concentration of 200,000 cells/disc was tested. The results showed (Fig. 2) a significant increase in the cellular adhesion on the treated surface during 24 h when compared to the non-treated one ($n=9$; $*p < 0.05$ for 6, 12 and 24 h). A clear decrease in the attached cells to the non-treated titanium discs was observed. The minimum was achieved 12 h after cell seeding. However, an almost constant and significantly higher number of cells were detected in the treated samples. Scanning electron micrographs of the cells on the substrates after 24 h of seeding showed a better adherence of the cells on the treated titanium surface than on the non-treated surface. This can be observed in Fig. 3 which shows a thin layer of cells appearing on

Fig. 4 Cell proliferation on the discs with an initial cellular concentration of 50,000 cells/disc during 28 days. A normal plastic culture surface was used as control (data not shown). Data are presented as mean $\pm$ S.E. of at least three independent experiments. Differences were analyzed by one-way analysis of variance, with $p < 0.05$ being considered statistically significant (*).

Fig. 5 Real-Time-PCR analysis of selected genes expressed by hMSCs under normal conditions in cell cultures on treated and non-treated surfaces. (a) Osteopontin expression (OPN) and (b) RUNX-2 expression. A normal plastic culture surface was used as control (data not shown). Values were normalized for GAPDH. Data are presented as mean $\pm$ S.E. of at least three independent experiments. Differences were analysed by one-way analysis of variance, with $p < 0.05$ being considered statistically significant (*).

the non-treated surface and cells spreading in different layers completely covering the treated surface of the discs.

Cell proliferation

Over 28 days, the treated surface samples showed an increased number of cells when compared to the non-treated samples. However, a non-significant decrease in cell numbers was observed after 14 days in both groups in comparison with previous time points. Proliferation on the BAS treated surfaces was observed when compared to the non-treated surfaces on day 7 and 28, this increase being significant on day 14 and 21 (Fig. 4).

Cell differentiation

Cells growing on common plastic culture surfaces were used as a control to normalize the results obtained in the RT-PCR analysis to also evaluate the osteogenic effect of the titanium. The expression of Osteopontin and Runx-2 in hMSCs isolated from the different surfaces was analyzed. Both titanium surfaces were able to increase the expression of the osteogenic differentiation markers. However, the BAS surface samples were able to enhance the expression levels of osteopontin (Fig. 5a) and Runx-2 (Fig. 5b) in comparison with the non-treated surface samples. These differences were significant on day 14 for OPN and on day 7 for RUNX-2. On day 28, both genes showed significant differences when compared with the non-treated surface samples.

DISCUSSION

It has been shown that a prerequisite for a successful implant is rapid osseointegration and long-term stability. In both cases, the implant surface is of paramount importance. Surface properties of biomaterials play a critical role in the establishment of the cell-biomaterial interface²⁸. The results presented in this study demonstrate that a biomimetic modified surface has beneficial effects on these osteoinduction-related processes *in vitro*. These positive effects observed in the mesenchymal cell behaviour are due to a combination of factors involving topography, microstructure and surface chemistry. It has been widely reported that surface roughness by itself has a great effect on osteoblast attachment, proliferation and differentiation^{21,32,36-38}. Most studies comparing the influence of surface modifications on titanium implants to determine adhesion, proliferation or differentiation properties have used osteoblast cell lines. However, in this study we have decided to use human mesenchymal stem cells because they are closer to the actual physiological conditions and play a key role in the osseointegration physiological process. Mesenchymal stem cells are undifferentiated pluripotential cells present in bone marrow, capable of differentiating into many cell types (chondrogenic, adipogenic, or osteogenic lineages) and may be a suitable autogenous cell source for tissue regeneration. Together with cells coming from the blood, the MSCs are the cells responsible for the first

contact with the surface of titanium implants³⁹⁻⁴¹. The adhesion of these cells to a biomaterial surface can be a major factor mediating its biocompatibility. A high degree of cell adhesion to the surface allows a strong attachment of the surrounding tissue to the biomaterial. In our study, we have observed that hMSCs were able not only to attach significantly better to treated surface titanium discs than to untreated ones, but also to significantly increase their number. This improvement in cell adhesion is in accordance with other authors⁴²⁻⁴³ and could be explained due to the greater surface area obtained in our surface modification. This increased area in the surface modified titanium discs could be coupled to a higher protein adsorption coming from the serum of the cell culture medium. Protein adsorption and matrix remodelling at the cell-material interface has been previously described as a potent cell adhesion inductor⁴⁴. Other previous works are also in concordance with our adhesion and proliferation results^{28,45}. SEM observation showed that cells cultured on the treated surfaces formed multicellular layers completely covering the whole sample while those cultured on the untreated ones proliferated but did not form layers. In our study we have also been able to induce the expression of osteogenic differentiation markers (Runx-2 and osteopontin (OP)) in the absence of any chemical differentiation inducer. Runx-2 is an early osteogenic marker and serves as a critical bone cell transcription factor. It has been previously demonstrated that Runx-2 is essential for osteoblast differentiation, gene expression of bone matrix proteins, bone formation, and tooth development and that it determines the lineage of osteoblasts from mesenchymal cells as well as regulating many bone and tooth related genes^{46,47}. A higher expression of this gene seems to be related with higher mineralization. However, OP is a late osteogenic marker and its expression is increased as a late event meanwhile Runx-2 increases as an early event on differentiation. It has been proposed that OP plays an important functional role not only in matrix calcification of hard tissues, but also in the adhesion, spreading, and migration of cells⁴⁸⁻⁵¹. However, the mechanism regulating its activity remains unclear⁵²⁻⁵⁷. In Fig. 5, it could be observed that the temporal distribution of the differentiation markers are in accordance with the previously described in literature⁴⁶. An early increase of Runx-2 in day 7 is followed by a higher OP mRNA expression in day 14. Both differentiation markers have been also described as important players in matrix mineralization^{46,47}. These could be the reason why both differentiation markers are overexpressed in day 28. This molecular sequence has been achieved, in our study, in the absence of any differentiation media but in contact with a biomimetic modified surface. Consequently, taking all these *in vitro* results together, it can be concluded that using Ti-implants with BAS treated surfaces for orthopaedic or dental prostheses could improve the osseointegration and osteoconduction processes in humans, stimulating the bone-marrow mesenchymal stem cell proliferation, adhesion and differentiation to osteoprogenitor fates

in the absence of any differentiation molecule. These results demonstrate that surface modification is an important parameter able to modify cell behaviour that should be taken into consideration for the improvement of implant design.

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