Bone 45 (2009) 17–26

Contents lists available at ScienceDirect

Bone

journal homepage: www.elsevier.com/locate/bone

Modified titanium surfaces promote accelerated osteogenic differentiation of mesenchymal stromal cells *in vitro*

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ARTICLE INFO

Article history:
Received 10 December 2008
Revised 12 March 2009
Accepted 16 March 2009
Available online 28 March 2009

Edited by: T. Einhorn

Keywords:
Gene arrays
Osseointegration
Mesenchymal stromal cells
Adult stem cells
Titanium implant surfaces

ABSTRACT

Titanium (Ti) is the material of choice for dental and orthopaedic implants due to its highly biocompatible nature. Modification of the implant surface, either topographically (as roughness) or chemically, can promote accelerated osteogenesis *in vivo* and greatly increase bone-implant contact and bonding strength. In this paper, we sought to characterise the cellular and molecular responses of human bone marrow-derived mesenchymal stromal cells (hMSCs) to two modified Ti surfaces: a rough hydrophobic surface that was sand-blasted and acid-etched (SLA) and an SLA surface of the same roughness that was chemically modified to have high wettability/hydrophilicity (SLActive). A smooth polished (SMO) Ti surface was used as a control. Whilst no differences in initial cell attachment to any of the surfaces were observed, we found that hMSCs cultured on the rough surfaces underwent a decrease in cell number early in culture, yet simultaneously expressed higher levels of the osteogenic markers SPP1, RUNX2 and BSP. Furthermore, deposits of calcified matrix were observed at earlier time points on both SLA and SLActive surfaces compared to SMO and this correlated with increased expression of the osteogenic promoter WNT5A in response to the rough surfaces. Osteogenic responses to SLActive were moderately better than the hydrophobic SLA surface and gene expression studies indicate that WNT5A activation may be responsible for this increased osteogenic differentiation.

Introduction

Functional restoration of bone following surgical intervention is crucial for the success and long term survival of orthopaedic and dental implants. Titanium (Ti) is the material of choice clinically, as bone regeneration occurs with no evidence of host rejection. Modifications to the surface of Ti implants (for instance by introducing topographical or chemical modifications) can greatly influence bone regeneration [1]. In particular, surface roughness is one parameter that has been extensively characterised, both *in vitro* and *in vivo*.

In vivo, rough surfaces such as that of the sand-blasted, acid-etched (SLA) Ti implant have generally proven superior to smooth surfaces in terms of promoting bone-implant contact [2–5] and the increased anchorage ultimately increases the removal torques of SLA implants [3–7], in spite of SLA being hydrophobic. *In vitro* data is rather more variable, presumably a reflection of the different cell types, material roughness values and methods employed by different researchers. Generally, soft tissue-derived cells, such as fibroblasts from the gingiva and periodontal ligament preferentially attach to smooth, rather than rough surfaces *in vitro* [8,9]. Conversely, osteoblast-like cells favour moderately rough surfaces to smooth surface [10–14]. It has been demonstrated that rough surfaces were also superior to smooth surfaces in promoting osteogenic induction of the human osteosarcoma cell line MG63, determined by increased osteocalcin, osteoprotegerin and TGF-β [15]. Increased osteogenic differentiation measured by elevated alkaline phosphatase activity and nodule formation is associated with decreased proliferation on rough versus smooth surfaces *in vitro* [16]. Moreover, osteoblast cell number on rough SLA surfaces has been reported to be much lower than on smooth surfaces or plastic 24 h after reaching confluence, yet concomitant elevation in osteocalcin and alkaline phosphatase production resulted from accelerated osteogenesis [17], indicating that cell number and proliferation is not a determinant of osteogenic responses to rough Ti surfaces. In addition, when a modified version of SLA with improved wettability/hydrophilicity (called SLActive) was used, even lower cell number and higher osteogenesis was observed than on the hydrophobic SLA [17].

Whilst much of this work has been conducted using human primary osteoblasts or transformed cells, little evidence has been published regarding the effect of different titanium surface roughness to human mesenchymal stromal cell (hMSC) populations. This effect is of importance during the early stages of osseointegration of titanium implants especially in areas where the bone quality is characterised by high percentage of bone marrow (termed type IV bone; [18]) that does not facilitate the mechanical primary stability of the implants. On the

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doi:10.1016/j.bone.2009.03.662

other hand, as the ageing population is prone to age-related degenerative disorders such as periodontal disease and loss of functional dentition, the use of hMSCs in promoting rapid osseointegration of implants is also highly significant in terms of its potential to integrate with and promote functional restoration of alveolar bone.

MSCs isolated from bone marrow have the ability to differentiate along several different pathways, including osteogenic, chondrogenic and adipogenic lineages [19–21]. hMSCs of osteogenic potential can be isolated from the fraction of cells expressing the stro-1 surface epitope [22]. The potential accelerating effect in terms of osteogenesis of a titanium surface might have a significant clinical impact when primary stability, which typically stabilizes the implant during the early healing events of osseointegration, is at risk due to the anatomic characteristics of the host bone. Parallel to this, the prospects for repairing bone defects using autologous populations of MSCs are promising, as they can form bone tissue when transplanted *in vivo* [23] and are easily differentiated *in vitro* [24].

In the present investigation, the effect of Ti surface roughness (SLA and SLActive) on osteogenic induction of hMSCs was assessed. In addition, modified surface chemistry that leads to greatly increased wettability/hydrophilicity of the Ti surface (SLActive) was evaluated for potential benefit to osteogenic responses.

Materials and methods

Cells

Populations of hMSCs were obtained from the Centre for Gene Therapy, Tulane University, New Orleans, USA. Cells were cultured in Minimum Essential Medium supplemented with 16% (v/v) foetal calf serum, L-glutamine (2 mM) and nonessential amino acids (1×) (all Invitrogen, Paisley, UK). Cells were seeded at a density of 50 cells cm^{-2} and maintained at 37 °C in a 5%CO_2 humidified atmosphere (standard culture conditions). Medium was changed every 3 days until confluency was achieved. Cells were then passaged by treatment with 0.05% (w/v) trypsin/0.53 mM EDTA (Sigma-Aldrich, Pooole, UK) and used for experiments or reseeded. All experiments were conducted using cells up to passage 4.

Titanium discs

Ti discs were kindly provided by Institut Straumann AG (Waldenburg, Switzerland). The discs were 1 mm thick and 15 mm diameter to fit into 24 well plates. Three different Ti surfaces: SMO, SLA and SLActive were examined. SMO cpTi discs were prepared using SiO grinding paper (grit size was 15–600 μm diameter) and then polished to a mirror. They were then treated with diamond paste in oil, before a final treatment with a SiO_2 suspension. For preparation of SLA, cpTi discs were alumina-blasted with large grit particles (average grit size of 250 μm) and then acid-etched with a hot solution of HCl/H_2SO_4 following a proprietary process of Institut Strauman AG. SLActive was produced following the same protocol as SLA, except after acid-etching, discs were rinsed in water under nitrogen protection then sealed in a glass tube (also under nitrogen protection) with an isotonic NaCl solution (pH 4–6).

Cell morphology

hMSCs were cultured on Ti surfaces for 3 or 24 h, then fixed in 3% glutaraldehyde in 0.14 M sodium cacodylate buffer (pH 7.3) at 4 °C overnight. They were then dehydrated using a graded series of alcohols (50%, 70%, 90% and 2×100% ethanol), washed with hexamethyldisilazane (TAAB Laboratories, Berkshire, UK) for 5 min and placed in a desiccator overnight. The Ti discs were then mounted onto stubs and sputter-coated with gold/palladium using a Polaron E5100 coating device (Polaron CVT, Milton Keynes, UK) and specimens viewed using a Cambridge Stereoscan S90B scanning electron microscope (Cambridge Instruments, Crawley, UK).

Cell attachment

Cell attachment was determined using the 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyltetrazolium bromide (MTT; Sigma-Aldrich) dye-reduction assay [25]. Briefly, cells were seeded onto Ti discs at a cell density of 1×10^5 cell per disc in 1 ml standard medium and cultured for 3 h, after which time non-adherent cells were gently washed off with PBS. Medium was replaced and 250 μl MTT was added per well and cells were incubated for 4 h at 37 °C. After this time solution was then discarded and 1 ml of extraction buffer (10% w/v sodium dodecyl sulphate/0.5 mol l^{-1} dimethylformamide; Sigma-Aldrich) was added to each disc and plates were incubated overnight at 37 °C. 100 μl aliquots of this solution were then transferred to a 96 well plate and absorbance of each well was assessed using a Dynex MRX spectrophotometer (Dynex Technologies, Billinghurst, UK) at 550 nm.

Cellular proliferation

Proliferation of hMSCs on Ti discs was also assayed using the MTT dye-reduction assay. Cells were seeded onto Ti discs at a cell density of 5×10^4 cell per disc in 1 ml standard medium and cultured for either 24 or 120 h. At these time points, 250 μl of MTT (5 mg ml^{-1}) was added to each disc and incubated at 37 °C for 4 h. The solution was then discarded and 1 ml of extraction buffer was added to each disc and plates were incubated overnight at 37 °C. 100 μl aliquots of this solution were then transferred to a 96 well plate and absorbance of each well was assessed as described above.

Flow cytometry

For determination of apoptosis, an apoptosis detection kit (Sigma-Aldrich) was used according to manufacturer's instructions. hMSCs were cultured on Ti discs (2×10^5cells/disc) for 24 or 120 h. Cells were then trypsinised, washed with PBS and resuspended in manufacturers binding buffer at 1×10^6cells/ml. 500 μl of cell suspension was then added to a flow tube and 5 μl annexin v-FITC and 10 μl propidium iodide was added to each cell suspension. Cells were then incubated in the dark at room temperature for 10 min, cell fluorescence was then measured immediately on a flow cytometer (BD Biosciences, Oxford, UK). hMSCs cultured on Ti surfaces were labelled with an antibody to Stro-1 and flow cytometry was conducted to address whether selection of cells of osteogenic potential occurred more favourably in response to roughness or wettability. For assessment of stro-1 positive cells on Ti surfaces, hMSCs were cultured on each surface for either 24 or 120 h. Cells were then trypsinised, washed with PBS and resuspended in 200 μl flow buffer (2% v/v FCS in PBS). 1 μg of mouse anti human stro-1 monoclonal antibody (MAB1038; R and D Systems, Abingdon, UK) was added and cells were incubated on ice for 1 h. The cells were then pelleted and washed twice with flow buffer, before being incubated with an Alexofluor rat anti mouse IgM 488 nm secondary antibody (1:200 in flow buffer; Invitrogen) for 30 min on ice.

Cells were then washed and analysed on a flow cytometer. A total of 10,000 events were recorded and total number of positive events was normalised against a negative control population of cells that was labelled only with the secondary antibody.

Gene expression analysis

hMSCs were cultured on Ti discs (2×10^5cells/disc) over a time course of 3, 24, 72 or 120 h. At these times, cells were trypsinised and RNA extracted using the RNeasy mini kit (Qiagen, Crawley, UK).

Samples were then processed using a commercially available service at the UCL Institute of Child Health Gene Microarray Centre, London,