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The effect of hydrofluoric acid treatment of titanium surface on nanostructural and chemical changes and the growth of MC3T3-E1 cells

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ABSTRACT

Fluoride-modification of dental titanium (Ti) implants is used to improve peri-implant bone growth and bone-to-implant contact and adhesion strength. In this study, the surface topography, chemistry and biocompatibility of polished Ti surfaces treated with hydrofluoric acid solution (HF) were studied. Murine osteoblasts (MC3T3-E1) were cultured on the different groups of Ti surfaces. Surfaces treated with HF had higher roughness, lower cytotoxicity level and better biocompatibility than controls. For short treatment times (40 and 90 s), fluorine was detected only within the first 5 nm of the surface layer (X-ray Photoemission Spectroscopy, XPS), whereas longer treatment time (120 and 150 s) caused fluoride ions to penetrate deeper (Secondary Ion Mass Spectrometry, SIMS). These results suggest that submerging Ti implants in a weak HF solution instigate time-dependant specific surface changes that are linked to the improved biocompatibility of these surfaces.

1. Introduction

In optimal cases, endosseous titanium (Ti) dental implants have reached the structural and mechanical strength necessary for a direct load of implant retained fixed partial dentures in the oral cavity [1]. However, an important goal in implantology is still to achieve a faster, stronger and more predictable bone-to-implant integration for early loading [2], even in bone of inferior quality and in the posterior maxilla as well as in bone weakened by disease [3].

The preferred body reaction of an implanted biocompatible endosseous device is osseointegration with the formation of a strong and mature bone matrix, creating a firm biomechanical bone–implant interface [4]. Bone cells attach to titanium surfaces through both chemical and mechanical bonds [5]. The first one includes ionic and covalent bonding between the biological environment and the adapted exposed surface [6]. The mechanical attachment is the physical interlocking of the strong mature bone matrix in the implant surface structure involving the presence of an appropriate implant surface topography [7–9].

Fluoride-modification of titanium dental implants has a documented potential for promoting implant attachment and improve bone response [6,10–15], reducing the healing time needed before loading [15,16], as well as significantly improve the bone-to-implant contact [16], and stimulating osteoblast gene expression at the implant surface [17]. A recent study showed that TiO_2 grit blasted cp Ti surfaces treated with HF enhances cell osteoblastic adhesion and expression of bone-specific mRNA [18]. Also, fluoride-treated implant show augmented trombogenic properties, resulting in a promoted fibrinogen activation and rapid coagulation [19].

One suggested mechanism of action for fluoride-treated implants is accelerated bone regeneration through a stimulatory effect on the bone cells. Another possibility is that fluoride in the surface makes a direct chemical bonding with the formed bone [3,20]. Additionally, surface structures at the nano-level caused by the treatment have also been suggested to have a positive effect on mechanical implant retention [21].

The aim of this study was to see if the structural and chemical changes of the titanium surfaces after various exposure times in weak hydrofluoric acid solution (HF) could be associated with the biological performance. The micro and nano-level topography were characterised, as well as the elemental chemical composition of the titanium surfaces. Surface topography and chemistry were linked to treatment duration. The HF concentration and the immersion time were selected based on previous experiments [22] as the most appropriate to slightly and gradually change the surface roughness

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and chemistry. To avoid the effect of micro roughness and in order to analyse surface changes at the nano-level, mirror polished surfaces were used.

In vitro experiments were performed to evaluate the biological response of MC3T3-E1 murine pre-osteoblast cells to the fluoride-modified titanium surfaces. Cytotoxicity, cell attachment and gene expression of osteoblast differentiation markers were analysed and related to the various surface characteristics.

2. Materials and methods

2.1. Titanium disks preparation and surface modification

Commercially pure (cp) titanium disks ($n = 75$) with a diameter of 6.2 mm and a height of 2 mm were grinded and polished (Phoenix 4000, Buehler GmbH, Duesseldorf, Germany) in seven sequences [20,23]. Silicon carbide papers from P800 to P4000, a porous neoprene for final polishing as well as the abrasive colloidal silica suspension (OP-S) were supplied by the same manufacturer (Struers GmbH, Willich Germany). After polishing, the disks were washed with NaOH at 40 vol.% and HNO_3 at 50 vol.% in an ultrasonic bath to remove contaminants, then washed with deionised water to reach a neutral pH, and stored at room temperature in 70 vol.% ethanol.

60 polished and cleaned titanium disks were submitted to fluoride-modification through HF treatment, whereas 15 disks were kept non-modified as control group. The 60 disks were divided into four groups of 15 each and immersed for 40, 90, 120 or 150 s (groups 1, 2, 3 and 4 respectively) in a 50 ml disposable high density polyethylene (HDPE) beaker filled with 0.2 vol.% HF at pH 2.1. Immediately after treatment, the disks were washed in autoclaved water for 10 s, air dried for 20 min in a bi-hepafiltered laminar sterile bench (Orion S-125 Safety, Kojair Tech Oy, Vilpulla Finland), and packed in autoclaved microcentrifuge tubes.

The grinding/polishing and washing steps were necessary to create uniform, clean surfaces for proper comparison between the groups.

2.2. Surface analyses

2.2.1. Surface topography

The micro and nano-level surfaces topography of the HF modified titanium disks were characterised by using three non-destructive techniques.

A blue light laser interferometer (Sensofar Plμ 2300, Terrassa, Spain) was utilised to scan 255 × 191 μm^2 areas with a 50× DI Nikon objective. Amplitude parameters (S_a, S_t, S_{sk} and S_{ku}), and fractal parameters (S_{dr} and S_{fd}) were calculated with advanced software (SensoMap Plus 4.1, Sensofar, Terrassa, Spain).

A Scanning Electron Microscope (Philips XL 30 ESEM, FEI Electron Optics, Eindhoven, The Netherlands) was used to detect surface changes at the limit micro, nano-level (100,000× of magnification).

An Atomic Force Microscope (MFP-3D Asylum research, Santa Barbara, U.S.) in alternative contact mode (AC mode) in air, with AC160 Olympus cantilevers (Olympus, Tokyo, Japan) were used to detect eventual surface changes at the micro and nano-level (scan size: 1 × 1 μm^2).

2.2.2. Surface chemistry

The surface elemental composition of one disk from each group was investigated by X-ray Photoemission Spectroscopy (XPS, Quantum 2000 Scanning XPS Microprobe, Physical Electronics, Chanhassen Minnesota, U.S.) and Secondary Ion Mass Spectroscopy (SIMS, Cameca IMS 7f, Paris, France). The crystalline configuration of the elements detected on the surfaces were analysed by X-ray diffraction (XRD, Panalatical X'pert, Almelo, The Netherlands).

With the XPS, monochromatic Al kα X-rays (1486.6 eV/15 kV) were shot at an angle of 45° toward the surface, with a spot size of 500 × 500 μm^2. The detection limit was about 0.1–0.5 atomic %, and the penetration of depth about 5 nm, therefore only the superficial surface layer.

The area analysed by the SIMS was 400 × 400 μm^2 using an 8 keV Cs^+ primary beam with 1 μA of current. Negative secondary ions were detected. The angle of incidence was set at 60 degrees. The depth profiles of the sputter craters were measured by blue light profilometry (Sensofar Plμ 2300, Terrassa, Spain) in order to convert sputter speed into erosion rate. The secondary ions were analysed until a penetrated depth in the surface structure of 4 μm, but only the first micron of data was shown.

The crystalline structure of the elements composing the surface was analysed by X-ray diffraction in Grazing Incidence (GI) mode on the Panalytical X'pert system, with parallel beam and incident angle of 0.9°. Data were collected from 8° to 80° in 2θ, with a step size of 0.03° in continuous mode.

2.2.3. Surface tension

The static contact angle was calculated following the sessile drop method using a video-based contact angle system (OCA 20, DataPhysics Instruments GmbH, Filderstadt, Germany) according to the Young–Laplace fitting at room temperature. The contact angle measurements were performed at three different places on one disk from each group using Ultra pure water (VWR, Oslo, Norway) as wetting agent.

2.3. Biological analyses

2.3.1. Cell culture

The murine osteoblast cell line MC3T3-E1 (DSMZ, Braunschweig, Germany) was used as in vitro model. Cells were routinely cultured at 37 °C in a humidified atmosphere of 5% CO_2, and maintained in alpha-MEM (PAA Laboratories GmbH, Austria) supplemented with 10% fetal calf serum (PAA Laboratories GmbH, Austria) and 50 IU penicillin/ml and 50 μg streptomycin/ml (Sigma, St. Louis, MO, USA). Cells were sub-cultured 1:4 before reaching confluence using PBS (PAA Laboratories GmbH, Austria) and trypsin/EDTA (Sigma, St. Louis, MO, USA). To test the surface modification of titanium implants with HF at different exposure times, disks from each group ($n = 6$) were placed in a 96-well plate and 10^4 cells were seeded on all the disks. The same number of cells was cultured in parallel in plastic in all experiments.

2.3.2. Cell viability

LDH activity was used as an index of cytotoxicity in the culture media. After 24 h, the culture media was collected, centrifuged at 500×g for 5 min at 4 °C, and the supernatant was stored at 4 °C. LDH activity was determined spectrophotometrically according to the manufacturer's kit instructions (Cytotoxicity Detection kit, Roche Diagnostics, Mannheim, Germany). Results were presented relative to the LDH activity in the medium of cells seeded on plastic (low control, 0% of cell death) and on plastic where 1% Triton X-100 was added (high control, 100% cell death), after subtracting the absorbance value obtained in the culture medium alone without cells (background control), using the equation: Cytotoxicity (%) = (exp.Value − low control)/(high control − low control) × 100.

2.3.3. Sample preparation prior to imaging with SEM

SEM analyses were performed to study the morphology of MC3T3-E1 cells grown on the surface of both control and test Ti disks. For this purpose, a first step of dehydration of the cells attached to the disk surface was done. The disks were placed in ethanol (Arcus, Oslo, Norway) at 30, 50, 70, 80, 90, 95 and 100%, immersed for 15 min at each step. A second step to substitute ethanol with acetone (Chemi-Teknik AS, Oslo, Norway) was fulfilled. Firstly with acetone:ethanol 25:75 for 15 min, then 50:50 for another 15 min, and finally acetone 100% for one hour.

The critical point drying of these samples were performed in an E3000 Critical Point Dryer (Quorum Technologies, Ashford, UK). A 10 nm thick gold-palladium layer was deposited on the samples with the use of a Cressington 108 Auto (Cressington Scientific Instrument Ltd, Watford, UK). SEM scans were taken (Philips XL 30 ESEM, FEI Electron Optics, Eindhoven, The Netherlands) to image the morphology of the cells attaching to the surfaces of the control disk and both a disk from group 2 and 4. Pictures at 600× of magnification were taken after 24 h and seven days.

2.3.4. Isolation of total DNA and RNA

Total DNA and RNA were isolated after seven days in cell culture using NucleoSpin® RNA/protein columns and DNA/RNA buffer set (Macherey-Nagel, Düren, Germany), following the instructions of the manufacturer.

2.3.5. Cell attachment

The number of cells attached to each disk after seven days was calculated after measuring DNA concentration at 260 nm using a Nanodrop spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) and taking into account that the DNA content in 10^6 mouse cells is about 5.8 μg [24].

2.3.6. Real-time reverse transcriptase polymerase chain reaction (RT-PCR) analysis

The same amount of total RNA (0.25 μg) from each sample was reverse transcribed to cDNA at 42 °C for 60 min in a final volume of 40 μl, using iScript cDNA Synthesis kit (BioRad, Hercules, CA, USA) that contains both oligo (dT) and random hexamers. Each cDNA was diluted 1/5 and aliquots were frozen (−20 °C) until the PCR reactions were carried out. Real-time RT-PCR analysis was performed for two housekeeping genes: 18S ribosomal RNA (18S rRNA), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and four target genes: collagen type I (coll-I), osterix (Osx), alkaline phosphatase (ALP) and osteocalcin (OC). Sequences of sense and antisense primers were as follows: 5′-GTAACCCGTTGAACCCCATT-3′ and 5′-CCATCCAATCGGTAGTAGCG-3′ for 18S rRNA; 5′-ACCCAGAAGACTGTGGATGG-3′ and 5′-CACATTGGGGGTAGGAAC-AC-3′ for GAPDH; 5′-AGAGCATGACCGATGGATTC-3′ and 5′-CCTTCTTGAGG-TTGCCAGTC-3′ for coll-I; 5′-ACTGGCTAGGTGGTGGTCAG-3′ and 5′-GGTAGGGAGCTGGGTTAAGG-3′ for Osx; 5′-AACCCAGACACAAGCATTCC-3′ and 5′-GAGAGCGAAGGGTCAGTCAG-3′ for ALP; 5′-CCGGGAGCAGTGTGAGC-TTA-3′ and 5′-TAGATGCGTTTGTAGGCGGTC-3′ for OC.

Real-time PCR was performed in the iCycler (BioRad, Hercules, CA, USA) using SYBR green detection. Each reaction contained 5 μl of cDNA (for 18S rRNA, GAPDH, OC, coll-I) or 10 μl of cDNA (ALP, Osx) 500 nm of the sense and antisense specific primers (for all, except for coll-I which was 300 nm and for Osx, 100 nm), 12.5 μl of 2X iQ SYBR Green Supermix in a final volume of 25 μl. The amplification program consisted of a pre-incubation step for denaturation of the template cDNA (3 min 95 °C), followed by 40 cycles consisting of a denaturation step (15 s 95 °C), an