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In vitro cellular response and in vivo primary osteointegration of electrochemically modified titanium

F. Ravanetti [a,*], P. Borghetti [b], E. De Angelis [b], R. Chiesa [d], F.M. Martini [c], C. Gabbi [a], A. Cacchioli [a]

[a] Anatomy Unit, Department of Animal Health, University of Parma, Via del Taglio 8, 43100 Parma, Italy
[b] Pathology Unit, Department of Animal Health, University of Parma, Via del Taglio 8, 43100 Parma, Italy
[c] Veterinary Surgery Unit, Faculty of Veterinary Medicine, Department of Animal Health, University of Parma, Via del Taglio 8, 43100 Parma, Italy
[d] Department of Chemistry, Materials and Materials Engineering "G. Natta", Politecnico di Milano, Via Mancinelli 7, I-20131 Milano, Italy

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ABSTRACT

Anodic spark deposition (ASD) is an attractive technique for improving the implant–bone interface that can be applied to titanium and titanium alloys. This technique produces a surface with microporous morphology and an oxide layer enriched with calcium and phosphorus. The aim of the present study was to investigate the biological response in vitro using primary human osteoblasts as a cellular model and the osteogenic primary response in vivo within a short experimental time frame (2 and 4 weeks) in an animal model (rabbit). Responses were assessed by comparing the new electrochemical biomimetic treatments to an acid-etching treatment as control. The in vitro biological response was characterized by cell morphology, adhesion, proliferation activity and cell metabolic activity. A complete assessment of osteogenic activity in vivo was achieved by estimating static and dynamic histomorphometric parameters at several time points within the considered time frame. The in vitro study showed enhanced osteoblast adhesion and higher metabolic activity for the ASD-treated surfaces during the first days after seeding compared to the control titanium. For the ASD surfaces, the histomorphometry indicated a higher mineral apposition rate within 2 weeks and a more extended bone activation within the first week after surgery, leading to more extensive bone–implant contact after 2 weeks. In conclusion, the ASD surface treatments enhanced the biological response in vitro, promoting an early osteoblast adhesion, and the osteointegrative properties in vivo, accelerating the primary osteogenic response.

1. Introduction

In the last decades endosseous biomaterials research has been focused on developing a novel surface modification of titanium and titanium alloys to achieve improved osteointegration [1,2]. Sandblasting, acid etching, titanium plasma spray deposition, sintering of titanium microspheres, and metallic filaments have been widely used for enhancing osteointegration of noncemented implants. By these approaches, the ability of the implant to bind to bone is improved through morphology optimization, which increases the roughness and provides a larger contact area between the implant and the tissue without significantly changing the chemical properties of the surface [3,4]. These surface treatments can improve osteointegration through a better mechanical interface but alone they do not provide a chemical interaction with bone, which would enable quicker healing. To overcome this limitation, biomaterials research has focused on osteoconductive materials, which can exert a physical, chemical or pharmacological effect on osteogenic activity. A titanium metal substrate can be coated with bioactive substances such as hydroxyapatite (HA), which is widely used and is usually applied by means of plasma spray techniques.

These kinds of coatings were found to chemically modify the implant surface and to promote bone apposition, accelerating chemical bonding between new bone and the implant surface [5–7]. The poor long-term performance of plasma-sprayed HA coatings has been linked to problems in the adhesion to the substrate and to an improperly controlled dissolution rate of the coatings, which depends on the level of HA crystallinity [8]. These concerns stimulated research for alternative deposition methods of HA coatings [9] and for the development of new approaches based on the nanoscale modification of the material surface [10]. Many and various techniques can be performed on titanium to enhance its biological response and tissue integration [11]. Among the many possible coating techniques [12], pulsed laser deposition [13], cathodic polarization [14], and magnetron sputtering [15] have been widely investigated and experimented. Among the electrochemical methods, an attractive technique that can be applied to titanium and titanium alloys to obtain a thin and porous surface layer enriched in calcium and phosphate is known as anodic spark

* Corresponding author. Tel.: +39 521032648; fax: +39 521032795.
E-mail address: francesca.ravanetti@nemo.unipr.it (F. Ravanetti).

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deposition (ASD) [16,17]. This is an electrochemical technique that can create a microporous morphology on titanium and titanium alloy surfaces by modifying the surface oxide layer. The first studies on ASD were performed by Kurze et al. [16,17] and then optimized by other research groups [18–23].

Since 2003 a promising surface treatment of titanium, involving ASD in combination with chemical treatments, has been under development [24,25]. In particular, the method consists of a double ASD process performed in solutions containing phosphate ions and calcium ions followed by a final alkali immersion treatment.

To test the osteoblast–biomaterials interaction in vitro, a variety of cell lines (SaOS-2, MG63, and MC3T3) have been used in several studies. Immortalized cell lines easily maintain phenotypic expression without senescence over a long period of time and after many passages; however, cell lines do not always exhibit stringent growth control and gene differentiation expression [26], thus supporting the biological relevance of primary osteoblast cultures. For this reason, despite the many previous studies [27–31] performed on this surface modification treatment of titanium using cell lines MG63 and SaOS-2, the present paper aims to evaluate biocompatibility using primary human osteoblasts as an in vitro model, which is believed to be much more correlated to the in vivo conditions [32].

Since, by definition, no bone is present on the surface of the implant when placed, the implant surface has to be colonized by bone cells before matrix formation can begin [5]. As to the implantable devices, studies on different animal models have concluded that the first steps of osteointegration, with new bone tissue growth at the bone–implant interface, takes place within 2 weeks after surgery [33,34].

Considering the in vivo performance of these surfaces, the present work aims to evaluate the osteogenic primary response to implanted surfaces within a short experimental time frame of 4 weeks, an extension on previous osteointegration studies [30,35–38]. For this purpose, the White New Zealand rabbit was chosen as the animal model, and histology, static and dynamic histomorphometry were considered. It is well known that rabbit bone is characterized by fast growth and rapid turnover [39]; this evidence suggested the choice of a short experimental time frame to better focus on the biological effect of the ASD-modified surfaces on primary osteogenic activity.

In the present study, the histological analysis is supported by quantitative histomorphometric measurements. In particular, a complete assessment of osteogenic activity is achieved by estimating static and dynamic histomorphometric parameters at several time points within the considered time frame. By this approach it is possible to evaluate the osteogenic activity both during and at the end of the selected experimental time.

2. Materials and methods

2.1. Titanium sample preparation

All titanium samples used in this work were obtained from commercially pure, grade 2 titanium (ISO 5832-2). Titanium sheets were used to prepare discs (12 mm diameter, 0.5 mm thick), and titanium bars were used to prepare cylindrical implants (3 mm diameter, 13 mm length); these were used for in vitro and in vivo analysis, respectively. All samples were cleaned by ultrasonic rinsing (Branson Automatic Cleaner) in acetone (RPE Carlo Erba) for 5 min, then in distilled water for a further 5 min, in order to degrease and clean the surface of contaminants.

Three different surface treatments were tested:

- ETC (acid-etching treatment) surfaces were obtained by a double-step etching process. An initial etching was carried out in a solution of 1 M NaOH, mixed with 2% v/v H_2O_2 kept at 80 °C. The second etching step consisted of an acid treatment performed at 28 °C for 1 h. After each etching step, specimens were rinsed three times for 5 min in distilled water by ultrasonic rinsing.
- BS (BioSpark™) and OS (OsseoSpark™) surface treatments involved two consecutive ASD processes carried out in different electrolyte solutions at different voltage ranges, and followed by an alkali etching processes. The first ASD was performed in a solution containing phosphate anions and calcium cations; the second ASD was performed in a solution containing only calcium cations. BS and OS differ only for a final chemical treatment: BS was obtained in NaOH solution, while OS was obtained by a final chemical treatment in KOH solution.

Samples were sterilized by ethanol (70% v/v in distilled water) followed by UV irradiation (254 nm) for the in vitro analysis and by γ-rays (25 kGy dose) for the in vivo study.

2.2. In vitro biological study

2.2.1. Cell culture

The human primary osteoblasts, kindly provided by Dr. C. Galli, were obtained from jaw bone specimens resulting from a surgical intervention on 5-year-old patient after the parents signed an informed consent, and isolated as previously described [40,41]; this was conducted in accordance with the Helsinki declaration of 1975, as revised in 2000 [42,43]. Briefly, bone fragments were cleaned from soft tissue remnants, thoroughly rinsed in phosphate-buffered saline (PBS), and maintained in Dulbecco's modified Eagle's medium (D-MEM, Sigma–Aldrich) containing 10% fetal bovine serum (FBS, EuroClone Ltd.), glutamine (4 mM), streptomycin (100 μg ml^{-1}) penicillin (100 U ml^{-1}) at 37 °C in modified atmosphere (5% CO_2, 95% air) until cell outgrowths appeared. Once confluent, cells were harvested by trypsin–EDTA 0.25% (Sigma–Aldrich) seeded in Petri dishes and fed three times a week with D-MEM complete medium with 250 μM ascorbic acid to maintain a differentiated osteoblastic phenotype. The cell populations were further characterized by evaluating alkaline phosphatase (ALP) activity [44] and osteocalcin production tested by enzyme-linked immunosorbent assay (ELISA osteocalcin kit, DAKO) using a normal skin fibroblast population as negative control. Afterwards the cells were frozen in liquid nitrogen and stored; for each experiments the cells were thawing, seeded and at confluence, the cells were harvested using trypsin–EDTA 0.25% and seeded in 24-well plates directly on the titanium surfaces at a density of 7×10^3 cells/100 μl/discs in D-MEM 10% FBS adding, after 2 h, 1 ml/well of complete medium, then were incubated for the time specified for each single analysis.

2.2.2. Cell proliferation

After 1, 2, 4, 7, and 14 days of culture, surfaces were washed with phosphate-buffered saline (PBS) and then incubated with 0.25% trypsin–EDTA for 7 min in order to obtain cell detachment. Obtained cells were counted with a Bürker hemocytometer. For cell viability evaluation, detached cells were incubated for 5 min with Trypan Blue and then counted. Complete removal of the attached cells from the surfaces with trypsin–EDTA was confirmed by scanning electron microscopy (SEM). Values reported in Fig. 1a represent the mean ± standard deviation of three independent analyses performed in triplicates.

2.2.3. MTT assay

Cell viability was evaluated by measuring the mitochondrial dehydrogenase activity using a modified MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide) reduction assay (Roche Applied Science). Briefly, the cells on day 1, 2, 4, 7, and 14