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Comparative molecular assessment of early osseointegration in implant-adherent cells

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ABSTRACT

Objective: The objective of our study is to identify the early molecular processes involved in osseointegration associated with a micro roughened and nanosurface superimposed featured implants.
Materials and methods: Thirty-two titanium implants with surface topographies exhibiting a micro roughened (AT-II) and nanosurface superimposed featured implants (AT-I) were placed in the tibiae of 8 rats and subsequently harvested at 2 and 4 days after placement. Total RNA was isolated from cells adherent to retrieved implants. A whole genome microarray using the Affymetrix Rat Gene 1.1 ST Array followed by validation of select genes through qRT-PCR was used to describe the gene expression profiles that were differentially regulated by the implant surfaces.
Results: While significant differences at the gene level were not noted when comparing the two-implant surfaces at each time point, the microarray identified several genes that were differentially regulated at day 4 vs. day 2 for both implant surfaces. A total of 649 genes were differentially regulated at day 4 vs. day 2 in AT-I and 392 genes in AT-II implants. Functionally relevant categories related to ossification, skeletal system development, osteoblast differentiation, bone development, bone mineralization and biomineral tissue development were upregulated and more prominent at AT-I (day 4 vs. day 2) compared to AT-II. Analysis of the downregulated gene lists (day 4 vs. day 2) with average fold change >2 (were not statistically significant) revealed the biological processes involved with the inflammatory/immune response gene expression. The number of genes that were associated with the inflammatory/immune response category was greater for AT-I than AT-II.
Conclusions: The presence of nanosurface features modulated in vivo bone response. Gene regulation implicating osteogenesis as well as the inflammatory/immune responses that occur as a function of surface topography may affect bone mass shortly after implant placement.

Introduction

The placement of endosseous implants is a common treatment option to treat edentulism. The success of dental implants is based on the concept of osseointegration introduced by Branemark [1]. Despite the high success rates achieved, implant failures that mandate implant removal do occur. Factors attributing to implant failures include local and systemic conditions such as reduced bone volume, reduced bone density, periodontitis, and impaired wound healing (e.g., diabetes, smoking, osteoporosis, radiation therapy, chemotherapy) [2–6]. Efforts to enhance osseointegration of dental implants allowing for faster prosthetic rehabilitation and improved success rates in clinically challenging situations, included modifications to the physical and chemical properties of the implant surfaces. It is well demonstrated that implants with moderately rough surfaces (average height deviation of 1–2 μm) [7] enhance the rate and quality of osseointegration with greater bone-to-implant contact and higher resistance to torque removal [8–12].

In contrast to micron-features of alloplastic materials, bone is composed of constituent nanofeatures [13]. Nanostructured materials are those with features less than 100 nm in at least one dimension [14]. Simulation of nanofeatures at implant surfaces has shown favorable bioactivities with titanium surfaces [15]. Enhanced in vivo bone responses to implants with nanofeatures compared to machined or micro roughened surfaces measured by histological and biomechanical means have been shown in several animal models (e.g., rabbits, dogs, rats) [16–23]. The topography of titanium surface at the nanolevel has been reported to modulate differentiation and proliferation, and increase expression of osteogenic markers [17,24,25]. Yet, the exact role of nanosurface topography on the molecular events occurring early in the process of osseointegration remains poorly understood. Prior studies mainly focused on select target genes; typically markers for osteoblasts including *Cbfa1/Runx2* [26,27], *Osterix*

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(Osx) [26–29], *Osteocalcin (Ocn)* [30–34], *Osteopontin (Opn)* [35–38], *Collagen I* [39–42] and *Alkaline phosphatase (Alp)* [27,29,43,44].

It is of interest to further investigate the overall gene expression profiles by implant adherent cells during the early phase of osseointegration. The advent of gene expression microarrays allows the rapid and high-throughput quantification of thousands of genes simultaneously [45]. Microarray analysis may unveil the regulation of individual genes that might not be identified otherwise. These molecular details may represent targets for future therapeutic improvement. Most recently, this approach has been applied in vitro in the analysis of MG63 osteoblastic-cell response to a nanoporous Ti6AL4V surface (produced by blasting with tricalcium phosphate and light nitric acid treatment; nanoPORE, Out-Link, Sweden and Martina, Due Carrare, Padova, Italy) [46]. However, we recognize that the biological environment in vivo is very different from the in vitro conditions and contains a variety of cells that each can respond to the implant surface and produce several cytokines and growth factors influencing each other's behavior. Few whole genome-wide profiling studies have been reported using in-vivo models [47–49]. Profiles of gene expression of in vivo bone healing with or without titanium implants (osteotomy sites) were described in a rat model [48,50] at different time points with 1 week time point representing the earliest analysis [50]. Moreover, Donos et al. [47] reported on the gene expression profiles associated with a moderately rough surface (SLA) compared to a chemically modified moderately rough surface (SLActive) in a human model. The microarray analysis was carried out at 4, 7 and 14 days post surgery.

Various methods have been developed in order to create a nanosurface. Typically applied techniques include lithography, ionic implantation, anodization, acid etching, alkali treatment, peroxidation and sol–gel deposition [15,51]. Recently, Johansson et al. [52], reported on a newly developed nanosurface (AT-I) produced by sequential chemical treatment with oxalic acid and hydrofluoric acid preceded by blasting with titanium oxide particles. These implants were tested in a rabbit model and compared with implants with micro-roughened surface (AT-II). The results demonstrated greater 2D bone-to-implant contact and 3D removal torque values for the nanosurface implants. The objective of the present study was to further investigate the effect of this newly developed nanosurface (AT-I) topography imposition on the whole genome expression profiles at early time points in the process of osseointegration using an in-vivo rat model and compare it to those of the micro-roughened surface (AT-II).

Materials and methods

Implants

Newly developed implant surfaces of commercially pure titanium grade IV screws (2.0 mm × 3.0 mm) were used in this study. The test samples were manufactured per protocol described previously by Johansson et al. [52]. Briefly, the samples were degreased, blasted with titanium oxide particles and rinsed in sterile water. These samples were then treated in a different sequential process resulting in two different surface structures. One sample group was treated in oxalic acid, named AT-II, while the other group was treated in oxalic acid and hydrofluoric acid sequentially (AT-I). All implants were washed by sterile water and beta-sterilized.

Surface characterization

The implant surfaces were examined by a high-resolution scanning electron microscope [ESEM XL30, FEI Company, 5651 GG Eindhoven, The Netherlands]. The three-dimensional surface parameters were determined using optic interferometry (MicroXam, Phase-Shift, Tucson, AZ). Three specimens of each surface type were analyzed, and each specimen was analyzed in three areas. Errors of form were removed using a Gaussian filter size of 50 × 50 mm. Surface roughness values were reported by the S_a and S_{dr}% values per the suggested guidelines by Wennerberg A and Albrektsson T for the topographic evaluation of implant surfaces [53].

The Sa represents the average height of the analyzed area.
The Sdr represents the developed interfacial area ratio %.

The chemical nature of the surfaces was examined using X-ray photoelectron spectroscopy (XPS). A Quantum 2000 ESCA Scanning Microscope A Quantum 2000 ESCA Scanning Microscope (Physical Electronics, Chanhassen,MN, USA) with an X-ray source of monochromatic A1*K*a was used to obtain a spectrum for each surface. The mean numbers and standard deviation (SD) were deduced from measurements of three implants per group with two regions per implants resulting in n = 6 per implant type. The wettability was measured by using the sessile drop technique on titanium coins with the AT-I and AT-II surfaces.

Model

A rat tibia model of osseointegration was used [18,54]. All procedures were approved by the Institutional Animal Care and Use Committee at the University of North Carolina, Chapel Hill (IACUC ID: 10–127.0). Eight male Sprague Dawley rats (326–350 g) were purchased from Harlan Laboratories and acclimated for 7 days prior to initiation of studies. Anesthesia was achieved using ketamine/xylazine (80–100 mg/kg and 10 mg/kg respectively) along with supplemental local anesthesia (lidocaine 2% with epinephrine (1:100,000)). The dorsal/medial aspect of the tibiae was identified, shaved, and disinfected using betadine and 70% ethanol scrub. Using aseptic technique, a full thickness myocutaneous flap was made and carefully retracted to reveal the medial aspect of the tibia bone. With sterilized stainless steel burs, two drill holes were created with copious irrigation. The drill holes were made approximately 5 mm apart. Two implants (cp titanium) were placed in each tibia to provide sufficient RNA for each experimental sample. For every time point (days 2 and 4), 4 rats were used. Each animal received two AT-I implants in one tibia and two AT-II implants in the contralateral tibia (randomly distributed). The periosteum was adapted over the site using interrupted 4–0 chromic gut sutures for subcuticular closure. The skin was then closed using vicryl sutures. Animals were monitored continuously following surgery. Ambulation was the defined criteria for immediate recovery. A postoperative analgesic was provided for 48 h after surgery, by means of subcutaneous ketoprofen injections (5 mg/kg) once daily. At the time of sacrifice, each animal was placed in a CO_2-saturated chamber. Death was assured by thoracotomy and severance of the inferior vena cava for exsanguination.

RNA isolation

At 2 and 4 days following surgery, animals were euthanized. Immediately thereafter, the tibia sites were isolated and the implant site was exposed using sterile technique and the entire tibiae were harvested and the implants were explanted by fracture of the tibia. For evaluation of mRNA expression in cells adherent to explanted endosseous implant surfaces, the implants were rinsed in cold PBS immediately following retrieval and then placed into 1000 μl of Tri-reagent. Total RNA was isolated from the lysates using the standard Tri-reagent protocol and collected by ethanol precipitation. This was followed by purification using RNeasy MinElute Clean up kit (Qiagen, Valencia, CA, USA). RNA was assessed for quality and quantity using a bioanalyzer (Agilent, Santa Clara, CA, USA) and nanodrop ND-1000 spectrophotometer (Nanodrop, Wilmington, DE) respectively. Samples were processed and hybridized to the Affymetrix Rat Gene 1.1