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Improved implant osseointegration of a nanostructured titanium surface via mediation of macrophage polarization

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ABSTRACT

The use of endosseous implanted materials is often limited by undesirable effects that may be due to macrophage-related inflammation. The purpose of this study was to fabricate a nanostructured surface on a titanium implant to regulate the macrophage inflammatory response and improve the performance of the implant. Anodization at 5 and 20 V as well as UV irradiation were used to generate hydrophilic, nanostructured TiO_2 surfaces (denoted as NT5 and NT20, respectively). Their surface characteristics and *in vivo* osseointegration as well as the inflammatory response they elicit were analyzed. In addition, the behavior of macrophages *in vitro* was evaluated. Although the *in vitro* osteogenic activity on the two surfaces was similar, the NT5 surface was associated with more bone formation, less inflammation, and a reduced $CD68^+$ macrophage distribution *in vivo* compared to the NT20 and polished Ti surfaces. Consistently, further experiments revealed that the NT5 surface induced healing-associated M2 polarization *in vitro* and *in vivo*. By contrast, the NT20 surface promoted the pro-inflammatory M1 polarization, which could further impair bone regeneration. The results demonstrate the dominant role of macrophage-related inflammation in bone healing around implants and that surface nanotopography can be designed to have an immune-regulating effect in support of the success of implants.

1. Introduction

Implantable materials are indispensable in modern regenerative medicine, specifically in the repair of bone defects. However, various types of implantable biomaterials display various undesirable effects *in vitro* and *in vivo*. For example, hydroxyapatite particles show excellent osteogenic activity *in vitro* but are unable to induce sufficient bone formation *in vivo* when mixed with bone marrow mesenchymal stem cells (bMSCs) [1]. Biomedical stainless steel shows good biocompatibility with osteoblasts *in vitro* but readily elicits formation of a surrounding inflammatory fibrous capsule *in vivo* [2]. Frequent early implant failure (47%) most often due to inflammation occurs even for Ti implants, which are the most widely used implant type in the clinic and the most recognized as ideal endosseous implantable devices [3]. Such findings remind us of the divergence of results obtained *in vitro* versus *in vivo* and inspire us to elucidate the causes of such differences in host responses to implanted materials.

The host immune response against implanted materials is a key factor contributing to the differences between *in vivo* and *in vitro* implant performance. Once implanted, all surgical implants adsorb proteins and simultaneously elicit an inflammatory foreign body reaction (FBR), which begins as an acute sterile inflammatory response and develops into a chronic fibrotic response that represents the first steps of tissue repair [4]. Macrophages and their precursor monocytes, key members of innate immunity, first recognize and attack foreign objects in addition to secreting inflammatory mediators to initiate inflammation [5]. Macrophages show remarkable plasticity with respect to the spatial structure of implants. For example, implantation of synthetic biomaterials such as polycaprolactone and silicone as films over solid shapes generally leads to a typical FBR with chronic inflammatory fibrotic

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encapsulation occurring as soon as 1 month post-implantation. However, when these same biomaterials are fabricated with uniform porous structures (30–40 μm pores), their implantation can induce vascularized tissue–biomaterial integration rather than encapsulation [6,7]. This phenomenon has been linked to differences in macrophage polarization, a process that plays an important role in the long-term performance of implants [8,9]. According to different phenotypes, polarized macrophages are divided into classically active M1 and alternatively active M2 types. M1 macrophages secrete cytokines such as interleukin (IL)-1β, IL-6, IL-8, inducible nitric oxide synthase (iNOS), tumor necrosis factor (TNF)-α, and interferon (IFN)-γ to promote inflammation and recruitment of polymorphonuclear neutrophils (PMNs), whereas M2 macrophages produce anti-inflammatory mediators such as IL-1Ra, IL-4, IL-10, and arginase-1 (ARG) to promote the resolution of inflammation and tissue regeneration. M2 macrophages also produce growth factors including vascular endothelial growth factor A, platelet-derived growth factor (PDGF), and transforming growth factor (TGF)-β to support the migration, homing, and osteogenic differentiation of bMSCs [10–14]. The presence of a greater proportion of M1 macrophages relative to M2 macrophages is highly correlated with the failure of artificial joints [15], which suggests that even though an implant may be tolerable to a host, a low-level, prolonged, and even chronic FBR elicited by macrophages can lead to eventual osteolytic loosening of implants.

For many years, biomaterial design focused on inert materials that could avert the host immune response by avoiding cell–material interactions. However, the control of specific cell–material interactions has proven beneficial for improving implant performance [16,17]. Because rapid physiological resolution of inflammation is beneficial for bone healing [18,19], the design of biomaterials for bone implants should attempt to use the inflammatory response to improve bone–implant integration while also avoiding perpetuation of chronic inflammation that results in loss of intended implant function [20]. Several strategies can be applied to modulate the inflammatory response elicited by implanted biomaterials. For example, whereas drug pre-coating often disrupts homeostasis within the peri-implant micro-environment, modification of the implant surface characteristics may offer an approach to regulate the inflammatory response and tissue healing due to the non-immunogenicity and durability of the implant. Previous studies have revealed that variation of the surface roughness and topography of implants can mediate altered macrophage functions such as survival, adhesion, and secretion [21–23]. Moreover, implant surfaces with unordered nanoscale roughness more strongly regulate inflammatory and osteogenic gene expression than do implant surfaces with micro-scale roughness [24]. In addition, the introduction of parallel gratings with widths ranging from micron to sub-micron scales (250 nm–2 μm) affects macrophage behavior, but with only a weak correlation between grating width and macrophage response [24,25]. These findings suggest that macrophages are sensitive to a certain nanoscale structure of implant surfaces, and we hypothesize that activated macrophages can further influence bone–implant interactions via M1/M2 polarization.

To test this hypothesis, Ti samples were anodized at 5 and 20 V to form multi-tubular, nanostructured surfaces on Ti implants (denoted as NT5 and NT20, respectively) in this study. Using a combination of *in vitro* histological analysis methods, including *in situ* semi-quantitative monocyte/macrophage morphologic analysis, flow cytometry, and enzyme-linked immunosorbent assay (ELISA), the host bone tissue and inflammatory responses to Ti implants with modified surfaces were characterized. The objectives of this study were to advance our understanding of the influence of implant surface topography on the host immune response and to provide a promising approach for improving bone–implant integration through immunomodulation.

2. Materials and methods

2.1. Nanostructured TiO_2 surface fabrication and characterization

Nanoscale textured Ti sheets were prepared using a multi-step procedure. Pure titanium (99.9%, Grade 1, Northwest Institute for Nonferrous Metal Research, Xi'an, China) was first machined to form circular disks (15 mm in diameter and 1 mm in thickness) and Ti screw implants (2.8 mm in diameter and 6 mm in length). Experimental samples were polished with 1500- to 8000-grit SiC sandpaper (Matador, Germany) and then subjected to ultrasonic cleaning (acetone, ethanol, and de-ionized water in sequence for 15 min each). Afterwards, samples were anodized in an aqueous electrolyte solution containing 0.5 wt% hydrofluoric acid and 1 M phosphoric acid at 20 °C for 1 h with a direct current power supply and a platinum cathode set at 5 or 20 V to fabricate the nanostructured, textured surfaces denoted as NT5 and NT20, respectively [26]. Polished Ti (P) samples served as the control group. All Ti samples were annealed at 450 °C and subjected to sonication again. Then, N_2 gas was used to dry the samples at 50 °C. Ti samples were sterilized by UVA/C irradiation ($\lambda = 365$(A)/254(C), Philips, Poland) at a 50 mm distance for 1 h. The morphology and roughness of the prepared surfaces were inspected by field emission scanning electron microscopy (FE-SEM; S-4800, Hitachi, Japan) and atomic force microscopy (AFM; Dimension Icon, Bruker, Germany). Using double distilled water, the hydrophilicity of the samples was assessed by analyzing water drop shape using the DSA1 System (Kruss, Germany). Solutions of 1 wt% bovine serum albumin (BSA; Sigma–Aldrich, USA), 1 wt % fibronectin (FN; Sigma–Aldrich), and 10% fetal calf serum (FCS; Gibco, USA) were used to investigate adsorption of BSA and FCS using the MicroBCA assay (Pierce, USA) [27] and of FN by ELISA (R&D Systems, USA). Prior to the addition of suspended cells for culture, Ti samples were placed in wells of 24-well plates (Corning, USA).

2.2. Behavior of human bMSCs on nanostructured TiO_2 surfaces

2.2.1. Human bMSC separation and culture

Human bMSCs were isolated from three healthy donors as follows. After maxillary third molar extraction, 0.5 ml bone marrow blood was obtained from the tooth socket and diluted 100% with RPMI 1640 medium (Cellgro, Corning, USA). One milliliter of diluted blood was layered over 3 ml Lymphocyte Separation Medium (LSM; MP, USA) in a 15-ml conical polystyrene tube (Corning, USA) and centrifuged at 450 g for 25 min without breaking at room temperature. The cells in the interphase band (buffy coat) were collected and resuspended at 1×10^6 cells/ml in α-Minimal Essential Media (α-MEM; Gibco, USA) containing 5% FCS (Gibco, USA) and 1% penicillin-streptomycin. Cells were seeded in 75-cm^2 culture flasks and then incubated in a humidified atmosphere of 5% CO_2 at 37 °C for 1 h. Cells that did not attach were discarded, and those that attached (including bMSCs and monocytes) were cultured. Cells were passaged using 0.1% trypsin (Sigma–Aldrich, USA). Because the monocytes are difficult to remove from the plate using trypsin, the bMSCs could be purified through passaging.

2.2.2. Biological responses of bMSCs on nanostructured TiO_2 surfaces

bMSCs at passages 4–7 were seeded onto the prepared Ti circular samples (2×10^4 cells/well) in 24-well plates and cultured for 0.5, 1, or 2 h or for 1, 3, or 7 days. For laser scanning confocal microscopy (LSCM; FV1000, Olympus, Japan) observation, samples were fixed in 4% paraformaldehyde and stained with fluorescein isothiocyanate (FITC)-phalloidin (1 μg/ml, Sigma–Aldrich) and DAPI. After glycerol mounting, samples were observed by LSCM. For FE-SEM observation, samples were fixed in 3% glutaraldehyde and dehydrated in a graded ethanol series (30%, 50%, 70%, 90%, and 100%). After lyophilization, desiccation, and platinum sputter-coating, samples were observed by FE-SEM (S-4800, HITACHI, Japan). The proliferation of bMSCs was assessed using the MTT assay. The osteogenic differentiation of bMSCs was measured by staining of alkaline phosphatase (ALP), mineralized extracellular matrix (ECM), and collagen. The procedures were described in detail previously [26]. Also, quantitative PCR (qPCR) was used to detect the mRNA expression of osteogenic genes such as ALP, bone morphogenetic protein 2 (BMP2), Runt-related transcription factor 2 (RUNX2), collagen 1 (COL1), osteopontin (OPN), and osteocalcin (OCN). Cells were collected for each condition, and total RNA was isolated using the RNAisoPlus system (TaKaRa, Japan). Aliquots of 1 μg total RNA were translated to cDNA using the PrimeScript™ RT reagent kit (TaKaRa). qPCR was performed using FastStart Universal SYBER Green Master (Roche, USA) on a CFX96™ PCR System (Bio-rad, USA). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a housekeeping gene, and the primers used are listed in Table 1.

2.3. In vivo implantation and histological analysis

2.3.1. Morphological observation and analysis of bone healing around implants

Eight-week-old male Sprague Dawley rats (specific pathogen-free) were obtained from the Lab Animal Centre of the Fourth Military Medical University. All rats were administered pentobarbital sodium (10 mg/100 g body weight, Sigma–Aldrich) via intraperitoneal injection as general anesthesia and lidocaine for local anesthesia (Sigma–Aldrich). The bone was exposed with a 1.5-cm incision through