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Soft tissue response to titanium dioxide nanotube modified implants

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ABSTRACT

Titanium is widely used clinically, yet little is known regarding the effects of modifying its three-dimensional surface geometry at the nanoscale level. In this project we have explored the in vivo response in terms of nitric oxide scavenging and fibrotic capsule formation to nano-modified titanium implant surfaces. We compared titanium dioxide (TiO_2) nanotubes with 100 nm diameters fabricated by electrochemical anodization with TiO_2 control surfaces. Significantly lower nitric oxide was observed for the nanostructured surface in solution, suggesting that nanotubes break down nitric oxide. To evaluate the soft tissue response in vivo TiO_2 nanotube and TiO_2 control implants were placed in the rat abdominal wall for 1 and 6 weeks. A reduced fibrotic capsule thickness was observed for the nanotube surfaces for both time points. Significantly lower nitric oxide activity, measured as the presence of nitrotyrosine ($P < 0.05$), was observed on the nanotube surface after 1 week, indicating that the reactive nitrogen species interaction is of importance. The differences observed between the titanium surfaces may be due to the catalytic properties of TiO_2, which are increased by the nanotube structure. These findings may be significant for the interaction between titanium implants in soft tissue as well as bone tissue and provide a mechanism by which to improve future clinical implants.

1. Introduction

It is well established that implanted biomaterials in soft tissue induce a cell-mediated inflammatory response and fibrotic encapsulation [1]. Immediately following implantation inflammatory processes are inherently present at biomaterials surfaces during wound healing events [2]. ED1-expressing macrophages recruited to the implant surface during early stage wound healing produce nitric oxide (NO) which is a key indicator of pro-inflammatory signal transduction in the inflammatory response and antimicrobial defense [3,4]. Oxygen is reduced by a pathway in inflammatory cells that generates superoxide anion and reactive oxygen intermediates [5]. Further degradation may yield hydroxyl radicals, singlet oxygen, and hydrogen peroxide. Such inflammatory cells and processes are always found at the implant surface. Therefore, the ability of a biomaterial to interact with inflammatory cells, and reactive oxygen and nitrogen species (i.e. biologically relevant radicals), may be relevant for the host response.

Titanium (Ti) is well established as a biomaterial for bone anchorage but is also used in soft tissues, e.g. in pacemakers, neurological implants, and metabolic sensors [6–8]. Ti has been used in a variety of clinical applications because it is corrosion resistant, light weight, and its surface is covered by a layer of titanium dioxide (TiO_2) believed to contribute to its overall biocompatibility [9]. This approximately 5 nm thick oxide layer forms spontaneously in air or water and exhibits favorable thermodynamic properties, and low ion formation and electrical conductivity in the physiological environment [10]. TiO_2 exhibits unique semiconductor-like properties which allow for the surface to change from Ti^{4+} to Ti^{3+}. Studies have shown that TiO_2 has the ability to inhibit reactive oxygen species involved in the inflammatory response [2,11,12]. The semiconductor-like properties of TiO_2 may act as an electron donor or acceptor and thereby actively participate in catalytic reactions with biologically relevant radicals.

We hypothesized that an increased TiO_2 surface area would exhibit greater catalytic degradation of nitric oxide species. One approach to increase the TiO_2 surface area is by modifying the implant surface with nanotube structures [13–15]. Specifically, TiO_2 nanotubes fabricated by electrochemical anodization provide patterned, strongly adherent nanostructures with an increased surface area compared with planar titanium [13]. In this study we have investigated the ability of the TiO_2 nanotube surface to:

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(1) quench nitric oxide in solution in a cell-free assay; (2) elicit fibrotic capsule formation in soft tissue. Minimally rough (Sa <1 μm) TiO_2 and SiO_2 surfaces were used as a control for the cell-free assays. For the in vivo studies a minimally rough (Sa <1 μm) TiO_2 surface and the nanotube modified surface were implanted in the abdominal walls of rats. The soft tissue response was measured in terms of fibrotic capsule thickness, macrophage density, and NO level using immunohistological methods.

2. Materials and methods

2.1. Surface modifications

All TiO_2 surfaces used in this study were modified with either nanotubes or by grit blasting. The surface was modified with a TiO_2 nanotube structure as follows. Vertically aligned TiO_2 nanotubes were fabricated by electrochemical anodization as described by Oh et al. [13]. Briefly, TiO_2 samples were cleaned for 5 min in 5.5 M nitric acid (HNO_3) containing a few drops of hydrofluoric acid (HF), rinsed in distilled water, and then dried at 60 °C. TiO_2 nanotubes were generated by anodization in 0.5% HF solution at 20 V for 30 min at room temperature, followed by annealing at ~550 °C, which produced anatase phase nanotubes with high bonding strength to the substrate [13]. The contact angle for the nanotubes, which is a measure of the hydrophilicity and surface energy, was 4° [16].

The TiO_2 control surface was grit blasted using size 22 mesh TiO_2 powders with a pore size of 0.8 μm (Alfa-Aesar, Ward Hill, MA). The particles struck the implant surface at a 90° angle from a blasting distance of approximately 30 mm. Each sample was blasted horizontally and vertically for a 10 s period at a pressure of 0.2 MPa. Following surface modification all samples were verified by scanning electron microscopy (SEM). The Ti control was more hydrophobic than the nanotubes, with a contact angle of 54° [16]. The bulk Ti sheets with a native TiO_2 oxide layer had a chemical composition analogous to that of the TiO_2 nanotube surface. Prior to experimentation the samples were autoclaved at 121 °C for 30 min and kept sterile.

2.2. Nitric oxide assay

A Ti sheet (0.25 mm thick, 0.5 × 5 cm, 99.5% pure, Alfa-Aesar, Ward Hill, MA) was subjected to either nanotube or grit blasted surface modifications as previously described. SiO_2 (MTT Crystals, Richmond, CA) plates with the same dimensions were used as a control. Four samples of each surface (nanotube, grit blasted, and silica) were placed in a 24-well plate. A NO donor DPTA-NO (Cayman Chemicals, Ann Arbor, MI) was used at 80 μmol in buffer at room temperature for 20 min to prepare a concentrated NO sample solution, the concentration of which was measured with a commercial colorimetric assay kit (Neogen Co., Lexington, KY). In brief, 0.5 ml of the sample solution was placed in each well. Nitrate reductase converted the existing nitrate into nitrite (NO_2). A Griess reagent was used to stabilize the NO_2. The measured concentration of NO_2 in each of the extracted sample duplicates correlated with the concentration of NO. Total nitrite concentration was measured at 550 nm wavelength and calculated from a standard dilution curve.

2.3. Implant manufacture

Forty commercial purity titanium implant disks with identical geometries (5 mm diameter, 2.5 mm thick) were manufactured by lathing (Wostab Finmekanik AB, Malmö, Sweden). The plane of the implant in contact with the tissue was subjected to nanotube or grit blasted surface modification as previously described.

2.4. Animals

Twenty adult male Sprague–Dawley rats (Taconic, Denmark) were used. Each rat was implanted with a grit blasted and a nanotube implant for comparison in the same animal. The rats weighed approximately 220 g before the experiment. All animals were housed under a 12 h light/dark cycle with free access to water and food. The study was conducted according to the Swedish legislation for animal research and approved by the local ethical committee.

2.5. Surgery

The rats were anesthetized by intraperitoneal (i.p.) injections of 6.3 ml kg body wt^{-1} of a solution containing fentanyl (50 g ml^{-1}) and Domitor vet (medetomidin hydrochloride) (1 mg ml^{-1}) in 20:1 volume proportions. After surgery the animals received subcutaneous injections of 1 ml kg body wt^{-1} of a mixture of Antisedan vet (atipamezole hydrochloride) (5 mg ml^{-1}) and sterile water in 1:9 volume proportions. The injection served as an antidote to the anesthesia. Simultaneously the animals received 1.7 ml kg body wt^{-1} analgesia subcutaneously, i.e. a solution containing 0.3 mg ml^{-1} Temgesic (buprenorfin) and sterile physiological sodium chloride in 1:9 volume proportions.

An established implantation model for soft tissue fibrotic capsule investigations was selected [17]. In brief, an incision was made through the shaved and cleaned abdominal skin of the rat. Two small openings or pockets in the rectus abdominis muscle sheath were made by dissection on each side of the linea alba. The implants were positioned in one opening on each side of the linea alba with the modified surface placed toward the muscle tissue. Implants were placed outside the peritoneum without injuring the peritoneal membrane. The rectus abdominis muscle openings were then closed and a suture placed in the muscle sheath secured the position of each implant. Finally, the skin was closed with surgical clips (Michell 7.5 × 1.75).

2.6. Tissue fixation

At either 1 or 6 weeks after implantation, the animals were killed by an i.p. injection of pentobarbital and the implants with the surrounding tissue attached were removed en bloc. The specimens were washed in ice cold saline, embedded in Tissue Tek O.C.T. compounds (Sakura, NL) and snap frozen for 30 s in 2-methylbutane at −70 °C. The implants were removed by dissection of the peritoneal membrane without specimen thawing. The frozen muscle tissue was sectioned in a cryostat (6 mm thick), collected on superfrost glass slides (Menzel-Glaser, Germany), and air dried before immunohistochemistry was performed.

2.7. Antibodies

The primary antibodies used were mouse monoclonal antibodies recognizing ED1 (CD68) (1:100, AbD Serotec, UK) or anti-nitrotyrosine (1:200, Upstate, USA). The ED1 antibody identifies membrane markers primarily associated with newly recruited blood monocytes and the anti-nitrotyrosine antibody identifies nitrotyrosine in tissues. Secondary antibodies used were a biotinylated horse anti-mouse IgG antibody (1:200, Vector Laboratories, USA) for the ED1 primary antibody.