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Simultaneous interaction of bacteria and tissue cells with photocatalytically activated, anodized titanium surfaces

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ABSTRACT

Photocatalytic-activation of anodized TiO_2-surfaces has been demonstrated to yield antibacterial and tissue integrating effects, but effects on simultaneous growth of tissue cells and bacteria in co-culture have never been studied. Moreover, it is unknown how human-bone-marrow-mesenchymal-stem (hBMMS) cells, laying the groundwork for integration of titanium implants in bone, respond to photocatalytic activation of anodized TiO_2-surfaces. Photocatalytically-activated, anodized titanium and titanium-alloy surfaces achieved 99.99% killing of adhering *Staphylococcus epidermidis* and *Staphylococcus aureus*, an effect that lasted for 30 days of storage in air. Surface coverage by osteoblasts was not affected by photocatalytic activation of anodized TiO_2-surfaces. Co-cultures of osteoblasts with contaminating *S. epidermidis* however, enhanced surface coverage on photocatalytically-activated, anodized titanium-alloy surfaces. hBMMS cells grew less on photocatalytically-activated, anodized titanium surfaces, while not at all on photocatalytically-activated, anodized titanium-alloy surfaces and did not survive the presence of contaminating staphylococci. This reduced surface coverage by hBMMS cells disappeared when photocatalytically-activated, anodized titanium-alloy surfaces were exposed to buffer for 60 min, both in absence or presence of contaminating *S. aureus*. Consequently, it is concluded that photocatalytically-activated, anodized titanium and titanium-alloy surfaces will effectively kill perioperatively introduced staphylococci contaminating an implant surface and constitute an effective means for antibiotic prophylaxis in cementless fixation of orthopaedic hardware.

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1. Introduction

Titanium and titanium alloys have been employed as biomedical implant materials since the 1940s, due to their superior biocompatibility, corrosion resistance and mechanical properties. At the same time, biomaterial-associated infections (BAI) have been reported for implants made of titanium and titanium alloys [1], especially in dental and orthopaedic applications. Dental implants are failing as a result of infection at an increasing incidence, currently estimated to be 5–10% [2], while the incidence of BAI in orthopaedic applications have ranged for many years between 2 and 5% [3]. Accordingly, there have been on-going attempts to improve the properties of titanium and titanium alloy surfaces in order to stimulate tissue integration and prevent biofilm formation [4].

The surfaces of titanium and titanium alloys are always covered with an oxide layer, which can have three different crystalline structures: anatase, rutile and brookite. Brookite is extremely difficult to synthesize, while anatase and rutile can be easily prepared by different oxidation processes, such as anodization [5,6]. When TiO_2 is exposed to UV light, electron-hole pairs are generated in the valence band that react with oxygen and atmospheric water to yield reactive oxygen species (ROS), capable of decomposing contacting organic molecules, including micro-organisms [7,8]. The antibacterial performance of TiO_2 surfaces is influenced by their crystalline phase and structure, porosity and surface area [9,10]. Anatase is generally considered the most photocatalytically active, crystalline TiO_2 phase because of its high adsorptive capacity and rate of hole trapping. However, the combination of anatase and rutile has been described to be more photocatalytically active than pure anatase or rutile [11]. Moreover, anatase enhances osteoblast adhesion, proliferation and differentiation [12]. The antibacterial and tissue integration promoting effects of photocatalytically activated, TiO_2 coatings have been amply demonstrated in monoculture studies [13,14]. Since around 30% of all orthopaedic implants are inserted in a bacterially contaminated state [15], tissue

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integration has to compete with bacterial colonization for estate on an implant surface, and this requires co-culture studies involving both bacteria and tissue cells. In the past, it has been demonstrated that mono-culture studies cannot be used to predict the outcome of the race for the surface in co-cultures. Extracellular bacterial substances for instance, can block tissue adhesive sites on implant surfaces discouraging tissue integration [16], but also unexpected, favourable effects of low numbers of contaminating bacteria on tissue integration of an implant surface have been observed, presumably through bacterial stimulation of integrin expression [17].

Therefore the aim of this paper is to determine whether tissue cells have better opportunities to win the race for the surface on photocatalytically activated, anodized titanium and titanium alloy surfaces than on non-activated, (non)-anodized titanium and titanium alloy surfaces. To this end, we will first compare the physico-chemistry of anodically oxidized titanium and titanium alloy surfaces. Secondly, we will determine the effects of photocatalytically activating the oxide surfaces on the killing efficacy of five different staphylococcal strains involved in BAI and the integration of the surfaces by osteoblasts and clinically more relevant human bone marrow mesenchymal stem cells. The behaviour of mesenchymal stem cells on photocatalytically activated, anodized titanium alloy surfaces has, to our knowledge, not been studied before. Since in clinical practice, implants are often inserted in a bacterially contaminated state [15], co-culture experiments will be carried out, simultaneously allowing the cells and *Staphylococcus epidermidis* ATCC 35983 to compete on photocatalytically activated, anodized titanium and titanium alloy surfaces in a peri-operative contamination model [18]. Finally, we will address hitherto neglected issues, such as the shelf-life of photocatalytically activated, anodized titanium and titanium alloy surfaces in air and the longevity of effects in a physiological fluid, both of major importance for ultimate clinical application of these materials.

2. Materials and methods

2.1. Anodic oxidation and photocatalytic activation of titanium and titanium alloy surfaces

Commercially pure titanium (grade 2) and titanium alloy (grade 5) discs (Salomon's Metalen, Groningen, The Netherlands) with a diameter of 5 mm and a thickness of 1 mm were used in this study. Discs were mechanically polished with 1200 grid SiC paper and ultrasonically cleaned with acetone, ethanol and ultra-pure water, three times and 5 min for each solution, prior to anodic oxidation.

Prior to anodic oxidation, titanium (Ti) and titanium alloy (Ti6Al4V) discs were immersed into 1 M H_2SO_4 solution for 5 min to dissolve their passivation layer. Next, titanium discs were connected to the anode of a 100 V DC (Ti−100 V) or 120 V DC (Ti−120 V) power supply for 1 min. Anodic oxidation of titanium alloy discs was done at a constant DC current density of 2 A/cm^2 for 1 min followed by 4 A/cm^2 for another min (Ti6Al4V-2A/4A), or by applying a DC current density of 4 A/cm^2 for 2 min (Ti6Al4V-4A). The cathodes were either of titanium or titanium alloy (60 mm × 15 mm × 1 mm) depending on whether titanium or titanium alloy was anodized. After treatment, the anodized discs were rinsed with ultra-pure water and dried in an oven at 40 °C for 24 h.

When appropriate, oxidized discs were photocatalytically activated with UV light (15 W, wavelength 254 nm) for 5 min or 30 min in a CleneCab for PCR workstation (CleneCab, Herolab GmbH Laborgeräte, Wiesloch, Germany) at a distance of 60 cm from the UV light tubes. Photocatalytically activated discs were always used within 30 min after UV irradiation, unless mentioned otherwise.

2.2. Physico-chemical characterization of photocatalytically activated, anodized titanium and titanium alloy surfaces

The crystalline compositions of the titanium and titanium alloy surfaces after anodic oxidation were determined using X-ray diffraction (XRD, Bruker D8, Karlsruhe, Germany) using CuKα radiation at 40 kV in the 2θ range of 10–80°. The surface roughness (R_a) of the disc surfaces was measured with Atomic Force Microscopy (AFM, Digital Instrument, Woodbury, USA) in the contact mode using a silicon nitride tip (Mountain View, CA, USA; probe curvature radius of 18 nm). Furthermore, scanning electron micrographs of the disc surfaces were taken at an accelerating voltage of 2 kV (SEM; JEOL JSM 6301F, Tokyo, Japan). The hydrophobicity of the surfaces was assessed using water contact angle measurements both prior to and after photocatalytic activation.

2.3. Bacterial strains, culture conditions and harvesting

Three slime-producing staphylococcal strains (*S. epidermidis* ATCC 35984, *S. epidermidis* 7388 (gentamicin-resistant), and *Staphylococcus aureus* ATCC 12600) and two non-slime producing ones (*S. epidermidis* ATCC 35983 and *S. epidermidis* ATCC 12228) were used in this study. Strains were streaked on blood agar plates from frozen stocks and grown overnight at 37 °C in ambient air. The agar plates were then kept at 4 °C for further use. For each experiment, one colony was inoculated from an agar plate in 10 ml tryptone soy broth (TSB, OXOID, Basingstoke, UK) and incubated for 24 h. This preculture was then added into 200 ml TSB to prepare a main-culture, which was grown for 16 h before harvesting. Bacteria were harvested by centrifugation at 4000 g for 5 min at 10 °C, and washed twice with 10 ml sterile phosphate buffered saline (PBS, 10 mM potassium phosphate, 0.15 M NaCl, pH 7.0). Subsequently, the bacterial concentration in PBS was fixed at the desired concentration using a Bürker-Türk counting chamber.

2.4. Antibacterial efficacy of photocatalytically activated, anodized titanium and titanium alloy surfaces

The antibacterial efficacies of the anodized titanium and titanium alloy surfaces were evaluated prior to and immediately after photocatalytic activation with the Petrifilm Aerobic Count plates (3 M Microbiology, St. Paul, Minnesota, USA). In addition, an experiment was done with one staphylococcal strain following storage of photocatalytically activated, anodized surfaces in air for different periods of time up to 30 days to determine their shelf-life. Briefly, the Petrifilm AC plate is a thin film, sample ready and dehydrated version of conventional agar plates. It contains a top film to enclose a sample within the Petrifilm system and a bottom film with standard nutrients, a cold water gelling agent and the dye triphenyl tetrazoliumchloride, which is able to stain bacterial colonies red. For enumeration of the number of colony forming units (CFUs) on a substratum surface, the bottom, nutrient-rich film first needs to be transferred to the top film, according to the manufacturer's instructions. Discs prior to and within 30 min after photocatalytic activation were put into a sterile petri-dish, together with a piece of wetted, sterile cotton to maintain a constant, high humidity. Subsequently, 5 μl of a bacterial suspension (1×10^4/ml) was placed on the disc surfaces for 1 h, corresponding with a bacterial challenge of 250/cm^2. Next, the discs were placed in the Petrifilm system at 37 °C for 48 h after which the number of CFUs was counted. In order to determine the exact challenge number, 5 μl bacterial suspension with the same concentration was placed in the Petrifilm system in absence of a sample disc. Killing efficacy was calculated as a percentage killing according to

$$\text{killing efficacy} = \frac{n_0 - n_1}{n_0} \times 100\% \quad (1)$$

in which n_0 is the number of CFUs formed within the Petrifilm system in absence of a sample disc, while n_1 is the number of CFUs formed on the sample discs.

All experiments were performed in triplicate for each strain with separately grown bacterial cultures.

2.5. Cell lines, culture conditions and harvesting

U2OS human osteosarcoma and human bone marrow mesenchymal stem (hBMMS) cells were employed in this study. U2OS human osteosarcoma cells were routinely cultured in Dulbecco's modified Eagle's medium supplemented with 1 g/l D-glucose (DMEM-LG), 10% fetal bovine serum (FBS) (both from Invitrogen, Breda, The Netherlands) and 0.2 mM ascorbic acid-2-phosphate (AA2P, Sigma–Aldrich Chemie B.V. Zwijndrecht, The Netherlands) at 37 °C in a humidified atmosphere with 5% CO_2. At 80–90% confluence, cells were detached using a trypsin-EDTA solution (Invitrogen) and harvested by centrifugation. Prior to each experiment, cells were resuspended and diluted to the desired density in culture medium. With informed consent, reaming debris of a 64-year old male patient (hBMMS #4; osteoarthritis) and of a 64-year old female patient (hBMMS #86; rheumatoid arthritis, not on medication) undergoing total hip replacement surgery at the Department of Orthopaedic Surgery of the University Medical Center Groningen, The Netherlands were obtained using aseptic procedures. hBMMS cells were isolated using Ficoll density gradient centrifugation [19] and cultured in α-Modified Eagle's medium (α-MEM, Invitrogen) supplemented with 10% heat-inactivated FBS, 2% APS (Amphotericin, Penicillin and Streptomycin from Invitrogen) and 0.2 mM AA2P at 37 °C in a humidified atmosphere with 5% CO_2. For co-culture experiments, medium was used without antibiotics. Culturing and harvesting procedures for hBMMS cells were similar as for U2OS cells. Both cell types were routinely counted using the Scepter™ cell counter (Millipore, Amsterdam, The Netherlands) yielding cell numbers and mean cell diameters. U2OS had a mean cell diameter of 15 μm and hBMMS cells of 17 μm.

2.6. Cellular adhesion and spreading on photocatalytically activated, anodized titanium and titanium alloy surfaces

Adhesion, spreading and growth of U2OS osteosarcoma and hBMMS #4 cells were determined on the anodized titanium and titanium alloy surfaces prior to and immediately after photocatalytic activation. In addition, experiments were done with hBMMS #86 cells on photocatalytically activated, anodized titanium alloy surfaces after storage for 60 min in PBS. One ml of a cellular suspension (2×10^4/ml) was added to a well of a 48-well plate containing one disc per well. After 24 h incubation, the discs were washed with PBS, fixed with 3.7% paraformaldehyde in

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