

In Vitro Biological Tests on AVINENT Dental Implants

PURPOSE

To determine the *in vitro* cellular response of AVINENT medical grade titanium implants, cell morphology and an evaluation of cellular proliferation and differentiation. The AVINENT surface is the result of a series of scientific investigations on the surface characteristics of dental implants for the purpose of achieving the best biological response possible. Specifically, the AVINENT surface is achieved through combining a *shot-blasting* process followed by an electrochemical treatment process. The *shot-blasting* (particle impact) process provides the surface of the implant with optimum structural roughness. Then the electrochemical process (anodizing) modifies the surface micro-topography by causing porosity in the titanium oxide while also enabling the incorporation of chemical elements (calcium and phosphorus) that provide the final surface with some fully biomimetic properties.

INTRODUCTION

The morphology of an implant surface, including microtopography and roughness, is intimately related to the success of the bone fixation process. The manufacturing process used to provide a certain surface texture on the implant can introduce chemical changes on the surface that will also have their own influence on good clinical behavior. Currently, implants made of titanium and titanium alloys for clinical use have variations in surface roughness and chemical composition, always starting from the premise that bone is formed more quickly on rough surfaces than on smooth or polished surfaces, which are more susceptible to the formation of fibrous connective tissue [1].

Studies that have been performed *in vitro* have enabled evaluation of the behavior of certain cell lines to variations in some surface properties, showing that surface roughness affects cellular response. Osteoblastic cells in particular tend to adhere more quickly to rough surfaces [4, 5 and 6].

Cell differentiation is also greater on rough surfaces when parameters such as cell morphology, the production of extracellular matrix, the specific activity of alkaline phosphatase and the production of osteocalcin are evaluated [1].

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Alkaline phosphatase is an enzyme that is generally found adhered to the external surface of the cell membrane, with anchoring through the carboxyl-terminal of phosphatidylinositol-glycan. In general there are two kinds of alkaline phosphatase coded by four different genes. The first kind is made up of tissue-specific phosphatase, expressed in the intestine and mature placenta. The second kind is non-tissue-specific phosphatase, which is expressed in numerous tissues, mainly including bone, liver and kidneys. Alkaline phosphatase is an early indicator of osteoblastic differentiation, and it plays an important role in mineralization of the extracellular matrix. The function of alkaline phosphatase is to hydrolyze organic phosphate and release inorganic phosphate for the formation of hydroxyapatite in bone. This enzyme is indispensable for bone formation, and its secretion or activity indicates bone formation, or the beginning of the differentiation stage [2].

MATERIALS AND METHODS

Specimen Preparation

Two types of specimens have been used in this study, smooth and treated with AVINENT surfacing. Both were obtained from medical grade Ti discs 10 mm in diameter and 2 mm thick.

Smooth discs were prepared through initial polishing with silicon carbide paper and then with aluminum oxide with a particle size of 1 μm and 0.05 μm . Once the discs had been polished they were given an ultrasound acetone wash.

Immediately thereafter the discs treated with AVINENT surfacing were also given an ultrasound wash.

All the specimens used in this study were sterilized by gamma radiation at an intensity of 25 kGy, and were placed in 24-well polystyrene plates.

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Cell Culture

Human osteoblasts from the MG63 cell line were used in performing this study. Cells were placed in culture plates with 15 ml of culture medium (DMEM, Dulbecco's Modified Tagle Medium, Gibco) with the addition of 10% fetal calf serum (FCS, Gibco), 1% penicillin/streptomycin, 1% pyruvate and 1% L-glutamine (all Gibco). The cells were maintained at 37° C in an incubator under one atmosphere with 5% CO₂. The culture medium was changed every 3 days. After the fourth renewal was performed, the cells were washed with phosphate-buffered saline (PBS) and trypsinized with Trypsin-EDTA (0.25%) in an incubator for 5 minutes at 37° C.

Cell Morphology

To determine the variation in cell morphology related to topography, the cultures were examined with an Electronic Scanning Microscope (Strata DB 235, FEI Company).

After 24 hours of incubation, the culture medium was removed and the specimens were washed three times with phosphate-buffered saline (PBS) solution, and the cells were fixed with OsO₄ (1%) in PBS (0.1 M) during 15-30 minutes. The specimens were dehydrated by successive immersion in different tert-butyl alcohol solutions (50, 75, 90 and 100%). The alcohol was removed during the critical point stage.

Proliferation

Cell proliferation was quantified by the WST test (Roche, Germany). 10,000 cells were cultured with 300 µl of DMEM in each one of the culture wells where the specimens and polystyrene discs as a positive control had been placed. After 1, 3 and 7 days the cells were washed with PBS and 200 µl of WST solution (Roche). WST is a tetrazolium salt (4-[3-(4-Iodophenyl)-2-(4-nitrophenyl)-3H-5-tetrazolium]-1,3-benzene disulfonate) that becomes transformed into formazan by a complex mitochondrial succinate - tetrazolium reductase system and is only active in the case of viable cells. Therefore, the amount of formazan produced during the test, which is evaluated through absorbency measurements

with a spectrophotometer, is related to the number of viable cells; that is, the number of metabolically active cells within the culture. The combination was left to incubate for one hour to achieve a good reaction level. After that time a change in color resulted that could be measured by using a PowerWaveX spectrophotometer from Bio-Tek Instruments with a wavelength of 450 nm.

Differentiation

100,000 cells were added to each one of the wells for performing cell differentiation tests. The culture medium was supplemented with 50mM of ascorbic acid (Sigma) and 10^{-8} mM of dexamethasone (Sigma). 3 μ l of each of these supplements was added to each well for 48 hours. The liquid floating on top of each of the wells was picked up and frozen at -80° C. The discs were washed twice with PBS (Phosphate Buffered Solution), and M-PER[®] (Pierce, USA) was used for lysating the cells. The cellular lysate was frozen at -80° C.

The cellular lysate obtained from these different specimens was thawed for quantifying total protein and the activity of the alkaline phosphatase present in the cells. For the quantification of total protein, the BCA test (Pierce, USA) was used according to the protocol specified by the manufacturer. In performing this test, 25 μ l of specimen was added to 200 μ l of working reagent on a 96-well plate. The plate was incubated at 37° C for 30 minutes and cooled to ambient temperature. Absorbency values at a wavelength of 562 nm were finally obtained. In obtaining the calibration curve, several dilutions of BSA (Bovine Serum Albumin) were prepared ranging from 0 a 2000 μ g/ml.

For a determination of alkaline phosphatase activity, 100 μ l from each specimen, 50 μ l of AMP buffered solution (0.5M 2-aminomethyl-propanol, 2mM MgCl₂ and 50 μ l of substrate solution (10mM p-nitrophenyl phosphate) were added to each well. For determining the calibration curve, several dilutions of p-nitrophenol were prepared with a concentration range of $2 \cdot 10^{-5}$ - $2.4 \cdot 10^{-4}$. The 96-well plate was incubated at 37° C for 30 minutes. The reaction was stopped by adding 100 μ l of NaOH 3M. Finally measurements were taken by spectrophotometry at 405 nm.

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RESULTS

Cell morphology

The osteoblasts cultured on the smooth medical grade Ti discs showed an extended shape with intimate contact with the material after 24 hours of culture. Weak dorsal activity was observed, with short cytoplasmic extensions (filopodia), indicating low phenotypical development activity (figs. 2, 3 and 4).

The osteoblasts cultured on the discs having an AVINENT surface showed a morphology that was significantly different from the morphology obtained on the polished substrate. The osteoblasts on the AVINENT treated surface were compact and extended cells that showed an abundance of filopodia intimately adhered to the treated surface. This high dorsal activity indicates high phenotypical development. A tendency was also observed in the positioning of cells in the valleys of the surface, with the cytoskeleton adapting to topographical irregularities (figs. 5, 6 and 7). Surface roughness does condition cell morphology and cell position.

Cell Proliferation Tests

The results showed good *in vitro* behavior by the treated AVINENT surface, because it provided proliferation levels similar to the positive control and the smooth specimens for the four times studied, thus emphasizing their cytocompatibility.

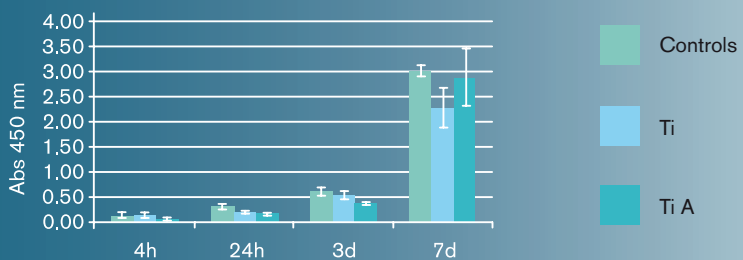


Figure 1. Results of cell proliferation. Ti: Polished discs and Ti A: treated discs.



Figure 2. Electronic microscope image of the osteoblast culture on smooth titanium after 24 hours of incubation. Flat morphology of osteoblasts is observed.



Figure 3. Electronic microscope image of the osteoblast culture on smooth titanium after 24 hours of incubation. Flat morphology of osteoblasts is observed.



Figure 4. Electronic microscope image of the osteoblast culture on smooth titanium after 24 hours of incubation. The presence of small cytoplasmic extensions (filopodia) is observed on the dorsal part of cells.

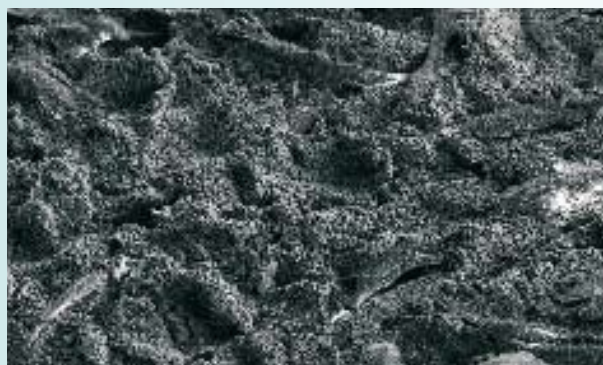


Figure 5. Electronic microscope image of the osteoblast culture on the AVINENT surface after 24 hours of incubation.



Figure 6. Electronic microscope image of the osteoblast culture on the AVINENT surface after 24 hours of incubation. The presence of cytoplasmic extensions (filopodia) is observed on the dorsal part of cells.



Figure 7. Electronic microscope image of the osteoblast culture on the AVINENT surface after 24 hours of incubation. The presence of cytoplasmic extensions (filopodia) is observed on the dorsal part of cells.

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Differentiation Tests

Results on the differentiation of osteoblasts on the two surfaces studied (smooth and treated) are presented in figure 8. It may be observed here that there is higher production of alkaline phosphatase on the discs treated with AVINENT surface than on the smooth disks.

The addition of vitamin 1, 25-(OH)₂D₃ caused a synergistic increase in the production of alkaline phosphatase in the treated specimens.

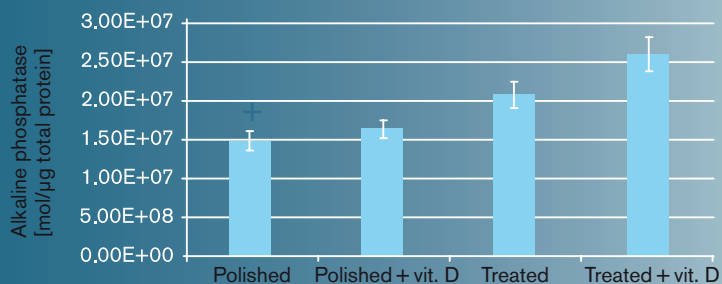


Figure 8. Results of alkaline phosphatase production.

DISCUSSION

This morphological study showed variations between smooth and treated specimens in the morphology of osteoblasts after 24 hours of incubation. On the smooth surfaces, cells showed a flat fibroblastic morphology with weak dorsal activity. This extended configuration decreases the cell's potential for expressing osteoblastic phenotypes, because these cells are characterized by having a columnar morphology in their *in vivo* secretion phase. On the other hand, for treated specimens the osteoblasts would become anchored to the surface by focal contacts between the cytoplasmic extensions and multiple peaks in the topography. This forces the cell to have a more osteoblastic morphology with increased dorsal activity.

The observations made in the morphological study were corroborated by cell differentiation results. Greater concentrations of alkaline phosphatase per micro-

gram of total protein were observed on the treated discs, indicating greater phenotypical expression of osteoblasts on the AVINENT surface.

These cell proliferation results clearly emphasized the good *in vitro* behavior of the treated surface, indicating proper material compatibility.

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