ATTACHMENT OF HUMAN MARROW STROMAL CELLS TO TITANIUM SURFACES

Mamle Mante, DMD, MS
Bina Daniels, BS
Eleanor Golden, BS
David Diefenderfer, VMD, PhD
Gwen Reilly, PhD
Phoebe S. Leboy, PhD

KEY WORDS

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The attachment of human bone marrow stromal cells to titanium alloy (Ti_6Al_4V) surfaces was investigated. Titanium disks were polished and modified by surface roughening and by passivation in nitric acid. Cell attachment to titanium surfaces and tissue culture plastic (TCP) was determined by tetrazolium bromide (MTT) assay at 2, 6, 24, and 48 hours after seeding. Cell proliferation was determined by thymidine incorporation. Attachment on titanium surfaces was 75.6% to 94.9% of attachment on TCP control. The difference between cell attachment on the TCP compared with smooth or rough titanium was statistically significant ($P < .05$). However, no statistically significant difference was found between attachment to TCP and passivated titanium. Cell proliferation on titanium surfaces after 24 hours was approximately 70% of proliferation on TCP. There was a statistically significant difference ($P < .05$) between proliferation on tissue culture and smooth and passivated titanium but not on rough titanium. These results indicate that titanium provides a surface that is conducive to cell attachment and that passivating titanium improves cell attachment, approaching levels seen with TCP, a surface specifically developed to enhance cell attachment. Increasing surface roughness results in improved cell proliferation on titanium.

Mamle Mante, DMD, MS, *is with the Department of Restorative Dentistry at the School of Dental Medicine at the University of Pennsylvania, 4001 Spruce Street, Philadelphia, PA 19104. Correspondence should be addressed to Dr Mante.*

Bina Daniels, BS; Eleanor Golden, BS; Gwen Reilly, PhD; *and* ***Phoebe S. Leboy, PhD,*** *are with the Department of Biochemistry in the School of Dental Medicine at the University of Pennsylvania in Philadelphia, PA 19104.*

David Diefenderfer, VMD, PhD, *is with the School of Veterinary Medicine at the University of Pennsylvania in Philadelphia, PA 19104.*

INTRODUCTION

Dental implants provide a means of anchoring various oral prostheses in the mandible and maxilla. The success of titanium and titanium alloy root form implants has resulted in their routine use in dentistry today. Implant success depends on the nature of the surrounding bone. The ideal bone condition is found where there is good cancellous bone surrounded by cortical bone of adequate thickness on both sides.[1]

Most polymer and ceramic implant materials, although bioactive in nature and able to stimulate osteogenesis, are of poor mechanical strength, which precludes their use as load-bearing implants. Titanium is not an osteoinductive material but has superior mechanical properties and the ability to ''bond'' to bone or osseointegrate, in a process similar to fracture healing, which has contributed to its clinical success.[2–4] When titanium is exposed to atmospheric conditions, or in vivo fluids, a thin tenacious film of titanium oxide spontaneously forms on the implant surface. This film renders the

otherwise reactive material bioinert and biocompatible by preventing it from further reactions, only minimal reaction products leach out into surrounding fluids, and toxicity to surrounding cells is very low.[5] An increase in the thickness of the oxide film results in improved bone formation at the implant surface[6–8]; implant manufacturers routinely passivate titanium implants by immersion in nitric acid to increase the thickness of the oxide layer.

Studies have shown that the surface roughness of the implant influences new bone formation at the implant surface regardless of implant composition, with rougher surfaces (at the micron level) showing better bone growth.[6–8] In vitro studies have demonstrated that surface roughness influences cell attachment, proliferation, differentiation, and matrix production.[9–12] Rougher implant surfaces provide an increased surface area for cell attachment and subsequent bone formation.[11] When cells were cultured on titanium, there was an increase in the production of Prostaglandin E_2 and TGF-$_{\beta 1}$, known promoters of osteoblast differentiation and bone growth, with increasing titanium surface roughness.[12–15]

Marrow stromal cells, also called mesenchymal stem cells, are precursor cells that can differentiate into bone, cartilage, and fat cells. Because of their ability to differentiate into osteoblasts (bone-forming cells), they play an important role in normal bone[16,17] repair and remodeling, as well as in osteogenesis around implant materials.[18–20]

Several investigators have studied the effects of implant surface treatment on the attachment of osteoblast or osteoblast-like cells[7,13,14,21–24] of vertebrate and human origin. These are cells that are already committed to osteogenesis. There is less extensive literature on the attachment patterns of marrow stromal cells (MSCs)[11,25] of nonhuman origin and the behavior of specifically human MSCs on implant materials.[10,19]

Greater knowledge of the attachment patterns of human MSCs is important in order to understand the processes that influence their proliferation, maturation, and differentiation at the implant surface at the cellular as well as the molecular level. Modification of implant surfaces to optimize the osteogenic capability of MSCs could lead to greater clinical implant success.

The aim of this project was to examine the attachment of human marrow stromal cells on a titanium alloy (Ti_6Al_4V) routinely used for dental implants and evaluate the effects of increasing surface roughness of titanium samples or passivation in nitric acid on cell attachment.

Materials and Methods

Preparation of titanium samples

Titanium alloy (Ti_6Al_4V) rods, 12.5 mm in diameter, were sectioned into discs 2 mm in thickness. To obtain differences in surface characteristics, the titanium discs were subjected to 1 of 3 different surface treatments. The first group, designated smooth Ti, were polished on a LECO polisher (LECO, St. Joseph, Mich), with 600-grit silicon carbide paper. The average particle size of the 600 grit used for final polish was 15.3 μm. The second group, designated rough Ti, was polished to final polish with 240-grit paper, average particle size 58.5 μm. The third group, passivated Ti, was polished to 600 grit and then passivated in 30% nitric acid for 60 minutes. The discs were then ultrasonically cleaned in acetone for 15 minutes and sterilized by immersion in 70% ethanol for 1 hour.

Cell culture

Primary human MSCs were obtained from human bone marrow following hip replacement surgery. The cells were obtained from a pool of randomly selected patients consisting of 5 women and 2 men ranging in age from 24 to 48 years. The samples were layered on Ficoll-Paque (Amersham Pharmacia Biotech, Piscataway, NJ) and centrifuged 30 minutes at 1900 relative centrifugal force to concentrate nucleated cells at the interface. The fraction was collected, washed once with αMEM media (Gibco), and suspended in αMEM with 15% fetal calf serum and antibiotics. Primary cultures of these cells were established at 5×10^5 cells/cm^2, and nonadherent cells were removed after 3 days. First- and second-passage cells were used for the experiment and were plated on tissue culture plastic (TCP) or titanium alloy (Ti_6Al_4V) in 24 well plates at a density of 10 000 cells/well.

The cells were seeded in a small volume of media on the respective surfaces and allowed to attach for 1 hour prior to the addition of αMEM medium containing 15% fetal calf serum (Atlanta Biologicals, Norcross, Ga). Cells were then harvested at 2, 6, 24, and 48 hours after seeding. The titanium disks with attached cells were removed from the plates they were cultured in and placed in clean wells prior to assay.

Scanning electron microscopy

Smooth, rough, and passivated Ti surfaces without cells were examined using a JEOL T300 scanning electron microscope (JEOL, Tokyo, Japan) at an accelerated voltage of 20 kV. Cells attached to Ti and tissue culture surfaces were prepared for microscopy by fixation in 2% formaldehyde followed by dehydration in graded alcohol. The samples were then carbon-sputtercoated and viewed by scanning electron microscopy.

Tetrazolium bromide (MTT) assay for viable cell number

Titanium samples were transferred to clean wells after an incubation period. Cell cultures were incubated in 0.5 mg/mL solution of tetrazolium bromide in Hanks buffered saline solution (HBSS, GIBCO, Grand Island, NY) diluted in 5 parts of clear media for 1 hour. After the incubation period, the media was aspirated, and 200 μL of dimethyl sulfoxide (DMSO) was placed in each well. Next, 190 μL of the solution was transferred into a microplate

and the absorbance was measured at 570 nm. Each experiment was done in triplicate.

Thymidine incorporation assay for proliferation

The cell cultures were treated with ^{3}H thymidine (1 μCi/mL) for 18 hours before harvesting. They were then washed in HBSS and lifted in 0.25% trypsin in 1 mM EDTA for 15 minutes. The cell suspensions were pipetted onto glass fiber filters prewetted with 5% trichloroacetic acid (TCA). The filters were allowed to dry and then washed for 5 minutes with ice cold 10% TCA followed by four 5-minute washes with ice cold 5% TCA and a final wash with ice cold 100% ethanol. The filters were allowed to dry and then transferred to vials, covered with 3 mL of scintillation fluid (ICN), and then counted. The average decay per minute (dpm) on the different titanium surfaces was expressed relative to control samples cultured on the TCP.

RESULTS

SEM of titanium surfaces

Smooth and passivated titanium samples showed shallow residual grooves 3–5 μm apart, whereas rough titanium specimens had deeper irregular grooves, which were approximately 1 μ apart (Figure 1). After 24 hours, cells attached to plastic and titanium had not yet reached confluence and exhibited a typical fibroblastic phenotype with centrally placed nuclei and elongated processes. There was no noticeable difference in cell morphology on the different titanium surfaces after 24 hours. Cells attached to a smooth titanium surface appeared to spread out across the residual polishing grooves (Figure 2). On rough titanium, the attached cells were attached in the same direction as the deeper and wider residual polishing grooves (Figure 3).

The attachment of human MSC to titanium was compared with the attachment to TCP (control) at 2, 6, 24, and 48 hours (Figure 4). The optical density of the MTT suspension read at 570 nm was proportional to the viable cell number. Although all surfaces showed an increase in cell number with time, the number of cells attached to the 3 titanium surfaces during the first 24 hours was approximately 80% of the number attached to the TCP control (Figure 5). When cell numbers were compared over the entire 48-hour period, by combining data from the 2, 6, 24, and 48 hour groups, cell numbers from TCP were significantly higher than smooth and rough titanium ($P < .05$). However, the smaller difference between passivated titanium and TCP ($P = .14$). was not statistically significant (Table). There was no statistically significant difference between cell attachment to the 3 different titanium surfaces at any of the time points investigated or over the 48-hour period.

Cell proliferation

The proliferation of human MSC on Ti surface was examined by measuring the incorporation of labeled thymidine during a 24-hour interval between days 1 and 2. The relative rates of proliferation on smooth, rough, and passivated titanium when compared with TCP were 68%, 74%, and 71%, respectively (Figure 6). The differences were statistically significant for smooth and passivated titanium, but not for rough titanium.

TABLE

Paired *t*-test human MSC cell number (optical density at 570 nm)

First 48 hours in culture	*P* value
control vs smooth	.04
control vs rough	.003
control vs passivated	.14
smooth vs rough	.92
smooth vs passivated	.59
rough vs passivated	.33

DISCUSSION

In this study, we used in vitro cell culture to characterize the attachment patterns of human marrow stromal cells on a titanium alloy (Ti_6Al_4V) routinely used for dental and orthopedic implants. The surface of the titanium was modified by increasing surface roughness and by passivation in nitric acid to increase the thickness of the surface oxide layer.

Results of the MTT assay show that during the initial 24 hours after seeding, cell attachment to the 3 titanium surfaces ranged from 75.6% to 94.9% of the attachment to the TCP control. Increasing surface roughness resulted in a higher cell count at 6 and 24 hours; however, the differences at these 2 time points and over the initial 24-hour period were not statistically significant from attachment to smooth Ti. The increase in attachment of cells to rougher titanium could be a direct effect of the titanium surface or an indirect effect of altered levels of adhesion proteins. Fibronectin and vitronectin are extracellular proteins that promote cell attachment and are present in the serum used in tissue culture. It has been shown that the serum proteins albumin, fibronectin, and vitronectin bind differently to various implant and bone substitute materials.[10,19] Changes in ti-

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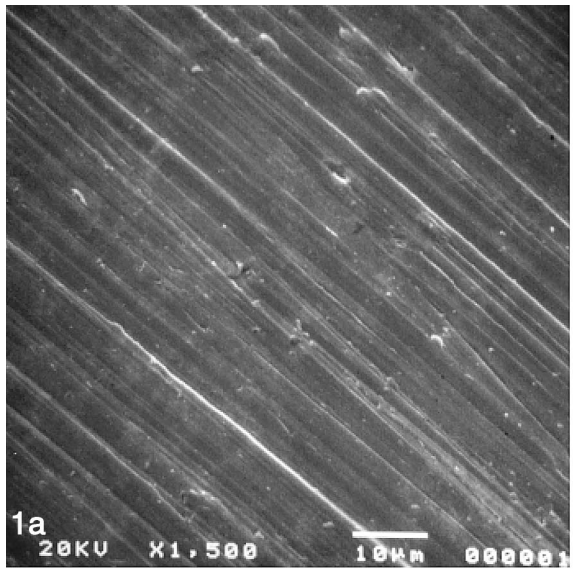
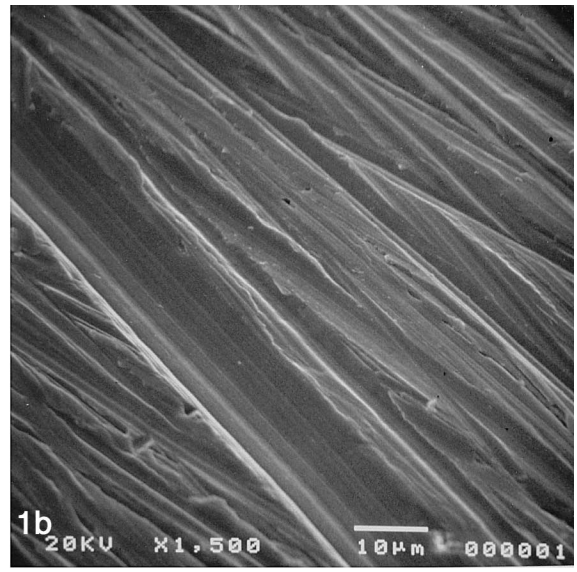
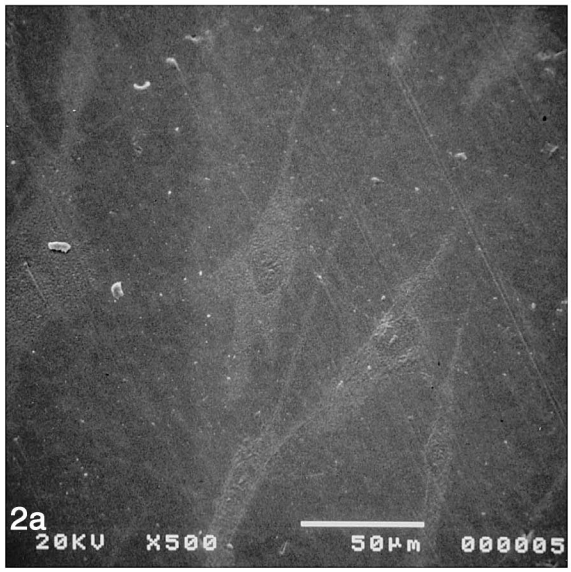
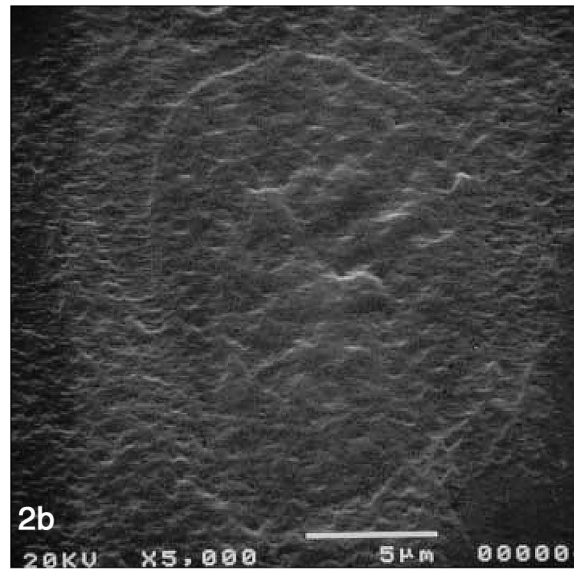
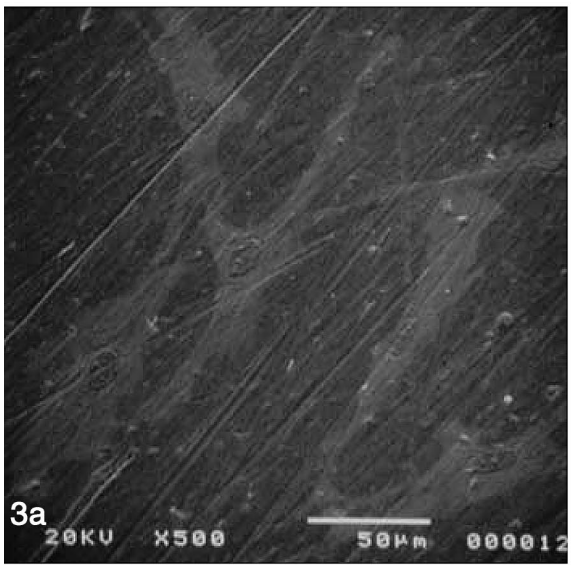
FIGURES 1–3. FIGURE 1. (a) SEM micrograph of titanium surface showing the surface morphology of a titanium disc polished to 600 grit on silicon carbide paper (smooth and passivated titanium). (b) SEM micrograph of titanium surface showing the surface morphology of a titanium disc polished to 240 grit on silicon carbide paper (rough titanium). FIGURE 2. (a) SEM micrograph (original magnification ×500) of titanium surface with attached marrow stromal cells on smooth titanium. (a) SEM micrograph (original magnification ×5000) of titanium surface with attached marrow stromal cells on smooth titanium. FIGURE 3. (a) SEM micrograph (original magnification ×500) of titanium surface with attached marrow stromal cells on rough titanium. (b) SEM micrograph (original magnification ×5000) of titanium surface with attached marrow stromal cells on rough titanium.

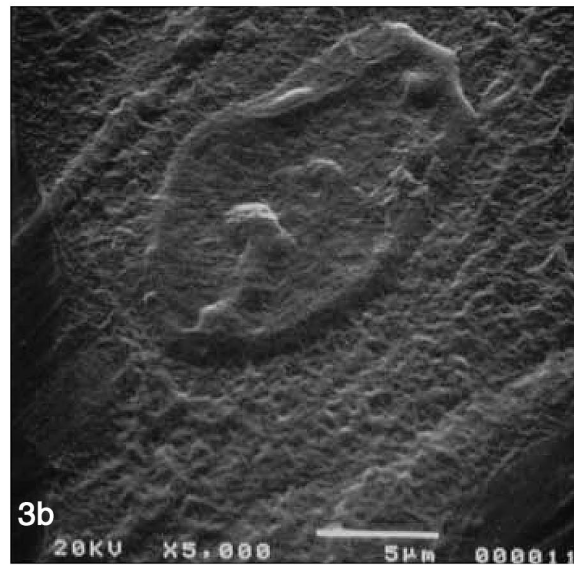


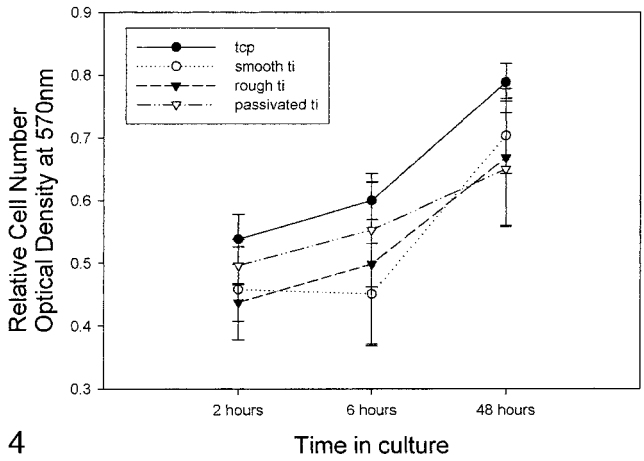


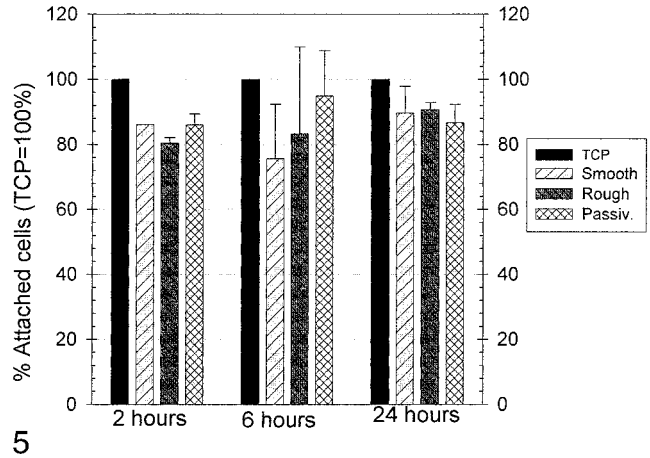


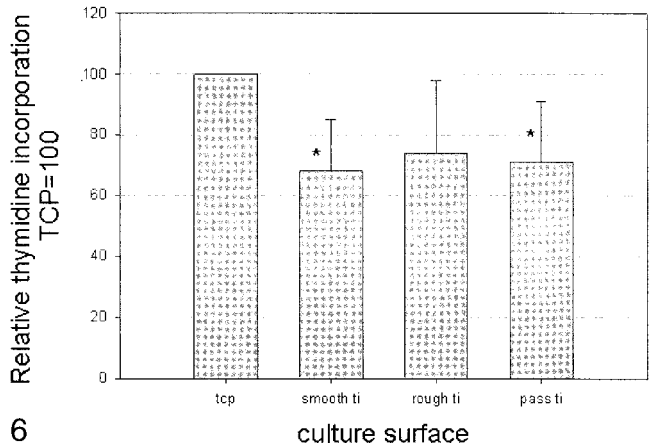












FIGURES 4–6. FIGURE 4. Human MSC change in number of human MSC attached to smooth rough and passivated titanium and tissue culture plastic (TCP) from 2 to 48 hours. The number of cells attached is expressed as the optical density of the MTT-cell suspension. FIGURE 5. Human MSC cell attachment expressed as a percentage of cells attached to TCP at 2, 6, and 24 hours. There was no statistically significant difference with TCP at each of the time points. FIGURE 6. Human MSC proliferation expressed as relative thymidine incorporation after 24 hours in culture. The asterisk (*) indicates a statistically significant difference ($P < .05$) with TCP control.

tanium surface roughness have been shown to alter the adsorption of these proteins,[10] with rougher surfaces binding a higher amount of total serum protein and fibronectin than smoother titanium surfaces.

Cell attachment to passivated titanium was not statistically significant from the TCP control. There are a few reports in the literature of the possible toxic effects of Al ions leaching out of Ti_6Al_4V alloys into the solution.[26] An increase in the thickness of the oxide layer from passivation would presumably decrease the potential for toxicity, and therefore result in improved cell attachment. Increasing the thickness of the titanium oxide layer results in an improvement in bone formation at the implant surface in vivo.[6,27]

In this experiment, marrow stromal cells grown on smooth and rough titanium formed a monolayer of cells of typical fibroblastic appearance with cell processes. Cells cultured on rough titanium appeared to spread out in the same direction as the residual grooves resulting from surface roughening. Cells on smooth titanium were orientated in different directions. Differences[26] in the amount and type of adhesion proteins adsorbed onto the deeper and rougher residual grooves could explain this difference in orientation of attaching cells. There was no noticeable difference in the overall cell shape between cells cultured on the 2 surfaces.

Protein coating presumably occurs in vivo after surgical placement; the implant surface is exposed to tissue fluids containing proteins and polysaccharides, as well as cells of mesenchymal origin. Adhesion proteins such as fibronectin and vitronectin, present in tissue fluids, as well as integrins expressed by cells, adhere to the implant surface, leading to complex and dynamic biochemical reactions occurring at the implant surface.[28–32] The surface characteristics of the implant surface affect the adsorption[19,20] of adhesion proteins, and consequently influence cellular attachment, proliferation, and differentiation around the implant.

Using different methods and a wider range of surface roughnesses, Deligianni et al[10] have reported that increasing the surface roughness of Ti_6Al_4V results in a significant increase in the attachment of human MSC at 0.5

and 2 hours. In the present study, there was an increase in cell attachment at 6 and 24 hours over a narrower range of surface roughness of titanium, indicating that even smaller increases in surface roughness improve cell attachment.

There was a statistically significant difference between cell attachment to TCP and attachment to smooth and rough titanium, indicating that these surfaces were less favorable for cell attachment. This is not surprising given that TCP is an idealized cell culture substrate. However, cell attachment to titanium was approximately 80% of attachment to TCP, and thus the difference in attachment is not very large. This confirms the biocompatibility of titanium. Presumably, high cell attachment to titanium will result in better bone formation on the implant surface, fixation or osseointegration of the implant, and a reduced chance of the formation of a fibrous capsule around the implant, which results in loosening and clinical failure. Due to its high mechanical strength, a well-anchored titanium implant is able to withstand the high forces encountered in dental and orthopedic applications.

All 3 titanium surfaces showed lower cell proliferation rates compared with TCP. As well as increasing attachment, compared with smooth nonpassivated surfaces, increasing surface roughness also resulted in an increase in cell proliferation when compared with all types of smooth titanium. Clinical reports indicate that increasing implant surface roughness results in improved bone formation and osseointegration of titanium implants in laboratory animals as well as humans.[33–35] The results of this study suggest that this may be because proliferation of osteoprogenitor cells are increased on rougher surfaces, which would result in the formation of more osteoblasts and therefore increased bone formation.

Conclusion

Titanium provides a surface that is conducive to the attachment of human marrow stromal cells. The rate of cell proliferation on titanium was lower than the rate on TCP; however, increasing surface roughness of titanium improved the proliferation rate, approaching the rate of TCP.

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