Nitric Acid Passivation Does Not Affect In Vitro Biocompatibility of Titanium

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Purpose: *In general, both chemical composition and surface features of implants affect cell response. The aim of this study was to evaluate the effect of titanium (Ti) passivation on the response of rat bone marrow cells, considering cell attachment, cell morphology, cell proliferation, total protein content, alkaline phosphatase (ALP) activity, and bonelike nodule formation.* ***Materials and Methods:*** *Cells were cultured on both commercially pure titanium (cpTi) and titanium-aluminium-vanadium alloy (Ti-6Al-4V) discs, either passivated or not. For attachment evaluation, cells were cultured for 4 and 24 hours. Cell morphology was evaluated after 4 days. After 7, 14, and 21 days, cell proliferation, total protein content, and ALP activity were evaluated. Bonelike nodule formation was evaluated after 21 days. Data were compared by analysis of variance and the Duncan multiple range test.* ***Results:*** *Cell attachment, cell morphology, cell proliferation, total protein content, ALP activity, and bonelike nodule formation all were unaffected by Ti composition or passivation.* ***Discussion and Conclusion:*** *Although the protocol for passivation used here could interfere with the pattern of ions released from Ti-6Al-4V and cpTi surfaces, the present study did not show any effect of this surface treatment on in vitro biocompatibility of Ti as evaluated by osteoblast attachment, proliferation, and differentiation.* INT J ORAL MAXILLOFAC IMPLANTS 2003;18:820–825

Key words: *biocompatibility, cell culture, nitric acid, osteoblast, titanium*

Since the introduction by Brånemark and coworkers[1] of osseointegrated dental implants, numerous in vivo and in vitro studies have been performed to find surface features that allow an improved tissue response. Several material properties condition the osseointegration of dental implants made of titanium (Ti), including the surface chemical composition and treatments modulating the chemical and physical characteristics of the metallic surfaces.[2]

At present, Ti implants in clinical use vary with respect to surface roughness and composition. Ti implants can be manufactured of commercially pure titanium (cpTi) or titanium-aluminium-vanadium alloy (Ti-6Al-4V). Both cpTi and Ti-6Al-4V develop a surface oxide layer because of the natural passivation of Ti.[3] However, differences in the crystallinity of the underlying metal, as well as the segregation of alloy components, may cause the oxide that forms on cpTi to be quite different from the oxide that forms on Ti-6Al-4V.[4] Several studies have shown that even subtle differences in surface composition, including Ti oxide crystallinity, can modify cell response, even when surface roughness is kept constant.[5–7] Although in vivo investigations of the tissue response to the materials have not revealed any qualitative difference between cpTi and Ti-6Al-4V, biomechanical tests have shown that cpTi is more stable in the bone bed than Ti-6Al-4V.[8,9] Lincks and associates[4] related that cpTi allows better cell responses than Ti-6Al-4V in experiments using cell lineage culture. However, mechanisms underlying cell responses to cpTi and Ti-6Al-4V using primary cultures are poorly understood.

In an attempt to improve the quantity and quality of the bone-Ti interface, surface treatments such as machining, acid etching, sandblasting, or plasma spraying may be undertaken to induce chemical modifications associated with alterations of the surface topography.[10] It has been shown that methods of implant surface preparation can significantly

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affect the resultant properties of the surface and subsequently the biologic responses and rates of cell attachment that occur at the surface.[11,12]

Implants made from cpTi or Ti-6Al-4V are customarily subjected to passivation treatment on the assumption that this will produce a more inert surface by increasing the thickness of the spontaneously produced oxide layer to which these materials owe their comparatively low reactivity.[13] ASTM-F86, which employs a 20% to 40% concentration of nitric acid (HNO_3), is such a surface passivation protocol. It was originally developed for stainless steel and chrome-cobalt alloy surgical implants, but it is suggested as an option for other implant materials.

In view of the afore mentioned and because the effect of such passivation of Ti on cell response has not been analyzed, it was decided to investigate the procedure using a cell culture system. The cpTi and Ti-6Al-4V surfaces were prepared so that passivated and nonpassivated surfaces were compared. The cell culture system used in this study was rat bone marrow directed in vitro to form osteoblastic cells and the following were evaluated: cell attachment, cell morphology, cell proliferation, total protein content, alkaline phosphatase (ALP) activity, and bone-like nodule formation.

MATERIALS AND METHODS

Preparation of Ti Discs

Discs of cpTi and Ti-6Al-4V were obtained from commercial bar stock with a diameter of 12 mm and were cut to a height of 4 mm. All discs were polished with silicon carbide papers in the sequence 280-600-1,200-2,400.

Cleaning and Passivation. This procedure has been described by Callen and coworkers,[13] and it is a modification of the ASTM-F86 protocol. Double-distilled high-purity (18.0 MΩ · cm) water (DDH_2O) was used throughout. The 34% HNO_3 was prepared by mixing 34 mL of HNO_3 with 66 mL of DDH_2O, and the protocol for passivation was as follows:

1. Cleaning: Sonication in 2% Sigmaclean solution (Sigma, St Louis, MO) for 1 hour
2. Rinsing: Sonication in 3 DDH_2O rinses (5 minutes each)
3. Passivation: Sonication in 34% HNO_3 for 1 hour
4. Final rinsing: Sonication in 5 rinses of DDH_2O (5 minutes each)

Nonpassivated discs were prepared using steps 1 and 2 only. All discs were autoclaved before they were used in the cell culture experiments.

Rat Bone Marrow Cell Culture. Rat bone marrow cells obtained from the femora of young adult male Wistar rats (5 weeks old, 120 g) were cultured for 14 days under conditions that allow osteoblastic differentiation.[14] The culture medium utilized was α-minimum essential medium (Gibco/Life Technologies, Grand Island, NY), supplemented with 15% fetal bovine serum (Gibco), 50 µg/mL gentamycin (Gibco), 0.3 µg/mL fungizone (Gibco), 10^{-7} mol/L dexamethazone (Sigma), 5 µg/L ascorbic acid (Gibco), and 7 mmol/L β-glycerophosphate (Sigma). After reaching subconfluence, first-passage cells were cultured in the same medium at a concentration of 2×10^4 cells per well on Ti discs in 24-well culture plates (Falcon, Franklin Lakes, NJ). During the culture period, cells were incubated at 37°C in a humidified atmosphere of 5% carbon dioxide and 95% air, and the medium was changed every 48 hours. In each plate, empty wells were used as a control of culture conditions.

Cell Attachment. For the evaluation of attachment, cells were cultured for 4 and 24 hours on Ti discs. The culture medium was removed and the wells were washed 3 times with phosphate-buffered saline (PBS) at 37°C to eliminate unattached cells. The adherent cells were then enzymatically released (1 mmol/L ethylenediamine-tetraacitic acid [EDTA] and 0.25% trypsin; Gibco) from the Ti discs and counted using a hemacytometer. Cell attachment was expressed as a percentage of the adherent cells.

Cell Morphology. For the evaluation of morphology, cells were cultured for 4 days on Ti discs and processed for scanning electron microscopy (SEM). Briefly, cells were fixed with 1.5% glutaraldehyde (Sigma), buffered in 0.1 mol/L sodium cacodylate (Sigma), and stained in 1% osmium tetroxide (Sigma). Subsequently, cells were dehydrated through a graded series of alcohol, followed by critical point drying at 40°C and 1,300 psi for 4 minutes. Once dry, the samples were sputter coated with gold before examination under SEM (JSM-5410; Jeol, Peabody, MA).

Cell Proliferation. For the evaluation of proliferation, cells were cultured for 7, 14, and 21 days on Ti discs. The cells were released and counted as described in the Cell Attachment section. After enzymatic treatment, Ti discs were observed using SEM to confirm completeness of the cells' release from discs' surface.

Total Protein Content. Total protein content after 7, 14, and 21 days was calculated according to a modification of the Lowry method.[15] The culture medium was removed, and the wells were washed 3 times with PBS at 37°C and were filled with 2 mL of 0.1% sodium lauryl sulfate (Sigma). After 30 minutes, 1 mL of this solution from each well was

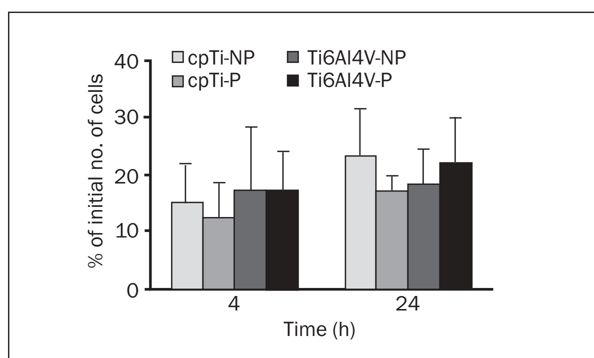

Fig 1 Cell attachment expressed as a percentage of the initial number of cells after 4 and 24 hours in culture on Ti discs. All data are reported as mean ± standard deviation (n = 5). NP = nonpassivated; P = passivated.

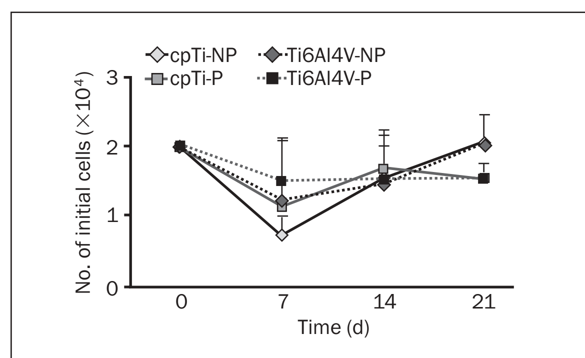

Fig 2 Proliferation of cells on Ti discs after 7, 14, and 21 days. All data are reported as mean ± standard deviation (n = 5). NP = nonpassivated; P = passivated.

mixed with 1 mL of Lowry solution (Sigma) and left for 20 minutes at room temperature. After this period, it was added to 0.5 mL of the solution of phenol reagent of Folin and Ciocalteau (Sigma). This stood for 30 minutes at room temperature to allow color development, and the absorbance was then spectrophotometrically measured (CE3021; Cecil, Cambridge, United Kingdom) at 680 nm. The total protein content (µg/mL) was calculated from a standard curve. These data were normalized by the number of cells counted after 7, 14, and 21 days, respectively.

ALP Activity. ALP activity was assayed as the release of thymolphthalein from thymolphthalein monophosphate using a commercial kit (Labtest Diagnostica SA, Belo Horizonte, Brazil), and specific activity was calculated. Aliquots of the same solutions used for calculating total protein content were assayed for measuring the ALP activity. The absorbance was spectrophotometrically measured at 590 nm and the ALP activity was calculated from a standard measure. Results were calculated (µmol thymophthalein/h) and data were expressed as the ALP activity normalized by the number of cells counted after 7, 14, and 21 days, respectively.

Bonelike Nodule Formation. After 21 days in culture, the cells were washed 3 times with PBS at 37°C. The attached cells were fixed in 3% glutaraldehyde in 0.1 mol/L sodium cacodylate buffer for 2 hours at room temperature and rinsed once in the same buffer. After fixation, the specimens were dehydrated through a graded series of alcohols and processed for staining with Alizarin red S (Sigma), which stains bonelike nodules, which are rich in calcium. The specimens were evaluated using an image analyzer (Image Tool; University of Texas Health Science Center, San Antonio, TX), and the amount of bonelike nodule formation was calculated as a percentage of the total Ti disc area.

Statistical Analysis

Data presented in this work are the representative results of 2 cultures with n = 5 for each group for each experiment. All data were submitted to an analysis of variance (ANOVA) and the Duncan multiple-range test when appropriate. Differences at $P \leq .05$ were considered statistically significant.

RESULTS

Cell Attachment

Cell attachment was not affected either by Ti chemical composition (ANOVA: F = 0.66; df = 1; P = .422) or by passivation (ANOVA: F = 0.29; df = 1; P = .592) (Fig 1). Cell attachment was a time-dependent event (ANOVA: F = 0.70; df = 1; P = .038), since there were more attached cells after 24 hours than after 4 hours.

Cell Proliferation

Cell proliferation was not affected either by Ti chemical composition (ANOVA: F = 0.58; df = 1; P = .453) or by passivation (ANOVA: F = 0.01; df = 1; P = .924) (Fig 2). Cell number was affected by period of culture (ANOVA: F = 6.78; df = 2; P = .003) in the following order: day 7 < day 14 = day 21 (Fig 2).

Cell Morphology

The evaluation of Ti discs with SEM after 4 days showed that cell morphology was not affected either by Ti chemical composition or by passivation (Fig 3). On all Ti surfaces, cells were relatively sparse, presenting an elongated morphology with extensions up to 50 µm, following the orientation of the residual machining grooves. Cells presenting dorsal ruffles were not seen on any of the surfaces tested.

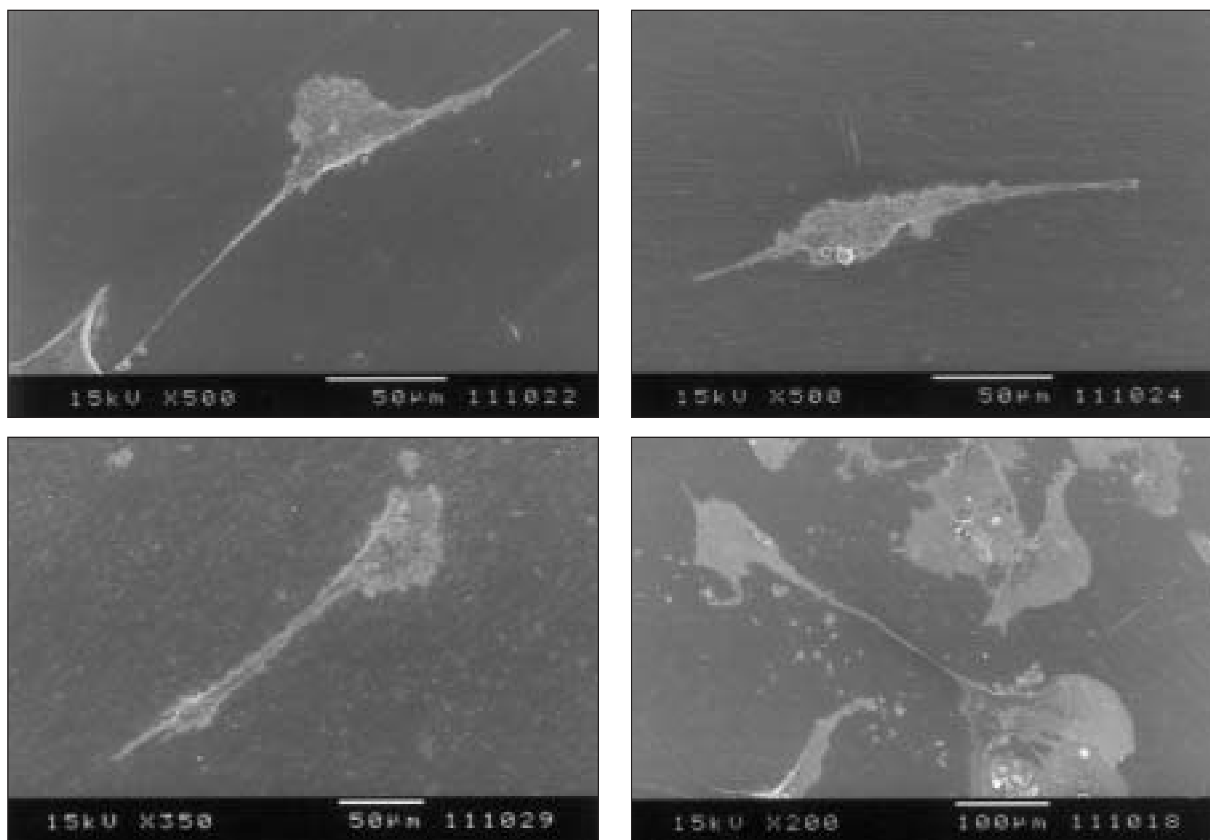

Fig 3 Scanning electron micrographs of rat bone marrow cells on Ti discs. *(Above left)* Nonpassivated cpTi; *(above right)* passivated cpTi; *(below left)* nonpassivated Ti-6Al-4V; *(below right)* passivated Ti-6Al-4V.

Total Protein Content

Total protein content was not affected by either Ti chemical composition (ANOVA: $F = 2.09$; df = 1; $P = .160$) or by passivation (ANOVA: $F = 1.69$; df = 1; $P = .202$) (Fig 4). Synthesis of total protein was affected by duration of culture (ANOVA: $F = 23.48$; df = 2; $P < .001$) in the following order: day 21 < day 14 < day 7 (Fig 4).

ALP Activity

ALP activity was not affected either by Ti chemical composition (ANOVA: $F = 0.005$; df = 1; $P = .946$) or by passivation (ANOVA: $F = 1.78$; df = 1; $P = .189$). ALP activity was affected by duration of culture (ANOVA: $F = 34.74$; df = 2; $P < .001$) in the following order: day 7 = day 14 < day 21 (Fig 5).

Bonelike Nodule Formation

Bonelike nodule formation was not affected by either Ti chemical composition (ANOVA: $F = 2.24$; df = 1; $P = .160$) or passivation (ANOVA: $F = 0.60$; df = 1; $P = .453$) (Fig 6).

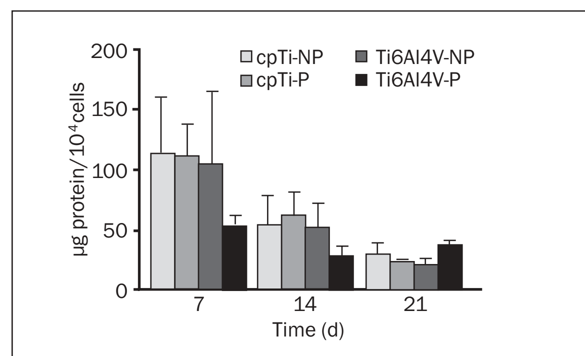

Fig 4 Total protein content normalized by the number of cells (µg protein/10^4 cells) after 7, 14, and 21 days. Data are reported as mean plus or minus standard deviation (n = 5). NP = nonpassivated; P = passivated.

DISCUSSION

Bone cell culture models are increasingly employed to study bone-biomaterial interactions.[16] Osteogenesis, induced by osteoblastic cells, is characterized by a sequence of events involving cell attachment and cell proliferation and followed by the expression of osteoblastic phenotype.[17] In this study, the response of rat bone marrow cells differentiated to

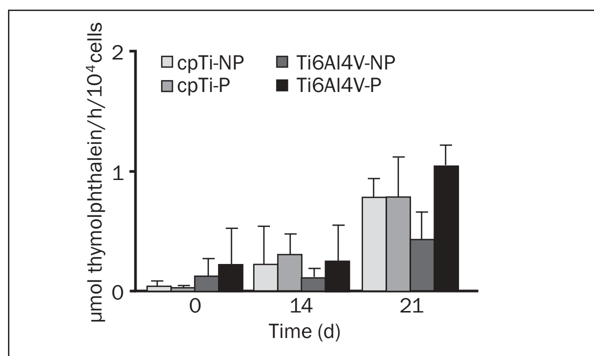

Fig 5 Alkaline phosphatase activity normalized by the number of cells (μmol thymolphthalein/h/10^4 cells) after 7, 14, and 21 days. All data are reported as mean plus or minus standard deviation (n = 5). NP = nonpassivated; P = passivated.

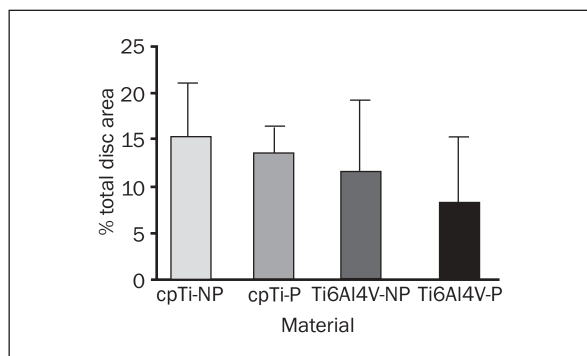

Fig 6 Bonelike nodule formation on Ti discs expressed as a percentage of total disc area after 21 days. All data are reported as mean plus or minus standard deviation (n = 5). NP = nonpassivated; P = passivated.

osteoblasts cultured on cpTi and Ti-6Al-4V discs, either passivated or nonpassivated, was evaluated. The results showed that all discs, independent of material chemical composition and surface treatment, allowed cell attachment, cell proliferation, and osteoblastic differentiation expressed as both ALP activity and bonelike nodule formation.

The surface conditions of the biomaterial are an important factor for implant acceptance in living bone.[18] Passivation is generally believed to render Ti-6Al-4V and cpTi surfaces more stable and thus less likely to release trace elements than their nonpassivated counterparts.[13] In the present work, Ti discs were passivated according to the procedure described by Callen and coworkers.[13] It has been observed that trace element release of the constituent elements of the Ti-6Al-4V is significantly increased, while cpTi is unaffected by HNO_3 passivation.[13,19] Also, it has been shown that changes on the Ti surface promoted by passivation are preserved after autoclaving.[13] Since the same protocol for passivation was used, it was assumed that the Ti surfaces in this study would present the same behavior as previously described.[13,19]

Cell adhesion probably is the single most important aspect of cell interaction with a biomaterial because it is the prerequisite for further cellular activity, such as spreading, proliferation, and differentiation.[20] In this study, some of the inoculated cells became attached to the Ti, whereas nonattached cells were removed by aspiration. The percentage of attached cells was not affected by Ti composition, as has been reported elsewhere.[21] Furthermore, cell attachment was not affected by passivation when the number of attached cells was counted after a 4-hour and 24-hour incubation period following cell inoculation. This might be because the ions released from Ti surfaces did not interfere with protein adsorption. As shown by Deligianni and associates,[17] in this study a time-dependent increase in cell attachment on biomaterials was observed, since there were more cells attached after 24 hours than after 4 hours.

It has been reported that the surfaces that show the best primary attachment characteristics are not necessarily the substrates on which cell differentiation is improved.[22] Therefore, it is important to investigate cell-biomaterial interactions at later time points. In the present study, evaluation of cell proliferation showed that both Ti chemical composition and passivation did not interfere with cell growth.

Recently, it was shown that cell morphology is unaffected by Ti surfaces submitted to machined and blasted treatments, whether acid-etched or not.[23] These authors also reported that when residual machining grooves are present on Ti surfaces, cells tend to follow the orientation of these grooves. In accordance with this, in the present study, cell morphology was unaffected by Ti surface treatments, and cells grew under orientation of the residual machining grooves.

In general, cell synthesis activity is sensitive to the type of material.[4] The present results showed that, for rat cells, neither Ti chemical composition nor passivation interfered with the amount of total protein produced. Considering the amount of protein that was normalized by the cells, these results reflect that the cell secretory activities were not affected by nitric acid passivation.

Lincks and colleagues[4] and Perizzolo and associates[24] showed a positive correlation between ALP activity and bonelike nodule formation, both markers of osteoblastic differentiation. This correlation was also observed in previous work by Rosa and Beloti[21] and confirmed in the present study. ALP activity was normalized by the number of cells to eliminate the effect of proliferation on this parameter. It was observed that neither Ti chemical composition nor passivation interfered with ALP activity.

Passivation did not affect bonelike nodule formation on either cpTi or Ti-6Al-4V. However, bonelike nodule formation was greater on cpTi than on Ti-6Al-4V, although the difference was not significantly significant. This is in agreement with previous work using in vitro and in vivo evaluations showing that cpTi is more biocompatible than Ti-6Al-4V.[8,9,21]

There is a possibility that the lack of difference between passivated and nonpassivated surfaces was a result of low sensitivity of the in vitro cell culture analysis. In contrast to this, use of the same methods was sensitive to discriminate biocompatibility of hydroxyapatite samples presenting slight surface modifications),[26] while in vivo analysis failed to provide such discrimination.[25,26]

Although Callen and coworkers[13] observed that the protocol for passivation used here interfered with the pattern of ions released from Ti-6Al-4V and cpTi surfaces, the present study did not show any effect of this surface treatment on the in vitro biocompatibility of Ti as evaluated by osteoblast attachment, proliferation, and differentiation. In keeping with this, Ku and coworkers,[27] using a different cell culture system, have shown that passivation has only minor effects on cell behavior.

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