In vitro biocompatibility evaluation of surface-modified titanium alloys

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Abstract: The present work is aimed to evaluate the effects of a surface modification process on the biocompatibility of three vanadium-free titanium alloys with biomedical applications interest. Chemical composition of alloys investigated, in weight %, were Ti-7Nb-6Al, Ti-13Nb-13Zr, and Ti-15Zr-4Nb. An easy and economic method intended to improve the biocompatibiblity of these materials consists in a simple thermal treatment at high temperature, 750°C, in air for different times. The significance of modification of the surface properties to the biological response was studied putting in contact both untreated and thermally treated alloys with human cells in culture, Human Umbilical Vein Endothelial Cells (HUVEC) and Human Peripheral Blood Mononuclear Cells (PBMC). The TNF-α release data indicate that thermal treatment improves the biological response of the alloys. The notable enhancement of the surface roughness upon oxidation could be related with the observed reduction of the TNF-α levels for treated alloys. A different behavior of the two cell lines may be observed, when adhesion molecules (ICAM-1 and VCAM-1 in HUVEC, ICAM-1, and LFA-1 in PBMC) were determined, PBMC being more sensitive than HUVEC to the contact with the samples. The data also distinguish surface composition and corrosion resistance as significant parameters for the biological response. © 2009 Wiley Periodicals, Inc. J Biomed Mater Res 92A: 1623–1634, 2010

Key words: titanium alloy; surface treatment; biocompatibility; endothelial cells; blood mononuclear cells

INTRODUCTION

The most important requirement for a biomaterial is its ability to exist in contact with tissues of the human body without causing an unacceptable degree of harm to that body. It is clear that an eventual metal release from the implant into the surrounding tissue is potentially harmful, and sometimes is associated with implant failure, osteolysis, cutaneous allergic reactions, and remote site accumulation.[1,2] Chronic inflammation and/or hypersensitivity are among the highly undesirable reactions. Hypersensitivity, either immediate or delayed, is fairly common, affecting more than 15% of the population.[3,4] Numerous metallic elements may act as sensitizers and most metal ions may form complexes with native proteins and these complexes can induce allergy or may act as allergens in the body.

Among biomaterials, titanium and titanium-based alloys are widely employed[5] for dental and orthopaedic implants, showing excellent corrosion resistance and mechanical properties. Although one of the most used metallic biomaterials is the well-known Ti-6Al-4V alloy, it has been reported the potential adverse effects of vanadium,[6] and the utility of developing an alternative titanium alloy, containing niobium instead of vanadium, but with comparable mechanical properties and with enhanced corrosion resistance. On the other hand, the reduced ion release and the good biocompatibility of titanium and its alloys are in great part attributed to the formation of an inert passive oxide film on the metallic surface, which is amorphous or poorly crystallized, thermodynamically stable and with a low solubility in the body fluid.[7,8] In the case of Ti alloys, this native oxide consists of a mixture of different titanium sub-oxides as well as other oxides that depend on the Ti alloy composition.[9] Body fluid is considered

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extremely corrosive to metallic materials, and dissolved metal ions can accumulate near the implant or may be transported to other parts of the body.[10] In the body, however, rupture or weakening of the protective oxide layer may occur as a consequence of mechanical friction and chemical influences, leading to the formation of wear debris and corrosion processes. Moreover, in *in vivo* environment, the regeneration of a passive surface layer on the titanium surface is slow. In some patients, after implantation, elevated levels of titanium in the serum were detected.[11]

Surface-modification techniques may be used in order to reduce wear and corrosion phenomena, which may occur at the implant level. Hence, a possibility to improve the corrosion resistance of metallic materials and, therefore, their biocompatibility, is to increase the thickness of the surface oxide layer. A simple and economical method is to treat the material thermally in an oxygen rich atmosphere, which produces a thicker surface oxide layer than the passive film.[12,13] For Ti alloys this procedure, in general, promotes Ti diffusion to the surface, reducing thus the presence of non-protective oxides.[14–16] In previous works, three Ti alloys without V, of composition (in wt %) Ti-7Nb-6Al, Ti-13Nb-13Zr, and Ti-15Zr-4Nb, have been evaluated as potential biomaterials both untreated and after a simple thermal treatment in air.[17–21] Electrochemical techniques were used to evaluate the corrosion rates of these alloys and to estimate the ion release.[17,18] While the oxidation process of Ti-7Nb-6Al improved the corrosion resistance due to the formation of a compact, dense and protective oxide layer, for the two TiNbZr alloys no improvement was observed due to the oxide layer porosity. In this sense, since the cells will be in direct contact with the alloy topmost surface, not only the corrosion behavior but also other physical and chemical properties related mainly to the material surface were studied (surface chemical composition, roughness, surface mechanical properties, etc.).[14–21] However, there is a lack of investigations on their biocompatibility, which is of high concern to evaluate new biomaterials.

In order to evaluate the biocompatibility of metallic biomaterials, cultured cells may be used.[22] *In vitro* models are suitable for clarifying several aspects of cell interactions with biomaterials,[23,24] and many cell lines have been employed for assessing the biocompatibility of titanium alloys as well as other biomaterials.[25–34]

In previous researches we have tested the biocompatibility of surface-treated Ti-6Al-4V titanium alloy and surface treated AISI 316L austenitic stainless steel, using as experimental model both Human Umbilical Vein Endothelial Cells (HUVEC) and Human Peripheral Blood Mononuclear Cells (PBMC).[35–37] The choice of HUVEC and PBMC cultures for verifying biocompatibility was justified by these considerations. Endothelial cells, which may interact with circulating cells to induce an inflammatory response, play a fundamental role in the phenomena present at the host implant interface, and, among endothelial cells, HUVEC are widely used for *in vitro* experiments. Human PBMC, a heterogeneous cell population including monocytes and lymphocytes, represent a model, which is similar to what is present *in vivo*. Vascular endothelial cells represent the interface between the blood-stream and tissues and regulate the adhesion of blood monocytes and their subsequent migration across the endothelium,[38] this process being mediated by endothelial surface adhesion molecules,[39,40] whose expression increases after stimulation with various biological modifiers, such as interleukin-1 (IL-1) and tumor necrosis factor-alpha (TNF-α).[41] On endothelial cells, intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) are involved in recognition and adhesion, and also E-selectin, an adhesion molecule expressed on activated endothelium, can support the adhesion of neutrophils and monocytes.[42,43] Moreover, leukocyte-endothelial interactions are pivotal steps in mediating inflammatory responses. Tissue integration of biomaterial implants depends not only on the repair processes, but also on the control of reactions like inflammation. Cells of circulating blood, resident inflammatory cells, and endothelial cells are all involved in the physiological response of inflammation. For this reason, blood cells have been also extensively used, in order to verify the biocompatibility of metal implants.[44–49]

Since the survival time of most metallic implants seems to be due to a lack of biocompatibility,[50] the evaluation of the effects exerted by these materials on suited cell culture models is of great importance. Therefore, taking into account the fundamental role exerted by both HUVEC and PBMC in response to inflammatory stimuli, it may be hypothesized that these cells represent a good experimental model to assess the biocompatibility of metallic samples.

The aim of this research was to evaluate the influence of the surface modification process based on a thermal treatment on the biocompatibility of three titanium alloys, Ti-7Nb-6Al, Ti-13Nb-13Zr, and Ti-15Zr-4Nb. For this goal, the effects of untreated and surface-modified alloys on HUVEC and PBMC in culture were investigated. The biocompatibility was assessed at the level of specific features of cells cultured in the presence of the sample types under study (lactate dehydrogenase, LDH, release in culture medium by HUVEC and PBMC, TNF-α release in culture medium by PBMC, expression of adhesion molecules in HUVEC and in PBMC).

MATERIALS AND METHODS

Preparation of the Samples

Three titanium alloys with compositions (in weight %) Ti-6Al-7Nb, Ti-13Nb-13Zr, and Ti-15Zr-4Nb have been prepared by arc melting and then casting in a copper coquille under high vacuum. Oxidation tests were carried out on specimens cut from as-cast ingots by electrospark erosion. Before the oxidation process, the major sample surfaces were abraded and polished using diamond pastes with successively smaller particle size. In the final stage of this process, colloidal silica was used to ensure a surface free of mechanical deformation. Finally, the samples were ultrasonically cleaned with acetone for 20 min before oxidation. Oxidation was carried out in air at 750°C for exposition times of 90 min, 6 and 24 h. The sample types were indicated as follows: Ti-7Nb-6Al as-received (T1-0), oxidized at 750°C for 1.5 h (T1-1.5), 6 h (T1-6), 24 h (T1-24); Ti-13Nb-13Zr as-received (T2-0), oxidized at 750°C for 1.5 h (T2-1.5), 6 h (T2-6), 24 h (T2-24); Ti-15Zr-4Nb as-received (T3-0), oxidized at 750°C for 1.5 h (T3-1.5), 6 h (T3-6), 24 h (T3-24).

The phase composition and the characteristics of the oxide layers were previously reported together with the values of the surface root mean square roughness (rms) of the metallic samples.[20] In that work, the rms roughnesses were obtained by using scanning force microscopy (SFM) in the contact mode of operation. These values were around 10–20 nm for the as-received samples and increased as the oxidation time increased up to around 100–250 nm for the 24 h oxidized specimens.

As reported in previous works,[17–20] the different alloying elements and their different percentage in each alloy have a clear influence on the morphology and surface composition of the thermally grown oxides. The three studied Ti alloys belongs to the α-β family of Ti alloys. The effect of the different alloying elements on the phase stabilization at room temperature is different. Whereas Al is an α-phase stabilizer, Zr is a neutral element and the β-phase is stabilized by Nb. The balance of alloying elements produces alloys with similar α-phase/β-phase ratio for the case of T1 and T3, presenting T2 a lower α-phase/β-phase ratio. Upon oxidation, different surface composition and morphology is promoted for the three alloys. Thus, the oxide layer of T1-24 is rich in Al_2O_3, whereas for oxidized T2 and T3 are mainly composed of TiO_2. Taking into account the surface topography, there seems to be a correlation between final rms values and α-phase/β-phase ratios of the alloys.[20]

HUVEC and PBMC Cultures

Samples, in untreated state or subjected to the various treatments, were sterilized in an autoclave at 120°C for 20 min.

Normal Human Umbilical Vein Endothelial Cells (HUVEC) cryopreserved at the end of the primary culture were obtained by Cascade Biologics. The phenotypic characterization was previously reported.[34] HUVEC were grown in phenol red free medium 200 (Medium 200PRF) supplemented with LSGS kit (complete medium), obtained by Cascade Biologics. The final concentrations of the components (from SIGMA) in the supplemented medium were: foetal bovine serum (FBS), 10% v/v; hydrocortisone, 1 μg/ml; human epidermal growth factor, 10 ng/ml; basic fibroblast growth factor, 3 ng/ml; heparin, 10 μg/ml; and antibiotics (penicillin/streptomycin/amphotericin B solution). Cultures were maintained at 37°C in humidified atmosphere containing 5% CO_2. In our experiments, HUVEC were used in passages three or four. When the cells were at 70% confluence, the cultures were divided in 13 groups (3×10^5 cells/group): the cells of the control group received only culture medium; 12 groups were maintained in direct contact with the 12 sample types (one sample type for each group) for 48 or 72 h.

Human Peripheral Blood Mononuclear Cells (PBMC) were obtained from healthy subjects by density gradient (1.077) centrifugation (30 min at 400*g*) of heparinized venous blood (different blood samples of 10 ml each) diluted 1:2 with PBS (phosphate buffered saline) on Ficoll-Paque (research grade, Amersham Pharmacia Biotech.). About 95% mononuclear cells at the interface, containing PBMC, was collected and washed twice with PBS. PBMC viability, checked by measuring Trypan blue dye exclusion, was >90%. PBMC were cultured in RPMI 1640 supplemented with 25 mM Hepes, 10% (v/v) heat inactivated FBS, 60 mg/ml (100 U/ml) penicillin, 100 mg/ml streptomycin, 0.29 g/l L-glutamine in the absence or in the presence of 10 μg/ml phytohemagglutinin (PHA). Culture medium and all the other components were from SIGMA. PBMC were divided in 13 groups (2×10^6 cells/group): the cells of the control group received only culture medium; 12 groups were maintained in direct contact with the 12 sample types (one sample type for each group) for 48 or 96 h, in the presence of 10 μg/ml PHA. For the determination of adhesion molecules, PBMC were incubated with the sample types for 48 h in the absence of PHA. Cultures were maintained at 37°C in humidified atmosphere containing 5% CO_2. In all the experiments when PBMC were collected, at the end of the incubation times, the adherent monocytes were scraped and added to the non-adherent cells. This method was selected over primary isolated monocytes, as it allowed obtaining quantities of cells sufficient for the programmed experiments.

Protein Determination

Protein concentration in total cell lysates was determined by the Bradford method,[51] using bovine serum albumin as standard.

LDH Assay

Cell viability was estimated by analysing lactate dehydrogenase (LDH) content in the culture media of HUVEC and PBMC. Duplicate or triplicate samples from the cell medium were taken after culture of the cells maintained in the culture medium alone (control HUVEC and control PBMC) or in contact with the sample types for 48 or 72 h (HUVEC) and 48 or 96 h (PBMC). LDH activity was

measured at 30°C by a continuous optical test based on the extinction change of pyridine nucleotide at 340 nm, using pyruvate as substrate.

TNF-α Measurement

For the assay of TNF-α, the medium from cultured PBMC (cells maintained in contact with the culture medium alone or with the samples) was collected after 48 or 96 h, immediately centrifuged free of cells and frozen at −80°C until analyzed. To induce cytokine production, the cells were stimulated with 10 μg/ml PHA, otherwise TNF-α levels were too low and sometimes undetectable. In culture media of PBMC the amount of TNF-α was quantified by an enzyme-linked immunosorbent assay (Biotrak ELISA System from Amersham Biosciences). The secreted levels of TNF-α, calculated as pg/ml culture medium, were normalized to the total protein amount of PBMC lysates.

Western Blot Analysis

At the end of the incubation periods, the cells were collected and then lysed for 60 min (HUVEC) or 30 min (PBMC) at 4°C in buffer containing 0.1% Nonidet P-40, 0.5% (wt/vol) sodium deoxycholic acid, 0.1% (wt/vol) SDS PBS, pH 7.4, and the following protease inhibitors: 0.5 mmol/l PMSF, 1 μg/ml leupeptin, 1 μg/ml aprotinin and 0.5 μg/ml pepstatin. All the products were from SIGMA. After centrifugation, the supernatant was used for protein determination and Western blot analysis. Electrophoresis was performed on 10% SDS-polyacrylamide gel, loading 10 μg protein per lane for ICAM-1 and LFA-1 (Lymphocyte Function Associated Antigen 1) determination, and 15 μg protein per lane for VCAM-1 and E-selectin. Electrophoresed proteins were transferred onto pure nitrocellulose membranes (Bio-Rad Laboratories), as reported by Laemmli.[52] After transfer, the membranes were washed, incubated overnight at 4°C with the opportune antibody (Santa Cruz Biotechnology) to ICAM-1 (4 μg primary antibody/membrane), VCAM-1 (8 μg primary antibody/membrane), E-selectin (8 μg primary antibody/membrane), LFA-1 (8 μg primary antibody/membrane), washed again, and then incubated for 60 min with the secondary horseradish peroxidase-linked antibody (Santa Cruz Biotechnology). The membranes, after two washes with TPBS, were treated with the chemiluminescent substrate and enhancer (ECL plus, Amersham). Blots were analyzed by Chemi-Doc (Bio-Rad), utilizing the Quantity One program (Bio-Rad). In some experiments LFA-1 was determined in the same membrane used for ICAM-1. In this case, the membrane was stripped and reprobed for LFA-1. Protein bands were normalized using the respective β-actin protein band. The data obtained by densitometric analysis are given relative to control (cells maintained in the culture medium alone), set equal to 100.

Statistical Methods

Statistical significance was determined by either one-way ANOVA, followed by Bonferroni *t* test or by Student's *t* test. One-way analysis of variance was used to determine significance among groups, after which the modified *t* test with the Bonferroni correction was used for comparison between individual groups. Differences were considered significant at $P < 0.05$.

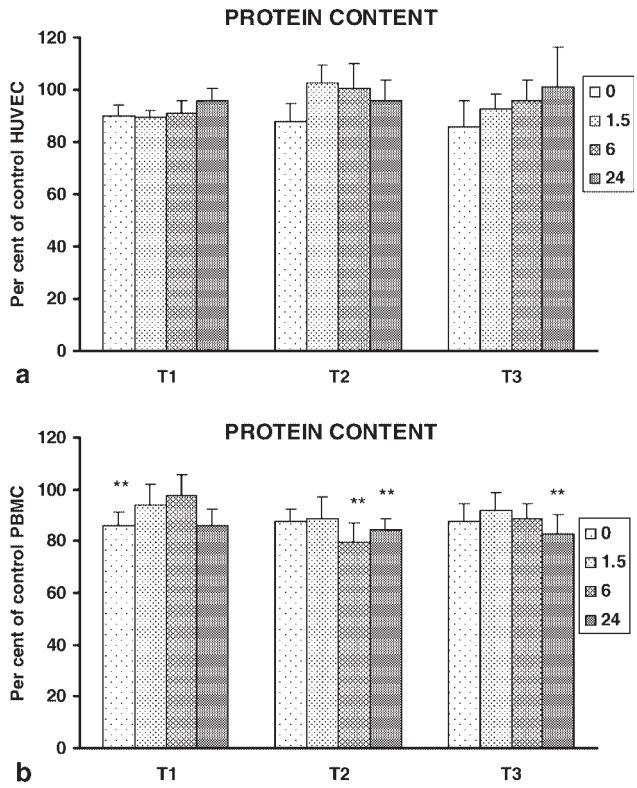

Figure 1. Effect of titanium sample types (indicated as reported in Materials and Methods) on protein content in HUVEC (a) and in PBMC (b). Data are given relative to control, set equal to 100. Each value represents the mean ± SD of three (HUVEC) or four (PBMC) separate experiments, each performed in duplicate. $^{**}P < 0.05$ (in comparison with control).

RESULTS

Protein Content

It is well known that total protein content is a good reflection of the general metabolic state of cells in contact with different surfaces. Protein content was determined in total lysates of HUVEC after 48- or 72-h contact with the sample types, and in total lysates of PBMC after 48- or 96-h contact with the sample types. For each cell line the data registered varying the time of incubation with the samples were not significantly different each other. In Figure 1(a,b), data are given relative to control (HUVEC or PBMC, respectively, incubated with culture medium alone), set equal to 100. In control HUVEC total protein content was 1.024 ± 0.118 mg/ml. In control

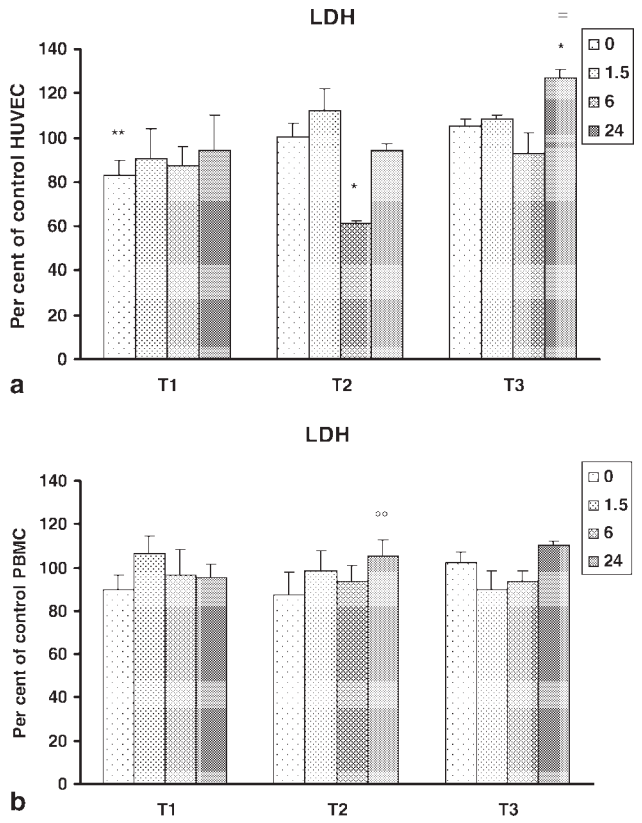

Figure 2. Effect of titanium sample types (indicated as reported in Materials and Methods) on LDH release in culture medium by HUVEC (a) and PBMC (b). Data are given relative to control, set equal to 100. Each value represents the mean ± SD of three (HUVEC) or four (PBMC) separate experiments, each performed in duplicate or triplicate. *$P < 0.01$ (in comparison with control); **$P < 0.05$ (in comparison with control); $^{=}P < 0.01$ (in comparison with T3-0); $^{\circ\circ}P < 0.05$ (in comparison with T2-0).

PBMC total protein content was 0.500 ± 0.053 mg/ml. None significant change in protein content was registered incubating HUVEC with the sample types, indicating that the general metabolic state of the cells was not critically affected. Otherwise, total protein content determined in PBMC maintained in contact with the sample types significantly, but not remarkably, decreased, in comparison with control PBMC, in the presence of T1-0, T2-6, T2-24, and T3-24. This result indicates that, considering protein content, the oxidation of the sample T1, at the three times, improves cell answer, whereas the oxidation for 24 h of T2 and T3 does not give a good result, together with the oxidation for 6 h of the sample T2.

LDH Assay

Cell viability was estimated by analysing the content of LDH in the culture medium. LDH activity was determined in medium of HUVEC cultures after 48- or 72-h contact with the sample types and in medium of PBMC cultures after 48- or 96-h contact with the sample types. For each cell line the data registered varying the time of incubation with the samples were not significantly different each other. In Figure 2(a,b), data are given relative to control (HUVEC or PBMC, respectively, incubated with culture medium alone), set equal to 100. In culture medium of control HUVEC, LDH activity was 0.034 ± 0.003 μmol/min/ml culture medium. In culture medium of control PBMC, LDH activity was 0.038 ± 0.002 μmol/min/ml culture medium. As regards HUVEC cultures, a slight, but significant increase was only registered in the presence of T3-24 in comparison with both untreated HUVEC and T3-0, whereas, in the presence of T1-0, and T2-6, LDH concentration was even lower than that detected in the medium of control HUVEC. None of the treatments affected LDH specific activity in culture medium of PBMC; a slight increase was only registered in the presence of T2-24 in comparison with T2-0.

TNF-α Release in Culture Medium by PBMC

To verify whether the contact of PBMC with the sample types might affect TNF-α release, the levels of this cytokine released in the culture medium by PBMC, maintained for 48 or 96 h in the presence of the sample types, were quantified and reported in Figure 3 relative to control (PBMC incubated with culture medium alone), set equal to 100. The data registered varying the time of incubation with the samples were not significantly different each other.

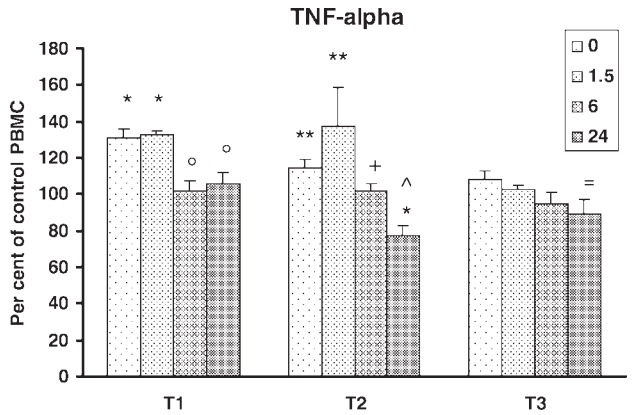

Figure 3. Effect of titanium sample types (indicated as reported in Materials and Methods) on TNF-α release in culture medium by PBMC. Data are given relative to control, set equal to 100. Each value represents the mean ± SD of four separate experiments, each performed in duplicate. *$P < 0.01$ (in comparison with control); **$P < 0.05$ (in comparison with control); $^{\circ}P < 0.01$ (in comparison with T1-0, and T1-1.5); $^{+}P < 0.01$ (in comparison with T2-0, and T2-1.5); ^$P < 0.05$ (in comparison with T2-0, T2-1.5, and T2-6); $^{=}P < 0.01$ (in comparison with T3-0).

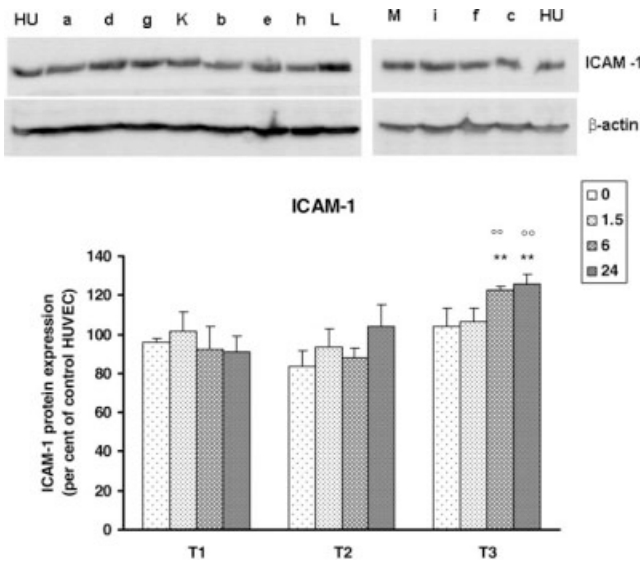

Figure 4. Effect of titanium sample types (indicated as reported in Materials and Methods) on ICAM-1 expression in HUVEC. ICAM-1 expression was analyzed by Western blot analysis. Protein bands were normalized using the respective β-actin protein band. Top, representative Western blot. Bottom, quantitative data. Blots were scanned by densitometry, and data, reported as a percentage of the control value (untreated HUVEC), set equal to 100, are expressed as the mean ± SD of three separate experiments. Abbreviations used in the representative Western blot: **HU**, untreated HUVEC; **a**, T1-0; **d**, T1-1.5; **g**, T1-6; **K**, T1-24; **b**, T2-0; **e**, T2-1.5; **h**, T2-6; **L**, T2-24; **c**, T3-0; **f**, T3-1.5; **i**, T3-6; **M**, T3-24. $^{**}P < 0.05$ (in comparison with control); $^{\circ\circ}P < 0.05$ (in comparison with T3-0).

In the culture medium of control PBMC, TNF-α concentration was 634.0 ± 43.0 pg/ml culture medium. In the presence of samples T1-0 or T2-0, TNF-α release significantly increased in comparison with that released by control PBMC. The oxidation of both T1 and T2 for 1.5 h was ineffective, whereas the oxidation of these samples for 6 or 24 h had a positive effect, cytokine levels decreasing up to those of control PBMC. On the other hand, in the presence of the sample T3, a significant change was only detectable comparing each other T3-24 and T3-0.

Adhesion Molecule Expression in HUVEC

The expression of ICAM-1, VCAM-1, and E-selectin in HUVEC incubated for 48 or 72 h with the sample types was studied. The data registered varying the time of incubation with the samples were not significantly different each other. Figures 4 and 5 report the densitometric values for ICAM-1 and VCAM-1 protein levels, respectively, registered after incubation of the HUVEC with the sample types, together with representative Western blots. The densitometric values reported in the graphic and expressed as per cent of the values registered in control cells (HUVEC maintained in the presence of culture medium alone) allow to calculate the fold increase (or decrease) for adhesion molecule levels. All the samples were ineffective in inducing an increase in ICAM-1 protein levels, with the exception of T3-6 and T3-24, which slightly, but significantly, increased the expression of this adhesion molecule in comparison with both control HUVEC and HUVEC incubated with the sample T3-0. None of the T1 samples (T1-0, T1-1.5, T1-6, and T1-24) affected VCAM-1 protein levels, whereas T2-0 induced a slight but significant increase in the expression of this adhesion molecule, which was reduced by the oxidation of T2 for 24 h. A significant increase was also registered in the presence of T3 (T3-0, T3-1.5, T3-6, T3-24), even if the three treatments lowered the expression of VCAM-1 in comparison with the untreated sample (T3-0). The levels of E-selectin were very low in control HUVEC and HUVEC maintained in contact with the sample types, so it was difficult to carry out a densitometric analysis, even if the data obtained seem to indicate

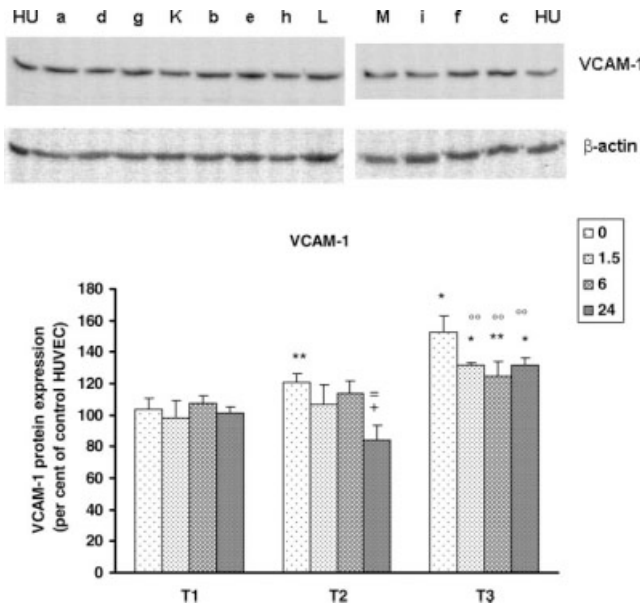

Figure 5. Effect of titanium sample types (indicated as reported in Materials and Methods) on VCAM-1 expression in HUVEC. VCAM-1 expression was analyzed by Western blot analysis. Protein bands were normalized using the respective β-actin protein band. Top, representative Western blot. Bottom, quantitative data. Blots were scanned by densitometry, and data, reported as a percentage of the control value (untreated HUVEC), set equal to 100, are expressed as the mean ± SD of three separate experiments. Abbreviations used in the representative Western blot: **HU**, untreated HUVEC; **a**, T1-0; **d**, T1-1.5; **g**, T1-6; **K**, T1-24; **b**, T2-0; **e**, T2-1.5; **h**, T2-6; **L**, T2-24; **c**, T3-0; **f**, T3-1.5; **i**, T3-6; **M**, T3-24. $^{*}P < 0.01$ (in comparison with control); $^{**}P < 0.05$ (in comparison with control); $^{=}P < 0.01$ (in comparison with T2-0); $^{+}P < 0.05$ (in comparison with T2-6); $^{\circ\circ}P < 0.05$ (in comparison with T3-0).

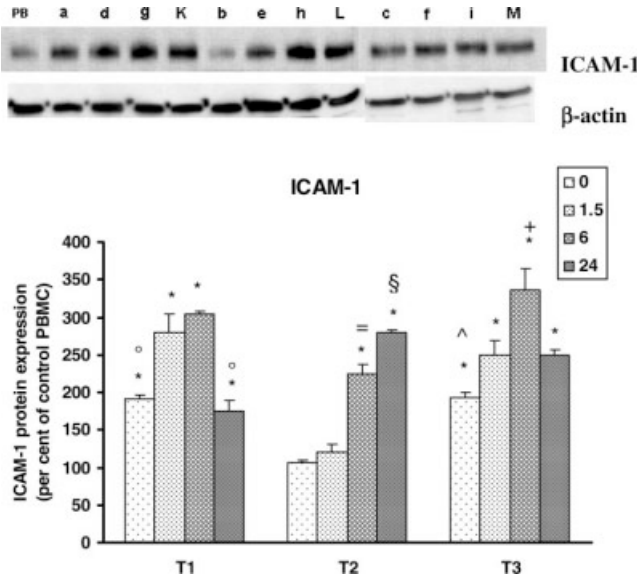

Figure 6. Effect of titanium sample types (indicated as reported in Materials and Methods) on ICAM-1 expression in PBMC. ICAM-1 expression was analyzed by Western blot analysis. Protein bands were normalized using the respective β-actin protein band. Top, representative Western blot. Bottom, quantitative data. Blots were scanned by densitometry, and data, reported as a percentage of the control value (untreated PBMC), set equal to 100, are expressed as the mean ± SD of three separate experiments. Abbreviations used in the representative Western blot: **PB**, untreated PBMC; **a**, T1-0; **d**, T1-1.5; **g**, T1-6; **K**, T1-24; **b**, T2-0; **e**, T2-1.5; **h**, T2-6; **L**, T2-24; **c**, T3-0; **f**, T3-1.5; **i**, T3-6; **M**, T3-24. $^{*}P < 0.01$ (in comparison with control); $^{\circ}P < 0.01$ (in comparison with T1-1.5, and T1-6); $^{=}P < 0.01$ (in comparison with T2-0, T2-1.5, and T2-24); $^{\S}P < 0.01$ (in comparison with T2-0, T2-1.5, and T2-6); $^{\wedge}P < 0.01$ (in comparison with the oxidized samples); $^{+}P < 0.01$ (in comparison with T3-0, T3-1.5, and T3-24).

an increase in E-selectin protein expression in the presence of T3-6 and T3-24 (data not shown).

ICAM-1 and LFA-1 Protein Expression in PBMC

The expression of ICAM-1 and LFA-1 in PBMC incubated for 48 h, in the absence of PHA, with the sample types was studied. Figures 6 and 7 report the densitometric values for ICAM-1 and LFA-1 protein levels, respectively, registered after incubation of the PBMC with the sample types, together with representative Western blots. The densitometric values reported in the graphic are expressed as per cent of the values registered in control cells (PBMC maintained in the presence of culture medium alone). With the exception of the samples T2-0 and T2-1.5, all the other sample types induced a significant increase in ICAM-1 protein levels in comparison with control PBMC. T1-0 and T1-24 induced a quite similar increase in ICAM-1, but this increase was significantly lower if compared to that registered in the presence of T1-1.5 and T1-6. The 6- and 24-h treatments of the sample T2 (T2-6 and T2-24) induced an increase in ICAM-1 protein levels more accentuated with respect to T2-0 and T2-1.5, the 24-h treatment inducing higher levels also if compared with the 6-h treatment. In the presence of the sample T3-6 the highest levels of ICAM-1 were detected, and, in the presence of T3-0, the lowest in comparison with the three treated sample types.

As concerns the other adhesion molecule tested, LFA-1, all the sample types, with the exception of T1-0 and T3-6, induced a significant increase in its protein levels. All the treatments of the sample T1 (T1-1.5, T1-6, T1-24) seem to significantly increase the levels of this adhesion molecule in comparison with T1-0, without differences among the three treatments. In the presence of the sample T2-24, LFA-1 levels were lower if compared to the other sample types, whereas, in the presence of T3-6, LFA-1 levels were significantly lower than in the presence of T3-0.

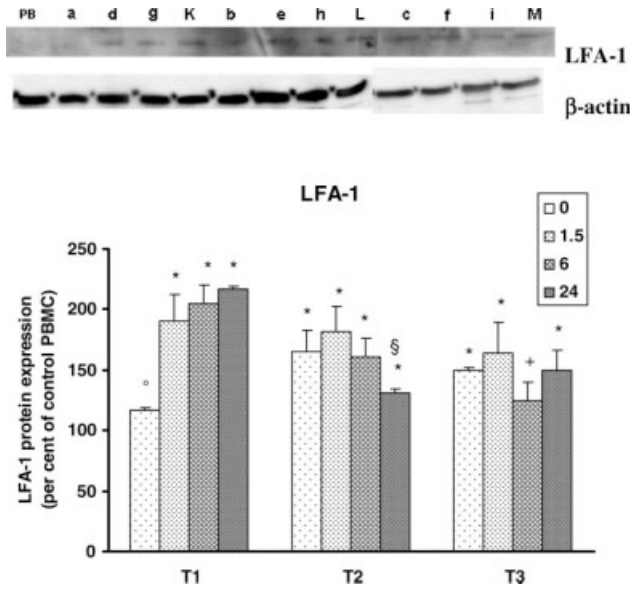

Figure 7. Effect of titanium sample types (indicated as reported in Materials and Methods) on LFA-1 expression in PBMC. LFA-1 expression was analyzed by Western blot analysis. Protein bands were normalized using the respective β-actin protein band. Top, representative Western blot. Bottom, quantitative data. Blots were scanned by densitometry, and data, reported as a percentage of the control value (untreated PBMC), set equal to 100, are expressed as the mean ± SD of three separate experiments. Abbreviations used in the representative Western blot: **PB**, untreated PBMC; **a**, T1-0; **d**, T1-1.5; **g**, T1-6; **K**, T1-24; **b**, T2-0; **e**, T2-1.5; **h**, T2-6; **L**, T2-24; **c**, T3-0; **f**, T3-1.5; **i**, T3-6; **M**, T3-24. $^{*}P < 0.01$ (in comparison with control); $^{\circ}P < 0.01$ (in comparison with the oxidized samples); $^{\S}P < 0.05$ (in comparison with T2-0, T2-1.5 and T2-6); $^{+}P < 0.05$ (in comparison with T3-0).

DISCUSSION

In the present research, we have studied the biocompatibility of titanium alloy samples Ti-7Nb-6Al, Ti-13Nb-13Zr and Ti-15Zr-4Nb, in both untreated state and treated at 750°C for 1.5, 6, and 24 h, in HUVEC and PBMC cultures, in order to verify whether the different sample types may, in any way, affect the behavior of these human cells.

The incubation time with the sample types under study was limited at a maximum of 72-h for HUVEC and 96-h for PBMC. No significant differences were registered in any of the parameters studied, varying the incubation time with the samples (48 and 72 h for HUVEC, 48 and 96 h for PBMC). It is possible that a more prolonged contact with the alloys could put in evidence different and/or more remarkable effects, but, as also suggested by Catelas et al.,[45] it was chosen not to analyze the PBMC response to exposure times longer than 96 h, to avoid nutrient depletion caused by prolonged incubation. As concern HUVEC cultures, also van Koten et al.[53] studied some characteristics of HUVEC in response to biomaterial contact after 48- and 72-h incubation periods, and many authors carried out biocompatibility studies after 24-h incubation.[44,54,55] The incubation periods for both HUVEC and PBMC may be considered a "short time", and the effects obtained register therefore the consequence of a contact limited to few days. The registration of early events in consequence of putting in contact the cells with the sample type is however very important, as the inflammatory events at the early stages of implant integration are necessary for efficient wound healing, and a dysregulation of inflammatory processes might contribute to aseptic loosening of the implant. The complexity of the host implant interface involves not only endothelial cells, but also cytokines, circulating cells, which may react with endothelial cells to generate an inflammatory response, and host growth factors. The use of two different human cell lines for the determination of biocompatibility of the alloys studied gave also some information about the possible different behavior of HUVEC and PBMC put in contact with the sample types.

Amongst different properties of the materials under study, surface composition, roughness and corrosion resistance are serious candidates to influence the biocompatibility behavior. Concerning the surface composition, the native oxide layers of the three untreated alloys are composed mainly of Ti_2O_3 with a small contribution of a mixture of different oxides.[9,14] However, the treated materials showed different behavior between both TiNbZr alloys and Ti-7Nb-6Al.[14–16] The two TiNbZr alloys show an oxide layer mainly composed of TiO_2 while Ti-7Nb-6Al exhibits for the first stages of the oxidation process an Al_2TiO_5 layer and for long oxidation times an Al_2O_3 layer. Concerning the surface roughness, a correlation between this property and the adhesion molecule expression may exist as deduced from the scientific literature (see below). It is well known that the mechanisms involved in cell-to-cell interactions are also sustained by cell surface molecules, termed adhesion molecules. Adhesion molecules are induced on the endothelial surface in response to various stimuli, including inflammatory cytokines like TNF-α[56] and the activation of adhesion molecules may be one of the consequences of the contact of biomaterials with the host cells, and in particular with endothelial cells.[28] ICAM-1 is a member of the immunoglobulin gene superfamily, which can be expressed both on non hematopoietic cells, such as endothelial cells, and hematopoietic cells. VCAM-1, a member of the immunoglobulin gene superfamily, supports the adhesion of lymphocytes, monocytes and other cells, and also participates in leukocyte adhesion outside the vasculature. VCAM-1 is not constitutively expressed on endothelium, but it can be up-regulated in response to several factors. E-selectin, which belongs to the selectin family of adhesion molecules, mediates the initial interactions of leukocytes and platelets with endothelial cells, and is expressed on cytokine-activated endothelial cells. LFA-1, a β2 leukocyte integrin involved in leukocyte adhesion to endothelium, is present on lymphocytes, monocytes and neutrofils and is the counter receptor of ICAM-1. Leukocyte adherence to endothelial cells is the result of a cascade of events where the participation of ICAM-1, probably together with VCAM-1, occurs in the later steps of the cascade.[28] We have therefore chosen to determine the levels of ICAM-1 and VCAM-1 together with E-selectin on HUVEC and those of ICAM-1 and LFA-1 on PBMC. ICAM-1 may be used *in vitro* as a marker of activation.[57] Therefore, the materials that present lower number of cells expressing ICAM-1 molecules are expected to induce less short-term inflammation. ICAM-1 is an activation marker of monocytes, regulates intercellular communication between leukocytes and, acting as a costimulatory molecule, is required but not sufficient for many inflammatory responses.[58] It is in fact well known that the events of the inflammatory response are very complex, as many elements are involved. Kirkpatrick et al.[22] have defined inflammation as a "complex orchestra of events".

The comparison between ICAM-1 and LFA-1 levels registered when PBMC were put in contact with the samples under study indicates a different behavior of the two adhesion molecules. This is not surprising, as, among blood mononuclear cells, monocytes present on their surface ICAM-1 and LFA-1, whereas lymphocytes LFA-1 only. In fact, as also reported by Pu et al.,[29] each adhesion molecule has distinctive patterns of expression. If we compare altogether the

results obtained assaying the adhesion molecules in HUVEC and PBMC lysates after the contact with the sample types under study, a different behavior may be observed, due to the higher sensitivity of PBMC if compared with that of HUVEC.

The results obtained for the adhesion molecule expression indicates for the ICAM-1 in HUVEC an increase of the measured values for high oxidation times. This result, only measurable for T3, could be related to the higher rms values of T3 upon oxidation as compared to oxidized T1 and T2 alloys. The effect of the surface roughness on the biocompatible behavior is not surprising, as the correlation between adhesion molecule expression and roughness of the samples has been demonstrated also by other authors. In a work of Mc Lucas et al.,[59] HUVEC were placed in contact with stainless steel discs of varying roughness. Similarly, the effect of surface roughness of the titanium alloy Ti-6Al-4V on human bone marrow cell response and on protein absorption, with the determination of cell attachment, cell proliferation and differentiation has been also reported.[60] Moreover, Marques et al.[61] concluded, in a previous study, that macrophages preferentially accumulate on rough and hydrophobic surfaces *in vitro*.

In contrast, the ICAM-1 expression in PBMC shows in the T1 case the best response for the fully oxidized T1 alloy, whereas for T2 and T3 the lowest ICAM-1 values are exhibited by the untreated materials. This result agrees with previous corrosion experiments where T1 alloy showed upon oxidation an improvement of the corrosion response due to the formation of a compact and dense oxide layer.[17,18] This layer reduces the ion release of the material with respect to the untreated alloy. However, the oxidation process leads to the formation of a porous oxide layer for T2 and T3 without improvement of the corrosion behavior as compared to the untreated alloys. Since the corrosion resistance is related to the ion release, its influence on the biological response could be expected. On the other hand, it is important to consider additionally that the oxide layer of treated Ti-7Nb-6Al alloy (Al_2O_3/Al_2TiO_5) and of treated TiNbZr alloys (TiO_2) are different, as discussed above.[14] A combined influence of corrosion resistance and chemical properties could be the responsible of the ICAM-1 expression results. However, the effect of other parameters such as thickness of the outer oxide layer on the biocompatibility is discarded being more decisive properties such as density, porosity and compact quality of the layer.

The cytokine TNF-α is known to be one of the most important signaling molecules involved in the response to foreign materials, and its up-regulation is considered to be a measure of inflammation.[62] This cytokine participates in the induction of adhesion molecule expression and, at higher doses, induces endothelial cell apoptosis. The assay of TNF-α released by PBMC put in contact with the sample types may represent a simple and rapid method to preliminary evaluate the biocompatibility of metallic alloys, as monocytes/macrophages are potent producers of this mediator. Bailey et al.[63] developed an *in vitro* model to measure the pro-inflammatory cytokine production useful for the quantification of cellular inflammatory responses to biomaterials and examined also the apoptotic pathway. These authors utilized a murine macrophage cell line, RAW 264.7. Although these cells retain many of the characteristics of macrophages *in vivo*, human PBMC represent, to our opinion, a more suitable experimental model. Human mononuclear cells were also used by Gretzer et al.,[44] who studied the influence of different material properties on the parameters of the inflammatory response. They did not observe any difference in cell viability as measured by LDH, but registered a significant increase in the levels of TNF-α released from cells stimulated with LPS and put in contact for 24 h with the material under study compared to unstimulated mononuclear cells. We registered only slight differences in LDH levels, but significant differences in TNF-α release by PBMC, with the exception of T-3 samples, which, considering this parameter, seem to have a good biocompatibility. However, it must be considered that the increases registered in TNF-α release, even if significant, are not so remarkable. We have previously observed that the contact of PBMC with Ti-6Al-4V titanium alloy[36] or with AISI 316L austenitic stainless steel[37] induced an increase in TNF-α levels up to about 10-fold and about 9-fold compared to control cells, respectively. In our experiments, in which TNF-α was assayed, PBMC were stimulated with PHA, as, in the absence of this substance, the levels of TNF-α were low, as also reported by Gretzer et al.,[44] and it was therefore difficult to compare each other the results obtained in the presence of the metallic samples. TNF-α tests suggest an improvement of the biological response for the long time heat-treated alloys with respect to untreated materials. From this point of view, considering the release of the proinflammatory cytokine TNF-α, the Ti alloys oxidation seems to decrease the inflammatory response. The decrease in TNF-α release in the presence of the samples subjected to the oxidation process could be related to the increase of the rms (root mean square roughness) values upon oxidation.[20]

CONCLUSIONS

The biocompatibility of three different vanadium-free Ti-alloys and their oxide layers was investigated

by putting their surface in contact with human cells in culture, HUVEC and PBMC. Interpretation of the results in combination with previous outcomes on the same materials has lead to the following conclusions:

1. PBMC are more sensitive than HUVEC to the contact with the sample types.
2. TNF-α tests reveal the amelioration of the biological response for the long time heat-treated alloys with respect to untreated materials, which could be related to the increase of the rms values upon oxidation.
3. The ICAM-1 in HUVEC indicates an increase of the adhesion molecule expression values for high oxidation times, only measurable for T3. The higher rms values for oxidized T3 as compared to oxidized T1 and T2 alloys could determine this behavior.
4. The ICAM-1 expression results in PBMC exhibit as the best result for T1, T2, and T3 samples the response obtained for the fully T1 oxidized alloy, the untreated T2, and the untreated T3, respectively. This outcome is in agreement with previous corrosion experiments.
5. Although different parameters could have an influence on the biocompatibility of biomaterials, surface chemical composition, corrosion resistance and surface roughness seem to be relevant properties in this topic.
6. The results obtained suggest that, to study biocompatibility, different cell lines must be used, and many biochemical parameters must be determined, in order to identify those metallic alloys, which combine a good corrosion resistance and a good biocompatibility.

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