

The Effects of Different Titanium Surfaces on the Behaviour of Osteoblast-Like Cells

Fabiano Ribeiro Cirano¹, Adriane Yaeko Togashi^{2*}, Márcia Martins Marques³,
Francisco Emílio Pustiglioni⁴, Luiz Antônio Pugliesi Alves Lima⁴

¹Department of Periodontology, Paulista University (UNIP), São Paulo, Brazil

²Department of Dental Implantology, State University of Western Parana (UNIOESTE), Cascavel, Brazil

³Department of Dentistry, University of São Paulo, São Paulo, Brazil

⁴Department of Periodontology, University of São Paulo, São Paulo, Brazil

Email: *adriane.togashi@unioeste.br, *adtogashi@gmail.com

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Abstract

This study investigated the influence of different titanium surfaces on the differentiation of rat osteoblast-like cells (osteoblast-1). Osteoblast-1 cells were cultured on the following titanium surfaces: 1) pretreated, smooth surface (PT); 2) sandblasted and acid etched surface (SLA); and 3) sandblasted and acid-etched surface rinsed under nitrogen protection to prevent exposure to air and preserved in isotonic saline solution (modSLA). Cell metabolism, total protein content, collagen content and alkaline phosphatase (AP) activity and the formation of calcified nodules were analyzed. The titanium surface did not influence cell metabolism, total protein content and collagen content. The SLA surface influenced cell differentiation, with the observation of a significant reduction of AP activity and formation of calcified nodules after 21 days compared to the PT surface. No difference was observed between the PT and modSLA surfaces. All titanium surfaces tested permitted the full expression of the osteoblast phenotype by osteoblast-1 cells, including matrix mineralization.

Keywords

Titanium, Dental Implants, Osteoblasts

1. Introduction

Various *in vitro* studies have shown that the surface roughness of titanium reduces the proliferation of osteoblast-like cells and increases cell differentiation by increasing alkaline phosphatase (AP) activity, protein synthesis, osteocalcin production and the formation of bone-like nodules [1]-[3]. *In vitro* studies also have shown that

*Corresponding author.

rough and chemically activated surfaces provide the ideal conditions for direct protein adsorption [4] and alter the adsorption of fibronectin and albumin [5] due to modifications in their ionic state [6]. Ramires *et al.* [7] demonstrated that titanium oxide acts as a nucleation substrate for calcium phosphate crystals. The biological activity of TiO₂ also influences protein adsorption to the titanium surface [6].

The quality of titanium surfaces can be described in terms of surface chemistry, which refers to the critical surface tension (CST) of a material [8]. The CST provides an indicator of the potential of cell adhesion (bioadhesiveness) or surface wettability [9]. Increased wettability thus improves the interaction between the implant surface and the biological environment [10]. In addition to changes in surface topography, some authors have proposed that the chemical modification of titanium surfaces, especially protection of sandblasted and acid etched (SLA) surfaces from air contamination, may improve osteoblast function [11]. This protection is achieved by immersing the surface in isotonic saline solution (modSLA) [4] [12].

Although many studies have reported improved results when cells were grown on rough titanium surfaces, there are reports showing that an increase in the roughness of titanium surfaces does not affect or even reduce cell function or bone formation [13]-[16].

The objective of the present study was to investigate the influence of SLA and modSLA surfaces on the metabolism and differentiation of rat osteoblast-like cells (osteoblast-1).

2. Experimental Methods

2.1. Sample Preparation

Sixty titanium discs with surfaces identical to those used in commercially available dental implants were fabricated. Titanium discs, measuring 15 mm in diameter and 1 mm in thickness, were manufactured from grade 2 unalloyed titanium (ASTM F67) by Institut. Straumann AG (Waldenburg, Switzerland). These discs had one of following surface topographies: 1) pretreated (PT) disc with a low mean roughness (Ra) which was degreased by washing in acetone, processing through 2% ammonium fluoride/2% hydrofluoric acid/10% nitric acid solution [17]; 2) sandblasted and acid-etched (SLA) disc which was further coarse grit-blasted with 0.25 - 0.50 mm corundum grit followed by acid etching, as described previously [17]; 3) sandblasted and acid-etched disc rinsed under nitrogen protection to prevent exposure to air and preserved in isotonic saline solution (modSLA) [4] [18]. Before use in the cell culture experiments, PT and SLA discs were washed and sterilized in an autoclave [17] [19]. The modSLA discs were sterilized by gamma irradiation and were provided in sealed wrappings and immersed in isotonic saline by the Straumann AG.

2.2. Cell Culture

A cell line derived from parietal bone tissue of newborn albino Wistar rats (osteoblast-1 cells) was obtained by enzymatic isolation. The culture medium was supplemented with sodium β -glycerophosphate, ascorbic acid and dexamethasone after semi-confluence as initially described by Deboni, Jaeger and Araújo [20] and Lavos-Valeto *et al.* [21] and recently characterized by Togashi *et al.* [22]. The cells were cultured in Dulbecco's modified Eagle medium (DMEM, Sigma Chemical Co., St. Louis, MO, USA) containing 10% fetal bovine serum (Cultilab, Campinas, SP, Brazil) and 1% antibiotic-antimycotic solution (Sigma). The cells were maintained in a humid atmosphere containing 5% carbon dioxide at 37°C. Cell growth was monitored at intervals of 24 h. The cells were plated onto the titanium discs at a concentration of 3×10^3 cells per well and the discs were placed in 24-well culture plates according to the growth curve of osteoblast-1 cells determined by the MTT method [23]. The media were changed every other day (Figure 1).

2.3. Cell Metabolism

For cell metabolism analysis, the cells were cultured for 24 h, enzymatically removed from the discs and counted in a hemocytometer [14]-[16].

2.4. Analysis of Total Protein Content

Total protein content was calculated after 7, 14 and 21 days of culture by the modified method of Lowry [24]. Total protein content was used to indirectly quantify the number of cells on the discs over time.

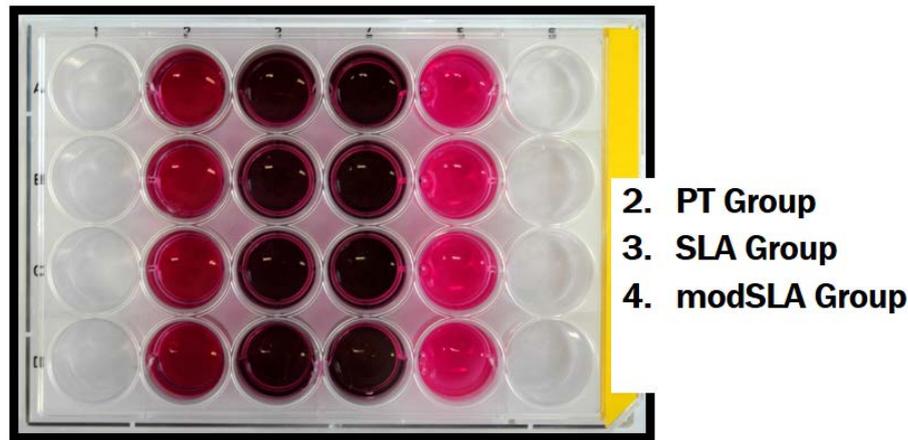


Figure 1. Osteo-1 cells plated onto the PT, SLA and modSLA discs at a concentration of 3×10^3 cells per well and the discs placed in 24-well culture plates.

2.5. Analysis of Collagen Content

Collagen content was calculated after 7, 14 and 21 days of culture according to the method of Reddy and Enwemeka [25] and normalized to total protein content.

2.6. Analysis of Alkaline Phosphatase Activity

AP activity was assayed after 7, 14 and 21 days of culture as the release of thymolphthalein from thymolphthalein monophosphate [14]-[16] using a commercial kit (Labtest Diagnostica AS, Lagoa Santa, MG, Brazil) and normalized to total protein content.

2.7. Evaluation of the Formation of Calcified Nodules

After 21 days of culture, the discs were processed for staining with Alizarin red (Sigma), which stains nodules rich in calcium. The specimens were analyzed with an image analyzer (Image Tool, University of Texas Health Science Center, San Antonio, TX, USA) as described previously [14]-[16].

2.8. Statistical Analysis

The data were submitted to analysis of variance (ANOVA), complemented by the Tukey test. The Kruskal-Wallis test was used when the variances were unequal. A level of significance of 5% ($p < 0.05$) was adopted.

3. Results

3.1. Cell Metabolism

Cell metabolism was not affected by the titanium surface topography ($p = 0.6407$) (Figure 2).

3.2. Total Protein Content

The topography of the titanium surface did not influence total protein content at any of the time points studied.

The highest total protein content was observed for the SLA group at 21 days. No significant differences between groups were observed after 7, 14 or 21 days of culture.

The results showed a time-dependent increase in total protein for the SLA group between 7 and 21 days. There was a time-dependent increase in total protein in the PT group and modSLA group between 7 and 14 days (Figure 3).

3.3. Collagen Content

The topography of the titanium surface did not influence collagen content at any of the time points studied ($p = 0.5502$) (Figure 4).

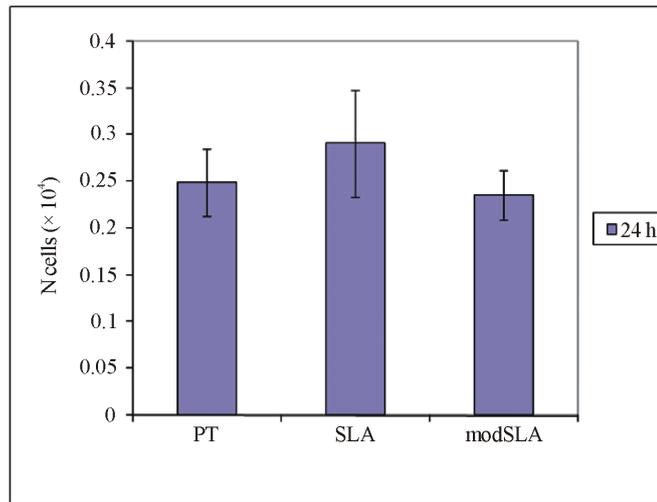


Figure 2. Effect of different titanium surfaces on the number of osteo-1 cells cultured for 24 h. Data are the mean \pm standard error of the mean of one experiment performed in quadruplicate (ANOVA, $p = 0.6407$).

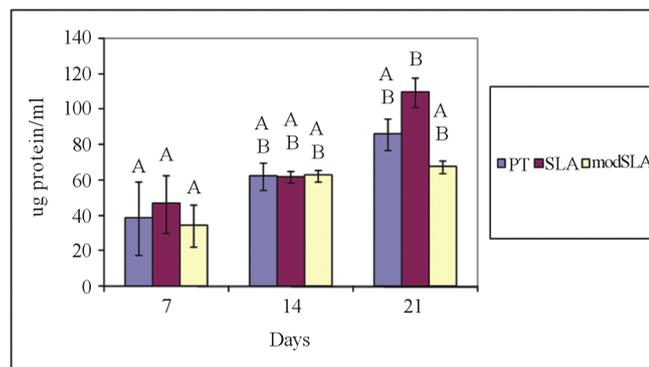


Figure 3. Total protein content of osteo-1 cells evaluated after 7, 14 and 21 days of culture. Data are the mean \pm standard error of the mean of one experiment performed in quadruplicate (ANOVA complemented by the Tukey test, $p = 0.0025$) and are expressed as μg protein/ml. Different letters indicate significant differences ($p < 0.05$).

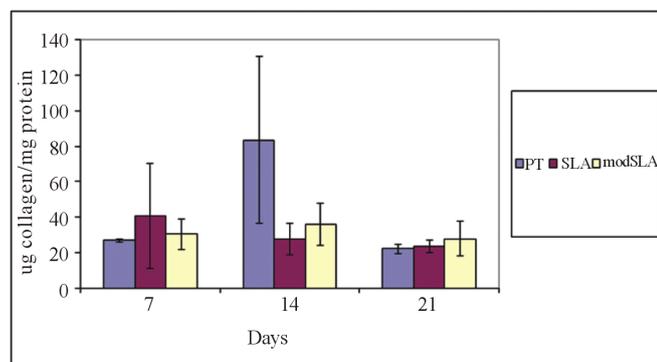


Figure 4. Collagen content of osteo-1 cells evaluated after 7, 14 and 21 days of culture. The results were normalized to total protein content (expressed as μg collagen/mg protein) and are reported as the mean \pm standard error of the mean of one experiment performed in triplicate (ANOVA, $p = 0.5502$).

3.4. Alkaline Phosphatase Activity

The topography of the titanium surface influenced AP activity after 14 and 21 days of culture. The activity of this enzyme was lower in the modSLA group compared to the PT and SLA groups after 14 days and in the SLA group compared to the PT group after 21 days.

The highest AP activity was observed in the PT group after 21 days of culture. There was a time-dependent increase in AP activity in the PT and modSLA groups at all time points studied. A time-dependent increase in AP activity was observed in the SLA group between 7 and 14 days and between 7 and 21 days (Figure 5).

3.5. Formation of Calcified Nodules

The topography of the titanium surface influenced the formation of calcified nodules at 21 days. A significant difference was only observed between the PT and the SLA groups (PT > SLA) (Figure 6).

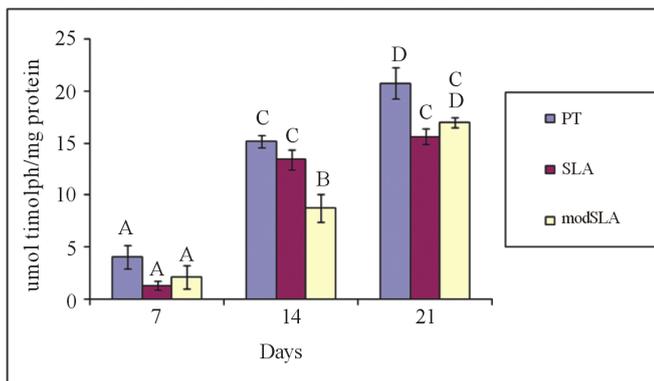


Figure 5. Alkaline phosphatase activity of osteo1 cells evaluated after 7, 14 and 21 days of culture. The results were normalized to total protein content (expressed as $\mu\text{mol thimolph/mg protein}$) and are reported as the mean \pm standard error of the mean of one experiment performed in quadruplicate (ANOVA complemented by the Tukey test, $p < 0.0001$). Different letters indicate significant differences ($p < 0.05$).

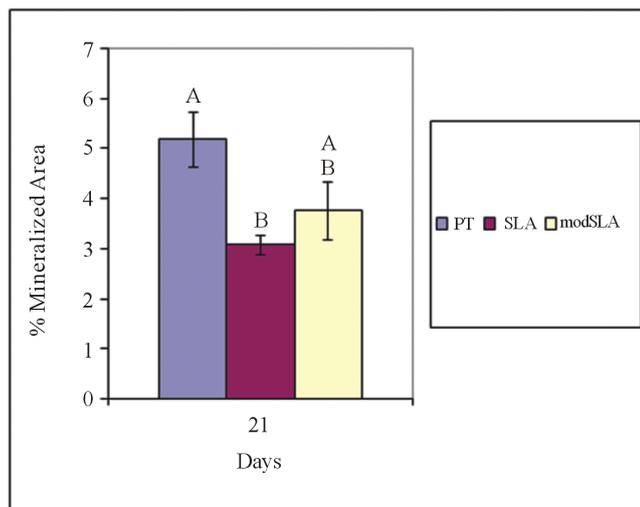


Figure 6. Formation of calcified nodules by osteo-1 cells evaluated after 21 days of culture. Data are the mean \pm standard error of the mean of one experiment performed in quadruplicate (Kruskal-Wallis test, $p = 0.0435$) and are expressed as percent mineralized area. Different letters indicate significant differences ($p < 0.05$).

4. Discussion

The topography of the titanium surface did not influence cell metabolism, total protein content or collagen content at any of the time points studied, but resulted in a significant reduction in AP activity in the modSLA group after 14 days of culture and in the SLA group after 21 days of culture. Furthermore, a significant reduction in the formation of calcified nodules was observed in the SLA group after 21 days of culture.

The results of this study showed that the titanium surface topography did not significantly affect cell metabolism. This finding can be explained by the fact that, although surface roughness seems to increase the area for cell adhesion, only the projected area is perceived by the cells; thus, no difference is observed between rough and smooth surfaces [26]. The cells seem to react to the morphology of the topography rather than to its roughness amplitude. Cells more easily attach to surfaces with an anisotropic roughness (machine-tooled and polished surfaces) than to isotropic surfaces (acid-etched and electro-eroded surfaces) [26]. Zhao *et al.* [4] [18] demonstrated a smaller number of adhered cells on modSLA surfaces compared to SLA surfaces and on SLA surfaces compared to machine-made surfaces; however, this was not observed in the present study. According to these authors, SLA surfaces have a high surface energy, a fact that may favor the selection of cells in a more advanced stage of differentiation. In contrast, Lai *et al.* [23] showed that the higher surface energy of titanium surfaces (modSLA) enhanced cell metabolism in the early stage of the cell response and may act by influencing the expression of adhesion-associated molecules. However, no significant differences in the gene expression of CK14, integrin 6, integrin 4, vinculin, TGF-1 or TGF-3 has been observed between epithelial cells (HSC-2) cultured on smooth, modSLA and SLA surfaces [27].

Titanium surface topography did not affect total protein content. This finding agrees with Rosa and Beloti [14] [15] who showed that surface roughness does not influence total protein since this parameter is unable to change the secretory potential of cells. The titanium surface topography also did not affect collagen content, in contrast to the literature showing an increase in collagen content with increasing surface roughness [1] [28]. According to Lincks *et al.* [1], higher collagen synthesis on rougher surfaces is positively correlated with the increased production of PGE₂ and latent TGF- β , which are markers of cell differentiation.

Studies investigating the associated biological mechanisms suggest that the TGF-b/BMP and WNT signaling pathways are triggered early in the interaction between osteoprogenitors and implant surfaces [29] [30] [31]. Chakravorty *et al.* [32] demonstrated that modified titanium implant surfaces induce differential regulation of miRNAs, which are potential regulators of the TGF-b/BMP and WNT/Ca²⁺ pathways during osteogenic differentiation on modified titanium implant surfaces.

Osteoblast-like cells cultured on titanium discs with SLA surfaces behave like cells cultured on other rough surfaces, with the observation of an increase in AP activity, an early marker of osteogenic differentiation [17] [19] [33] [34]. The present study showed a significant reduction in AP activity for cells grown on the modSLA surface compared to the PT and the SLA surfaces at 14 days and on the SLA surface compared to the PT surface at 21 days. Similarly, Zhao *et al.* [18] demonstrated lower AP activity of cells grown on modSLA surfaces compared to smooth surfaces, a finding suggesting the presence of mature secretory osteoblasts. According to these authors, the high surface energy of modSLA surfaces had no effect on AP activity, but increased the production of osteocalcin.

The topography of the titanium surface influenced the formation of calcified nodules at 21 days, with the observation of a significant reduction in the formation of calcified nodules in the SLA group. This finding agrees with Xavier *et al.* [16] who showed that formation of calcified nodules was significantly reduced on blasted and acid etched surfaces. One explanation for this reduction could be the release of constituent ions of the titanium, which negatively interfere with the mineralization process. The same does not apply to the modSLA surface since the sandblasted and acid-etched surface conditioned with nitrogen and preserved in isotonic saline solution may improve osteoblast function.

Although AP activity and the formation of calcified nodules after 21 days of culture were higher in the PT group compared to the SLA group, no difference was observed between the PT and modSLA groups. However, AP activity and the formation of calcified nodules after 21 days of culture tended to be lower in the SLA and modSLA groups when compared to the PT group. This finding is probably due to the selection of more differentiated cells by these surfaces, with a consequent earlier onset of differentiation characterized by elevated AP activity and subsequent expression of other markers of more advanced stages of differentiation (osteocalcin, osteopontin and bone sialoprotein) during this period and a consequent reduction in AP. Ong *et al.* [35] and Ku *et al.* [36] showed an early expression of osteocalcin on rough surfaces, an observation confirming the faster diffe-

rentiation of cells adhered to this type of surface.

The topography (micron and submicron scale roughness) and hydrophilicity (SLA and modSLA surfaces) of implant surfaces promote a microenvironment around the implant that may enhance osseointegration. Chemical modification decreases further cell attachment and proliferation, but upregulates early osteoblastic differentiation genes [2] [3]. Genes of osteoblastic differentiation are differentially expressed by osteoprogenitor cells cultured on modSLA and SLA surfaces. Chakravorty *et al.* [32] showed that BMP2, BMP6, ACVR1 and FZD6 were significantly up-regulated on the modSLA surface compared to the smooth surface. BMP6 was also significantly increased on the SLA compared with the smooth surface, whereas significantly higher expression of ACVR1 was observed on the modSLA surface compared to the SLA surface. In that study, the expression of BMP2, BMP6, ACVR1 and FZD6 was measured after 24 h of culture on modSLA, SLA and smooth surfaces.

Boyan *et al.* [17] also suggested that increased AP activity results in an increase of phosphate and calcium ion levels, which may represent an increase in the area of mineralized nodules but is not accompanied by non-physiological mineral deposition on the extracellular matrix. Thus, the finding of higher AP activity and calcified nodule formation in the PT group at 21 days does not necessarily indicate a better response. The SLA and modSLA surfaces may have selected more differentiated cells, triggered an earlier onset of differentiation and induced the formation of a better organized extracellular matrix with physiological mineral deposition.

This study was not designed with sufficient power to demonstrate other markers of bone formation such as osteocalcin, bone sialoprotein, osteopontin and the earlier expression of differentiation and formation of nodules around 10 - 12 days.

Further studies evaluating other markers of bone formation and osteogenic gene expression on SLA and modSLA surfaces may help to clarify cell metabolism. In this respect, Chakravorty *et al.* [32] compared the early pattern of expression of a panel of miRNAs associated with human cell development and differentiation in osteoprogenitor cells cultured on three titanium implant surfaces (modSLA, SLA and smooth polished) and evaluated their prospective regulation of the initial molecular interactions on modified titanium implant surfaces. Osteogenic genes such as COL5A1, RUNX1, FOXN1, IGF1 were potential targets for miR-215. Similarly, TargetScan also predicted SCARB1, MAP2K7, VDR, NCOR2, BMPR1B, SEL1L, SMAD2, SMAD4, PPP2CA, COL4A3 genes to be possible targets for miR-125b. The miRNAs, miR-503 and miR-214 were significantly down-regulated on modSLA surface, and not on SLA surface. The miR-215 and miR-125b were the most highly down-regulated miRNAs on both the SLA and modSLA surfaces.

5. Conclusion

Within the limitations of this study, we conclude that the SLA and modSLA surfaces did not influence cell metabolism. The SLA surface influenced cell differentiation, with a significant reduction in AP activity and the formation of calcified nodules after 21 days of culture compared to the PT surface. However, no difference was observed between the PT and modSLA surfaces. All titanium surfaces tested permitted the full expression of the osteoblast phenotype by osteo-1 cells, including matrix mineralization.

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