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Effect of cpTi Surface Roughness on Human Bone Marrow Cell Attachment, Proliferation, and Differentiation

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There is general agreement that rough surfaces improve both biologic and biomechanical responses to titanium (Ti) implants. The aim of this investigation was to study the effect of Ti surface roughness on the response of human bone marrow cell culture evaluating: cell attachment, cell proliferation, total protein content, alkaline phosphatase (ALP) activity, and bone-like nodule formation. Cells were cultured on commercially pure titanium (cpTi) discs with four different average roughnesses (R_a). For attachment evaluation, cells were cultured for 4 h. After 21 days, cell proliferation, total protein content, and ALP activity were evaluated. For bone-like nodule formation, cells were cultured for 28 days. Data were compared by ANOVA and Duncan's multiple range test. Cell attachment was not affected by surface roughness. For cells cultured on Ti with R_a ranging from 0.80 µm to 1.90 µm, proliferation was reduced while total protein content, and ALP activity were increased. There was a non-statistically significant increase of bone-like nodule formation on a surface with R_a near 0.80 µm. These results suggest that for Ti an R_a ranging from 0.80 µm to 1.90 µm would optimize both intermediary and final cellular responses but not affect the initial response, and a smoother surface would not favor any evaluated response.

Key Words: surface roughness, titanium, human bone marrow, cell culture, biocompatibility.

INTRODUCTION

Currently, most dental implant systems are made of commercially pure titanium (cpTi) because of its high *in vitro* and *in vivo* biocompatibility. This material allows direct bone-to-implant contact that has also been called "osseointegration" (1). To improve the bone integration of Ti implants, surface treatments such as surface machining, acid etching, electropolishing, anodic oxidation, sand blasting or plasma-spraying may be undertaken to induce chemical modifications associated with alterations of the surface topography (2). *In vitro* studies have shown that surface roughness is an important parameter influencing basic biologic responses (3-5). Several studies have shown that cell response is improved by rough Ti surfaces. Wennerberg et al. (6) evaluated implants with different surface roughness obtained by blasting with particles of Al_2O_3, and reported that rough implants have greater bone contact compared with a turned surface and that the surface blasted with 75-µm particles showed more bone-to-implant contact than either a 25-µm or a 250-µm blasted surface. These results suggested that an intermediary average roughness (R_a) would optimize bone formation in close contact with the implant.

Evaluations of *in vitro* biocompatibility of Ti using osteoblast cell culture have also indicated that rough surfaces would favor the development of some cell activities. Cell attachment increases on rough surfaces (3,7). Collagen synthesis, extracellular matrix, cytokines such as PGE2, growth factors and bone-like formation are also favored by rough surfaces (4,8). However, differences in the origin of the cells and the experimental methods make direct comparisons of results difficult or even questionable. Evaluations of biocompatibility through cell culture would have to be made using primary culture because the biomaterials will interact with these kinds of cells after *in vivo*

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implantation (9). Cells derived from osteosarcoma cannot present total differentiation *in vitro* while immortalized lineage can present different phenotypic expression of the cells from which they were originated (10). The cell culture system used in this study was human bone marrow directed *in vitro* to form osteoblastic cells. This culture system contains mesenchymal stem cells (progenitor cells) that have the potential to differentiate into various cell types depending on the culture condition (11). Until the present time, there are no studies evaluating the *in vitro* biocompatibility of Ti blasted with particles of Al_2O_3 using human bone marrow cell culture.

The aim of this investigation was to study the effect of Ti surface roughness on the response of human bone marrow cell culture evaluating cell attachment, cell proliferation, total protein content, alkaline phosphatase (ALP) activity, and bone-like nodule formation.

MATERIAL AND METHODS

Ti discs

Discs of Ti 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 SiC papers in the sequence 280-600-1200. Discs were subsequently submitted to the following treatments: Ti-smooth, polished with Al_2O_3 cloths to a final grain of 0.05 µm; Ti-25, blasted with 25-µm particles of Al_2O_3; Ti-75, blasted with 75-µm particles of Al_2O_3; Ti-250, blasted with 250-µm particles of Al_2O_3. All discs were cleaned in an ultrasonic bath and autoclaved before use in the cell culture experiments. The Ti surfaces were evaluated by scanning electron microscopy (SEM) and the R_a was measured using a profilometer (Prazis Rug-03, Arotec, São Paulo, SP, Brazil).

Culture of human bone marrow cells

Human bone marrow cells obtained from a healthy donor were cultured in α-MEM (Gibco, Life Technologies, Grand Island, NY, USA), supplemented with 15% fetal bovine serum (Gibco), 50 µg/ml gentamicin (Gibco), 0.3 µg/ml fungizone (Gibco), 10^{-7} M dexamethazone (Sigma, St. Louis, MO, USA), 5 µg/l ascorbic acid (Gibco) and 2.16 g/l ß-glicerophosphate (Sigma). First passage cells were cultured in the same medium at a concentration of 2 x 10^4 cells/well on Ti discs in 24-well culture plates (Falcon, Franklin Lakes, NJ, USA). During the culture period, cells were incubated at 37ºC in a humidified atmosphere of 5% CO_2 and 95% air, and the medium was changed every 48 h. In each plate, empty wells were used as a control of culture conditions.

Cell attachment

For attachment evaluation, cells were cultured for 4 h on Ti discs. The culture medium was removed and the wells were washed three times with PBS at 37ºC to eliminate unattached cells. The adherent cells were then enzymatically (1 mM EDTA + 0.25% trypsin; Gibco) released from the Ti discs and counted using a hemacytometer. Cell attachment was expressed as percentage of the initial number of cells.

Cell proliferation

For proliferation evaluation, cells were cultured for 21 days on Ti discs. The culture medium was removed and the wells were washed three times with PBS at 37ºC to eliminate unattached cells. The adherent cells were then enzymatically (1 mM EDTA + 0.25% trypsin; Gibco) released from the Ti discs three times, pooled, and counted using a hemacytometer. Data were used for calculating the doubling time as described by Patterson Jr. (12).

Total protein content

Total protein content was calculated according to the modified Lowry method after 21 days in culture. The culture medium was removed, the wells were washed three times with PBS at 37ºC and were filled with 2 ml of 0.1% sodium lauryl sulfate (Sigma). After 30 min, 1 ml of this solution from each well was mixed with 1 ml of Lowry solution (Sigma) and left for 20 min 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 min at room temperature to allow color development, and the absorbance was then spectrophotometrically measured (CE3021, Cecil, Cambridge, UK) at 680 nm and the total protein content was calculated from a standard curve and expressed as µg/ml. These data were normalized by the number of cells counted after 21 days.

ALP activity

ALP activity was assayed as the release of ρ-nitrophenol from ρ-nitrophenyl using a commercial kit (Sigma), and specific activity was calculated. Aliquots of the same solutions used for calculating total protein content were assayed for measuring ALP activity. Absorbance was spectrophotometrically measured at 410 nm and ALP activity was calculated from a standard curve. Results were calculated as µmol ρ-nitrophenol/hour and data were expressed as ALP activity normalized by the total protein content and by number of cells counted after 21 days.

Bone-like nodule formation

After 28 days in culture, the cells were washed three times with PBS at 37ºC. The attached cells were fixed in 3% glutaraldehyde in 0.1 M sodium cacodylate buffer for 2 h at room temperature and rinsed once in the same buffer. After fixation, the specimens were dehydrated through a graded series of alcohol and processed for staining with Alizarin red S (Sigma), that stains bone-like nodules rich in calcium. The specimens were evaluated using an image analyzer (Image Tool, University of Texas Health Science Centre, San Antonio, TX, USA) and the amount of bone-like nodule formation was calculated as a percentage of total Ti disc area.

Statistical analysis

All data were submitted to analysis of variance (ANOVA) and Duncan's multiple range test when appropriate.

RESULTS

Average roughness (R_a)

The surface evaluation showed that polishing or blasting with Al_2O_3 particles of different sizes produced discs with different rough surfaces. Five discs of each group (5 areas/disc) were used for R_a measurement: Ti-smooth = 0.24 µm, Ti-25 = 0.69 µm, Ti-75 = 0.83 µm, and Ti-250 = 1.91 µm. ANOVA showed that R_a was affected by the treatment (F=63.38; DF=3; p=0.0001). It was possible to observe from SEM micrographs that both polishing and blasting treatments produced different surfaces (Figure 1).

Cell attachment

Cell attachment was not affected by surface roughness (ANOVA: F=0.64; DF=3; p=0.56). Data are presented in Table 1.

Cell proliferation

Doubling time was affected by surface roughness (ANOVA: F=27.65; DF=3; p=0.0001), in the following order: Ti-25 > Ti-smooth > Ti-75 = Ti-250. Data are presented in Table 1.

Total protein content

Total protein content was affected by surface roughness (ANOVA: F=6.39; DF=3; p=0.047), in the following order: Ti-25 < Ti-smooth = Ti-75 = Ti-250. Data are presented in Table 1.

ALP activity

ALP activity was affected by surface roughness (ANOVA: F=9.96; DF=3; p=0.0009), in the following order: Ti-25 < Ti-smooth < Ti-75 = Ti-250. Data are presented in Table 1.

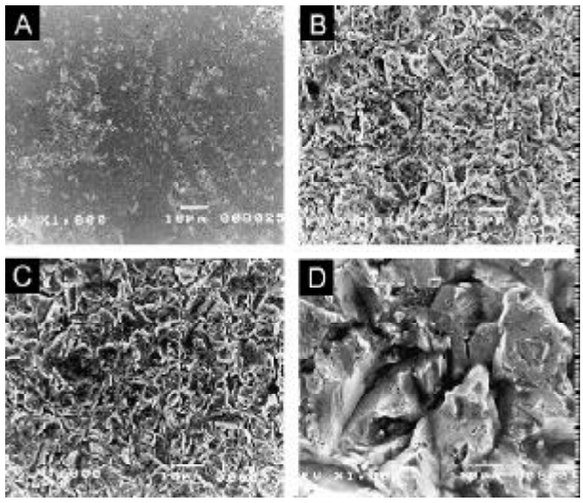

Figure 1. Scanning electron micrograph of Ti discs. (A) Ti-smooth; (B) Ti-25; (C) Ti-75; (D) Ti-250. Original magnification 1000X. See Material and Methods for explanation of Ti disc treatments.

Bone-like nodule formation

After 28 days in culture, bone-like nodule formation coating some areas of the Ti disc surfaces was observed. There was a non-statistically significant difference among surfaces (ANOVA: F=0.75; DF=3; p=0.54). Data are presented in Table 1.

DISCUSSION

Osteogenesis, induced by osteoblastic cells, is characterized by a sequence of events, involving cell attachment, cell proliferation and followed by the expression of osteoblast phenotype (9). In the present study, the response of human bone marrow cells cultured on Ti discs with different surface roughness was evaluated. The results showed that all discs, independent of the surface roughness, allowed cell attachment, cell proliferation, and osteoblastic differentiation expressed as both ALP activity and bone-like nodule formation.

The surface conditions of the biomaterial are an important factor for implant acceptance in vital bone (13). By blasting the surface with particles of a material other than that of the implant itself, the surface composition may be altered and, thus, affect biocompatibility (14). However, Wennerberg et al. (13) did not observe any statistically significant differences in torque measurements or histomorphometric analyses of implants blasted with the same sized particles, even though the particles were comprised of different materials (Al_2O_3 and TiO_2). This suggests that the surface was not affected by the composition of the blasting particles. Based on these results, it was decided to blast with Al_2O_3 particles using the same parameters as Wennerberg et al. (6). However, in this study, different values for R_a were obtained. Wennerberg et al. (6) observed that blasting with a certain size of blasting material or other surface preparation method did not automatically result in identical surface roughness from one experiment to another, suggesting that several factors can influence surface roughness. It is therefore important to measure the surface roughness in every study. Besides surface roughness, the formation of an oxide layer on the Ti surface, mainly its thickness, influences the cell response to Ti implants (2,4). The formation of this layer undoubtedly originates from chemical alteration of Ti as the result of stresses generated by blasting, and not only from the Al_2O_3 particles used for blasting (2).

The effect of surface roughness on cells may be the result of the surface roughness itself or the result of the reaction that occurs as the material surface is conditioned by the media and serum (4). This initial interaction produces a layer of macromolecules that modify cell behavior. Fibronectin, a cell adhesion protein present in serum, has been shown to mediate cell attachment and spread on artificial substrates by interacting with glycosaminoglycans and the cytoskeleton (15). It is possible that the rough surfaces may have absorbed more fibronectin than other surfaces (16), increasing cell attachment. This study has failed to show any effect of surface roughness on cell attachment. While it may occur, four hours, as used in this evaluation, is a very short time to allow a significant difference in protein adhesion that could affect cell attachment. Besides the period of incubation, it is also possible that cell attachment is not affected by any characteristic of material, because materials with different degrees of biocompatibil-

Table 1. Cell attachment (% of initial number of cells) after 4 h, cell proliferation expressed as doubling time (hours) between 0 and 21 days, total protein content (µg protein/10^4cell) normalized by the number of cells counted after 21 days, ALP activity (µmol ρ-nitrophenol/h/µg protein/10^4cell) normalized by the total protein content and by the number of cells, after 21 days, and bone-like nodule formation (% of total disc area) after 21 days in culture on Ti discs. All data are reported as mean ± standard deviation (n=5).

	Ti-smooth	Ti-25	Ti-75	Ti-250
Cell attachment	31.30 ± 9.72	32.50 ± 16.08	23.80 ± 6.92	26.30 ± 14.22
Cell proliferation	211.44 ± 10.67	238.95 ± 13.91	183.21 ± 14.70	174.35 ± 9.83
Total protein	7.67 ± 2.41	2.87 ± 2.50	5.62 ± 1.06	6.99 ± 0.95
ALP activity	0.20 ± 0.04	0.08 ± 0.03	0.34 ± 0.07	0.28 ± 0.09
Bone-like nodule formation	25.24 ± 8.40	32.63 ± 18.44	40.36 ± 18.60	29.60 ± 17.93

See Material and Methods for explanation of Ti disc treatments.

ity do not show differences in cell attachment (17).

Cells cultured on rougher surfaces tend to exhibit attributes of more differentiated osteoblasts including reduced cell proliferation and increased ALP activity (5). Protein production on rougher surfaces tends to be greater than on smoother surfaces (4). These parameters were all affected by surface roughness in this study. Cell proliferation, total protein content, and ALP activity were significantly decreased by the 25-µm blasted surface. Hatano et al. (18) studied the role of surface roughness in promoting osteoblastic differentiation using tissue culture polystyrene as a substrate excluding factors other than roughness, and observed that cell proliferation and ALP activity of the calvarial cells increased on rough surfaces. Culture of MC3T3-E1 cells showed a similar increase in proliferation on rough surfaces (19), while human osteoblastic cloned MG63 cells showed a decrease in proliferation rate in response to surface roughness (4). The explanation for this opposite response could be the difference in the degree of osteoblastic differentiation. Both human cells used in this study and MC3T3-E1 cells are osteoblastic progenitor cells whereas MG63 cells are mature osteoblastic cells.

Castellani et al. (1) did not find significant differences in protein production among different surfaces, whereas Martin et al. (4) observed inhibition of the protein production on smoother surfaces. This could occur because of the use of cells of different origins and also the use of different methods. The present results showed that for human cells surface roughness did not influence the amount of total protein produced. Considering that the amount of protein was normalized by the number of cells, these results evaluated the cell secretory activities that were not affected by surface roughness.

ALP activity is increased in cells cultured on rough surfaces. This seems to be a general characteristic of osteoblastic cells because it has been reported using MG63 cells (5), embryonic chick osteoblasts (8), and, in the present study, human bone marrow cells. Normalizing the ALP activity by total protein content and number of cells eliminates the effect of proliferation on this parameter, so the observed difference among surfaces could result from both a higher differentiated number of cells and a higher cellular activity.

This study did not show a marked difference in bone-like nodule formation related to surface roughness. However, there was a non-statistically significant increase of bone-like nodule formation on the surface with R_a near 0.80 µm. Because a similar result was found for ALP activity, this suggests that surface roughness ranging from 0.80 µm to 1.90 µm allows osteoblastic differentiation. This result is in agreement with those of Perizzolo et al. (20) that showed a positive correlation between ALP activity and bone-like nodule formation. Despite surface roughness, bone-like nodule formation did not follow any apparent organization. This seems to be characteristic of human osteoblastic cells, because Anselme et al. (2) reported that rat cells generally follow surface orientation, while human osteoblasts do not follow any surface orientation.

These results suggest that for Ti an R_a ranging from 0.80 µm to 1.90 µm would optimize both intermediary cellular responses such as proliferation, total protein content and ALP activity, and final cellular responses such as bone-like nodule formation, but not affect initial responses such as cell attachment, and a smoother surface would not favor any evaluated response.

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RESUMO

Sabe-se que superfícies rugosas melhoram as respostas biológica e biomecânica dos implantes de titânio (Ti). O objetivo desse estudo foi investigar o efeito da rugosidade de superfície do Ti sobre a resposta de células de medula óssea humana, avaliando: adesão e proliferação celulares, síntese de proteína total, atividade de fosfatase alcalina (ALP), e a formação de nódulos de matriz mineralizada. Células foram cultivadas sobre discos de titânio comercialmente puro (cpTi) com quatro diferentes rugosidades de superfície (R_a). A adesão foi avaliada após 4 horas. Após 21 dias, a proliferação, a quantidade de proteína total e a atividade de ALP foram avaliadas. A formação de nódulos de matriz mineralizada foi avaliada após 28 dias em cultura. Os dados foram comparados por ANOVA e teste de Duncan. A adesão não foi afetada pela R_a. Para as células cultivadas sobre Ti com R_a variando entre 0,80 µm e 1,90 µm a proliferação foi reduzida enquanto o conteúdo de proteína total e atividade de ALP foram aumentadas. Houve um aumento estatisticamente não-significante na formação de nódulos de matriz mineralizada sobre superfícies com R_a próxima de 0,80 µm. Esses resultados sugerem que, para o Ti, uma R_a variando entre 0,80 µm e 1,90 µm otimizaria respostas celulares intermediárias e finais, mas não afetaria respostas iniciais, e superfíces lisas não favoreceria nenhuma das respostas avaliadas.

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