Role of rhBMP-2 and rhBMP-7 in the Metabolism and Differentiation of Osteoblast-Like Cells Cultured on Chemically Modified Titanium Surfaces

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This study analyzed the role of recombinant human bone morphogenetic protein 2 (rhBMP-2) and recombinant human bone morphogenetic protein 7 (rhBMP-7) in the adhesion and differentiation of rat osteoblast-like (osteo-1) cells cultured on chemically modified titanium surfaces. Osteo-1 cells were cultured on chemically modified (modified sandblasted and acid-etched) titanium surfaces in 3 different types of medium: control, medium supplemented with 20 ng/mL rhBMP-2, and medium supplemented with 20 ng/mL rhBMP-7. The following parameters were evaluated: cell adhesion after 24 hours; total protein content; collagen content and alkaline phosphatase (AP) activity after 7, 14, and 21 days; and calcified nodule formation after 21 days. The addition of rhBMP-2 or rhBMP-7 did not influence cell adhesion ($P = .1175$). Cell differentiation was influenced by rhBMP-2, as demonstrated by a significant increase in collagen content after 7 days of culture ($P < .0001$) and a significant decrease in AP activity after 21 days ($P < .0001$). The addition of rhBMP-7 only influenced AP activity, and a significant increase was observed after 21 days ($P < .0001$). Within the limitations of the study, we conclude that the presence of rhBMP-2 or rhBMP-7 did not influence cell adhesion to chemically modified titanium surfaces but provided an additional stimulus during the differentiation of rat osteo-1 cells cultured on this type of surface.

Key Words: bone morphogenetic protein, titanium surface, implants, osteoblasts

INTRODUCTION

Chemical modification of titanium surfaces may stimulate osteoblast function, improving the adhesion and differentiation of osteoblasts cultured on these surfaces. Modified sandblasted and acid-etched (modSLA) titanium surfaces present a change in their reactivity characterized by a substantial increase in their hydrophilic character.[1,2] This surface may promote a better bone-implant contact during the first weeks of osseointegration, favoring faster bone formation.

In parallel, studies have shown that bone morphogenetic proteins (BMPs) are extremely important for the process of bone formation.[3] In addition, BMPs, especially recombinant human bone morphogenetic protein 2 (rhBMP-2), play a crucial role in the proliferation and differentiation of osteoblasts.[4–6] Another relevant BMP, recombinant human bone morphogenetic protein 7 (rhBMP-7), has the capacity to stimulate bone metabolism,

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modulating the proliferation and differentiation of mesenchymal cells in bone-forming cell lines.[7–9]

In this respect, chemically activated (modSLA) titanium surfaces and BMPs (rhBMP-2 and rhBMP-7) may favor the differentiation of osteoblasts, and it would be interesting to determine the extent to which this combination improves the process. Therefore, the objective of the present study was to investigate the role of rhBMP-2 and rhBMP-7 in the metabolism and differentiation of rat osteoblast-like (osteo-1) cells cultured on chemically modified (modSLA) titanium surfaces.

Materials and Methods

Sixty titanium disks (Straumann AG, Waldenburg, Switzerland) with surfaces identical to those used in commercially available dental implants were fabricated. The disks consisted of grade 2 unalloyed titanium (ASTM F67),were 1 mm thick, and measured 15 mm in diameter. The titanium disks had the following surface topography: sandblasted and acid-etched surface rinsed under nitrogen protection to prevent exposure to air and preserved in isotonic saline solution (modSLA).[2] The modSLA disks were sterile and were provided in sealed wrappings and immersed in isotonic saline solution.

A cell line derived from parietal bone tissue of newborn rats (osteo-1 cells), initially described by Lavos-Valareto et al[10] and again characterized by Togashi et al,[11] was used for the experiment. The cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Sigma Chemical Co, St Louis, Mo) containing 10% fetal bovine serum (Cultilab, Campinas, Brazil) and 1% antibiotic-antimycotic solution (Sigma). The cells were incubated in a humid atmosphere containing 5% carbon dioxide at 37°C. Cell growth was monitored at intervals of 24 hours. For the experiment, the cells were cultured in 3 different media: DMEM alone (control), DMEM supplemented with 20 ng/mL rhBMP-2 (Sigma), and DMEM supplemented with 20 ng/mL rhBMP-7 (Sigma). The concentrations of rhBMP-2 and rhBMP-7 were evaluated in a pilot study. The cells were plated onto the titanium disks at a concentration of 3×10^3 cells, and the disks were placed in 24-well culture plates. The control and experimental media were changed every 2 days.

For the analysis of metabolism (number of cells adhered), the cells were cultured for 24 hours, enzymatically removed from the disks and counted in a hemocytometer.[12] Total protein content was calculated after 7, 14, and 21 days of culture by the modified method of Lowry et al.[13] This parameter was used to indirectly quantify the number of cells on the disks for the different experimental periods.

Collagen content was calculated after 7, 14, and 21 days of culture according to the method of Reddy and Enwemeka[14] and normalized to total protein content. Alkaline phosphatase (AP) activity was assayed after 7, 14, and 21 days of culture by the release of thymolphthalein from thymolphthalein monophosphate[12] using a commercial kit (Labtest Diagnostica AS, Lagoa Santa, MG, Brazil) and normalized to total protein content.

After 21 days of culture, the disks 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) as described previously.[12]

Statistical analysis

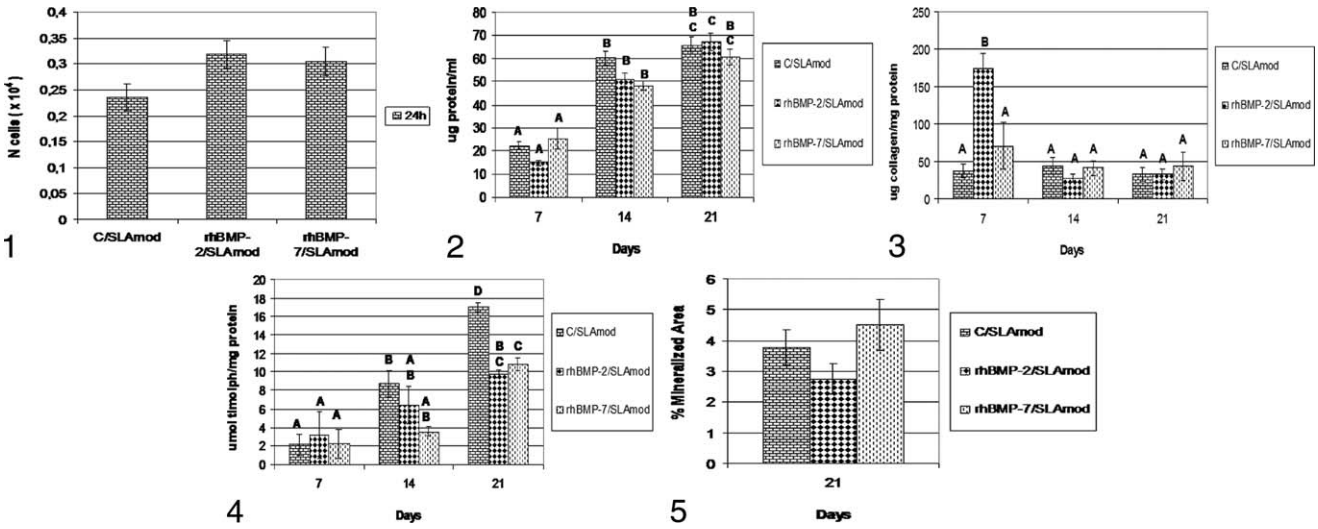
The data ($n = 4$) were submitted to analysis of variance (ANOVA), complemented by the Tukey test. A level of significance of 5% ($P < .05$) was adopted.

Results

The presence of rhBMP-2 or rhBMP-7 did not influence number of cells adhered to the modSLA surface ($P = .1175$) (Figure 1). The addition of rhBMP-2 or rhBMP-7 did not influence total protein content at any of the times analyzed. An increase in total protein content was observed over the study period in all groups. A time-dependent increase throughout the study period was only observed in the rhBMP-2/modSLA group. In the C/modSLA and rhBMP-7/modSLA groups, a time-dependent increase was observed between 7 and 14 days and between 7 and 21 days (Figure 2).

The addition of rhBMP-2 resulted in a significant increase of collagen content in cells grown on the modSLA surface after 7 days of culture (C/modSLA and rhBMP-7/modSLA < rhBMP-2/modSLA). In contrast, the presence of rhBMP-7 did not influence collagen content.

The highest collagen content was observed in the rhBMP-2/modSLA group at 7 days, followed by



Figures 1–5. Figure 1. Effect of rhBMP-2 and rhBMP-7 on the number of osteo-1 cells cultured on the modSLA surface for 24 hours. Results are reported as the mean ± standard error of one experiment carried out in quadruplicate (analysis of variance [ANOVA], $P = .1175$). **Figure 2.** Total protein content (μg protein/mL) of osteo-1 cells evaluated after 7, 14, and 21 days of culture. Results are reported as the mean ± standard error of one experiment carried out in triplicate (ANOVA complemented by the Tukey test, $P < .0001$). Different letters indicate significant differences ($P < .05$). **Figure 3.** Collagen content (μg collagen/mg protein) of osteo-1 cells evaluated after 7, 14, and 21 days of culture. The data were normalized to total protein content and are reported as the mean ± standard error of one experiment carried out in quadruplicate (ANOVA complemented by the Tukey test, $P < .0001$). Different letters indicate significant differences ($P < .05$). **Figure 4.** Alkaline phosphatase activity (μmol thymolphthalein/mg protein) of osteo-1 cells evaluated after 7, 14, and 21 days of culture. The data were normalized to total protein content and are reported as the mean ± standard error of one experiment carried out in quadruplicate (ANOVA complemented by the Tukey test, $P < .0001$). Different letters indicate significant differences ($P < .05$). **Figure 5.** Formation of calcified nodules by osteo-1 cells evaluated after 21 days of culture. Results are reported as the mean ± standard error of one experiment carried out in quadruplicate (ANOVA, $P = .21$) and are expressed as percent mineralized area.

a time-dependent decrease between 7 and 14 days and between 7 and 21 days. No significant differences between the time points analyzed were observed for the other groups (Figure 3).

The addition of rhBMP-2 or rhBMP-7 led to a significant reduction of AP activity at 21 days of culture (C/modSLA > rhBMP-2/modSLA and rhBMP-7/modSLA). This reduction was also noted at 14 days but the difference was not significant.

The highest AP activity was observed in the C/modSLA group at 21 days. This group was the only one presenting a time-dependent increase in AP activity throughout the study period. There was a time-dependent increase of AP activity in the rhBMP-2/modSLA group between 7 and 21 days and in the rhBMP-7/modSLA group between 7 and 21 days and between 14 and 21 days (Figure 4). The addition of rhBMP-2 or rhBMP-7 did not influence the formation of calcified nodules by osteo-1 cells grown on modSLA disks for 21 days (Figure 5).

Discussion

The objective of the present study was to evaluate the influence of rhBMP-2 and rhBMP-7 on the metabolism and differentiation of rat osteo-1 cells cultured on chemically modified (modSLA) titanium surfaces. The addition of rhBMP-2 did not influence metabolism (number of cells adhered, total protein content, or formation of calcified nodules) but led to a significant increase in collagen content after 7 days of culture and to a significant reduction in AP activity after 21 days. The presence of rhBMP-7 only affected AP activity; a significant reduction was observed after 21 days of culture.

Treatment with rhBMP-2 did not influence cell adhesion, which is in agreement with the findings of Chaudhari et al[15] and Van den Dolder et al,[6] who suggested that rhBMP-2 might not be an important modulator of cell adhesion in these cells. The same was observed for the addition of rhBMP-7, which

seems to be more involved in the process of cell differentiation.[7,8]

The addition of rhBMP-2 or rhBMP-7 did not affect total protein content. A time-dependent increase in this parameter over the period studied was only observed in the rhBMP-2/modSLA group. In the C/modSLA and rhBMP-7/modSLA groups, total protein content increased significantly between 7 and 14 days but not between 14 and 21 days. Despite the significant increase of total protein content between 14 and 21 days, the rhBMP-2/modSLA group presented a behavior similar to that of the other groups, demonstrating that the cells acquired an adequate level of total protein for cell differentiation.

Collagen synthesis was affected by the action of rhBMP-2, which promoted a significant increase after 7 days of culture. Similarly, Chen et al[16] observed the highest synthesis of collagen stimulated by rhBMP-2 between days 5 and 7 of culture. Although not significant, rhBMP-7 induced a numerical increase in collagen content after 7 days compared with the C/modSLA group. No differences between groups were observed after 14 days, indicating that the collagen matrix is ready for mineralization after 7 days.[17,18]

The present results showed a more marked effect of treatment with rhBMP-2 and rhBMP-7 on AP activity; this was demonstrated by a significant reduction in this parameter after 21 days of culture (C/modSLA $>$ rhBMP-2/modSLA and rhBMP-7/modSLA). This decrease was also noted after 14 days but the difference was not significant. In contrast, the literature has shown that rhBMP-2 induces the differentiation of osteoblasts cultured on titanium disks; these cells respond to rhBMP-2 with an increase in AP activity but within shorter periods of culture.[6,19] Chaudhari et al[20] and Martinovic et al[18] observed that rhBMP-7 did not increase AP activity during differentiation, whereas an increased synthesis was demonstrated by Eichner et al.[8] Martinovic et al[18] suggested that one BMP may functionally replace another BMP in an autocrine/paracrine manner or mediate a response to an endocrine action on osteoblasts.

These results should not be analyzed separately but need to be interpreted together with the other assessments. An increase in collagen content was observed after 7 days of culture in the rhBMP-2/modSLA and rhBMP-7/modSLA groups. This difference was significant for the rhBMP-2/modSLA group; however, no differences between groups were observed after 14 days. This finding indicates that the period of 7 days is an adequate time for the initiation of differentiation, when the collagen matrix is ready for mineralization.[17,18] Chaudhari et al[20] reported that the same concentration of rhBMP-2 is necessary to produce a maximum effect on the stimulation of AP activity and collagen synthesis, suggesting that the induction of AP is directly or indirectly related to the synthesis or accumulation of collagen matrix.

Treatment with rhBMP-2 and rhBMP-7 influenced AP activity, and a significant reduction was observed after 21 days of culture and a nonsignificant decrease after 14 days. This reduction in AP production suggests that the activity of this enzyme, an early differentiation marker, is reaching its plateau at 14 days. Subsequently, late markers of differentiation, such as bone sialoprotein, osteocalcin, and osteopontin, which are calcium-binding proteins, start to be synthesized by the cells. This interpretation is supported by the lack of differences in the formation of calcified nodules, the final stage of differentiation, between the groups treated with rhBMP-2 and rhBMP-7.[4,5]

Thus, the present results suggest that the addition of rhBMP-2 or rhBMP-7 stimulates the early differentiation of rat osteo-1 cells cultured on chemically modified (modSLA) titanium surfaces. To confirm this hypothesis, further studies are necessary to evaluate other markers of bone formation, such as osteocalcin, osteopontin, and bone sialoprotein, and to assess the formation of calcified nodules during an earlier period of culture.

Within the limitations of the study, we conclude that the presence of rhBMP-2 or rhBMP-7 did not influence the number of cells adhered to chemically modified (modSLA) titanium surfaces but provided an additional stimulus during the differentiation of rat osteo-1 cells cultured on this type of surface.

Abbreviations

ANOVA: analysis of variance
AP: alkaline phosphatase
BMP: bone morphogenic protein
DMEM: Dulbecco's modified Eagle's medium
modSLA: modified sandblasted and acid-etched
osteo-1: osteoblast-like

rhBMP-2: recombinant human bone morphogenic protein 2
rhBMP-7: recombinant human bone morphogenic protein 7

References

1. Buser D, Broggini N, Wieland M, et al. Enhanced bone apposition to a chemically modified SLA titanium surface. *J Dent Res*. 2004;83:529–533.

2. Zhao G, Schwartz Z, Wieland M, et al. High surface energy enhances cell response to titanium substrate microstructure. *J Biomed Mater Res A*. 2005;74:49–58.

3. Urist MR, DeLange RJ, Finerman GAM. Bone cell differentiation and growth factors. *Science*. 1983;220:680–686.

4. Puleo DA. Dependence of mesenchymal cell responses on duration of exposure to bone morphogenetic protein-2 in vitro. *J Cell Physiol*. 1997;173:93–101.

5. Hay E, Hott M, Graulet AM, Lomri A, Marie PJ. Effects of bone morphogenetic protein-2 on human neonatal calvaria cell differentiation. *J Cell Biochem*. 1999;72:81–93.

6. Van den Dolder J, de Ruijter AJ, Spauwen PH, Cansen JA. Observations on the effect of BMP-2 on rat bone marrow cells cultured on titanium substrates of different roughness. *Biomaterials*. 2003;24:1853–1860.

7. Açil Y, Springer ING, Broek V, Terheyden H, Jepsen S. Effects of bone morphogenetic protein-7 stimulation on osteoblasts cultured on different biomaterials. *J Cell Biochem*. 2002;86:90–98.

8. Eichner A, Brock J, Heldin CH, Souchelnytskyi S. Bone morphogenetic protein-7 (OP-1) and transforming growth factor-β1 modulate 1,25 (OH)2-vitamin D3-induced differentiation of human osteoblasts. *Exp Cell Res*. 2002;227:132–142.

9. Yeh LCC, Tsai AD, Zavala MC, Lee JC. Cartilage-derived morphogenetic proteins enhance the osteogenic protein-1-induced osteoblastic cell differentiation of C2C12 cells. *J Cell Physiol*. 2004;2001:401–408.

10. Lavos-Valareto IC, Deboni MCZ, Azambuja N Jr, Marques MM. Evaluation of the titanium Ti-6Al-7Nb alloy with and without plasma-sprayed hydroxyapatite coating on growth and viability of cultured osteoblast-like cells. *J Periodontol*. 2002;73:900–905.

11. Togashi AY, Cirano FR, Marques MM, Pustiglioni FE, Lima LAPA. Characterization of bone cells obtained from the calvaria of neonatal rats (osteo-1) after serial subculture. *J Appl Oral Sci*. 2007; 15:442–447.

12. Rosa AL, Beloti MM. Rat bone marrow cell response to titanium and titanium alloy with different surface roughness. *Clin Oral Implants Res*. 2003;14:43–48.

13. Lowry OH, Rosebrough NJ, Farr Al, Randall RJ. Protein measurement with the Folin phenol reagent. *J Biol Chem*. 1951;193: 265–275.

14. Reddy GK, Enwemeka CS. A simplified method for the analysis of hydroxyproline in biological tissues. *Clin Biochem*. 1996; 29:225–229

15. Chaudhari A, Ron E, Rethman MP. Recombinant human bone morphogenetic protein-2 stimulates differentiation in primary cultures of fetal rat calvarial osteoblasts. *Mol Cell Biochem*. 1997; 167:31–39.

16. Chen D, Harris MA, Rossini G, et al. Bone morphogenetic protein 2 (BMP-2) enhances BMP-3, BMP-4, and bone cell differentiation marker gene expression during the induction of mineralized bone matrix formation in cultures of fetal rat calvarial osteoblasts. *Calcif Tissue Int*. 1997;60:283–290.

17. Cooper LF, Zhou Y, Tabeke J, et al. Fluoride modification effects on osteoprogenitor differentiation. *Bone*. 2006;34:402–411.

18. Martinovic S, Borovecki F, Miljavac V, et al. Requirement of a bone morphogenetic protein for the maintenance and stimulation of osteoblast differentiation. *Arch Histol Cytol*. 2006;69:23–36.

19. Boyan BD, Bonewald LF, Paschalis EP, et al. Osteoblast-mediated mineral deposition in culture is dependent on surface microtopography. *Calcif Tissue Int*. 2002;71:519–529.

20. Chaudhari LR, Hofmeister AM, Hruska KA. Differential growth factor control of bone formation through osteoprogenitor differentiation. *Bone*. 2004;34:402–411.