Tissue Engineering and Regenerative Medicine, Vol. 7, No. 1, pp 64-68 (2010)

| Original Article |

Initial Osteoblast-like Cell Response to Titanium Coated with Recombinant Human Bone Morphogenic Protein-2 (rhBMP-2)

Su Young Lee[1], Jai Young Koak[1]*, Seong Joo Heo[1], Seong Kyun Kim[1], In Kyung Shim[2], and Seung Jin Lee[2]

[1]*Department of Prosthodontics & Dental Research Institute, School of Dentistry, Seoul National University, 275-1 Yeongun-Dong, Chongno-Gu, Seoul, 110-749, Korea*

[2]*College of Pharmacy, Ewha Womans University,11-1, Daehyun, Seoul, 120-750, Korea*

(Received: November 20th, 2009; Accepted: December 8th, 2009)

Abstract : The aim of this study was to investigate the initial bone cell response to titanium discs coated with a biodegradable polymer, poly(D,L-lactide-co-glycolide) (PLGA) incorporated recombinant human Bone Morphogenic Protein-2(rhBMP-2). Titanium discs were fabricated and divided into three groups. Group 1 was anodized under 300 V and not coated. Group 2 was machined and coated with PLGA/rhBMP-2 (500 ng/ml) solutions. Group 3 was anodized then coated with the same solution. Surface topography was examined by scanning electron microscopy (SEM). The osteoblast-like Human Osteogenic Sarcoma (HOS) cells were seeded and cultured. MTS based cell proliferation assay for 1, 3 days and alkaline phosphatase activity test were carried out for 3 and 7 days. It was observed that PLGA/rhBMP particles were observed as smooth and round onto the coated titanium surfaces. Cell proliferation and ALP activity were the highest on anodized and coated Ti surfaces (group 3). In this study, biodegradable PLGA polymers incorporated with rhBMP could enhance proliferation and differentiation of cells on the titanium surface.

Key words : *bone morphogenic protein (BMP), PLGA, titanium, osteoblast-like cell*

1. Introduction

Titanium is the material of choice for load-bearing, bone contacting devices. The orthopedic and dental implant is based on the favorable interfacial interaction at the bone-titanium interface.[1] It is clear that osseointegration is a well-established property of titanium implant surfaces and that the current success rate is satisfactory. Nevertheless, surface modification of titanium is still a very active area of research, because definite interest exists in surface treatments that can induce acceleration of normal bone healing phenomena. An accelerated stable fixation between bone and implant would allow early or immediate loading of implants.

Most manufacturers strive to enhance implant stability and load-bearing capability for sub-optimal bone qualities by modifications of the implant surface, thus several surface variations have been developed using blasting, plasma spraying, etching and anodic oxidization. Recently, biochemical methods of surface modification offer an alternative or adjunct to physicochemical or morphological methods. Biochemical methods are aimed at the control of the bone-implant interface by immobilization and/or delivery of proteins for the purpose of inducing specific cell.[2] They rely on the current understanding of the biology and biochemistry of cellular function and differentiation and on suitable modification.

Various studies have shown that growth factors like PDGF, BMP, IGF, TGF have osteoinductive effects.[3,4] However, the local application of these factors at the places of interest without additional devices still remains a problem. Until now, no practicable technique in the field of dental implant has been described for the local application of growth factors without the need of further application devices than implant itself. A controlled drug release with high local, but low systemic concentrations could be achieved by biodegradable drug carrier. The incorporated growth factors must remain stable in the coating layer and carrier itself are no negative effects on bone metabolism and complete absorption without any side effects.

Different techniques and materials for the coating biomaterials and local drug release have been described so far. Poly(lactic acid)(PLA) and poly(glycolic acid)(PGA) or their copolymers are widely used as biodegradable implants and

*Tel: +82-2-2072-3816; Fax: +82-2-2072-3860
e-mail: young21c@snu.ac.kr (Jai Young Koak)

drug delivery system.[5,6]

The bone morphogenic proteins (BMPs) are multifunctional growth factors that belong to the TGF-β superfamily. Among BMPs, BMP-2 is known as the most effective cytokine. The BMP was introduced by Urist in 1965[7] and recombinant BMPs were developed by Wozney in 1988.[8] Among BMPs, BMP-2 is known as the most effective cytokine, while recombinant human BMP-2(rhBMP-2) is useful in treating bony defects when combined with an adequate carrier system or matrix. Coating of implants with locally active growth factors could influence bone-implant interface.

The aim of the study was to investigate the initial cell response of a thin PLGA coating with incorporated BMP-2 of anodized titanium disc. The physical characteristics examined are the surface morphology of titanium disc. The biological characteristics investigated are the cell proliferation and differentiation.

2. Materials and Methods

2.1 Titanium Disc Preparation

Titanium discs (Warantec Co., Seoul, Korea) were fabricated using grade 4 commercially pure titanium, with dimensions of 25 mm diameter and 1 mm thickness. Prior to use, degreasing and acid prepickling of all discs were performed. All discs were ultrasonically degreased in trichloroethylene for 20 min, followed by soaking in 99% ethanol for twice cycles of 20 min each and distilled water overnight. The machined samples were dried and sterilized by Ehylene Oxide gas. The anodic oxidation treatment of the titanium discs was performed at 300 V in an aqueous electrolytic solution of 0.02 M/L calcium glycerophsphate and 0.15 M/L calcium acetate. All procedures were done in room temperature, and the total time for anodization of one disc was three minutes.[9] The anodized discs were washed with distilled water, dried and then sterilized in Ethylene Oxide (E.O.) gas before the experiment.

2.2 Surface Modification

The titanium discs were further processed, as described below.

Poly(lactide-co-glycolide) (PLGA, PURAC Biochem BV, Gorinchem, Holland) was used for a growth factor carrier and growth factor was employed a manufactured recombinant human Bone Morphogenic Protein-2 (rhBMP-2, Cowellmedi, Pusan, Korea). Poly(lactide-co-glycolide) was dissolved in 0.3% w/v acetone (Duksan pure chemicals co., LTD, Kyungkido, Korea) and the solution was passed through a sterile filter (DISMIC, 0.45 μm, ADVANTEC, Japan). rhBMP-2(0.15 mg/ml) in sterile water was incorporated into PLGA solution just before coating. The doses of rhBMP-2 used in this study are similar to those in previous study[10] which indicated that 500 ng/ml is effective for inducing osteoblast differentiation.

To coat the discs, 200 μl of the mixed solution was dropped on the sterilized titanium discs and dried under sterile conditions.

Group 1: Anodized under 300 V

Group 2: Machined then, coated with 0.02 ml PLGA/ rhBMP-2 [3.75 μg per disc]

Group 3: Anodized under 300 V then, coated with 0.02 ml PLGA/ rhBMP-2 [3.75 μg per disc]

2.3 Observation of the Ti Disc Surface

Discs were coated with gold in a vacuum condition using a sputter coater (Polaron SC7620, VG Microtech, England) to look at the samples easily and clearly. The morphology of PLGA/rhBMP particles coated onto Ti discs was observed using scanning electron spectroscopy (SEM, Jeol Model JSM5200, Jeol, Tokyo, Japan) operated at 10 kV.

2.4 Cell Culture

HOS cells achieved from Korean Cell Line Bank. The cells were maintained as subconfluent monolayers in RPMI-1640 (Gibsco BRL, Grand Island, NY, USA), supplemented with 10% fetal bovine serum, 10 U/ml penicillin-streptomycin (Sigma, MO,USA), and at 37°C in a humidified atmosphere of 5% CO_2/95% air. The medium was changed twice a week and the cells were passaged at 80-90% confluence with the use of 0.05% trypsin-EDTA (Gibco BRL, Grand Island, NY, USA). After subculturing, the cells were washed with phosphate-buffered saline (PBS), detached with trypsin/EDTA solution (0.25% trypsin) at 37°C for 10min, and centrifuged and resuspended for further reseeding and growth tests.

2.5 Colorimetric MTS Assay

For proliferation study, incubation of Ti discs was performed for 2 h with cells of 1×10^5/ml iDentical with the attachment study. Thereafter, discs were washed with PBS, changed to new plates and cultured for 1, 3days. Cells were treated with CellTiter 96® AQueous Assay (Promega Corp., WI, USA). The assay is based on the reduction of a tetrazolium compound to a coloured formazan product by viable cells (or metabolic activity). The absorbance at 490 nm is directly proportional to the cell proliferation.

2.6 Alkaline Phosphatase Activity

For alkaline phosphatase (ALP) activity test, cells cultured for 3 and 7 days were rinsed three times with PBS and extracted

with 0.5% Triton X in 25 mM Glycine-NaOH. 100 ul aliquots of the extracts were added to 50 ul ALP solution (pNPP; Sigma Steinheim, Germany) in 96 well culture plate for 30 min at 37°C. After development of color, the time was recorded and reaction stopped by adding 50 ul of 2N-NaOH, and the final absorbance was read at 405 nm using a microplate reader.

2.7 Statistical Analysis

SPSS ver. 12 package for Windows was used. Five samples were used for each experimental group. ANOVA and Scheffe's post hoc test were used to determine the statistical significance of the differences among observed groups. Statistical significance was established at $p<0.05$.

2.8 In Vitro Release Test

In our study, the in vitro experiment of rhBMP release was performed in phosphate-buffered saline solution at 37°C. The rhBMP-incorporating PLGA coated Ti disc was further placed at 37°C for 12hr, 1d, 2d, 3d, 5d in the incubator for cell culture to prevent the solution evaporation. The resulting rhBMP-incorporating discs were placed in 2 ml of PBS, and at different time intervals, the sample solution was exchanged by the fresh PBS at the same volume. The concentration of rhBMP was analyzed by enzyme linked immunosorbent assay (ELISA) using kit (Quantikine BMP-2 Immunoassay; R&D systems, Minneapolis, USA).

3. Results

3.1 Morphology of the Titanium Disc

Fig 1(a) displays the uniformly porous anodized titanium disc surface which was composed of small craters. In coated groups, titanium discs were coated with smooth, nonporous PLGA nanospheres and microspheres. (Fig 1. b,c)

3.2 Cell Proliferation

The proliferation of human osteoblast-like cells cultured on titanium disc after 1 day and 3 days is presented in Fig 2. The number of osteoblast-like cells in each sample increased with the incubation time. After 1 day and 3 days, there were significantly higher in coated titanium discs (group 2, 3) than in noncoated discs (group 1). Also, the anodic oxidized and PLGA/rhBMP coated group was significant difference in the number of osteoblast-like cells between the two coated groups. ($p<0.05$)

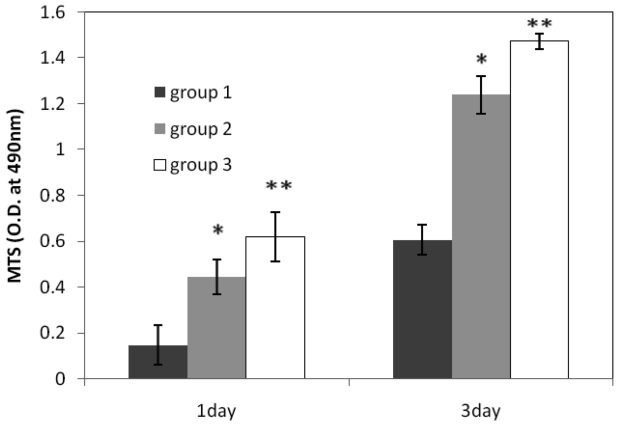


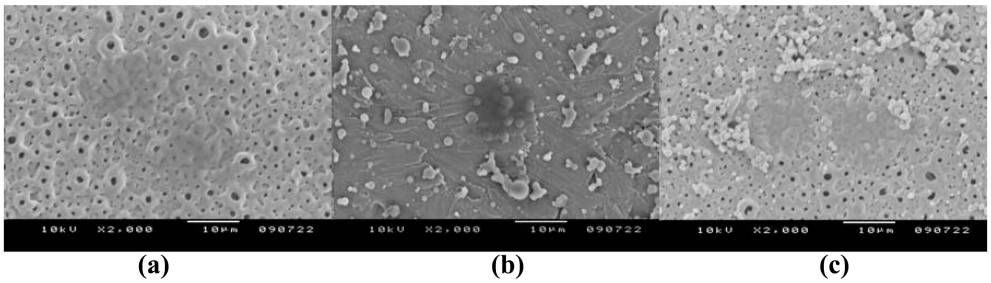
Figure 2. Cell proliferation of osteoblast-like Human Osteogenic Sarcoma (HOS) cells grown on anodized (group1), machined and PLGA/rhBMP coated (group 2) and anodized and PLGA/rhBMP coated titanium discs by MTS assay (3day). *,**denotes significant difference compare with each groups.($p<0.05$)

3.3 Cell Differentiation

Alkaline phosphatase is an early- stage marker of osteoblast differentiation. Measured optical density increased significantly during the 7-day cell culture period in all groups (Fig 3). The ALPase optical density value on group 3 was significantly higher than the group 1 and 2. Group 3 showed the greatest cellular differentiation, followed by group 2 and 1. ($p<0.05$)

3.4 In Vitro Release

The result showed controlled release of rhBMP and 50% of



(a) (b) (c)

Figure 1. SEM image of titanium discs (×2000). (A) anodized, (B) machined and PLGA/rhBMP coating, (C) anodized and PLGA/rhBMP coating.

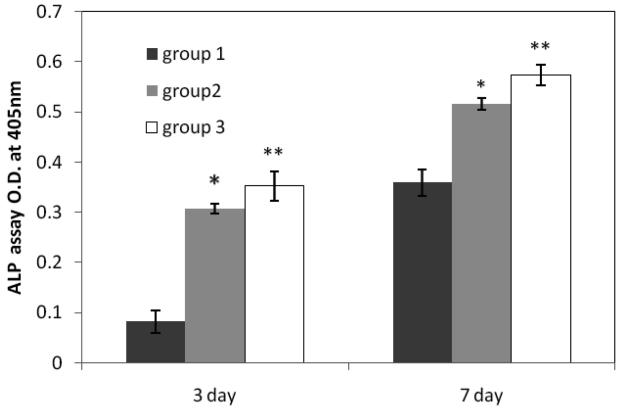


Figure 3. ALP assay of osteoblast-like Human Osteogenic Sarcoma (HOS) cells grown on anodized (group1), machined and PLGA/rhBMP coated (group 2) and anodized and PLGA/rhBMP coated titanium discs. *,** denotes significant difference compare with each groups.($p<0.05$)

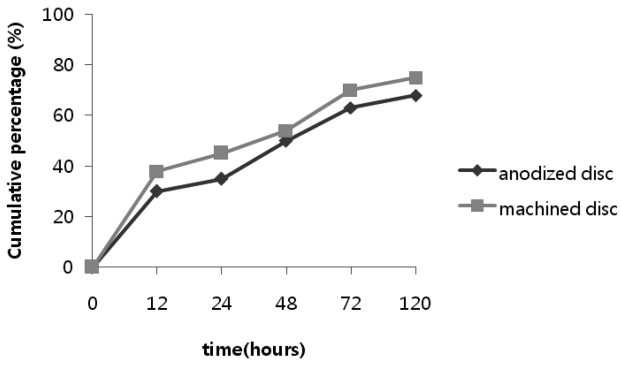


Figure 4. Cumulative percentage of rhBMP released from the coated titanium discs at 37°C in PBS.

incorporated rhBMP was released into PBS within the initial 48hrs of release test. (Fig 4) Release characteristics of incorporated growth factor showed controlled release profile.

4. Discussion

The aim of this study was to investigate the initial bone cell response to titanium surface coated with a biodegradable polymer, poly(D,L-lactide-co-glycolide)(PLGA) incorporated recombinant human Bone Morphogenic Protein-2(rhBMP-2). The coating was performed in a cold coating technique, [11] which allowed to incorporate proteins in a biologically active form.

BMP-2 has strong bone-inductive activity and is being evaluated as a bone growth inducer for dental and orthopedic indications. Despite its strong osteoinductive activity, clinical use of BMP-2 has been hampered by the lack of suitable delivery systems. Systems evaluated as carriers to localize BMP-2 include porous hydroxyapatite, absorbable collagen, polylactic acid (PLA), PLGA, demineralized bone powder and bovine collagen type sponges. Hydroxyapatite is a biocompatible but not biodegradable material and therefore remains at the surgical site. Collagen sponges can be immunogenic and demineralized bone powder suffers from insufficient supply and poor characterization as a delivery system. Synthetic polymers like PLA and PLGA offer many advantages over biological materials, e.g., biocompatibility, minimal immunogenicity, biodegradability, and the fact they can be manufactured with high reproducibility.[12,13] An efficacious delivery system is still needed to localize BMP-2 at the implant site with appropriate dose.

In this study, controlled release systems for BMP-2 release were formulated using combinations of surface modification both anodic oxidization and prepared PLGA polymer. The final goal of this research was to address whether release of BMP-2 based on biodegradable PLGA polymer coating enhances cell growth and differentiation more effectively than the control group. The growth and differentiation of osteoblasts, the bone forming cells, is central to the regeneration of bone around dental implants. The initial response of osteoblasts with implant surfaces is an essential event for osseointegration of dental implants.

In this study, cellular proliferation and differentiation were measured in vitro. MTS-based cell proliferation assay is a colorimetric method for determining the number of viable cells in proliferation or cytotoxicity assays.[14] ALP activity is widely used as an osteoblast marker, and an increase in ALP activity is associated with osteoblastic differentiation, bone formation, and matrix mineralization.[15] Therefore, the ALP specific activity of the osteoblast-like HOS cells was measured to determine effect on the differentiation of osteoblast cells. The results of this study shows that the optical densities of MTS assay and the specific activity of ALP were increased in time for all groups and showed significant differences among the groups. This means that cells not only grow and proliferate, but also differentiate better on the PLGA/rhBMP coated discs.

The functional activity of cells close to the implant surface is influenced by the implant surface properties. The osseointegration of anodized titanium was increased compared to machined implant in previous studies.[16,17] Nevertheless, this study showed that the PLGA/rhBMP coated machined specimens are more biocompatible than noncoated anodized specimens. It is proposed that biostimulation by rhBMP-2 may enhance effectively the osteogenic potential of these cells.

The coated groups showed improved cellular responses such as proliferation and differentiation. Especially, the anodic oxidized and PLGA/rhBMP coated group was significant difference in

the number of osteoblast-like cells between the two coated groups. (p<0.05) Anodic oxidation of titanium occurs forming a TiO_2 surface layer by applying a positive voltage to a Ti specimen immersed in an electrolyte. When the applied voltage is increased to a certain point, a micro-arc occurs resulting from dielectric breakdown of TiO_2. The newly formed TiO_2 layer is both porous and firmly adhered to the substrate, which is beneficial for the biological performance of the implants.[9,18]

The anodized titanium surface had uniformly porous oxide layers, which, in fact, was composed of small craters with holes at the center. The pores of the anodized oxide layers are the result of micro arcs, which occur on the surface of the titanium anode.[16,17] These crater shape surfaces could have served as the carrier of the growth factors, that enabled the cellular differentiation and proliferation. Therefore, the anodized and coated implants group may have retained rhBMP-2 on its surface for a certain period time.

Further studies for considering specific markers for differentiation such as osteocalcin to confirm if the osteoblasts differentiate to generate, and for confirming the results such as surface spreading morphology are needed.

Within the limit of our study, it can be concluded that PLGA can be a good carrier for rhBMP-2 and PLGA incorporated rhBMP-2 coating can increase the cell proliferation and differentiation. This new biomimetic strategy may be applied in treatment of implant therapy.

Acknowledgements: The authors wish to thank, Cowellmedi, for providing the rhBMP-2 used in this study.

References

1. DM Brunette, P Tengvall, M Textor, *et al*., Titanium in Medicine. Berlin: Springer, 2001.
2. DA Puleo, A Nanci, Understanding and controlling the bone-implant interface, *Biomaterials*., **20**, 2311 (1999).
3. M Lind. Growth factor stimulation of bone healing. Effects on osteoblasts, osteomies, and implants fixation, *Acta Orthop Scand Suppl*., **283**, 2 (1998).
4. S Trippel, RCoutts, T Einhorn, *et al*., Growth factors as therapeutic agents, *J Bone Joint Surg*., **78A**, 1272 (1996).
5. G Schmidmaier, B Wildemann, H Bail, *et al.,* Local application of growth factors (IGF-I and TGF-b1) from a biodegradable Poly(D,L-lactide) coating of osteosynthetic implants accelerates fracture healing in rats, *Bone*., **28**, 341 (2001).
6. C Laurencin, JM Lane, Poly(lactic acid) and Poly(glycolic acid): orthopaedic surgery applications. In Bone formation and repair, *Am Acad Orthop Surg*., 325 (1994).
7. MR Urist, Bone formation by autoinduction, *Science.,* **150**, 893 (1965).
8. AH Reddi, Role of morphogenetic proteins in skeletal tissue engineering and regeneration, *Nat Biotechnol*., **16**, 247 (1998).
9. JW Choi, SJ Heo, JY Koak, *et al*., Biological responses of anodized titanium implants under different current voltages, *J Oral Rehabil*., **33**, 889 (2006).
10. T Katagiri, A Yamaguchi, T Ikeda, *et al*., The non-osteogenic mouse pluripotent cell line C3H10T1/2, is induced to differentiate into osteoblastic cells by recombinant human bone morphogenetic proteins-2, *Biochem Biophys Res Commun.*, **172**, 295 (1990).
11. G Schmidmaier, B Wildemann, A Stemberger, Biodegradable poly(D,L-lactide) coating of implants for continuous release of growth factor, *J Biomed Mater Res*., **58**, 449 (2001).
12. SS Duggirala, RC Mehta, PP DeLuca. Interaction ofrecombinant morphogenetic protein-2 with poly (d,l-lactide-coglycolide) microspheres, *Pharm. Dev. Technol.,* **1**, 11 (1996).
13. JA Schrier, PP DeLuca, Recombinant human bone morphogenetic protein-2 binding and incorporation in PLGA microsphere delivery systems, *Pharm Dev Technol*., **4**, 611 (1999).
14. AH Cory, TC Owen, JA Barltrop, *et al*., Use of an aqueous soluble tetrazolium/formazan assay for cell growth assays in culture, *Cancer Commun*., **3**, 207 (1991)
15. TA Owen, M Aronow, V Shalhoub, *et al*., Progressive development of the rat osteoblast phenotype in vitro: Reciprocal relationship in expression of genes associated with osteoblast proliferation and differentiation during formation of the bone extracellular matrix, *J Cell Physiol.*, **143**, 420 (1990).
16. LH Li, YM Kong, HW Kim, *et al*., Improved biological performance of Ti implants due to surface modification by micro-arc oxidation, *Biomaterials*., **25**, 2867 (2004).
17. YT Sul, C Johansson, A Wennerberg, *et al*., Optimum surface properties of oxidized implants for reinforcement of osseointegration: Surface chemistry, oxide thickness, porosity, roughness, and crystal structure, *Clin Oral Implants Res*., **20**, 349 (2005).
18. LH Li, HW Kim, SH Lee, *et al*., Biocompatibility of titanium implants modified by microarc oxidation and hydroxyapatite coating, *J Biomed Mater Res A*., **73**, 48 (2005).