


# Cellular Response of Anodized Titanium Surface by Poly(Lactide-co-Glycolide)/Bone Morphogenic Protein-2

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## Abstract

**BACKGROUND:** The purpose of this study is to examine physical characteristics of and initial biological properties to anodized titanium treated with poly(D,L-lactide-co-glycolide) (PLG) mixed with recombinant human bone morphogenic protein-2 (rhBMP-2).

**METHODS:** Titanium specimens were prepared in groups of four as follows: group NC was anodized under 300 V as control; group PC was anodized then dropped and dried with solution 0.02 ml PLG; group D was anodized then dropped and dried with solution 0.02 ml PLG/rhBMP-2 (3.75 µg per disc); and group E was anodized then coated with 0.02 ml PLG/rhBMP-2 (3.75 µg per disc) by electrospray. Human osteoblastic-like sarcoma cells were cultured. Cell proliferation and alkaline phosphatase (ALP) activity test were carried out. Runx-2 gene was investigated by the reverse transcription-polymerase chain reaction. Immunofluorescence outcome of osteogenic proteins was observed.

**RESULTS:** After 3 days, there were significantly higher proliferations compared rhBMP-2 loaded titanium discs with rhBMP-2 unloaded discs. The ALPase activity on rhBMP-2 loaded titanium discs was significantly higher than in rhBMP-2 unloaded discs. The expression level of Runx2 mRNA presented the highest on the PLG/rhBMP-2-coated surface.

**CONCLUSION:** PLG polymers mixed with rhBMP-2 might improve proliferation, differentiation and osteogenic protein formation of cells on the anodized titanium.

**Keywords** Bone morphogenic protein · Polymer · Anodized titanium · Cell proliferation · Cell differentiation

## 1 Introduction

Enhancing bone formation around dental implant is the key factor in shortening the treatment period. A variety of physical and chemical processes for surface treatment of Ti have been studied for this purpose [1]. The biochemical

methods of surface treatment of Ti implants offer a substitute or addition to physicochemical and morphologic methods. The purpose of biochemical surface treatment is to regulate the growth factors released to the interface [2].

Bone morphogenic proteins modulate the cell differentiation and proliferation associated with osteogenesis [3]. Human bone morphogenic protein-2 (BMP-2) initiates the differentiation of mesenchymal stem cells into osteoblasts [4]. Due to the positive responses on the growth of bone cells, BMP-2 has been practically used to promote bone regeneration in healing of bone fracture and spinal fixation [5]. Also, an intriguing concept of applying BMPs onto titanium for improved local bone formation has been observed in animals [6–8].

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However, one of the important considerations regarding the utilization of BMPs is that no suitable vehicle has been identified that this protein keeps biologically active at the application area. BMP is a soluble protein and spreads readily in the body fluids. Moreover, the implant surface has a limitation to deliver appropriate amounts of an osteoinductive or osteoconductive factors. A biologic factor must be adequately effective to initiate and improve osteogenesis, and must also be allowed to release sufficient concentration and duration in the adjacent tissues for appropriate bone formation. Therefore, a suitable carrier system is required to effectively deliver BMP-2 so that it will have a confined local effect at the bone healing site.

The BMP carriers can be widely classified into natural polymers, synthetic polymers, and composites of polymers. Systems evaluated as carriers to localize BMP-2 include inorganic biomaterials from porous hydroxyapatite [9], calcium phosphates [10], and organic polymers, including polylactic acid (PLA) [11], poly(D,L-lactide-co-glycolide) (PLG) [12], absorbable collagen [13]. Hydroxyapatite is a biocompatible but not biodegradable material and therefore remains at the defect 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 PLG offer many advantages over biological materials, e.g., biocompatibility, minimal immunogenicity, biodegradability, and high reproducibility during manufacturing. However, to our knowledge there is no study about applications of these polymers as carriers for BMP-2 to titanium.

The purpose of present study was to evaluate the early response of human osteoblast like sarcoma (HOS) cell to a loading PLG with rhBMP-2 on anodized titanium disc. The physical characteristics were examined by the surface morphology and roughness of titanium disc. Besides, the release condition of rhBMP-2 was assessed and its biological action was evaluated using the cell proliferation, differentiation, Runx2 gene and osteogenic protein expression.

## 2 Materials and methods

### 2.1 Titanium disc

The samples were prepared 25 mm titanium discs in diameter and 1 mm in thickness (Warantec Co., Seoul, Korea). All discs were ultrasonically de-greased for 20 min and soaked in 99% ethanol for 2 cycles of 20 min each. The anodization of the specimens was conducted at 300 V in a solution (0.02 M/L calcium glycerophosphate and 0.15 M/L calcium acetate) [14].

### 2.2 Surface treatment

Poly(lactide-co-glycolide), PLG polymer was applied as vehicle and growth factor was employed a manufactured recombinant human bone morphogenic protein-2 (rhBMP-2) (Cowellmedi, Pusan, Korea). PLG Polymer was soluble in 0.3% acetone and the solution was filtered. rhBMP-2 (0.15 mg/ml) in sterile water was mixed with PLG solution just before coating. To coat the discs, 200  $\mu$ l of the solution was dropped on the sterilized anodized discs and dried. Uncoated anodized titanium discs were used as a control. Group E was coated by an electrospray method.

The 500 ng/ml of rhBMP-2 was used in this experiment. That was known to be effective for inducing osteoblast differentiation in the previous study [15].

The titanium specimens were prepared, as below.

Group NC: Anodized under 300 V as control.

Group PC: Anodized under 300 V, then dropped and dried with solution 0.02 ml PLG.

Group D: Anodized under 300 V, then dropped and dried with solution 0.02 ml PLG/rhBMP-2 (3.75  $\mu$ g per disc).

Group E: Anodized under 300 V, then coated with PLG/rhBMP-2 by electrospray (3.75  $\mu$ g per disc).

The solution of PLG/rhBMP for electrospray was put into a syringe with a needle 27G. A positive electrode was connected to the syringe needle and Ti disc was connected to the ground. The 15 k voltage was applied. The distance of needle-to-disc and the speed were installed 20 cm at 0.02 ml/min.

### 2.3 Surface characteristics

The surface characteristics of the treated Ti discs was identified with a scanning electron microscopy (FE-SEM, Hitachi S-4700, Tokyo, Japan) at 15 kV accelerating voltage. An optical interferometer (Accura 2000, Intekplus Co., Seoul, Korea) was used for measurement of the surface roughness.

### 2.4 Cell culture

The HOS cells were cultured as subconfluent monolayers in RPMI-1640 (Gibco BRL, Grand Island, NY, USA) with 10% fetal bovine serum at 37 °C in a humid atmosphere of 5% CO<sub>2</sub>/95% air. The cells were passaged at 80–90% confluence with the use of 0.05% trypsin-EDTA (Gibco BRL, Grand Island, NY, USA).

## 2.5 Methyl tetrazol sulfate assay (MTS assay) for cell proliferation

The cell proliferation was measured with the MTS assay (Cell Titer 96<sup>TM</sup>AQ Nonradioactive Cell Proliferation Assay, Promega, WI, USA) after 1, 3 and 7 days. The specimens were incubated with the MTS reagent for 2 h in the incubator at 37 °C and the optical density (OD) was examined at 490 nm by a spectrophotometer.

## 2.6 Alkaline phosphatase activity

The  $1 \times 10^5$  cells/mL of cultured cells was inoculated on each specimen. At the 3 and 7 days, cells were prepared and the extracts with 0.5% Triton X in 25 Mm Glycine-NaOH put into the ALP solution in culture plate for 30 min at 37 °C. The absorbance was observed at 405 nm by a spectrophotometer for ALP activity.

## 2.7 Release test

The profile of rhBMP-2 from Ti surface loaded in PLG was examined. The rhBMP/PLG-coated Ti disc (group D) and the rhBMP/PLG-coated Ti disc (group E) soaked into plates with 2 mL PBS. These plates were then incubated at 37 °C with gentle shaking at 100 rpm. The supernatant was collected and the plates were refilled with a PBS buffer after the decided time interval. The amount of rhBMP-2 was evaluated with a BMP-2 Immunoassay kit (R&D systems, Minneapolis, USA). The absorbance of specimens was recorded by spectrophotometer at 495 nm.

## 2.8 Runx2 gene expression

The osteoblastic differentiation of HOS cells was observed by the reverse transcription-polymerase chain reaction (RT-PCR) examination of Runx-2. The cells were seeded on Ti discs of  $5 \times 10^5$  cells/ml and incubated for 3 days. Cells plated on the discs were collected using Trysin-EDTA. Total RNA was extracted from the collected cells using TRIzol (Invitrogen, Carlsbad, CA). The forward primer (5-TCTGGCCTTCCACTCTCAGT-3) and reverse primer (5-TATGGAGTGCTGCTGGTCTG-3) of Runx-2 were fabricated according to the Runx-2 mRNA sequence. The first-strand synthesis of single-strand cDNA from RNA was conducted with SuperScript<sup>TM</sup> Reverse transcriptase First-Strand cDNA Synthesis Kit (Invitrogen, Carlsbad, CA). Subsequently, cDNA was synthesized by 1 µg of total RNA using AccuPower<sup>®</sup> RT PreMix (Bio-ner, Daejeon, Korea). The temperature of thermal cycler for amplifications set as follows: 94 °C for 30 s (denaturation), 58 °C for 30 s (annealing), 72 °C for 30 s: 26

cycles at 72 °C for 5 min (final extension). PCR products were obtained by 1.2% agarose gel with ethidium bromide.

## 2.9 Immunofluorescence analysis

The cells were cultured at  $1 \times 10^4$ /ml on titanium discs. After 24 h, the cells were rinsed with PBS and fixed by 4% formaldehyde (Sigma, St. Louis, MO, USA). After washing with PBS incorporating 0.1% BSA for 10 min each, the cells were penetrated with 0.3% Triton X-100 and obstructed in 1% bovine serum albumin (BSA) in 10% FBS for 40 min. The specimens were treated with primary antibodies for type I collagen (Bio genesis, Brentwood, NH, USA), osteonectin (Zymed, San Francisco, CA, USA), and osteocalcin (Zymed, San Francisco, CA, USA) at room temperature for 90 min, rinsed thrice with PBS incorporating 0.1% BSA for 10 min each, and then responded with fluorescein isothiocyanate (FITC)-conjugated secondary antibodies (R&D, Minneapolis, USA or pharmigen, Brentwood, NH, USA) for 90 min. The cell nuclei were stained with DAPI (1:1000, Sigma, St. Louis, MO, USA) for 10 min at room temperature. After staining, the titanium discs were equipped in gel mount (Biomeda, Bulington, CA, USA) on cover glass plates. All the marked cells on the titanium discs were identified directly utilizing the Confocal Laser Scanning Microscope (Olympus FV-300, Leeds Precision Instruments, Inc., MN, USA).

## 2.10 Statistical analysis

SPSS ver. 12.0 package for Windows was utilized for statistical analysis. Data were recored as mean value  $\pm$  standard deviation (SD) of three independent experiments.

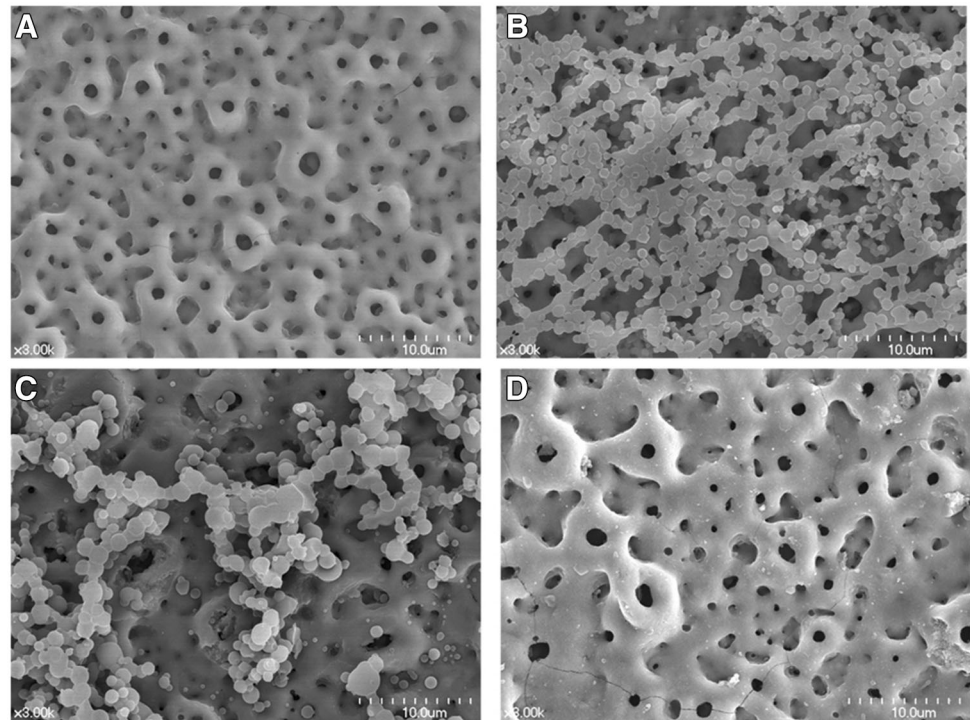
Each experimental group used five samples ( $n = 5$ ). One-way analysis of variance (ANOVA) and Scheffe's post hoc test were accomplished for the statistical significance of the differences among the groups.

## 3 Results

### 3.1 Surface morphology and roughness of prepared Ti discs

Figure 1A shows the even porous anodized disc surface with small craters. In coated groups, titanium discs display smooth, nonporous PLG microspheres (Fig. 1B, C) and nanospheres (Fig. 1D). Image analysis identified submicron-sized particles on the coated titanium surfaces. The size of the PLG microspheres was  $1.4 \pm 0.52$  µm (mean). The average diameter of group E was  $256 \pm 0.24$  nm and ranged from 100 to 500 nm. Table 1 presents the values of the surface roughness. The Ra values of the film-coated

**Fig. 1** SEM surface morphology of Ti discs ( $\times 3000$ ). **A** Anodized Ti (group NC). **B** PLG-coated Ti (group PC). **C** PLG/rhBMP-2-coated Ti (group D). **D** PLG/rhBMP-2 eletrospray-coated Ti (group E)



**Table 1** The surface roughness of Ti discs

Groups	Average roughness (Ra, $\mu\text{m}$ )	10 Point roughness (Rq, $\mu\text{m}$ )	Peak-to-valley height (Rmax, $\mu\text{m}$ )
NC	$0.70 \pm 0.38$	$1.07 \pm 0.25$	$9.22 \pm 1.63$
PC	$1.53 \pm 0.10^*$	$1.47 \pm 0.35$	$11.76 \pm 2.06$
D	$1.18 \pm 0.11^*$	$2.00 \pm 0.13^*$	$15.67 \pm 1.56^*$
E	$1.08 \pm 0.08$	$1.41 \pm 0.11$	$10.85 \pm 1.37$

Data were expressed as mean  $\pm$  SD

NC: anodized under 300 V as control

PC: anodized under 300 V, then dropped and dried with solution 0.02 ml PLG

D: anodized under 300 V, then dropped and dried with solution 0.02 ml PLG/rhBMP-2 (3.75  $\mu\text{g}$  per disc)

E: anodized under 300 V, then coated with PLG/rhBMP-2 by eletrospray (3.75  $\mu\text{g}$  per disc)

\*Denotes significant difference compare with each groups ( $p < .05$ )

groups (PC and D) were significantly higher than those of the control and the eletrospray group ( $p < .05$ ). The average surface roughness between PLG-coated group (Ra:  $1.53 \pm 0.10 \mu\text{m}$ ) and PLG/rhBMP-2-coated group (Ra:  $1.18 \pm 0.11 \mu\text{m}$ ) showed no significant difference ( $p < .05$ ).

### 3.2 Cell proliferation

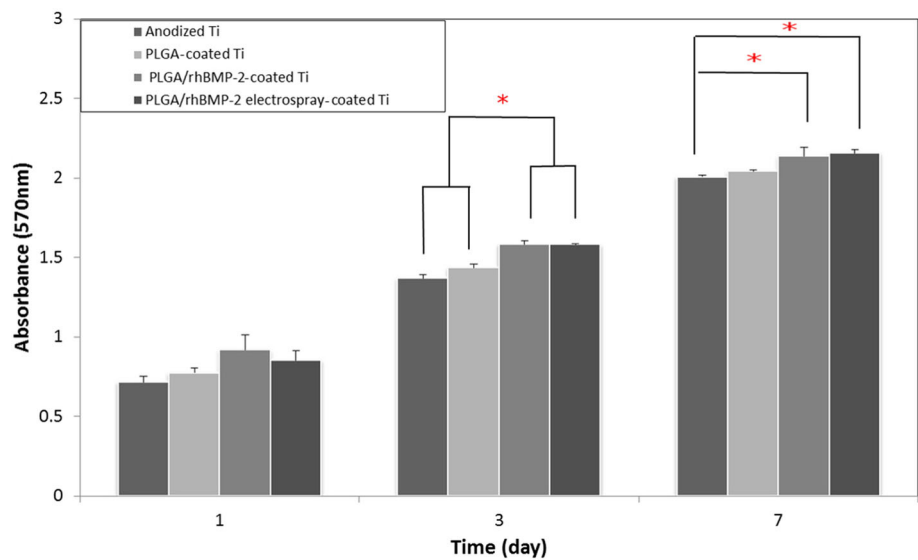
Figure 2 shows the proliferation of human osteoblast-like cells cultured on titanium discs after 1, 3 and 7. The number of osteoblast-like cells in each sample increased with incubation time. At 1 day, there were no significant differences between groups. However, there was

significantly higher proliferation in rhBMP-2 loaded titanium discs (group D and E) than rhBMP-2 unloaded discs (group NC and PC) at 3 days. Also, the anodic oxidized Ti surface group showed a significantly lower proliferation of osteoblast-like cells than the two rhBMP-2-coated groups at 7 days ( $p < .001$ ).

### 3.3 Alkaline phosphatase activity

Alkaline phosphatase activity increased significantly in all of the groups at the 7 days (Fig. 3). The ALPase activity on rhBMP-2 loaded titanium discs (group D and E) was significantly higher than in nonloaded discs (group NC and

**Fig. 2** Cell proliferation of HOS cells cultured on different Ti surfaces (n = 5). Data were expressed as mean ± SD. \*Denotes significant difference compare with each groups (p < .001)



PC) at 3 days. At 7 days, the significant difference between the groups was not found (p < .05).

### 3.4 Release test

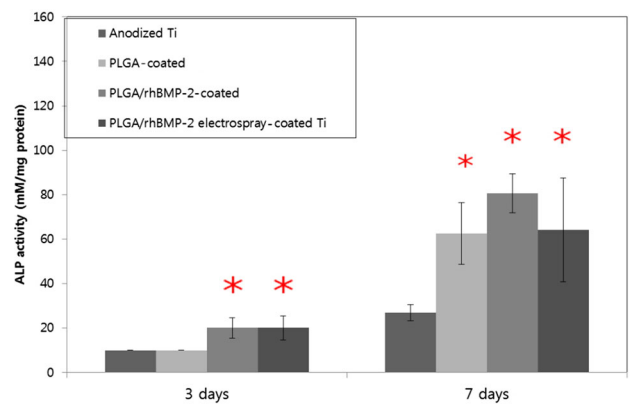
rhBMP-2 was released from the coated surface into PBS. 60% of rhBMP from PLG/rhBMP-2-coated Ti was released and 50% of rhBMP from PLG/rhBMP-2 electro-spray-coated Ti during the 5 days of release test (Fig. 4). The release characteristics of the protein showed a controlled release profile in both groups.

### 3.5 RT-PCR

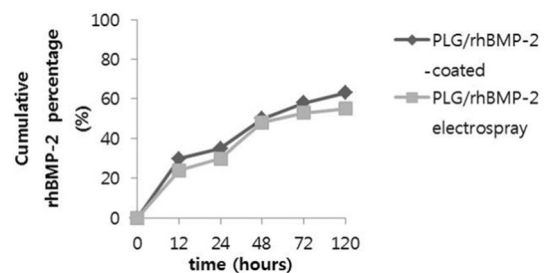
Figure 5A shows the results of electrophoresis. Intensity relative to the GAPDH gene for the Runx2 (Fig. 5B) was identified with these results. The Runx2 mRNA from on the PLG/rhBMP-2-coated surface presented the highest level.

### 3.6 Immunofluorescence analysis

Type I collagen was deposited on the intracellularly cytoplasm but rarely in the nucleus at 24 h. We could not find the distinct differences among the groups (Fig. 6A–C). It was found that osteocalcin was predominantly presented in the perinuclear area and the cytoplasm of HOS cells cultured during 24 h. There were no determinant differences among the groups (Fig. 6D–F). Immunostaining pattern for osteonectin in the control and the PLG-coated group exhibited mainly in the perinuclear area. It was not obvious different between the anodized only discs (control group) and the PLG-coated discs (Fig. 6G–F). However, the level of osteonectin expression was highest in the PLG/BMP-2-



**Fig. 3** Alkaline phosphatase activity of the series of anodized Ti surfaces at 3 and 7 days (n = 5). Data were expressed as mean ± SD. \*Denotes significant difference compare with each groups (p < .05)

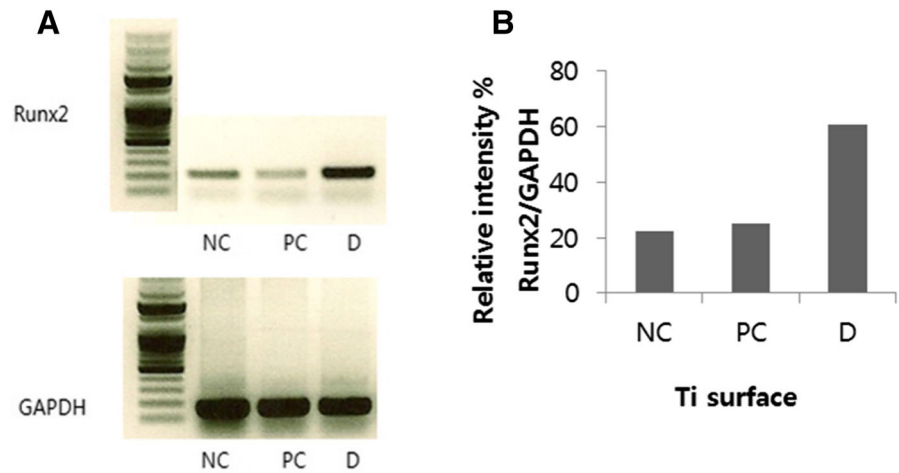


**Fig. 4** Release kinetics of rhBMP-2 from PLGA/rhBMP-2-coated Ti and PLGA/rhBMP-2 electro-spray-coated Ti (n = 3)

coated group. Cytoplasmic osteonectin was expressed in the nucleus and the cytoplasm as mesh-like frame which reveals that the osteonectins were positioned along the cytoskeleton (Fig. 6I).



**Fig. 5** The effect of modified Ti surface on Runx2 expression in HOS cells. **A** Ethidium bromide-stained agarose gel analysis of RT-PCR products. **B** Intensity relative to the GAPDH gene for the Runx2 gene. NC: Anodized under 300 V as control, PC: Anodized under 300 V, then dropped and dried with solution 0.02 ml PLG, D: Anodized under 300 V, then dropped and dried with solution 0.02 ml PLG/rhBMP-2



#### 4 Discussion

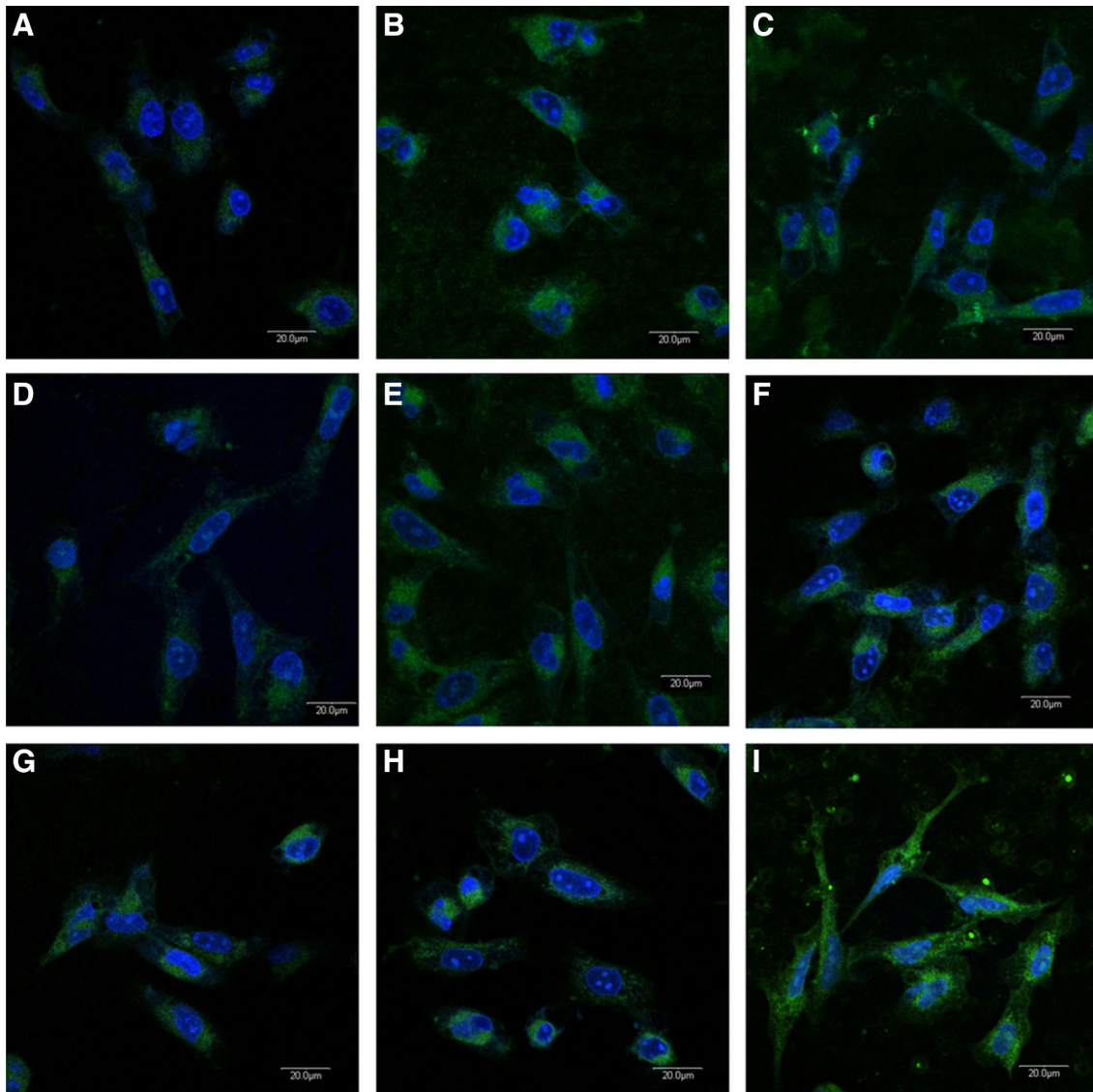
The purpose of present study was to examine the physical characteristics of and the initial biological cell reaction to anodized titanium treated with a PLG mixed with rhBMP-2. Ti surfaces have been altered by electrochemical anodization to increase the surface roughness or modify the physical structure and chemical property [16]. Ti discs utilized in this study had a uniformly porous oxide layer with holes at the center (Fig. 1A). These cratered surfaces may have provided as a vehicle for the rhBMP-2, allowing cellular differentiation and proliferation. These surfaces may have held rhBMP-2 during a certain period (Fig. 1C, D). Hall et al. [8] compared titanium porous oxide (TPO) surfaces with machined titanium (MT) surfaces. TPO maintained more rhBMP-2 than MT. The small crater structures provide storage when the material is implanted at the surgical site.

This study investigated two methods to control the coating size of particles on Ti surface using an electro spray method, or modified cold film coating on Ti surface. Growth factors should be carried through a “cold coating technique”. This technique, which is simple, experimentally proven, and eliminates the risk of heat degeneration, was used to incorporate growth factors into titanium [17]. Schmidmaier et al. [17] identified the stability of incorporated proteins during the cold coating process. In this study, we used a modified cold film coating technique to coat rhBMP-2 onto Ti discs. By another coating method, we used conventional electrostatic spraying (CES), which is a solvent system consisting of applying solvent system consisted of acetone and water. In the previous studies, an electrostatic spray technique was applied for both gene vehicle and protein carrier [18]. Electro spray produced the structures of nanoparticles by a high electric field. By controlling solution properties and dealing with parameters, the size and the pore of the structure can be adjusted

during electro spraying [19]. By modulating the polymer molecular weight and/or the polymer ratio, the release rate and duration can be efficiently manipulated. It has been reported that nanoparticles incorporate better into the cell compared to microparticles [20]. We hypothesized that BMP of PLG smaller particles would be effective uptake in the target cell. In this study, using an electrostatic spray method, submicron-sized ( $\sim 500$  nm) PLG/rhBMP-2 particles with uniform sphere shape and smooth, nonporous surfaces were fabricated (Fig. 1D). We obtained a smaller particle size by electro spray method in comparison to the modified cold coating on titanium surfaces. The average surface roughness between PLG-coated group and poly(lactic-co-glycolic acid) (PLGA)/BMP-2-coated group showed no significant difference ( $p < .05$ ). The Ra values of the PLG/BMP-2 electro spray-coated group ( $1.08 \pm 0.08 \mu\text{m}$ ) were significantly smaller than the film-coated PLG group ( $1.53 \pm 0.10 \mu\text{m}$ ) and PLG/BMP-2 group ( $1.18 \pm 0.11 \mu\text{m}$ ,  $p < .05$ ). It was significant difference of cell proliferation and ALPase activity between only PLG-coated group PC and BMP incorporated groups D and E. However, we could not find a significant difference of cell proliferation and ALPase activity between the BMP incorporated group D and group E.

Even though we obtained a smaller particle size by electro spray method in comparison to the modified cold coating on titanium surfaces, we did not find significant differences in cell proliferation and differentiation after culturing osteoblasts-like cells for 7 days on coated disc versus electro spray disc with rhBMP-2 using PLG. Despite the use of the same solution of polymer/BMP for coatings in groups D and E, the possibility of scattering of a portion of solution into the air during the electro spray process cannot be excluded.

Consequently, there was no difference in cell responses depending on the coating method but there was a difference according to the insertion of BMP. In this context, it can be



**Fig. 6** Immunofluorescence labeling of type I collagen (A, B, C), osteocalcin (D, E, F) and osteonectin (G, H, I) of human osteoblast-like cells grown on Ti surface at 24 h ( $\times 1000$ ). Cytoplasm (green), nucleus (blue). Cytoplasmic osteonectin was expressed particularly in

the perinuclear area appearing as mesh-like structure (I). A, D, G anodized Ti surface. B, E, F PLG-coated Ti surface. C, F, I PLGA/rhBMP-2-coated Ti surface

speculated that an effect of the bioactive material, BMP may be more important than a particle size of the coating surface. Also, additional studies are required on methods of electrospray more efficiently to better manipulate the BMP-2 release rate and duration by adjusting the polymer molecular weight and/or the polymer ratio.

BMP-2s are multifunctional molecules that can promote bone formation and modulate the expression and organization of osteoblastic cell protein. They may act as growth and differentiation factors, and as chemotactic agents. Also, they stimulate angiogenesis, and migration, proliferation, and differentiation of osteoblast-progenitor cells into bone-forming cells. BMP-2 treatment of osteoblastic

cells has been shown to significantly affect the cytoskeletal and extracellular matrix organization, and promote cellular adhesion to titanium surfaces by increasing the expression of fibronectin and integrin receptor subunits. A mechanistic explanation for the enhanced proliferation and differentiation of osteoblastic cells after BMP-2 treatment may be their enhanced ability to regulate extracellular matrix (ECM) and integrin synthesis and organization. It seems to be upregulated during migration and proliferation and may involve in the cell-matrix interaction.

BMP signals are mediated by BMP receptors and their downstream molecules. BMP signaling pathways have been described through the elaborate network of mediators

[21]. Increase of the presentation of BMP receptors during bone formation has been found [22] and many binding sites for BMP-2 have been identified on osteoblasts [23]. BMP signals are regulated by serine/threonine kinase BMP receptors and their downstream molecules. Phosphorylated Smad1, 5 and 8 make a compound with Smad4 and then are moved into the nucleus where they communicate with other transcription factors, such as Runx2 [23].

To evaluate the cellular responses to PLG/rhBMP-2-coated Ti surfaces, the MTS assay was used as the marker for cellular proliferation. MTS assay presented that cells proliferated continuously until 7 days on the disc. At 1 day, it did not show significantly different in cell proliferation between the groups. However, after 3 days, cells on PLG/rhBMP-2-coated Ti specimens (group D and E) showed more active proliferation compared to control and PLG-coated Ti specimens without rhBMP-2. Also, the anodic oxidized Ti surface group showed a significantly lower proliferation of cells than the two rhBMP-2-coated groups at 7 days ( $p < .001$ ). Therefore, biodegradable PLG nanoparticles mixed with rhBMP-2 positively might affect initial proliferation of HOS cells on the anodized titanium.

The osteoblast differentiation was assessed by the alkaline phosphatase (ALP) activity and Runx2 gene expression. The ALP is well known as an osteoblast marker, and an expression in ALP is related to osteoblastic differentiation. In this study, the ALP activity increased over the experimental time. At 3 days, the cells on rhBMP-2 incorporated Ti presented a higher activity than the control and PLG-coated Ti specimens. Also, at 7-day culture, the anodic oxidized only Ti surface presented a significantly lower level of ALP activity of osteoblast-like cells than the other groups ( $p < .05$ ). In general, the cells cultured on PLG/rhBMP-2-coated discs showed a higher proliferation and differentiation. Based on these findings, we proposed that PLG/rhBMP-2-coated Ti surfaces might have significant functions in controlling early proliferation and differentiation of osteoblast lineage cells and in regulating osteogenesis.

Controlled and sustained release is crucial for rhBMP-2 delivery system, so early burst and short term release course should both be avoided. Furthermore, a very low concentration of release is not wanted if it is below the effective (therapeutic) concentration level. Although BMPs can be potent osteoinductive growth factors, their administration is complicated by their short biological half-lives, localized actions and rapid local clearance. To overcome these problems, effective BMP treatments require their incorporation into a biomaterial for its local sustained delivery at the target site. The delivery vehicle should maintain a local BMP concentration within the therapeutic window for a sufficient period of time to allow osteoprogenitor cells to migrate to the target site and differentiate

into osteoblasts. In this study, controlled release systems for BMP-2 release were formulated using combinations of surface modification both anodic oxidization and prepared PLG polymer. The release pattern of BMP-2 on group D and E showed an S-shaped sustained release for 5 days. This result is similar to previous study of release system for BMP-2 based on PLG microspheres [24]. In particular, group E exhibited a slower BMP-2 release than Group D.

Runx2 operates the presentation of osteocalcin, and modulates osteoblast differentiation [25]. Runx2 upregulates the presentation of bone matrix genes, including type I collagen, osteopontin, bone sialoprotein (BSP), and fibronectin [26]. Therefore, Runx2 is an important gene both osteoblast differentiation and osteoblast function. In this study, the amounts and patterns of Runx2 gene expressions were different depending on the rhBMP-2 surface treatments. It was identified that initial Runx2 gene expression was favorable in HOS cells on Ti surface coated with PLG/rhBMP-2 (Fig. 5). From this result, rhBMP-2 coating of titanium surfaces may influence on the osteoblast differentiation by controlling the level of gene expression of major osteogenic factors.

In the present study, we observed the osteogenic protein expression by immunofluorescence assay. The proteins of extracellular matrix, such as collagen, osteonectin and other glycoproteins, are produced by osteoblasts and most are engaged in adhesion. Also, osteoblasts produce and release bone matrix protein such as osteonectin, osteocalcin, osteopontin, and bone sialoprotein. These proteins have been known to be effective osteogenic markers. Among these proteins, we observed the type I collagen, osteonectin, osteocalcin as osteogenic markers (Fig. 6). The immunoresponses of osteonectin were more active in cells on PLG/BMP-2-coated Ti surface than other groups (Fig. 6I). Osteonectin is a significant element of the non-collagenous matrix of bone. It is concerned with binding  $\text{Ca}^{2+}$  ions and hydroxyapatite during mineralization and is an important indicator and regulator of these processes [27]. It seems to be upregulated during migration and proliferation and may involve in the cell-matrix interaction. However, we could not observe distinct differences between the surfaces with regard to initial cytoplasmic and extracellular distribution and fluorescence intensity of type I collagen and osteocalcin. Therefore, it might be thought that the presentation of osteocalcin and collagen protein was modulated at the late phases of osteoblast differentiation. Following incorporation of BMP-2, the osteonectin production was significantly increased compared to the BMP-2 non-treated group. It can be explained that the rhBMP-2 promote the differentiation of osteoblast.

Overall, these results propose that PLG might be utilized for an appropriate carrier for rhBMP-2 to improve bone formation. Surface treatments of anodized Ti with PLG/



rhBMP-2 contribute to a clinical model of rhBMP-2 for developing dental implant.

In conclusions, this study examined the biological responses of the PLG/rhBMP coating on an anodized titanium surface. PLG particles mixed with rhBMP-2 were distributed on titanium surfaces. Under the cell proliferation and differentiation test, the PLG/rhBMP-2-coated Ti groups were significantly greater than the control and the PLG-coated group. The PLG/rhBMP-2-coated group showed a significantly higher Runx-2 gene expression and osteonectin production than the control and the PLG-coated group.

PLG mixed with rhBMP-2 has positive outcomes on the biologic responses of osteoblast like cells with respect to cell proliferation, differentiation, and extracellular matrix formation.

#### Compliance with ethical standards

**Conflict of interest** The authors declare that they have no conflict of interest.

**Ethical statement** There are no animal experiments carried out for this article.

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