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CELLULAR RESPONSES ON ANODIZED TITANIUM DISCS COATED WITH 1α,25-DIHYDROXYVITAMIN D_3 INCORPORATED POLY (D,L-LACTIDE-CO-GLYCOLIDE) (PLGA) NANOPARTICLES

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INTRODUCTION

A goal of current implant research is to design a device that induces controlled, guided, and rapid healing. In addition to acceleration of normal wound healing process, the implant should result in an interfacial matrix with surrounding bone in composition and structure characteristics, and the matrix should have adequate biomechanical properties. Different approaches are possible to obtain the desired bone-implant interface. The methods of modifying surfaces of existing biomaterials to achieve desired biological responses can be classified as physicochemical, morphological, or biochemical.[1] A biochemical approach of surface modification can offer an alternative or adjunct to physicochemical and morphological methods. The goal of the biochemical surface modification is to immobilize proteins, enzymes, or peptides on biomaterials for the purpose of inducing specific cell and tissue responses or, in other words, to control the tissue-implant interface with molecules delivered directly to the interface.

Vitamin D is one of the major factors involved in calcium homeostasis, and biologically most active vitamin D molecule is 1α,25-dihydroxyvitamin D_3 (1α,25-$(OH)_2D_3$).[2] It has been reported that 1α,25-$(OH)_2D_3$ directly stimulates in vitro mineralization of bone and differentiation of human osteoblasts.[2,3] Coatings incorporating biomolecules are also being explored for delivering biomolecules to the tissue-implant interface. In light of the dependence of cell and tissue responses on the duration of exposure and concentration of biomolecule, this approach is attractive because it can be used to control release of biomolecules.[4]

Polymers that belong to the Poly(D,L-lactide-co-glycolide) (PLGA) family are now widely used for fabricating implantable drug delivery systems.[5] These materials are popular because of their adequate biocompatibility and their structure-property relationships have been examined in detail. In addition, their *in vivo* and in vitro biodegradation characteristics have also been studied extensively.[5-7]

In this study, the anodized titanium discs were coated with a PLGA solution incorporated with 1α,25$(OH)_2D_3$ via electrospray technique. The purpose of the present study was to investigate the responses of osteoblast-like cells to PLGA/ 1α,25-$(OH)_2D_3$ coated titanium surface with reference to cellular proliferation and differentiation *in vitro*.

MATERIAL AND METHODS

1. Titanium disc preparation

Ninety six titanium discs were fabricated using

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commercially pure titanium (Warantec Co., Seoul, Korea), with dimensions of 25 mm diameter and 1 mm thickness. Prior to use, degreasing and acid prepickling of all discs were done by washing them in acetone, processing through 2% ammonium fluoride/2% hydrofluoric acid/10% nitric acid solution at 55℃ for 30 seconds, and pickling in 2% hydrofluoric acid/10% nitric acid at room temperature for 30 seconds. The pretreated discs were further processed, as described below, to produce anodized surface. 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.[8,9] The anodized discs were washed with distilled water, dried and then sterilized in Ethylene Oxide (E.O.) gas before the experiment.

2. Titanium disc surface modification

Poly(lactide-co-glycolide) (PLGA) (PURAC Biochem BV, Gorinchem, Holland) was used for a drug carrier. PLGA was dissolved in 0.2% w/v acetone (Duksan pure chemicals Co. LTD, Kyungkido, Korea) and 1,25$(OH)_2D_3$ (Sigma-Aldrich Co., St. Louis, MO, USA) in acetone (12.5 ul/ml) was incorporated. All the solvents were used without further purification. Seventy two titanium discs in 96 anodized discs were coated with the solution by an electrospray technique as follows:

Group 1: Anodized under 300 V

Group 2: Anodized under 300 V then, coated with 3 ml [PLGA/1α,25-$(OH)_2D_3$ (2 ul/disc)] solution

Group 3: Anodized under 300 V then, coated with 3 ml [PLGA/1α,25-$(OH)_2D_3$ (20 ul/disc)] solution

Group 4: Anodized under 300 V then, coated with 3 ml [PLGA/1α,25-$(OH)_2D_3$ (200 ul/disc)] solution

The apparatus for the electrospray experiments was presented on previous studies.[10,11] The polymer solution that contained PLGA and 1α,25-$(OH)_2D_3$ was placed into a glass syringe with needle gauge 27. A positive electrode was connected to the syringe needle and a titanium disc, used as the collector directly, was connected to the ground. A high voltage power supply (Nano NC, Seoul, Korea) was employed to generate the electric field (0 - 30 kV) and the applied voltage was fixed at 17 kV. The distance of tip-to-collector and the flow rate were settled 6 cm and at 20 - 40 ml/min, respectively. For even distribution, the disc was connected with a rotating motor. The rotation speed of motor was 30 rpm. The electrospray was performed at room temperature and coated implants were packaged aseptically and stored moist free at -20℃ in a freezer.

3. Cell proliferation and differentiation assay

The osteoblast-like Human Osteogenic Sarcoma (HOS) cells (ATCC, Rockville, MD) were used in the experiment. The cells were cultured in Dulbecco's modified Eagle's medium (D-MEM) supplemented with 10% fetal bovine serum (FBS). Cells were seeded onto the surface modified titanium discs in 6-well plates at a density of 5×10^5 cells/ml and cultured at 37℃ in 5% CO_2. Prior to culture each titanium discs were transferred to new culture plates and cells were cultured for 1, 3 and 7 days. After 1 day, 3 discs in culture medium for each group were treated with MTS-based cell proliferation assay (CellTiter 96® AQueous One Solution Cell Prolifertion Assay, Promega Corp., WI, USA) and another 3 discs were used for ALPase activity test. After 3 days and 7 days, same procedures were done. The MTS-based cell proliferation assay is based on the reduction of a tetrazolium compound to a colored formazan product that is soluble in tissue culture medium by viable cells (or metabolic activity).[12] Small amount of the CellTiter 96® AQueous One Solution Cell Prolifertion Assay reagent was directly added to culture wells, incubated for 3 hours and then the absorbance was recorded at 490 nm. For alkaline phosphatase (ALP) activity test, cells cultured for 1, 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℃. 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.

4. Field Emission Scanning Electron Microscopy (FE-SEM)

Twenty four discs were used for surface analysis. After 1, 3 and 7 days, one titanium disc for each group was selected for this examination. The samples were observed using a field emission scanning electron microscopy (FE-SEM, S-4700, Hitachi, Ltd., Tokyo, Japan) at 15 kV accelerating voltage.

5. Statistical analysis

A one-way analysis of variance and Tukey' s b post hoc test were carried out to determine the statistical significance of the differences among observed groups.

RESULTS

1. Characteristics of titanium disc surface and HOS cells

The surfaces of titanium discs and representative morphologies of osteoblast-like HOS cells are shown in Figs. 1, 2 and 3. PLGA nanoparticles were observed as fine, smooth and round particles. They were got tangled with one another and the cells attached and spread well on all of the surfaces. Fig. 1 displays the uniformly porous anodized titanium disc surface which was composed of small craters and HOS cells attached to the anodized surfaces and PLGA/1α,25-$(OH)_2D_3$ coated surfaces at 1-day culture. HOS cells were observed to attach to titanium discs through strand-like and sheet-like filopodia. After 3 days of culture, the dendritic filopodia were exaggerated and sheet-like cytoplasmic projections covered the coated titanium surfaces. In groups 3 and 4, nuclei of cells were more round and cytoplasms were fused one another and covered larger surfaces than in groups 1 and 2.

2. Cellular proliferation and differentiation

The MTS tetrazolium compound reduction as an indicator of cell vitality has been used to quantify the proliferation capacity of the osteoblast-like HOS cells seeded onto the different substrates. The resulting graph in Fig. 4 shows the average optical density values of the measured absorbance. The cells proliferated actively on all substrates. After 3 days culture, all of the groups showed increased cellular proliferation than day 1 ($P < .05$) and the smallest proliferation rate was measured on group 2. Higher amount of incorporated 1α,25-$(OH)_2D_3$ improved optical densities but the difference was significant statistically only between group 2 and the others. After 7 days of culture, all of the groups showed slight increase of proliferation rate except group 1 ($P > .05$).

Fig. 5 shows the ALP-specific activity of each group of osteoblast-like cells. The experimental groups presented greater increase rate of ALP activities than the control group ($P < .05$) except group 2. Group 2 exhibited higher ALP activity at 1 day and 3 days of culture, however significantly lower activity was observed at day 7. On the other hand group 3 and 4 showed lower ALP activities than anodized disc group at day 1, but they were increased after 3 days culture and demonstrated greater optical density values. The differences were statistically significant at day 3 ($P < .05$).

DISCUSSION

1α,25-dihydroxyvitamin D_3 (1α,25-$(OH)_2D_3$) is one of the degradation products of vitamin D. 1α,25-$(OH)_2D_3$ is formed after two hydroxylation steps of vitamin D_3 (cholecalciferol). It is presumed that the vitamin D effects not only on mineralization indirectly via control of calcium (re)absorption in intestine and kidney, but the vitamin D receptor (VDR) is present in osteoblasts and 1α,25-$(OH)_2D_3$ has direct effects on osteoblasts as shown by regulation of the expression of several genes.[2,3,13] van Driel *et al*. observed the stimulatory effect of 1α,25-$(OH)_2D_3$ on ALP activity and this is in accordance with previous findings of increased ALP activity by 1α,25-$(OH)_2D_3$ at proliferative stages of human primary osteoblasts.[14]

There are several reports showing that 1α,25-$(OH)_2D_3$ regulates osteoblast differentiation and mineralization. Human osteoblast cells cultured in the continuous presence of 1α,25-$(OH)_2D_3$ displayed significantly increased ALP activity at all time-points during culture and the effects on ALP activity were dose-dependent.[2] 1α,25-$(OH)_2D_3$ also significantly increased osteocalcin content in the matrix and mineralization (calcium content) of the extracellular matrix during culture.[2] The expression of integrins, which serve as

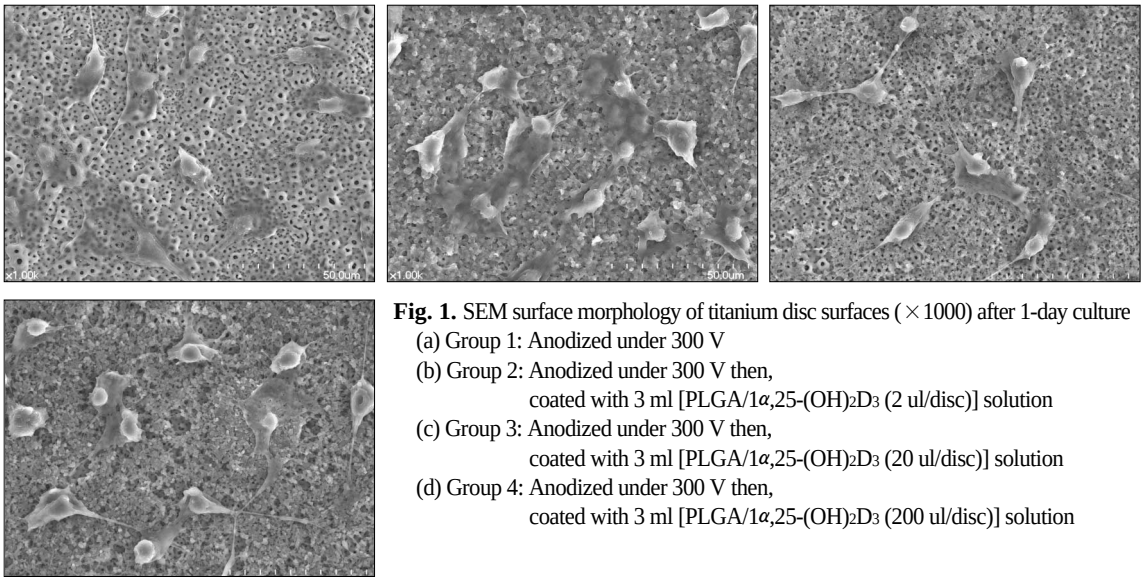

Fig. 1. SEM surface morphology of titanium disc surfaces (×1000) after 1-day culture
(a) Group 1: Anodized under 300 V
(b) Group 2: Anodized under 300 V then,
coated with 3 ml [PLGA/1α,25-$(OH)_2D_3$ (2 ul/disc)] solution
(c) Group 3: Anodized under 300 V then,
coated with 3 ml [PLGA/1α,25-$(OH)_2D_3$ (20 ul/disc)] solution
(d) Group 4: Anodized under 300 V then,
coated with 3 ml [PLGA/1α,25-$(OH)_2D_3$ (200 ul/disc)] solution

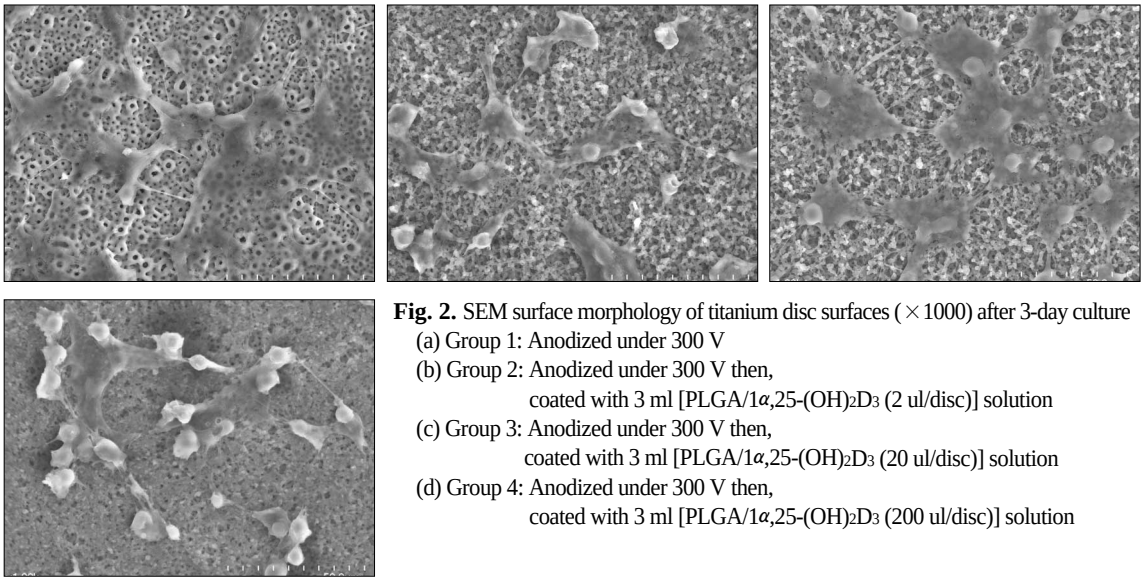

Fig. 2. SEM surface morphology of titanium disc surfaces (×1000) after 3-day culture
(a) Group 1: Anodized under 300 V
(b) Group 2: Anodized under 300 V then,
coated with 3 ml [PLGA/1α,25-$(OH)_2D_3$ (2 ul/disc)] solution
(c) Group 3: Anodized under 300 V then,
coated with 3 ml [PLGA/1α,25-$(OH)_2D_3$ (20 ul/disc)] solution
(d) Group 4: Anodized under 300 V then,
coated with 3 ml [PLGA/1α,25-$(OH)_2D_3$ (200 ul/disc)] solution

transducer of extracellular information to the cell, in human osteoblast-like cells was differentially modulated by 1α,25-$(OH)_2D_3$.[15] Osteocalcin level, a marker of a differentiated osteoblast, was sensitive to vitamin D metabolite. Osteocalcin mRNA increased with 1α,25-$(OH)_2D_3$ treatment and these change were greater in cultures on the titanium discs than on culture plastic.[15]

In this study, we used nanoparticles formulated using a FDA approved biodegradable and biocompatible polymers, poly (D,L-lactide-co-glycolide) (PLGA), for vitamin D metabolite carrier. The hypothesis was that vitamin D metabolites released from PLGA nanoparticles could enhance osteogenic differentiation and matrix mineralization by osteoprogenitor cells by activation of cellular vitamin D receptors. PLGA has been most extensively investigated biodegradable polymer for drug delivery.[16] As polyesters in nature, these polymers undergo hydrolysis upon implantation into the body, forming biologically compatible and metabolizable moieties (lactic acid and glycolic acid) that are eventually removed from the body by the citric acid cycle. Polymer biodegradation products are formed at a very slow rate, and hence they do not affect the normal cell function.[7] The drug entrapped in PLGA matrix is released at a sustained rate through

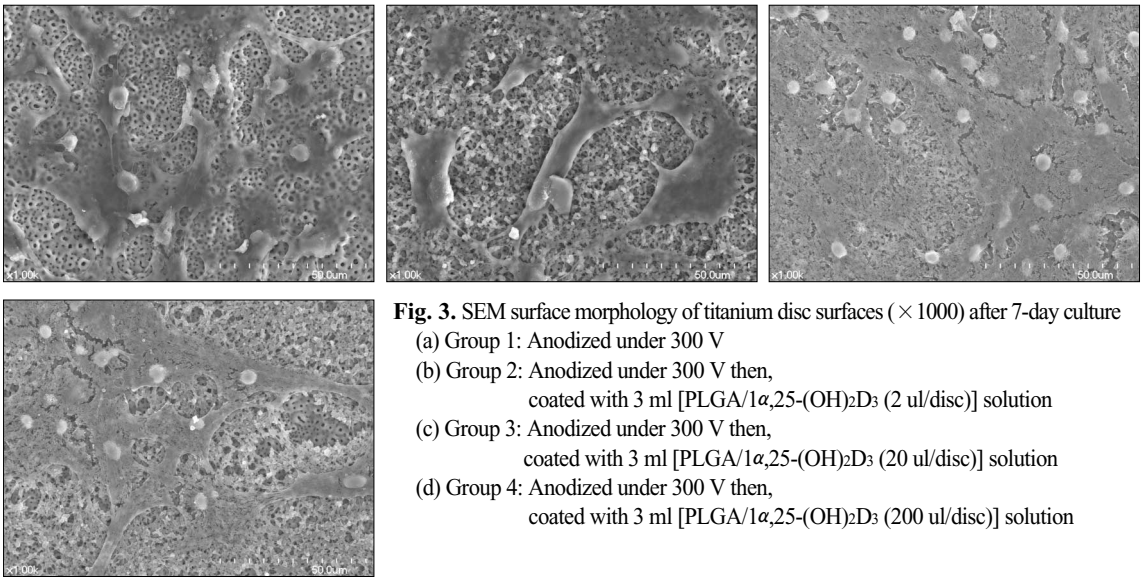

Fig. 3. SEM surface morphology of titanium disc surfaces (×1000) after 7-day culture
(a) Group 1: Anodized under 300 V
(b) Group 2: Anodized under 300 V then, coated with 3 ml [PLGA/1α,25-$(OH)_2D_3$ (2 ul/disc)] solution
(c) Group 3: Anodized under 300 V then, coated with 3 ml [PLGA/1α,25-$(OH)_2D_3$ (20 ul/disc)] solution
(d) Group 4: Anodized under 300 V then, coated with 3 ml [PLGA/1α,25-$(OH)_2D_3$ (200 ul/disc)] solution

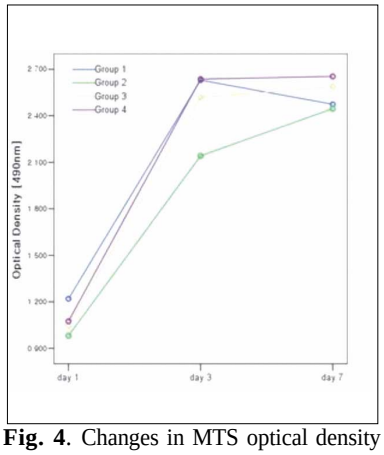

Fig. 4. Changes in MTS optical density values of osteoblast-like HOS cells grown on the discs after culturing 1, 3 and 7 days (mean + SD, n = 3).

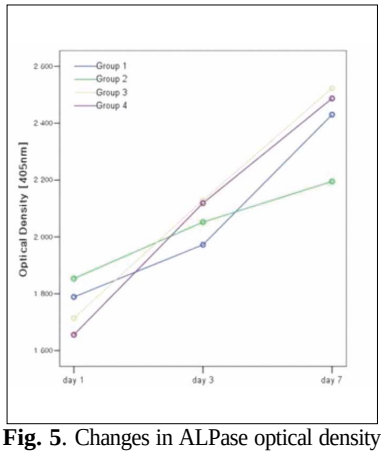

Fig. 5. Changes in ALPase optical density values of osteoblast-like HOS cells grown on the discs after culturing 1, 3 and 7 days (mean + SD, n = 3).

diffusion of the drug in the polymer matrix and by degradation of the polymer.[17] Thus, it was expected that the localized and sustained release of vitamin D metabolite would aid cellular differentiation and matrix mineralization.

In the present study, cellular proliferation and differentiation were measured after PLGA/1α,25-$(OH)_2D_3$ coating by electrospray technique in vitro. MTS-based cell proliferation assay is a colorimetric method for determining the number of viable cells in proliferation or cytotoxicity assays.[12] Alkaline phosphatase activity is widely used as an osteoblast marker, and an increase in ALP activity is associated with osteoblastic differentiation, bone formation, and matrix mineralization.[18,19] Therefore, the ALP specific activity of the osteoblast-like HOS cells was measured to determine effect to the differentiation of osteoblast cells.

The control group of this study was anodized titanium surface. The anodized titanium has improved biocompatibility and rougher surface compared to turned surface.[9,20] Surface roughness plays a role in the response of osteoblast-like cells to 1α,25-$(OH)_2D_3$. The response of MG63 cells to 1,25-dihydroxyvitamin D_3 is enhanced on rougher surfaces, and for some parameters, the effects of surface roughness and 1α,25-$(OH)_2D_3$ are synergistic.[21] On smooth cp titanium surfaces, 1α,25-$(OH)_2D_3$ had no effect

on alkaline phosphatase activity, osteocalcin production, or release of TGF-b1 or PGE2 into the media, but when the cells were cultured on rough cp titanium, 1α,25-$(OH)_2D_3$ caused a marked increase for all parameters over the effect of surface roughness alone.[21] That was the reason we chose the anodized surface as the control group.

In the present study, cellular proliferation was not changed significantly after day 3 and group 2 revealed lower values than other groups. ALP activity, cellular differentiation marker, was increased in each group until day 7 at dose-dependent manner. But group 2 presented lower ALP activity at day 7 than the control group and except this value significant differences did not exist. The influence of 1α,25-$(OH)_2D_3$ treatment to cellular proliferation of osteoblast-like cells was reported in a few studies. The number of MG63 cells cultured on tissue culture plastic, chemically modified polished titanium, grit-blasted and acid-etched titanium (SLA), and modified SLA surfaces was decreased at smooth surfaces (plastic, polished titanium) and was not significantly different at rough surfaces (SLA, modified SLA) after 1α,25-$(OH)_2D_3$ treatment.[22] And 1α,25-$(OH)_2D_3$ treatment to titanium surface had no effect on cell number or decreased cellular proliferation according to maturation state of osteogenic cells.[23]

As mentioned above, the responsiveness of osteoblasts to the vitamin D metabolite 1α,25$(OH)_2D_3$ is synergistically increased with roughness. In rough surface, osteoblasts respond to 1α,25-$(OH)_2D_3$ in a number of ways.[24,25] 1α,25-$(OH)_2D_3$ inhibits or has no effect on cell proliferation, increases alkaline phosphatase expression and activity, and increases osteocalcin production, indicating that 1α,25-$(OH)_2D_3$ promotes osteoblastic differentiation.[2,21,22] The results of the present study were in accordance with these studies. Our matter of concern is to extend the effect of 1α,25-$(OH)_2D_3$ by sustained release capacity of PLGA nanoparticles. Biodegradable polymers like PLGA are widely used and popular in various fields, but there are few studies about applications of these polymers incorporated with bioactive molecules as 1α,25-$(OH)_2D_3$ to titanium material.

In this study, we showed that biodegradable PLGA nanoparticles incorporated with vitamin D metabolite 1α,25-$(OH)_2D_3$ affects proliferation and differentiation of osteoblast-like cells on the anodized titanium surface. And higher concentration of incorporated 1α,25-$(OH)_2D_3$ improved cellular proliferation and differentiation. Further *in vitro* and *in vivo* studies on the detailed mechanism of actions of PLGA nanoparticles and 1α,25-$(OH)_2D_3$ are needed.

CONCLUSION

Within the limitation of this in vitro study, we concluded that biodegradable PLGA nanoparticles incorporated with vitamin D metabolite 1α,25-$(OH)_2D_3$ positively affected proliferation and differentiation of osteoblast-like HOS cells on the anodized titanium surface.

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STATEMENT OF PROBLEM: A biochemical approach for surface modification has offered an alternative for physicochemical and morphological methods to obtain desirable bone-implant interfaces. **PURPOSE:** The purpose of the present study was to investigate cell responses to poly (D,L-lactide-co-glycolide) (PLGA)/1α,25-$(OH)_2D_3$ coating with reference to cellular proliferation and differentiation in vitro. **MATERIAL AND METHODS:** 96 titanium discs were fabricated and divided into four groups. Group 1 was anodized under 300 V as control. Group 2, 3 and 4 were anodized then coated with 3 ml PLGA/1α,25-$(OH)_2D_3$ solutions. Amount of the solutions were 2 ul, 20 ul and 200ul respectively. The osteoblast-like Human Osteogenic Sarcoma (HOS) cells were seeded and cultured for 1, 3 and 7 days. MTS-based cell proliferation assay and ALPase activity test were carried out. **RESULTS:** PLGA nanoparticles were observed as fine, smooth and round and HOS cells attached to the anodized surfaces through strand-like and sheet-like filopodia. After 3 days of culture, the dendritic filopodia were exaggerated and sheet-like cytoplasmic projections covered the coated titanium surfaces. After 3 days of culture, all of the groups showed increased cellular proliferation and the lowest proliferation rate was measured on group 2. Higher amount of incorporated 1α,25-$(OH)_2D_3$ (Group 3 and 4) improved cellular proliferation but the differences were not significant statistically ($P > .05$). But they increased the rate of ALP activities than the control group at day 3 ($P < .05$). **CONCLUSION:** Biodegradable PLGA nanoparticles incorporated with vitamin D metabolite positively affected proliferation and differentiation of cells on the anodized titanium surface.

KEY WORDS: 1α,25-dihydroxyvitamin D_3, PLGA, Nanoparticle, Anodized titanium, Cell proliferation, Differentiation

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