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Alendronate–calcium phosphate hybrid films promoted the osteoblast differentiation and inhibited osteoclastogenic activity

Won-Kyue Yang [a], Eun-Ju Chang [b], Woo-Kul Lee [a,*]

[a] Department of Chemical Engineering, College of Engineering, Institute of Tissue Regeneration Engineering (ITREN), Dankook University, Cheonan, South Korea
[b] Department of Biomedical Sciences, College of Medicine and Asan Medical Center, University of Ulsan, Ulsan, South Korea

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ABSTRACT

Surface property of biomaterials is a crucial factor determining the biocompatibility of a biomaterial. In this study, we aimed at evaluating the feasibility of alendronate (ALN) and CP hybrid films (CPAs) to promote bone regeneration. Experimental analysis elucidated that ALN incorporation promoted osteoblast proliferation and differentiation, phenotype gene expression, phase shift from differentiation into mineralization, and bone-like nodule formation. Inhibitory effect on the formation of multinucleated osteoclast was substantial. Therefore, the hybrid films can enhance osteogenic responses while inducing anti-osteoclastic effects. We expect the hybrid films can be potentially utilized for the promotion of bone regeneration.

Introduction

The elderly population is rapidly increasing due to the advances in medical and pharmaceutical technologies. Bone-related diseases including osteoporosis have become one of the major problems deteriorating the quality of life. It is, therefore, desired to develop more efficient and advanced methods for the treatment of bone defects. Bisphosphonates (BPs) are a family of drugs that has been widely used for the treatment of bone-related diseases such as osteoporosis, Paget's disease and hypercalcemia of malignancy since 1970s [1]. BPs possess a common chemical structure of P–C–P and display a strong affinity toward cations such as calcium ion. Accordingly, BPs strongly bind to mineralized bone, bone matrix, and hydroxyapatite [2]. Dependent on properties of chemical groups substituted into its side chains, namely R1 and R2, physicochemical and biological functionalities of bisphosphonate can be significantly altered [3].

Major pharmacological action of BPs includes the prevention of bone loss by inhibiting bone resorption activity by OC [1]. Especially, nitrogen-containing BPs inhibit farnesyl pyrophosphate synthases in mevalonate pathway which results in the reduction of geranylgeranyl diphosphonate (GGPP) [4,5]. GGPP is a substance essential to prenylation of guanosine triphosphate-binding proteins which are required for the survival and activation of osteoclast. Although PBs are primarily acting on OC, these drugs also promote proliferation and differentiation of OB and bone marrow stromal cells [6–8] and can inhibit bone metastases from breast cancer in addition to bone loss [9]. BPs also inhibit OB apoptosis [6,10] and stimulate OB precursors and mineralized nodule formation in murine and human bone marrow cultures [11]. ALN is a member of BPs and contains nitrogen and side chains of –OH and $-(CH_2)_3NH_2$. It also displays considerably stronger effects, more than hundreds times, on anti-resorptive activity compared to other BPs [12]. Therefore, ALN can be used for the treatment of osteoporosis [13] and positively act on bone regeneration activity [8,14].

Biomaterial surface property determines not only osteoblastic responses but also can modulate osteoclastic activities. Bone forming activity of OB in bone remodeling process is coupled with bone resorbing activity of OC. OB synthesizes extracellular matrix proteins including collagen and non-collagenous proteins and regulates matrix mineralization [15]. OC is a highly specialized multinucleate giant cell differentiated from monocyte/macrophage lineage of bone marrow hematopoietic cells and responsible for bone resorption [16,17]. Interestingly, two factors including macrophage colony-stimulating factor (M-CSF) and receptor

* Corresponding author. Tel.: +82 31 8005 3540; fax: +82 31 8005 3536.
E-mail address: leewo@dankook.ac.kr (W.-K. Lee).

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activator of NF-κB ligand (RANKL) required for OC formation, survival and function, are provided by bone marrow OBs [18]. After implantation, in general, the primary bone marrow osteoblastic cell lineage rather than mature OBs appears on implant surface in vivo mouse model and which leads to OB differentiation and subsequent bone matrix deposition in close proximity to the implant surface [18]. At that moment, these conditions can provide RANKL source for osteoclastic differentiation in vivo. Previous report showed that areas of peri-implant tissue are associated with osteolysis by excessive OC activity, suggesting that we should speculate how different chemical modification of biomaterial surface regulates OC activity and select biomaterials less likely to induce bone lysis [19,20].

In this study, we prepared hybrid CP coatings co-precipitated with ALN at different concentrations and evaluated the physical/chemical properties. And the effects of co-precipitated ALN on OB and OC responses including proliferation, differentiation and matrix mineralization were examined.

Materials and methods

Film preparation

CP was prepared using an ionic solution saturated with calcium and phosphate ions at the concentration of 299 mM of calcium and 605.6 mM of phosphate using $Ca(NO_3)_2{\cdot}HPO_4$ (C4955, Sigma) and $(NH_4)_2{\cdot}HPO_4$ (A5764, Sigma) according to our earlier report [23]. The ALN solutions were prepared at two different ALN concentrations (10^{-6} and 10^{-4} M) using alendronate sodium trihydrate (molecular formula: $C_4H_{12}NaNO_7P_2{\cdot}3H_2O$, Sigma). For the preparation of ALN-incorporated CPs (CPAs), the ionic solution mixed with ALN solutions at the ratio of 1:8 (v/v) was used. The mixed solutions was filtered using syringe filter with pore size of 0.2 μm (Minisart, Sartorius, Germany) in order to remove homogeneous precipitates and then filtrate was applied to tissue culture plate for the film formation. Culture plates were incubated at 4 °C for 60 min followed by incubating at 37 °C where incubation time was adjusted to obtain a similar film structure: 60 min for CP and 75 min for CPAL and CPAH. The treated plates were washed with the excess amount of DDW and then dried overnight in a clean bench. CPAs prepared at 10^{-6} and 10^{-4} M were designated as CPAL and CPAH, respectively. The uncoated culture plate was used as a control (CTL). All the coating procedure prior to incubation was performed at low temperature.

Physical and chemical examination

The surface morphology of the prepared films was examined using field-emission scanning electron microscopy (FE-SEM) (Hitachi S-3000, Japan). The microscopic images were obtained at a magnification of 20,000×. Acceleration voltage was set at 15 kV. X-ray photoelectron spectroscopy (XPS, AXIS-NOVA, Kratos) was used for the analysis of the chemical composition of the films. The X-ray source of XPS was a mono-chromated Al Kα (150 W). The base pressure was set at 5.0×10^{-9} Torr. The resolution (pass energy) was 20 eV and scan step was at 0.05 eV/step.

Cell culture

In this study, we used a human osteoblast-like MG63 cell-line. The cells were cultured in Dulbecco's modified eagle's medium (DMEM) supplemented with 10% fetal bovine serum (16000-044, Gibco) and 1% (v/v) antibiotic antimycotic solution (15240-062, Gibco). The cells were cultured under 5% CO_2 at 37 °C. The culture media was changed at every two days.

OB proliferation assay

For the examination of cell proliferation on various specimens, i.e. CTL, CP, CPAL, and CPAH, 1×10^5 cells were plated on each specimen and cultured consecutively in GM for 3, 5, and 7 d. The degree of cell proliferation was determined using cell counting kit-9 (CCK-9, Dogindo, CK04, Japan) according to manufacturer's instruction. Briefly, each specimen was washed with serum-free media and treated with cell counting solution for 3 h. The absorbance of each specimen was determined at a wavelength of 450 nm using microplate reader. Reference wavelength was set at 600 nm. All measurements were performed in triplicate. The result was displayed with a mean value of three measurements with standard deviation.

OB differentiation assay

ALP activity has been widely used as an index of OB differentiation [21,22]. The differentiation of MG63 cells cultured on various specimens was characterized by ALP activity measurement. Osteoblast-like MG63 cells were seeded at the cell density of 1×10^4 cells per sample. After 5, 10, and 15 d of culture in culture media supplemented with dexamethasone, vitamin C, and β-glycerophosphate, the culture medium was aspirated, and the cells were washed with 0.9% PBS solution. Then, the cells were lysed using 300 μL of lysis buffer and the insoluble cell debris was removed by centrifugation. 100 μL of the supernatant was treated with 100 mL of *p*-nitrophenyl phosphate solution for 1 h at 37 °C. ALP activity was measured in terms of optical density by using a microplate reader at a wavelength of 405 nm.

Analysis on osteoblastic gene expression pattern

MG63 cells were seeded on each specimen at a density of 1×10^5 cells/specimen and cultured in OM for 5, 10, 15, and 20 d. At the appointed culture periods, total RNA was extracted from the MG63 cells from each specimen using Tissue RNA PreMate (K-3080, Bioneer, Korea). Briefly, cultured cells were washed with 1× phosphate buffer solution and lyzed with 1 mL of lysis buffer. After adding 200 mL of chloroform, the lysate was vortexed and centrifuged. The supernatant was mixed with 600 mL of phenol-chloroform 5:1 solution and centrifuged. The resultant supernatant was removed and the remnant was mixed with 600 mL of isopropanol followed by centrifugation. After discarding the supernatant, the remaining was diluted with 80% EtOH. The supernatant after centrifugation was discarded and the remaining pellet was dried in a clean bench. The dried pellet was rehydrated with RNA hydration solution to prepare a stock solution. The obtained RNA was quantified using UV/Vis spectrophotometer (Optizen 2120UV, Mecasys, Korea) by measuring the optical density at 260 nm. The 2 μg of the extracted RNA and 20 pmol of olig dT were mixed and incubated at 70 °C for 5 min and then mixed with RT premix followed by synthesizing cDNA using thermal cycler (FTGENE2D, Techne, UK). In order to amplify the osteogenic genes such as ALP and OCN, PCR was performed using 5 μg cDNA mixed with 20 pmol of forward and reverse primers together with PCR premix supplemented with 2.5 unit of Taq DNA polymerase, 250 mM dNTP mix, 10 mM Tris–Cl (pH 9.0), 40 mM KCl, 1.5 mM $MgCl_2$, stabilizer and tracking dye. PCR product was loaded on 2% agarose gel for the examination of the expressed genes using nucleic acid staining kit. After electrophoresis for 1 h, the gel was stained using TAE buffer solution containing 2 μg/mL ethidium bromide. The band image was obtained from stained gel using UV at a wavelength of 312 nm. The base sequences of the primers used in this study were listed in Table 1 with PCR conditions.