Osteoblast-like Cell Growth and Differentiation Behaviors on the Phospholipid Vesicle-Interacted Calcium Phosphate Films

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The culture behavior of the osteoblast-like cells on the calcium phosphate (CP) films interacted with phospholipid vesicle (PV) were investigated. The different CP crystalline phases were prepared from the biological solutions such as simulated body fluid (SBF) and unique SBF, which were called as CP/PV and U-CP/PV films, respectively, and these were used after the sterilization. The protein adsorption amounts on the CP/PV films were higher than that of the PV film. The osteoblast-like cells cultured on the U-CP/PV film had the longest length along with the extension direction. The density of the osteoblast-like cells adhered on the CP/PV and U-CP/PV films were higher, suggesting the preferential adhesion on the surfaces. The differentiated osteoblasts on the films were visualized by the alkaline phosphatase and Alizarin-red-S staining methods. It was suggested that the osteoblasts cultured on the CP/PV and U-CP/PV films exhibited the possibility of the high osteogenic activities. Therefore, the PV-interacted CP films prepared from the biological solutions are useful for the osteoblast culture plates. [DOI: 10.1380/ejssnt.2018.156]

Keywords: Biological compounds; Biological aspects of nano-structures; Hydroxyapatite; Bioceramics

I. INTRODUCTION

The cell culture substrates serve as the cell adhesion and growth. There are some characteristics to be required, including the cytocompatibility and transparency. Based on the points, tissue culture polystyrene (TCPS) has mainly been used so far [1–3]. The TCPS has been produced by hydrophilic treatment of the PS surfaces, which is an important approach to improve the cell adhesion [4–8]. The treatment methods are plasma [5, 6] and corona treatments [7], and hydrophilic molecular coating [8]. The sterilization process is also necessary for the cell culture which include light-radiation, high pressure steam, and so on. However, TCPS has low heat resistance in the sterilization process, and the survival rate was low (e.g., 10% for 72 h) when the cultured cells on TCPS were transplanted into animal body [9]. Thus, the development of surface modification technology by cytocompatible materials of TCPS is important for promoting stability against sterilization treatment and providing environment like in vivo.

As the surface coating, the synthetic polymer grafting and the extracellular matrix (ECM) coating on TCPS have been investigated [11–17]. The poly-N-isopropylacrylamide was grafted with the layer thickness of 20–30 nm for providing hydrophilic surfaces, suggesting the cell adhesion at the monolayer [10]. The polyethylene oxide and polypropylene oxide block copolymers were used for the surface modifiers which can limit the bacterial attachment [11]. However, the synthetic polymers are lack of the stability against the sterilization. On the other hand, the ECM components (e.g., laminin, fibronectin, and collagen) coating was useful to promote the cell adhesion [12–14]. Thus, the ECM component has been shown to improve cell proliferation and differentiation [15–17]. However, there was a potential limitation that might lead to pathogenicity and virus infection. In addition, the thermal denaturation was a trouble spot.

Therefore, the surface modification technology utilizing the material which similar to animal body hard tissues (e.g., teeth, bone) in vivo is required for good cell affinity.

Calcium phosphate (CP) is an important inorganic component in the human body and plays an indispensable role in the hard tissue formation. The main CP mineral phase is hydroxyapatite (HAp) [18, 19] to provide the high biocompatibility [20–22] and high affinity with biomolecules [23, 24]. Amorphous calcium phosphate (ACP) is known as the HAp precursor in the bone formation [25, 26], which has good absorbability by the body. In addition, calcium pyrophosphate dehydrate (CPPD), octacalcium phosphate (OCP) and β-tricalcium phosphate coexist in the body [27]. Simulated body fluid (SBF) is the aqueous solution with an inorganic ion concentration close to human blood plasma, which has been used as the biomimetic HAp deposition and has been made to synthesize HAp on the various substrates under mild condition [28–30]. The obtained HAp exhibited the bone-like apatite with high biological affinity [31, 32]. Thus, it is suggested that the HAp precipitation process from SBF can be applied for the TCPS coating.

Phospholipids are amphiphilic molecules that are the main component of the cell membrane [33, 34]. The phospholipid molecule has two groups of hydrophobic fatty acid tails and hydrophilic phosphate head in the structure. In water, the hydrophilic parts self-assemble on the outside and the hydrophobic parts gather together on the inside to form the vesicle structures, which be called as phospholipid vesicle (PV). We have already suggested that it was useful for the effective CP formation on the PV surfaces by electrostatic interactions of calcium and phosphate ions from SBF. It has been proved that CP was successfully deposited on the PV surfaces to synthesize the unique CP and PV hybrid (CP/PV) structures [35, 36], which had transparency and good stability against sterilization treatments.

In this study, we cultured the osteoblast-like cells on the CP/PV films created on TCPS. The different CP crystal phases and protein adsorption ability on the surfaces were
evaluated after the sterilization treatment against the CP/PV films. Furthermore, the differentiated morphologies and activities of the osteoblast cells on the CP/PV films were investigated using alkaline phosphatase (ALP) and Alizarin staining methods after the culture for 14 days.

II. MATERIALS AND METHODS

A. Preparation of SBF and unique SBF

The SBF with 1.5 times ion concentrations (1.5SBF: Na+, 213 mM; K+, 7.5 mM; Mg2+, 2.5 mM; Ca2+, 3.8 mM; Cl–, 222 mM; HCO3–, 6.3 mM; HPO42–, 1.5 mM; SO42–, 0.75 mM; Tris, 75 mM) was prepared according to the previous report [37] and hydrochloric acid (HCl) was used to adjust the solution pH at 7.4. In contrast, we creatively prepared a unique SBF (USBF) for the first time by adding excessive tris(hydroxymethyl)aminomethane (Tris) to 1.5SBF [38]. Firstly, 10 mM of Tris was prepared to adjust the solution pH at 7.4 by HCl, which is called as Tris-HCl buffer. Then, the USBF was prepared by adding 50 mL of the Tris-HCl buffer into 250 mL of 1.5SBF to be the novel ion concentrations (Na+, 177.4 mM; K+, 6.3 mM; Mg2+, 1.9 mM; Ca2+, 3.1 mM; Cl–, 184.7 mM; HCO3–, 5.2 mM; HPO42–, 1.3 mM; SO42–, 0.6 mM; Tris, 64.2 mM).

B. Fabrication of films

According to previous reports of the PV preparation and CP precipitation methods [35, 36, 39], the aqueous PV dispersion was prepared at the concentration of 1.0 mg/mL. 0.3 mL of the dispersion was casted on the TCPS (BD Falcon easy-grip cell culture dish, 35 mm) at the density of 0.03 mL/cm2 and subsequently was lyophilized by a freeze-dryer (EYELA FDU-1200) for 1 day to obtain the PV film. Then, the PV film on TCPS was statically immerced into 3.0 mL of 1.5SBF for 5 days and 10 days at 37.5°C, and the films were washed by ultrapure water (pH = 7.4) and lyophilized by a freeze-dryer for 1 day to obtain the named “CP/PV-5d” and “CP/PV-10d” films, respectively. In contrast, the PV film on TCPS was statically immerced into 3.0 mL of 1.5USBF for 5 days and 10 days at 37.5°C, and the films were washed by ultrapure water (pH = 7.4) and lyophilized by the freeze-dryer for 1 day to obtain the named “U-CP/PV-5d” and “U-CP/PV-10d” films, respectively.

C. Adsorption of fetal bovine serum proteins on the CP/PV film surfaces

According to the Bradford method [40], the protein adsorption amount from fetal bovine serum (FBS) was measured by utilizing the reaction of the proteins with Coomassie Brilliant Blue G-250 [Fig. S1 (Supplementary Material)] under acidic condition. The Bradford Dye Reagent exhibit the maximum absorption wavelength at 465 nm and alter to that at 595 nm by combining with the proteins. The absorption change is attributed to the ionic bond with basic amino acid residues as well as the hydrophobic bond with aromatic amino acid residue [41]. The calibration curve was prepared using the aqueous bovine serum albumin (BSA) solution with the concentrations at 25, 125, 250, 500, 750, and 1000 μg/mL, which were measured by adding 1 mL of Bradford Dye Reagent into 20 μL of aqueous BSA solution. The result was shown in the Fig. S2 (Supplementary Material). By adding 50 mL of FBS into 500 mL of alpha minimal essential medium (α-MEM), FBS-α-MEM solution was prepared. The CP/PV-5d, CP/PV-10d, U-CP/PV-5d, U-CP/PV-10d, TCPS, and PV films were statically immerced into 3.0 mL of FBS-α-MEM for 1 h and subsequently the Bradford Dye Reagent was added into FBS-α-MEM. The protein adsorption amount was determined on the UV-Visible absorption spectrophotometer (JASCO Co., Ltd., V-750).

D. Culture of the osteoblast-like cells on the films

The osteoblast-like MC3T3-E1 cells were added into the cell culture flask containing 15 mL of FBS-α-MEM and incubated at 37°C under a humidified atmosphere of CO2 for 7 days. Then, the cultured cell layer was washed with 5 mL of phosphate buffered saline (PBS) and treated with 1 mL of 0.05 vol% Trypsin-EDTA for 10 min. The medium for the cell differentiation was prepared with 100 mL of FBS-α-MEM and 1 mL of aqueous solution containing 266 mM of disodium β-glycerophosphate tetrahydrate and 25 mM of L (+)-ascorbic acid sodium salt. The cells were dispersed into 20 mL of the differentiation medium to obtain the cell suspension. 2 mL of the cell suspension was seeded on CP/PV-5-10d, U-CP/PV-5d, U-CP/PV-10d, TCPS, and PV films at the density of 8.0 × 103 cells cm−2 and cultured for 14 days. At the initial adhesion states (culture time: 6, 24, 48, and 72 h), the cell densities and shapes were characterized by a light microscope (Olympus Co., Ltd., CKX41N). The cell density was counted in 20 different places with the area of 1 × 1 mm2 to be calculated as the number of cells per unit area. The area of the adhered cells was averaged between 50 cells.

E. Alkaline phosphatase activity staining of the osteoblasts on the films

The osteoblasts differentiated from osteoblast-like cells secrete alkaline phosphatase (ALP) enzyme. The differentiation degree was evaluated through the ALP activity staining [TRACP & ALP double-stain Kit (MK300)]. The differentiated cells on the films at the culture times of 11 days and 14 days were washed with 2 mL of PBS, 1 mL of the citrate buffer containing 45 vol% of acetone and 10 vol% of methanol (pH = 5.4) were added to fix the cells. The cells were repeatedly washed with 2 mL of sterile ultrapure water. Then, 1 mL of reactive substrate (aqueous ALP premix ground substrate solution) was added into the cell surfaces and the enzyme reaction was allowed at 37°C for 45 min. The chemical reaction mechanism in ALP staining process was demonstrated in the Fig. S3.
(Supplementary Material). In brief, 5-bromo-4-chloro-3-indolyl phosphate react with ALP to resultingly produce the blue precipitate of indigo dye and the absorbance band is at 605 nm. The ALP-stained cells were washed with 2 mL sterile water, and observed using digital camera and light microscope and evaluated by UV-Visible absorption spectrophotometer.

F. Alizarin-red-S staining of the osteoblasts on the films

The calcium phosphate forming ability of the osteoblasts was evaluated by Alizarin-red-S staining method [42]. The staining mechanism in the osteoblasts was shown in Fig. S4 (Supplementary Material). 29 mM of Alizarin-red-S solution was prepared with sterile ultrapure water. The differentiated cells on the films at the culture times of 11 and 14 days were washed with 2 mL of PBS. Then, 1 mL of 70 vol% of ethanol in water was added into the cell surfaces to fix the cells, and the surfaces were washed with 2 mL of sterile ultrapure water. 2.5 mL of Alizarin-red-S solution was added into the cells at room temperature for 30 minutes. The stained cells were washed with 2 mL sterile water, and observed using digital camera and light microscope and evaluated by UV-Visible absorption spectrophotometer.

III. RESULTS AND DISCUSSIONS

A. Adsorption behavior of serum proteins on the precipitated CP films

The XRD patterns and FE-SEM images of CP/PV-5d, CP/PV-10d have been reported [35], where the mixed phases of HAp and CPPD were confirmed, and the morphologies of crystals were petal-shaped. With the sterilization treatment, the phases were converted to the single phase of HAp, indicating that the crystal growth effectively occurs on PV surfaces. In contrast, the XRD patterns of U-CP/PV-5d and U-CP/PV-10d exhibited the mixed phases of HAp and OCP, which were converted to the phases of HAp and ACP with the sterilization treatment [38]. Moreover, the crystallite sizes for the 200 and 211 planes of HAp decreased, indicating that some crystals were dissolved at the sterilization processes.

The adsorbed amounts of the FBS proteins on TCPS, PV, CP/PV-5d, CP/PV-10d, U-CP/PV-5d, and U-CP/PV-10d, which were 8, 96, 214, 173, 156, and 116 µg/cm², respectively, as shown in Fig. 1. The proteins in the FBS mainly contain BSA and immunoglobulin [43, 44]. When the films were immersed in the cell culture medium containing FBS, a plurality of proteins competitively adsorbed on the surfaces. Immunoglobulin was hydrophobic, thus not easy to adsorb on the hydrophilic surfaces. In contrast, BSA was negatively charged in the cell culture medium containing FBS, and was adsorbed on the surfaces. Immunoglobulin was hydrophobic, thus not easy to adsorb on the hydrophilic surfaces. In contrast, BSA was negatively charged in the cell culture medium containing FBS, which were heavily adsorbed on the CP/PV and U-CP/PV film surfaces through the interaction with the calcium ions on the film surfaces. However, some crystals on the U-CP/PV films were dissolved at the sterilization processes, so that the amount of the proteins that adsorbed on U-CP/PV film surfaces were less than CP/PV films. Therefore, the amount of the proteins adsorbed on the CP/PV films were more than those of the TCPS, PV, and U-CP/PV films.

B. Culture behavior of osteoblast-like cells on the films

Optical microscope images of the osteoblast-like cells on TCPS, PV, CP/PV-5d, CP/PV-10d, U-CP/PV-5d, and
U-CP/PV-10d at the culture times of 6 h and 24 h were shown in Fig. 2, and their higher magnification images were shown in Fig. S5 (Supplementary Material). There was no obvious change in the density of the cells adhered on the films at 6 h. The area of cell increased for 24 h on all the samples. In the viewpoint of the cellular shape, the fusiform cells were observed on the CP/PV-5d, CP/PV-10d, U-CP/PV-5d, and U-CP/PV-10d films and the flat cells were observed on the TCPS and PV surfaces, indicating the preferential extension on the CP films. Generally, the osteoblasts realized and extended along with the collagen fibers and then formed bone tissue. Thus, it was thought that the fusiform cells on the CP films have the higher differentiation function. Especially, the cells cultured on U-CP/PV-10d film surface have the longest length along with the extension direction.

Optical microscope images of TCPS, PV, CP/PV-5d, CP/PV-10d, U-CP/PV-5d, and U-CP/PV-10d at the culture time of 48 h and 72 h were shown in Fig. 3, and their higher magnification images were shown in in Fig. S6 (Supplementary Material), in which the density of cells on the CP/PV-10d and U-CP/PV-10d films were higher, suggesting the preferential adhesion on the surfaces.

The (a) adhered cell density and (b) cellular area changes on TCPS, PV, CP/PV-5d, CP/PV-10d, U-CP/PV-5d, and U-CP/PV-10d with the culture time were shown in Fig. 4. It was revealed that the density of cells on the films increased with the passage of cultured time on all the samples, and that on U-CP/PV-10d film was highest. On the other hand, the cellular areas on TCPS, PV, CP/PV, and U-CP/PV film surfaces increased firstly and then decreased with the increase of culture time, suggesting that when the cells were adjacent to each other, they cannot become larger anymore and subsequently were self-aligned so that their area was minimized. According to these results, the U-CP/PV-10d film could provide the most benefit for the osteoblast-like cell growth environment.

C. Evaluation of differentiation behaviors to osteoblasts on the films

The photographs of the osteoblasts, which were differentiated from osteoblast-like cells, were shown in Scheme 1. We observed that the white products gradually generated in the cells on the PV, CP/PV-5d, CP/PV-10d, U-CP/PV-5d, and U-CP/PV-10d films as the culturing time progressed from 7 days to 14 days. It has been reported that osteoblast-like cells express an osteoblast phenotype and produce a highly mineralized extracellular matrix, when grown in medium containing ascorbic acid and β-glycerophosphate [45]. There are there stages in the osteoblast-like cell differentiation [46, 47]. Firstly, osteoblast precursors showed activity proliferation (1–9 days of culture). Secondly, the alkaline phosphatase was produced and collagenous extracellular matrix was increasingly deposited (9–16 days of culture). The final stage of osteoblast phenotypic development was the mineralization of extracellular matrix which began approximately 16 days of culture. Therefore, this result suggested that the osteoblasts were successful differentiated from osteoblast-like cells and the formation of extracellular matrix.

The optical microscope images and digital camera photographs of the ALP-stained osteoblasts on the PV, CP/PV-5d, CP/PV-10d, U-CP/PV-5d, and U-CP/PV-10d films at the culture time of 11 days and their UV-visible adsorption spectra were shown in Fig. 5. All the stained films were blue, indicating the ALP secretions by the osteoblasts on the films. Especially, the U-CP/PV-5d film indicated the highest absorbance intensity at 605 nm, indicating the densely-differentiated cells at the center position (ϕ: 12 mm). The optical microscope images of the ALP-stained osteoblasts on the PV, CP/PV-5d, CP/PV-10d, U-CP/PV-5d, and U-CP/PV-10d films at the culture time of 14 days and their UV-visible adsorption spectra were shown in Fig. 6. The bright blue values of the cells on the CP/PV and U-CP/PV films increased, whereas that on the PV film decreased. The U-CP/PV-10d film indicated the highest absorbance intensity at 605 nm, indicating the densely-differentiated cells at the center position. From the above results, ALP activity staining of the osteoblasts on the films were different, suggesting that the differentiation cycles were different among the films when osteoblasts were differentiated from osteoblast-like cells. Nonetheless, the culturing of the osteoblast-like cells on the U-CP/PV films were effectively differentiated.

![Graph](image-url)

**FIG. 4.** (a) Adhered cell density and (b) cellular area changes on TCPS, PV, CP/PV-5d, CP/PV-10d, U-CP/PV-5d, and U-CP/PV-10d with the culture time.

**SCHEME 1:** Illustration of the preparation processes of CP/PV-Aft-5d, CP/PV-Aft-10d, U-CP/PV-Aft-5d, and U-CP/PV-Aft-10d, and their osteoblast-like cell culture ability for investigating the differentiation behaviors.
FIG. 5. Optical microscope images of the ALP-stained cells on (a) PV, (b) CP/PV-5d, (c) CP/PV-10d, (d) U-CP/PV-5d, and (e) U-CP/PV-10d at the culture time of 11 days [inset: photographs of the films on TCPS (dish diameter: 40 mm)], and (f) their UV-visible adsorption spectra.

FIG. 6. Optical microscope images of the ALP-stained cells on (a) PV, (b) CP/PV-5d, (c) CP/PV-10d, (d) U-CP/PV-5d, and (e) U-CP/PV-10d at the culture time of 14 days [inset: photographs of the films on TCPS (dish diameter: 40 mm)], and (f) their UV-visible adsorption spectra.

The optical microscope images and digital camera photographs of the Alizarin-red-S-stained osteoblasts on the PV, CP/PV-5d, CP/PV-10d, U-CP/PV-5d, and U-CP/PV-10d films at the culture time of 11 days and their UV-visible adsorption spectra were shown in Fig. 7. The stained PV, CP/PV-5d, CP/PV-10d, and U-CP/PV-5d films partially turned out red, whereas the stained U-CP/PV-10d film turned out at the larger area, indicating the effective coverage of the calcification by the osteoblasts on the film. In addition, the UV-Visible adsorption spectra of the CP/PV-10d film exhibited the highest absorbance intensity at 450 nm, indicating the dense calcification of the cells at the center position. The optical microscope images and digital camera photographs of the stained osteoblasts on the PV, CP/PV-5d, CP/PV-10d, U-CP/PV-5d, and U-CP/PV-10d films at the culture time of 14 days and their UV-visible adsorption spectra were shown in Fig. 8. The stained cells on the CP/PV-10d film not only completely turned out red but also exhibited the highest absorbance intensity at 450 nm. Above all, the results of Alizarin-red-staining of the osteoblasts on the films were different between the culture time of 11 days and 14 days, suggesting that the timing of bone tissue formation among the films were different, but the CP/PV-10d film showed the effectively bone tissue formation. Thus, the culturing of the osteoblasts on the CP/PV-10d film was helpful for the calcification based on the bone tissue formation, which would be attributed to the crystallinity and morphology of the CP/PV-10d film.

Figure 9 showed the absorbance changes at 605 and 450 nm of the osteoblasts on the PV, CP/PV-5d, CP/PV-10d, U-CP/PV-5d, and U-CP/PV-10d films with the culture time, which were stained by ALP and Alizarin-red-S, respectively. Phospholipid as the main component of cell membranes is convenient for adsorption and growth of cells. On the other hand, ALP activity component and

FIG. 7. Optical microscope images of the Alizarin-red-S-stained cells on (a) PV, (b) CP/PV-5d, (c) CP/PV-10d, (d) U-CP/PV-5d, and (e) U-CP/PV-10d at the culture time of 11 days [inset: photographs of the films on TCPS (dish diameter: 40 mm)], and (f) their UV-visible adsorption spectra.

FIG. 8. Optical microscope images of the Alizarin-red-S-stained cells on (a) PV, (b) CP/PV-5d, (c) CP/PV-10d, (d) U-CP/PV-5d, and (e) U-CP/PV-10d at the culture time of 14 days [inset: photographs of the films on TCPS (dish diameter: 40 mm)], and (f) their UV-visible adsorption spectra.

FIG. 9. Absorbance changes at (a) 605 nm and (b) 450 nm of the cells on PV, CP/PV-5d, CP/PV-10d, U-CP/PV-5d, and U-CP/PV-10d films with the culture time, which were stained by ALP and Alizarin-red-S, respectively.

J-Stage: https://www.jstage.jst.go.jp/browse/ejssnt/
Alizarin-red-staining of the osteoblasts on the CP/PV, U-CP/PV films and PV films were similar. Especially, the ALP-stained osteoblasts on the U-CP/PV-10d film and the Alizarin-red-S-stained osteoblasts on CP/PV-10d film at the culture time of 14 days indicated the highest absorbance intensities, where the blue precipitate of indigo dye and red chelate complex were generated [48, 49]. Therefore, the cells cultured on the CP precipitated derived from the biological solutions of this study exhibited the high osteogenic activity.

In this study, we cultured the osteoblast-like cells on the TCPS, PV, CP/PV-5d, CP/PV-10d, U-CP/PV-5d, and U-CP/PV-10d films. After the sterilization, the protein adsorption amounts on the CP/PV films were higher than those on the TCPS and PV films. The osteoblast-like cells cultured on U-CP/PV-10d film surface have the longest length along with the extension direction. The density of the osteoblast-like cells adhered on the CP/PV-10d and U-CP/PV-10d films were higher, suggesting the preferential adhesion on the surfaces. The differentiated osteoblasts on the CP/PV-10d and U-CP/PV-10d films were stained by the ALP and Alizarin-red-S methods. It was suggested that the osteoblasts cultured on the CP/PV-10d and U-CP/PV-10d films exhibited the possibility of the high osteogenic activities. Therefore, the PV-interacted CP films prepared from the biological solutions are useful for the osteoblast culture plates.

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APPENDIX

Molecular structure of Coomassie Brilliant Blue G-250, calibration curve between the absorbance at 595 nm and BSA concentration, chemical reaction mechanism in the ALP staining process, staining mechanism of the Alizarin-red-S with the calcium ions in the osteoblasts, and optical microscope images of the cells adhered on TCPS, PV, CP/PV-5d, CP/PV-10d, U-CP/PV-5d, U-CP/PV-10d in the higher magnification are available in Supplementary Material at https://doi.org/10.1380/ejssnt.2018.156.


