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Bone Morphogenic Protein-2 (BMP-2) Immobilized Biodegradable Scaffolds for Bone Tissue Engineering

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Abstract: Recombinant human bone morphogenic protein-2 (rhBMP-2), which is known as one of the major local stimuli for osteogenic differentiation, was immobilized on the surface of hyaluronic acid (HA)-modified poly(ε-caprolactone) (PCL) (HA-PCL) scaffolds to improve the attachment, proliferation, and differentiation of human bone marrow stem cells (hBMSCs) for bone tissue engineering. The rhBMP-2 proteins were directly immobilized onto the HA-modified PCL scaffolds by the chemical grafting the amine groups of proteins to carboxylic acid groups of HA. The amount of covalently bounded rhBMP-2 was measured to 1.6 pg/mg (rhBMP/HA-PCL scaffold) by using a sandwich enzyme-linked immunosorbant assay. The rhBMP-2 immobilized HA-modified-PCL scaffold exhibited the good colonization, by the newly differentiated osteoblasts, with a statistically significant increase of the rhBMP-2 release and alkaline phosphatase activity as compared with the control groups both PCL and HA-PCL scaffolds. We also found enhanced mineralization and elevated osteocalcin detection for the rhBMP-2 immobilized HA-PCL scaffolds, in vitro.

Keywords: recombinant human bone morphogenetic protein-2, bone tissue engineering, scaffolds, surface modification, human mesenchymal stem cells.

Introduction

Traditionally, autologous bone grafts have been used for various orthopedic, plastic, and dental procedures that required reconstruction of lost bone and healing of ununited fractures. However, these procedures have major limitations including: limited supply, harvest site morbidity, additional blood loss, and prolongation of the operative time. Allogenic bone grafts carry the potential risk of disease transmission and inflammatory response. With the advancements in the field of tissue engineering, it has now become possible to differentiate human bone marrow derived stem cells to multiple mesenchymal tissue lineages such as: osteogenic, chondrogenic and adipogenic tissues with the proper stimulation.[1]

Recent new approaches of bone tissue engineering are based on the use of biodegradable scaffolds in combination with bone marrow-derived mesenchymal stem cells. Biodegradable synthetic polymers, such as poly(lactic acid) (PLA), poly(glycolic acid) (PGA), poly(ε-caprolactone) (PCL), and

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polyimides, have been used as three dimensional scaffolds and they served as a template for tissue regeneration.[2-7] A major step towards biodegradable scaffolds have been the development of biomimetic scaffolds by incorporating or immobilizing biological active agents, such as, hyaluronic acid (HA), transforming growth factor-β (TGF-β), or fibroblast growth factor (FGF).[8,9] Biomimetic scaffolds containing various biological agents have provided the microenvironment for cell-matrix interactions that resulted in rapid and stable cell colonization.

In particular, biomimetic scaffolds containing bone morphogenic proteins (BMPs), which are known as one of the major local stimuli for osteogenic differentiation, have provided a bony microenviroment that mimics the structure and biological function of the extracellular matrix (ECM).[10-12] To mimic the microenvironment of bone ECM, the BMPs have been delivered with the aliphatic polyester (PLA, PLGA) scaffolds and they have elicited the healing of bone defects in a variety of animal model. However, BMPs-loaded scaffolds lead to unwanted release profiles that resulted in the systemic side effects.[13,14] Also, the aliphatic polyesters of PLA and PLGA tend to under go rapid degradation of the biodegradable polymers, which consequently produce the acid monomers that is known to be deleterious to proteins and cells.[15,16]

In this study, we have developed a rhBMP-2 immobilized scaffold which was prepared by grafting biological active agent, rhBMP-2, to the HA- PCL scaffold. The covalently bound rhBMP-2 proteins on the scaffold surface can be stable and it will minimize an unwanted protein release profiles. We also used the PCL scaffold because PCL, which is highly hydrophobic and crystalline polymer, can prevent rapid degradation and deleterious acid monomer production. Finally, the in vitro approaches to the attachment, proliferation, and differentiation of human bone marrow stem cells (hBMSCs) using rhBMP-2 immobilized HA-PCL scaffolds are described.

Experimental

The research protocol was reviewed and approved by the Ethical Care Committee (ECC) at our hospital. All patients provided informed written consent.

Materials. Poly(ε-caprolactone) (PCL) was purchased from Sigma Chemical (St. Louis, MO). Dexamethasone, ascorbic acid, *N,N*-(3-dimethylaminopropyl)-*N'*-ethyl carbodiimide (EDC), 1-hydroxybenzotriazole (HOBt), ethylenediamine (EDA), and glycerol-2-phosphate were purchased from Sigma Chemical (St. Louis, MO). Dulbecco's modified Eagle's medium (DMEM) and Fetal bovine serum (FBS) were purchased from Invitrogen (Grand Island, NY). Ammonium bicarbonate and acetic acid were purchased from Sigma Chemical (St. Louis, MO). Hyaluronic acid was obtained from Huons Co (Seoul, Korea). rhBMP-2 was purchased from R&D systems Inc. (Minneapolis, MN). All other chemicals were of the purest analytical grade.

PCL Scaffold Fabrication. Scaffolds were fabricated as described by Lin *et al.*.[17] Briefly, 10% (w/v) PCL was dissolved in 50 mL of methylene chloride and then by precipitating it into an excess volume of cold ethanol solution with gentle mixing. Sieved ammonium bicarbonate particulates (size was 150-250 μm) were added to the PCL gel paste and mixed homogeneously by spatula. The weight ratios of ammonium bicarbonate to PCL were 10:1. The homogeneous gel paste was then cast to 10 mm in diameter with 5 mm in thickness Teflon mold. Semi-solid scaffolds were obtained after partial solvent evaporation and immersed into an aqueous 20% (v/v) acetic acid solution. And then the samples were kept at room temperature to induce gas foaming as well as salt leaching within the polymer/salt matrices. Finally, the porous scaffolds were taken out of Teflon molds after the completion of the effervescence and washed with distilled water several times, and then freeze-dried for several days.

Preparation of rhBMP-2 Immobilized HA-PCL Scaffolds. The rhBMP-2 immobilized HA-PCL scaffold was prepared by three processes, as shown in Figure 1. First, amino groups were immobilized onto the PCL scaffolds. The PCL scaffolds were immerged into 1 N NaOH solution at 50 °C for 48 h, followed by dipping them into 30 mM EDC solutions at room temperature for overnight. After the reaction, 10% ethylenediamine was added in the solution and it was reacted for 4 h. The amine-terminated PCL scaffolds were thoroughly washed in distilled water and dried at room temperature. Second, the freshly prepared amine-terminated PCL scaffolds were immersed in 30 mM of EDC and HOBt solution overnight followed by addition of 1% HA (Huons Co., Seoul, Korea) for 4 h at room temperature. The produced HA-PCL scaffolds were completely washed with running distilled water for 10 times and lyophilized. Finally, the bioactive protein, rhBMP-2, was immobilized into the HA-PCL scaffolds by using EDC and HOBt coupling agent in the PBS condition. The HA-PCL scaffold was dipped into 30 mM EDC solution with gentle agitation at room temperature for 24 h, followed by adding 1 μg of rhBMP-2 in PBS (pH 7.4), and the reaction was allowed to continue for 24 h at 4 °C. The produced rhBMP-immobilized HA-PCL scaffold was washed with PBS (pH 7.4) for 10 times to remove completely non-specifically adsorbed rhBMP-2 and then it was immediately used for further experiments without any exposure to air environment.

Cell Isolation and Cultivation. Bone marrow samples were obtained from hematologically normal patients undergoing lumbar discectomy. Primary cultures of bone marrow cells were harvested as described by Kim *et al.*.[24] Briefly, bone marrow samples (10 mL) were mixed with 0.3 mL of heparin to prevent coagulation, and then was diluted with 20 mL of phosphate buffered saline (PBS). The cells were

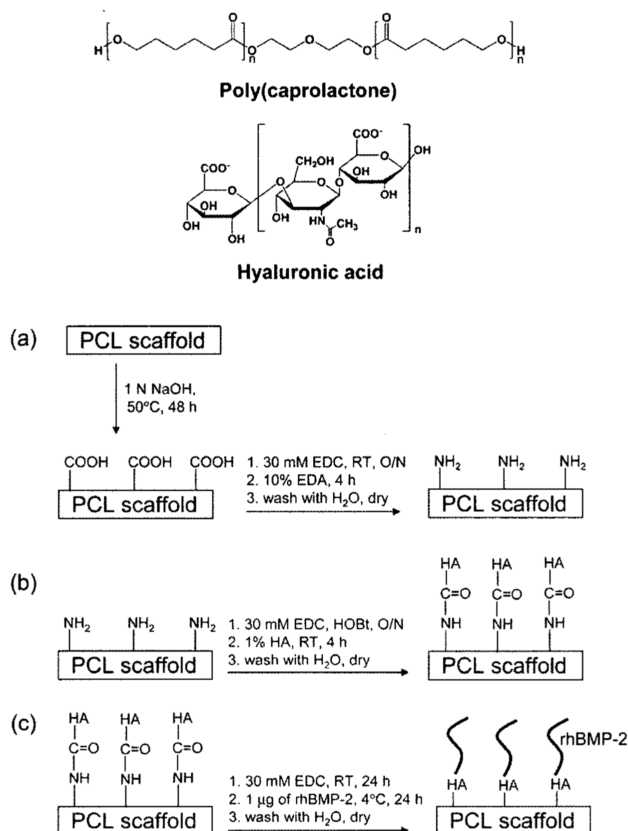

Figure 1. Scheme of surface modification of PCL scaffold.

harvested by centrifuging at 600 × g for 10 min. The interface mononuclear cells were isolated, washed with PBS, and then erythrocyte (RBC) lysis buffer [0.154 M NH_4Cl, 10 mM $KHCO_3$, 0.1 mM ethylenediamine tetra-acetic acid (EDTA)] was added to destroy the RBC contaminants. The cells were washed twice by centrifugation (600 × g) in PBS. One million cells were seeded in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum (FBS) and 1% antibiotics (penicillin 100 U/mL, streptomycin 0.1 mg/mL) at 37 °C in a humidified atmosphere with 5% CO_2.

Cell Culture. After three passages, cultured hBMSCs were detached using trypsin/EDTA (etylenediaminetetraacetic acid) (0.05% (w/v) trypsin, 0.02% (w/v) EDTA) and seeded onto pre-hydrated PCL scaffolds (DMEM, overnight) at a concentration of 5×10^5 cells/scaffold. After 24 h, the media was removed and scaffolds were transferred into a 24-well plate where they were maintained in DMEM supplemented with 10% FBS, 50 μg/mL ascorbic acid, 10 nM dexamethasone, and 10 mM β-glycerolphosphate in the presence of 100 U/mL penicillin, 100 μg/mL streptomycin, and 0.25 μg/mL fungizone. Culture was maintained at 37 °C in a humidified incubator supplemented with 5% CO_2. Half of the media was changed every 3 days. The scaffolds were cultured for 28 days and examined for cell morphology and differentiation. The amount of covalently bounded rhBMP-2 on the HA-PCL scaffold was measured using a sandwich enzyme-linked immunosorbant assay (R&D System, Minneapolis, MN) as described by Kim *et al.*.[18]

Scanning Electron Microscopy. After 21 days of culture, hBMSCs grown on the scaffolds were gently rinsed with PBS buffer (pH 7.4). They were then fixed with 2.5% glutaraldehyde solution in distilled water overnight at 4, dehydrated in a gradual ethanol/distilled water mixture from 50 to 100% in steps of 10% for 10 min each, and lyophilized for 3 days. The resulting specimens were observed using the scanning electron microscopy (SEM) (S-2460N, Hitachi, Tokyo, Japan).

Alkaline Phosphatase Assay. The level of alka line phosphatase (ALPase), a marker of osteoblast activity, was measured spectroscopically as described by Yang *et al.*.[19] The differentiated osteoblasts on the different scaffolds were washed with PBS, homogenized with 1 mL Tris-HCl buffer (1 M, pH 9.0, Sigma Chemical, St. Louis, MO) and then sonicated using the Sonic Dismembrator 550 (Fisher, Pittsburgh, PA) for 1 min at 110 watts (50/60) in ice. Aliquots of 50 μL were incubated with 0.5 mL of 0.1 M glycin-NaOH buffer, 0.5 mL of 15 mM *p*-nitrophenyl phosphate solution (16 mM, Sigma Chemical, St. Louis, MO), 0.1% Triton X-100/saline (Sigma Chemical, St. Louis, MO), and 0.5 mL of distilled water for up to 30 min at 37 °C. The reaction was quenched by adding 1.25 mL of 1 N NaOH and placing the mixture in ice. The level of *p*-nitrophenol production in the presence of ALPase was measured by monitoring the light absorbance of the solution at 405 nm using a microplate reader (Spectra Max 250, Molecular Devices, Sunnyvale, CA).

Calcium Assay. The amount of calcium deposited by the cultured cells on the different scaffolds was measured as previously described by Jaiswal *et al.*.[20] The cell-scaffolds were rinsed twice with PBS and homogenized with 0.6 N HCl in ice. Calcium was extracted by shaking for 4 h at 4 °C and then centrifuged at 1000 × g for 5 min, and the supernatant was used for determination of calcium content. The calcium concentration of cell lysates was quantified spectrophotometrically with cresolphthalcin complexone (Sigma Chemical, St. Louis, MO). Five minutes after the addition of the reagents, the absorbance of samples was read at 570 nm using a microplate reader (Spectra Max 250, Molecular Devices, Sunnyvale, CA).

Calcium Staining. Alizarin Red S staining was performed after three weeks of incubation to detect calcium deposition. The cultures were fixed with 70% ethanol for 1 h at -20 °C, covered with 2% Alizarin Red S solution (adjusted to pH 4.2 with 10% ammonium hydroxide) for 10 min and then rinsed with water and PBS. Calcium deposits were stained red.

Phosphate Staining. Calcium phosphate deposits were detected by the von Kossa's silver nitrate-staining method after three weeks of incubation. Phosphate deposits were

stained black. The sections were deparaffininzed and hydrated with distilled water. Freshly prepared 2% silver nitrate was added to sections which were incubated in the dark for 10 min. The sections were rinsed with distilled water and exposed to bright light for 15 min. The reaction was terminated by rinsing thoroughly with distilled water.

Immunohistochemistry. The cell/scaffolds were rinsed with PBS and fixed in a 4% paraformaldehyde solution. The constructs were then rinsed with PBS, cut in half, embedded in an optimal freezing temperature compound (Tissue-Tek, Torrance, CA) and frozen at -70 °C. Serial sections (5 μm in thickness) were prepared and stored under -70 °C until staining. The sections were obtained from all scaffolds for immunohistochemical analyses. For this study, goat polyclonal antibodies (SC-18319, Santa Cruz Inc, Delaware, CA) were selected against osteocalcin. The goat polyclonal antibodies were used in conjunction with an anti-goat ABC kit (both Vectastain Elite ABC kit, Vector Laboratories, Burlingame, CA). The experimental staining procedure used was as described previously by Fisher *et al.*.[21] The sections were first incubated in a hydrogen peroxide solution to block against endogenous peroxidase activity and then incubated with normal serum (provided in ABC kit) to block against random secondary antibody binding. The sections were then incubated with primary antibody for the antigen of interest, followed by an avidin-biotin secondary antibody system; they were then examined with an inverted microscope (CK40-F200, Olympus Optical Co., Tokyo, Japan) after three weeks of incubation.

Statistical Analysis. All the measured values were expressed as a mean±SD. One-way analysis of variance (ANOVA) was employed to assess the statistical significance of the results. A p-value of less than 0.05 was accepted as statistically significant. Experiments were performed at least three times, and results of representative experiments are presented except where otherwise indicated.

Results and Discussion

Surface Characterization of rhBMP-2 Immobilized HA-PCL Scaffolds. The covalent immobilizing rhBMP-2 onto HA-PCL scaffolds was processes by three processed as shown in Figure 1; (a) preparation of amine-terminated PCL scaffolds, (b) preparation of HA-immobilized PCL scaffolds (HA-PCL scaffolds), and (c) direct grafting of rhBMP-2 onto the HA-PCL scaffolds.

First, ethylenediamine was used to functionalize carboxylated PCL scaffolds and it gave rise to uniform amine-terminated functional groups on the scaffolds.[10] The reaction condition was optimized by measuring the hydrophilicity of amine-terminated surface as compared with the hydrophobic PCL scaffolds. When the reaction time increased to 4 h, the water contact angle of amine-terminated PCL scaffolds were minimized to 70 ± 5.4°, while the contact angle of the PCL scaffolds were 120 ± 3.4°. Second, HA was covalently immobilized onto the amine-terminated PCL scaffolds in the presence of EDC and HOBt for 4 h. After the reaction, the water contact angle of HA-PCL scaffolds further decreased to 30 ± 3.4°, due to the hydrophilic and covalently bound HA on the surface of PCL scaffolds.[22] Finally, rhBMP-2 was directly immobilized onto the carboxylic acid groups of HA in the PBS (pH 7.4) condition at 4 °C for 1day, wherein the bioactivity of rhBMP-2 was maintained.[12] The amount of covalently bound rhBMP-2 on the HA-PCL scaffolds was measured to 1.6 pg/mg (rhBMP/HA-PCL scaffold) by using a sandwich enzyme-linked immunosorbant assay. It means that 40% of rhBMP-2 in the reaction solution was covalently bound on the HA-PCL scaffolds. Moreover, covalently bound rhBMP-2 maintained its bioactivity about 90% for 2 weeks in the cell culture condition (PBS, pH 7.4, 37 °C). By using our reaction processes, biological active agent, rhBMP-2 could be covalently bound to HA-PCL scaffolds and it still maintained its bioactivity for 4 weeks.

Morphology of the Cultured Human Bone Marrow Stem Cells (hBMSCs) on the Scaffolds. To evaluate the effect of covalently bound BMP-2 on the cultured hBMSCs on the scaffolds, we examined the morphology and density of hBMSCs on the scaffolds using the scanning electron microscopy (Figure 2). After the cell culture time of 21 days, the cells on the PCL scaffolds presented irregular and discontinuous morphologies. And the cells were aggregated to make a large cell mass at the local site (presented by arrow in Figure 1(a)). This deduced that the highly hydrophobic surface of PCL scaffold prevents the colonization, proliferation, and differentiation of hBMSCs.[23] HA-PCL and BMP-2 immobilized HA-PCL scaffolds showed the good colonization with the highest number of proliferated cells on the surface of each scaffold, because both HA and BMP-2 play an important role in cell hydration, cell recognition, cell differentiation, proteoglycan organization and wound healing.[22] All the cultured cells were flattened and spread. This can be attributed to the effect of surface immobilized HA and BMP-2 on the proliferation of hBMSCs, compared to PLC scaffolds.

Alkaline Phosphatase Activity and Mineralization of BMp-2 Immobilized HA-PCL Scaffolds. To evaluate the osteoblastic differentiation of the mesenchymal stem cells, we measured the alkaline phosphatase (ALPase) activity using spectrophotometry (Figure 3). After the culture time of 1 week and 2 weeks, the ALPase activity of HA-PCL and rhBMP-2 immobilized HA-PCL scaffolds was found to be higher ($^*p<0.05$) than that of the PCL scaffolds. But, after 4 weeks, the ALPase activity of rhBMP-2 immobilized HA-PCL scaffolds was found to be significantly higher ($^*p<0.05$) than the HA-PCL and PCL scaffolds only scaffolds. It means that the covalently bounded rhBMP-2 on the scaffolds are stable for prolonged culture system for 4 weeks and the stabilized rhBMP-2 greatly enhance the ALPase activity.

To detect the occurrence of mineralization in the differen-

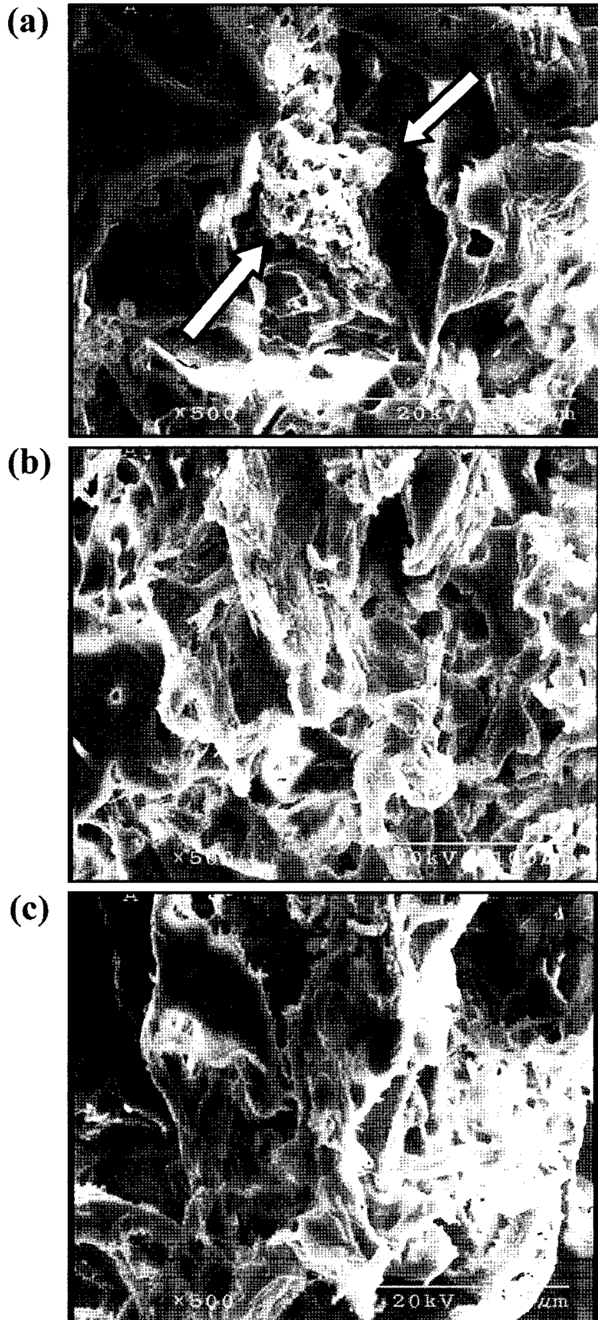

Figure 2. SEM photographs of the cultured cells on the PCL-only scaffold (a), HA immobilized PCL scaffold (HA-PCL) (b), and rhBMP-2 immobilized HA-PCL scaffold (c) at 3 weeks of incubation. The arrows indicate the attached cells.

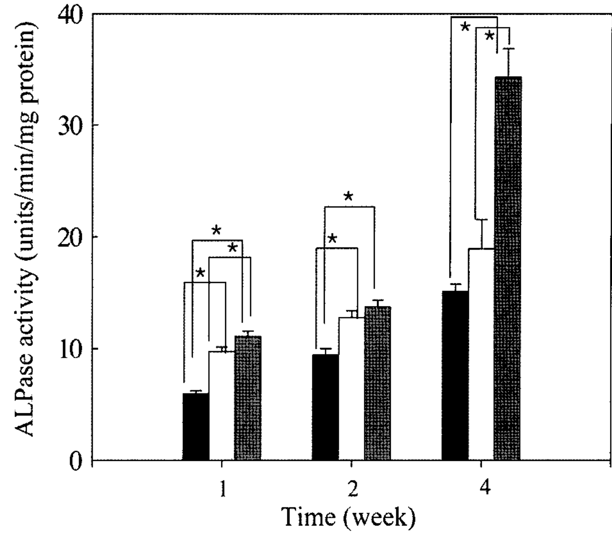

Figure 3. Alkaline phosphatase (ALPase) activity for (■) PCL scaffold, ( ) HA-PCL scaffold, and (▩) rhBMP-2 immobilized on HA-PCL scaffold. Initial seeding was with 5×10^5 cells/scaffold and measured at 7, 14, and 28 days. The results are presented as a mean±SD (n=3) (*p<0.05).

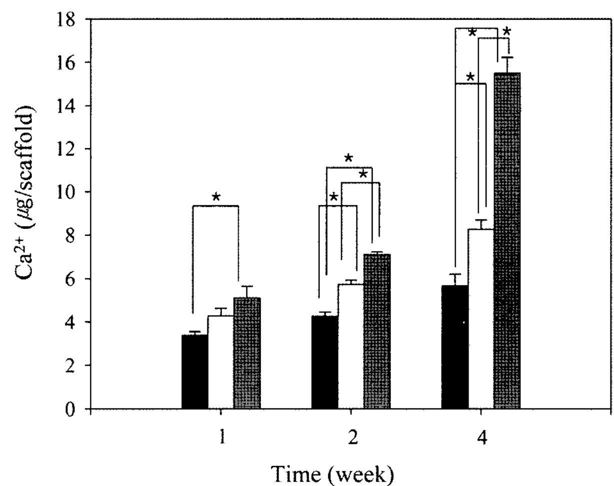

Figure 4. Calcium deposition for (■) PCL scaffold, ( ) HA-PCL scaffold, and (▩) rhBMP-2 immobilized HA-PCL scaffold at 7, 14, and 28 days. The results are presented as a mean±SD (n=3) (*p<0.05).

tiated tissues, we performed Alizarin Red S and von Kossa's staining to identify calcium and phosphorus deposition respectively. Calcium deposits were stained as dense orange spots by Alizarin Red S whereas phosphorus deposits were stained as black spots. We observed a larger area of densely packed orange and black spots in the tissues differentiated on rhBMP-2 immobilized HA-PCL scaffolds compared to the control groups (Figures 5, 6). Furthermore, we quantified the amount of calcium deposited on the scaffolds spectrophotometrically. The calcium deposition of rhBMP-2 immobilized HA-PCL scaffolds was found to be significantly higher (*p<0.05) than that of PCL scaffolds during the first, second and fourth weeks of culture, and that of the HA-PCL scaffold during the second and fourth weeks of culture (Figure 4). All of these findings suggest a greater degree of mineralization

in the tissues differentiated on the rhBMP-2 immobilized HA-PCL scaffolds compared to the PCL and HA-PCL scaffolds.

Assessment of Osteocalcin. To verify the presence of osteoblastic differentiation, we performed immunohistochemical staining for osteocalcin, that is a specific marker for osteoblasts. A dark brown color represented immunostaining for osteocalcin in all three groups (Figure 7). The rhBMP-2 immobilized HA-PCL scaffolds demonstrated more abundant staining for osteocalcin than that of PCL and HA-

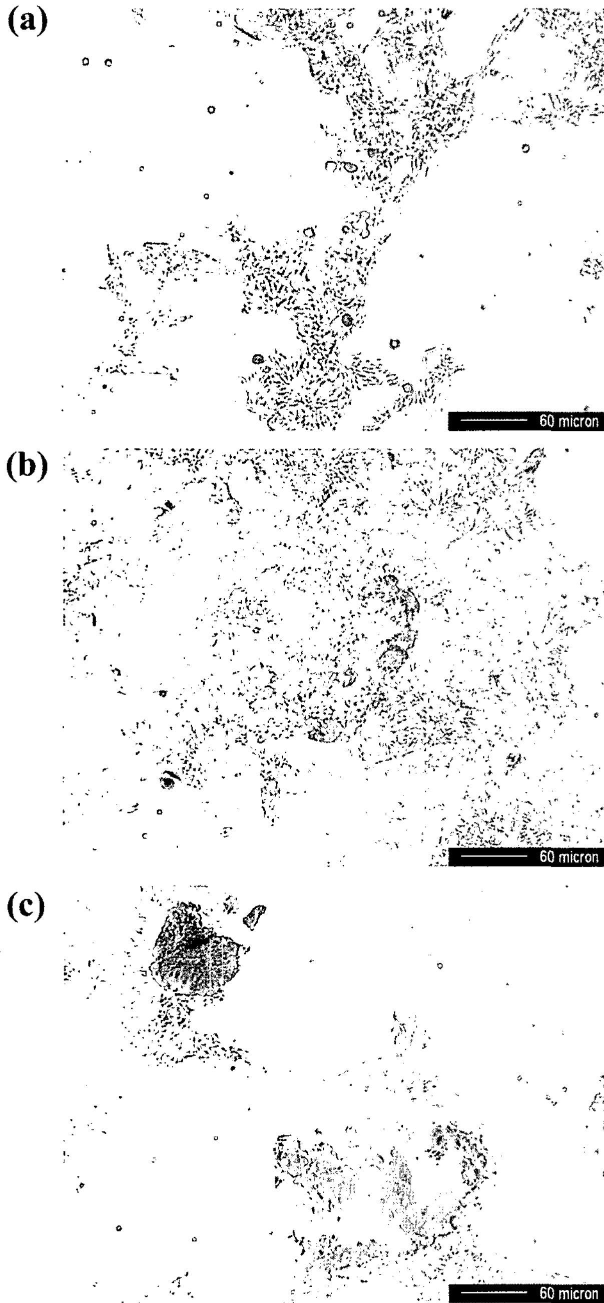

Figure 5. The Alizarin Red S stains of the PCL scaffold (a), HA-PCL scaffold (b), and rhBMP-2 immobilized on HA-PCL scaffold (c) at 3 weeks of incubation. Calcium deposits were observed throughout PCL scaffold, HA-PCL scaffold, and rhBMP-2 immobilized HA-PCL scaffold, but the amount of calcium deposits from the rhBMP-2 immobilized on HA-PCL scaffold was the greatest.

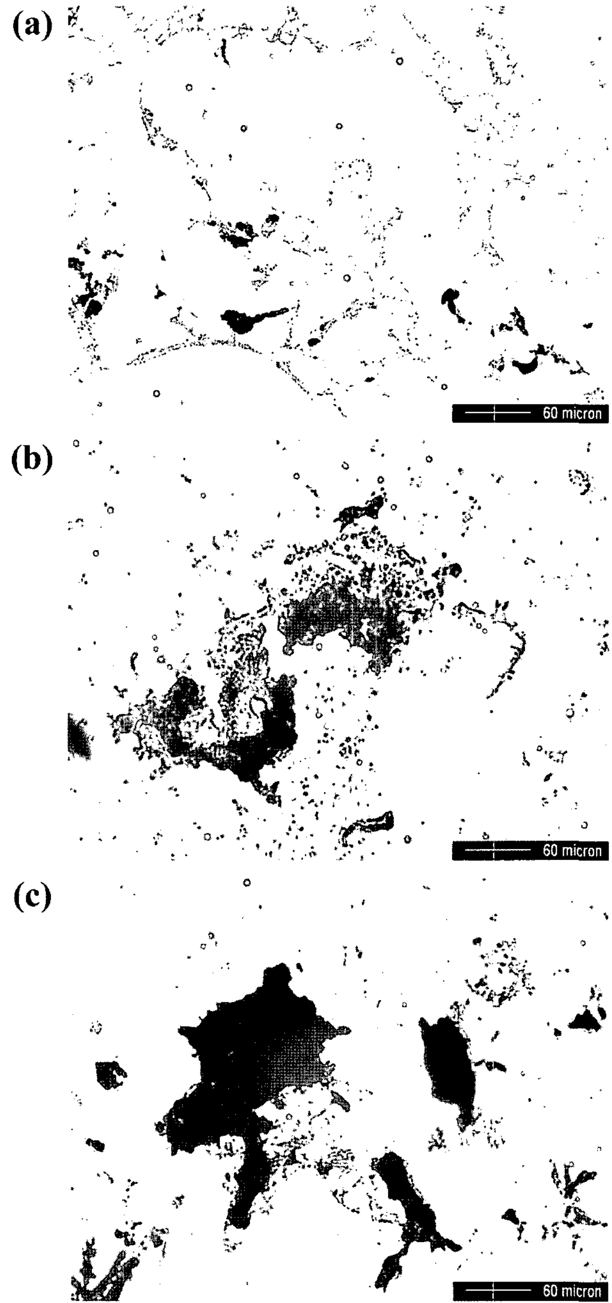

Figure 6. The von Kossa's staining of PCL scaffold (a), HA-PCL scaffold (b), and rhBMP-2 immobilized on HA-PCL scaffold (c) at 3 weeks of incubation. Mineralization nodules were observed throughout PCL scaffold, HA-PCL scaffold, and rhBMP-2 immobilized on HA-PCL scaffold, but the size of calcium deposits for the rhBMP-2 immobilized on HA-PCL scaffold was the greatest.

PCL scaffolds. We also found that the staining of osteocalcin in the rhBMP-2 immobilized HA-PCL scaffolds was more prominent towards the external parts of the scaffold, reflecting a denser cellular localization and extracellular matrix deposition at these areas.

In summary, we observed an enhanced differentiation for osteogenic tissues on the rhBMP-2 immobilized HA-PCL

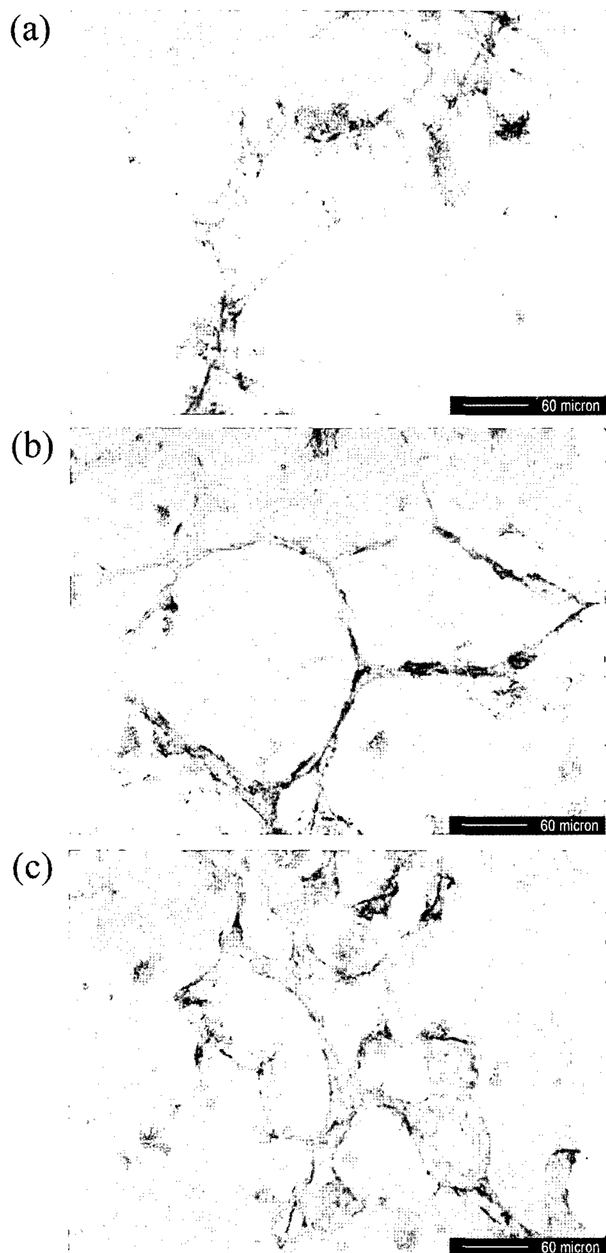

Figure 7. The immunohistochemistry (osteocalcin) stains of PCL scaffold (a), HA-PCL scaffold (b), and rhBMP-2 immobilized on HA-PCL scaffold (c) at 3 weeks of incubation. Positive immunostaining was observed among PCL scaffold, HA-PCL scaffold, and rhBMP-2 immobilized on HA-PCL scaffold, but osteocalcin immunostaining for the rhBMP-2 immobilized on HA-PCL scaffold was the greatest.

scaffolds compared to control scaffolds. The covalently bounded rhBMP-2 on the scaffolds was stable for 4 weeks in the culture condition. The effect of covalently bounded rhBMP-2 on the scaffolds on the osteogenic differentiation of the hBMSCs in vitro was confirmed by presenting greatly enhanced ALPase activity, mineralization and osteocalcin production as compared with bare PCL and HA-PCL scaffolds. Our findings demonstrated that the rhBMP-2 immobilized HA-PCL scaffolds are capable of providing enhanced osteoblastic differentiation from the hBMSCs, in vitro. Further in vivo studies are needed to better understand the biomechanical properties and potential uses of the rhBMP-2 immobilized scaffolds.

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