Nutritional Sciences 9(4), November, 2006, pp.233-239

Bioactive Polyglycolic Acid (PGA) or Polylactic Acid (PLA) Polymers on Extracellular Matrix Mineralization in Osteoblast-like Mc3T3-E1 Cells*

Young-Eun Cho, Hye-Jin Kim, Yong-Ha Kim[1], Jae-Won Choi[1], Youn-Jung Kim[2], Gab-Joong Kim[2], Jin-Su Kim[2], Sik-Young Choi[2] and In-Sook Kwun§

Dept. of Food Science and Nutrition, Andong National University, Kyungpook 760-749, Korea
[1]Dept. of Plastic & Reconstructive Surgery, College of Medicine, Yeungnam University, Kyungpook 712-749, Korea
[2]Dept. of Chemistry, Andong National University, Kyungpook 760-749, Korea

Porous matrices of bioactive polymers such as polyglycolic acid (PGA) or polylactic acid (PLA) can be used as scaffolds in bone tissue growth during bone repair process. These polymers are highly porous and serve as a template for the growth and organization of new bone tissues. We evaluated the effect of PGA and PLA polymers on osteoblastic MC3T3-E1 cell extracellular mineralization. MC3T3-E1 cells were cultured in a time-dependent manner -1, 15, 25d as appropriate - for the period of bone formation stages in one of the five culture circumstances, such as normal osteogenic differentiation medium, PGA-plated, fetal bovine serum (FBS)-plated, PGA/FBS-coplated, and PLA-plated. For the evaluation of bone formation, minerals (Ca, Mg, Mn) and alkaline phosphatase activity, a marker for osteoblast differentiation, were measured. Alizarin Red staining was used for the measurement of extracellular matrix Ca deposit. During the culture period, PGA-plated one was reabsorbed into the medium more easily and faster than the PLA-plated one. At day 15, at the middle stage of bone formation, cellular Ca and Mg levels showed higher tendency in PGA- or PLA-plated treatments compared to non-plated control and at day 25, at the early late stage of bone formation, all three cellular Ca, Mg or Mn levels showed higher tendency as in order of PGA-related treatments and PLA-plated treatments, compared to control even without significance. Medium Ca, Mg or Mn levels didn't show any consistent tendency. Cellular ALP activity was higher in the PGA- or PLA-plated treatments compare to normal osteogenic medium treatment. PGA-plated and PGA/FBS-plated treatments showed better Ca deposits than other treatments by measurement of Alizarin Red staining, although PLA-plated treatment also showed reasonable Ca deposit.
The results of the present study suggest that biodegradable material, PGA and also with less extent for PLA, can be used as a biomaterial for better extracellular matrix mineralization in osteoblastic MC3T3-E1 cells.

Key words: Polyglycolic acid (PGA), Polylactic acid (PLA), MC3T3-E1 cells, Osteoblast, Extracellular mineralization

Received August 30, 2006; Revised October 30, 2006; Accepted November 8, 2006

INTRODUCTION

Recently, polymeric materials which include biodegradable compounds such as polyglycolic acid or polylactic acid, have received much attention from bone researchers because of their biocompatibility, good mechanical properties, and easy handling for bone health.[1,2] Current trends in bone health suggest that polyglycolic acid (PGA, polyglicolide), polylactic acid (PLA, polylactide) or their copolymers with other substance such as fetal bovine serum (FBS) would be beneficial bioactive compounds for bone formation.[3] The most common degradable polymers used for these applications are polyesters derived from lactic acid, glycolic acid, and carprolactone monomers.[4] Porous matrices of biodegradable polymers, such as PGA or PLA, can be used as scaffolds in bone tissue repair process. Absorbable fixation devices of PGA have been proved to be good in low-stress-bearing cancellous bone fixation.[5-8] The other polymers, PLA has also known to be

* This work was suported in part by a grant from the 2004 Special Research Grant of Andong National university for KIS, and for CSY (grant No. 2004-0185).
§ To whom correspondence should be addressed.
(E-mail : iskwun@andong.ac.kr)

absorbable, too. Potential applications for these biocompounds include bone forming fixation devices as biodegradable materials, such as plates, for bone tissue health maintenance and formation.[9]

The biodegradable polymers, such as PGA or PLA can be used as degradable biocompounds for bone tissue growth during bone repair process. These polymers are highly porous and serve as a template for the growth and organization for new bone tissues. We evaluated whether PGA or PLA biomaterials would beneficially affect osteoblastic bone formation by measuring bone-related mineral contents for extracellular mineral deposits, alkaline phosphatase activity as an osteoblast differentiation marker and measured extracellular matrix bone nodule formation using Alizarin Red stain. Thus, we examined whether PGA or PLA or their copolymers could increase the extracellular mineralization in osteoblastic MC3T3-E1 cells.

MATERIALS AND METHODS

1. Materials (fabrication of the PGA/FBS films and PLA plate)

Polyglycolic acid (PGA, Aldrich, USA) was dissolved in hexafluoroisopropanol (Aldrich, USA) at a concentration of 50 mg/mL at room temperature. Fetal bovine serum (FBS, JRH, USA) was added in 0.1% concentration by weight based on the weight of PGA. The PGA/FBS films were fabricated by mixing the PGA polymer solution and FBS. The PGA/FBS films for the cell culture were prepared on cover glasses, which were placed in the Petri dishes. The PGA/FBS film was thinly and evenly covered on the cell culture dishes and then, produced the multi-pore via vacuum using PGA removing solvent at room temperature. The biodegradable PGA or PGA/FBS material was coated on the dish as thin glass plate. PLA (BioSorbFX 2.0 mm System, USA), as commercial product, was used as half size of the commercial plate.

2. Cell Culture

Preosteoblast cell line, MC3T3-E1 (ATTCC, CRL-2593) was seeded at a density of 3×10^4 cells/㎠ (100 mm culture dish, PA, USA) and cultured in growth culture medium consisting of α-minimum essential medium (α-MEM: Gibco, USA) supplemented with 10% fetal bovine serum (Gibco, USA) and 100 units/mL penicillin in a humidified atmosphere of 5% CO_2 at 37 ℃: At 70-80% confluence, the cells were cultured in osteogenic media (regular media described above plus 10 mM β-glycero-phosphate (Sigma, USA) and 50 μg/mL L-ascorbic acid (sigma, USA))on one of 5 different biodegradable polymer-treated dishes (normal differentiation medium, PGA-plated, FBS-plated, PGA/fetal bovine serum (FBS)-plated, and PLA-plated) for 5, 15, and 25 days. The purpose for the addition of FBS to PGA on PGA/FBS plate composition was whether the additional FBS supplement could affect the improvement of the osteoblast activity, compared to PGA only. Normal differentiation media were changed every 3 days and cells were harvested at 5, 15, and 25 days.

3. Cellular and Medium Mineral (Ca and Mg, Mn) Measurement

Cells were wet-digested using trace element free concentrated nitric acid (Fluka, Switzerland). The wet-digested samples were diluted with trace element free 0.125 M HCl (Fluka, Switzerland). The diluted samples were filtered using 0.45 μm syringe filters (Corning, USA) and measured using inductively coupled plasma emission spectroscopy (Boschstra*Be* 10, Spectro Analytical Instruments, Germany) for Ca and bone-related minerals (Mg, Mn). The analytical accuracy for mineral analysis was tested using a standard reference material (SRM) obtained from the National Institute of Standards and Technology (NIST SRM 1577b, bovine liver, USA). The certified Ca, Mg, and Mn values of SRM were 116±4 μg/g, 601±28 μg/g, and 10.5±1.7 μg/g, respectively, and the measured Ca, Mg, and Mn value were 147 μg/g, 469 μg/g, and 8 μg/g, which corresponded to 126%, 78%, and 79% of the reference values, respectively. The range of 78-126% of validation for standard reference material analysis would be considered being acceptable for mineral analysis in the present study.

4. Cellular and Medium Alkaline Phosphatase (ALP) Activity Assay

Cellular (internal and anchored) and medium (secreted) alkaline phosphatase was measured by enzymatic activities. Cells were washed with PBS and extracellular membrane and internal alkaline phosphatase were lysed in 1 mL of 0.02% Nonident P-40 (Sigma, USA). The lysates were sonicated for 30 s twice on ice. The sonicated lysates were centrifuged for 15 min at 12,000 g. The supernatant was kept at −20 ℃ until analysis. The activity of ALP in cell lysates and medium was measured by using p-nitrophenyl phosphate as a substrate and the optical of 405 nm was determined as previously described.[10] Protein concentration was estimated by the

method of Lowry et al.,[11] with bovine serum albumin as the substandard. The activity of ALP was expressed as mU/mg of protein.

5. Alizarin Red Staining and Mineralized Nodule Formation

Since calcium usually co-precipitates with phosphate ions in *in vitro* culture condition, the mineralization of nodules in the cultures was assessed using Alizarin Red. The matrix was washed with ice-cold PBS and fixed for 60 minutes at 4 ℃ with 70% ethanol (Sigma, USA). Fixed cultures were rinsed with ice-cold PBS and stained with 40 mM Alizarin Red (pH 7.2, Sigma, USA) for 10 minutes at room temperature. The cultures were washed 3 times with water for 5 minutes, so as PBS to reduce non-specific Alizarin Red. The culture plate was exposed for few minutes to develop color. Mineralized and unmineralized nodules could be distinguished separately: mineralized nodules by their Alizarin-Red-positive staining (dark red center and light red peripheral area), and unmineralized nodules by their surface layer of cuboidal cells, light red staining, and three-dimensional structure. To evaluate bone nodule area precisely, the Alizarin-Red-stained areas were viewed by light microscopy. For each experiment, a minimum of two dishes were counted and the experiments were repeated two times.

6. Statistical Analysis

Data were analyzed with SPSS program and differences were considered significant at $p<0.05$. Statistical analysis of the data was performed by one-way ANOVA to test the effect of the different plate levels and time factors. Significance was detected among plate levels and time factors due to the small sample size.

RESULTS

1. Cellular and Medium Mineral Levels

The concentrations of bone-related minerals (Ca, Mg, and Mn) in osteoblastic MC3T3-E1 cells and the media at day 15 and 25 were measured, and the results are

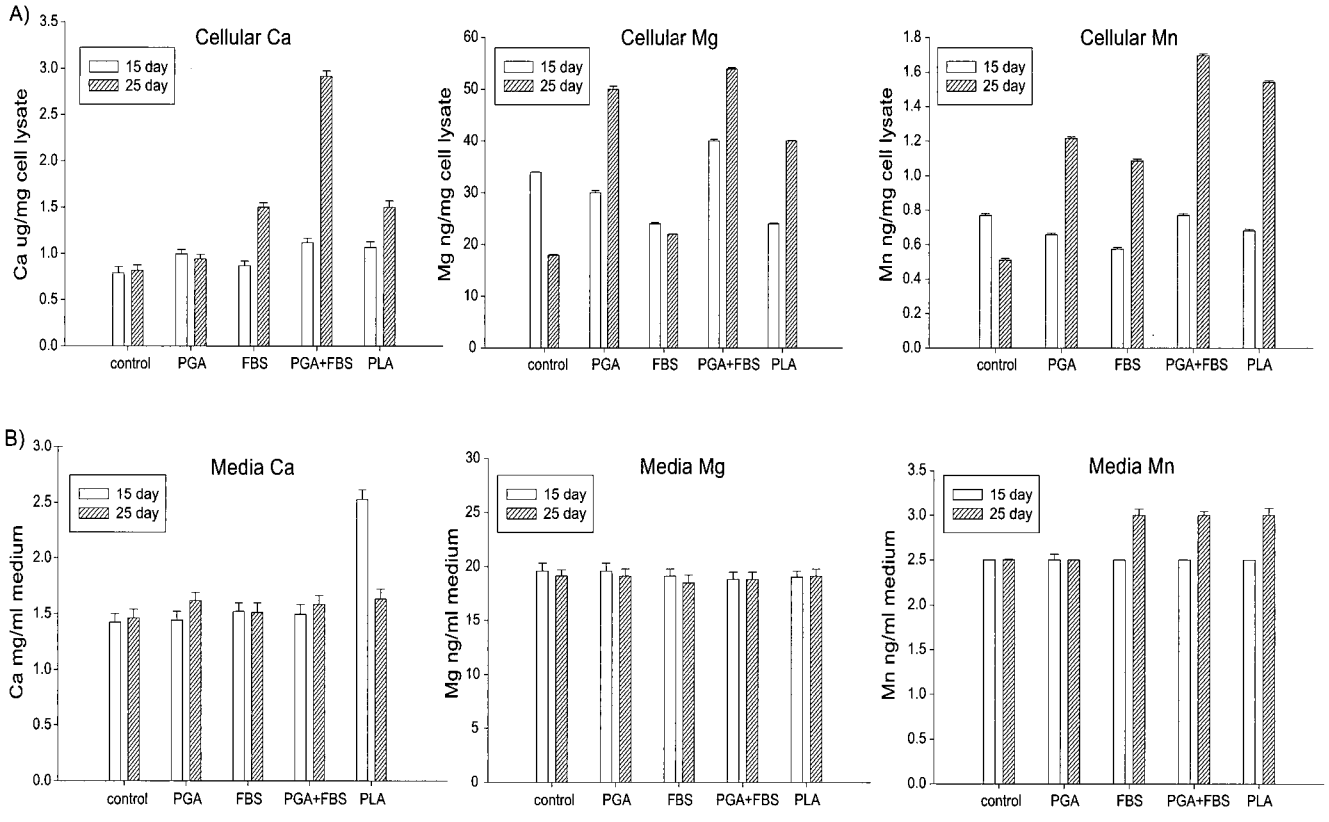

Fig. 1. Bone-related mineral (Ca, Mg and Mn) level in osteoblastic MC3T3-E1 cells and the media

The MC3T3-E1 cells were cultured on the various plated-treatment, such as normal osteogenic differentiation medium (control), PGA-, FBS-, PGA/FBS- or PLA-plated for 15 and 25 days. Cellular (A) and medium (B) bone-related mineral such as Ca, Mg and Mn levels were measured.
The value are mean ± SD (n=2 for per group).
The statistical analysis by ANOVA didn't show any significance, probably due to the limited sample replications.

shown in Figure 1. During culture period, PGA-plated biodegradable material was reabsorbed into the medium more easily and faster than the PLA-plated one (data not shown). For the cellular mineral levels, at day 15, presumably the middle stage of bone formation period, cellular Ca level showed a higher tendency in PGA- or PGA/FBS-, or PLA-plated treatments, compared to the control, even without statistical significance, due to the limited replications. Cellular Mg showed a tendency of higher level in PGA/PLA-coplated treatment, however, cellular Mn didn't show any prominent tendency at day 15 among the treatments. At day 25 which would be the early late stage of bone formation period, cellular Ca, Mg or Mn levels showed more prominent tendency of higher mineral levels in PGA- or PLA-plated treatment. All three minerals of Ca, Mg, and Mn level showed higher tendency in biodegradable materials treatments in order of PGA-related treatments (PGA/PLA- and PGA-plated) and PLA-plated treatment, compared to FBS-plated or non-plated control. PGA/FBS-coplated treatment tended to show much higher bone-related mineral contents in the cells, compared to other singly PLA- or PGA-plated treatment.

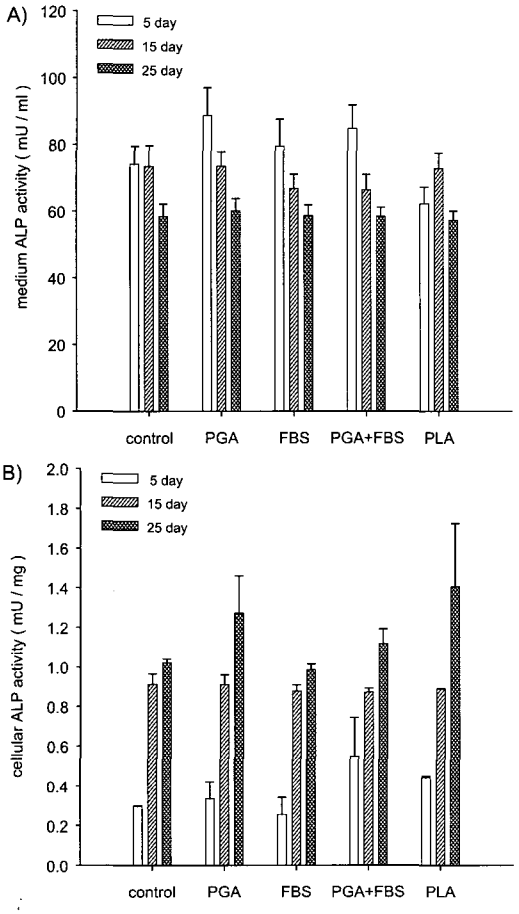

Fig. 2. Alkaline phosphatase (ALP) activity in MC3T3-E1 cells which were plated-treatments with various biodegradable materials (PGA and PLA) and FBS (control normal osteogenic differentiation medium, PGA-, FBS-, PGA/FBS- and PLA-plated treatments)

Internal cellular ALP activity (cellular ALP, A) and secreted extracellular matrix ALP activity (medium ALP, B) were determined at the indicated times (5, 15, 25 days). Control was treated with normal differentiation medium only without any biodegradable material-plated treatment.
The value are mean ± SD (n=4 per group).
Statistical analysis by ANOVA among treatment × time didn't show significance due to the limited replications.

Medium Ca, Mg or Mn levels didn't show any consistent tendency at both day 15 and 25. The results showed that PGA- or PLA-plated treatment tended to increase cellular minerals (Ca, Mg and Mn) and PGA/FBS-coplated treatment showed a pattern of more positive effect on the bone-related mineral (Ca, Mg, and Mn) levels in MC3T3-E1 cells. This would be a good sign for extracellular matrix Ca in osteoblasts including other bone-related minerals such as Mg and Mn deposits, too.

2. Cellular Alkaline Phosphatase (ALP) Activity Assay

Alkaline phosphatase activity (ALP) is supposed to play a key role in the bone formation and calcification. Once ALPs are synthesized in the osteoblasts, they are secreted outside the cells, and being deposited at the extracellular bone matrix to increase extracellular matrix mineralization. The data obtained from the measurement of ALP is shown in Figure 2. Cellular ALP (intracellular ALP) activity was higher in the PGA- or PLA-treated cells compared to control osteogenic differentiation medium cells at day 25, even without statistical significance due to the limited replications. Cellular ALP activity was not much affected on day 5 or 15 (Figure 2). Medium ALP (secreted extracellular ALP) activity was not affected by PGA-, PLA-, or PGA/PLA-coplated treatment (Figure 2). In the present study, both cellular and medium ALP activity didn't show prominent tendency among the biodegradable materials treatments.

3. Alizarin Red Staining and Mineralized Nodules Formation

Mineralized nodules were visualized by Alizarin Red staining after 5, 15, and 25 days of incubation. The osteoblasts were stained by Alizarin Red, which monitors areas of mineralized extracellular matrix, and thus can recognize calcium deposit at extracellular matrix of bone tissue. Nodules appeared to have dark red spots in cell morphologically and cell dish morphologically, which is

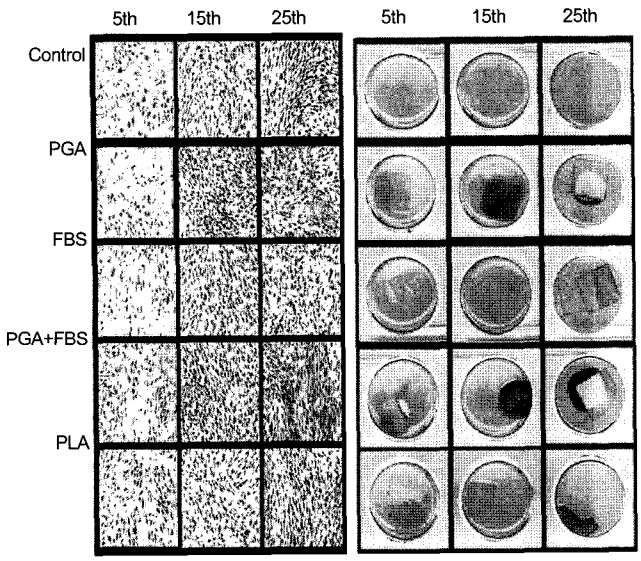

Fig. 3. Extracellular matrix mineralization measured by Alizarin red staining in MC3T3-E1 cells treated with different PGA or PLA-plated treatment at the indicated times (5, 15, 25 days)

The osteogenic MC3T3-E1 cells were cultured on the biodegradable PGA or PLA or FBS only or combination of PGA and FBS as plated treatment on the cell culture dish for as indicated time period.
Control was treated with normal differentiation medium without any biodegradable material.

shown in Figure 3. PGA- and PGA/FBS-coplated treatment showed better Ca deposits than other treatments. At day 15, PGA- and PGA/FBS-coplated treatment showed the darkest spot which means those two treatments showed higher Ca deposit in the middle stage of bone formation at day 15. Again, as time went on, Ca deposit on PGA- or PGA/FBS-coplated was being absorbed into the medium at day 25. PLA-plated one showed late degradable response, which was shown on day 25 as being the darkest spot. The order of being degradable, which was absorbed into the medium, was FBS-, PGA-, PGA/FBS- and PLA-plated. Therefore, PGA-plated treatment would be more degradable and absorbable than PLA-plated treatment in these osteoblastic cells.

Alizarin Red staining can bind with nodules and Ca deposits of osteoblast on extracellular matrix layers. We stained the cultures with Alizarin Red stain to examine the extracellular mineralization on MC3T3-E1 cells. PGA-plated and PGA/FBS-plated treatments showed more Ca deposits measured by Alizarin Red staining, compare to other treatments, although PLA-plated treatment also showed reasonable Ca deposits (Figure 3). We also examined both PGA-plated and PGA/FBS-coplated cell culture status with cells and without cells, to verify if cells grown on PGA-plated or PGA/FBS-plated could bind the Alizarin Red stain, which gives much stronger, darker red staining (Figure 4). This experiment would clarify the PGA availability for binding medium or cellular Ca. In other words, under PGA-plated cell culture circumstance might hold osteoblasts within the plate placement and can be beneficial to the bone formation. PGA-plated treatment with cells, compared to without cells, showed darker Alizarin Red staining, which means higher Ca deposits when the cells were grown on PGA- or PGA/FBS-coplated treatment in extracellular matrix mineralization. These results mean that PGA- or PGA/FBS-coplated biomaterials can be used for better extracellular matrix mineralization in osteoblastic MC3T3-E1 cells.

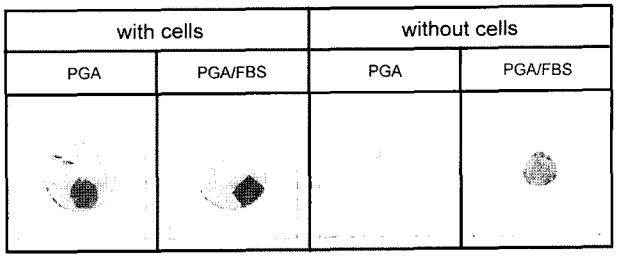

Fig. 4. Alizarin red staining for the PGA and FBS treatment with cells and without cells

To evaluated whether the cells treated with PGA would combine to Alizarin red stain or not. Alizarin red stain was tested with or without the cells under PGA and PGA/FBS treatment.

DISCUSSION

The need for the repair of bone fracture due to osteoporosis or large bone defects due to osteomalacia and osteopenia can be a serious problem not only for degenerated bone health matter but also for medical matter.[12-16] Biodegradable polymers, such as PGA and PLA can be used for supplement biodegradable biocompound which can be utilized for the bone repair. In general, these polymers can be degraded by pores scaffolds in bioactivity agents.[17]

The biodegradable fracture fixation device, such as PGA or PLA or PGA/FBS copolymers in the present study is particularly attractive because such biocompounds can be useful for repairing bones.[18] Before their wide-scale, the osteogenical benefits of these biomaterials should be addressed. The use of self-reinforced PLA and PGA rods has also been reported in some other studies.[19-20] However, so far, the osteogenically beneficial effect of these PGA or PLA polymers on osteoblastic cell models has not been reported, such as for

alkaline phosphate activity for the marker of onset of osteoblast differentiation, or extracellular matrix Ca deposit which is for the measurement of extracellular matrix mineralization on the bone tissue. In the present study, we evaluated the beneficial function of PGA, PLA and new formation of PGA/FBS copolymer materials in the osteoblast model on bone formation.

The results of the present study showed that various combinations of PGA- or PLA-plated (PGA-plated, FBS-plated, PGA/FBS-coplated, and PLA-plated) treatments positively affected osteoblastic cell differentiation and extracellular matrix mineralization. MC3T3-E1 cells, a mouse clonal osteoblastic cell line, are known to form multiple layers and accumulate mineralized extracellular matrices.[21] Generally, osteoblast cell development along osteogenic lineage consists of cell proliferation with extracellular matrix maturation, and finally extracellular matrix mineralization.[22] One of the drawbacks of using bridgeable materials, such as PGA or PLA in this study, for many biomedical applications is the fact that their biodegradable materials can be degradable in short time period, and these biomaterial nature challenges the adhesion of cells to their surface.[23] In this study, in Figure 3, cell adhesion to the surface of biodegradable polymers was observed on day 15, but this cell adhesion disappeared on day 25 which means the PGA or PGA/FBS biomaterials was degraded and thus could be absorbed into the bone tissue to enhance the bone formation.

ALP is the key enzyme for bone formation and can be a marker for the onset of osteoblast differentiation in osteoblastic cell lines. The preosteoblastic MC3T3-E1 cells are differentiated into osteoblasts by adding vitamin C and glycerophosphate, both of them can induce the production of alkaline phosphate.[24] As the mineralization of the bone forming cells progress, osteoblatic cells such as MC3T3-E1 cells in this study, increase alkaline phosphatase in the medium.[10] In the present study, cellular ALP activity showed a tendency of increase as mineralization period went by, while the medium ALP activity showed in the opposite pattern. This implies that as mineralization period goes by, less ALP synthesis would be needed as the previous study reported.[10] In the present study, especially MC3T3-E1 cellular ALP activity was higher in the PGA-plated or PGA/FBS-coplated treatment, compared to normal control which was not treated with any of PGA or PLA biomaterials. PLA-plated treatment also showed increased cellular ALP activity (Figure 2). In the present study, both cellular and medium ALP activities didn't show prominent tendency among the biodegradable materials treatments.

The mineral phase of bone is responsible for both mechanical and homeostatic functions.[25] Mineralization of bone at particular remodelling site is largely dependent on its stage of secondary mineralization, at the stage of bone-related mineral (Ca, Mg, P, Mn, etc.) attachment to the extracellular matrix deposit.[26] PGA or PLA-plated treatment in this study showed the increased cellular bone-related mineral (Ca, Mg, Mn) contents. This result confirmed that PGA or PLA-plated treatment would increase osteoblastic extracellular matrix mineralization.

The common histological assay to determine extracellular matrix mineralization is Alizarin Red stain for calcium. Cell matrix interactions are crucial for the regulation of cytoskeletal structures, growth and diffraction.[27] Cell survival depends on extracellular matrix (ECM), with other cells and with soluble growth factors in serum.[28] Extracellular matrix layers and Alizarin Red stain can bind with nodules pattern and Ca deposition mineralization of osteoblast cultures in vitro. In the present study, PGA-, PGA/FBS- and PLA-plated treatments showed stronger Ca deposits by these biomaterials in extracellular matrix circumstances by the measurement of Alizarin Red stain for Ca deposit, particularly on these osteoblasts.

In the present study, we didn't evaluate any potential toxicity or resistance for using these biodegradable materials, PGA or PLA, for the application to osteoblast mineralization. PGA and PLA have already being reported as biodegradable materials in the tissue and cell model.[1,4] Therefore, any serious harmfulness would be expected if the treatment level would be within certain ranges. However, depending cell types and each experimental design for evaluating the benefits of these biodegradable materials on bone cells and tissue application, more detailed toxicity evaluation would be strongly considered.

Taken all together, in conclusion, the results showed that biodegradable biocompounds, such as PGA- or PLA- or PGA/FBS-coplated biomaterials can increase alkaline phosphatate activity and extracellular matrix mineralization. Therefore, PGA or PLA biomaterials can be used as a biocompound for bone formation in osteoblasts or for bone repair for better extracellular matrix mineralization. Industrial application using PGA or PLA biocompound which is degradable would be considered further. Also further study for any anticipated toxicity or resistance for using these biodegradable materials for bone tissue would be necessarily needed.

Acknowledgment

The authors thank for the help of the mineral analysis done by Korea Basic Science Institute.

Literature Cited

1) Garg AK. Grafting materials in repair and restoration. In; Lynch SE, Genco RJ, Marx RE, eds. Tissue engineering: Application in maxillofacial surgery and periodntics. pp.83-101, Quintessence International Co, Chicago, 1992
2) Griffith LG. Polymeric biomaterials. *Acta Mater* 48:263-277, 2000
3) Agrawal L. Polyostotic osteonecrosis in a patient with celiac disease. *Endocr Pract* 2(6):385-388, 1996
4) Lia Rimondini DD. In vivo experimental study on bone regeneration in critical bone defects using an injectable biodegradable PLA/PGA copolymer. *Oral surg oral med oral path radiol endod* 99:148-154, 2005
5) Rokkanen P, Böstma O, Vainiopää S, Vihtone K, Törmälä P, Laiho J, Kilpikari J, Tamminmäki M. Biodegradable implants in fracture fixation: early results of treatment of fractures of the ankle. *Lancet* 22(i):1422-1424, 1985
6) Hirvensalo E. Fracture fixation with biodegrable rods. Forty-one cases of severe ankle fractures. *Acta Orthop Scand* 60(5):601-606, 1989
7) Partio EK, Böstma O, Hirvensalo E, Pätiälä H, Vainionpää S, Vihtonen K, Törmälä P, Rokkanen P. The indication for the fixation of fractures with totally absorbable SR-PGA srews. *Acta Orthop Scand* 61(Suppl 237):43-44, 1990
8) Dijkema ARA, van der Elst M, Breederveld RS. Surgical treatment of factures with biodegradable implants. *A prospective randomised study. J Trauma* 34:82-84, 1993
9) Kyriacos A, Anthanasiou, Gabriele G, Niederauer C, Mauli Agrawal. Sterization, Toxicity, biocompatibility and clinical applications of polylactic acid/polyglyclic acd copolymers. *J Biomaterials* 17:93-102, 1996
10) Bessey OA, Lowry OH, Brock MJ. A method for the rapid determination of alkaline phosphatase with fibecubic millimetres of serum. *J Biol Chem* 164:321-329, 1946
11) Lowry OB, Rosenbrough MJ, Farr AL, Rebar RW. Protein measurement with folin phenol reagent. *J Biol Chem* 193:255-260, 1951
12) Muschler GF, Lane J. Orthopedic surgery. In: Habal MB, Reddi AH, eds. Bone graft bone substitutes, pp.375-413, WB Saunders Company, Philadelphia, 1992
13) Perry CR. Bone repair techniques, bone graft substitutes. *Clin Orthop* 360:71-86, 1999
14) Phelps JB, Hubbard GB, Wang X, Argawal CM. Microstructural heterogeneity and the fracture toughness of bone. *J Biomed Mat Res* 51:735-741, 2000
15) Russell JL, Block JE. Surgical harvesting of bone graft from the ileum; point of view. *Med Hypotheses* 55:474-479, 2000
16) Daniels AU, Cgang MKO, Andriano KP. Mechanical properties of biodegradable polymerase and composites proposed for internal fixation of bone. *J Appl Biomater* 1:57-78, 1990
17) Gabriela AS, Olga PC, Paul Ducheyne, Irving M, Shapiro, Rui LR. The effect of starch and starch-bioactive glass composite microparticles on the adhesion and expression of the osteoblastic phenotype of a bone cell line. *Biomaterials* (in press) 2006
18) Vinipää S, Kilpikari J, Laiho J, Helevirta P, Rokkanen P, Törmälä P. Strengh retention in vitro, of absorbable, self-reinforced polyglycolide (PGA) rods for fracture fixation. *Biomaterials* 8:46-48, 1987
19) Törmälä P, Vasenius J, Vainiompää S, Pohjonen T, Rokkanen P, Kaiho J. Ultra high strength absorbable self-reinforced polyglycolide (SR-PGA) coposite rods for internal fixation of bone fractures: in vitro and in vivo study. *J Biomed Mater Res* 25:1-22, 1991
20) Ruuskanen MM, Kallioinen MJ, Jaarela OI, Laiko JA, Törmälä PO, Waris TJ. The role of polyglycolid acid rods in the regeneration of cartilage from perichon-drium in rabbits. *Scand J Plast Reconstruct Hand Surg* 25:15-18, 1991
21) Yamaguchi M, Yamaguchi R. Action of zinc on bone metabolism in rats. Increases in alkaline phosphatase activity and DNA content. *Biochm Pharmacol* 35:773-777, 1986
22) Herzberg M, Foldes J, Steinberg R, Menczel J. Zinc excretion in osteoporotic women. *J Bone Minner Res* 5:251-257, 1990
23) Verderio E, Coombes A, Jones RA, Li X, Heath D, Downes S. Role of the cross-linking enzyma tissue transglutaminase in the biological recognition of synthetic biodegradable polymerase. *J Biomed Mater Res* 54:294-304, 2001
24) Beck Jr Gr, Sulivan EC, Moran E, Zeler B. Relationship between alkaline phosphatase levels, oeteopontin expression, and mineralization in differentiating MC3T3-E1 osteoblasts. *J Cell Biochm* 68:269-280, 1998
25) Davison KS, Siminoski K, Adachi JD, Hanley DA, Goltiman D. Bone strength whole is great than the sum of its parts. *Semin Arthritis Rheum* 36:22-31, 2006
26) Roschger P, Fratzl P, Eschberger J, Klaushofer K. Validation of quantitative backscattered electron imaging for the measurement of mineral density distribution in human bone biopsies. *Bone* 23:319-326, 1998
27) Storrie H, Stupp SI. Cellular response to zinc-containing organoapatitie: an vitro study of proliferation, alkaline phosphatase activity and biomineralization. *Biomaterials* 26:5492-5499, 2005
28) Suzuki T, Ishihara K, Magaki H, Matsurra W, Kohda A, Okumura K, Nagao M, Yamaguchi-Iwai Y, Kambe T. Zinc transports, Zn5 and ZnT7, are required for the activation of alkarine phosphatase, zinc-requiring enzymes that are glycosylphosphatidylinositol-anchored to the cytoplasmic membrane. *J Biol Chem* 280:637-643, 2005