Transforming Growth Factor (TGF)-β1 Releasing Tricalcium Phosphate/Chitosan Microgranules as Bone Substitutes

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Purpose. Tricalcium phosphate (TCP)/chitosan composite microgranules were developed as bone substitutes and tissue engineering scaffolds with the aim of obtaining a high bone forming efficacy. The microgranules have the ability to fill various types of defect sites with closer packing. In addition, the transforming growth factor-beta 1 (TGF-β1) was added to the microgranules in order to improve bone-healing efficacy.

Methods. TCP/chitosan microgranules were fabricated by dropping a TCP suspended chitosan solution into a NaOH/ethanol solution. TGF-β1 was incorporated into the TCP/chitosan microgranules by soaking the microgranules into the TGF-β1 solution. Scanning electron microscopy (SEM) observations as well as experiments examining the release of TGF-β1 from chitosan and TCP/chitosan microgranules were performed. SEM was used to examine the cell morphologies on the microgranules, and the extent of cell proliferation was evaluated using a dimethyl-thiazol tetrazolium bromide (MTT) assay. The differentiated cell function was assessed by measuring the alkaline phosphatase activity as well as performing an osteocalcin assay.

Results. The size of the prepared microgranules was 350–500μm and TCP powders were observed on the surface of the microgranules. TGF-β1 was released from the TCP/chitosan microgranules at a therapeutic concentration for 4 weeks. The proliferation of osteoblasts on the TGF-β1 loaded microgranules was the highest among the microgranules. SEM indicated that the seeded osteoblastic cells were firmly attached to the microgranules and proliferated in a multilayer fashion. The ALPase activity and osteocalcin content of all the samples increased during the culture period.

Conclusions. These results suggest that the TCP/chitosan microgranules are potential bone substitutes with a drug releasing capacity and a osteoblastic cells culture scaffold.

KEY WORDS: bone substitutes; chitosan; TGF-β1; tricalcium phosphate.

INTRODUCTION

Bone substituting materials must satisfy a number of biologic criteria, such as biocompatibility, controlled biodegradability, and osteoconductive potential (1). For clinical use, bone substitutes must be malleable and adaptable to the size and shape of defects because the dimensions of a defect cannot always be precisely determined preoperatively. Spherical shape bone substitutes have the flexibility in filling different geometric cavities with closer packing than nonspherical shaped substitutes. Therefore, microgranular bone substitutes allow it to fill an irregular bone defect easy. The void spaces formed between the microgranules facilitate osteoblast migration and conduction. It was previously reported that chitosan inhibited fibroblast proliferation and attachment, and doubled the number of bone forming colonies derived from mesenchymal stem cells when compared to a culture plate (2). Chitosan, which is composed of glucosamine and *N*-acetylglucosamine residues linked by 1-4 glycosidic bonds, has been proven to be biologically renewable, biodegradable, biocompatible, nonantigenic, and biofunctional (3). Chitosan appears to favor the differentiation of osteoprogenitor cells and bone formation. The osteoconductivity can be improved by combining it with calcium phosphates. β-TCP has been shown to have good biocompatibility and biodegradability and is extremely osteoconductive (4). Therefore, β-TCP appears to be suitable for combining polymer constructs, chitosan and β-TCP.

Optimization and the control of the growth and differentiation of ingrown cells can be achieved by the administration of growth factors either *in vitro* or *in vivo* (5–7). Among the growth factors, TGF-β1 has been used as a potential induction factor for bone tissue engineering. The major activity of the transforming growth factor-beta (TGF-β) on osteoblasts is to trigger rapid differentiation, which includes the secretion of the bone matrix protein followed by the mineralization of this matrix. Indeed, it has also been suggested that an increased in bone formation can be due to the effects of TGF-β in stimulating the proliferation of the osteoblast precursors to create a large pool of cells in the osteoblastic lineage (8). Among the TGF-β superfamily, TGF-β1 is the most abundant isoform in mammalian species and it has been used in most *in vivo* and *in vitro* studies (9). However, even if a high dose of growth factors are injected in an aqueous solution form, it might be difficult to maintain the desired biologic effects *in vivo* for a certain period of time, because of the short half-life of the growth factor in the body. Therefore, it is indispensable for an improved high efficacy *in vivo* to achieve the controlled release of growth factors. To this regard, controlled release of TGF-β1 at the bone defect may be beneficial for the induction of bone regeneration. The TCP/chitosan microgranules designed in this study exhibited a drug releasing capacity over a 4-week period to enhance bone regeneration. The controlled release of TGF-β1 from the TCP/chitosan microgranules may be highly beneficial for enhancing osteoblasts proliferation and differentiation.

The aim of this study was to develop chitosan microgranules combined with TCP as bone substitutes and a tissue-engineering scaffold with a drug releasing function. Chitosan microgranules, composite microgranules of TCP/chitosan, and TGF-β1 loaded TCP/chitosan microgranules were fabricated. The level of TGF-β1 released from each sample and MC3T3-E1 cells proliferation and differentiation were performed.

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MATERIALS AND METHODS

Materials

Chitosan (98% deacetylated) was purchased from Tae-Hoon Bio (Kyong-Book, Korea). The β-tricalcium phosphate size (TCP) was purchased from Showa Chemicals (Osaka, Japan). The size distribution of TCP powder is 1–10 μm and the ratio of Ca/P is 1.5. The human recombinant TGF-β1 was purchased form R&D Systems (Minneapolis, MN, USA). The human recombinant TGF-β1 labeled with [^{125}I]-Bolton Hunter reagent (^{125}I-labeled TGF-β1, 185 kBq/ml in 0.05 M sodium acetate buffer) and *N*-succinimidyl-3-(4-hydroxy-3-[^{125}I] iodophenyl) propionate {[^{125}I]-Bolton-Hunter Reagent (diiodinated)} were purchased from (Amersham Co., Buckinghamshire, UK). The collagenase, β-glycerol phosphate, L-ascorbic acid, and glutaraldehyde were obtained from Sigma-Aldrich (St. Louis, MO, USA). The trypsin-EDTA, fetal bovine serum (FBS), and α-minimum essential medium (MEM) were purchased from Gibco (Grand Island, NY, USA). All the solvents used were of analytical grade.

Preparation of Chitosan Microgranules

Chitosan (4 g) was dissolved in a 3% v/v acetic acid solution, and the solution was filtered to remove undissolved chitosan. Then, the TCP (2 g) was dispersed in the chitosan solution, and the resulting solution followed by drop-wise extrusion into a coagulation solution of a 4% NaOH ethanol/water mixture (20/80) (% v/v) at a rate of 0.45 ml/min through a flat-end needle with gentle stirring. The size of the microgranule was controlled by an air stream following coaxial to the needle tip, thereby cutting the microgranules off at the tip of the needle. The airflow was controlled by a pressure regulator, which was monitored by a gauge pressure. The resulting microgranules were washed until the pH approached neutral. Chitosan microgranules of 350–500 μm were selected and freeze-dried. TGF-β1 (100 ng in 4 mM HCl) was added to 20 mg of the TCP/chitosan microgranules, which was stored overnight at 4°C and freeze-dried.

Scanning Electron Microscopy Observation

The microgranules were coated with gold using a sputter coater (POLARON SC 7620, VG Microtech, England) and their morphology was examined by scanning electron microscopy (SEM; JEOL Model JSM 5200, JEOL Ltd., Tokyo, Japan) operated at 20 kV.

TGF-β1 Release Experiments

^{125}I-labeled TGF-β1 (free ^{125}I level was less than 5% at the initial analysis) was used as a tracer in order to measure the amount of TGF-β1 released from the device. The ^{125}I-labeled TGF-β1 (10μCi, Amersam, Buckinghamshire, UK) was diluted with nonradioactive TGF-β1 solution to reach a radioactivity of 1 μCi. These radioactive mixtures were incorporated into the TCP/chitosan microgranules at a amount of 100 ng/20 mg of microgranules. The loading efficiency of TGF-β1 was measured by counting the level of ^{125}I-labeled TGF-β1 from newly prepared microgranules. The microgranules (ca. 20 mg by weight) were placed into pH 7.4 phosphate buffer as a releasing medium (10 ml). The sealed vials were placed in a shaking water bath at 37°C and shaken at 15 rpm.The samples were withdrawn from the vial at predetermined time intervals and the volume was replenished with fresh medium. The concentration of the TGF-β1 released from the samples was assayed using a gamma counter (Cobra II, Packard Instrument, Meriden, CT, USA).

Cell Proliferation

Twenty milligrams of the microgranules were incubated with 1 ml of α-MEM containing the MC3T3-E1 cells (2×10^5 cells/ml) and supplemented with 10 mM 15% FBS, 1% antibiotic-antimycotic solution, 10 mM sodium β-glycerol phosphate, 50 μm/ml L-ascorbic acid, and 10^{-7} M dexamethasone. The microgranules were transferred to another Petri dish 1 day after incubation, and the medium was changed once every 3 days. The cultures were maintained in a humidified atmosphere consisting of 95% air and 5% CO_2 at 37°C. At various times after incubation, the microgranules were washed gently using HBSS to remove the unattached cells and remaining media, and then treated with 2.5% trypsin in 4 mM EDTA to detach the cells from the matrix.

Cell Morphology

The cell morphology on the microgranules was observed by SEM. The cells were fixed with 2.5% glutaraldehyde in 0.1 M PBS (pH 7.4) for 30 min and then rinsed with 0.1 M PBS. The cells were then stained with 1 ml of cold 1% OsO_4 (Polyscience Inc., Pittsburgh, PA, USA), placed on ice for 30–40 min, and then rinsed with deionized water. The fixed specimens were dehydrated through a graded series of ethanol up to 100% (v/v). The dehydrated samples were then freeze-dried, sputter coated with gold, and examined by SEM.

MTT Assay

The extent of cell proliferation on each specimen was measured by a MTT assay, which is based on the mitochondrial conversion of tetrazolium salt, 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT). Briefly, after the cells were incubated on different samples for 1, 7, 14, and 28 days, a 250 μl MTT solution (2 mg/ml in PBS) was added to each sample, and incubated at 37°C for 4 h in order to allow for MTT formazan formation. The medium and MTT were replaced with a dimethyl sulfoxide solution. The solution was then transferred to 96 wells, and the optical density of the solution in each well was measured using a microplate reader (THERMOmax, Molecular Devices, Menlo Park, CA, USA) at a wavelength of 540 nm.

Alkaline Phosphatase (ALPase) Assay

The level of alkaline phosphatase (ALPase) production was measured spectroscopically. The osteoblast-seeded microgranules were washed with PBS, treated with 2.5% trypsin in 4 mM EDTA to detach the cells from the chitosan microgranules and centrifuged at 70 × *g* for 5 min. The cells removed were then sonicated using a sonic dismembrator 550 (Fisher, Pittsburgh, PA, USA) for 1 min at 110 watts (50/60 Hz) 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, 0.1% Triton X-100/saline, and 0.5 ml of DDW

for up to 30 min at 37°C. The reaction was quenched by adding 1.25 ml of 0.1 M NaOH and placing the mixture on ice. The level of p-nitrophenol production in the presence of ALPase was measured by monitoring the light absorbance by the solution at 405 nm. Protein concentrations of the cell lysates were determined by Bradford protein assay reagent (Bio-Rad Laboratories, Hercules, CA, USA). The data were expressed as nmol μg protein^{-1} 30 min^{-1}.

Osteocalcin Assay

Aliquots of the culture medium from each well were sampled, frozen immediately, and stored at −70°C until needed. The osteocalcin level was measured using a mouse osteocalcin ELISA kit (Biomedical Technologies Inc., Stoughton, MA, USA). The osteocalcin levels are expressed as ng/ml of supernatant.

Statistical Analysis

The results are expressed as a mean ± SD for each group of samples. Two-way variance analysis (ANOVA) was used to measure the statistical significance of all measurements of cell proliferation in order to compare the results of the TGF-β1 loaded TCP/chitosan microgranules with those from the TGF-β1 unloaded TCP/chitosan microgranules. A p value <0.05 was considered significant.

RESULTS

Morphology of TCP/Chitosan Microgranules

Figure 1 shows SEM micrographs of the prepared microgranules. TCP powder was observed on the surface and interior of the TCP/chitosan microgranules. The diameter of the microgranules could be controlled by altering the airflow rate. In this study, the selected size distribution of the microgranules was 350–500 μm in order to be considered as commercialized bone substitutes. The size of bone substitutes currently used such as BioGran and Bio-Oss are 300–355 μm and 250–1000 μm, respectively (10). Osteoblasts (10–30 μm) might easily migrate into the void space of the microgranules and be expected to proliferate (11).

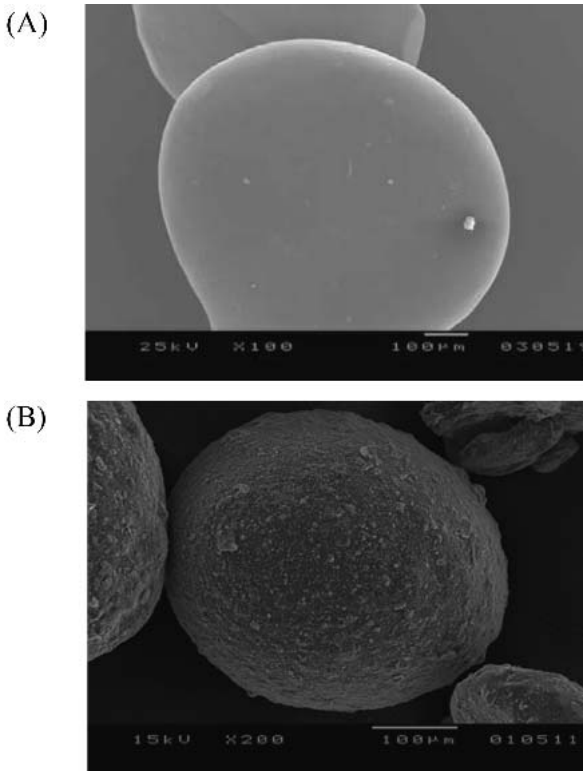

Fig. 1. Scanning electron micrographs of the surface of the (A) chitosan and (B) TCP/chitosan microgranules.

Release of TGF-β1 from the TCP/Chitosan Microgranules

The loading content of TGF-β1 in chitosan microgranules was 4.9 ng/mg of chitosan microgranules and the loading efficiency was 98% (w/w). The amount of TGF-β1 released from the TCP/chitosan microgranules is shown in Fig. 2. The extents of TGF-β1 released at the first day from chitosan and TCP/chitosan microgranules were 45% and 55%, respectively. The release profile comprised three phases: an initial burst occurring during the first 24 h, a linear steady release phase that lasted to day 12, and second longer lasting linear release phase release phase for the rest of the period (day 13 to 28).

Cell Viability

The degree of cell proliferation on the chitosan microgranules was determined from the cell viability test based on the results from the MTT assay. Figure 3 shows the absorbance of formazan produced by the proliferated cells growing on the chitosan microgranules after 1, 7, 14, and 28 days. The absorbance of all the samples increased with increasing incubation time. The optical density of the TCP/chitosan microgranules at 14 days was 134% higher than that of the chitosan microgranules. The proliferated cells on the TGF-β1 loaded TCP/chitosan microgranules persistently increased with time and was highest among the samples.

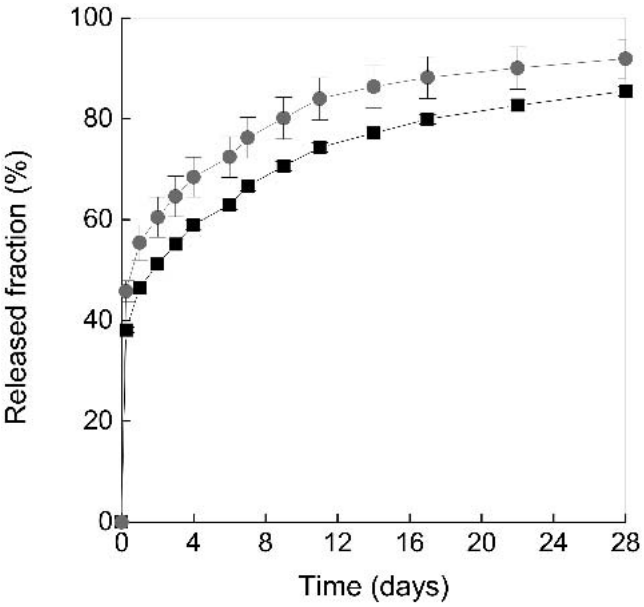

Fig. 2. Cumulative release of TGF-β1 from the (■) chitosan, (●) TCP/chitosan microgranules as a function of time (100 ng of TGF-β1 was loaded into 20 mg of microgranules).

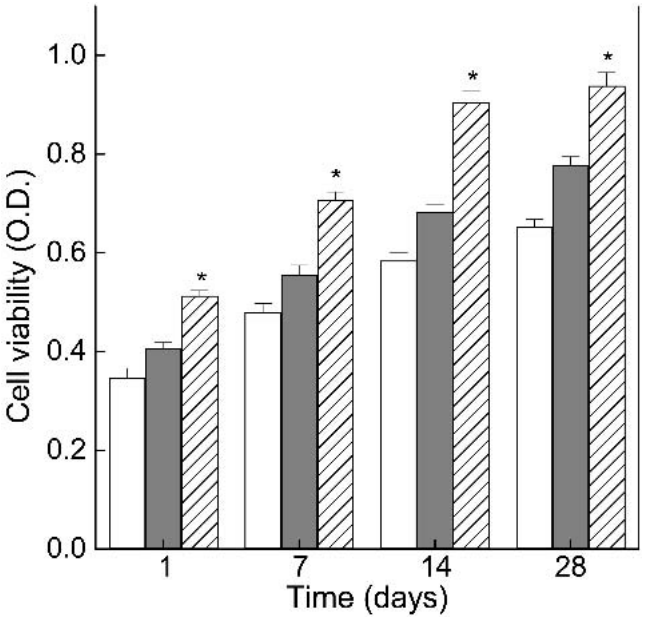

Fig. 3. MTT assay formazan absorbance is a measure of the cell viability of the MC3T3-E1 cells seeded on (□) chitosan, (■) TCP/chitosan, and (▨) TGF-β1 loaded TCP/chitosan microgranules. Initial seeding was with 2×10^5 cells/sample and cultured for 1, 7, 14, and 28 days. The results are presented as a mean ± SD (n = 6). *$p < 0.05$ as compared with that of chitosan microgranules and TGF-β1 unloaded TCP/chitosan microgranules.

Cell Morphology

Figure 4 shows the morphology of the osteoblasts cultured on microgranules at 1 day. The cells adhering to the TCP/chitosan microgranules were even more flattened than those found on the chitosan microgranules. The cells appeared polygonal, spindle-shaped, and spread well on the TGF-β1 loaded TCP/chitosan microgranules. Figure 5 shows SEM images of the cells cultured on the microgranules for 7 days. The morphology of the cells on the chitosan and TCP/chitosan microgranules was similar. It showed that many cells had spread and colonized patches of the material surfaces within 7 days. The spread cells maintained physical contact with each other through the filopodia or lamellopodia. More polygonal and spindle-shaped cells attached and spread well on the TGF-β1 loaded microgranules.

Alkaline Phosphatase Activity

Figure 6 shows the ALPase activity of the cells cultured on the microgranules. All groups showed an increase in ALPase activity during the incubation period. However, the ALPase activity did not increase significantly after 14 days. The ALPase activity of the TGF-β1 loaded TCP/chitosan microgranules was significantly higher that the activity on the TCP/chitosan and chitosan microgranules over a 28-day culture period.

Osteocalcin Assay

Osteocalcin synthesis was detected all groups at 14 days and 28 days incubation. In the TCP/chitosan, only a slightly increase from 0.25 ng/ml to 0.59 ng/ml was observed respectively (Fig. 7). However, in the group with the TGF-β1 loaded

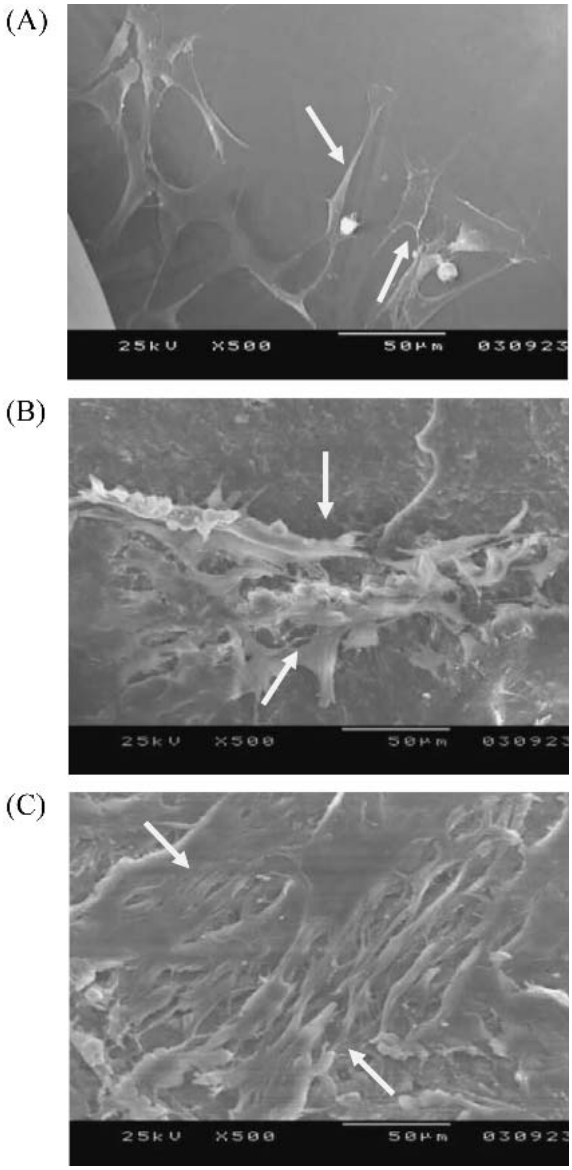

Fig. 4. Scanning electron micrograph of the osteoblasts on the (A) chitosan, (B) TCP/chitosan, and (C) TGF-β1 loaded TCP/chitosan microgranules for 1 day. The arrows indicate attached osteoblasts.

TCP/chitosan microgranules, the osteocalcin content increased significantly from 0.51 ng/ml to 1.01 ng/ml. The controlled release of TGF-β1 from the TCP/chitosan microgranules effectively stimulated the osteoblastic cells.

DISCUSSION

Successful bone regeneration requires a combination of many factors; 1) cells that can undergo differentiation to form osteoblasts; 2) biological factors that control the growth and differentiation of these cells; and 3) osteoconductive bioresorbable materials that promote cellular attachment, migration, and proliferation (12). Although single factors (hydroxyapatite, bone morphogenetic proteins, osteogenic cells) have been shown to stimulate bone formation both *in vitro* and *in vivo* (13), therapeutically useful bone replacement materials are most likely to result from the combined use of all these factors (14). The objective of this study was to develop TCP/

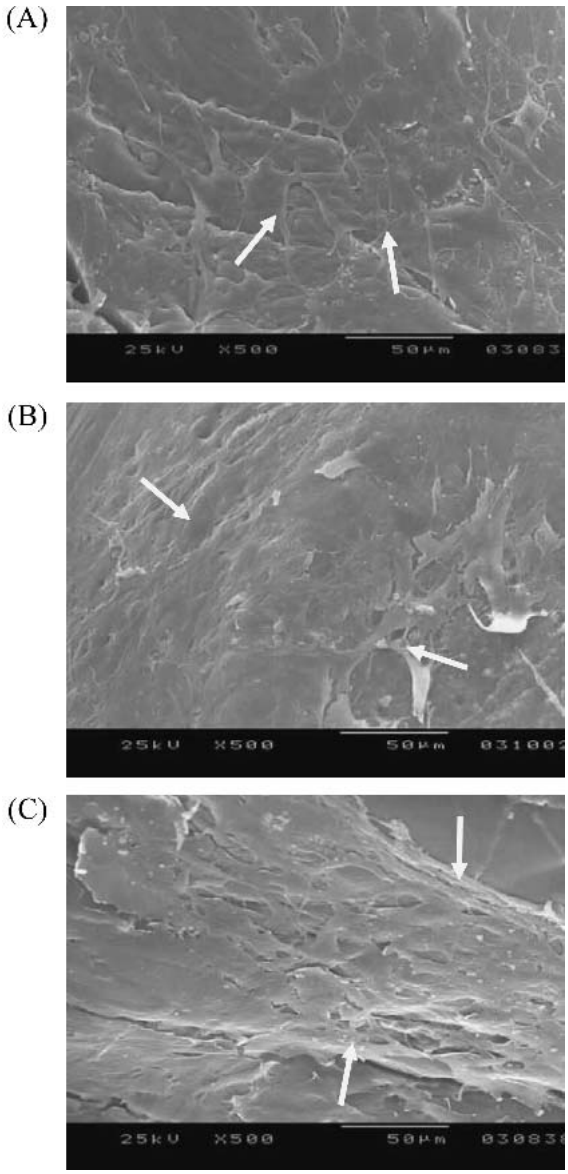

Fig. 5. Scanning electron micrographs of the osteoblasts on (A) chitosan, (B) TCP/chitosan, and (C) TGF-β1 loaded TCP/chitosan microgranules for 7 days. The arrows indicate the attached osteoblasts.

chitosan microgranules as bone substitutes and a tissue-engineering scaffold with a drug releasing function. The chitosan microgranules were fabricated by gelation of chitosan in basic solution and the size of the microgranule was controlled by an airflow following coaxial to the needle tip.

The high loading efficiency of TGF-β1 into TCP/chitosan microgranules was obtained from the adsorption of TGF-β1 at the surface of microgranules and diffusion into chitosan matrix. The diffusion can be achieved by the low pH of TGF-β1 solution (TGF-β1 was dissolved in 4 mM HCl). The acidic condition can loose chitosan chain network, thus probably TGF-β1 can be diffused inside of chitosan microgranules.

The initial burst was caused by desorption of exposed TGF-β1 at the surface of the microgranules. The rapid release at the initial step did not appear to be good in the aspect of a controlled release. However, obtaining a high concentration of TGF-β1 at a local site in a short time can be beneficial to

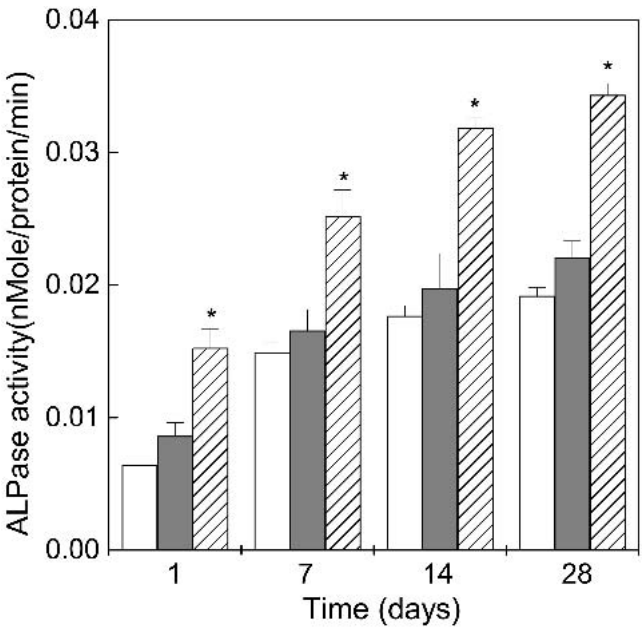

Fig. 6. Alkaline phosphatase (ALPase) activity for (□) chitosan, (■) TCP/chitosan, and (▨) TGF-β1 loaded TCP/chitosan microgranules. Initial seeding was with 2×10^5 cells/sample and cultured for 1, 7, 14, and 28 days. The results are presented as a mean ± SD (n = 6). *$p < 0.05$ as compared with that of chitosan microgranules and TGF-β1 unloaded TCP/chitosan microgranules.

the early healing and regenerative effect. In addition, the following continuous release can help the osteoblasts to proliferate. The release of TGF-β1 after the initial burst was maintained at the concentration of 0.3–0.4 ng/ml for 2–12 days and 0.1 ng/ml for the remained of the study. In our preliminary

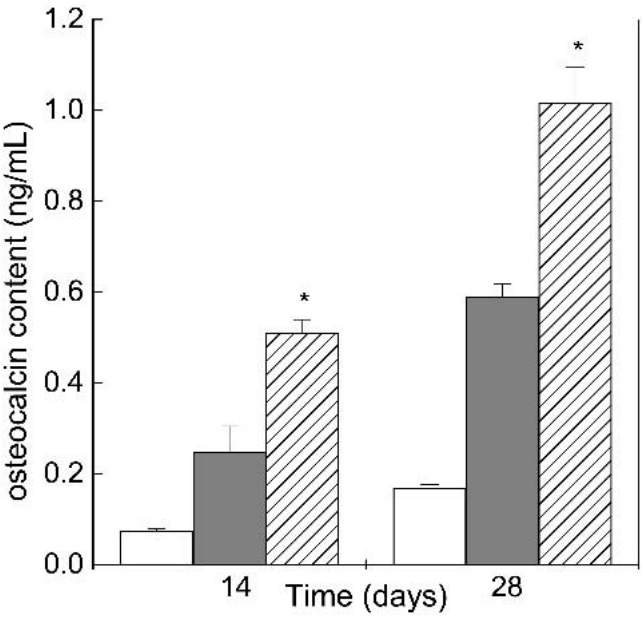

Fig. 7. Osteocalcin content (□) chitosan, (■) TCP/chitosan, and (▨) TGF-β1 loaded TCP/chitosan microgranules. Initial seeding was with 2×10^5 cells/sample and cultured for 1, 7, 14, and 28 days. The results are presented as a mean ± SD (n = 6). *$p < 0.05$ as compared with that of chitosan microgranules and TGF-β1 unloaded TCP/chitosan microgranules.

study, the ALPase activity of MC3T3-E1 cultured for 3 days was the highest value in case that TGF-β1 solution was added in a concentration of 0.1 ng/ml. There are a wide variety of stimulatory and inhibitory effects depending on the TGF-β1 dose applied to the used cells. Centrella *et al.* found that TGF-β1 had a biphasic effect in subconfluent fetal rat calvarial cell cultures. Treatment with 1.5 ng/ml of TGF-β1 significantly increased DNA synthesis and collagen synthesis, whereas treatment with 0.15 ng and 15 ng/ml decreased both parameters (15). The local therapeutic concentrations for TGF-β1 during bone regeneration at local defect site are not completely understood. According to the above investigation, the concentration of released TGF-β1 from the TCP/chitosan microgranules for 28 days is sufficient to induce biological effect.

Considering the isoelectric points of chitosan and TGF-β1 are 4.5 and 8.5, the forming of ionic interaction between chitosan and TGF-β1 is possible, which traps TGF-β1 inside the chitosan. The ionic interaction was corroborated by differential scanning calorimeter (data not shown). The retarded release of TGF-β1 could be attributed by the ionic interaction between chitosan and TGF-β1. The need for the continuous release of TGF-β1 may be due to the short half-life of TGF-β1. The systemic clearance of the TGF-β1 occurs within 5 min at low doses ($\ll$1 μg), whereas it occurs within 11 min at a high doses ($\ll$300 μg) (16). In other studies, the growth factor was adsorbed to HA/TCP ceramics in order to enhance bone formation more efficiently (17,18). However, the maintenance period of the therapeutic range was only up to 1–2 weeks. In contrast, TCP/chitosan microgranules could prolong the therapeutic period for up to 4 weeks. It is anticipated that the steady release of TGF-β1 from the TCP/chitosan microgranules would stimulate osteoblastic cell proliferation and differentiation during the culture period.

The degree of cell proliferation on the chitosan microgranules was measured by a MTT assay instead of counting cell number. The osteoblasts cultured in long term periods produced ECM matrix and differentiated into calcified bone matrix. It is difficult to detach the all adhered cells from the matrix and count cell number using hemocytometer. Rather, MTT assay was chosen as an alternative method to evaluate the proliferation level of cells (19). The reason we chose MTT assay to measure proliferation was that it is based on the fact of binding only to the live mitochondria of live cells. Therefore, increased intensity of absorbance reflects the increased cell numbers.

TCP is a biodegradable material and is believed to have osteocompatibility (20). It was reported that a composite of highly pure porous beta-TCP and bone marrow-derived osteoprogenitor cells could improve bone formation (21). Therefore, TCP/chitosan microgranules can be developed for use as bone substitutes. The absorbance of cells on the TGF-β1 loaded TCP/chitosan microgranules persistently increased with time and was highest among the samples. This result showed that the activity of TGF-β1 released from the microgranules was maintained during the culture period and the sustained release of TGF-β1 was obtained using the TCP/chitosan microgranules.

The cellular behavior on the biomaterials is an important factor when evaluating the biocompatibility of a biomaterial, and cell attachment and spreading is essential for cell growth and differentiation. The cells appeared polygonal, spindle-shaped, and spread well on the TGF-β1 loaded TCP/chitosan microgranules in compared to TGF-β1 unloaded microgranules. Cellular attachment to the bone graft surface is essential for stabilizing the filling material at the bone defect sites. The TCP powders observed at the surface of the microgranule provided a roughness that should assist the attachment of the adjacent cells. Osteoblasts tend to attach more rapidly to surfaces with a rougher microtopography (22). The adhesion of anchorage-dependent cells, such as osteoblasts, is a prerequisite for the successful proliferation of cells (23). By incorporating β-TCP in the chitosan, there is a potential for using this osteoconductive material for regenerating periodontal defects. These results suspected that osteoblasts around the microgranules might freely migrate into the void space when applied in bone defect sites.

The differentiation of osteoblasts was demonstrated by ALPase activity and osteocalcin. The activity of ALPase, which is one of the marker enzymes confirming the differentiating osteoblast functions, was tested during the proliferation study. The ALPase activity of the TGF-β1 loaded TCP/chitosan microgranules was significantly higher that the activity on the TCP/chitosan and chitosan microgranules over a 28-day culture period. Increased ALPase activity dose not present increased cell attachment onto the microgranules because the data (Fig. 3) of increased cell attachment by TGF-β1 loaded microgranules was not in the same magnitude as that of ALPase activity although it has statistically significant. Instead, the increased ALPase activity at 1st day rather indicates the increase metabolic activity of cells triggered by released TGF-β1 resulting in the increased osteocalcin production through 28 days. Osteocalcin is one of the major noncollagenous proteins of the bone matrix and is secreted by the osteoblasts (24). Thus, osteocalcin is widely used as a biochemical marker for bone formation because it is only synthesized by osteoblasts. The controlled release of TGF-β1 from the TCP/chitosan microgranules effectively stimulated the osteoblastic cells. The increase in the ALPase activity and osteocalcin content clearly indicated that the released TGF-β1 enhanced cellular metabolic bone forming activity in addition to the physical supports by the microgranules during culture period. Therefore, TCP/chitosan microgranules were considered to provide as a potential tool for the proliferation of osteoblasts and controlled release of TGF-β1. The bone regeneration *in vivo* is currently being in progress in our laboratory.

CONCLUSIONS

TCP/chitosan microgranules were developed as bone substitutes and tissue engineering scaffolds with a drug releasing function. The TCP/chitosan microgranules were effective for the controlled release of TGF-β1 for 28 days. The microgranules supported the proliferation of seeded osteoblasts as well as their differentiation, as indicated by the ALPase activity and osteocalcin content. The TCP/chitosan based microgranules can be used as potential bone substitutes that combine a drug releasing capacity with an improved bone forming efficacy.

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