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Controlled release of platelet-derived growth factor-BB from chondroitin sulfate–chitosan sponge for guided bone regeneration

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Abstract

Platelet-derived growth factor-BB (PDGF-BB) releasing porous chondroitin-4-sulfate (CS)–chitosan sponge was designed with an aim of controlling growth factor delivery in order to improve bone formation. Porous CS–chitosan sponge was fabricated by freeze drying and crosslinking aqueous CS–chitosan solution. PDGF-BB was incorporated into the CS–chitosan sponge by soaking CS–chitosan sponge into the PDGF-BB solution. CS–chitosan sponge retained a porous structure with a 150–200-μm pore diameter that was suitable for cellular migration and osteoid ingrowth. Release rate of PDGF-BB could be controlled by varying the composition of CS in the sponge or initial loading content of PDGF-BB. CS–chitosan sponge induced increased osteoblast migration and proliferation as compared with chitosan sponge alone. Furthermore, the release of PDGF-BB from CS–chitosan sponge significantly enhanced osteoblast proliferation. These results suggest that PDGF-BB-releasing CS–chitosan sponge may be beneficial to enhance bone cell adaptation and regenerative potential when applied in wound sites.

Keywords: Chondroitin sulfate–chitosan sponge; Bone regeneration; Platelet-derived growth factor-BB; Controlled release

1. Introduction

Porous matrices made of biodegradable polymers have been playing a crucial role as bone substitutes and as tissue-engineered scaffolds in bone regenerative therapy [1–5]. Porous chitosan matrix may be suggested as a potential candidate as a bone regenerative material due to its proper biological and physical properties [5]. Biological activity of chitosan on bone regeneration has been well demonstrated in many reports [6–13]. Chitosan is a biodegradable cationic polysaccharide comprised of *N*-acetylglucosamine residue, and is known to accelerate wound healing and bone formation [6,7]. Chitosan has structural characteristics similar to glucosaminoglycan (GAG) found in extracellular

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matrices (ECM) of several human tissues [7,8]. Previous reports corroborated enhanced wound healing and hemostatic effects of chitosan [9,10].

Implanted chitosan matrix in vertebrates can function as a substrate that enhances the migration and differentiation of specific types of progenitor cells [8–10]. Chitosan matrix has also been noted for its bone-forming ability in in vivo experiment. However, chitosan itself acts physically in bone defects as bone substitutes and tissue-engineered scaffolds and has some limitations in inducing rapid bone regeneration at initial status of bone regeneration. Bone formation after grafting these matrices occurs over a long period (several months or years). Additional functions for chitosan matrices are necessary to shorten the bone forming period and improve bone forming efficacy. In addition to the biomedical applications, chitosan has been demonstrated to be useful in regulating release of bioactive agents [11–13]. Incorporation of bioactive materials such as growth factors may be highly beneficial for obtaining improved bone-forming efficacy.

Growth factors have been utilized in enhancing periodontal regeneration efficacy [14–23]. Growth factors such as platelet-derived growth factor-BB (PDGF-BB) is a potent mitogen and chemotactic factor for cells of mesenchymal origin, including periodontal ligament cells and osteoblasts [14–18]. Enhancement of periodontal tissue regeneration using PDGF-BB has been demonstrated in beagle dogs and monkeys [19–23]. Despite the superior activity of PDGF-BB in tissue regeneration, however, rapid clearance of PDGF-BB due to short half-life (below 4 h in in vivo) resulting in a difficulty in maintaining therapeutic concentrations from injection, has lead to the administration of extremely high doses (above 10 μg) [19–21].

It is essential that a carrier system is developed to maintain PDGF-BB at therapeutic concentration levels (1–10 ng/ml) [19–21] at wound sites for a healing period of up to 4 weeks to obtain enhanced bone regeneration. Several biomaterials have been used as carriers or supports to deliver growth factors in wound sites; however, most of these systems have exhibited burst-like release characteristics [24–26]. Sponge-like porous chitosan matrix (chitosan sponge) has been used to deliver PDGF-BB, but it also showed initial burst effect. The burst release was probably due to ionic repulsion between chitosan and PDGF-BB. To obtain steady release of PDGF-BB, use of additional materials such as chondroitin-4-sulfate (CS) is proposed considering its ionic interaction with PDGF-BB or chitosan. CS is one kind of GAG located in ECM such as bone, skin, and cartilage [27–29]. GAGs are known to facilitate the migration and proliferation of progenitor cells promoting tissue regeneration [27,28]. Since CS is negatively charged, interaction with positively charged chitosan or PDGF-BB is anticipated. Interaction between PDGF-BB and CS is expected to induce prolonged release of PDGF-BB from the sponge. More steady release of PDGF-BB may be obtained by using CS–chitosan system than by using chitosan alone. Also, ionic coacervation between chitosan and CS may cause phase separation followed by an increase in porosity.

The aims of employing CS in this study are: (1) to achieve steady release of PDGF-BB via ionic interaction between PDGF-BB and CS; and (2) to control the porosity of the sponge due to ionic coacervation between chitosan and CS. Fabrication of a CS–chitosan sponge, release kinetics of PDGF-BB, activity of PDGF-BB, and osteoblastic cell proliferation in PDGF-BB loaded CS–chitosan sponge are discussed.

2. Materials and methods

2.1. Materials

Chitosan was purchased from Showa Chemicals (Tokyo, Japan). Chondroitin-4-sulfate was obtained from Sigma–Aldrich (St. Louis, MO, USA). Sodium tripolyphosphate was purchased from Showa Chemicals. PDGF-BB and ^{125}I-labeled PDGF-BB were purchased from Genzyme (Cambridge, MA, USA) and Amersham (UK), respectively. Collagenase, β-glycerol phosphate, L-ascorbic acid were obtained from Sigma–Aldrich. Trypsin–EDTA, fetal bovine serum, α-minimum essential medium were purchased from Gibco (NY, USA). All solvents used were of analytical grade.

2.2. Fabrication of CS–chitosan sponge

Chitosan solution (3%, w/v) in acetic acid was freeze-dried, crosslinked with 5% (w/v) tripolyphos-

phate and freeze-dried again to obtain spongeous matrix. CS was added into the chitosan solution with the ratio of 20–40% to chitosan weight (w/w), then followed by freeze-drying, crosslinking and refreeze-drying process. Constant amount of PDGF-BB solution was added to chitosan sponge, it was kept overnight at 4°C and freeze-dried. PDGF-BB-loaded chitosan sponges were prepared in a plate forms (10×10×1 mm in size, ca. 25 mg by weight) containing 100, 200 and 400 ng of PDGF-BB, respectively. Surface and cross-section of the sponge were examined using scanning electron microscopy (SEM) (Hitachi S-2460N, Tokyo, Japan).

2.3. Release experiments

To investigate the release kinetics of PDGF-BB from the sponge, ^{125}I-labeled PDGF-BB was utilized as a tracer. ^{125}I-Labelled PDGF-BB (5 μCi, Amersham, UK) was diluted with non-radioactive PDGF-BB to reach the radioactivity of 1 μCi. These radioactive mixtures were loaded into the chitosan sponge content of 100, 200 or 400 ng per sponge. Each chitosan sponge was immersed in a glass vial containing pH 7.4 phosphate buffer as releasing medium (10 ml). The sealed vials were placed in a shaking water bath at 37°C and were shaken at a frequency of 15 rpm. At predetermined time intervals over a period of 4 weeks, samples were withdrawn from the vials and replenished with fresh medium. The concentrations of released PDGF-BB in the samples were assayed using a gamma counter (Cobra II, Packard Instrument, CT, USA).

2.4. Chemotactic activity of released PDGF-BB

Chemotactic effects of PDGF-BB on gingival fibroblast cells were tested for both released samples from chitosan sponge and standard PDGF-BB solution. This experiment was based on the methods reported by Adelmann-Grill et al. [30]. In brief, 48-well microchemotaxis, composed of upper and lower compartments divided by polycarbonate membrane filters, was utilized. The lower compartment of each well was filled with released sample of PDGF-BB from the membranes or standard PDGF-BB solution. Gingival fibroblast suspension (1×10^5 cells/ml) in cultural media (DMEM) was placed in the upper compartment and incubated in an atmosphere consisting of 5% CO_2–95% O_2 for 4 h at 37°C. The filter was collected and the cells on the bottom surface of the filter were stained with Diff-Quick staining solution. The amounts of migrated cells were determined by counting the number of nuclei using a light microscope (Olympus Optical, Osaka, Japan).

2.5. In vitro culture of cell–sponge construct

2.5.1. Isolation of fetal rat calvarial osteoblasts

Primary cultures of rat calvarial cells were obtained by sequential enzymatic treatment of embryonic rat calvariae, based on the method described by Bellows et al. [31]. In brief, calvariae were dissected aseptically from 21-day-old Sprague–Dawley rat fetus, and then striped off periosteum and loosely adherent tissue. Calvariae were minced and digested at 37°C in the enzyme mixture of 0.1% collagenase and 0.25% trypsin in 4 mM EDTA with magnetic stirring in Reacti-vial (Pierce, Rockford, IL, USA). After the first 10 min of stirring, the enzyme mixtures containing the released cells were collected and mixed with an equal volume of ice-cold fetal bovine serum (FBS). Fresh enzyme mixture was added to the calvariae fragments, and the process was repeated at 20, 30, 50 and 70 min after the start of the digestion procedure. These five sequential digestions were designated populations I–V. Populations IV and V were gathered and the cells were collected by centrifugation for 10 min at 400×*g*. The cell pellets were resuspended into 35 mm tissue culture dishes at a density of 4×10^4 cells/cm^2 in α-minimum essential medium (MEM) containing 10% FBS and 1% antibiotic–antimycotic solution. Cultures were maintained in a humidified atmosphere consisting of 95% air and 5% CO_2 at 37°C.

2.5.2. Cell seeding into the sponge and culture in vitro

When confluent monolayers were reached, the cells were enzymatically lifted from the dishes using 0.25% trypsin in 4 mM EDTA. The cells were concentrated by centrifuging at 400×*g* for 10 min and resuspended in 10 ml media. Cells were counted using a hemocytometer and diluted to 5×10^7 cells/ml in complete media consisting of α-MEM supplemented with 15% FBS, 1% antibiotic–an-

timycotic solution, 10 mM sodium β-glycerol phosphate, 50 μg/ml L-ascorbic acid and 10^{-7} M dexamethasone. Aliquots of 20 μl of cell suspension were seeded on the top of prewetted chitosan sponges with complete media placed in the wells of 24-well plates (Nunc, Rochester, NY, USA), resulting in a seeding density of 10^5 cells/sponge. The sponge was left undisturbed in an incubator for 3 h at 37°C to allow cell attachment to the sponge, after which an additional 1 ml of complete media was added to each well. Medium was changed every 2–3 days.

2.5.3. Osteoblastic cell proliferation

After 7 days of incubation period, the sponge was gently washed using HBSS to remove any unattached cells and remaining media. The adherent cells were removed from the substrate by incubation in 1 ml of 0.25% trypsin in 4 mM EDTA for 5 min, and then the sponges were thoroughly washed twice using 1 ml of HBSS. Cells in trypsin solution and HBSS were centrifuged together, and then resuspended in fresh HBSS. An aliquot of the resulting cell suspension was counted with a hemocytometer.

Cell morphology on the chitosan sponge was observed histologically. The samples were washed, fixed with glutaraldehyde, and embedded in paraffin. Tissue blocks were sectioned to 5 μm thickness and stained using hematoxylin–eosin to investigate the cell layers within the sponge matrix. The prepared specimens were examined using a light microscope (Olympus Optical).

2.6. Statistical analysis

All measurements were collected in triplicate and expressed as means±standard error ($n=6$). A two-way analysis of variance with post hoc using Tukey at $P<0.05$ was employed to assess the statistical significance of results for all measurements.

3. Results and discussion

3.1. Fabrication of CS–chitosan sponge

Fig. 1 demonstrates the morphology of chitosan sponges. The CS–chitosan sponge demonstrated a three-dimensional porous structure with 150–200 μm pore size and an anastomosing network of chitosan matrix. Incorporation of CS increased the porosity of the sponge (150–200 μm porosity of both surface and sublayer). Ionic interaction between CS and chitosan led to coprecipitation and, consequently, efficient phase separation in the polymer solution. Optimum porosity could thus be obtained by varying CS content in the chitosan sponge. Besides the biological effect of adding CS in respect to bone cell proliferation and maturation into chitosan sponge [29,32–34], CS in chitosan sponge played an important role in regulating the pore structure and porosity. Since bone is a vascular tissue, porosity may be an important factor in providing sufficient space for growth of vascular tissue and invasion of blood vessels around newly formed tissue in the sponge [35]. Osteoblasts (10–30 μm) [36] and osteoids could easily migrate into the chitosan sponge and be expected to properly proliferate within the sponge. In general, it has been considered that osteoblast proliferation and function was enhanced in three-dimensional cultures with spongeous matrices of pore diameter above 100 μm [33].

3.2. Release experiments of PDGF-BB from CS–chitosan sponge

The effect of CS in the chitosan sponge on the release of PDGF-BB is demonstrated in Fig. 2. Initial burst release was observed from chitosan sponge without CS during the first day, followed by slow release up to 6 days followed by leveling off. Rapid release at the initial step and maintenance of proper concentration at the local site could be favored for growth factor delivery [37]. However, consistent stimulation by prolonged release of PDGF-BB was needed to enhance osteoblast cell migration and proliferation within the chitosan sponge. Incorporation of CS was thus required to obtain steady PDGF-BB release. Release of PDGF-BB was efficiently sustained as the content of CS in chitosan sponge increased. Initial burst was also reduced and the following release rate after initial burst was higher and more steady than that of chitosan sponge devoid of CS. Ionic interaction between anionic CS with cationic PDGF-BB (isoelectric point of 9.8) might occur in the sponge, so that dissociation of PDGF-

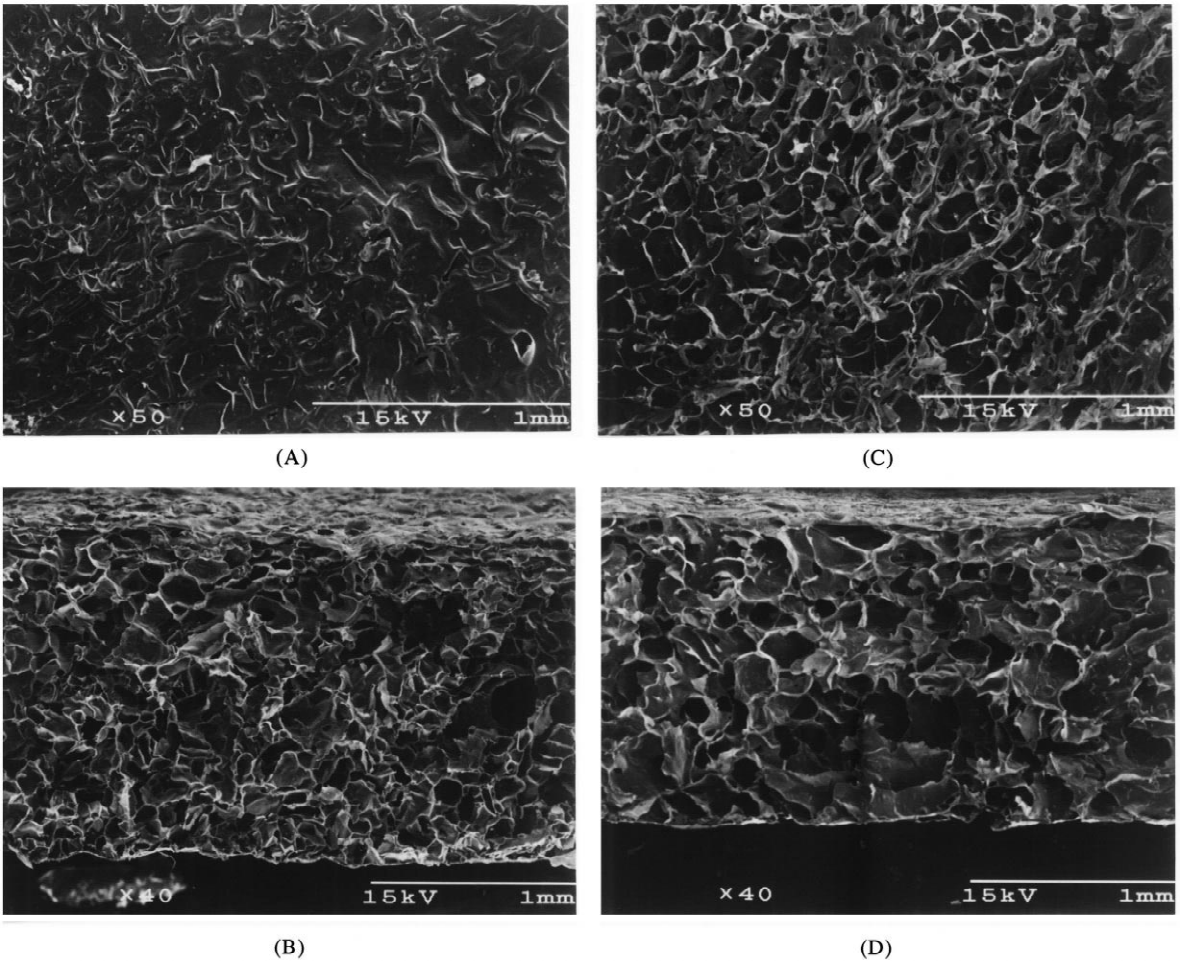

Fig. 1. Scanning electron micrograph of chitosan and CS–chitosan sponges. (A) Surface and (B) cross-section of chitosan sponge. (C) Surface and (D) cross-section of CS–chitosan sponge.

BB from CS controlled the release of PDGF-BB. After an initial release of 90 ng, PDGF-BB was consistently released from CS (20%)–chitosan at a rate of 4–5 ng/day. An initial 90 ng may properly induce osteoblast attachment and proliferation, and the following steady release of PDGF-BB would efficiently stimulate osteoblast migration and proliferation as well. Significant levelling off in PDGF-BB release was not observed from this sponge. PDGF-BB is reported to increase mitogenesis and chemotaxis of bone cells proportionally to a concentration within the 0.1–100 ng/ml range [15,16]. It is anticipated that steady release of PDGF-BB within its therapeutic range achieved in this study would be highly advantageous for osseous regeneration, including osteoblast migration and proliferation over a critical period of time. Bone formation and tissue engineering using PDGF-BB-loaded CS–chitosan sponge is now progressing in our laboratory. Fig. 3 shows the release profile of PDGF-BB from the CS–chitosan sponge varying the loading content. Release rate increased proportionally as the loading content increased. The above results revealed that the release rate of PDGF-BB can be controlled for optimum bone-forming efficacy in tissue regenerative therapy.

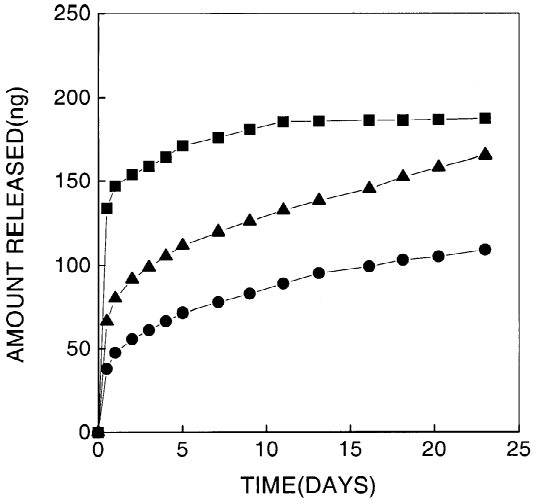

Fig. 2. Release of PDGF-BB from the CS–chitosan sponge. Effects of CS content in chitosan sponge on PDGF-BB release: (■) 0%, (▲) 20% and (●) 40% of CS content. PDGF-BB content in the sponge was fixed at 200 ng/sponge.

3.3. Chemotactic activity of released PDGF-BB from chitosan sponge

The loading procedure of PDGF-BB into the CS–chitosan sponge may cause alteration of the activity of PDGF-BB. In this study, chemotactic activity was measured to examine the stability and the activity of PDGF-BB that was released from the chitosan sponge. Activity of trace amounts of protein in the ng range could be tested by this method. This method is more suitable to determine the activity/stability of trace amounts of potent peptide than sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) [38] and enzyme-linked immunoabsorbant assay (ELISA) [39]. Chemotactic activity of PDGF-BB appears at a concentration above 0.1 ng/ml [15,16]. Since the chitosan sponge was designed to release PDGF-BB with an initial burst of 90 ng and a steady release rate of 1–10 ng/day, this test was well suited for stability measurement.

Chemotaxis test was performed for both PDGF-BB loaded CS–chitosan sponge and standard PDGF-BB. The concentration of PDGF-BB extracted from the CS–chitosan sponge was initially 90 ng/ml, and standard PDGF-BB solution was thus prepared to have the same concentration. In Fig. 4, chemotactic activities in various media are presented as the degree of cell migration. High degrees of cell

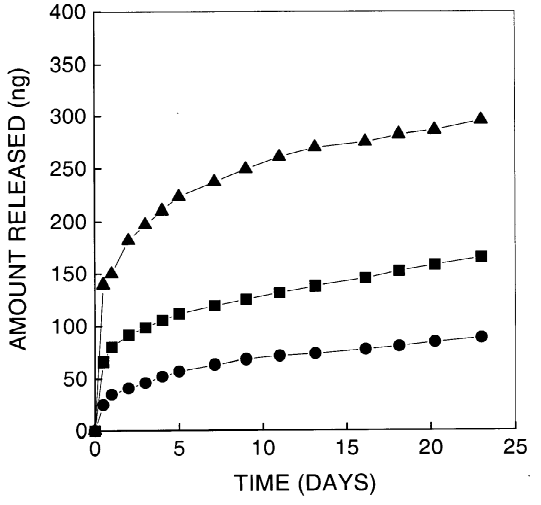

Fig. 3. Release of PDGF-BB from CS–chitosan sponge. Effects of loading content on PDGF-BB release: (●) 100 ng, (■) 200 ng and (▲) 400 ng PDGF-BB in chitosan sponge. CS content in the chitosan sponge was 20%.

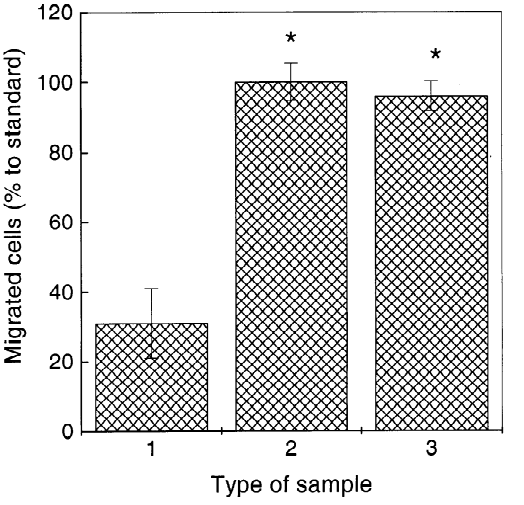

Fig. 4. Chemotactic activity of PDGF-BB loaded in the CS–chitosan sponge to gingival fibroblasts. Chemotactic activity was expressed as the amount of migrated cells to PDGF-BB solutions; (1) α-MEM, (2) standard PDGF-BB and (3) released PDGF-BB from CS–chitosan sponge. Data are presented as the ratio to standard PDGF-BB and as mean±standard error ($n=6$). *$P<0.05$, as compared with that of control, α-MEM. There was no significance between standard PDGF-BB and released PDGF-BB.

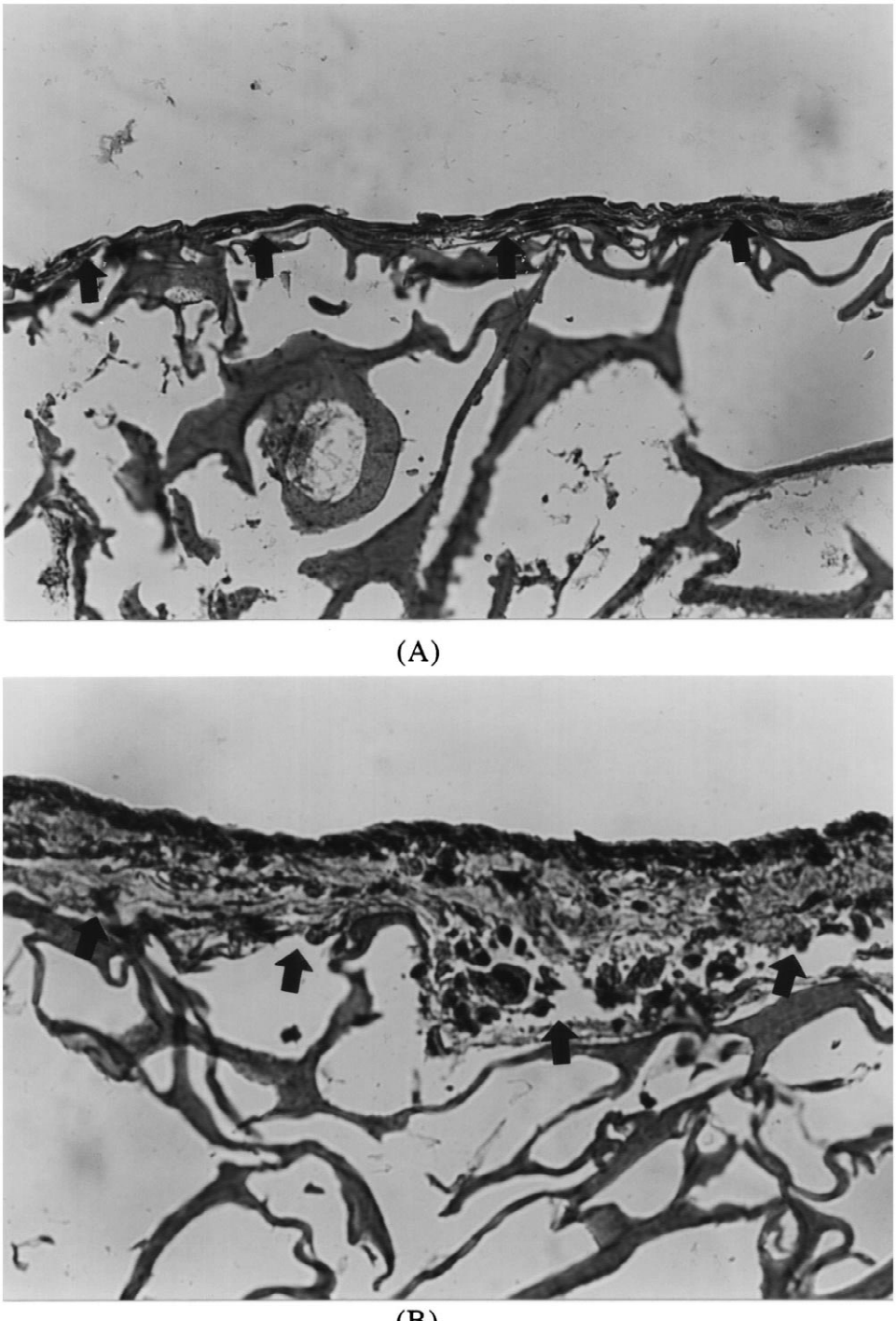

Fig. 5. Light micrograph of chitosan sponge seeded with 1×10^5 cells/mm^2 and cultured for 7 days. Arrows indicate cell layer (hematoxylin–eosin staining; magnification, ×100). (A) CS–chitosan sponge, and (B) PDGF-BB-loaded CS–chitosan sponge.

migration were observed from PDGF-BB solutions in contrast to that from α-MEM control media. In addition, PDGF-BB released from CS–chitosan sponge showed almost the equivalent cell migration activity (98.3%) as compared to standard PDGF-BB solution. This result revealed that PDGF-BB in the sponge maintains its biological activity, and that the fabrication procedure did not affect the stability of PDGF-BB.

3.4. Osteoblast proliferation in CS–chitosan sponge

Fig. 5 shows histological observations of proliferated osteoblasts in the CS–chitosan sponge and PDGF-BB-loaded CS–chitosan sponge at the 7-day culture period. Light microscopic examination indicated that seeded osteoblasts were well attached on and proliferated in a multi-layered fashion over the CS–chitosan sponge (Fig. 5A). All adhered osteoblasts show typical lamellar morphology within the chitosan sponge. When PDGF-BB was incorporated into the CS–chitosan sponge, much enhanced osteoblast proliferation within the sponge was observed. Proliferation was fast and thick multilayer cells formed in PDGF-BB-loaded CS–chitosan sponge (Fig. 5B). This implies that tissue-engineered bone formation may optimally occur with PDGF-BB-loaded CS–chitosan sponge. In addition, it could be anticipated that osteoids and osteoblasts around the sponge can freely migrate into PDGF-BB-loaded CS–chitosan sponge when applied in a bony defect site. Quantitative results determined using hemocytometry indicated that osteoblasts grew more rapidly in the sponge up to 14 days (Fig. 6). The CS–chitosan sponge showed similar or slightly higher density of osteoblasts compared with that of chitosan sponge at 14 days. A significant degree of proliferation of osteoblasts were observed in PDGF-BB-loaded CS–chitosan sponge compared with that of unloaded CS–chitosan sponge.

In vitro experiment revealed that seeded osteoblasts proliferated throughout the 14-day period. Moreover, PDGF-BB-loaded CS–chitosan sponge markedly enhanced cell proliferation rate up to 14 days. Steady stimulation by PDGF-BB from the CS–chitosan sponge might cause rapid cell proliferation within the sponge. These results demonstrated

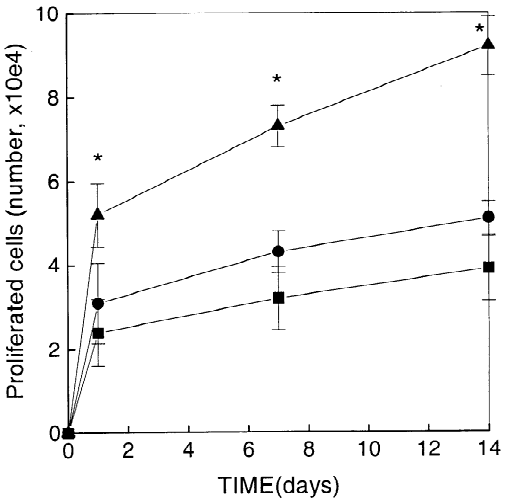

Fig. 6. Proliferation kinetics for osteoblasts cultured in sponge. (■) Chitosan, (●) CS–chitosan and (▲) PDGF-BB-loaded CS–chitosan sponges. Data are presented as mean±standard error ($n=6$). *$P<0.05$, as compared with that of chitosan sponge and CS–chitosan sponge at each time point.

that the CS–chitosan sponge can be utilized as a bone substitute material with the release-regulating ability of PDGF-BB and cellular growth-increasing potential. PDGF-BB-loaded CS–chitosan sponge might be a useful tool in improving bone-regenerating efficacy when used as both bone substitute and tissue engineering scaffold.

4. Conclusion

PDGF-BB-loaded CS–chitosan sponge may potentially control PDGF-BB release and physically serve as a scaffold for osteoblast proliferation. Incorporated CS effectively controlled the release of PDGF-BB from the sponge and increased the porosity of the sponge. PDGF-BB in the sponge retained its biological activity. PDGF-BB released from the sponge enhanced osteoblast migration and proliferation. The PDGF-BB-loaded CS–chitosan sponge might be a valuable modality in bone regenerative therapy.

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