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Growth Factor Releasing Porous Poly (ε-caprolactone)-Chitosan Matrices for Enhanced Bone Regenerative Therapy

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Drug releasing porous poly(ε-caprolactone) (PCL)-chitosan matrices were fabricated for bone regenerative therapy. Porous matrices made of biodegradable polymers have been playing a crucial role as bone substitutes and as tissue-engineered scaffolds in bone regenerative therapy. The matrices provided mechanical support for the developing tissue and enhanced tissue formation by releasing active agent in controlled manner. Chitosan was employed to enhance hydrophilicity and biocompatibility of the PCL matrices. PDGF-BB was incorporated into PCL-chitosan matrices to induce enhanced bone regeneration efficacy. PCL-chitosan matrices retained a porous structure with a 100-200 μm pore diameter that was suitable for cellular migration and osteoid ingrowth. $NaHCO_3$ as a porogen was incorporated 5% ratio to polymer weight to form highly porous scaffolds. PDGF-BB was released from PCL-chitosan matrices maintaining therapeutic concentration for 4 week. High osteoblasts attachment level and proliferation was observed from PCL-chitosan matrices. Scanning electron microscopic examination indicated that cultured osteoblasts showed round form and spread pseudopods after 1 day and showed broad cytoplasmic extension after 14 days. PCL-chitosan matrices promoted bone regeneration and PDGF-BB loaded matrices obtained enhanced bone formation in rat calvarial defect. These results suggested that the PDGF-BB releasing PCL-chitosan porous matrices may be potentially used as tissue engineering scaffolds or bone substitutes with high bone regenerative efficacy.

Key words: Poly(ε-caprolactone), Chitosan, Porous matrix, PDGF-BB, Controlled release, Bone substitutes

INTRODUCTION

Current therapy in bone reconstruction with bone graft materials uses autograft (Langer, R., Vacanti, J.P., 1993) or allograft (Peltzman *et al.*, 1988; Sepe *et al.*, 1978; Pulio *et al.*, 1991) in spite of limitations on these bone transplants exist (Damien *et al.*, 1991). The restrictions include donor site morbidity and donor shortage for autograft and immunologic response and risk of disease transmission for allograft. Numerous bone substitutes using metals, ceramics (such as hydroxyapatite and calcium phosphate), natural polymers (such as collagen (Liu *et al.*, 1999; Du *et al.*, 1999), gelatin) have been developed to promote bone regeneration. These substitutes have demonstrated their periodontal regenerative potency. Also, biodegradable polymers such as poly (glycolic acid) (PGA), poly (L-lactic acid) (PLA), and poly (DL-lactic-co-glycolic acid) (PLGA) have been widely investigated as bone substitutes. However, most of these materials revealed insufficient bone regenerative effects due to their drawbacks including resorption, low biodegradability, poor adaptation, malleability, immunogenecity, weak mechanical strength.

The properties required in ideal bone composite materials are biocompatibility, biodegradability, ability to initiate osteogenesis, and mechanical properties. Biodegradability with biocompatibility and suitable mechanical properties is found only in a small group of materials.

In this study, composite matrices of PCL and chitosan were designed PCL is frequently used for fabrication of porous scaffolds for tissue engineering (Corden *et al.*, 2000; Vandamme *et al.*, 1995). Hydrophobicity of PCL can be modulated by adding hydrophilic chitosan, thus composites of PCL and chitosan enhance biocompatibility and cellular affinity. The resemblance of chitosan to glycosaminoglycans, which are major components of the extracellular matrix of bone and cartilage, may be beneficial to

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cell attachment and promoting function. PDGF-BB was incroporated into PCL-chitosan matrices to improve bone forming efficacy. PDGF-BB is a potent mediator of bone regeneration owing to its stimulating activity of bone cell proliferation and increased tissue attachment of newly formed osteoblasts and fibroblasts to periodontal tissue (Lynch *et al.*, 1991). The fabrication of PCL-chitosan matrices, release kinetics, *in vitro* biodegradation, osteoblast attachment and proliferation will be discussed in this paper.

MATERIALS AND METHODS

Materials

Poly(ε-caprolactone) (m.w. 70,000) was obtained from Dongkook pharm. (Seoul, Korea). Chitosan (70% deacetylated), sodium bicarbonate, span 80 were purchased from Showa Chemicals (Osaka, Japan). PDGF-BB and ^{125}I-labelled 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.

Fabrication of PCL-chitosan matrix

PCL (1g) was dissolved in 6 ml of methylene chloride and mixed with span 80 (5% v/v). 3 mL of 3% chitosan solution in 1% lactic acid were added to the PCL solution, and emulsified for 30s using a vortex mixer (Fisher Vortex Genie 2, Fisher Scientific, USA). For PDGF-BB loading PCL-chitosan matrices, aqueous PDGF-BB solution (50 μl) in place of BSA was added to 3 mL of 3% chitosan solution in 1% lactic acid. PDGF-BB was incorporated into PCL-chitosan mixture in the amount of 10 μg per 1 g PCL. Then, $NaHCO_3$ was mixed to this solution by homogenous dispersing for 15-20s. PCL-chitosan emulsions were frozen in liquid nitrogen for 24 hrs and dried under vacuum for 48 hrs to remove solvents. Drug loaded PCL-chitosan matrix was cut to have a plate form ($0.5 \times 0.5 \times 0.1$ cm in size, ca. 10 mg by weight) for release test.

Scanning electron microscopy

The external and internal morphology of matrices was analyzed by scanning electron microscopy (SEM) (JEOL Model JSM 5200, JEOL Ltd., Tokyo, Japan). The matrices were fixed on supports with carbon-glue, and coated with gold-palladium under an argon atmosphere using a gold sputter module in a high-vacuum evaporator.

Release experiments

To investigate the release kinetics of PDGF-BB from the PCL-chitosan matrix, ^{125}I-labelled PDGF-BB was utilized as a tracer. ^{125}I-labelled PDGF-BB (5 μCi, Amersham Co., UK) was diluted with non-radioactive PDGF-BB to reach the radioactivity of 1 μCi. These radioactive mixtures were incorporated into the PCL-chitosan mixture by the content of 200 ng. Each PCL-chitosan matrix 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 shaken at a frequency of 15 rpm. At predetermined time intervals, samples were withdrawn from the vials and replenished with fresh medium. The concentration of released PDGF-BB in the samples was assayed by using a gamma counter (Cobra II, Packard Instrument Company, CT, USA).

In vitro degradation test

For *in vitro* degradation experiments, 20 mg of PCL-chitosan matrices were immersed into pH 7.4 phosphate buffer (10 ml) containing lysozyme at a concentration of 20 μg/mL. Degradation experiment of these matrices was performed under the same condition employed for the release experiments. At each time point, samples were withdrawn, washed 3 times with buffer and freeze-dried. Weight loss of the samples was measured. The results reported are an average of three measurements.

Osteoblasts proliferation

MG63 cells were counted using hemocytometer and diluted to 5×10^7 cells/ml in complete media consisting α-MEM supplement with 15% FBS, 1% antibiotic-antimycotic 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 onto the top of pre-wetted porous matrix with complete media described above placed in the wells of 24-well plates (Nunc, Rochester, NY, USA) resulting in a seeding density of 10^5 cells/device. The matrices were left undisturbed in an incubator for 3 hrs at 37°C to allow for cell attachment to the matrix, after which an additional 1mL of complete media was added to each well. Medium was changed every 2-3 days. Cultures were maintained in a humidified atmosphere consisting of 95% air and 5% CO_2 at 37°C. At the designated time point, the matrices were gently washed using HBSS to remove 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 and 1 ml of trypan blue for 5 minutes, and then the matrices were thoroughly washed twice by 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 device was observed by using SEM. Cells were fixed with 2.5% glutaraldehyde (Sigma) in 0.1 M PBS (pH 7.4) for 30 min and the rinsed with 0.1 M PBS.

The cells were then stained with 1 ml of cold 1% osmium tetroxide (Polyscience Inc., PA, USA), placed on ice for 30-40 min, then rinsed with deionized water. The fixed and stained cell samples were freeze-dried and sputter-coated with gold and were then examined using a scanning electron microscope.

Bone regeneration

Sprague-Dawley rats (250 mg in average body wt.) were anesthetized by intraperitoneal injection of ketamine (30 mg/kg body wt.). After wiping the surgical site with 0.5% chlorhexidine, a linear incision was formed around head. The soft tissue was reflected and the periosteum was dissected from the site. A craniotomy defect (8 mm in diameter) was formed by a trephine needle in a dental handpiece while supplemented with physiologic saline. After dissecting the calvarial disc, porous PCL-chitosan matrices placed into the defect, and soft tissue and skin were then closed using 5-0 chromic gut and 4-0 silk (Ethicon, Somervile, NJ, USA). PCL-chitosan matrices were prepared for disc form of 8 mm in diameter and 1 mm in thickness. For control, membranes were placed into the defect. Animals were sacrificed 4 weeks post-implantation. Retrieved specimens were fixed in formalin solution, decalcified in 5% trichloroacetic acid solution, and embedded in paraffin. Coronal sections (5 μm in thickness) were sliced and stained using Goldner-Masson trichrome for photomicrography. Microscopical examination was undertaken using Olympus BH-2 light microscope (Olympus Optical Co., Osaka, Japan).

Statistical analysis

Triplicate experiments were performed for each sample, expressed as means and standard deviation. A two tailed unpaired *t*-test was employed to assess the statistical significance ($p<0.05$) of results for all measurements.

RESULTS AND DISCUSSION

Morphology of PCL-chitosan matrix

Fig. 1 showed the morphology of the PCL-chitosan porous matrix. PCL-chitosan matrices demonstrated a three-dimensional porous structure with 100-200 μm pore size. Incorporation of chitosan solution lead to phase separation in the polymer solution which generates porous structure. $NaHCO_3$ was incorporated 5, 10% ratio to polymer weight (w/w) and pore size increase was investigated by SEM. $NaHCO_3$ acted as a gas-generating component and enhanced porosity. In order to foaming to occur, the lactic acid reacted with the $NaHCO_3$ to release CO_2 (Eiselt *et al.*, 2000). Initially, the CO_2 created gas bubbles in the PCL-chitosan mixture and exposure of the mixture to vacuum drew entrapped gas bubbles out and created a highly

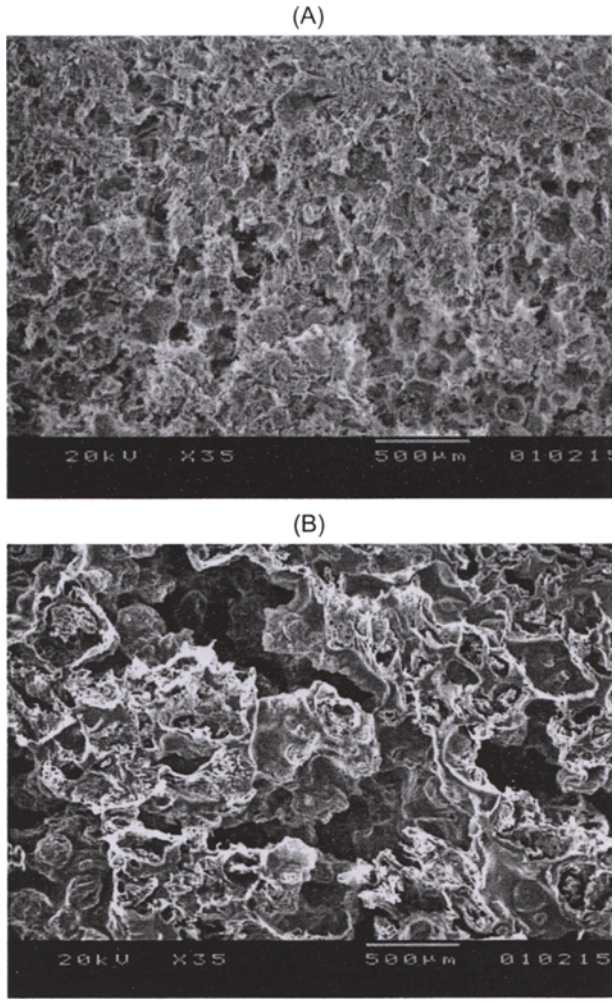

Fig. 1. Scanning electron micrographs of PCL-chitosan matrices. Effect of $NaHCO_3$ on pore size and morphology. (A) PCL-chitosan matrix, $NaHCO_3$ 0% and (B) PCL-chitosan matrix, $NaHCO_3$ 5%.

porous matrices. Optimum porosity could be obtained by varying $NaHCO_3$ content in the PCL-chitosan matrix. Compared to 5% of $NaHCO_3$ loaded matrix, the pore size of 10% of $NaHCO_3$ loaded matrix was not increased (Data not shown).

Porosity may be an important factor in providing sufficient space for growth of newly developed tissue in the matrix. Osteoblasts (10-30 μm) and osteoids could easily migrate into the PCL-chitosan matrix and be expected to properly proliferation within the matrix. It is established that the minimum pore size required for effective bone ingrowth into biodegradable polymer scaffolds (Ishaug *et al.*, 1997) or porous ceramic structures is approximately 100 μm (Klawitter *et al.*, 1971).

Release experiments

Fig. 2 demonstrated the release of PDGF-BB from PCL-chitosan matrices. Initial burst at first day was 63% of

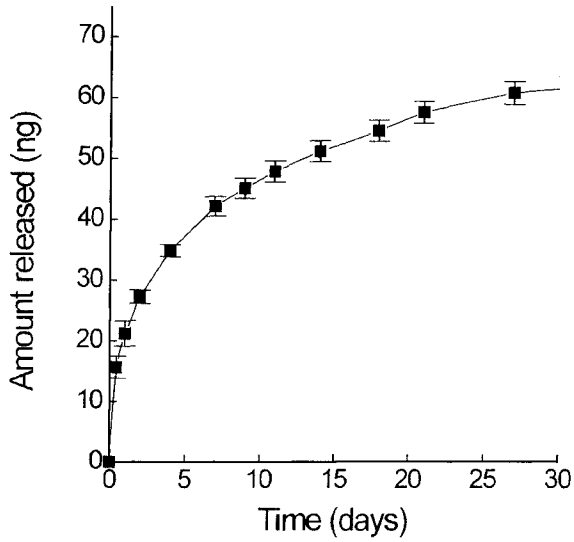

Fig. 2. Release profile of PDGF-BB loaded PCL-chitosan matrices.

loading amount (100 ng) and released at a rate of 1-2 ng/day for 28 days. Rapid release at the initial step and maintenance of proper concentration at the local site could be favored for growth factor delivery. PDGF-BB is reported to increase mitogenesis and chemotaxis of bone cells proportionally to a concentration within the 0.1-100 ng/ml range (Park *et al.*, 1995; Canalis *et al.*, 1995). 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. This combination of controlled drug delivery concept with bone substitute technique can be highly beneficial for bone regeneration.

In vitro biodegradation test

Fig. 3 demonstrates the degradation profile of PCL-chitosan matrices *in vitro*. After 56 days, PCL-chitosan matrices were decreased 34% of their original weight. Degradation of $NaHCO_3$ contained PCL-chitosan matrix was faster than that of $NaHCO_3$ not contained PCL-chitosan matrix. In general, PCL degraded over a longer time period. Though PCL has slow degradation kinetics, increased porosity by adding $NaHCO_3$ might be accelerate degradation rate of PCL-chtosan matrix.

Osteoblasts proliferation

One of the important requirements for bone-substituting materials would be adaptation to a wide variety of bone tissue defects. Cellular attachment and migration over the bone substituting material surface are essential to obtain effective wound filling and bone tissue adaptation. For this reason, the cellular attachment and proliferation were examined onto PCL-chitosan matrix. Fig. 4 demonstrated

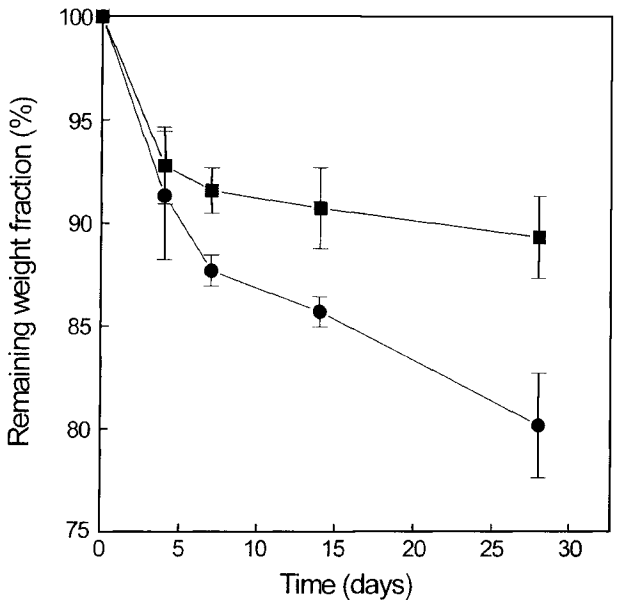

Fig. 3. *In vitro* degradation profile of PCL-chitosan matrices. (■) PCL-chitosan matrix, (●) PCL-chitosan matrix, $NaHCO_3$ 5%.

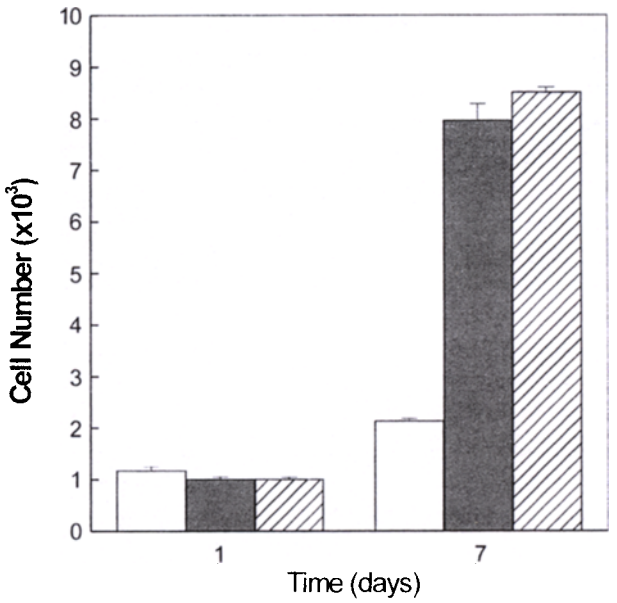

Fig. 4. Number of osteoblasts attached and proliferated to each PCL matrix. ( ) PCL matrix, (■) PCL-chitosan matrix, and ( ) PCL-chitosan matrix, $NaHCO_3$ 5%.

cell attachment and proliferated levels to each PCL matrices after 14 days culture period. In the present study, PCL-chitosan matrix exhibited consistently growing osteoblast proliferation profile. Cell numbers on PCL-chitosan matrix were higher than that on the PCL matrix. It was confirmed that osteoblasts were well attached and proliferated in PCL-chitosan matrix (Fig. 5). Attached cells were rounded and satellite morphology that is typical shape of initially attached osteoblast cells (Fig. 5(A)). After 7 days, osteo-

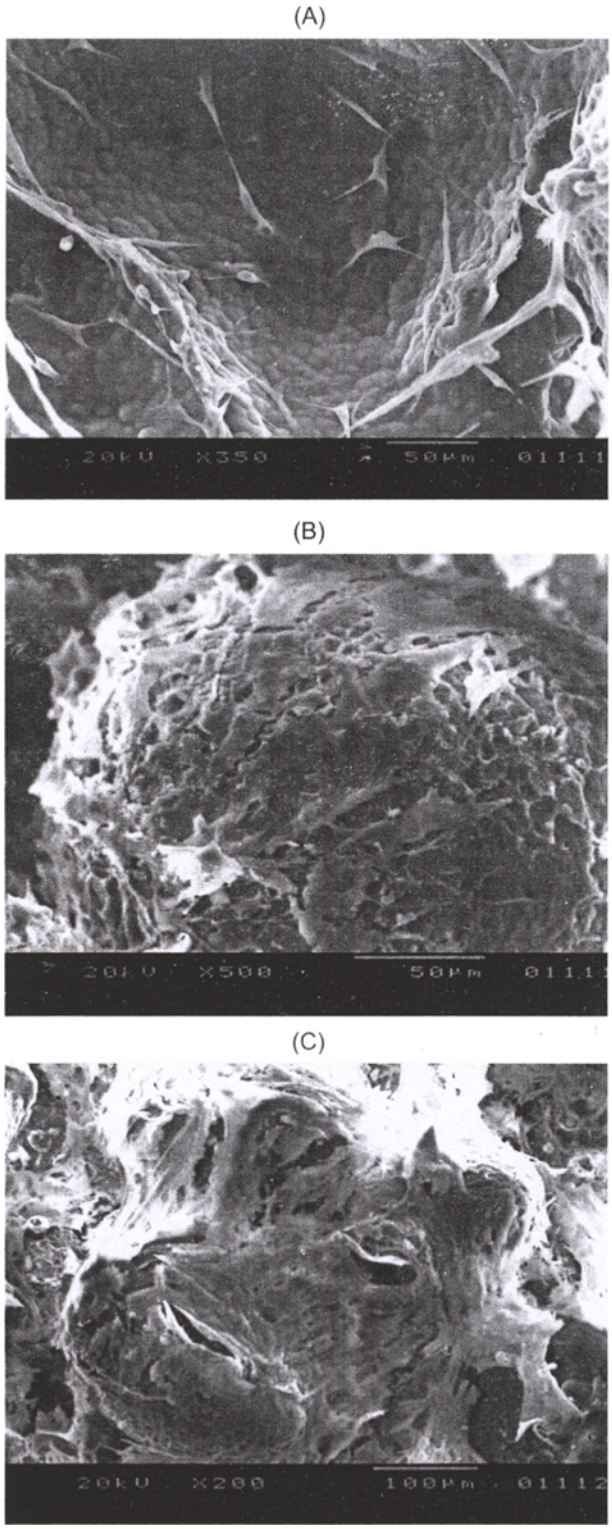

Fig. 5. Scanning electron micrographs of osteoblast on PCL-Chitosan matrices after (A) 1 day, (B) 7 days, and (C) 14 days of culture.

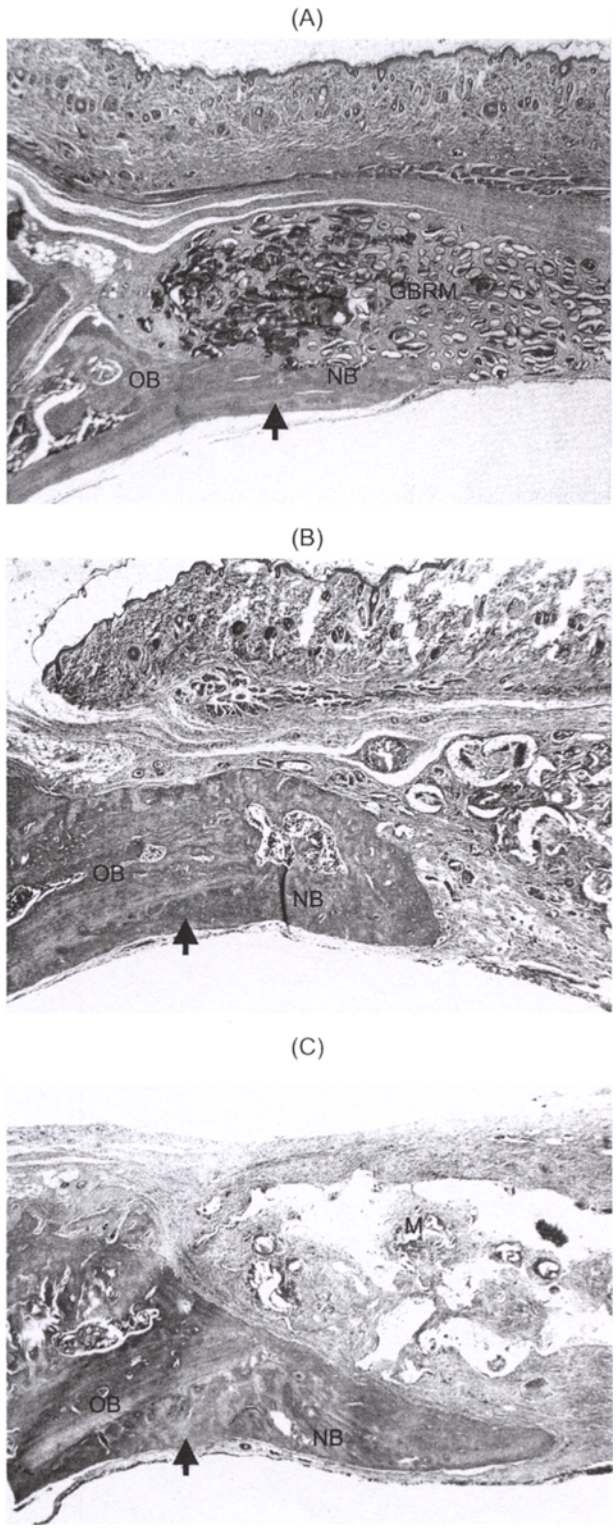

Fig. 6. Histologic evaluation of PCL-chitosan matrices in rat calvarial defects after 8 weeks implantation. (A) No treat, (B) PCL-chitosan matrix, (C) PDGF-BB loaded PCL-chitosan matrix. Edge of original host bone margins was indicated by the arrow (↑). The mark GBR M, M, OB and NB depict GBR membrane, PCL-chitosan matrix, old bone and new bone, respectively (Masson and trichrome staining ×40).

blastic cells assumed a spindle-shaped, elongated and flattened appearance (Fig. 5(B)). After 14 days, the continuous cell layer spread over the surface of the scaffold and

formed osteoids (Fig. 5(C)).

In general polymer-derived scaffolds lack cell-recognition signals, and their hydrophobic property hinders smooth cell seeding. The fact that these polymers do not match the target tissues extracellular matrix well may be a key limitation to the success of bone tissue engineering (Zhang *et al.*, 2001). Chitosan structurally resembles glycosaminoglycans can be a useful modification tool by enhancing hydrophilicity and cell affinity. The unique material properties and ability of chitosan to support viable and functioning osteoblasts make the material an attractive candidate for future use in bone and cartilage tissue engineering. These results demonstrated that PCL-chitosan matrices could be utilized as tissue engineering scaffold.

Bone regeneration

Critical size defect (CSD) is defined as the minimal size defect would not allow spontaneous healing unless treated with regenerative materials and known to be helpful for assessing new bone forming ability of regenerative materials (Schmitz *et al.*, 1986; Matsuda *et al.*, 1992). Circular defect with diameter of 8mm in the rat calvaria was prepared as CSD in this study. Outcomes from treatments were measured at 8 weeks postoperatively.

Fig. 6 demonstrated bone regenerative potential of the porous PCL-chitosan matrix and PDGF-BB loaded PCL-chitosan matrix in rat calvarial defect. In membrane treated defect, membrane prevented soft tissue migration into bone defect and only a limited amount of new bone formation was observed (Fig. 6(A)). In contrast, histological observation confirmed bone regeneration in both matrix treated site. Osteogenesis was increased in PCL-chitosan matrix treated defect (Fig. 6(B)). Bone growth took place centripetally toward the matrices. Porous PCL-chitosan matrices were embedded in newly formed osseous tissue without fibrous tissue invasion. This may be explained by inhibitory effect of chitosan against fibroblasts while stimulating osteoblast activity. Significantly increased bone regeneration capacity was observed in PDGF-BB loaded PCL-chitosan matrix treated defects compared to control (Fig. 6(C)). Bone growth took place centripetally toward the matrix. The potency of PDGF-BB at wound healing and tissue regeneration has been extensively investigated; however, the obstacle to obtain optimal therapeutic efficacy is its short biological half-life. For this reason, extremely high amount of growth factor has been administered to compensate the loss of biologic activity. Therefore, the development of delivery system of PDGF-BB that release PDGF-BB in controlled manner is essential for optimal clinical efficacy. The experimental results suggested that porous PCL-chitosan matrix properly function as local PDGF-BB delivery devices and physical bone substituting materials. Therefore, PDGF-BB loaded PCL-chitosan matrix might be a useful tool to improve bone regeneration efficacy when used as both bone substitute and tissue engineering scaffold.

CONCLUSION

Porous PCL-chitosan matrix composite was constructed as a bone regenerative material due to its proper biological and physical properties. Release rate of incorporated PDGF-BB could be controlled and enhanced bone formation. Three-dimensional porous PCL-chitosan matrices may be attractive candidates for future use in tissue engineering and bone substitutes.

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