Platelet derived growth factor releasing chitosan sponge for periodontal bone regeneration

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Abstract

With an aim of improving bone regeneration, chitosan sponge containing platelet-derived growth factor-BB (PDGF-BB) were developed. For fabrication of chitosan sponge, chitosan solution was freeze-dried, crosslinked and freeze-dried again. PDGF-BB was incorporated into the chitosan sponge by soaking chitosan sponge into the PDGF-BB solution. Release kinetics of PDGF-BB, cell attachment, proliferation capacity and bony regenerative potentials of PDGF-BB-loaded chitosan sponge were investigated. Prepared chitosan sponge retained porous structure with 100 μm pore diameter that was suitable for cellular migration and growth. Release rate of PDGF-BB could be controlled by varying initial loading content of PDGF-BB to obtain optimal therapeutic efficacy. PDGF-BB-loaded chitosan sponge induced significantly high cell attachment and proliferation level, which indicated good cellular adaptability. PDGF-BB-loaded chitosan sponge demonstrated marked increase in new bone formation and rapid calcification. Degradation of the chitosan sponge was proceeded at defect site and subsequently replaced with new bone. Histomorphometric analysis confirmed that PDGF-BB-loaded chitosan sponge significantly induced new bone formation. These results suggested that chitosan sponge and PDGF-BB-loaded chitosan sponge may be beneficial to enhance periodontal bone regeneration. © 1999 Elsevier Science Ltd. All rights reserved.

Keywords: Chitosan sponge; Periodontal bone regeneration; Platelet-derived growth factor-BB; Controlled release

1. Introduction

Periodontal regenerative therapy with bone-substituting materials has gained favorable clinical efficacy by enhancing osseous regeneration in periodontal bony defects [1–9]. As bone-substituting materials, bone powder [1,2], calcium phosphate ceramic [3,4], modified forms of hydroxyapatite (Osteogen®) [5,6], and hard tissue replacement polymer (HTR polymer®) [7–9] have demonstrated their periodontal bony regenerative potency. Bone-substituting materials should fulfill several requirements such as biocompatibility, osteogenecity, malleability, biodegradability [1–3,7]. However, these materials revealed some drawbacks including bone resorption, immune response, disease transmission, low biodegradability, poor adaptation. Several attempts have been made to overcome these problems; use of biodegradable polymers and combination of ceramics with bioactive polymers such as collagen and polylactides [10–12]. These attempts have been aimed at developing new bone substitutes that resemble bone more closely than other materials. However, these techniques have some limitations in inducing bone regeneration within whole therapeutic period. These materials act only as physical scaffolds for bone tissue. Bone formation after grafting these materials occurred over a period of several months or years [1,4,6,7,10]. Additional function to these materials should be imparted to shorten bone forming period and enhancing bone forming efficacy. As an effective approach, drug incorporation within these materials might be suggested to obtain this purpose.

Early healing concept of bone formation by some bioactive agents such as flurbiprofen and tetracycline has been recently suggested in periodontal regeneration [13,14]. Especially, growth factors including platelet-derived growth factor-BB (PDGF-BB), insulin-like

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growth factor (IGF), transforming growth factor-β (TGF) function as modulators of chemotaxis, proliferation, and differentiation of pluripotent cells concerning bone regeneration [15,16]. Overall healing periods might be significantly shortened by using these agents. It would thus be more advantageous for the substituting material to have release-controlling capacity of bioactive agents. This combination of controlled drug delivery concept with bone substitute technique can be highly beneficial for bone regeneration.

In this study, chitosan sponge were developed as an osteoconductive material which induces or stimulates bone formation. Chitosan is a biodegradable cationic polysaccharide composed of *N*-acetylglucosamine residues which is known to accelerate wound healing and bone formation [17]. Many previous reports corroborated enhanced wound healing and hemostatic effect of chitosan [18–20]. In addition to these biomedical applicability, chitosan can regulate release of bioactive agents [21–23]. Platelet-derived growth factor-BB (PDGF-BB) was used in this study. PDGF-BB is reported as a potential mediator of bone regeneration [24]. It stimulates proliferation and differentiation of mesenchymally derived cells including fibroblasts, smooth muscle cells, ligament cells and osteoblasts [24–26]. Also, it has revealed significant neoosteogenic effect in beagle dogs and monkeys [27–29]. However, as yet, extremely high dose of PDGF-BB (above 10 µg/ml) has been applied in clinical trials owing to rapid clearance of PDGF-BB in vivo and inability to maintain therapeutic concentration during implantation period [25–28]. Therefore, controlled PDGF-BB delivery from the sponge may be highly beneficial for the treatment of periodontal disease.

The objective of this study is to develop growth factor releasing chitosan sponge matrices as a osteoconductive materials for bone regeneration. This paper reports on the fabrication of chitosan sponge, release kinetics of growth factor, osteoblast attachment and proliferation and bone regeneration capacity of growth-factor-loaded chitosan sponge.

2. Materials and methods

2.1. Fabrication of chitosan sponge

Chitosan solution was freeze-dried, crosslinked with tripolyphosphate and freeze-dried again to obtain spongeous matrix. Constant amount of PDGF-BB (Genzyme Co., Cambridge, USA) solution was added to chitosan sponge, kept overnight at 4°C and freeze-dried. PDGF-BB-loaded chitosan sponge was prepared to be of plate form ($1 \times 1 \times 0.1$ cm in size, ca. 30 mg by weight) containing 100, 200 and 400 ng of PDGF-BB per each sponge, respectively. Surface and cross-section of the

sponge were examined by using a scanning electron microscope (SEM) (Hitachi Model S-2460N, Hitachi Ltd., Tokyo, Japan). The sponge was cut into small fragments for calvarial defect filling.

2.2. Release experiments

To determine the release kinetics of PDGF-BB from the sponge, ^{125}I-labeled PDGF-BB was utilized as a tracer. Consistent ratio of ^{125}I-labelled PDGF-BB (5 µCi, Amersham Co., UK) was diluted with non-radioactive PDGF-BB to have final radioactivity of 1 µCi. These radioactive mixtures were incorporated into the chitosan sponge by the content of 200–400 ng per each sponge. Each chitosan sponge was immersed in glass vials 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 interval over a period of 4 weeks, 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).

2.3. Isolation of fetal rat calvarial osteoblastic cells

The cell isolation experiment was performed using the method described by Bellows et al. [30]. The calvaria of 17–19 day-old fetal rats (Sprague-Dawley) were obtained and all soft tissue was removed including the periosteum. The frontal and parietal bones were then minced into pieces approximately 2×2 mm using a sterile scalpel. Bone cell isolation was performed with an enzymatic digestion: Dulbecco's modified Eagle's medium (DMEM; Gibco, Grand Island, NY, USA) containing 3 mg/ml dispase and 0.5 mg/ml collagenase (Sigma Chemical Company, St. Louis, MO, USA). Following 20 min exposure, the media and released cells were removed and discarded. The same enzymes were then used in a 3 h digest. Contents of this digest were spun down, then the pellet was resuspended and the resulting cells were incubated at 37°C in an atmosphere containing 5% CO_2, 95% air, with 99% relative humidity. Osteoblasts were maintained in growth medium containing DMEM, 15% heat-inactivated fetal bovine serum (FBS), 1% penicillin–streptomycin, 1% fungizone, HEPES buffer (15 mM), sodium pyruvate (1 mM) and ascorbic acid (5 µg/ml). Isolated cells were characterized by alkaline phosphatase staining and Von Kossa staining for mineralization.

2.4. Osteoblastic cell attachment and proliferation

Osteoblastic cells (ca. 10^5 cells/cm^2) were placed on top of each chitosan sponge. Tissue culture plate (polystyrene) well was used as a control. The cultures were

placed in the incubator for 1 day, and upon removal were washed with phosphate buffered saline (PBS) and trypsinized. Aliquots of the resulting dissociated cell suspensions were counted on a Coulter counter multisizer (Model 0646, Coulter Electronics, Hialeah, FL, USA). Only counts between 8 and 32 μm in diameter were used. Cell proliferation was also determined by cell counts as described above after 1, 3, 7 and 10 days in culture. In this experiment, six replicate samples were examined.

Attached and/or proliferated osteoblastic cells were fixed with 2.5% glutaraldehyde (Sigma, MO, USA) in 0.1 M PBS (pH 7.4) for 30 min and then rinsed with 0.1 M PBS. The cells were stained with 1 ml of cold 1% osmium tetroxide (Polyscience, WI, USA), placed on ice for 30–40 min, rinsed with deionized water, and stored in a deep freezer ($-70°$C). The fixed and stained cell samples were freeze-dried and sputter-coated with gold and were examined by using a SEM.

2.5. Bone regeneration capacity of PDGF-BB-loaded chitosan sponge

In surgical procedure, 24 male Sprague-Dawley rats (250 gm in average body weight) were divided into three groups of eight each. The three groups included untreated sample group, chitosan-sponge-treated group, and PDGF-BB-loaded chitosan-sponge-treated group. Sprague-Dawley rats were anaesthetized by intraperitoneal injection of ketamine 30 mg/kg body weight). A linear incision was formed after wiping the surgical site with 0.5% chlorhexidine. A craniotomy defect (8 mm in diameter) was prepared using a trephine needle in a dental handpieces supplemented with physiologic saline. After dissecting the calvarial disc, chitosan sponge, PDGF-BB-loaded chitosan sponge were placed into the defect, and soft tissues and skins were then closed. Rats were sacrificed 4 weeks after implantation. Retrieved specimens were fixed in formalin solution, decalcified in 5% nitric acid solution and embedded in paraffin. Coronal sections (5 μm in thickness) were prepared and stained with Goldner-Masson trichrome for photomicrography. Microscopical examination was undertaken using Olympus BH-2 light microscope (Olympus Optical Co., Osaka, Japan). Histomorphometric analysis for these specimens was performed using a microscope coupled with a high resolution video camera and a semi-automated image analysis system.

2.6. Statistical analysis

All measurements were collected in six replicate and expressed as means ± standard deviation. A two tailed unpaired *t*-test was employed to assess the statistical significance of results for all measurements.

3. Results and discussion

3.1. Fabrication of chitosan sponge

Prepared chitosan sponge demonstrated porous structure with ca. 100 μm in pore diameter (Fig. 1). Since the average size of bone cells is 10 μm [31], bone cells were expected to easily migrate into the chitosan sponge and to properly proliferate within the sponge. The sponge showed brittle property at dried state, but provided good malleability after being wetted with saline.

3.2. Release of PDGF-BB from chitosan sponge

Fig. 2 demonstrates the release of PDGF-BB from chitosan sponge. Initial burst release was observed with
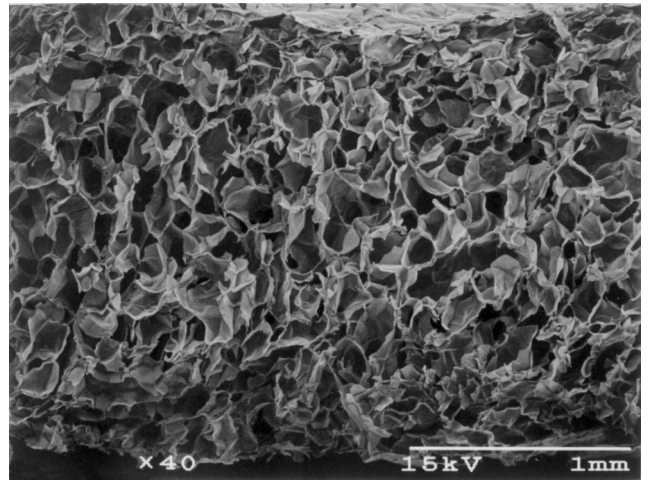

Fig. 1. Scanning electron microscopy of cross-section of a chitosan sponge.
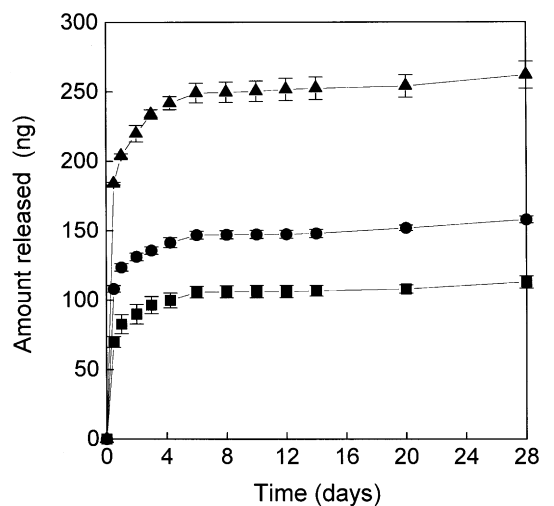

Fig. 2. Release profile of PDGF-BB from chitosan sponge. Effects of initial PDGF-BB loading on release rate: (■) 100 ng loaded, (●) 200 ng loaded, (▲) 400 ng loaded.

rapid release during the first day, followed by sustained release up to 6 days and a levelling off of the release rate. The burst effect indicates rapid water uptake of chitosan sponge and dissolution of the exposed PDGF-BB particles at the surface of the chitosan sponge. As the PDGF-BB-loading content increased, release rate increased correspondingly for the 6 days. Rapid release at the initial step and maintenance of proper concentration at the local site could be favored for growth factor delivery. Furthermore, considering the chemotactic effect of PDGF-BB, the initial burst release of PDGF-BB is perhaps advantageous in bone cell migration, mitogenesis and proliferation. Especially, consistent release of PDGF-BB at the rate of 6 ng/day was observed from 200 ng PDGF-BB-loaded chitosan sponge up to 6 days after initial burst of 100 ng. The release of PDGF-BB after 6 days maintained therapeutic level (1–2 ng) for 3 weeks. PDGF-BB is reported to increase mitogenesis and chemotaxis of bone cells proportionally to its concentration within 0.1–100 ng/ml range [32]. The range of release rate of PDGF-BB is anticipated to stimulate regeneration of bony defects during implantation periods. Also, increased release rate as the increase in loading content revealed that the release rate of PDGF-BB can be controlled for optimum therapeutic efficacy in bone regenerative therapy.

3.3. Osteoblastic cell attachment and proliferation

One of the important requirements for bone-substituting materials would be adaptation to a wide variety of bone tissue defects. Bone-substituting materials appear to have been suited in those case where wound filling could be obtained over the wound site. Cellular attachment and migration over the bone substituting material surface are essential to obtain effective wound filling and bone tissue adaptation [33,34]. For this reason, the authors intended to examine the cellular attachment and proliferation onto chitosan sponge.

Fig. 3(a) shows the SEM of the initial attachment of osteoblastic cells to chitosan sponge 1 day after incubation. Attached cells showed rounded and stellate morphology that is typically observed in initially attached osteoblastic cells [35]. Higher density and amount of cells were observed from PDGF-BB-loaded chitosan sponge (Fig. 3(b)). Alkaline phosphatase activity confirmed the viability of attached osteoblasts over the sponge (data not shown). Fig. 4 demonstrates the proliferation level to chitosan sponge after 10 day incubation period. Chitosan sponge demonstrated increase in bone cell attachment and proliferation to compare with that of control, polystyrene dishes. The surface of polystyrene dish has been known to have good cellular attachment and show rapid cellular confluency in incubation period [33]. The extent in cellular attachment and proliferation implies that chitosan sponge have good cellular
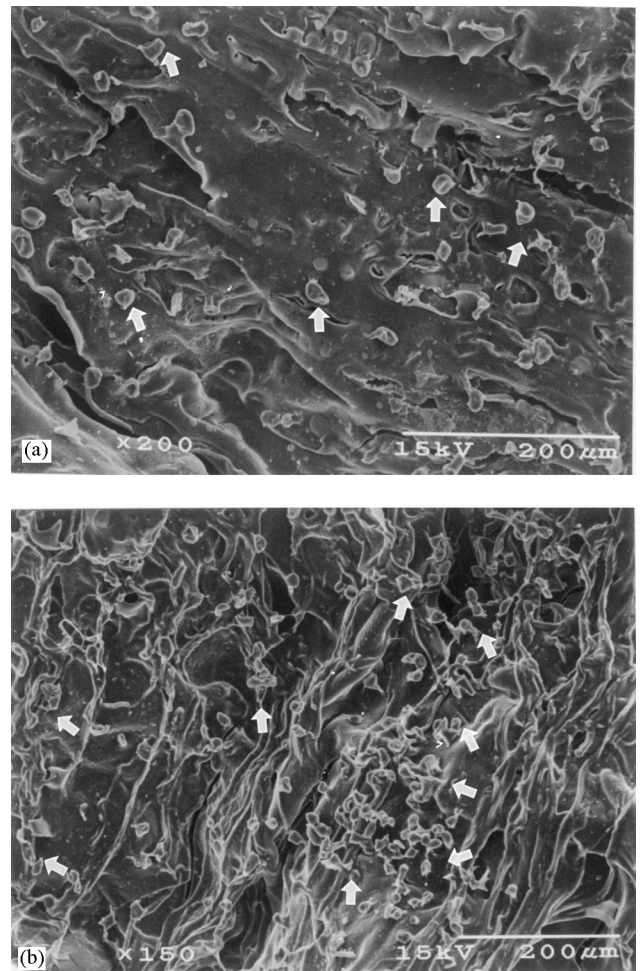

Fig. 3. Scanning electron microscopy of osteoblastic cells cultured on chitosan sponge 1 day after incubation: (a) attached cells onto chitosan sponge and (b) onto PDGF-BB-loaded chitosan sponge.
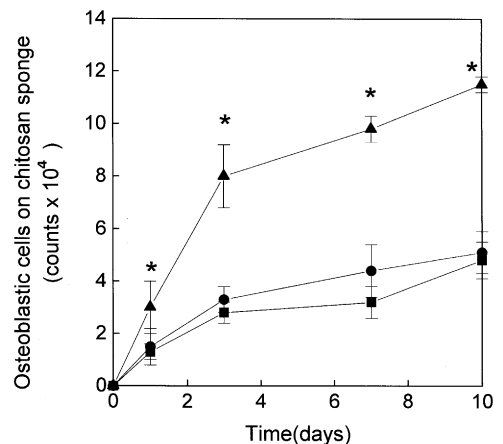

Fig. 4. Proliferation profile of osteoblastic cells cultured on chitosan sponge for 10 day incubation period: (■) control polystyrene dish, (●) chitosan sponge, (▲) PDGF-BB-loaded chitosan sponge. Data are presented as mean ± SD, $N = 6$; *: $P < 0.05$, as compared with chitosan sponge at same time point.

adaptability. Moreover, PDGF-BB-loaded chitosan sponge revealed significantly increased cell attachment and proliferation. High degrees of cell attachment and proliferation were observed from PDGF-BB-loaded chitosan sponge in contrast to that from a control or unloaded chitosan sponge. Controlled release of PDGF-BB from chitosan sponge effectively stimulated osteoblastic cells. PDGF-BB has been known to stimulate proliferation, chemotaxis and collagenous protein synthesis of osteoblastic cells and ligament fibroblasts [26]. Chitosan was reported to enhance the proliferation of progenitor cells [36]. The combination of chitosan and PDGF-BB may be highly beneficial to increase cellular proliferating response.

3.4. Bone regeneration by PDGF-BB-loaded chitosan sponge

Figs. 5–8 show regenerative potential of chitosan sponge and PDGF-BB-loaded chitosan sponge in rat calvarial defect. In chitosan sponge untreated defect at 4 weeks (Fig. 5), connective tissue spanned the host bone margin and defect was completely filled with fibrous connective tissue (CT). Negligible amount of new bone was observed. In contrast, osteogenesis was significantly increased in chitosan sponge treated defect at 4 weeks (Fig. 6). Marked formation of inner and outer bone tables was observed. At this period, degradation of chitosan sponge was proceeded at defect site and degraded site was filled with fibrous tissue and new bone. Bone growth took place centripetally toward the sponge. Although new bone was rapidly formed, complete reunion of new bone was not occurred. Other studies suggested that chitosan interfere with the function of inhibitory cells

Fig. 6. Histologic evaluation of chitosan sponge in rat calvarial defects after 4 weeks implantation ($\times 40$). Chitosan sponge was degraded and indicated by the arrow ($\uparrow$). The marks OB and NB depict old bone and new bone, respectively (Goldner–Masson trichrom staining).
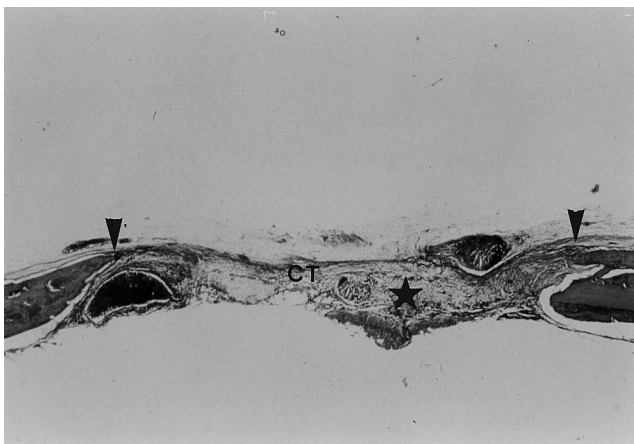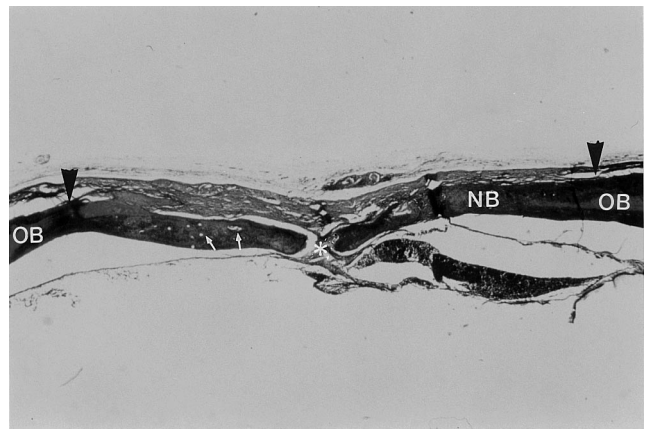

Fig. 5. Histologic evaluation of rat calvarial defects with no treatment of chitosan sponge after 4 weeks implantation. Edge of original host bone margins was indicated by the arrow ($\downarrow$). Fibrous bridges was formed between host bones and indicated by the star ($\bigstar$). The mark CT depicts connective tissue (Goldner–Masson trichrom staining, original magnification $\times 40$).
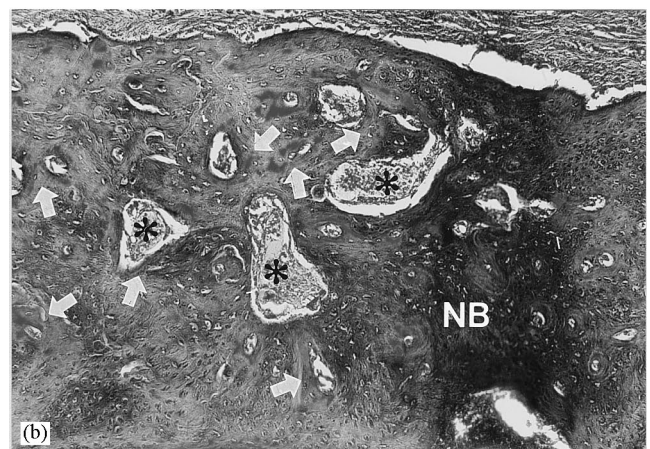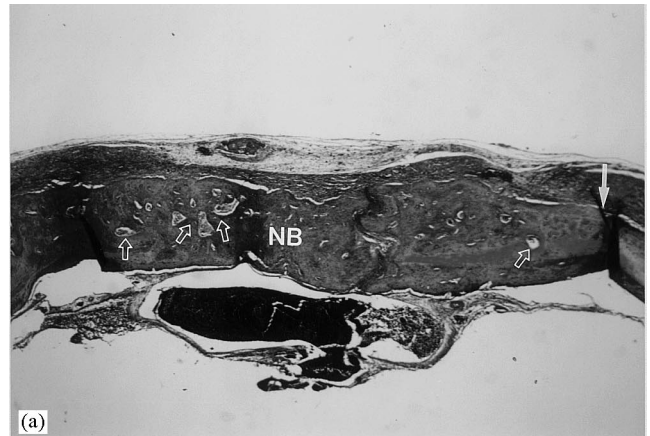
Fig. 7. Histologic evaluation of PDGF-BB-loaded chitosan sponge in rat calvarial defects after 4 weeks implantation. (a) lower magnification ($\times 40$), (b) higher magnification ($\times 200$). Chitosan sponge was degraded and indicated by the asterisk ($*$). The marks OB and NB depict old bone and new bone, respectively (Goldner–Masson trichrom staining).
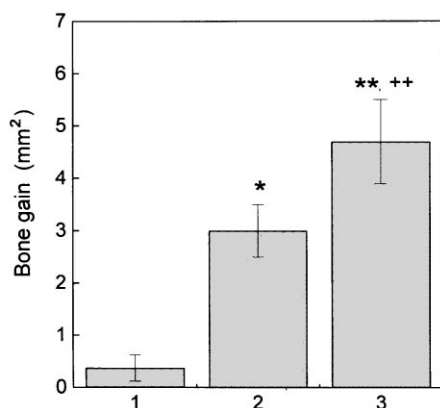
Fig. 8. Histomorphometric data (area of new bone in a defects) for calvarial defects treated with PDGF-BB loaded or unloaded chitosan sponge after 4 weeks implantation. 1: untreated defect, 2: chitosan sponge treated defect, 3: PDGF-BB-loaded chitosan sponge treated defect. Data are presented as mean $\pm$ SD, $N = 6$; $*$,$**$: $P < 0.05$, as compared with control at same time point; $++$: $P < 0.05$, as compared with chitosan sponge at same time point.

including fibroblasts and stimulate the function of osteoprogenitor cells such as osteoblasts, which may enhance osteogenesis [37,38]. Especially, Klokkevold reported that chitosan potentiates the differentiation of osteoprogenitor cells and can facilitate bone formation [34]. In present study, degraded chitosan sponge 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.

Fig. 7 shows the result of bone regenerative capacity of PDGF-BB-loaded chitosan sponge. Thick osteoid tissue and new bone formation was noticeably increased (Fig. 7(a)). Rapid calcification of new bone was observed and new bone formation was still continued nearby the chitosan sponge. Prominent bony bridges consolidating calvarial defects were also observed. Bony contour was completely restored with new bone and neoosteogenesis was still proceeded. Newly formed bone tissue were placed at the site that was previously occupied by the sponge. Higher magnification (Fig. 7(b)) of this specimen shows the diffused neoosteogenesis through the remnants of chitosan sponge. No separation between new bone and the sponge by fibrous encapsulation could be observed. Bone regenerative effects of PDGF-BB-loaded chitosan sponge at 4 weeks are quantitatively shown by histomorphometric data (Fig. 8).

This study aimed to test chitosan sponge carrying PDGF-BB in restoring osseous tissue to a critical sized defect of 8 mm in diameter in the rat calvaria. Osseous regeneration with normal contouring was evident histologically in chitosan sponge treated defect. Furthermore, PDGF-BB-carrying chitosan sponge showed much improved bone regenerative capacity histologically

and histometrically. Histometric data proposed that PDGF-BB-loaded chitosan sponge provide a better response than that of PDGF-BB-unloaded chitosan sponge. 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 [25–28]. 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 chitosan sponge properly function as local PDGF-BB delivery devices and physical bone substituting materials.

4. Conclusion

PDGF-BB-loaded porous chitosan sponge was effective for controlled PDGF-BB release to obtain bone regenerative effects. Chitosan sponge, as an osteoconductive material which induces or stimulates bone formation, showed sufficient cellular adaptability and high bone healing efficacy. Especially, PDGF-BB in combination with chitosan sponge significantly enhanced bone healing and regeneration. PDGF-BB-loaded chitosan sponge may be a valuable tool in periodontal bone regenerative therapy.

Acknowledgements

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