＜原　著＞

Activation of Osteoblastic Differentiation through Calcium Supplementation by Beta-Tricalcium Phosphate Collagen Composite Leading to Initial Inner Bone Formation

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初期内部骨形成をもたらすベータ型リン酸三カルシウムによるカルシウム供給を介した骨芽細胞分化の活性化

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BACKGROUND：Composite comprising bovine dermis-derived soluble collagen sponge and porous beta-tricalcium phosphate（β-TCP）small particles has the potential to combine favorable clinical handling properties, excellent osteoconductivity and biodegradation capability.

PURPOSE：The purpose of this study was to determine cell biological properties of the β-TCP−collagen composite and histologic characteristics during new bone formation using *in vivo* and *in vitro* experimental models.

MATERIALS AND METHODS：The β-TCP−collagen composite or control collagen sponge was implanted in rat calvarial defects with a 5.0-mm diameter. Histologic evaluation was performed at 2, 4 and 8 weeks postoperatively. Rat bone marrow-derived osteoblastic cells were co-cultured with the composite or control collagen sponge on a polystyrene plate without physical contact.

RESULTS：The composite initially underwent osteoblastic cellular invasion and connective-like tissue ingrowth from inside and outside the material together with biodegradation. Eventually, most parts of the composite were replaced with densely packed, thick and mature bone tissue without reduction in the size of the implantation site, whereas only thin, fibrous-like tissue formation was observed in the defect implanted with the control collagen sponge. ALP activity, von Kossa-positive mineralizing nodule area and intracellular calcium level were higher in osteoblastic culture under the composite than under the control collagen sponge.

CONCLUSIONS：The β-TCP−collagen composite allowed initial trabecular formation inside the material followed by replacement of the material with mature bone tissue during the healing process in rat calvarial critical-size bone defect. Moreover, it was suggested that the composite stimulated osteoblastic cellular differentiation through supplementation of calcium. These observations supported the excellent osteoconductivity, biodegradation property and osteostimulating activity of the composite.

Key words：*rat, bone substitute, bone regeneration, calvarial model, critical-size defect*

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Introduction

A wide range of properties is required in the bone substitute used for alveolar augmentation. If the intraosseous defect to be filled is narrow and has a complex geometry, then flexibility will be required. However, pressure from the surrounding soft tissue such as mucosal/gingival flap or Schneider's membrane will mean that the bone substitute must have sufficient mechanical strength to maintain the integrity of the site. In addition, it would be ideal if the bone substitute could eventually be bio-absorbed and replaced by *de novo* bone tissue without reduction in the volume of augmented bone[1,2]. However, the contradictory nature of these various properties has meant that no bone substitute to date has been capable of fulfilling all of them.

Beta-tricalcium phosphate (β-TCP) has a higher water solubility than stoichiometric synthetic hydroxyapatite (HA); this results in its dissolution in tissue fluid and absorption by osteoclasts *in vivo*[2]. The material also has sufficient physical strength to resist the pressure of the soft tissues covering the implantation site and serves as a three-dimensional scaffold for bone regeneration[3]. These characteristics allow slow biodegradation, which is in harmony with bone formation and remodeling[2,4]. Moreover, β-TCP has the potential to function as a source of calcium and phosphate ions for the local tissue as a result of the degradation[5]. Extracellular free ionized calcium (Ca^{2+})taken up by osteoblasts modulates their function[6,7] through increased intracellular Ca^{2+} concentration[8,9].

Recently, a composite material that combines inorganic and organic components has attracted attention as a possible candidate[10~12]. Bio-Oss Collagen®, which consists of a combination of bovine bone mineral particles and a purified procaine collagen matrix sponge, is clinically available and exhibits substantial osteoconductivity and space-making properties. Bovine mineral provides substantial mechanical properties to the composite, whereas the collagen spongy form allows ingrowth of newly formed tissue into the inner structure. However, bovine bone mineral particles have low bio-absorbability. Therefore, artificial biodegradable calcium phosphate has attracted attention as an alternative[10~12].

A composite material fabricated by a combination of a β-TCP with collagen sponge, which is a well-known biocompatible scaffold with excellent flexibility, has been expected to exhibit osteostimulation and biodegradation in conjunction with sufficient mechanical properties to support bone formation. In fact, a β-TCP-collagen composite material completely closed a moderate-sized cortical defect on a beagle dog's tibia with little residual material[13]. The composite with a weight ratio of 4∶1 yielded substantial new bone formation in the extraction socket of a dog to a level equivalent to that acquired by filling with β-TCP granules[14]. A β-TCP-collagen composite material with a weight ratio of 4∶1 far surpassed Bio-Oss Collagen® in healing a critical-sized cortical defect on rat calvarial bone[15]. However, it remains to be fully clarified how the β-TCP-collagen composite material could exhibit such favorable osteostimulating capability without collapse of the implanted region, which is hypothetically attributed to tissue ingrowth into the inner structure and activation of osteoblastic function through supplementation of calcium ions. The purposes of this study were to (1) histologically and histomorphometrically evaluate initial bone formation on β-TCP-collagen composite during the healing of a critical-size defect in rat calvarial bone, and (2) determine whether the β-TCP-collagen composite modulated the function of osteoblastic cells in association with supplementary calcium ions.

Materials and Methods

1. Preparation of β-TCP-collagen composite material

The β-TCP-collagen composite material was prepared by Olympus Terumo Biomaterials Corp. (To-

kyo, Japan). The β-TCP particles were prepared by mechanochemical synthesis. Briefly, a slurry was prepared by wet-mixing calcium hydrogen phosphate and calcium carbonate to a calcium/phosphate molar ratio of 3 : 2. The slurry was then ground down into powder, resulting in the formation of calcium-deficient hydroxyapatite by mechanochemical reaction. After drying and modeling with deflocculant, a β-TCP block was synthesized by sintering the modeled material at 1,050℃. The β-TCP had a calcium/phosphate molar ratio of 3 : 2 with 75% porosity. The block was crushed and sieved into a powder with particles measuring 0.15～0.8 mm.

The collagen component of the β-TCP-collagen composite was prepared according to the methodology previously described in the literature[16]. Briefly, original collagen fibers were obtained from bovine dermal connective tissue. Atelocollagen, which was first subjected to protease (pepsin) treatment to remove teropeptides, was arranged into either fibrous collagen by neutralization with phosphate-buffered saline at 37℃ or heat-denatured collagen by high-temperature heat treatment at 60℃. The β-TCP particles were subsequently blended into a collagen mixture in which the heat-denatured collagen was mixed with the fibrous collagen at a volume ratio of 1 : 9. After lyophilization, the β-TCP-collagen composite was crosslinked into a spongy form by heat dehydration at 110℃ for 6 h. The ratio of β-TCP particles in the composite was adjusted according to the dry-weight ratio, with ratios of 4 : 1, 10 : 1, 20 : 1, and 30 : 1 to verify the validity of the material design. Density of β-TCP content in the composite was evaluated with soft X-ray apparatus (SOFTEX-M 60, Softex Corp., Kanagawa, Japan) at 40 kV with a current of 20 mA for 60 s. In addition, the composites were scanned with a three-dimensional X-ray microscopic CT scanner (micro-CT) (TDM-1000, Yamato Scientific Co., Ltd., Tokyo) at 60 kV with a current of 60 μA and an isotropic resolution of 19.7 μm. Volume rendering images were constructed according to given values of bone mineral density obtained from the

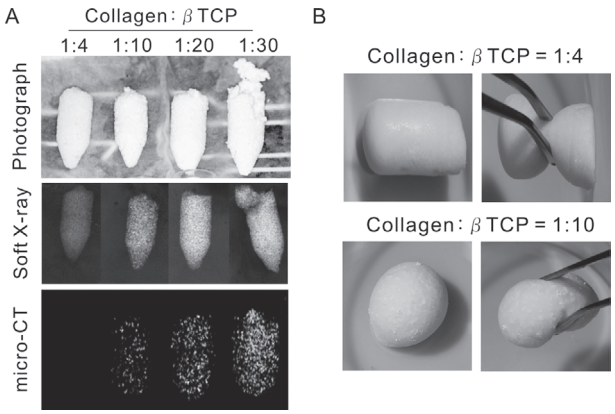

Fig. 1 (A) Photographs, soft X-ray and micro-CT images of the beta-tricalcium phosphate (β-TCP)-collagen composite materials with various β-TCP particle-to-collagen ratios. Shown is the structural collapse of composites with β-TCP particle-collagen ratios of 20 : 1 and 30 : 1. (B) Photographs of β-TCP-collagen composite materials with β-TCP particle-to-collagen ratios of 4 : 1 and 10 : 1 were pinched with forceps exerting equal force.

HA calibration phantom (β-TCP : 719 mg/cm^3, bone : 120 mg/cm^3). The β-TCP particle-to-collagen ratios of 20 : 1 and 30 : 1 resulted in the collapse of the composite structure (Fig. 1A). The soft X-ray images and micro-CT based 3D images clearly showed a gradual reduction of radiopacity in composite according to the reduction in content ratio of β-TCP. The flexibility obtained with a ratio of 4 : 1 was clearly superior to that with a ratio of 10 : 1 (Fig. 1B). This indicated that the composite with a ratio of 4 : 1 had better operability for defect filling. In addition, a previous dog study demonstrated that the β-TCP-collagen composite with a ratio of 4 : 1 preserved the volume for new bone formation against muco-gingival pressure to a level equivalent to that of β-TCP granules alone in the extraction socket model of dog premolars[14]. This indicated that the composite with a ratio of 4 : 1 might have both flexibility and volume maintenance capability, therefore, this ratio was employed in this study. The composite was prepared in the shape of a disc 5.0 mm in diameter and 2.0 mm in thickness. As a control, collagenous sponges without β-TCP (control collagenous sponge) were also pre-

pared in the same shape as that of the composite.

2. Animal surgery

Fourteen-week-old male Sprague-Dawley rats (Charles River Japan, Inc., Kanagawa, Japan) were anesthetized by inhalation of 1.2% isoflurane. The parietal region was shaved and scrubbed with 10% povidone iodine solution and the cranium carefully exposed by skin and periosteal incision. The flat surfaces of the cranium were selected for the creation of critical-size bone defects. Two circular, bicortical cranial bone defects of 5.0 mm in diameter, which were shown to be critical-size defects in a previous experiment[15], were created across the sagittal suture between the coronal and lambdoid sutures with a trephine bur. Care was taken to avoid injury to the dura mater and other deeper tissues. The β-TCP–collagen composite was subsequently placed in one defect and the control collagenous sponge in the other. The defects were then covered with an absorbable collagenous barrier membrane (Bio-Mend®, Zimmer, Inc., IN, USA) prior to placing the skin sutures (Fig. 2A). This study was conducted at the laboratory of Hamri Co., Ltd. (Ibaraki, Japan).

3. Histologic specimen preparation and histologic and histomorphometric analysis

Histologic specimen preparation and histomorphometric analysis were contracted out to the laboratory of Hamri Co., Ltd. The animals were sacrificed at 2, 4, or 8 weeks postoperatively. The cranial bones were removed and fixed in 10% neutral buffered formalin for 1 week and then decalcified in 10% ethylenediaminetetraacetate for 10 days. Decalcified specimens were dehydrated in ascending grades of ethanol and embedded in paraffin wax. Embedded samples were sectioned (3.5-μm serial slices) using a microtome in the sagittal direction of the artificial defect and cranial bone (Fig. 2B). Sections were stained with hematoxylin and eosin. Histologic observation and photography were performed on sections of the middle portion of each cavity using a light microscope (BX51, Olympus, Tokyo, Japan) and a digital still camera (DP72, Olympus) (Fig. 2B). The histologic images were color coded and histomorphologically measured by visual judgment by a disinterested histological expert in the laboratory of Hamri Co., Ltd. according to the following structures in the defect : new bone (green), original bone (red), new tissue apart from new bone (light blue), and remnant β-TCP particles (blue). These measurements represented a percentage of the total defect area, which was sectioned by lines connecting the edges of the defect margin (Fig. 2C) using an image analyzer (ImageJ, NIH, Bethesda, MD, USA). The image analyzer was used on specific areas of the defect as described below.

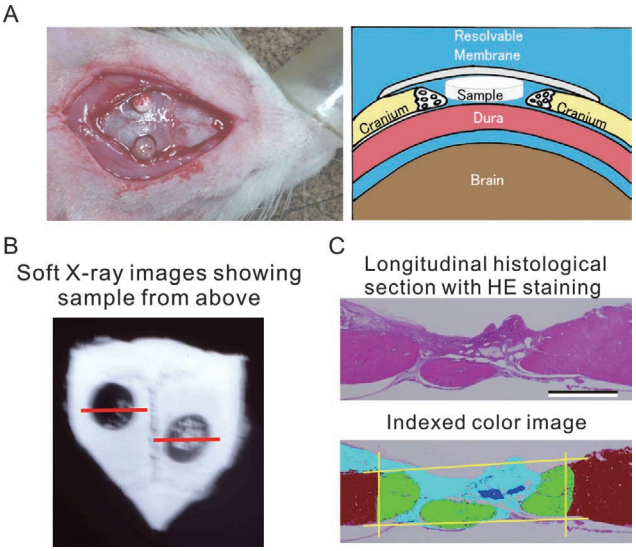

Fig. 2 (A) Left image shows two rat calvarial critical-size defects across a sagittal suture ; the right schema shows the positioning of material in the bone defect. (B) View of sample from above by soft X-ray. (C) Histologic section stained with hematoxylin and eosin in the sagittal direction of an artificial defect ; (upper image) calvarial bone and (lower image) color coding example for histomorphologic measurements based on the following structures in the defect : new bone (green), original bone (red), new tissue apart from new bone (light blue), and remnant β-TCP particles (blue).

New tissue area : Area comprising tissues other than generated bone.

New bone area : Area of newly formed bone. New

bone at the edge of the cortical bone was distinguished from the existing cortical bone according to the difference in course of the lamellar structure.

Remnant defect area：Area devoid of any tissue or material.

Augmented area：Area occupied by new bone, tissue, and remnant-implanted material.

4. Rat osteoblastic culture

To evaluate the effect of the β-TCP−collagen composite on osteoblastic function, a noncontact co-incubation model incorporating a culture insert chamber with a submicron porous bottom (0.04-μm pore filter) (BD Japan, Tokyo, Japan) was used. Bone marrow cells isolated from the femurs of 14-week-old, male, Sprague-Dawley rats were grown in alpha-modified Eagle's medium supplemented with 15% fetal bovine serum, 50 μg/mL ascorbic acid, 10 mM Na-β-glycerophosphate, 10^{-8} M dexamethasone and antibiotic-antimycotic solution according to a previously established method[17]. Cells were incubated in a humidified atmosphere of 95% air and 5% CO_2 at 37℃. When 80% confluent, the cells were detached using 0.25% trypsin, 1 mM EDTA, 4 Na. They were then seeded onto substrates at a density of 3×10^4 cells/cm^2 in 700 μL osteoblastic media on a 24-well polystyrene plate. The chamber containing the composite was 5.0 mm in diameter and 2.0 mm in thickness, and was suspended above the bottom of the culture plate substrate where the cells were seeded (Fig. 5A). The bottom of the chamber was submerged at a distance of 0.8 mm from the bottom of the culture plate. This co-culture model allowed the cells on the culture plate to receive substances from the material in the chamber without physical contact[18]. A polystyrene culture under the chamber with the control collagenous sponge was also prepared as a negative control culture. The cells were cultured for up to 14 days, with 50 μL fresh medium being added every 3 days.

The animals were maintained according to the Guide for the Care and Use of Laboratory Animals (National Institute of Health), and the *in vitro* and *in vivo* animal experiment protocols were approved by the Laboratory Animal Care Committee of Hamri Co., Ltd. (Ibaraki, Japan) (Approval number：10-H009).

5. Cell attachment and density

Initial cell attachment and proliferation were evaluated by measuring the amount of attached and propagated cells on the polystyrene at days 4 and 7. Quantification was performed through colorimetry using a tetrazolium salt (WST-1) (Roche Applied Science, Mannheim, Germany). A culture well was incubated at 37℃ for 4 h with the WST-1 reagent (70 μL) and the amount of formazan produced was measured using a microplate reader at 420 nm.

6. Alkaline phosphatase activity

Alkaline phosphatase (ALP) activity in the osteoblastic cells was examined at day 7 using a colorimetry-based assay. For colorimetry, the cells were rinsed with Dulbecco's phosphate-buffered saline (D-PBS；Life Technologies Japan Ltd., Tokyo, Japan) and incubated at 37℃ for 15 min in the presence of p-nitrophenylphosphate (250 mL) (LabAssay ALP®, Wako Pure Chemicals, Osaka, Japan). ALP activity was evaluated as the amount of nitrophenol released by enzymatic reaction and measured using an ELISA reader at 405 nm.

7. Intracellular calcium level

Intracellular calcium level was evaluated through Fluo-4 fluorescent-based analyses. Cells co-incubated with the β-TCP−collagen composite or control collagenous sponge for 4 days were incubated with a loading buffer consisting of 1.25 mmol Probenecid, an anion transporter inhibitor, and Fluo-4 AM (Calcium Kit Ⅱ-Fluo-4, Dojindo Laboratories, Kumamoto, Japan), the latter of which is rapidly taken up and subject to de-esterification by cells, was used to label intracellular Ca^{2+}, for 60 min at 37℃. The fluorescence of Fluo-4 was determined with an ELISA reader at

an excitation wavelength of 495 nm and an emission wavelength of 518 nm.

8. Mineralization assay

Mineralization capability of the culture at day 21 was examined by image-based assays using von Kossa staining to visualize the mineralized area of the culture. The cells were fixed using a 50% ethanol/18% formaldehyde solution for 30 min. The cultures were then incubated with 5% silver nitrate under UV light for 30 min. Finally, the cultures were washed twice with ddH2O and incubated with 5% sodium thiosulfate solution for 2–5 min. The mineralized nodule area defined as [(stained area/total dish area)×100] (%) was measured using Image J.

9. Statistical analyses

Four animals were used for the *in vivo* histomorphologic analysis ($n=4$). Three independent cultures using different cell batches were evaluated in the *in vitro* culture experiment ($n=3$). The statistical analysis was performed using a commercial computer program (SPSS, Standard Version, SPSS Japan, Tokyo, Japan). The Bonferroni multiple comparison and Student's *t*-test were used after a repeated two-way analysis of variance (two-way ANOVA) to compare defects filled with the β-TCP–collagen composite with those filled with the control collagen sponge. A comparison was also made between healing periods with regard to the percentages of new tissue, new bone, remnant defect, and augmented area. Student's *t*-test was used to evaluate differences in cell number, intracellular calcium level, ALP activity and mineralizing nodule area between osteoblastic cultures on polystyrene under the chamber with the composite and control collagenous sponge. Statistical significance was set at $p<0.05$.

Results

1. Histologic observation of defect healing

At 2 weeks postoperatively, little tissue formation was observed in the defect implanted with collagen sponge alone (Fig. 3A), whereas polygonal, white regions of thick, densely packed fibrous-like tissue showing strong staining for eosin at the edge of the bone defect and around some remnants of the composite were seen in the defect implanted with the β-TCP–collagen composite (Fig. 3B). After 4 weeks of healing, only thin and poor fibrous-like tissue containing a small number of trabeculae-like structures continuous with the edge of the defect was observed in the defect implanted with collagen sponge alone (Fig. 3C). Newly formed trabeculae-like structures with a thickness similar to that of existing bone occupied the main portion of the defect implanted with the composite, connecting the edge of the defect with the remnant composite (Fig. 3D). After 8 weeks of healing, sparse trabecular formation was observed in the defect implanted with collagen alone, with dense but thin fibrous-like tissue occupying the greater portion of the defect (Fig. 3E). Relatively highly mature bone formation was observed only near the edge of the defect. In contrast, the defect implanted with the composite showed a large amount of thick and dense trabecular bone formation not only at the edge, but also at the center, where the remnant composite particles appeared smaller than before (Fig. 3F).

2. Histologic observation of composite degradation process

At 2 weeks postoperatively, higher magnification at the white rectangular region in Fig. 3B revealed that the remnant composite was surrounded by dense connective tissue comprising abundant flat cells with oval nuclei (Fig. 3G, arrowheads). This remnant composite showed partial cellular invasion together with ingrowth of tissue with a string-like structure (Fig. 3G, triangles). After 4 weeks, dense connective-like tissue (Fig. 3H, asterisks) was observed within the remnant composite, with progressive ingrowth of string-like structures throughout. Cubic cells with large round nuclei and distinct nucleoli were attached to the inner surface of the rem-

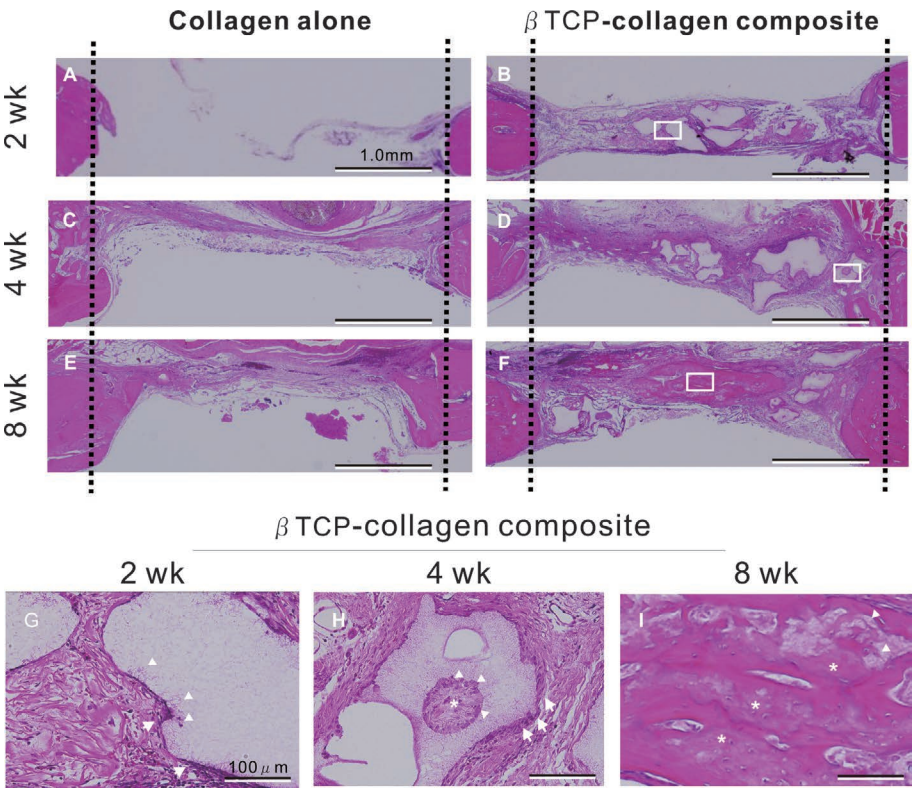

Fig. 3 Representative histologic sections stained with hematoxylin and eosin at 2, 4, and 8 weeks postoperatively.

Lower magnification images (2.0×magnification) showing entire bone defect implanted with control collagen sponge or β-TCP-collagen composite (from A to F), and higher magnification images (20×magnification) showing the center of the bone defect implanted with β-TCP-collagen composite (from G to I) at 2, 4, and 8 weeks postoperatively, corresponding to white rectangular regions in lower magnification images (B, D, F) ; note dense connective tissue comprising abundant flat cells with oval nuclei (G, arrowheads) and cellular invasion together with ingrowth of tissue with a string-like structure into the remnant composite (G, triangles) at 2 weeks postoperatively, dense connective-like tissue within the remnant composite (H, asterisks) and cubic cells with large round nuclei (H, triangles) and a wavy bone-like structure (H, arrowheads) on the inner and outer surfaces of the remnant composite at 4 weeks postoperatively, and replacement of the composite into newly formed trabeculae with a dense lamellar structure containing osteocytes (Fig. 3I, asterisks) and ingrowth of trabecular bone into the composite (I, triangles).

nant composite (Fig. 3H, triangles). In addition, the formation of a wavy bone-like structure was seen on the outer surface of these remnants (Fig. 3H, arrowheads). After 8 weeks, most parts of the composite had been replaced by newly formed trabeculae with a dense lamellar structure and cells in the lacunae (Fig. 3I, asterisks). Ingrowth of trabecular bone into the remnant composite was observed (Fig. 3I, triangles).

3. Histomorphologic evaluation of tissue augmentation in defects

An interaction was observed between the type of implanted material and healing period in areas of new bone ($p<0.05$, two-way ANOVA). At 4 weeks postoperatively, the percentage of new tissue area in the defect implanted with the composite (40%) was twice that in the defect implanted with the control collagen sponge ($p<0.05$, Student's t-test) (Fig. 4A).

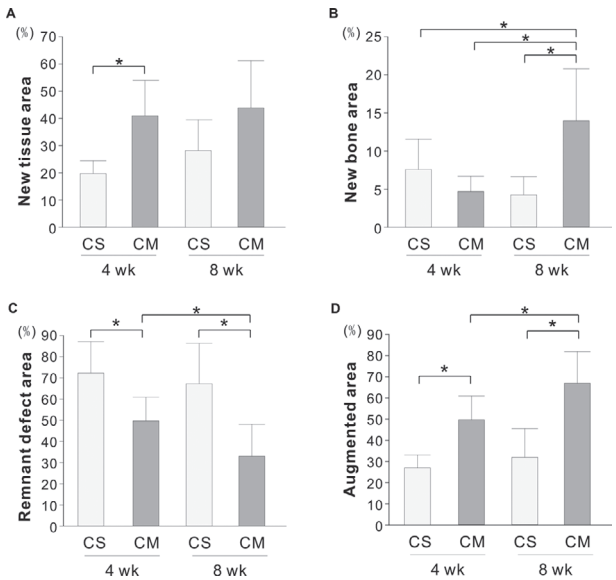

Fig. 4 Histograms showing 4- and 8-week histomorphologic results for the mean percentage of new tissue area (A), new bone area (B), remnant defect area (C), and augmented area (D) in defects implanted with control collagen sponge (CS) or β-TCP–collagen composite (CM). Data represents mean ± SD ($n=4$). *$p<0.05$, significant difference between materials or between healing periods for each material (Bonferroni multiple comparison or Student's *t*-test).

The percentage of new bone area in the defect implanted with composite (15%) was 3 times that in the defect implanted with control collagen sponge at 8 weeks postoperatively ($p<0.05$, Bonferroni) (Fig. 4B). The percentage of remnant defect area was consistently lower in the defect implanted with composite (50% and 30% after 4 and 8 weeks of healing, respectively) than that in the defect implanted with collagen alone (approximately 70% at any point of time) (Fig. 4C). The percentage of augmented area was consistently higher in the defect implanted with composite (50% and 67% after 4 and 8 weeks of healing, respectively) than that in the defect implanted with collagen alone (30% at any point of time) (Fig. 4D). The defect implanted with composite showed no significant difference in new tissue area between 4 and 8 weeks (Fig. 4A), but did exhibit higher values in new bone and augmented areas and lower values in the remnant defect area at 8 weeks than at 4 weeks, respectively (Figs. 4B, C and D). No significant difference was observed in any parameter between 4 and 8 weeks in the defect implanted with the control collagen.

4. Effect of β-TCP–collagen composite on osteoblastic cellular proliferation, osteoblastic differentiation and intracellular calcium level

At day 4 of culture, no significant difference was observed in osteoblastic cell number between the composite and control collagenous sponge within the chamber above the culture. At day 7, however, the cellular number in the culture was lower under the chamber with the composite than that with the control collagen sponge ($p<0.05$, Student's *t*-test) (Fig. 5B). ALP activity at day 7 increased by 27% in the culture under the chamber with the composite compared to that with the control collagen sponge ($p<0.05$) (Fig. 5C). Intracellular calcium level at day 4 was 1.3 times greater in the culture under the chamber with the composite than that with the control collagen sponge ($p<0.05$) (Fig. 5D). At day 21, the culture under the composite exhibited apparently expanded von Kossa-positive mineralizing nodule formation compared to that in the culture under the control collagen sponge (Fig. 5E, left images). The percentage of von Kossa-positive area in the culture at day 21 was higher under the composite than under the control ($p<0.05$) (Fig. 5E).

Discussion

To be of clinical benefit, bone substitute must be capable of supporting tissue-forming cells while preventing the collapse of the region to be augmented. In this rat calvarial critical-size defect model, the composite material substantially contributed to new bone formation while maintaining the integrity of the site to a level equivalent to that of the original cortical bone. On the other hand, only poor and sparse connective tissue formation accompanied by compression of the site from the dura mater side was ob-

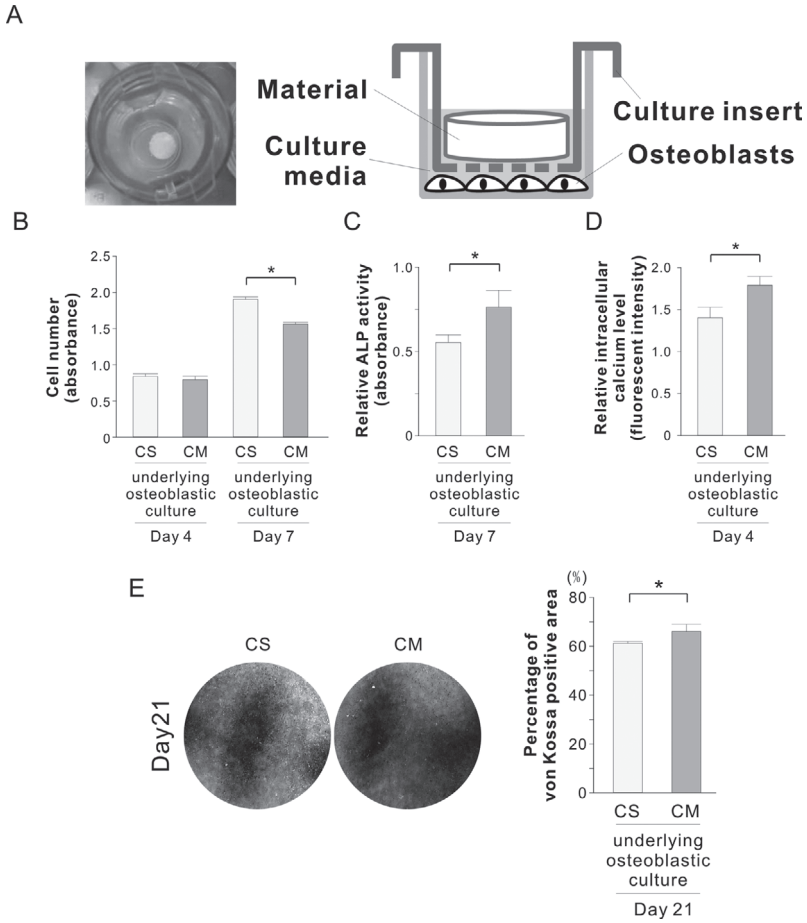

Fig. 5 (A) Schema of co-culture model without physical contact between bone marrow-derived osteoblastic cells and β-TCP–collagen composite or control collagen sponge : cells were seeded onto polystyrene underlying an insert-containing material ; both were separated by a 0.04-μm pore filter suspended at 0.8 mm distance from the polystyrene substrate. Results of WST-1-based cell number at days 4 and 7 (B), colorimetry-based quantification of alkaline phosphatase activity at day 7 (C), fluorescent-based quantification of intracellular calcium level at day 4 (D) and image analysis for mineralizing nodule formation using von Kossa staining at day 21 in osteoblastic culture under β-TCP–collagen composite or control collagen sponge. Data represents mean ± SD ($n=3$). $^{*}p<0.05$, significant difference between materials (Student's *t*-test).

served with collagen alone. Histomorphometric analysis demonstrated that the composite yielded substantial defect filling and bone formation without collapse of the implantation site, whereas the control collagen sponge only induced nonprogressive healing.

The weight ratio of β-TCP to collagen was set as 4 : 1 in this study. In addition to the flexibility, it could be considered that the composite with the weight ratio of 4 : 1 had sufficiently favorable mechanical properties to support the bone forming process against the pressure from surrounding tissue. A previous study showed that the mechanical properties of β-TCP–collagen were strengthened according to the increase of β-TCP content and that a β-TCP–collagen composite with an approximate weight ratio of 3 : 1 had a Young's modulus of 1.8 kPa (approxi-

mately 13.5 mmHg) under wet conditions[13]. The value was higher than the intracranial pressure (approximately 10 mm Hg in rat)[19] to which implant material in the created defect through the calvarium would be subjected. Therefore, the composite used in this study must have exhibited substantial mechanical properties against the surrounding pressure because of higher β-TCP content.

Bone formation in the defect implanted with the composite was characterized not only by bone elongation from the existing bone walls, but also progressive direct bone deposition on the remnant material in the center of the defect. Higher magnification of histologic images revealed invasion by tissue with a string-like structure and flat cells with oval nuclei. Later, at 4 weeks, islands of dense connective-like tissue and cubic cells with large round nuclei and distinct nucleoli were observed within the composite material. These findings indicate that the composite material induced cellular attachment, extracellular matrix synthesis and matrix mineralization by activation of internal and external osteoblastic cells together with inner biodegradation during the initial process of bone formation. The remnant composite was eventually replaced with mature bone tissue. Earlier bone formation inside porous material more rapidly reinforces and stabilizes the material. This may explain how the composite was able to maintain the integrity of the implantation site to a level equivalent to that of existing cortical bone. Measurement of the mechanical strength of the composite during the healing period should be of great interest for future research.

The physicochemical and enzymatic characteristics of the collagen components may have been responsible for promoting tissue formation within the composite material.

The collagen component of this composite is spongiform and has been demonstrated to allow internal infiltration by cells in an *in vitro* model[16]. The collagen components in the composite used in this study included both insoluble fibrous and water-soluble heat-denatured collagen. Heat-denatured collagen undergoes a dissolution reaction with tissue fluids, and fibrous collagen is decomposed by collagenase, which is secreted by many types of cell, including neutrophils, macrophages, endothelial cells, fibroblasts, and osteoblasts[20, 21]. Thus, the degradation of these two types of collagen fiber may have provided the space for cellular invasion and subsequent tissue growth within the composite.

The calvarium mainly comprises cortical bone and a small amount of bone marrow cells, so the source of osteogenic lineage is limited. Other studies, on the other hand, have used different bone sites such as the femur, where periosteal, endosteal, or bone stromal cells and their interactions are in abundance[22]. In addition, the defect size was confirmed as a critical value in our previous study using the same experimental model without defect filling[15]. This indicates that the β-TCP−collagen composite was able to induce excellent osteostimulating property in a biologically severe bone defect in the present study. The present culture results demonstrated that the composite material influenced osteoblastic cellular function under co-culture conditions where there was no physical contact between the material and the cells, which were separated by a 0.04-μm pore filter. Previous culture study using β-TCP−collagen composite showed that the attached cell number and ALP activity of MG63 cell culture were increased on the composite with a weight ratio of 2 : 1 compared to that of the control collagen[23]. This direct culture model did not distinguish the influence of physical contact from the inherent chemical properties of β-TCP on osteoblastic function, which was the reason why co-culture conditions without physical contact between the material and the cells were employed in this study. Osteoblastic cellular number on the polystyrene under the composite was comparable to that under the control collagen at day 4, but was lower at day 7. This indicates that the composite material did not affect cellular attachment, but reduced osteoblastic cellular proliferation. ALP activity and mineraliz-

ing nodule formation in the culture at days 7 and 21 were higher under the composite than under the control collagen. An inverse relationship between proliferative activity and progress of cellular differentiation is a basic cellular biologic rule[24]. Moreover, the intracellular calcium level in the osteoblasts was higher under the composite than under the control collagen. These observations demonstrated that the composite material enhanced osteoblastic differentiation and the intracellular calcium level through the release of certain agents from the inside.

The β-TCP components in the composite had a relatively small diameter (0.15-0.8 mm) and high porosity (75%), which might efficiently facilitate initial dissolution of β-TCP particles by tissue fluid. Extracellular free ionized calcium plays a critical role in regulating osteoblastic cellular function[6,7] by causing an increase in concentration of intracellular free calcium through calcium channels[8,9]. The present findings demonstrate that the composite material enabled the activation of osteoblastic differentiation through supplementation of calcium followed by dissolution of β-TCP components. Moreover, it was demonstrated that the β-TCP-collagen composite aggressively stimulates, rather than just passively supports, the bone-forming capability of osteoblastic cells by enhancing extracellular matrix formation and mineralization. Accurate quantification of the calcium ions released from the composite into local tissue would be needed to determine its pharmacodynamic properties.

Collagen offers excellent biocompatibility, flexibility and workability as a scaffold. It also possesses excellent excipient properties, which facilitate compressing or trimming to adapt to the shape of a defect. Incorporation of granular material within a collagen matrix is more likely to keep the particles at the grafting region than direct loading of particles only. A spongy form of collagen matrix may help to retain blood clots from the host bone ; these clots contain autogenous growth factors and extracellular matrix. As referred to in Fig. 1B, these characteristics should greatly facilitate clinical handling, which may indirectly lead to successful outcomes in bone augmentation procedures. Moreover, spongiform collagen is also favorable in terms of sustained release, which would be advantageous for localized delivery of molecular agents such as recombinant bone morphogenetic protein and inorganic chemical compounds[18,25~27]. Evidence suggests that micron-submicron particles and an excessive local concentration of calcium ions potentially cause biologically adverse effects such as inflammatory response, cell death or cellular dysfunction as a result of activation of immune response[28] or distortion of the metabolism of cellular calcium[27,29]. Collagen-based composite material might reduce the risk of undesirable side effects in local tissue. The present results demonstrated that the β-TCP-collagen composite allowed initial trabecular formation inside the material together with biodegradation followed by replacement of the material with mature bone tissue during the healing process in rat calvarial critical-size bone defects. They also showed that the composite stimulated osteoblastic cellular differentiation through calcium supplementation, which explains the excellent osteoconductivity of the composite. It should be of great interest for future research to determine whether this composite material yields a favorable outcome in bone augmentation prior to implant placement.

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背景：ウシ真皮由来の生体吸収性コラーゲンスポンジと，多孔性ベータ型リン酸三カルシウム（β-TCP）小顆粒からなる複合体は良好な臨床的操作性，優れた骨伝導能と生体内崩壊性を示す可能性がある．

目的：本研究の目的は，動物実験と細胞培養実験モデルを用いて，β-TCP コラーゲン複合体の組織学的，細胞生物学的性質を示すことである．

材料と方法：β-TCP コラーゲン複合体もしくは対照のコラーゲンスポンジを，直径5.0 mm のラット頭蓋骨欠損へ埋入した．術後2, 4および8週に組織学的評価を行った．ラット大腿骨骨髄由来骨芽細胞様細胞を，ポリスチレン培養皿上で物理的接触がないように，β-TCP コラーゲン複合体もしくは対照コラーゲンスポンジとともに共培養した．

結果：治癒初期に，β-TCP コラーゲン複合体は，生体内崩壊とともに，材料内外から骨芽細胞と結合組織様組織の侵入を受けた．最終的に，β-TCP コラーゲン複合体は埋入部位の体積を減少させずに緻密で厚い成熟骨組織に置換された．一方，対照コラーゲンスポンジを埋入した骨欠損内では，薄い線維様組織のみ観察された．β-TCP コラーゲン複合体は，対照コラーゲンスポンジよりも，共培養した骨芽細胞様細胞のアルカリフォスファターゼ活性や von Kossa 陽性石灰化基質産生量，細胞内カルシウムレベルを上昇させた．

結論：β-TCP コラーゲン複合体は，ラット頭蓋骨クリティカル・サイズ骨欠損において，治癒初期には材料内部で骨梁形成をうけ，その後，成熟骨組織に完全に置換された．さらに，β-TCP コラーゲン複合体はカルシウムの供給を通じて骨芽細胞様細胞の分化を刺激することが示唆された．これら所見は，β-TCP コラーゲン複合体の優れた骨伝導性，生体内崩壊性および骨刺激活性を支持するものであった．