

## $\beta$ TCP/Collagen Sponge Composite Enhances the Osteogenic Differentiation of Mesenchymal Stem Cells

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**Abstract:** For the bone regeneration, tissue engineering approach combines cells capable of osteogenic activity with an appropriate scaffold. We developed a biodegradable sponge composite (b-TCP/CS) by combining  $\alpha$ -tricalcium phosphate ( $\alpha$ -TCP) granules and collagen. Human mesenchymal stem cells (MSCs) were cultured within b-TCP/CS scaffolds and collagen sponge (CS) scaffolds, we investigated the alkaline phosphates (ALP) activity and the expression of osteogenic markers using RT-PCR. Furthermore, MSCs- loaded b-TCP/CS and CS were implanted under the back skin of nude mice for 4 and 12 weeks, and then removed for histological evaluation of bone formation. *In vitro* studies demonstrated that b-TCP/CS with MSCs of the ALP activity and the expression of osteogenic markers was higher than CS. *In vivo*, no bone formation was observed in CS, but newly bone formation was observed around  $\alpha$ -TCP granules of b-TCP/CS. These results suggest that b-TCP/CS composites enhance the osteogenic differentiation of MSCs and new bone formation.

**Key words:** bone regeneration;  $\alpha$ -tricalcium phosphate; collagen; sponge composite; mesenchymal stem cells; Osteogenic differentiation

### Introduction

For the repair of bone defects, a tissue engineering approach is to combine cells capable of osteogenic (i.e. bone-forming) activity with an appropriate scaffolding material to stimulate bone regeneration and repair<sup>1</sup>.  $\alpha$ -TCP possess high tissue compatibility and new bone can form directly on these apatites. Moreover, the regular and uniform surface morphologies of a  $\alpha$ -TCP are important factors that affect cell proliferation and differentiation<sup>2,3</sup>. Collagen has been conventionally applied in the clinic, since it shows good biocompatibility and absorbability. Therefore we developed a  $\alpha$ -TCP/collagen sponge composite, designated b-TCP/CS. MSCs are multipotential cells that are capable of differentiating into, at a minimum, osteoblasts, chondrocytes, adipocytes, tenocytes and myoblasts<sup>4</sup>.

In this study, culture of MSCs within b-TCP/CS was performed to determine whether the scaffolds provide an environment that enhances the osteogenic differentiation of MSCs. In addition, we implanted b-TCP/CS with MSCs subcutaneously into the backs of mice, and investigated *in vivo* the potential of b-TCP/CS to sustain the osteogenesis of transplanted MSCs.

### Materials and methods

#### Preparation of b-TCP/CS composites

The atelocollagen preparation obtained consisted of types I (70-80%) and III (20-30%) collagen. The  $\alpha$ -TCP granules used were OS fermions Type G1 (Olympus Optical Co., Tokyo, Japan) of 0.5-1.5 mm in diameter. A 1% (w/v) atelocollagen hydrochloride

solution and  $\alpha$ -TCP granules were mixed at ratios of 0.2 g/mL ( $\alpha$ -TCP granules/collagen solution). The resulting  $\alpha$ -TCP/collagen mixtures were poured into plastic molds, frozen at -80°C and then freeze-dried for 24 h. The freeze-dried  $\alpha$ -TCP/collagen composites resembled sponge-like structures, and were subsequently crosslinked *in vacuo* at 140°C for 24 h. Collagen sponges without  $\beta$ -TCP granules (CS) were used as controls.

#### *In vitro* study of differentiation

MSCs<sup>5</sup> were seeded at  $2 \times 10^5$  cells/ml into b-TCP/CS and CS using syringe with needle, and cultured tissue culture plastic in 24-well culture plate containing DMEM supplemented with 10% FBS and  $3 \times 10^{-6}$  M b-FGF. The cells were incubated for 3 days in a CO<sub>2</sub> incubator at 37°C. The medium was removed from the wells, and the cells were cultured in DMEM containing 10% fetal bovine serum,  $3 \times 10^{-6}$  M b-FGF and osteogenic supplements (10 mM  $\beta$ -glycerophosphate, 50 mg/l ascorbic acid,  $10^{-8}$  M dexamethasone) for 14 days.

Total RNA was isolated from the cell cultures using a Total RNA extraction kit. Single strand cDNA was synthesized from 1  $\mu$ g of total RNA. For quantitative real-time PCR, sequences for the ALP and osteocalcin (OCN) probes and primers were designed. This allowed simultaneous determination of the concentration of both the target message and GAPDH within a single reaction. Thermal cycling and fluorescence detection were performed in a real-time Light Cycler. Quantities of the target were determined by comparison of the signals with a standard curve. Values were normalized to the expression of the GAPDH housekeeping gene.

The lysates obtained from the cells were homogenized with Tris buffer and sonicated 15 min on ice. A fluorimetric assay was performed to ascertain the total amount of DNA. Immediately prior to analysis, Hoechst 33528 solution was added to the sample

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tubes, and the contents were mixed. The fluorescence was then quantified using a fluorescence plate reader. Cell suspension was extracted as described above. ALP activity was assessed colorimetrically using p-nitrophenyl phosphate as the substrate. Conversion of the substrate by ALP was quantified. The amount of ALP activity was normalized by the amount of total DNA in the cell lysate.

#### *In vivo study of bone formation*

MSCs without osteogenic culture were injected at  $2 \times 10^5$  cells/ml into b-TCP/CS and CS using syringe with needle. Cells were allowed to attach to scaffolds for 12 h at 37°C prior to implantation. MSCs-loaded b-TCP/CS and CS were implanted subcutaneously into the backs of 8 nude mice. The wounds were then closed with 4-0 silk sutures. Implants were harvested at 4 and 12 weeks and processed for routine decalcified and subjected to hematoxylin and eosin (H&E) staining for histological examination. All procedures were performed in accordance with the *Guidelines for Animal Experiments of Osaka Dental University*.

### Results

#### *b-TCP/CS composites*

The microstructure of the b-TCP/CS (0.2 g/mL) composite is shown in Fig. 1. The composites were composed of  $\beta$ -TCP granules and a three-dimensional porous structure that created an anastomosing network.

#### *In vitro differentiation*

We assessed the expression of ALP (Fig. 2) and OCN (Fig. 3) genes by quantitative real-time PCR. Expression of both mRNA was detected in all MSCs cultures. The mean value of ALP mRNA was significantly higher in cultures on b-TCP/CS than in CS, but there was no significant difference of OCN mRNA between both materials. Figure 4 shows the results from an assay of ALP activity. The cultures on b-TCP/CS had higher ALP activity than that on CS.

#### *In vivo bone formation*

At 4 weeks post implantation, newly formed vessels were seen at the collagen sponge regions in the MSCs loaded b-TCP/CS. In the control group, collagen sponge was degraded gradually, and fibrous tissue was formed. By 12 weeks after implantation, new bone and osteocytes were dotted along the layers of the lamina bone. Regenerated bone marrow areas were also observed in association with the newly formed bone among the  $\beta$ -TCP granules (Fig 5). But with the implantation of CS groups, collagen sponge was degraded completely, resulting in the formation of fibrous tissue. No bone formation was seen in MSCs loaded CS.

### Discussion

MSC have long been recognized as the source of osteoprogenitor cells, and they can be induced to differentiate with various factors, including ascorbic acid, sodium  $\beta$ -glycerophosphate, and dexamethasone<sup>6</sup>. In the present study, b-TCP/CS enhanced the expression of ALP mRNA and protein compared to CS in the presence of the above differentiation medium. WANG et al.<sup>7</sup> supports the findings of our study that b-TCP not only provides a favorable environment for osteoblast attachment and growth, but also acts as a nucleation site for the deposition of calcium and phosphate ions and the formation of apatite crystals so that an apatite layer is easily formed and bone formation improved. On the other hand, a similar tendency was observed between two materials in the expression of OCN mRNA. The reason is unclear, but *in vitro* studies have demonstrated that ALP is expressed during the proliferative period of extracellular

matrix maturation and that the expression of OCN occurs later during the period of extracellular matrix mineralization<sup>8</sup>). Until 14 days, there was little induction of OCN and the difference of OCN induction in between the two materials might be observed with increasing culturing day.

In the *in vivo* study, new bone formation was present within b-TCP/CS loaded with MSCs. But only fibrous tissue was seen at the implantation of MSCs loaded CS. Collagen sponges are considered to be a useful scaffold matrix for several tissues. And extra cellular matrix molecules such as laminin and fibronectin can be adsorbed onto the collagen scaffold, in order to aid cell migration and proliferation<sup>9</sup>). Furthermore, Type I collagen is the major component of the bone matrix and is useful as a carrier of osteoblasts<sup>10</sup>). But the implantation of CS with MSCs subcutaneously into the backs of mice did not induce bone formation. We developed b-TCP/CS which was composed of  $\beta$ -TCP granules and collagen sponge. This novel biomaterial is not only useful as a carrier of MSCs and growth factors, but also enhances the osteogenic differentiation of MSCs and new bone formation. These results suggest that the osteogenesis of b-TCP/CS with MSCs acted on b-TCP.

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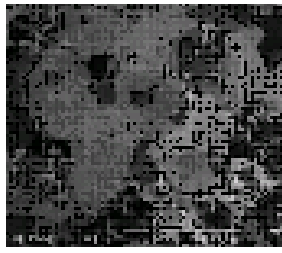


Fig. 1. The microstructure of the b-TCP/CS (0.2 g/mL) composite

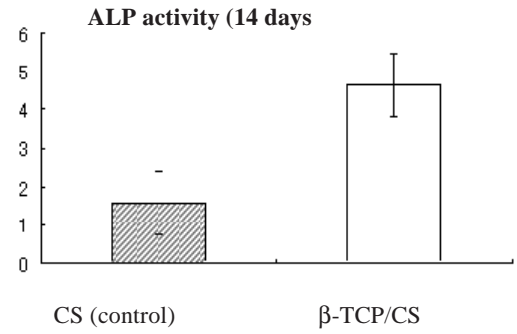


Fig. 4. The assay of ALP activity

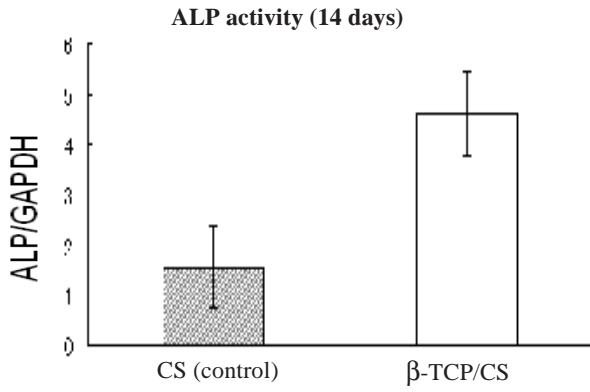


Fig. 2. The expression of ALP gene

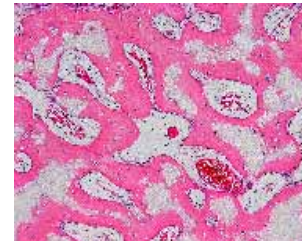


Fig. 5. The implantation of MSCs loaded b-TCP at 12 weeks (H-E stain  $\times 20$ )

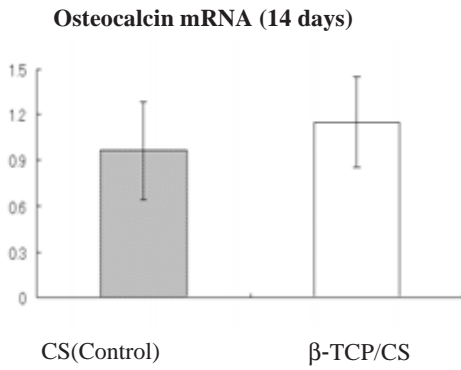


Fig. 3. The expression of OCN gene