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Behavior of β-tricalcium phosphate granules composed of rod-shaped particles in the rat tibia

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Porous spherical granules of β-tricalcium phosphate (β-TCP) composed of rod-shaped particles were prepared via a hydrothermal route. The biological significance of implantation of the spherical β-TCP granules for healing of bone defects was analyzed by implantation into 2 mm diameter and 3 mm deep defects created in tibiae of 9-week-old Wistar rats. Implantation of spherical β-TCP contributed to regeneration of bone tissue. At 2 and 4 weeks after implantation, numerous alkaline phosphatase-positive cells appeared around the implant and newly formed bone. At 8 weeks after implantation, residual implants were mostly embedded in bone tissue. Without implantation, bone defects healed with a much lower amount of bone. In addition, bone marrow adipocytes were considerably fewer in tibiae with implants compared to tibiae without implants at 4 and 8 weeks after the operation. These data suggested that implanted β-TCP granules worked as scaffolds to maintain alkaline phosphatase-positive cells and also resulted in less fatty change of bone marrow.

Key-words : Beta-tricalcium phosphate, Hydrothermal route, Bone substitute, Alkaline phosphatase, Healing of bone defect

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1. Introduction

Biodegradable bone substitutes have been used with the expectation of being replaced with bone. The varied results in previous reports suggest that the prognosis of implanting biodegradable bone substitutes largely depends on the site and size of bone defects, excluding the age and species of the host.[1)–4)] The clinical application of biodegradable bone substitutes in the fibula was reported to result in successful exchange of the implant for bone tissue. The fibula might be one of the most applicable bones for biodegradable bone substitutes,[5)] possibly because the fibula is a slender long bone which supports the body weight with the tibia and has an active metabolism, but the reason remains unclear at the cellular level. It may be partly because of a difference in the number and activity of osteoblasts or osteoblast progenitor cells in the implanted portion. Biodegradable bone substitutes implanted into large defects in other bones are usually difficult to replace with bone tissue.[3),4),6)] This may also be explained by an insufficiency of osteoblasts or osteoblast progenitor cells.

We recently developed porous beta-tricalcium phosphate (β-TCP) ceramic, which was constructed of micrometer-sized rod-shaped particles via a hydrothermal route.[7)] This β-TCP ceramic demonstrated favorable biodegradation and replacement by bone tissue in bone defects created at the distal end of rabbit femurs.[8)] When β-TCP composed of rod-shaped particles was implanted, hard tissue volume was significantly higher than that of animals implanted with β-TCP composed of micrometer-sized globular-shaped particles prepared by a normal sintering method. These data suggested that microstructure affects the biological behavior of β-TCP when implanted into bone defects.

In order to clarify the biological significance of microstructure on healing of bone defects, it is important to examine the influence of implantation of biodegradable bone substitutes not only on bone tissue, but also on bone marrow cells, which remains unclear. In this study, we implanted spherical granules of β-TCP composed of rod-shaped particles into bone defects created in rat tibiae and analyzed the histological findings focusing not only on bone, but also on bone marrow cells compared with healing of bone defects without implantation.

2. Materials and methods

2.1 Preparation and characterization of ceramic granules

Spherical β-TCP composed of rod-shaped particles was prepared by our unique methods as follows.[9)] Briefly, powder of α-tricalcium phosphate [α-$Ca_3(PO_4)_2$: α-TCP; Taihei Chemical Ind., Japan] was mixed with 10% gelatin (Wako Pure Chemical Ind., Japan) solution. An aqueous slurry of α-TCP and gelatin was dropped into vegetable oil stirred at 70°C. The oil was then chilled on ice to 4°C with stirring and spherical α-TCP/gelatine granules formed. These granules were separated from the oil, washed with ethanol and dried in air. To remove organic matter and maintain the crystal phase of α-TCP, the granules were heated at 1200°C for 5 min. The resultant α-TCP granules were then placed in a 50 cm^3 autoclave (**Fig. 1**) with 10 cm^3 water at 160°C under saturated water vapour pressure for 10 h, and then the granules were heated at 900°C for 3 h in air to form β-TCP. Synthesized

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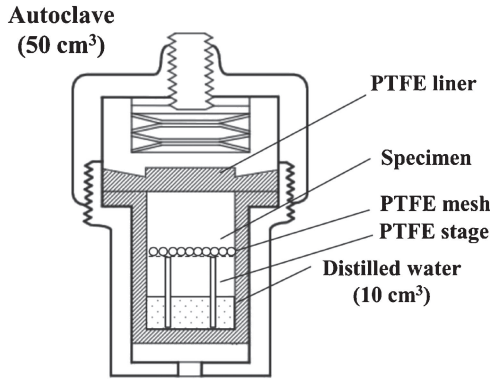

Fig. 1. Schematic illustration of apparatus for hydrothermal treatment.

Fig. 2. Created bone defect and implanted granules in the tibia.

spherical β-TCP granules were sieved, and the granules with 0.3–0.6 mm diameters were used for experiments. The produced phases were identified by powder X-ray diffractometry with graphite-monochromatized Cu Kα radiation, operating at 40 kV and 40 mA (XRD; RINT-2200VL; Rigaku, Japan). The microstructure of specimens was observed using a scanning electron microscope (SEM; JSM-T300; JEOL, Japan). Pore volume and the distribution of pore diameter were measured by mercury intrusion porosimetry (MIP; Poremaster 33P; Quantachrome Co., USA). Specific surface area of samples was measured by the BET method (Autosorb-1, AS-1MP/XP, SYSMEX Co., Japan).

2.2 Animals and operative procedures

Thirty-six female 9-week-old Wistar rats were anesthetized with an intraperitoneal injection of ketamine (40 mg/kg body weight) and xylazine (3 mg/kg body weight) before surgery. A dead-end bone defect 2 mm in diameter and 3 mm in depth was created in the medial cortex of the tibia just distal to the epiphyseal growth plate using a Kirschner's wire.[10] The orientation of the defect was perpendicular to the sagittal axis of the tibia. The defect was irrigated with saline, 15 mg β-TCP granules were implanted into the defect, and the wound was sutured layer by layer (**Fig. 2**). Defects without implantation created in other animals were used as the control. Two, 4 and 8 weeks after the operation, animals were euthanized, the affected bones were resected and undecalcified tissue sections were made from the resected bones. Animal rearing and experiments were performed at the Biomedical Research Center, Center for Frontier Life Sciences, Nagasaki University, following the Guidelines for Animal Experimentation of Nagasaki University (Approval No. 0703010564).

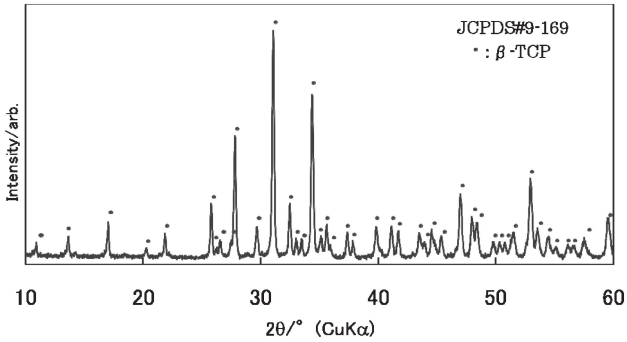

Fig. 3. XRD pattern of spherical β-TCP granules used in this study.

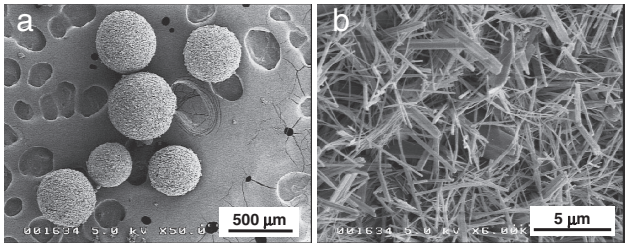

Fig. 4. SEM photographs of spherical β-TCP granules used in this study. Overview (a) and microstructure (b). Microstructure shows unique rod-shaped particles of β-TCP prepared via a hydrothermal route.

2.3 Histological analyses

All harvested tissue specimens were fixed in 4% formaldehyde in 0.1 M phosphate buffer (pH 7.2), embedded in 2-hydroxyethyl methacrylate/methyl methacrylate/2-hydroxyethyl acrylate mixed resin, and 3 μm thick sections were prepared by adherent sectioning using Cryofilm Type I (Finetech, Japan).[8] These sections were stained by the Giemsa method or histochemically stained for alkaline phosphatase (ALP) activity or tartrate-resistant acid phosphatase (TRAP) activity. ALP activity was stained as described previously using 0.2 M Tris–HCl buffer (pH 8.5), and staining for TRAP activity was performed using 0.1 M sodium acetate–acetic acid buffer (pH 5.0).[11] In this study, fast blue BB salt (F3378; Sigma, St. Louis, MO) and fast red RC salt (F5146; Sigma) were used as couplers to detect ALP and TRAP activities, respectively. Sections stained for ALP and TRAP activity were counterstained with nuclear fast red and hematoxylin, respectively.

3. Results

3.1 Properties of β-TCP granules

Figures 3 and **4** show XRD pattern and SEM photographs, respectively, of the obtained granules. The crystal phase was assigned to β-TCP. The spherical β-TCP granules 0.3–0.6 mm in diameter were composed of rod-shaped particles of about 10 μm in length with an aspect ratio of around 15. The β-TCP granules had about 70% porosity, and the mean diameter of sub-micro-sized pore of the β-TCP granules was about 0.2 μm in size. The specific surface area of the β-TCP granules was about 5 m^2/g.

3.2 Healing of bone defects with or without β-TCP granules

Two weeks after implanting spherical β-TCP granules in bone defects created in tibiae, although the degradability of implanted β-TCP granules was recognized, a large amount of β-TCP

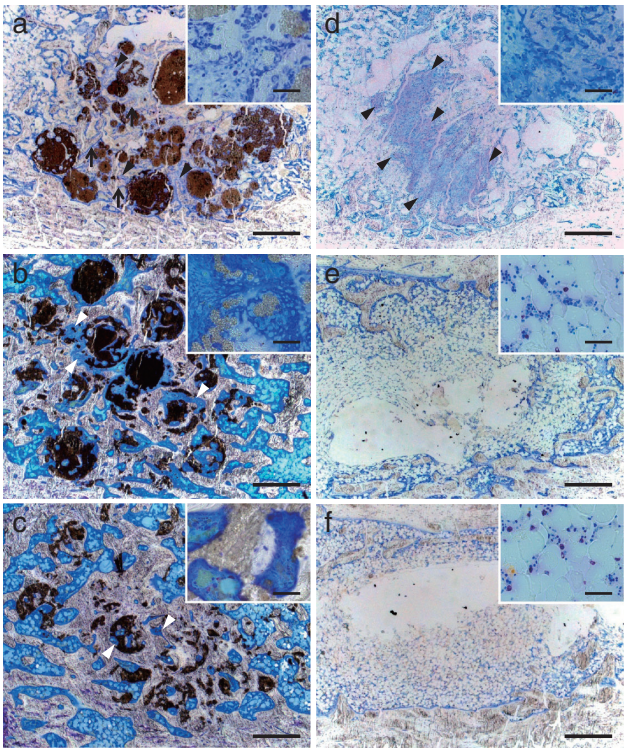

Fig. 5. Histological appearance of sections of tibial bone defects with (a–c) or without (d–f) implants 2 weeks (a, d), 4 weeks (b, e) and 8 weeks (c, f) after the operation. Arrowheads indicate mesenchymal cells. Arrows in (a) indicate newly formed bone. Scale bars represent 500 µm. Insets show high-power magnification views of mesenchymal cells in intergranular spaces or bone marrow cavities (a–d) and fatty bone marrow cells in the specimen without implant (e, f). Scale bars in insets represent 50 µm.

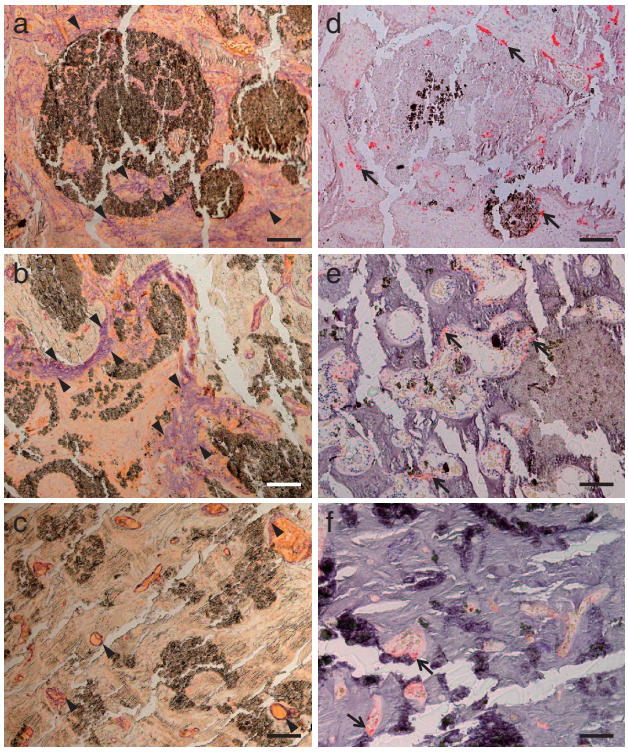

Fig. 6. Histological appearance of ALP activity (a–c) and TRAP activity (d–f) 2 weeks (a, d), 4 weeks (b, e) and 8 weeks (c, f) after implantation. Purple staining in (a–c) indicates ALP-positive cells and red staining in (d–f) indicates TRAP-positive cells. Arrowheads in (a) and (b) indicate ALP-positive mesenchymal cells and arrowheads in (c) indicate ALP-positive osteoblasts aligned in small bone marrow cavities. Arrows in (d), (e) and (f) indicate TRAP-positive cells. Scale bars represent 100 µm.

granules remained in the bone. The intergranular space was rich in mesenchymal cells [**Fig. 5**(a), arrowheads and inset], and newly formed bone was seen [Fig. 5(a), arrows]. At 4 weeks after implantation, the intergranular space was composed of trabecular bone and bone marrow rich in mesenchymal cells compared to surrounding normal bone marrow of the tibia [Fig. 5(b), arrowheads and inset]. At 8 weeks after implantation, most residual β-TCP granules were embedded in abundant bone [Fig. 5(c)]. Hypercellular bone marrow cavities were scattered throughout the area [Fig. 5(c), arrowheads], and at higher magnification, osteoblast-like cells were aligned in them [Fig. 5(c), inset]. Without implantation of β-TCP granules, numerous mesenchymal cells were seen in bone defects at 2 weeks after the operation [Fig. 5(d), arrowheads and inset]; however, bone regeneration was poor and the defect were filled with fatty bone marrow with considerably fewer bone trabeculae at 4 and 8 weeks after implantation [Figs. 5(e), 5(f)].

3.3 Distribution of ALP-positive cells and TRAP-positive cells

At 2 weeks after implantation, numerous ALP-positive cells were detected in intergranular space of tibiae [**Fig. 6**(a)]. At 4 weeks after implantation, mesenchymal cells seen in the limited area of bone marrow cavities stained positively for ALP activity [Fig. 6(b)]. At 8 weeks after implantation, most residual implants were embedded in bone and ALP-positive cells were detected in osteoblasts aligned in bone marrow cavities [Fig. 6(c), arrowheads].

TRAP-positive cells were seen on the surface of bone and implanted β-TCP granules at 2 weeks after implantation [Fig. 6(d)]. Four weeks after implantation, some β-TCP granules were surrounded by bone tissue, and TRAP-positive cells on the surface of implants decreased compared to that seen at 2 weeks after implantation [Fig. 6(e)]. At 8 weeks after implantation, TRAP-positive cells were distributed only in bone marrow cavities surrounded by bone or β-TCP/bone composite hard tissue [Fig. 6(f)].

4. Discussion

Bone substitutes are used for various purposes. Hydroxyapatite (HA), a non-biodegradable bone substitute, is useful to fuse bones. To fill bone defects, both non-biodegradable bone substitutes and biodegradable bone substitutes have been used.[12] Biodegradable bone substitutes have been developed with the hope that they will be replaced with bone tissue over a certain period. Hence, biodegradable bone substitutes are clinically applied to accelerate bone regeneration or to help large defects to be filled with bone. We have studied the significance of biodegradability of bone substitutes for healing of bone defects. We found that the microstructure of β-TCP and HA affected the biological behavior and that the biodegradability of bone substitutes affected the healing of bone defects.[8),10),13)]

In this study, we analyzed the histological findings of healing bone defects with and without implantation of biodegradable bone substitutes focusing not only on bone, but also on bone marrow cells. Fibroblastic mesenchymal cells were seen in the

implanted region and most were ALP-positive cells. Numerous mesenchymal cells appeared in bone defects without implantation of β-TCP granules at 2 weeks after the operation [Fig. 5(d), arrowheads] and were ALP-positive cells, just like the mesenchymal cells in tibiae with β-TCP implants (data not shown). However, the bone defects healed with a much lower amount of bone [Figs. 5(e), 5(f)]. In this model, implantation of β-TCP granules greatly helped healing of bone. At 4 and 8 weeks after the operation, fatty changes of bone marrow were obviously increased compared to bone marrow at 2 weeks after the operation in animals without implantation [Figs. 5(d)–5(f)]. In animals implanted with β-TCP granules, fatty changes of bone marrow were not significant compared to in animals without implantation at 4 and 8 weeks after the operation [Figs. 5(b), 5(c), 5(e) and 5(f)]. Mesenchymal cells in bone marrow cavities in the specimen 4 weeks after implantation and osteoblast-like cells in the specimens 8 weeks after implantation were positively stained for ALP activity [Figs. 6(b), 6(c)]. These data suggested that implanted β-TCP granules worked as scaffolds to maintain ALP-positive cells and also prevented fatty changes of bone marrow cells. Although the biological mechanism is uncertain, studies of β-TCP-associated osteoblastic differentiation under in vitro conditions have been reported. It has been suggested that osteoprogenitor cells do not differentiate into osteoblasts simply by culturing on β-TCP.[14),15)] However, β-TCP was strongly suggested to stimulate the differentiation of osteoprogenitor cells under three-dimensional culture using osteoblast differentiation medium.[16)–18)] In vivo results of this study agreed with these in vitro data. In addition, we have reported that culture supernatants of osteoclasts cultured on β-TCP or HA helped osteoblastic differentiation of C2C12 myoblastic cells.[10)] Hence, TRAP-positive cells on the surface of β-TCP granules might contribute to bone formation, and implanted β-TCP granules might work as scaffolds for TRAP-positive cells, which stimulated osteoblastic differentiation. Within light of these in vitro studies, the microstructure of β-TCP granules composed of rod-shaped particles might contribute to stimulating osteoblastic differentiation and preventing adipocytic differentiation of bone marrow mesenchymal cells, and may improve biodegradation and replacement by bone tissue. Fatty change is one of the major findings of aging in bone marrow, and implantation of our β-TCP granules composed of rod-shaped particles might also contribute to prevent aging of bone marrow cells.

5. Conclusion

Implantation of β-TCP granules composed of rod-shaped particles contributed to healing of bone defects created in rat tibiae. Much less fatty change of bone marrow cells around implanted β-TCP granules compared to the control was also observed.

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