Effects on bone regeneration when collagen model polypeptides are combined with various sizes of alpha-tricalcium phosphate particles

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We evaluated the effects on bone formation of combining synthesized collagen model polypeptides consisting of a Pro-Hyp-Gly [poly(PHG)] sequence and alpha-tricalcium phosphate (α-TCP) particles with various median sizes (large: 580.8 µm; small: 136.2 µm; or large and small mixed: 499.3 µm) in a skull defect model in mini-pigs. Quantitative image analyses for the volume density (VD) of new bone revealed that the VD in each α-TCP group was significantly higher than that in the poly(PHG) control group, with the mixed group showing the highest VD among all the groups at 4 weeks after implantation. Histological assessments revealed that the small α-TCP particles were almost completely degraded at 8 weeks. At 12 weeks, all sizes of α-TCP particles were completely degraded and remodeling of the lamellar bone was observed. The present findings suggest that particle size may influence the success of bone formation in defects.

Keywords: Bone regeneration, Biomaterials, Alpha-tricalcium phosphate, Collagen model polypeptides

INTRODUCTION

Autogenous bone grafting for patients with cleft lip and palate has become a well-accepted treatment modality to restore the function and structure of the maxillary arch at the cleft site¹. However, the procedure is very invasive and the amount of collectable bone is limited. Therefore, it is desirable that the scaffold is bioabsorbable and ultimately replaced by autologous tissue ². In addition, outstanding handling characteristics for surgical applications are required, because conventional granular materials tend to leak from pores and are difficult to apply to bone defects.

Collagen is known to be a promising material for transplantation, owing to its diverse applications in tissue engineering and its excellent biocompatibility and biodegradability³. It is possible to easily form a collagen gel that can be either injected or implanted into the defect site. Tanihara et al.⁴ previously reported the chemical synthesis of collagen model polypeptides consisting of a Pro-Hyp-Gly [poly(PHG)] sequence that forms a triple-helical structure. The materials for implantable scaffolds for tissue regeneration need to be biodegradable, have no ability to induce inflammation and to act as a scaffold for cell proliferation and differentiation⁵.

Hydroxyapatite (HAp), tricalcium phosphate (TCP) and tetracalcium phosphate have been widely used as substitutes for autologous bone in orthopedic and maxillofacial surgery, since these materials show high biocompatibility and osteocompatibility. Among the different calcium phosphate materials, previous studies have demonstrated a role for α-TCP particles as bone-rebuilding materials by gradual biodegradation and the formation of bone around them⁶,⁷. Such characteristics of α-TCP particles would be suitable for bone graft materials.

In light of these reported benefits, we prepared collagen model polypeptide gels combined with various sizes of α-TCP particles. The particle size of bone graft materials is an important determinant of osteogenic activity⁸,⁹. The particle size also affects the quantity of the newly formed bone. However, the most effective particle size is unclear when collagen model polypeptides are combined with α-TCP particles. In the present study, the effects on bone formation of combining poly(PHG) and various sizes of α-TCP particles were evaluated in a calvarial defect model in mini-pigs.

MATERIALS AND METHODS

X-ray diffraction measurements of α-TCP

X-ray diffraction measurements were performed using a Geigerflex diffractometer (Ultima IV; Rigaku, Tokyo, Japan), with Cu-Kα radiation generated at 40 kV and 40 mA. The scan rate was 4 degrees/min with a step size of 0.02 degrees over a 2θ range from 5–70 degrees.

Characterization of α-TCP particles

Large and small α-TCP particles were provided by Taihei Chemical Industrial Co. Ltd. (Osaka, Japan). The large and small α-TCP particles were mixed at mass ratios of 50:50 mass%. The ranges of the particle sizes present in the α-TCP powders were analyzed using a conventional laser diffraction particle analyzer (LS13 320; Beckman Coulter Inc., Miami, FL, USA).
Synthesis of poly(PHG)
Poly(PHG) was synthesized according to previously published methods. Briefly, PHG was dissolved in 10 mM phosphate buffer (pH 7.4) at a concentration of 165 mM, and mixed with 1-hydroxybenzotriazole (33 mM; Peptide Institute, Osaka, Japan) and 1-ethyl-3-(3-dimethyl-aminopropyl)-carbodiimide hydrochloride (825 mM; Peptide Institute). After sequential stirring for 2 hours at 48°C and 46 hours at 20°C, the reaction mixture was dialyzed against water for 48 hours to remove any residual reagents. To form the poly(PHG) sponge, an aqueous solution at a concentration of 5 mg/mL was poured into a polystyrene dish and freeze-dried. The poly(PHG) sponge was sterilized with ethylene oxide gas at 40°C.

Test groups
Four groups were produced for this study. In the large and small groups, the large and small α-TCP particles were combined with poly(PHG), respectively. In the mixed group, a mixture of the large and small α-TCP particles was combined with poly(PHG). The control group was poly(PHG) sponge only.

Selection of the model animal
The adult mini-pig was the animal of choice because it is especially suitable for the evaluation of bone healing and bone remodeling. The bone regeneration rates in mini-pigs have a strong correlation with those in humans (1.2–1.5 µm/day in mini-pigs vs. 1.0–1.5 µm/day in humans). Mini-pig bone also shows similar bone mineral densities and bone mineral concentrations to human bone. Furthermore, the bone microstructure in mini-pigs is described as a lamellar bone structure, which is similar to that in humans.

Transplantation procedures
All animal experiments were performed in accordance with the regulations for animal experimentation at the Institute of Biomedical Research and Innovation (IVTeC Animal Experiment Committee: approval number 190901-1). Nine adult female Clown mini-pigs (30–34 weeks of age; Japan Farm Ltd., Kagoshima, Japan) were used. All surgical procedures involved anesthesia with isoflurane. Following anesthesia, an incision was made in the skin and periosteum of the skull, which created access to the neurocranium. The surgically prepared area of the frontal skull allowed us to create four identical defects that were sectioned (5–7 µm) in the coronal plane and stained with hematoxylin and eosin (HE) for qualitative bright-field light microscopy analysis. Each section was observed using a BZ9000 All-in-One Fluorescence Microscope (Keyence, Tokyo, Japan).

Statistical analysis
All data were expressed as the mean and standard error. Differences were evaluated by analysis of variance (ANOVA) with Scheffe’s F test. Differences were considered significant at $p<0.05$.

RESULTS
X-ray diffraction measurements and characterization of α-TCP particles
Scatter plots of the data obtained by X-ray diffraction are shown in Fig. 1. The data for the scatter plots of the synthesized α-TCP particles were checked against α-TCP data registered with the Joint Committee on Powder Diffraction Standards (JCPDS), which confirmed that the peaks appeared at the same angles (Fig. 1). The particle size distribution was analyzed by laser diffraction and is shown in Fig. 2. In general, the samples were dispersed in ethanol to minimize aggregation during the measurements. In the present study, we confirmed that the measurement data in the drying process did not change between the dry and wet processes. The median size of the particles was 580.8, 136.2 and 499.3 µm in the large, small and mixed groups, respectively. Two peaks of particle sizes were observed in the mixed group.

Microradiography
Representative 3D reconstructions of the filled defects in the control, large, small and mixed groups at 4, 8 and 12 weeks are presented in Fig. 3. In the experimental
Fig. 1  X-ray diffraction patterns of the α-TCP particles.

Fig. 2  Laser particle analyses detected the cumulative distribution (grey bars) and relative frequency (black lines) of the particle sizes within the large (a), small (b) and mixed (c) groups.
groups, the amount of new bone increased with time and the whole defects were filled with newly formed bone with a trabecular structure at 12 weeks. In the control group, the empty defect showed dense ring formation from the edge of the defect toward the center, with thickening from 4 to 12 weeks.

The VDs of each group at the three time points are shown in Fig. 4. At 4 weeks post-transplantation, the VDs in the mixed and control groups were the highest and lowest, respectively ($p<0.05$). No significant differences in the VDs were observed between the large and small groups. At 8 weeks post-transplantation, no significant differences in the VDs were observed between the small and mixed groups, although each VD was higher than that in the large group. At 12 weeks post-transplantation, there were no significant differences in the VDs among the experimental groups. However, the threshold was difficult to establish for the $\alpha$-TCP particle scaffolds, because they responded to micro-CT in the same way as bone HAp.

Histological assessment at 4 weeks

In the control group, the poly(PHG) was not degraded, and it maintained the external form and was surrounded by connective tissue. However, the cellular density and staining pattern of the connective tissue were different from those in the other three groups (Fig. 5-a). It is considered that the connective tissue contained a lot of granulation tissues in the early stage of the bone formation. New bone was observed on the surface of the poly(PHG), and a few osteoblast-like cells were also present on its surface (Fig. 5-a). In the experimental groups, the defects were compactly filled with $\alpha$-TCP particles, in contrast to the control group. A reticulate structure was observed inside the $\alpha$-TCP particles (Fig. 5-b, -c and -d). However, there were no significant changes in the form or contour of the $\alpha$-TCP particles and the poly(PHG) was not degraded in the large group (Fig. 5-b). In the small group, a few capillaries were present between the $\alpha$-TCP particles and the poly(PHG) and some poly(PHG) remained. The spaces within the $\alpha$-TCP particles were almost completely filled with osseous tissue composed of mature and immature bone (Fig. 5-c). In the mixed group, connective tissue and new bone were observed among the $\alpha$-TCP particles. Immature bone was observed on the $\alpha$-TCP particle surfaces, and a fair amount of connective tissue remained between them (Fig. 5-d).
Histological assessment at 8 weeks
In the control group, reticulate new bone or bone-like structures were observed, with osteoblast-like cells around the new bone. The size of the poly(PHG) at 8 weeks was smaller than that at 4 weeks (Fig. 6-a). In the experimental groups, osteoblast-like cells were located at the periphery of the α-TCP particles and the poly(PHG) was almost completely degraded (Fig. 6-b, -c and -d). In the large group, connective tissue and a few capillaries were present among the α-TCP particles. In addition, some of the α-TCP particles were partially connected with new bone and the α-TCP particles more or less retained their original form. The size of the α-TCP particles was smaller than that at 4 weeks (Fig. 6-b). In the small group, a significant amount of connective tissue was present within the new bone and α-TCP particles were not observed. Osteoblast-like cells were seen at the edge of the new bone (Fig. 6-c). In the mixed group, various forms of α-TCP particles were observed and some particles had been deeply invaded by new bone. The space between the dividing α-TCP particles was sporadically filled with new bone. Most α-TCP particles had lost their original shape and become smaller. New bone was formed on and within most of the α-TCP particles (Fig. 6-d).

Histological assessment at 12 weeks
In the control group, poly(PHG) still remained at 12 weeks, and connective tissues were present together with the new bone (Fig. 7-a). In the experimental groups, the α-TCP particles and poly(PHG) were almost completely substituted by the newly formed bone. The newly formed bone was mature bone, and possessed the intact structure of calvarial bone. Connective tissues were present among the new bone (Fig. 7-b, -c and -d). In the large group, α-TCP particles were slightly visible, but their original shape was lost and a reticulate structure was present (Fig. 7-b).

DISCUSSION
Synthetic biomaterials have gained wide acceptance as synthetic bone grafts in many orthopedic and dental applications as alternatives to autografts and allografts. Tanihara et al. previously reported the synthesis of collagen model polypeptides consisting of a poly(PHG) sequence that formed a triple-helical structure. We evaluated the effects on bone formation of new bone grafts combining poly(PHG) and various sizes of α-TCP particles in a skull defect model of mini-pigs. The results of our study suggest that the particle size of...
alpha-TCP might influence the success of bone formation in defects.

Polymer materials used as scaffolds are generally expected to degrade in the early stage in vivo, and become substituted by newly formed tissue. Among the widely used natural biopolymers, collagen has been conventionally applied in the clinic in various forms, such as gels or sponges, since it shows good biocompatibility and absorbability. Atelocollagen molecules do not contain telopeptides, and show extremely low antigenicity when adopted as basic materials for medical use. Schlegel et al. reported that transplantation of bovine collagen alone into mini-pig skull defects showed higher degrees of de novo bone formation comparable with that of autogenous bone grafts. However, the major disadvantages of using natural collagens of animal origin, such as atelocollagen, are that the potential for antigenicity may exist, along with pathogen pollution and nonspecific cell adhesion.

In contrast, poly(PHG) removes the danger of pathogen pollution associated with animal-derived collagens. Our previous study investigated the usefulness of a poly(PHG) sponge as a scaffold. We combined the poly(PHG) sponge with mesenchymal stem cells (MSCs), and transplanted the combination into mini-pig calvarial defects. The histological findings indicated that poly(PHG) did not invoke any tissue reactions. In addition, the study showed that transplantation of...
MSCs in a poly(PHG) sponge can significantly enhance bone formation in mini-pig calvarial defects, compared with transplantation of the poly(PHG) sponge alone. Stem cell therapies may theoretically be applicable to all fields of tissue engineering\(^\text{19}\). However, their clinical applications are not widespread at present, because of cost/effectiveness considerations and regulatory restrictions for cell manipulation and therapies.

HAp is a biomaterial with a lower degradation rate than the rate of new bone formation \textit{in vivo}\(^\text{20,21}\). On the other hand, TCP possesses higher biodegradability than HAp \textit{in vivo}\(^\text{20}\). The physical characteristics of \(\alpha\)-TCP result in higher and earlier remodeling activity compared with \(\beta\)-TCP\(^\text{6}\). Biopolymer scaffolds have often been combined with calcium phosphate as described above. In the present study, bone ingrowth was observed because of the osteoconductive properties of the combination of poly(PHG) and \(\alpha\)-TCP particles. Naturally, collagen fibers and other starting materials in implant materials should begin to degrade soon after implantation. Our histological assessments revealed that the absorption of implant materials was accompanied by decreases in the poly(PHG) and \(\alpha\)-TCP particles. In the experimental groups, the absorption of poly(PHG) had started by 4 weeks and was completely finished by 8 weeks. On the other hand, poly(PHG) still remained at 12 weeks in the control group. Osteoclast-like cells have been recognized to absorb \(\alpha\)-TCP\(^\text{2}\) and mineralized collagen I scaffolds\(^\text{22}\).

Fig. 6 Light microscopic images of HE-stained sections of defects at 8 weeks. In the control group (a), connective tissue is present among the poly(PHG). In the large group (b), connective tissue and a few capillaries are present among the \(\alpha\)-TCP particles. In the small group (c), a significant amount of connective tissue is present within the new bone and \(\alpha\)-TCP particles are not observed. In the mixed group (d), new bone has formed on and within most of the \(\alpha\)-TCP particles. Original magnification: \(\times200\). Asterisk: poly(PHG); TCP: \(\alpha\)-TCP particle; CT: connective tissue; NB: new bone; black arrow: capillary; white arrow: osteoblast-like cell. Scale bars: 100 µm.
Osteoclast-like cells may induce the mineralization of poly(PHG) in the presence of α-TCP. Thus, the absorption behaviors of poly(PHG) differed between the experimental and control groups. The present findings suggest that new bone is formed before the implant materials are absorbed, and may therefore indicate that the composites we fabricated show faster and better bone induction capacities.

There has been insufficient research into bone regeneration with dried bone of different particle sizes. It has been reported that freeze-dried bone allografts with particle sizes of 250–750 µm are able to promote osteogenesis, whereas bone allografts with particle sizes of <125 µm are more likely to be rapidly resorbed\(^{22}\). Xu et al.\(^{24}\) compared the osteoconductive capabilities of deproteinized bone particles of two different sizes (300–500 µm and 850–1,000 µm) in rabbits undergoing a maxillary sinus lift. They reported a significantly higher density of newly formed bone in the small particle group than in the large particle group at both 4 and 8 weeks after implantation. On the other hand, within the parameter of rhBMP-4 combined with either small or large β-TCP particles, varying the particle size did not seem to have any significant effect on bone formation\(^{25}\).

A particle diameter that is too large or too small might have some advantages and disadvantages. In the present study, the large and small α-TCP particles were mixed at mass ratios of 50 : 50 weight %. As expected, the diameter of the α-TCP particles in the mixed group showed a wide range from 10 µm to <1,500 µm. The bone formation and healing processes were faster in the mixed group than in the large and small groups in the early
phase. The reasons for the promotion of bone formation in the mixed group are unclear. Xu et al. demonstrated that the interparticle spaces of small particles were significantly larger than those of large particles and that, as a result, the density of the newly formed bone in the small particles was higher than that in the large particles. In the present study, the VD in the small group was higher than that in the large group at 8 weeks. On the other hand, the histological assessments revealed that the small α-TCP particles were completely absorbed at 8 weeks. Therefore, the small α-TCP particles might initiate new bone formation, and induce absorption of the large particles and replacement by mature bone.

Secondary bone grafting of the residual alveolar cleft in patients with cleft lip and palate is a widely-accepted treatment regimen. Successful grafting allows eruption of teeth into the former cleft area and further orthodontic expansion of a typically collapsed maxilla. It is ideally carried out at a developmental age between 9 and 11 years, before eruption of the maxillary canine, to allow the canine to erupt through the grafted site. With the advent of tissue engineering techniques, alternatives to autologous bone graft techniques have become available. However, harvesting of an autologous bone graft has several disadvantages. For example, the donor site surgery requires a prolonged operating time for young children and may cause morbidity.

The present study investigated a new bone graft material for closure of alveolar cleft defects with poly(PHG) and α-TCP particles. The created defects were non-critical-size defects, and the bone was obviously repaired at 8 weeks after poly(PHG) and α-TCP particles were implanted into the skull defect model of mini-pigs. The bone regeneration rates in mini-pigs are similar to non-critical-size defects, and the bone was obviously repaired at 8 weeks after poly(PHG) and α-TCP particles. The created defects were non-critical-size defects, and the bone was obviously repaired at 8 weeks after poly(PHG) and α-TCP particles. The clinical application of poly(PHG) and α-TCP particles as bone graft materials for younger patients with alveolar cleft defects should be successful. Further studies of bone regeneration will be required in an alveolar cleft defect model in a larger animal.

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