Hydroxyapatite/Collagen Bone-Like Nanocomposite

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Our group has succeeded to synthesize material with bone-like nanostructure and bone-like inorganic and organic composition via self-organization mechanism between them using simultaneous titration method under controlled pH and temperature. The hydroxyapatite/collagen (HAp/Col) bone-like nanocomposite completely incorporated into bone remodeling process to be substituted by new bone. Cells cultured on the HAp/Col revealed very interesting reactions. Osteoblast-like MG63 cells showed upregulation of alkaline phosphatase 3 times greater than MG63 cells cultured on tissue culture polystyrene (TCPS). MG63 cells 3-dimensionally cultured in a “HAp/Col sponge,” a porous HAp/Col having sponge-like viscoelasticity, accumulated calcium phosphate nodules on extracellular matrices they secreted. Bone marrow cells co-cultured with osteoblasts on HAp/Col differentiated to osteoclasts without differentiation supplements. This phenomenon is not found in cells cultured on hydroxyapatite ceramics and TCPS, and rarely in cells cultured on dentin. These results suggest that HAp/Col is a good candidate for tissue engineering of bone as well as bone filler. In a clinical test as a bone filler, the HAp/Col sponge was significantly better than porous β-tricalcium phosphate. The HAp/Col sponge has been approved by the Japanese government and will be used as greatly needed bone filler in patients. In addition to the above, HAp/Col coating on titanium revealed higher osteoconductivity than HAp-coated titanium and bare titanium and improved direct bonding between titanium and newly formed bone. The HAp/Col coating may be used for metal devices requiring osseointegration.

Key words  hydroxyapatite; collagen; nanocomposite; nanostructure; chemical composition; biological reaction

1. INTRODUCTION

Bone is a typical nanocomposite composed of non-stoichiometric carbonate containing hydroxyapatite (HAp) nanocrystals as main inorganic phase and type-I collagen molecules as main organic phase. The HAp nanocrystals are regularly aligned along collagen molecules to form fibrils and fibers. The composite fibers of HAp and collagen further form a membrane that forms osteon by random oriented tubular lamination. Bone nanostructure has an appropriate structure and chemical composition to play two important roles of bone. The first role is as a “hard” material to support the body and to protect important organs. The second is as a “soft” material to maintain calcium homeostasis. As hard material, nanocomposite of HAp and collagen apparently behave as a single material macroscopically; that is, they retain the viscoelasticity of collagen and hardness of HAp. In addition, nanocomposite allows microscopic lamination of composite bundles of HAp and collagen. As soft material, the large specific surface area of HAp nanocrystals allows rapid dissolution by osteoclasts for maintenance of calcium ions in body fluids as well as appropriate bone remodeling rate.

The first system to prepare bone-like material is very simple. HAp or related calcium phosphate granules or powders were mixed with collagen sol followed by gelation of collagen, freeze-drying, and lyophilization.1) The merits of this process are its ease to control the HAp:collagen mass ratio similar to or higher than bone (approximately 7:3); however, this process does not allow formation of nanostructure of bone even if HAp nanocrystals are used in the process. Accordingly, bone tissue reactions of the mixture material are macrophage phagocytosis for collagen and osteoconductivity for HAp, and are not similar to autologous bone transplantation.

The second system is precipitation of HAp nanocrystals on collagen fibers previously prepared by conventional gelation-freeze drying method.2) This method partially allows bone-like nanocomposite at the very initial stage and/or low HAp:collagen mass ratio; however, obtaining composite of bone-like HAp:collagen mass ratio similar to bone requires a long time-period (>1d) and HAp nanocrystals completely cover collagen fibers by that time. Accordingly, bone tissue reactions to the materials are almost similar to HAp nanocrystal coatings, with osteoconductivity in the initial stage and possibly reaction with collagen if the HAp nanocrystals formed on the collagen fiber are resorbed/dissolved.

Recently, an improved modification of the above method has been reported3) using polylysine as a polymer-induced liquid precursor (PILP) to form aligned HAp nanocrystals on both inner and outer sides of collagen fibers. The PILP method is one of the best methods to mimic natural bone formation process but is not suitable for mass production of materials with bone-like HAp:collagen mass ratio.

The third system is simultaneous precipitation system proposed first by us,4) followed by others, which involves...
increasing pH of acidic mixture of calcium, orthophosphate, and collagen and aging of 1.5X simulated body fluid with collagen. The composite obtained by the method described by Nassif and coworkers seems very similar to bone in both nanostructure and chemical composition; however, homogeneity of the products obtained depends on homogeneous increase of pH in the whole reaction vessel, especially for mass production. The method described by Xia’s group is unsuited to obtain bone-like mass ratio of HAp and collagen and is also not suitable for mass production. Our method forms composite fibers of HAp nanocrystals and collagen molecules using simultaneous titration of calcium hydroxide suspension and orthophosphoric acid solution with collagen into a reaction vessel while maintaining pH and temperature. In the reaction vessel, the presence of collagen allows HAp nucleation at lower calcium and phosphate concentrations to form HAp, and formation of HAp allows collagen fibrillogenesis at significantly lower ion concentrations compared with regular fibrillogenesis; therefore co-precipitation is the key factor in forming composite fibers. Our method, even if it is the first “simultaneous precipitation” system, realizes bone-like nanostructure, a variety of HAp:collagen mass ratios (at least between 3:2 and 9:1), a variety of fiber lengths (micrometers to centimeters), and rapid preparation (approximately 10–20 g dry mass/h). In addition, the HAp/Col bone-like nanocomposite obtained by our method is the first recognized synthetic material that is completely incorporated into bone remodeling process; that is, the HAp/Col is resorbed by osteoclasts followed by formation of bone by osteoblasts at the Howship’s lacunae. In the present paper, advances in the development of HAp/Col materials are presented.

2. CELL REACTIONS

Osteoblastic Cells Activation of osteogenic functions of cells is the aim of artificial bone materials with bioactivity. The HAp/Col membrane was prepared by filtration of short and long fibers of HAp/Col followed by freeze-drying, pressing, and dehydrothermal cross-linking for 2-dimensional scaffold. The membrane showed filter paper-like flexibility. Human osteoblastic MG63 cells were cultured on the membrane for up to 14 d. Cell proliferation was measured by a total DNA measurement and osteogenic activity evaluated by alkaline-phosphatase (AlkP) gene expression measured by a real-time polymerase chain reaction (PCR). Tissue culture polystyrene (TCP) was used as control. According to the DNA measurements, MG63 cells on the HAp/Col membrane proliferated at the same rate as cells on TCPs over 7–14 d incubation under a confluent condition. The HAp/Col membrane is translucent and allows observation of the cells on the membrane stained by Giemsa by optical microscope. Gene expression of AlkP of cells on the HAp/Col membrane statically analyzed by the ΔΔCT method showed significantly 3.2 and 5.8 times greater than that on the TCPs on days 10 and 14, respectively, suggesting that the HAp/Col activated osteogenic function of MG63 cells.

For more dynamic evaluation, 3-dimensional culture was performed under pressure-perfusion conditions. The MG63 cells cultured in the HAp/Col porous body of 95% porosity, so-called HAp/Col sponge because of its sponge-like viscoelasticity in wet condition, showed similar cell proliferation but lower AlkP gene expression in comparison to that in the collagen sponge of 1% porosity at days 7 and 10. Cell number in the HAp/Col porous body became greater than that of collagen porous body at day 14, although AlkP gene expression remained lower. Osteocalcin gene expression was the same for both systems during the test period. The lower gene expression was probably due to cell density on the surface of the scaffolds. In the porous collagen, cells proliferated only at the surface approximately 500 μm; however, cells in the porous HAp/Col migrated to the center. Thus cell number drastically increased after 14 d. In addition, only in the porous HAp/Col, calcium phosphate nodules were formed on the extracellular matrices secreted by cells. The results suggest that HAp/Col has a high potential for tissue engineering scaffold of bone as well as bone filler to repair large bone defects.

3. OSTEOCLASTIC DIFFERENTIATION OF BONE MARROW CELLS

Osteoclast differentiation of bone marrow cells on Hap/Col self-organized bone-like nanocomposite disk has described by Kikuchi and Irie. Animal studies have shown that bisphosphonate, an inhibitor of osteoclast activities, prevents bone formation through inhibition of osteoclastic resorption of HAp/Col. The effect of HAp/Col material on the bone remodeling process has gained significant interest from researchers and surgeons alike. To investigate the influence of HAp/Col on osteoclastic differentiation of bone marrow cells, HAp/Col disks measuring 4 mm in diameter were used as substrate instead of dentin (ivory) slice, generally used in osteoclast research, for co-culture system for osteoclastic differentiation of bone marrow cells. In brief, bone marrow cells and osteoblasts were independently isolated from CL57BL/6 mice and co-cultured on the HAp/Col disk for 6 d in 96-well plates in α-minimum essential medium containing 10% fetal bovine serum with or without osteoclast differentiation inducers, 10 nM 1,25-dihydroxyvitamin D3 (1,25(OH)2 D3) and 1 μM prostaglandin E2 (PGE2). The cells on the HAp/Col disk were then fixed and stained for tartrate resistance acid phosphatase (TRAP), a marker enzyme of osteoclastic differentiation. Dentin slices, sintered HAp disk, and TCPs plates were used as controls.

Bone marrow cells cultured on all substrates with the osteoclastic differentiation inducers differentiated into osteoclasts. Contrarily, negative controls (bone marrow cells without the inducers) showed no differentiation except for cells on the HAp/Col. These results suggest that the HAp/Col could activate osteoclast differentiation factors such as RANK, OSCAR and/or inhibit suppressor activity of osteoclast differentiation such as OPG.

4. CLINICAL TRIAL

Bone tissue and osteochondral defects were successfully repaired using the HAp/Col sponge. In addition, HAp/Col was also used as BMP carrier. Thus a clinical trial was conducted in 7 hospitals using commercially available β-tricalcium phosphate porous ceramics (β-TCP) as control. The test conditions included 65 patients for each material. Cases were selected based on filling of the curettage part of bone tumor, bone defect by bone fracture or after reconstruc-
tion of bone fracture, and bone defect at donor site of autograft. Efficacy was evaluated at 24 weeks after surgery by summation of scores of “continuity with surrounding bone” (0–2 points) and “bone regeneration at the defect, including resorption of materials followed by bone formation” (0–2 points). A perfect score, 4, meant “remarkable efficacy.” The remarkable efficacy ratio for the HAp/Col was 20.6% better than that for the β-TCP, a statistically significant result, starting from 4 weeks after surgery. Interestingly, the remarkable efficacy ratio for large implantation (10–30 cm³) for the HAp/Col was 71.4% (5/7) and 0% (0/7) for β-TCP. However, in the HAp/Col group, side effects such as small inflammatory reactions were observed in 9.5% of subjects compared with 0% for the β-TCP group. The reactions were not due to allergic reaction to the collagen used in the HAp/Col but were simply attributed to the body’s immune reaction to foreign material. However, weak and non-severe inflammation is assumed to be necessary for the damaged tissue’s regeneration process, and the efficacy results support this assumption. Accordingly, the HAp/Col sponge was approved and is now commercially available in selected hospitals; it will be widely available in the near future as “Refit®” (HOYA, Japan).

5. COATING ON TITANIUM FOR ORTHODONTIC SUBPERIOSTEAL DEVICE

The use of HAp/Col nanocomposite-coated titanium (Ti) rod for orthodontic subperiosteal device was successfully described by Uezono’s group. Ti and its alloys are recognized as highly osseointegrative materials; however, successful osseointegration depends on the implant conditions including clinical circumstances and cell activities. Thus HAp coatings on artificial tooth roots are commonly used to improve bone-material bonding. Thin HAp sputter coated implants show good clinical results due to initial osteoconduction by the HAp layer followed by transferring to good osseointegration by Ti. This concept is used in subperiosteal devices in the orthodontic field as minimally invasive anchorage devices in comparison with microscrews. Even with HAp coating, such devices require 3–4 months until start of orthodontics due to device shape and properties of coating. Recently, bone-Ti direct bonding at periosteal site was improved by HAp/Col dip coating. HAp/Col was dip-coated on Ti wire and inserted into the subperiosteal site of cranial bone of Sprague-Dawley (SD)-rat for 4 weeks. Titanium wire coated with HAp by biomimetic method and bare Ti wire were used as controls. Bone contact ratio for the HAp/Col-coated Ti, 62%, was significantly higher than that for HAp-coated Ti (20%) and bare Ti (0%). Newly formed bone height around the HAp/Col coated Ti, 380 µm, was higher than that of HAp-coated Ti (330 µm) and significantly higher than that of bare Ti (230 µm). Accordingly, bone bonding strength evaluated by shear stress for the HAp/Col coated Ti, 16.4 N, was significantly higher than that for the HAp-coated Ti (6.0 N) and for the bare Ti (2.8 N). Considering the results, HAp/Col coatings improved osteoconduction followed by osseointegration of Ti to enhance direct bonding between bone and Ti. HAp/Col coating with appropriate device shape would be useful for novel subperiosteal devices to reduce waiting time before treatment. Moreover, the HAp/Col coating could be used for other implant devices.

6. CONCLUSION

HAp/Col has been demonstrated to be a highly useful bone-like material with a variety of successful clinical applications. In addition to the results mentioned above, HAp/Col is also expected to be used as a gene transfer bioreabsorbable substrate based on calcium phosphate-plasmid DNA composite coating method and as an injectable material including raw material for rapid prototyping using sodium alginate as lubricant and gelation reagents. Considering its useful properties as a bone substitute, HAp/Col material used for applications discussed in this paper promises far better outcomes for patients than currently used materials.

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