Effect of addition of hyaluronic acids on osteoconductivity and biodegradability of
synthetic octacalcium phosphate

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Abstract

The present study was designed to investigate whether three sodium hyaluronic acid (HyA) medical products, Artz®, Suvenyl® and a chemically-modified derivative of sodium HyA Synvisc®, can be used as suitable vehicles for an osteoconductive octacalcium phosphate (OCP). OCP granules (300-500 μm diameter) were mixed with these sodium HyAs with molecular weights of 90×10^4 (Artz®), 190×10^4 (Suvenyl®), and 600×10^4 (Synvisc®) (referred to as HyA90, HyA190, and HyA600, respectively). OCP/HyA composites were injected using a syringe into polytetrafluoroethylene ring, placed on the subperiosteal region of mouse calvaria, for 3 and 6 weeks, and then bone formation was assessed by histomorphometry. Capacity of the HyAs on osteoclast formation from RAW264 cells with RANKL was examined by TRAP staining in vitro. Bone formation was enhanced by the OCP composites with HyA90 and HyA600, compared to OCP alone, through enhanced osteoclastic resorption of OCP. HyA90 and HyA600 facilitated in vitro osteoclast formation. The results suggest that the osteoconductive property of OCP was accelerated by the HyAs-associated osteoclastic resorption of OCP, and, therefore, that HyA/OCP composites are attractive bone substitutes which are injectable and bioactive materials.

Keywords: octacalcium phosphate, hyaluronan products, injectability, biodegradation, bone regeneration

1. Introduction

Octacalcium phosphate (OCP; $\text{Ca}_8\text{H}_2(\text{PO}_4)_6 \cdot 5\text{H}_2\text{O}$) is an osteoconductive ceramic used as a bone substitute that has demonstrated quantitative bioactivity in several *in vitro* studies [1-3]. Osteoblastic differentiation of mouse bone marrow stromal cells is enhanced when they are in contact with OCP crystals [1, 2]. Differentiation is augmented by OCP in a dose-dependent manner based on the quantification of mRNA expression of osteoblast differentiation markers, such as alkaline phosphatase (ALP) and osterix [1]. Another particular feature of OCP is the capability to induce osteoclast formation from a co-culture of bone marrow cells (osteoclast precursor cells) with osteoblasts on OCP crystals even in the absence of $1,25(\text{OH})_2\text{D}_3$, which is an agent that increases the osteoclast differentiation factor receptor activator of NF- κ B ligand (RANKL) in osteoblasts [3]. Hydroxyapatite (HA) does not possess this bioactive property [1, 2]. OCP tends to progressively convert to the most thermodynamically stable HA phase when implanted in bone defects [2, 4] or subcutaneous tissue [4-6]. This transition process is thought to induce the bioactive property of OCP that is associated with physicochemical changes [2], such as the controlled diffusion of calcium and inorganic phosphate ions around the crystals [7] as well as the progressive increase in affinity of serum protein adsorption onto the crystals [8, 9]. OCP appears to have a greater ability for enhancing bone regeneration *in vivo* compared to HA materials [2, 4, 10-12]. In addition, OCP is biodegraded by osteoclastic cells when implanted in various bone defects [10, 12-15] through various forms, such as granules [2, 4, 10, 12, 13, 16], even in critical-sized defects [2, 11, 16], which are defined as defects not spontaneously repaired

during the lifetime of the animal [17].

We have found that the capability of OCP in a granule form to regenerate bone in vivo can be markedly controlled not only by the conversion process of OCP to HA [2] but also by physical and chemical factors, including the dose [18], the microstructure of the individual crystals assembling the each granule [19], the granule size [16] and the stoichiometry of OCP (Ca/P molar ratio of OCP) [14], which may be important factors to design the OCP-based materials for bone regeneration. One disadvantage of using OCP as a filling material for bone defects, however, is the difficulty to form the correct shape [20-22], not only for filling large defects, but also for injecting into randomly-shaped defects. This is due to the presence of large water molecules in the OCP structure [23, 24], indicating that OCP cannot be molded through a general sintering process, unlike HA or β -tricalcium phosphate (β -TCP) ceramics [25, 26]. Sintering of OCP leads to the decomposition of the OCP structure as the temperature increases [23, 24]. In order to overcome the poor moldability of OCP, the granules or crystals of OCP need to be combined with natural polymers, such as collagen [21], gelatin [20], and alginate [27]. Using this approach, the composites are able to form a three-dimensional scaffold and can be used in large bone defects with relatively better handling performances and excellent bone regenerative properties compared to OCP granules without a polymer [20, 21, 27]. However, the composites still cannot be used to fill randomly-shaped defects by injection, which may result in less bone formation reaction compared to defects filled with a sufficient amount of material [28-32]. Therefore, improvements in the injection characteristics of OCP without loss of bioactivity or osteoconductivity

characteristics could lead to the wide use of these materials in bone tissue engineering fields.

Hyaluronic acid (HyA) is an extracellular matrix that is involved in most connective tissues of the body [33, 34]. HyA is a linear natural polymer with repeating unsulfated glycosaminoglycans consisting of D-glucuronic acid and N-acetyl-D-glucosamine [34]. Due to the involvement of HyA in several biological processes, such as osteoconduction and wound healing [35-39], several studies have assessed the utility of injectable HyA gels in bone-related diseases, such as osteoarthritis [40, 41], which have a relatively high molecular weight determining the viscoelastic properties [33]. Other studies have explored developing scaffold materials with or without cross-linking of HyA [42-45]. Special attention has focused on maintaining the osteoconductive property of HyA by adding calcium phosphate materials through the deposition of HA crystals biomimetically onto the HyA matrix [44, 45] or simple by mixing β -TCP granules with HyA gels [42, 46, 47]. These studies suggest that HyA-containing scaffold materials are efficacious in bone repairing processes. Other studies have attempted to promote the binding between abundant carboxyl groups present in HyA and the calcium site in calcium phosphate crystal surfaces [45], which allows for the formation of relatively firm composites. The structure of OCP stacks apatite layers alternatively with hydrated layers [48, 49], which is why OCP has been proposed as a precursor of HA formation in experimental calcium and phosphate solutions *in vitro* [48, 50] and hypothesized to be a precursor of bone apatite crystals *in vivo* [51]. Some properties of OCP, such as the serum protein adsorption affinity [9], resemble those of HA. Although the

chemical compatibility of HyA with OCP crystals has never been assessed, OCP may work as a better adsorbent for HyA molecules in a similar manner as HA.

The aim of the present study was to examine whether the viscoelastic properties of HyA gels with relatively high molecular weights (ranging from 90×10^4 to 600×10^4), using commercially available HyA medical products, can be used as suitable vehicles for the OCP granules from the point of view of osteoconductivity and also the handling properties during surgical procedures. Unexpectedly, the HyA gels increased not only the handling property of OCP but also the osteoconductivity of OCP granules, accompanied by the increase of osteoclastic activity to resorb OCP, when the gels were combined with OCP and implanted onto mouse calvaria as composite materials consisting of OCP and HyA. It is likely that the OCP-HyA combination may have a synergistic effect with regard to the osteoconductivity of OCP.

2. Materials and methods

2.1. Preparation of OCP and OCP/HyA composites

OCP was prepared by mixing calcium and phosphate solutions as previously described [4]. The granules, consisting of an OCP crystal aggregate, were prepared from OCP precipitates by passing them through a standard testing sieve. Granules with diameters ranging from 300 to 500 μm were used (Figure 1A). The sieved OCP granules were sterilized by heating at 120 °C for 2 h. As shown previously, such heating does not affect the physical properties, such as the crystalline structure or specific surface area of OCP granules [6, 52], although it has been reported that temperatures above 100 °C induced a gradual

collapse of the OCP structure due to dehydration [23, 24]. Three kinds of medical-grade high molecular weight HyAs, including HyA90 (Artz[®], MW = 600-1200 kDa), HyA190 (Suvenyl[®], MW = 1900 kDa), and chemically cross-linked HyA600 (Synvisc[®], MW = 6000 kDa), were purchased from Seikagaku Kogyo Co. (Tokyo, Japan), Chugai Pharmaceutical Co. Ltd. (Tokyo, Japan), and Genzyme Biosurgery (Cambridge, MA, USA), respectively. OCP granules (100 mg) were added to the 1 ml HyA gels and mixed (Figure 1B).

2.2. Characterization of OCP/HyA gel

OCP/HyA gels after drying at room temperature and OCP itself were characterized by XRD. The XRD patterns were recorded by step scanning at 0.05° intervals from 3.0° to 60.0°, with Cu K α X-rays on a diffractometer (Mini Flex; Rigaku Electrical Co., Ltd., Tokyo, Japan) at 30 kV and 15 mA. The 2 θ range measured included the primary peak (100) of OCP at 4.7°. Joint Committee for Powder Diffraction Standard (JCPDS) numbers 26-1056A9 for OCP and 9-432 for HA were utilized to identify their crystalline phases. FTIR spectrum of the composites was obtained by FTIR spectroscopy (FREEXACT-2; HORIBA, Kyoto, Japan) with the sample diluted in potassium bromide over a range of 4,000-400 cm⁻¹ with 4 cm⁻¹ resolution. The morphology of OCP and OCP/HyA were examined using a JEOL analytical scanning electron microscope JSM-6390LA (Tokyo, Japan) operating at an accelerating voltage of 10 kV. Gold sputtering was performed before the observation. The viscosity of HyA solutions was measured using an electromagnetically spinning viscometer (Kyoto electronics manufacturing Co., Ltd., Kyoto, Japan).

2.3 Experimental animal model

Eight-week-old male ICR mice weighing 30-36 g (SLC, Hamamatsu, Shizuoka, Japan) were used in this study. The principles of laboratory animal care and use as well as national laws were followed. All procedures were approved by the Animal Research Committee of Tohoku University. The experimental mice were anesthetized with intraperitoneal injection of sodium pentobarbital (50 mg/kg) supplemented by ether inhalation. When they felt pain, ether inhalation was administered additionally during the operation. The skin and periosteum of the calvarium were carefully ablated. A polytetrafluoroethylene (PTFE) ring (inner diameter 6 mm, thickness 1 mm) was placed on the center of calvaria across the suture with a medical tissue glue (Aron Alpha A, Sankyo, Inc., Tokyo, Japan). After placement of the PTFE ring on the calvarium, the OCP/HyA composite was injected using a syringe with a 14G polymer catheter (Figure 1C). In order to prevent an outflow of the composite, the ring was then covered by a 100 μ m-thick PTFE sheet with a medical tissue glue (Figure 1D). The mice were sacrificed by using an excess dose of anesthetic drug (sodium pentobarbital, 200 mg/kg) intra-peritoneally at 3 and 6 weeks (six mice respectively) post-implantation respectively. Therefore twelve mice in each of the OCP granule, OCP/HyA composites, and PTFE (untreated) groups were prepared for histomorphometric examination.

2.4. Tissue preparation and histomorphometric analysis

Calvaria were resected and kept in 4% paraformaldehyde in 0.1 M

phosphate-buffered saline (PBS) overnight at 4 °C. The fixed specimens were decalcified in 10% EDTA in 0.01 M PBS, pH 7.4, for 2 weeks at 4 °C. The samples were then dehydrated in a graded series of ethanol and embedded in paraffin. Serial sections with 4 µm thickness were cut coronally and stained with hematoxylin and eosin (H&E) and Alcian blue for light micro-microscopy, and images were procured using a photomicroscope (Leica DFC300 FX, Leica Microsystems Japan, Tokyo, Japan). Light micrographs of the H&E-stained sections were used for histomorphometric measurements. The area rate of newly formed bone was calculated as the area of newly formed bone divided by the total implanted area. Likewise, the percentage of remaining implant in the defect was calculated as the area of remaining implant divided by the total area. The area rates were quantified on computer using Adobe Photoshop Elements 8 and Image J 1.45 (NIH Image, Bethesda, MD, USA).

2.5. Tartrate-resistant acid phosphatase and alkaline phosphatase double staining

Tartrate-resistant acid phosphatase (TRAP) and alkaline phosphatase (ALP) of the tissue sections were evaluated by TRAP/ALP double staining kit (Wako Code 294-67001, Osaka, Japan). The sections were soaked into TRAP buffer for thirty minutes. After washing by distilled water, there were soaked into 2-amino-2-methylpropane-1,3 diol for ten minutes, then incubated with ALP buffer for thirty minutes. Counterstaining was performed by hematoxylin.

2.6. Immunostaining of osteoclastin

After deparaffinization, the serial sections were incubated with 3% (vol/vol) H₂O₂ to eliminate endogenous peroxidase activity and blocked with 10% normal goat serum (Histofine® blocking reagent, Nichireibiosciences, Tokyo, Japan). The prepared slides were incubated with primary antibody for osteocalcin (Takara Code M188 R21C-01A, dilution 1:400) overnight at 4°C. They were incubated with the polymer prepared by combining amino acid polymers with peroxidase and goat anti-rat IgG (Simple Stain Mouse MAX PO (RAT), Code 414311F, Histofine® immunohistochemical staining reagent, Nichireibiosciences, Tokyo, Japan). The signal was detected using 3,3'-Diaminobenzidine tetrahydrochloride (DAB, Sigma-Aldrich Corp.). Counterstaining was performed with hematoxylin. Negative control staining was also examined without using the primary antibody of osteocalcin after the same procedure with experimental group including the incubation with 3% (vol/vol) H₂O₂ and the blocking with 10% normal goat serum.

2.7 In vitro assay

2.7.1 Cells and reagents

RAW264 cells were obtained from Riken Cell Bank (Tsukuba, Japan). DMEM/F-12 was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Gentamicin was purchased from Invitrogen Corp (Carlsbad, CA, USA). Fetal bovine serum (FBS) was purchased from Biological Industries, Ltd. (Kibbutz Beit Haemek, Israel). Recombinant human RANKL(rhRANKL) was purchased from Oriental Yeast Co., Ltd.(Tokyo, Japan)

2.7.2 TRAP staining

RAW264 cells were cultured in a DMEM/F-12 containing 10%FBS and 10µg/mL gentamicin (growth medium). Cells were inoculated into 48-well plates at a density of 5×10^3 cells/well and cultured in a growth medium containing 50 ng/ml rhRANKL and 0.5mg/ml HyA. After five days, cells were fixed with 4% paraformaldehyde and then stained with a TRAP staining kit (Sigma) following the manufacturer's protocol. After washing the wells with pure water, five regions in each well were photographed and counted for TRAP positive multinuclear cells.

2.8 Statistical analysis

Results were expressed as the mean $\pm$ standard deviation (SD). The in vitro experiments were performed at least three times. The histomorphometric analysis was carried out using three sections obtained from each animal at each time point. They showed reliable reproducibility. Statistical differences among specimens were evaluated by Tukey-Kramer multiple comparison analysis. A value of $p < 0.05$ was regarded as statistically significant.

3. Results

3.1 Characterization of OCP/HyA gels

Figure 1A and B show an image of sieved OCP granules and OCP/HyA90 composites, respectively. Figure 2A shows the X-ray diffraction (XRD) pattern of the OCP and OCP/HyA composites after drying. All reflections of XRD observed for the synthetic OCP, including the characteristic (100) reflection at $2\theta = 4.9^\circ$

and (700) reflection at $2\theta = 33.6^\circ$, corresponded well with those expected from the OCP structure [49]. The characteristic (100) reflection of HA at $2\theta = 10.8^\circ$ or reflections other than those from OCP was not detected, revealing that the synthesized OCP consisted of a single OCP crystalline phase. Figure 2B shows the Fourier transform infrared spectroscopy (FTIR) spectra of the OCP and OCP/HyA composites after drying. The FTIR spectrum of OCP corresponded well with that reported previously [23]. Three distinct bands originating from ν_3 P-O in the orthophosphate stretch absorption around $1,030\text{-}1,130\text{ cm}^{-1}$ and two sharp absorption bands of ν_4 PO_4 at $560\text{-}600\text{ cm}^{-1}$ were identified. Figure 2C and D shows scanning electron microscopy (SEM) photographs of OCP granules and OCP/HyA composite after drying. The granules showed an irregular morphology consisting of an aggregate form of OCP crystals. OCP crystals exhibited a platy morphology of several μm in length. These data demonstrated that the addition of HyA did not affect any structural properties in OCP. Measurement of viscosity using an electromagnetically spinning viscometer demonstrated that the viscosity of HyA gels was dependent on their molecular weight (Table 1).

3.2 Histological examination

Figure 3 shows overviews and magnified view including calvaria surfaces (Fig.3a) and magnified views (Fig.3b) of histological sections with H&E staining of OCP itself and OCP/HyA gels implants 3 and 6 weeks post-implantation. A previous study demonstrated that OCP granules can be recognized as hematoxilinophilic materials, which mainly occur due to the presence of

accumulated matrix proteins within the spaces between OCP crystals that are already absent as a result of decalcification during histological section preparation [6]. Most of the OCP granules tended to be encapsulated with the newly formed bone in all groups. New bone formation from the calvaria surface was observed but limited in the OCP group (Fig. 3aA). However, the combination of OCP with HyA, in particular HyA90 and HyA600, enhanced bone formation compared to the OCP alone group. Bone marrow tissue was also formed within the newly formed bone. In the OCP/HyA600 group, the remaining HyA gels were clearly recognized. The magnified images for the host bone zone, the interface zone and the material zone are shown in Figures 3aE, 3aF, 3aG, 3aH, 3aM, 3aN, 3aO and 3aP. Bone formation was recognized around OCP granules directly independent of the calvaria (Fig. 3aG and Fig. 3aH) in addition to the reactive bone formation from the calvaria (Fig. 3aF) in 3 weeks. The bone formation increased and became integrated with the host calvaria bone at 6 weeks (Fig.3aM-Fig.3aP). Figure 4 shows overviews and magnified views of histological sections stained with Alcian blue from the OCP/HyA600 implant group at 3 (A and C) and 6 (B and D) weeks post-implantation. Although there was no remaining HyA gel in the OCP/HyA90 and OCP/HyA190 groups even after 3 weeks (data not shown), HyA600 remained partially unresorbed at 3 and 6 weeks post-implantation.

3.3 Detection of TRAP-positive osteoclast-like cells and ALP-positive osteoblastic cells around OCP/HyA implants

Figure 5 shows TRAP and ALP staining of the calvaria tissues at 3 (Fig.5a)

and 6 (Fig.5b) weeks after the implantation of OCP and OCP/HyA composites. TRAP was detected closely associated with OCP granules regardless of the presence of HyAs (Fig.5aE-Fig.5aH). It seems likely that the TRAP of the OCP/HyA90 (Fig.5aF) and OCP/HyA600 (Fig.5aH and 5bP) were relatively higher than those by OCP/HyA190 (Fig.5aG and 5bO). TRAP of the control (PTFE ring only) was much lower than those of OCP/HyA composites (Fig.5aA and 5bI). ALP was detected on the calvaria surfaces of HyA90 (Fig.5aB) and HyA600 (Fig.5aD) without OCP at 3 weeks, and those of HyA190 (Fig.5bK) and HyA600 (Fig.5bL) at 6 weeks. ALP was also detected around OCP granules in OCP/HyA90 at 3 weeks (Fig.5aF) and in OCP/HyA90 at 6 weeks (Fig.5bN).

3.4 Localization of osteocalcin around bone formation

Figure 6 shows the immunostaining of osteocalcin at 6 weeks. The implanted OCP was stained positively for osteocalcin. Osteocalcin positive cells were observed around new bone. HyA remaining (HyA600, Fig.6D) was not stained by osteocalcin. Neither OCP granules nor bone tissues were stained in the control group (Fig. 6I, J, K and L).

3.5 Histomorphometric examination

The histomorphometric findings regarding the percentage of new bone formation are shown in Figures 7A and 7C. By week 3, the mean new bone area in the PTFE ring, OCP granule, HyA90, OCP/HyA90, HyA190, OCP/HyA190, HyA600, and OCP/HyA600 was 3.9 ± 4.2 , 5.6 ± 4.3 , 13.6 ± 6.2 , 17.1 ± 12.5 , 0.9 ± 0.7 , 9.9 ± 8.0 , 15.5 ± 9.2 , and 15.0 ± 10.2 %, respectively. A significant

difference was observed between the PTFE ring group and HyA600 and OCP/HyA600 groups. By week 6 post-implantation, the mean new bone area in the PTFE ring, OCP granule, HyA90, OCP/HyA90, HyA190, OCP/HyA190, HyA600, and OCP/HyA600 was 5.6 ± 7.6 , 20.8 ± 7.1 , 13.4 ± 5.1 , 33.0 ± 8.3 , 3.6 ± 5.9 , 21.5 ± 8.4 , 27.4 ± 7.9 , and 36.0 ± 5.6 %, respectively. A significant difference was observed between the PTFE ring group and the OCP/HyA90, OCP/HyA190, HyA600, and OCP/HyA600 groups. The new bone area rate of OCP/HyA90 and OCP/HyA190 was significantly higher compared to that of HyA90 and HyA190, respectively. It is noteworthy that the percentage of newly formed bone in the OCP/HyA90 and OCP/HyA600 groups was significantly higher than that of the OCP granule group. Histomorphometric findings regarding the percentage of remaining implant are shown in Figure 7B. By week 3 post-implantation, the mean percentage of remaining implant in the OCP granule, OCP/HyA90, OCP/HyA190, and OCP/HyA600 groups was 21.7 ± 16.3 , 20.8 ± 10.4 , 14.9 ± 5.2 , and 16.8 ± 11.9 %, respectively. No significant difference in the percentage of remaining implant was observed among the OCP/HyA composites. By week 6 post-implantation, the mean percentage of remaining implant in the OCP granule, OCP/HyA90, OCP/HyA190, and OCP/HyA600 groups was 12.7 ± 5.1 , 8.3 ± 3.5 , 13.7 ± 6.2 , and 14.4 ± 6.8 %, respectively, which was also not significantly different between the OCP/HyA composite groups.

3.6 Effect of HyA and OCP on osteoclast formation from RAW264 cells with RANKL

Figure 8 shows TRAP-staining (Fig.8A-8E) and the number of TRAP-positive cells estimated (Fig.8F) in RAW264 cells in the presence and the absence of RANKL and HyAs. TRAP positive cells were formed when RANKL was present in the absence of OCP (Fig.8B). The appearance of TRAP positive cells was enhanced in the presence of HyAs in general (Fig.8C-8E), in particular, and significantly increased in the presence of HyA 90 and HyA 600, respectively (Fig.8F).

4. Discussion

The present study provides evidence that OCP combined with HyAs having a molecular weight of 90×10^4 (HyA 90) and 600×10^4 (HyA 600) enhances osteoconductivity after implantation of the composites, particularly during the early stage of bone formation. The addition of HyAs to OCP granules, including HyA 120, made it possible to inject OCP into in a model defect (space) using a PTFE ring placed onto the mouse calvaria. HyA 90 and HyA 600 alone also induced new bone formation, although the extent of formation was less than that induced by the OCP/HyA composites. Histological examination found that while HyA 90 and HyA 120 combined with OCP granules were almost resorbed, HyA 600 remained completely unresorbed. The osteoconductivity of HyA 600 may be due to the matrix acting as a scaffold [45] for new bone formation together with the OCP granules [2]. In contrast, HyA 90 in combination with OCP granules may involve another stimulatory capacity in bone formation during the dissolution of HyA.

Injectable ceramics and putties, which are mostly aimed at forming the HA or dicalcium phosphate dihydrate (DCPD) phase, have been widely investigated [46] since the mechanism of cementation was elucidated in calcium phosphate ceramics [28, 31]. A cement consisting of raw calcium phosphate materials, such as DCPD and α -TCP, for forming OCP has also been reported [53, 54], although the cement products were composed of multi-phase calcium phosphates, including calcium phosphate crystals other than OCP or HA. We have previously shown that the osteoconductivity of OCP is markedly dependent on the stoichiometry and the crystallinity of OCP [14]; the process of progressive conversion from OCP to HA between the two phases seems to be required for the osteoconduction of OCP [55].

The injectability of calcium phosphate cements has been reported to be dependent on the liquid-to-powder ratio, the particle size, the plastic limit, and in some cases physical factors [56, 57]. The viscosities of OCP/HyA composites could not be measured in the present study most likely because of the relatively large OCP granule size present in HyA (300-500 μm diameter). The viscosities of HyAs themselves increased depending on the molecular weight of the HyAs (Table 1). Therefore, it is reasonable to postulate that the injectability of OCP/HyA is most likely based on the viscosity of the HyAs used. HyA has been previously assessed with β -TCP for improving injectability [46, 47] using granule sizes of 250-500 μm in diameter, which is a range that includes the OCP granule size assessed in our study.

The addition of HyA did not affect the structure of OCP based on XRD analysis. OCP is known to hydrolyze to HA if soaked under physiological pH

[58-60]. Once the hydrolysis is initiated, it proceeds spontaneously and irreversibly. The OCP crystals used in this study were well grown during the preparation [2, 4, 9, 55] and maintained a stable structure even when in contact with the HyAs used, which had a neutral pH. Previous studies have posulated that the HA and OCP structures can form chelates with carboxylate ions due to the presence of calcium ions in their structures [44, 45]. The carboxyl ions in HyA molecules are expected to form such an interaction with calcium ions in the calcium phosphate ceramics [61-65], resulting in relatively firm bonding. FTIR spectroscopy of the OCP/HyA composites also did not detect any evidence of interactions between the two molecules. However, the morphology of OCP crystals is markedly regulated if precipitated in the presence of natural polymers, such as gelatin [20, 66], suggesting a possible interaction between the carboxylate in the molecules with the OCP surface, though nothing in the IR band spectra changed. Therefore, a precise adsorption study of HyA molecules is required to make a definitive conclusion regarding the possible interaction using OCP as an adsorbent.

Our histological findings re-confirmed that OCP is an osteoconductive material that induces new bone formation closely associated with the OCP granule surface, which might be induced by the osteoconduction from calvaria and the direct deposition initiated from the OCP surface, as previously reported in studies that have implanted OCP into various bone defects [2, 4, 10, 12, 14, 15, 21, 67]. The biodegradation of OCP granules through TRAP-positive osteoclast-like cellular resorption was also ascertained around OCP, confirming that the addition of HyA did not inhibit the appearance of the osteoconduction in

OCP. Interestingly, in vitro study on the formation of TRAP-positive osteoclast like-cells from RAW246 cells with RANKL showed that HyA90 and HyA600 enhances its formation, suggesting that the biodegradability of OCP could be augmented if these HyAs are present with OCP. We have previously reported that OCP itself is capable of inducing osteoclast formation from the bone marrow cells in co-culture of osteoblasts with increasing its RANKL expression even in the absence of vitamin D₃ [3]. Taken together, it is reasonable to assume that HyAs in HyA/OCP composites in vivo take indirectly part in the biodegradation of OCP through the osteoclastic resorption, which results in enhancing bone formation by OCP. The present study, however, did not observe complete resorption of OCP granules in the limited periods. The previous study using similar experimental mouse calvaria model without HyAs confirmed the OCP granules can almost be replaced with new bone until 24 weeks [12]. The resorption rate may be affected by the presence of HyAs through the possible interaction with OCP crystals thereby controlling the OCP-HA conversion process although OCP can in fact be converted into HA in the presence of natural polymers, such as alginate [27] and collagen [68]. It seems likely that the composite of OCP combined by HyA90 or HyA600 may form a functional architecture to stimulate osteogenesis through synergistic effect on osteoclast formation around OCP.

Osteocalcin was demonstrated to be localized onto OCP regardless of the presence of HyA. Osteocalcin was also detected in osteoblasts forming new bone including the bone around OCP granules or HyA600 but not in the HyA itself. It has been reported that non-collagenous circulating serum proteins,

including osteocalcin, can be accumulated onto OCP if implanted in bone defects [4, 14] and subcutaneous tissue [4] or if incubated in rat serum in vitro [69]. The osteoconductivity of OCP was found for the first time by analyzing the implanted OCP into the subperiosteal area of mouse calvaria [4] using a model similar to the model used in the present study, demonstrating that the structure consists of OCP and the accumulated matrix proteins, including α_2 HS-glycoproteins, can be a nucleation site to initiate new bone deposition by osteoblasts [4, 6]. The present observation of the osteocalcin accumulation onto OCP may result from the accumulation of this protein circulating in blood stream or synthesized by osteoblasts onto OCP in spite of the presence of HyAs, which is consistent with the previous observation of OCP to show the osteoconductivity.

Remarkably, we found that HyA increases the osteoconductivity of OCP, especially during the early stage of implantation (3 weeks post-implantation). However, the efficacy of HyA was highly dependent on the molecular weight of the material. The lowest (HyA 90) and highest (HyA 600) molecular weight HyAs induced different levels of bone formation: HyA 90 was resorbed and tended to be replaced with new bone around OCP granules, while HyA 600 remained partially unresorbed, thereby providing a scaffold for new bone together with the OCP granules. Preliminary *in vitro* analysis showed that the incubation of HyAs with osteoblastic cells in the absence of OCP crystals did not enhance proliferation or differentiation of the cells even after prolonged incubation for up to 3 weeks (data not shown). Two studies have shown that HyAs with a molecular weight less than several hundred kDa are capable of facilitating

osteoblastic differentiation *in vitro* by binding to the receptor of HyA in the cell membrane [35, 37]. Other studies suggest that such HyAs with a low molecular weight regulate osteoclast activity to initiate bone matrix resorption through hormone-related biological processes [70]. These studies suggest that HyA 90 used in the OCP/HyA 90 composite in this study may be enzymatically degraded by hyaluronidase *in vivo* and that the degraded small molecules from HyA90 may play a role in enhancing bone formation. Another possibility is that growth factors, such as BMP-2 [37], are incorporated into the HyA at the early stage of implantation and are involved in the bone formation around OCP granules, which is coupled with HyA degradation and the re-release of the growth factors. Another possibility to be involved in raising the osteoconductivity of OCP by HyAs is that the osteoblastic cell migration could be controlled by HyAs depending on the molecular weight. HyA with higher molecular weight (HyA600) may work to inhibit the immigration of the osteoblasts within its own mass of HyA, resulting in the enhancement of bone formation around the HyA600 near OCP granules as observed. It is proposed that HyA could be forming a diffusion barrier between bone surface and osteoclasts around the sealing zone by the large hydrodynamic volume [70]. Furthermore, it is known that short-chain HyA (6.5kDa) with 3-10 disaccharide units is involved in displaying the migratory capacity of the angiogenic cells through providing the cell focal adhesion [71, 72], suggesting a possible involvement of the degraded small molecules from HyA90 to stimulate osteoblastic cell migration. The preliminary additional data (unpublished) suggested that the enhanced bone formation observed is derived from the synergistic effect of OCP and HyAs. Further studies are currently under

way to establish a link between the synergistic effect of OCP and HyAs and enhanced bone formation, and between the degradation of the remaining HyA, depending on the molecular weight of HyA, and enhanced bone formation in association with OCP crystals involving the intrinsic property to convert to HA.

5. Conclusion

In this study OCP/HyA composites with injectability and higher osteoconductivity, particularly in the early stage of implantation, were successfully generated. Previous studies have confirmed the effects of the granule size and the dose of OCP on osteoconductivity [16, 18, 68]. The osteoconductivity of OCP/HyA can be relatively easily controlled through the adequate selection of OCP preparations [14, 55] that are well established. The OCP/HyA composites tested in this study could be used extensively in various bone defects due to the injectable nature, including defects that are large or have complex shapes that often occur in orthopedic and oral surgeries.

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Figure captions

Figure 1. A photograph of the sieved OCP granules (300-500 μm) (A) and the injectable OCP/HyA90 composite (B). A photograph (C) and schematic illustration (D) of the experimental animal model.

Figure 2. XRD patterns (A) and FTIR spectra (B) of OCP and OCP/HyA composites. Scanning electron micrographs of OCP (C) and OCP/HyA90 composite (D). The marks asterisks (*) and arrows ($\downarrow$) represent the characteristics (100) XRD peaks at $2\theta = 4.9^\circ$ and three distinct bands originating from ν_3 P-O in the orthophosphate stretch absorption around $1,030\text{-}1,130\text{ cm}^{-1}$. Bars = 5 μm .

Figure 3. Overview and magnified view around calvaria (3a(A-P)) and magnified images around the implants (3b(Q-X)) of the sections stained with hematoxylin and eosin. (A, E, I, M, Q and U) OCP granules at 3 (A, E and Q) and 6 (I, M and U) weeks, (B, F, J, N, R and V) OCP/HyA90 at 3 (B, F and R) and 6 (J, N and V) weeks, (C, G, K, O, S and W) OCP/HyA190 at 3 (C, G and S) and 6 (K, O and W) weeks, and (D, H, L, P, T and X) OCP/HyA600 at 3 (D, H and T) and 6 (L, P and X) weeks. The letters (C), (B), (*), (V), and (HyA) represent mice calvaria, newly formed bone, OCP granules, blood vessel, and HyA gel, respectively. Bars = 1 mm (A-D, I-L), 200 μm (E-H, M-P) and 100 μm (Q-X).

Figure 4. Overview (A and B) and magnified images (C and D) of the sections stained with Alcian blue from the OCP/HyA600 implant group at 3 (A and C) and

6 (B and D) weeks post-implantation. The letters (*) and (HyA) represent OCP granules and HyA gel, respectively. Bars = 1 mm (A and C) and 75 μ m (B and D).

Figure 5. TRAP and ALP staining of the sections at 3 (5a(A-H)) and 6 (5b(I-P)) weeks. (A, E, I and M) Control by ring only (A and I) and by ring and OCP granules (E and M) but without HyA, (B, F, J and N) HyA90 without OCP granules (B and J) and with OCP granules (F and N), (C, G, K and O) HyA190 without OCP granules (C and K) and with OCP granules (G and O), and (D, H, L and P) HyA600 without OCP granules (D and L) and with OCP granules (H and P). The marks asterisk (*), arrow heads ($\blacktriangle$), arrows ($\rightarrow$) and letters HyA represent OCP granules, TRAP-positive cells, ALP-positive cells and HyA gels, respectively. Bars = 250 μ m.

Figure 6. Osteocalcin immunostaining of the sections at 6 weeks. (A and E) Control by ring only (A) and by ring and OCP granules (E) but without HyA, (B and F) HyA90 without OCP granules (B) and with OCP granules (F), (C and G) HyA190 without OCP granules (C) and with OCP granules (G), and (D and H) HyA600 without OCP granules (D) and with OCP granules (H). The marks asterisk (*) and arrows ($\rightarrow$), and letters B and HyA represent OCP granules and osteocalcin positive-positive cells, and newly formed bone and HyA gels, respectively. The control sections without using the primary antibody in the OCP group (I, J, K and L) corresponding to those with using the antibody (E, F, G and H). Bars = 25 μ m.

Figure 7. Histomorphometric analysis of bone formation by OCP/HyA composites and remaining implant. (A) Area rate of new bone formation of composites, OCP and control. (B) Area rate of remaining implant. (C) Area rate of new bone formation of HyAs, OCP and control.

Figure 8. Effect of HyAs and RANKL on the formation of TRAP-positive cells from RAW264 cells in vitro. (A) Control culture in the absence of RANKL and HyAs, (B) Culture in the presence of 50ng/ml of RANKL, (C) Culture in the presence of 50ng/ml of RANKL and 0.5mg/ml of HyA60, (D) Culture in the presence of 50ng/ml of RANKL and 0.5mg/ml of HyA190, (E) Culture in the presence of 50ng/ml of RANKL and 0.5mg/ml of HyA600, (F) Cell number of TRAP-positive cells estimated from the culture. Arrows (→) represent TRAP-positive cells. Bars = 200 μ m (A-E).

Figure 1

Figure 1

Figure 2

Figure 2

Figure 3a

Figure 3b

Figure 4

Figure 4

Figure 5a

Figure 5b

Figure 6

Figure 6

Figure 7

Figure 8

Figure 8

Table 1. Molecular weight and viscosity of HyA

	Molecular weight	Viscosity (mPa · s)	SD (mPa · s)
HyA90	90 x10 ⁴	834*	1
HyA190	190 x10 ⁴	17700*	500
HyA600	600 x10 ⁴	31100**	1000

Spinning speed, *; 2000rpm, **; 4000rpm