Reconstruction of Critical-Sized Bone Defect in Dog Skull by Octacalcium Phosphate Combined with Collagen

Tadashi Kawai, DDS, PhD;[*†] Keiko Matsui, DDS, PhD;[‡] Shinya Iibuchi, DDS, PhD;[§] Takahisa Anada, PhD;[¶] Yoshitomo Honda, DDS, PhD;[**] Kazuo Sasaki, BSc;[††] Shinji Kamakura, DDS, PhD;[‡‡] Osamu Suzuki, PhD;[§§] Seishi Echigo, DDS, PhD[¶¶]

ABSTRACT

Purpose: The present study was designed to investigate whether synthetic octacalcium phosphate (OCP) combined with collagen (OCP/collagen) can repair a critical-sized defect in dog skull. OCP/collagen has been shown to biodegrade and to tend to be replaced by newly formed bone if implanted in rat calvaria defects.

Materials and Methods: An OCP/collagen disk was prepared from pepsin-digested atelocollagen isolated from porcine dermis and synthetic OCP. Two critical-sized defects (20 mm in diameter) were made in a dog skull. Ten disks of OCP/collagen or collagen (control) were implanted in the bone defects and resected with surrounding tissues at 3, 6, or 12 months after the implantation. The specimens were analyzed radiographically, crystallographically, histologically, and histomorphometrically.

Results: X-ray diffraction and FTIR analyses showed that OCP tended to convert to a poorly crystallized hydroxyapatite, similar to that of biological apatite, by 3 months. Radiographic and histologic analyses showed that the implantation of OCP/collagen disks initiated new bone formation in the defects at 3 months after implantation. However, there was no promotion of bone formation by control collagen disks even with prolonged implantation up to 12 months. Histomorphometric analysis revealed that the percentage of newly formed bone in the defect implanted with OCP/collagen increased significantly, from 30.91 ± 6.65 at 3 months to 51.22 ± 5.99 at 12 months, although the value tended to reach a plateau at 6 months (44.49 ± 3.34). On the other hand, the percentage of remaining OCP was estimated at approximately 10% at 3 months and remained nearly unchanged thereafter.

Conclusion: The results suggest that bone regeneration of a critical-sized bone defect of dog calvaria by OCP/collagen can be enhanced for 3 to 6 months and that OCP/collagen holds potential as a bone substitute material.

KEY WORDS: bone regeneration, collagen, critical-sized defect, octacalcium phosphate (OCP), translational research

INTRODUCTION

Reconstruction of a large bone defect is an important issue in oral and orthopedic surgeries.[1–3] Today, although synthetic bone substitute materials such as hydroxyapatite (HA) or beta-tricalcium phosphate (β-TCP) have been utilized clinically,[4–7] autogenous

*Postdoctoral researcher, Division of Oral Surgery, Tohoku University Graduate School of Dentistry, Sendai, Japan; †postdoctoral researcher, Division of Craniofacial Function Engineering, Tohoku University Graduate School of Dentistry, Sendai, Japan; ‡assistant professor, Division of Oral Surgery, Tohoku University Graduate School of Dentistry, Sendai, Japan; §postdoctoral researcher, Division of Oral Surgery, Tohoku University Graduate School of Dentistry, Sendai, Japan; ¶assistant professor, Division of Craniofacial Function Engineering, Tohoku University Graduate School of Dentistry, Sendai, Japan; **assistant professor, Division of Craniofacial Function Engineering, Tohoku University Graduate School of Dentistry, Sendai, Japan; ††team leader, Nippon Meat Packers, Inc., Tsukuba, Ibaraki, Japan; ‡‡professor, Division of Bone Regenerative Engineering, Tohoku University Graduate School of Biomedical Engineering, Sendai, Japan; §§professor, Division of Craniofacial Function Engineering, Tohoku University Graduate School of Dentistry, Sendai, Japan; ¶¶professor, Division of Oral Surgery, Tohoku University Graduate School of Dentistry, Sendai, Japan

Reprint requests: Prof. Shinji Kamakura, 2-1 Seiryo-machi, Aoba-ku, Sendai 980-8574, Japan; e-mail: kamakura@m.tains.tohoku.ac.jp; Prof. Osamu Suzuki, 4-1 Seiryo-machi, Aoba-ku, Sendai 980-8575, Japan; e-mail: suzuki-o@m.tains.tohoku.ac.jp

DOI 10.1111/j.1708-8208.2009.00192.x

bone is still the first choice in bone reconstruction because it can repair the bone defect more effectively than other biomaterials can.[8] However, the use of autogenous bone has disadvantages, including limited availability and morbidity associated with harvesting bone from a second operative site.[5] Furthermore, the application of autogenous bone is not easy in common medical offices due to the large-scale surgery required. Synthetic biomaterial, therefore, is widely used as a bone substitute to overcome the disadvantages of using autogenous bone.

Octacalcium phosphate (OCP) is a precursor of initial mineral crystals of biological apatite in bone and teeth.[9] In fact, direct evidence of the presence of OCP was found in the central part of human dentine crystals, and apatite was found in the outermost layers of the same crystal,[10] in porcine enamel,[11] and in the sutures of mouse calvaria during intramembranous osteogenesis.[12] The biological responses have also been investigated in forms of coatings on metallic implants[13–16] by estimating the longevity of the coatings.[13] We established a method of OCP synthesis in 1991 on a relatively large scale[17] and found its osteoconductive characteristics of synthetic OCP in murine bone.[17,18] Recent in vitro studies have revealed that synthetic OCP facilitates osteoblastic cell differentiation.[19–21] The effect of OCP on the promotion of in vitro osteoblastic cell differentiation has been reported to be dose dependent.[22] It has been demonstrated that OCP enhances bone regeneration more than nonsintered stoichiometric HA,[17,23] Ca-deficient HA,[17,19] sintered HA ceramic,[24] or biodegradable β-TCP ceramic.[23] However, unlike HA or β-TCP, OCP cannot be sintered because of the large number of water molecules in its structure.[9] β-TCP is a biodegradable ceramic and, therefore, is recognized as a material that is progressively replaced by bone.[6] However, the implanted OCP is resorbed by osteoclast-like cells[24–26] and is more resorbable than HA and β-TCP.[23] Synthetic OCP has been shown to convert to apatite when implanted in various sites, including muscle pouches,[27] the subperiosteal region of calvaria[17,18] and the subcutaneous region in mouse,[18] as well as critical-sized calvarial bone defects[19] in rat. The conversion from OCP to HA has been proved in some in vitro physiological environments, such as culture media,[19] simulated body fluid,[28] serum[29] and so forth.[30–34]

A scaffold constructed from synthetic OCP and porcine collagen sponge has been engineered to overcome the poor moldability of OCP.[25,35] OCP combined with collagen (OCP/collagen) has been shown to enhance bone regeneration more than OCP alone in a rat skull defect[35] or in rabbit bone marrow.[25] It has been demonstrated that OCP/collagen augments bone regeneration correlated with OCP content in vivo.[36] However, it is still unclear whether OCP/collagen is capable of stimulating bone regeneration in large defects created in the bones of large animals. In the present study, we investigated the bone regenerative properties of OCP/collagen disks in a critical-sized bone defect of dog skull. Critical-sized bone defects of skulls have been used in the quantitative analysis of bone regeneration by biomaterials.[3,35,37] The experimental model used in the present study was developed for a pre-clinical test of treatment of human bone defects with OCP/collagen.

MATERIALS AND METHODS

Animals

Eighteen-month-old male beagle dogs (SLC, Shizuoka, NARC Co., China) were used. The principles of laboratory animal care, as well as national laws, were followed. All procedures were approved by the Animal Research Committee of Tohoku University.

Preparation of Collagen and Composite of Octacalcium Phosphate/Collagen Disks

OCP was prepared by mixing a calcium and phosphate solution as described previously.[17,38] The sieved granules (particle size of 300–500 μm) of OCP, obtained from dried OCP, were sterilized by heating at 120°C for 2 hours. Our previous study showed that such heating does not affect physical properties such as the crystalline structure or specific surface area of OCP granules,[18,30] although it was reported that increasing the temperature above 100°C induced the collapse of the OCP structure because of dehydration.[39,40] Collagen was prepared from NMP collagen PS (Nippon Meat Packers, Tsukuba, Ibaraki, Japan), a lyophilized powder of pepsin-digested atelocollagen isolated from porcine dermis. NMP collagen PS was dissolved with distilled water and adjusted to 3% of the final concentration at pH 7.4. The concentrated collagen was lyophilized, and then a disk was molded (9 mm diameter, 1 mm thick). OCP/collagen was prepared from NMP collagen PS and OCP granules.[35,36] OCP was added to the concentrated collagen and mixed. The OCP/collagen mixture was then lyophilized, and the disk was molded (9 mm diameter,

1 mm thick). The molded OCP/collagen and collagen underwent dehydrothermal treatment (150°C, 24 hours) in a vacuum drying oven DP32 (Yamato Scientific, Tokyo, Japan) and were then sterilized using electron beam irradiation (5 kGy).

Surgical Procedure

General anesthesia was administered with intravenous sodium pentobarbital (0.5 mL/kg), followed by intramuscular atropine sulfate (0.5 mg) and ketamine hydrochloride (20 mg/kg). After the head area was disinfected and local anesthesia was applied, an arc-shaped incision was made from the front region of the ear to the other side through the forehead region, and the skin, muscle, and periosteum were ablated. Two holes were made on the left and right regions of the exposed skull under continuous saline buffer irrigation. The diameter of the skull defect was 20 mm (Figure 1A). It has been reported that there is insufficient bone regeneration in the skull defect when the diameter of the defect is more than twenty millimeters in mesofauna.[41] Ten OCP/collagen disks were implanted in the skull defect (see Figure 1B), and 10 collagen disks were implanted in the other side. After that, periosteum, muscle, and skin were repositioned and sutured. To prevent infection, flomoxef sodium (Flumarin®, Shionogi & Co., Ltd., Osaka, Japan) was used by intravenous drip during the operation, and cefcapene pivoxil hydrochloride hydrate (Flomox®, Shionogi & Co., Ltd., Osaka, Japan) was used by oral administration for 3 days postsurgically. Three dogs were sacrificed 3 months after implantation, five dogs were sacrificed at 6 months, and the other five dogs were sacrificed at 12 months after implantation by intravenous injection of an overdose of sodium pentobarbital (20 mL). After sacrifice, the implants were resected along with the surrounding bones and tissues and were fixed with 10% formalin neutral buffer solution, pH 7.4, by infiltration for 4 weeks at 4°C.

Radiographic Analysis

The skulls were radiographed by means of a microradiography unit (Softex CMBW-2; Softex, Tokyo, Japan) with X-ray film (FR; Fuji Photo Film, Tokyo, Japan) under standardized conditions (30 KV, 5 mA, 20 seconds) in which neither OCP nor OCP/collagen showed radiopacity (Figure 2).

XRD and FTIR of Implanted OCP/Collagen

The OCP/collagen implants in the dog skulls were examined with X-ray diffraction (XRD) and Fourier transform infrared spectroscopy (FTIR). The implants were collected from one dog skull bone from either the 3- or 6-month implantation group. Tweezers were used for the collection to exclude as much of the soft tissue around the implanted OCP/collagen as possible. The implants were immediately washed in deionized water, lyophilized, and reduced to powder. Then, the samples were collected for XRD and FTIR. An XRD pattern of each sample was obtained by scanning with Cu Kα

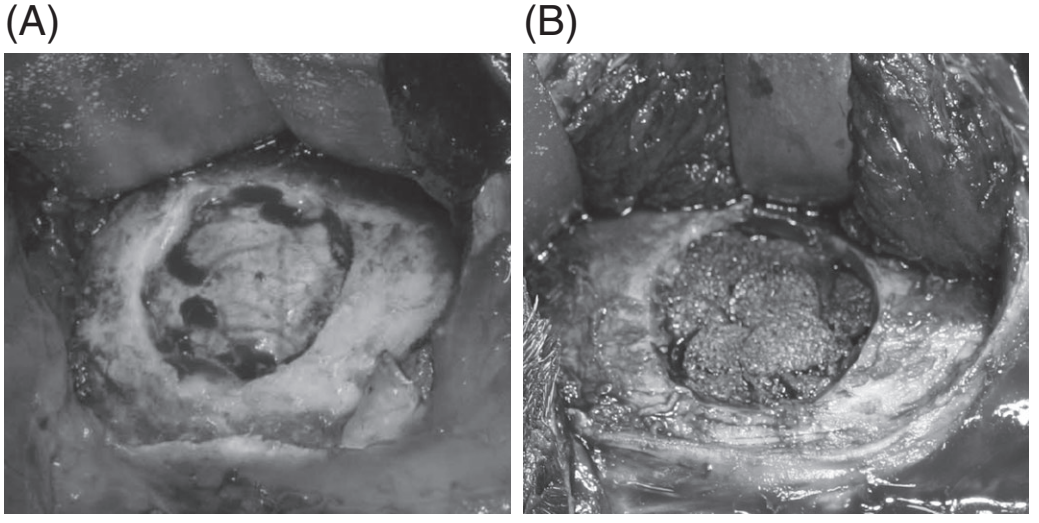

Figure 1 Photographs of (A) a defect created on the dog skull and (B) the defect filled with ten OCP/collagen disks. The diameter of the skull defect was 20 mm.

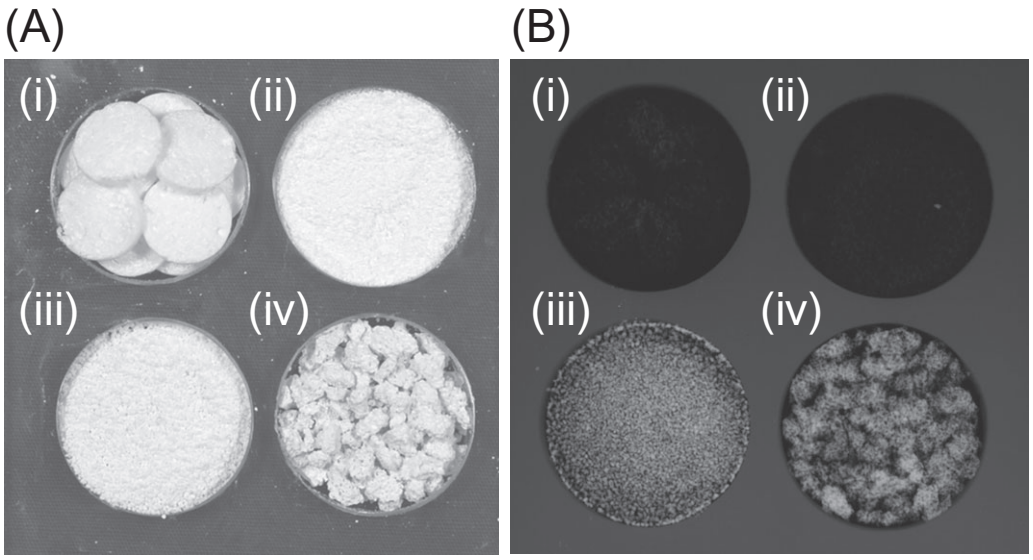

Figure 2 Radiopacity of (i) OCP/collagen disks, (ii) OCP, (iii) HA, and (iv) β-TCP. HA and β-TCP for comparison. *A*, Each material was put in a hole (diameter 20 mm, depth 2 mm). *B*, X-ray of each material. HA or β-TCP has radiopacity, but OCP/collagen disks and OCP have no radiopacity. X-ray condition was 30 KV, 5 mA, 20 seconds.

X-rays on a Rigaku Electrical Co., Ltd. (Tokyo), RAD-2B diffractometer at 40 KV, 20 mA. The FTIR spectrum of each sample was obtained by a HORIBA FTIR FREEXACT-2 (HORIBA, Kyoto, Japan), with the sample diluted with KBr over a range of 4000–400/cm with a 4/cm resolution. Collagen, OCP, OCP/collagen, hydroxyapatite, and dog bone were examined with XRD and FTIR for comparison with the implanted OCP/collagen.

Tissue Preparation

After radiographs were taken, the samples were cut coronally into two pieces at the center of the defect. One piece of each sample was dehydrated in a graded series of ethanol, embedded in methyl methacrylate (MMA) and sectioned coronally using a low-speed saw machine (Isomet 5000: Buehler, Lake Bluff, IL) with a diamond wafer blade. The sectioned wafers were mounted on plastic slides and were ground and polished until they were 200–300 μm thick. Microradiographs of undecalcified sections were made with a microradiography unit (Softex CMR Unit; Softex, Tokyo, Japan) for 60 seconds exposure at 20 KV, 5 mA. The other pieces of each sample were decalcified in formic acid and sodium citrate solutions for six weeks at 4°C. The samples were dehydrated in a graded series of ethanol, embedded in paraffin, and sectioned coronally at a thickness of 4 μm. The sections were taken with hematoxylin and eosin, and photographs were taken with a photomicroscope (Leica DFC300 FX, Leica Microsystems Japan, Tokyo, Japan).

Quantitative Micrograph Analysis

Light micrographs of the sections stained with hematoxylin and eosin were used for the histomorphometric measurement. Photographs projecting the overall defect were taken of each specimen. The percentage of newly formed bone in the defect (n-Bone%) was calculated as the area of newly formed bone per area of defect originally created by trephination × 100.[23,35,36] Likewise, the percentage of remaining implant in the defect (r-Imp%) was calculated as the area of remaining implant per area of defect originally created by trephination × 100.[23,36] The n-Bone% and r-Imp% were quantified on a computer using Scion Image public domain software (Scion Corporation, Frederick, MD, USA).

Statistical Analysis

Histomorphometric data were analyzed using a computer software package (Excel v.X, Microsoft Co., Redmond, WA, USA). All values were reported as the means ± standard deviation (SD). The statistical analysis was performed for histomorphometric experiments. One-way analysis of variance (ANOVA) was used to compare the means among groups. If the ANOVA was significant, Tukey multiple comparison analysis was used as a post hoc test.

RESULTS

Radiographic Analysis

The results of the radiographic examination are shown in Figure 3. There was little radiopacity along the margins of the defects with implanted collagen disks at 3, 6, and 12 months. Radiopacity was observed in the defects implanted with OCP/collagen disks at 3, 6, and 12 months. The shape of the OCP/collagen disk and the implanted state of 10 OCP/collagen disks were maintained throughout the implantation terms. The radiopacity was almost the same as that of the host bone around defects implanted with OCP/collagen disks for both 6 and 12 months, and there were few differences in the results of radiographic analysis between the OCP/collagen disks implanted for 6 months and those implanted for 12 months. The radiopacity of OCP/collagen disks implanted for 6 or 12 months increased compared with that of disks implanted for 3 months.

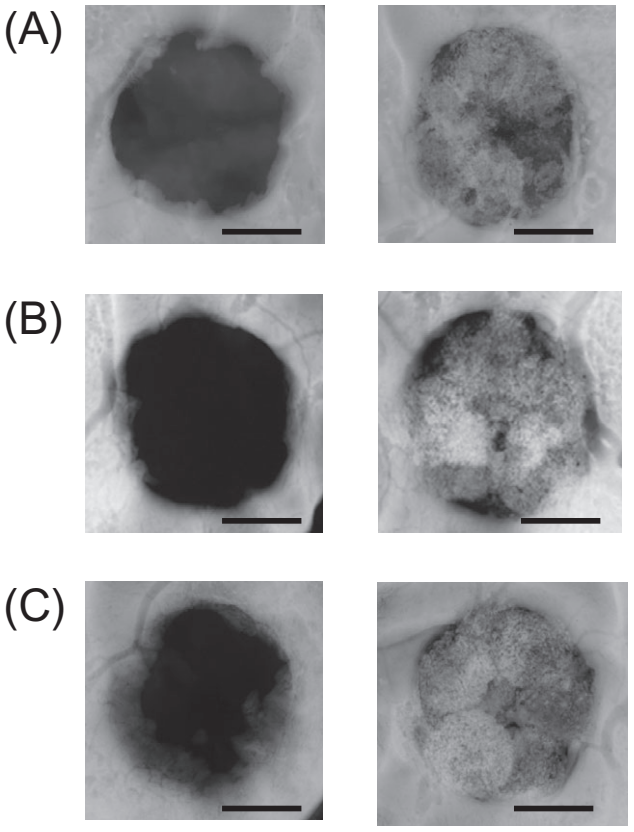

Figure 3 Radiographic analysis of the implants. The implantation terms were (A) 3 months, (B) 6 months, and (C) 12 months. In each radiograph, the left X-ray shows a defect implanted with collagen disks, and the right X-ray shows a defect implanted with OCP/collagen disks. OCP/collagen disks have changed to hard tissue with radiopacity. Bars = 10 mm.

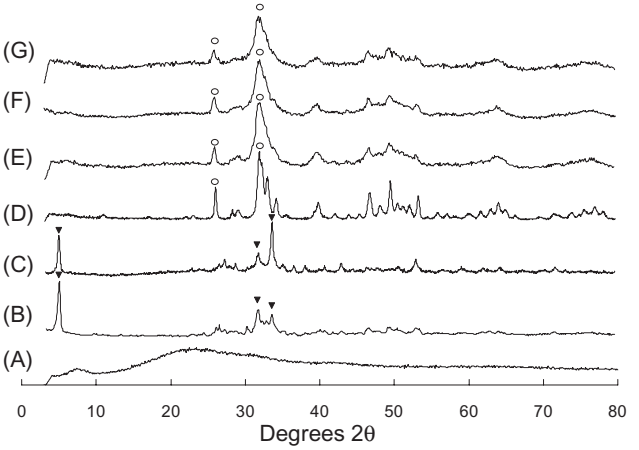

Figure 4 XRD patterns of (A) collagen, (B) OCP, (C) OCP/collagen, (D) HA, (E) dog bone and OCP/collagen implanted for (F) 3 or (G) 6 months. (▼) OCP reflection. (○) Apatite reflection.

Structural Changes of OCP/Collagen Implanted in Dog Skull Defects

Figure 4 shows the XRD patterns of OCP/collagen implanted for 3 and 6 months, collagen, OCP, OCP/collagen, HA, and dog bone. The patterns of OCP/collagen implanted for 3 and 6 months were almost the same and different, respectively, from that of OCP/collagen before implantation. No features of OCP or OCP/collagen were seen in the patterns of OCP/collagen implanted in the defects, whereas features of HA were seen in those patterns. There was no difference between the patterns of OCP/collagen implanted in the defects and those of dog bone.

Figure 5 shows the FTIR spectra of OCP/collagen implanted for 3 and 6 months, collagen, OCP, OCP/collagen, HA, and dog bone. The features of FTIR spectra of OCP/collagen implanted for 3 and 6 months indicate the conversion of OCP/collagen into HA in the IR spectral bands around 1030–1130/cm. The features of CO_3^{2-} were observed at bands around 870 and 1410–1460/cm in the spectra of OCP/collagen implanted in the defects. The features of FTIR spectra of HA and CO_3^{2-} were also observed in the FTIR spectrum of bone. These results confirm that the structure of OCP in OCP/collagen converted to the structure of biological apatite.

From the results of XRD and FTIR, it was apparent that the characteristic of OCP in OCP/collagen disk remained after the vacuum heating.

Histological Analysis

Microradiographs of undecalcified sections are shown in Figure 6. The defect mostly remained in the sections

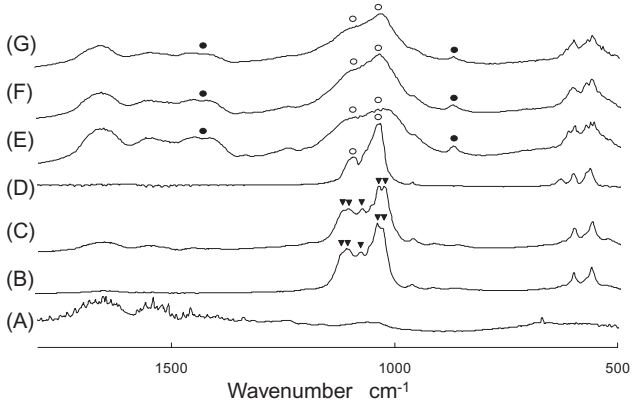

Figure 5 FTIR spectra of (A) collagen, (B) OCP, (C) OCP/collagen, (D) HA, (E) dog bone and OCP/collagen implanted for (F) 3 or (G) 6 months. (▼) The feature of FTIR spectra of OCP. (○)The feature of FTIR spectra of HA. (●) The feature of FTIR spectra of CO_3^{2-}.

implanted with collagen disks (see Figure 6, A–C), whereas the area corresponding to the radiopacity was occupied by newly formed bone and the remaining OCP of OCP/collagen disks and both sides of the host bone were connected with hard tissue in each implantation term (see Figure 6, D–F).

Figure 7 shows an overview of the sections of OCP/collagen disks and collagen disks stained with hematoxylin and eosin implanted in the defects for 3, 6, and 12 months. The defects implanted with collagen disks for each term remained, muscle came into the defects, and a small amount of newly formed bone was observed along the margins of the defects (see Figure 7, A–C). The defects implanted with OCP/collagen disks for each term were filled with newly formed bone and the remaining OCP of OCP/collagen (see Figure 7, D–F).

Figure 8, A–C shows magnified histological sections of OCP/collagen disks stained with hematoxylin and eosin implanted in the defects for each term. Normal bone tissue was observed in the bone defects implanted with OCP/collagen. No inflammatory cell infiltration was observed at the implantation site.

The margins of the defects implanted with OCP/collagen disks for each term are shown in Figure 8, D–F. Newly formed bone and host bone were close together in each term, and there was an unclear border between host bone and newly formed bone implanted for 12 months.

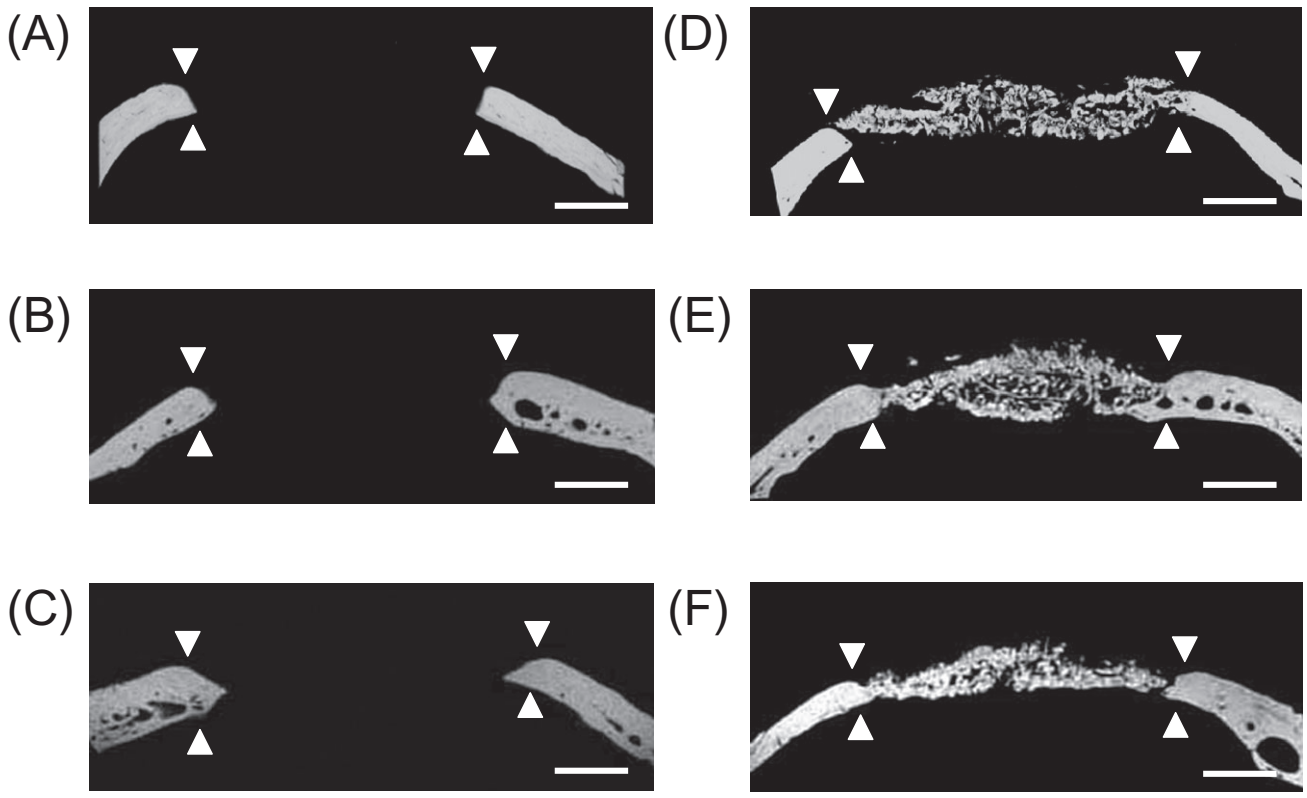

Figure 6 Microradiographs of undecalcified sections cut coronally. *A–C*, Defects implanted with collagen disks. *D–F*, Defects implanted with OCP/collagen disks. The implantation terms were (A and D) 3 months, (B and E) 6 months, and (C and F) 12 months. Bars = 5 mm. ▼ = defect margin.

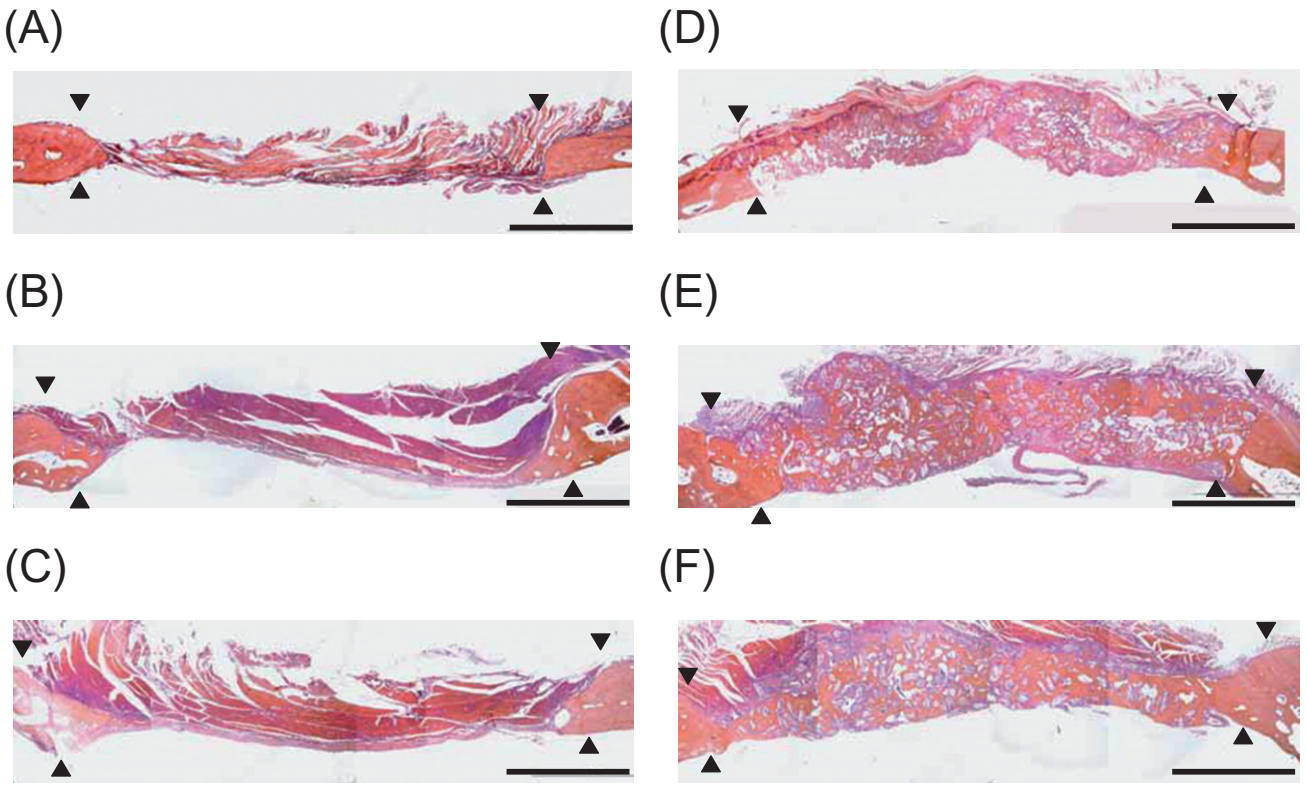

Figure 7 Overview of the sections stained with hematoxylin and eosin. *A–C*, Defects implanted with collagen disks. *D–F*, Defects implanted with OCP/collagen disks. The implantation terms were (A and D) 3 months, (B and E) 6 months, and (C and F) 12 months. Bars = 4 mm. ▼ = defect margin.

Histomorphometrical Analysis

Histomorphometrical findings regarding n-Bone% of the defect with implantation of OCP/collagen, that of the defect with implantation of collagen and r-Imp% of the defect with implantation of OCP/collagen are shown in Figure 9. In 3 months, n-Bone% ± SD of the defect with implantation of OCP/collagen, that of the defect with implantation of collagen and r-Imp% ± SD of the defect with implantation of OCP/collagen were 30.91 ± 6.65, 4.42 ± 0.29, and 12.20 ± 6.80. In 6 months, those were 44.49 ± 3.34, 7.98 ± 2.20, and 11.59 ± 4.24. In 12 months, those were 51.22 ± 5.99, 7.19 ± 1.42, and 11.51 ± 3.79. N-Bone% of defects implanted with OCP/Collagen at 6 or 12 months increased compared with that at 3 months. No significant difference was observed between the n-Bone% of the defects implanted with OCP/collagen at 6 and 12 months and r-Imp% of the defects implanted with OCP/collagen for each term. A significant difference was observed between the n-Bone% of OCP/collagen and that of collagen in each term.

DISCUSSION

The results of the present study provide evidence that the implantation of OCP/collagen disks in dog skull defects raises the radiopacity at 6 or 12 months to nearly that of the host bone around the defects. The CMR of undecalcified sections of the defects implanted with OCP/collagen disks also shows that the defect was connected to the host bone and was almost without a radiolucent zone. In the histological analysis, the defect implanted with OCP/collagen disks for each term was filled with newly formed bone or the remaining OCP of OCP/collagen, which may have corresponded to the newly formed bone and the remaining OCP of OCP/collagen. The results confirm that OCP/collagen can repair a critical-sized bone defect of a large animal. There was a significant difference between the n-Bone% of defects implanted with OCP/collagen and that of defects implanted with collagen in each implantation term. The n-Bone% of the defects implanted with OCP/collagen for 6 or 12 months increased compared with that at 3 months, which suggests that the

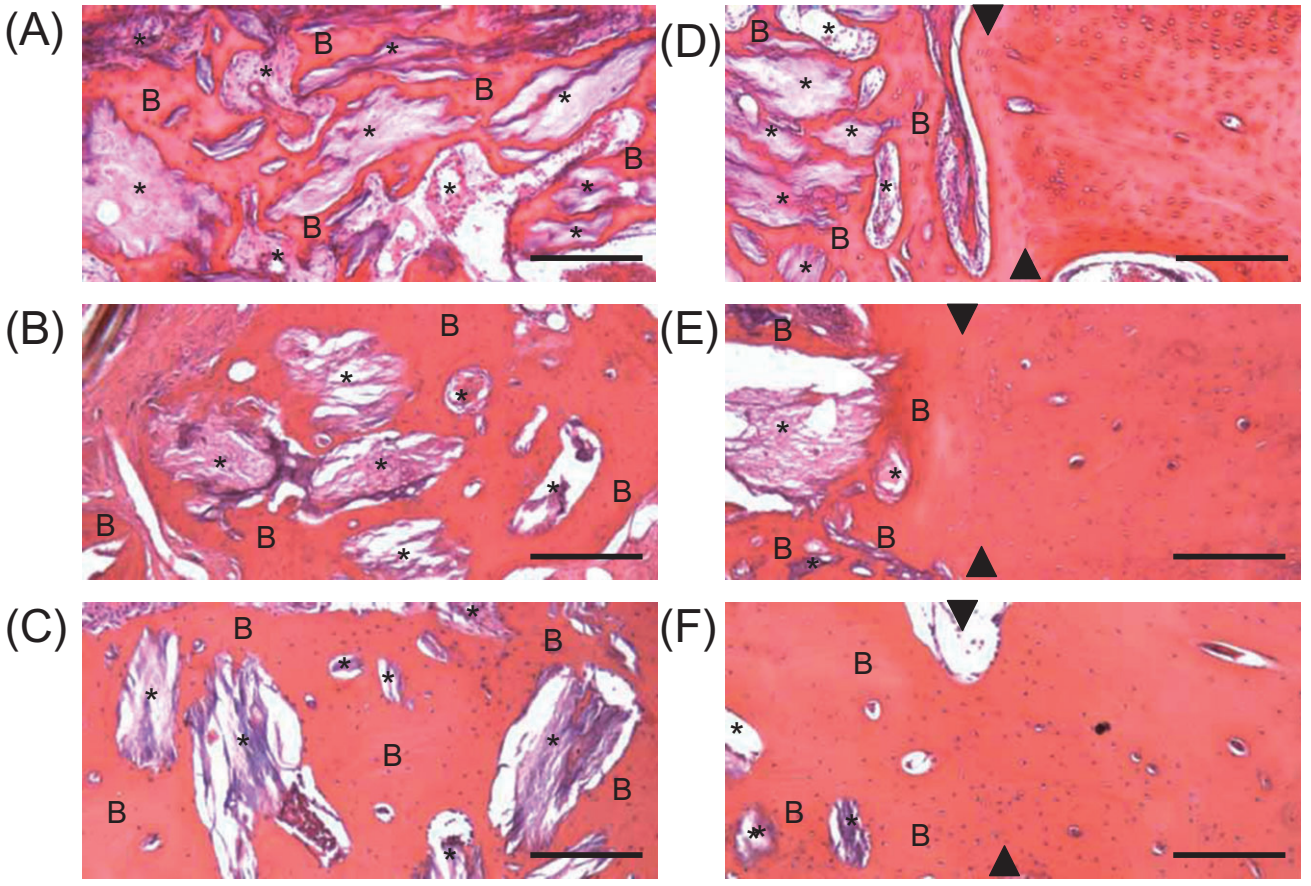

Figure 8 Magnified histological sections of OCP/collagen disks implanted for (A) 3 months, (B) 6 months, and (C) 12 months and the margins of the defects implanted with OCP/collagen disks for (D) 3 months, (E) 6 months, and (F) 12 months. B = newly formed bone. Asterisks = remaining OCP of OCP/collagen. Bars = 200 μm. ▼ = defect margin.

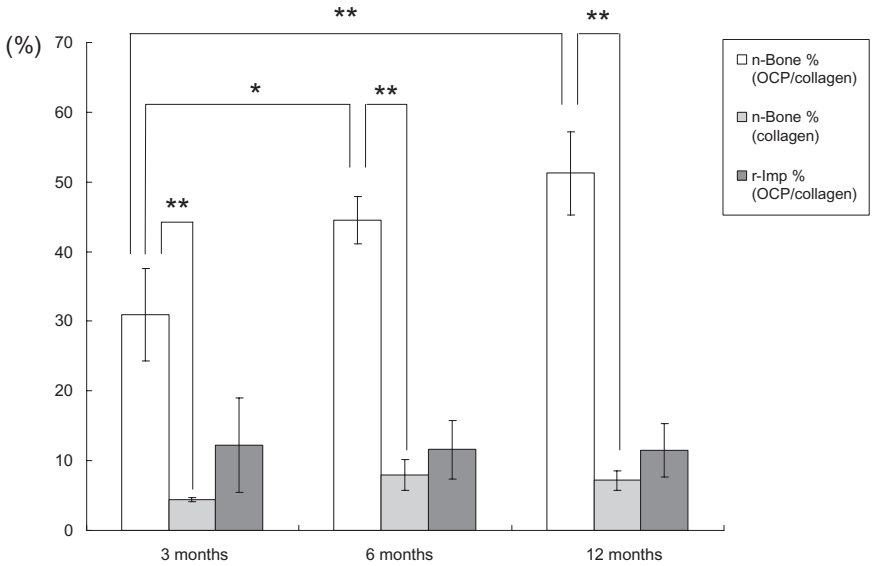

Figure 9 Histomorphometric analysis of the sections stained with hematoxylin and eosin. *$p < 0.05$, **$p < 0.01$.

bone regeneration by OCP/collagen was attained 6 months after implantation. However, the r-Imp% of the OCP/collagen was virtually unchanged, suggesting that the biodegradable characteristic of OCP was enhanced during the 3 months after implantation. The histological features of the newly formed bone surrounding the remaining OCP indicate that the OCP within OCP/collagen may help to initiate bone formation. The present histological examination showed that there is some radiolucent area at the margin of the bone defects, while the deposition of bone is enhanced in center of the defect. The previous studies reported that OCP works as a starting locus of intramembranous bone formation[17] and that osteoblasts present in bone not in periosteum are involved in the bone formation.[42] The current and previous studies suggested that the bone formation took place in the implanted OCP/collagen is promoted by osteoblasts present in the calvaria near the defect.

It has been suggested that OCP is more soluble than HA and β-TCP.[43] In fact, studies of physicochemical reactions of OCP showed that OCP tended to convert to HA by accompanying phosphate ion release and calcium ion uptake in both physiological conditions, reflecting the higher solubility of OCP[34,44] in culture media.[22] The results of previous and the present studies, however, do not explain whether the implanted OCP exhibits biodegradability even after conversion to HA by prolonged implantation. In the present study, the results of FTIR analysis reveal that the HA that converted from OCP was carbonate apatite, which is more soluble than stoichiometric sintered HA ceramic.[45] It is anticipated that the remaining OCP of OCP/collagen can be resorbed in the long term if the converted HA displays the solubility of carbonate apatite in vitro.[46] In our previous studies, we demonstrated that OCP converted HA in vitro and that bone regeneration of HA converted from OCP was less than that of OCP.[19] Because it is thought that the HA converted from OCP is considered as the carbonate apatite converted from OCP in OCP/collagen in the present study, it is suggested that OCP facilitates bone regeneration more than carbonate apatite. In other words, it is suggested that the conversion of OCP to HA, which corresponds to calcification, is important in bone regeneration. This is supported by an experimental result that OCP facilitates osteoblastic differentiation during the conversion to HA.[19,22]

Our previous study revealed that the control collagen disk was capable of forming new bone if implanted in rat calvaria, although the amount of new bone was significantly lower than that formed by OCP/collagen.[35,36] However, bone formation was only marginal when the control collagen disk was implanted in the dog skull defect in the present study. A possible explanation for this inhibitory reaction may be the result of the invasion of muscle of the calvaria into the defect, which may have reduced the space available for bone formation by the collagen disk. Nevertheless, the fact that the defects implanted with the OCP/collagen disks were filled with newly formed bone suggests that the osteoconductive characteristics of OCP/collagen can be displayed by the intrinsic bone regenerative property of OCP in concert with the collagen matrix. In a previous study, calcium deposition was observed on the collagen of OCP/collagen before implantation, and the collagen of OCP/collagen supported cell attachment and proliferation in vitro.[36]

In the present study, 10 OCP/collagen disks were arranged like pavement in order to cover fully the dog skull defect at the implantation. The shape of the newly formed bone in the defects with the implantation of OCP/collagen disks was in accordance with the disk shape. Histologic examination revealed that the remnants of OCP granules were surrounded by newly formed bone. These results suggest that bone can be formed along the bone defect if the shape of OCP/collagen can adjust to the shape of the bone defect or if OCP/collagen disks is arranged to cover as much of the defect as possible.

The mechanical strength of the newly formed bone was not analyzed in the present study. However, manual inspection verified that the newly formed bone had sufficient stiffness to resist compression. This qualitative estimation supports the radiographic and histologic observations. It seems likely that there are some advantages to OCP/collagen in clinical use. OCP/collagen disk has good handling performance compared with OCP granules. Moreover, OCP/collagen shows styptic characterization by collagen.[47,48] In oral surgery, it is sometimes difficult to stop bleeding after the extraction of a tooth or of a large-size cyst in a bone. If OCP/collagen can be used in these cases, bleeding should be reduced and bone repair facilitated.

OCP/collagen may be applicable to implantation in a tooth extraction hole which maintains the alveolar

crest.[49] In oral surgery, a large bone defect such as that in an alveolar cleft requires orthodontic therapy after bone reconstruction.[50,51] OCP/collagen could likely be used also as a bone repair material in the alveolar cleft. The mechanism that tooth moving in orthodontic therapy is considered to proceed with bone resorption by osteoclasts and subsequent new bone formation by osteoblasts in osseous tissue. However, the present results indicate that OCP within OCP/collagen cannot biodegrade completely. The remaining OCP may prevent the tooth moving because it was apparent that the OCP is converted to the most thermodynamically stable salt HA in physiological environment. Therefore, the solubility of the remaining OCP may decrease and not be resorbed by osteoclasts easily. Although the results of the present FTIR analysis demonstrate that OCP within OCP/collagen can be converted to biodegradable carbonate apatite, further study is required to clarify the biodegradable characteristics of the converted apatite from OCP. Our recent study demonstrated that OCP facilitated osteoblastic cell differentiation of OCP in vitro in a dose-dependent manner.[22] Furthermore, it was confirmed that OCP augmented bone regeneration with its content in a collagen scaffold.[36] Further study is under way to establish the optimal conditions for an OCP/collagen composite, which may lead to the development of a bone substitute material compatible with autogenous bone.

CONCLUSION

The results of the present study demonstrate that OCP/collagen can be a scaffold for bone reconstruction even if the bone defect is of critical size in a large animal. The bone-regenerating ability of OCP/collagen was observed up to 6 months after implantation. OCP/collagen has the potential to be used as a bone substitute material in clinical treatment.

ACKNOWLEDGMENTS

This study was supported in part by Grants in Aid (16591979, 17076001, 19390490, 20300165, and 20659304) from the Ministry of Education, Science, Sports and Culture of Japan.

REFERENCES

1. Petite H, Viateau V, Bensaid W, et al. Tissue-engineered bone regeneration. Nat Biotechnol 2000; 18:959–963.
2. Alsberg E, Hill EE, Mooney DJ. Craniofacial tissue engineering. Crit Rev Oral Biol Med 2001; 12:64–75.
3. Cowan CM, Shi YY, Aalami OO, et al. Adipose-derived adult stromal cells heal critical-size mouse calvarial defects. Nat Biotechnol 2004; 22:560–567.
4. Bucholz RW. Nonallograft osteoconductive bone graft substitutes. Clin Orthop Relat Res 2002; 95:44–52.
5. LeGeros RZ. Properties of osteoconductive biomaterials: calcium phosphates. Clin Orthop Relat Res 2002; 395:81–98.
6. Ogose A, Kondo N, Umezu H, et al. Histological assessment in grafts of highly purified beta-tricalcium phosphate (OSferion) in human bones. Biomaterials 2006; 27:1542–1549.
7. Chow LC. Calcium phosphate cements. In: Chow LC, Eanes ED, eds. Octacalcium phosphate. Basel: KARGER, 2001:148–163.
8. Wozney JM, Rosen V, Celeste AJ, et al. Novel regulators of bone formation: molecular clones and activities. Science 1988; 242:1528–1534.
9. Brown W, Smith J, Lehr J, Frazier A. Crystallographic and chemical relations between octacalcium phosphate and hydroxyapatite. Nature 1962; 196:1050–1055.
10. Bodier-Houlle P, Steuer P, Voegel JC, Cuisinier FJ. First experimental evidence for human dentine crystal formation involving conversion of octacalcium phosphate to hydroxyapatite. Acta Crystallogr D Biol Crystallogr 1998; 54:1377–1381.
11. Tohda H, Yamada M, Yamaguchi Y, Yanagisawa T. High-resolution electron microscopical observations of initial enamel crystals. J Electron Microsc 1997; 46:97–101.
12. Crane NJ, Popescu V, Morris MD, Steenhuis P, Ignelzi MA, Jr. Raman spectroscopic evidence for octacalcium phosphate and other transient mineral species deposited during intramembranous mineralization. Bone 2006; 39:434–442.
13. Barrere F, van der Valk CM, Dalmeijer RA, van Blitterswijk CA, de Groot K, Layrolle P. In vitro and in vivo degradation of biomimetic octacalcium phosphate and carbonate apatite coatings on titanium implants. J Biomed Mater Res A 2003; 64:378–387.
14. Bigi A, Bracci B, Cuisinier F, et al. Human osteoblast response to pulsed laser deposited calcium phosphate coatings. Biomaterials 2005; 26:2381–2389.
15. Barrere F, van der Valk CM, Dalmeijer RA, et al. Osteogenecity of octacalcium phosphate coatings applied on porous metal implants. J Biomed Mater Res A 2003; 66:779–788.
16. Dekker RJ, de Bruijn JD, Stigter M, Barrere F, Layrolle P, van Blitterswijk CA. Bone tissue engineering on amorphous carbonated apatite and crystalline octacalcium phosphate-coated titanium discs. Biomaterials 2005; 26:5231–5239.
17. Suzuki O, Nakamura M, Miyasaka Y, Kagayama M, Sakurai M. Bone formation on synthetic precursors of hydroxyapatite. Tohoku J Exp Med 1991; 164:37–50.

18. Suzuki O, Nakamura M, Miyasaka Y, Kagayama M, Sakurai M. Maclura pomifera agglutinin-binding glycoconjugates on converted apatite from synthetic octacalcium phosphate implanted into subperiosteal region of mouse calvaria. Bone Miner 1993; 20:151–166.
19. Suzuki O, Kamakura S, Katagiri T, et al. Bone formation enhanced by implanted octacalcium phosphate involving conversion into Ca-deficient hydroxyapatite. Biomaterials 2006; 27:2671–2681.
20. Liu Y, Cooper PR, Barralet JE, Shelton RM. Influence of calcium phosphate crystal assemblies on the proliferation and osteogenic gene expression of rat bone marrow stromal cells. Biomaterials 2007; 28:1393–1403.
21. Shelton RM, Liu Y, Cooper PR, Gbureck U, German MJ, Barralet JE. Bone marrow cell gene expression and tissue construct assembly using octacalcium phosphate microscaffolds. Biomaterials 2006; 27:2874–2881.
22. Anada T, Kumagai T, Honda Y, et al. Dose-dependent osteogenic effect of octacalcium phosphate on mouse bone marrow stromal cells. Tissue Eng Part A 2008; 14:965–978.
23. Kamakura S, Sasano Y, Shimizu T, et al. Implanted octacalcium phosphate is more resorbable than beta-tricalcium phosphate and hydroxyapatite. J Biomed Mater Res 2002; 59:29–34.
24. Imaizumi H, Sakurai M, Kashimoto O, Kikawa T, Suzuki O. Comparative study on osteoconductivity by synthetic octacalcium phosphate and sintered hydroxyapatite in rabbit bone marrow. Calcif Tissue Int 2006; 78:45–54.
25. Sugihara F, Oonishi H, Minamigawa K, Maidai Y, Tsuji E, Yoshikawa M, Toda T. Bone tissue reaction of octacalcium phosphate-collagen conjugated sponge. Bioceramics 1996; 9:399–402.
26. Kamakura S, Sasano Y, Homma-Ohki H, et al. Multinucleated giant cells recruited by implantation of octacalcium phosphate (OCP) in rat bone marrow share ultrastructural characteristics with osteoclasts. J Electron Microsc (Tokyo) 1997; 46:397–403.
27. Ban S, Jinde T, Hasegawa J. Phase transformation of octacalcium phosphate in vivo and in vitro. Dent Mater J 1992; 11:130–140.
28. Lu X, Leng Y. Theoretical analysis of calcium phosphate precipitation in simulated body fluid. Biomaterials 2005; 26:1097–1108.
29. Eidelman N, Chow LC, Brown WE. Calcium phosphate phase transformations in serum. Calcif Tissue Int 1987; 41:18–26.
30. Suzuki O, Yagishita H, Amano T, Aoba T. Reversible structural changes of octacalcium phosphate and labile acid phosphate. J Dent Res 1995; 74:1764–1769.
31. LeGeros RZ, Daculsi G, Orly I, Abergas T, Torres W. Solution-mediated transformation of octacalcium phosphate (OCP) to apatite. Scanning Microsc 1989; 3:129–138.
32. Tung MS, Tomazic B, Brown WE. The effects of magnesium and fluoride on the hydrolysis of octacalcium phosphate. Arch Oral Biol 1992; 37:585–591.
33. Ban S, Matsuura M, Arimoto N, Hayashizaki J, Itoh Y, Hasegawa J. Factors affecting the transformation of octacalcium phosphate to apatite in vitro. Dent Mater J 1993; 12:106–117.
34. Suzuki O, Kamakura S, Katagiri T. Surface chemistry and biological responses to synthetic octacalcium phosphate. J Biomed Mater Res B Appl Biomater 2006; 77:201–212.
35. Kamakura S, Sasaki K, Honda Y, Anada T, Suzuki O. Octacalcium phosphate combined with collagen orthotopically enhances bone regeneration. J Biomed Mater Res B Appl Biomater 2006; 79:210–217.
36. Kawai T, Anada T, Honda Y, et al. Synthetic octacalcium phosphate augments bone regeneration correlated with its content in collagen scaffold. Tissue Eng Part A 2009; 15:23–32.
37. Schmitz JP, Hollinger JO. The critical size defect as an experimental model for craniomandibulofacial nonunions. Clin Orthop Relat Res 1986; 205:299–308.
38. LeGeros RZ. Preparation of octacalcium phosphate (OCP): a direct fast method. Calcif Tissue Int 1985; 37:194–197.
39. Fowler BO, Moreno EC, Brown WE. Infra-red spectra of hydroxyapatite, octacalcium phosphate and pyrolysed octacalcium phosphate. Arch Oral Biol 1966; 11:477–492.
40. Nelson DG, McLean JD. High-resolution electron microscopy of octacalcium phosphate and its hydrolysis products. Calcif Tissue Int 1984; 36:219–232.
41. Frame JW. A convenient animal model for testing bone substitute materials. J Oral Surg 1980; 38:176–180.
42. Sasano Y, Kamakura S, Homma S, Suzuki O, Mizoguchi I, Kagayama M. Implanted octacalcium phosphate (OCP) stimulates osteogenesis by osteoblastic cells and/or committed osteoprogenitors in rat calvarial periosteum. Anat Rec 1999; 256:1–6.
43. Brown WE, Mathew M, Chow LC. Crystal chemistry of octacalcium phosphate. Prog Cryst Growth Charact 1981; 4:59–87.
44. Suzuki O, Kamakura S, Katagiri T. Bone regeneration by synthetic octacalcium phosphate and its role in biological mineralization. Curr Med Chem 2008; 15:305–313.
45. LeGeros RZ, Tung MS. Chemical stability of carbonate- and fluoride-containing apatites. Caries Res 1983; 17:419–429.
46. Okazaki M, Takahashi J. Synthesis of functionally graded CO3 apatite as surface biodegradable crystals. Biomaterials 1999; 20:1073–1078.
47. Cameron WJ. A new topical hemostatic agent in gynecologic surgery. Obstet Gynecol 1978; 51:118–122.
48. Abbott WM, Austen WG. The effectiveness and mechanism of collagen-induced topical hemostasis. Surgery 1975; 78:723–729.

49. Sela J, Jaffe A. The role of bone remodeling in the healing of extraction socket in rats. Acta Anat (Basel) 1977; 97:241–247.
50. Yilmaz S, Kilic AR, Keles A, Efeoglu E. Reconstruction of an alveolar cleft for orthodontic tooth movement. Am J Orthod Dentofacial Orthop 2000; 117:156–163.
51. Kokkinos PP, Ledoux WR, Kinnebrew MC, Weinberg R. Iliac apophyseal cartilage augmentation of the deficient piriform rim and maxilla in alveolar cleft grafting. Am J Orthod Dentofacial Orthop 1997; 112:145–153.