Original

The Effect of Partial Dissolution-Precipitation Treatment on Calcium Phosphate Ceramics in the Release of BMP-2 and Osteoinduction

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Abstract: Biomimetic calcium phosphate ceramics with excellent bio-absorption and osteoinduction were designed by the partial dissolution-precipitation (PDP) treatment. Beta-tricalcium phosphate (β -TCP) ceramics with single phase were transformed into monetite, hydroxyapatite (HAp) and β -TCP phases by the PDP treatment. The PDP- β -TCP ceramics had a specific surface area of 22.5 m²/g and porosities of 60-80 %. Changes in microstructure of the ceramics before and after PDP treatment influenced body fluid permeation, release characteristics of bone morphogenetic protein-2 (BMP-2) and osteoinduction. ¹²⁵I-labeled BMP-2 was loaded on the four different ceramics, such as spongy-derived bulk of HAp (b-HAp), functionally graded HAp (fg-HAp), β -TCP, and PDP- β -TCP. The four kinds of ceramics were implanted into the back region of subcutaneous tissue in rats and the analyses of the release of BMP-2 and BMP-2 dose-response of bone formation were investigated. In the fg-HAp/BMP-2 and PDP- β -TCP/BMP-2 ceramics, the release time corresponding to the half amount ($t_{1/2}$) of BMP-2 were prolonged about 14 and 7 times respectively compared to the ceramics before PDP treatment. BMP-2 dose-response of bone formation study showed the areas of bone tissues were significant higher in the fg-HAp/BMP-2 (5.0 μ g) and PDP- β -TCP/BMP-2 (0.5 μ g) ceramics compared to the ceramics before PDP treatment. These results indicated the PDP treatment enhanced BMP-2 release and osteoinduction.

Keywords: Calcium phosphate ceramic, Partial dissolution-precipitation treatment, BMP-2, Osteoinduction, Bio-absorption

Introduction

A human clinical trial using bone morphogenetic protein-2 (BMP-2) was incorporated in an absorbable collagen sponge (ACS) for augmentation of localized osseous defect. Statistically, significant dose-response relationships were detected using CT scan for the measurement of bone height responses. However, no bone formation was observed without adding BMP-2 over 150 μ g¹⁾. The *in vivo* release of ¹²⁵I-labeled BMP-2 from ACS was investigated using rat ectopic model. The ACS showed high release rate of BMP-2 (the release time corresponding to the half amount ($t_{1/2}$) of BMP-2: day 2.1)²⁾. An optimized delivery system might improve the osteoinduction of the device, while reducing the loading amount of BMP as much as possible, which offers double

advantages of being safer as well as less expensive³⁾. Several reports indicated that too rapid release of BMP-2 decreased osteoinduction while high retention of BMP-2 elicited more bone formation^{4,5)}. It is likely that an extremely low dose of BMP-2 is enough to enhance osteoinduction if BMP-2 is appropriately delivered to the site of action⁶⁾.

Thus, it is necessary to develop a carrier for the controlled release of biologically active BMP-2 over an extended time period. Furthermore, we believe that ideal scaffolds for bone engineering should be bioabsorbable, mimetic and hydrophilic which can allow the permeation of liquid components, such as blood and/or extracellular fluid and thus can be replaced by new bone⁷⁾. Responding to these requirements, we developed a functionally graded hydroxyapatite (fg-HAp) ceramics. We previously described that HAp obtained by the calcination of spongy-derived bulk of HAp (b-HAp) from bovine bone at 800-1100°C had good crystallinity and appropriate mechanical strength for handling and

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Figure 1. SEM Photographs of B-TCP and PDF-B-TCP ceramics

operation^{8,9}). The b-HAp ceramics with a porosity of 60-80% and specific surface area of 1-2 m²/g were partially dissolved and HAp nano-microcrystals were reprecipitated on the pore walls of b-HAp to fabricate fg-HAp ceramics (porosity: 60-80%, pore diameter: macropores: 100-600μm, nano-micropores: 10-160nm, specific surface area: 30m²/g). The treatment is called partial dissolution-precipitation (PDP). PDP treatment produced a structure of fg-HAp decreasing the ratio of reprecipitated HAp (r-HAp)/b-HAp from the surface layer of pore walls to the mass of b-HAp in the nano-micropores matrix and forming gradations in both crystallinity and grain size of HAp. In the *in vivo* release profile of ¹²⁵I-labeled BMP-2, the *t*_{1/2} of BMP-2 was day 14 for fg-HAp/BMP-2 ceramics and day 1 for b-HAp/BMP-2 ceramics¹⁰). Moreover, ectopic bone formation occurred in fg-HAp/BMP-2 (0.5, 1.0, 5.0μg) ceramics at 3 weeks, while only bone induction was found in b-HAp/BMP-2 (5.0μg) ceramics. Histological observation showed body fluid permeation into fg-HAp as densely stained areas and nano-microcracks were noted on the fg-HAp surface. The densely stained areas were immunopositive for albumin⁷). Thus, PDP treatment enhanced osteoinduction, body fluid permeation and gradual release of BMP-2. As a result, fg-HAp/BMP-2 ceramics had an ideal BMP-2 release curve and osteoinductive property at a low dose of BMP-2. However, the practice of using a material derived from bovine bone has significant hurdle to overcome.

In experimental and clinical cases, porous HAp and beta-tricalcium phosphate (β-TCP) have been widely used because of their inherent osteoinductivity^{11,12}). However, since synthetic HAp is not practically degraded under physical condition, it remains inside the regenerated bone tissue. One of the advantages of β-TCP is its biodegradability, though it is brittle compared to HAp. Therefore, we tried to improve a commercial bioceramic into a

more effective biomaterial called partially dissolved and precipitated beta-tricalcium phosphate (PDP-β-TCP) with the properties of fg-HAp by adding PDP treatment. The purpose of this study was to modify the microstructure of bioceramics and enhance osteoinduction by adopting specific PDP treatment.

Materials and Methods

Preparation and physicochemical characterization of calcium phosphate ceramics

fg-HAp ceramics were fabricated in the same method as described previously^{8,9}). Briefly, bovine spongy and cortical bones were cut into cubic samples, which were boiled and calcined at 800-1100°C for 24 h in air to remove organic components. The spongy-derived bulk of HAp (b-HAp) was partially dissolved into a nitric acid aqueous solution, while the cortical-derived bulk of HAp was completely dissolved into another nitric acid aqueous solution. By adding the treatment of partial dissolution, nano-micropores were formed on the material surface.

After the two solutions were mixed, an ammonium aqueous solution was added with stirring, and nano-microcrystals were carefully reprecipitated on the nano-micropores of the spongy b-HAp at pH 10.5 and 25°C for 24 h. The artificial modified spongy b-HAp was filtrated and washed with distilled water and dried to fabricate the fg-HAp ceramics (porosity: 60-80%, pore diameter: macropores 100-600mm, nano-micropores 10-160nm). Precipitated HAp particles formed spherical moss like-grains (1-2μm) with needle-like nano-microcrystals (10nm) on the fg-HAp surface. The new ceramics were fabricated based on same concept.

Commercial β-TCP (Osferion®, sintering temperature: 1050 °C, porosity: 60-80%, pore size: 100-400μm, surface area: 4m²/g) was kindly provided by OLYMPUS Co., Tokyo, Japan (Fig.1). Cortical bone of bovine femur (Holstein bull, Hokkaido prefecture,

Japan) was used as a starting material to synthesize crystal surfaces substituted by small amounts of metal ions derived from a living body. The bovine bone was boiled in distilled water, calcined at 500°C-1100°C for 48h in air, and completely dissolved in HNO_3 and H_3PO_4 aqueous solutions. The commercial and spongy β -TCP ceramics were soaked in the acid solution to partially dissolve for 1h while stirring. Ammonium aqueous solution was added in a dropwise manner to precipitate calcium phosphate nanomicrocrystals carefully on the surfaces and pore walls of the ceramics at pH 7, at 25°C for 24h. Then after, the modified β -TCP ceramics were filtrated, washed with distilled water and dried at 50-120°C for 24h. The fabricated ceramics are now called PDP- β -TCP ceramics (porosity: 60-80%, surface area: 22.5m²/g).

Analysis of the release of BMP-2

Radioiodination of BMP-2

Human recombinant BMP-2 (rhBMP-2, Astellas Pharmaceutical Co., Tokyo, Japan) underwent radioiodination according to the standard method¹³⁾. Briefly, 10 μ l of Na¹²⁵I was added to 30 μ l of 1mg/ml BMP-2 solution in 5mM glutamic acid with 2.5wt% Tween 80 (pH 4.5). Then, 200 μ l/ml of chloramine-T in 0.5M potassium phosphate-buffered solution (pH 7.5) containing 0.5M potassium phosphate-buffered solution in 5mM glutamic acid, 2.5wt% glycine, 0.5wt% sucrose, and 0.01wt% Tween 80 (pH 4.5) were added to the mixture. Then, 200 μ g/ml of chloramine-T in 0.5M potassium phosphate-buffered solution (pH 7.5) containing 0.5M sodium chloride (100 μ l) was added to the mixture. After agitation at room temperature for 2 min, 100 μ l of phosphate-buffered saline solution (PBS, pH 7.5) containing 400 μ g of sodium metabisulfate was added to the reaction solution to stop radioiodination. The reaction mixture was passed through an anionic exchange column to remove uncoupled, free ¹²⁵I molecules from the ¹²⁵I-labeled BMP-2.

Figure2. XRD patterns of β -TCP and PDP- β -TCP ceramics

In vivo evaluation of the release of BMP-2

A volume of 10 μ l of ¹²⁵I-labeled BMP-2 solutions (0.5 μ g in 10 μ l of PBS solution) was added to b-HAp, β -TCP, and PDP- β -TCP blocks (3 $\times$ 3 $\times$ 3mm) in sterilized culture dishes. The implants were called b-HAp/BMP-2, β -TCP/BMP-2 and PDP- β -TCP/BMP-2 ceramics. Forty-five ddy mice (female, 6 week-old, body weight 30g) were subjected to intraperitoneal anesthesia with pentobarbital sodium (4mg/100g body weight). Each ceramic was implanted into the subcutaneous tissue at the back of the mice, at the central position 15mm away from the tail root. The mice were sacrificed and the ceramics were explanted at different time interval (day 1, 3, 7, 14 and 28).

The skin on the back of mice around the implanted site of BMP-2 was cut into a strip of 3 $\times$ 5cm² and the corresponding fascia was thoroughly wiped off with a filter paper and measured on the gamma counter (ARC-301B, Aloka Co., Tokyo, Japan) to assess the time profile of BMP-2 retention. All radioactivities were compensated for the natural decay of ¹²⁵I. At different time intervals, the radioactivity of explanted ceramics and the

Figure 3. pore size distribution curves of β -TCP and PDP- β -TCP ceramics

Increase of pore diameter and accumulated pore volume by adding PDP treatment in PDP- β -TCP ceramics compared to the untreated ceramics

Figure 4. *In vitro* release profiles of BMP-2 from ceramics N=3 $p < 0.04^*$ $p < 0.03^{**}$ $p < 0.01^{***}$
In vivo decrement patterns of the remaining radioactivity at the skin of the back of ^{125}I -labeled BMP-2 combined ceramics blocks. The initial BMP-2 dose : $0.5\mu\text{g}$

Figure 5. Photomicrographs of HE sections at 3 weeks after implantation of b-HAp and fg-HAp ceramics

(a) b-HAp/BMP-2 $5.0\mu\text{g}$ ceramics. Localized bone (★) formation occurred in b-HAp/BMP-2 $5.0\mu\text{g}$ ceramics. No body fluid permeation into mass of b-HAp (*). Bar indicate $200\mu\text{m}$.
 (b) fg-HAp/BMP-2 $5.0\mu\text{g}$ ceramics. Active bone formation (★) and bone marrow in fg-HAp/BMP-2 $5.0\mu\text{g}$ ceramics. Nano-microcracks(→) and body fluid permeation into mass of fg-HAp (*). Bar indicate $200\mu\text{m}$.
 (c) fg-HAp/BMP-2 $5.0\mu\text{g}$ ceramics. Body fluid permeation into fg-HAp (*) and r-HAp (▲). fg-HAp (*) divided by induced bone. Phagocytosis from inside of r-HAp (▲) by osteoclasts and bone formation from r-HAp surface by osteoblast. Nano-microcracks (→) of fg-HAp. Bar indicate $50\mu\text{m}$.
 (d) fg-HAp/BMP-2 $0.5\mu\text{g}$ ceramics. The fragmentation of fg-HAp (*) with body fluid by new bone formation (★) with mosaic appearance. Nano-microcracks (→) in mass of fg-HAp. Bar indicate $100\mu\text{m}$.

surrounding tissues were measured to evaluate the time profile of *in vivo* BMP-2 retention of ceramics. Statistical analysis was done using Student t-test. A p-value < 0.05 was considered significant. The data of fg-HAp/BMP-2 used was from a previous study ¹⁰.

Analysis of BMP-2 dose-response bone formation

Implantation and histopathological evaluation of the ceramics

Ceramics/BMP-2, rhBMP-2 (Astellas Pharmaceutical Co., Tokyo, Japan) solution was prepared in the following manner. A total of 10 ml of various concentrations of BMP-2 solution (0.05,

Figure 6. Histological observation of the β -TCP/BMP-2 ceramics

(a) Active bone formation (★) and bone marrow in β -TCP/BMP-2 5.0 μ g ceramics. Body fluid permeation into mass of β -TCP (*). Bar indicate 200 μ m.

(b) Body fluid permeation into β -TCP (*). β -TCP (*) divided by induced bone (★). Bar indicate 100 μ m.

(c) β -TCP/BMP-2 0.5 μ g ceramics. Bone formation (★) and body fluid permeation into mass of β -TCP (*). Bar indicate 100 μ m.

Figure 7. Histological observation of the PDP- β -TCP/BMP-2 ceramics

(a) Active bone formation (★) and bone marrow in the PDP- β -TCP/BMP-2 5.0 μ g ceramics. Body fluid permeated into mass of PDP- β -TCP (*). Bar indicate 200 μ m.

(b) Body fluid permeation into mass of PDP- β -TCP (*). Mosaic appearance of mass of PDP- β -TCP. The reprecipitated TCP (r-TCP) (▲) observed as densely stained areas on the PDP- β -TCP surface. Phagocytosis by osteoclasts and bone formation (★) on PDP- β -TCP surface. Bar indicate 100 μ m.

(c) Body fluid permeation into mass of PDP- β -TCP (*). Mosaic appearance of mass of PDP- β -TCP. Observation of body fluid permeation as densely stained areas. Active bone formation (★) in the PDP- β -TCP /BMP-2 0.5 μ g ceramics. Bar indicate 100 μ m

Figure 8. SEM photographs of b-HAP and f-HAP ceramics.

Figure 10. Without PDP-treated surfaces with BMP-2.

Figure 9. PDP-treated surfaces with BMP-2

0.1, 0.5, 1.0, 5.0 μ g in 10 μ l PBS solution) was added to the ceramics blocks (3 $\times$ 3 $\times$ 3mm) in sterilized culture dishes. The implants were called fg-HAP/BMP-2 ceramics, b-HAP/BMP-2 ceramics, β -TCP/BMP-2 ceramics and PDP- β -TCP/BMP-2 ceramics and the corresponding ceramics without adding BMP-2 served as negative controls for a total of 6 groups. Thirty-six Wistar rats (male, 4 week-old, 70g body weight, 6 rats per group) were subjected to intraperitoneal anesthesia with pentobarbital sodium (4mg/100g body weight). Under sterile conditions, vertical incisions were made on the skin at the back region, and two subcutaneous pockets were prepared by blunt dissection. The

Table 1 Bone formation at 3 after implantation

Dose of BMP-2 (μg)	Bone			
	fg-HAp	b-TCP	β -TCP	PDP- β -TCP
	Ceramics	Ceramic	Ceramics	Ceramics
0.0	0/3	0/3	0/3	0/3
0.05	0/3	0/3	0/3	0/3
0.1	0/3	0/3	0/3	0/3
0.5	3/3	0/3	1/3	3/3
1.0	3/3	0/3	3/3	3/3
5.0	3/3	3/3	3/3	3/3

Ratio of bone and carrier implanted materials
(values are given as numbers of implants containing bone/total
numbers of implanted materials)

incisions were sutured with a nylon thread. All implanted materials were explanted 21 days after implantation. The specimens were fixed in 10% neutral buffered formalin, decalcified with 10% formic acid, embedded in paraffin, sectioned (4 μm in thickness) and stained with hematoxylin and eosin (H-E). For morphometric analysis, bone tissues were measured by Weibel's method^{14, 15)} at 3 separate points 100 μm apart, the midpoint being at the center of the implants. The average values of the 3 points were used as the mean area of each implant. Statistical analysis was done using Student t-test. P-values <0.05 were considered significant. This project was approved by the Ethical Committee for Animals Care at the Health Sciences University of Hokkaido. NIH guidelines (or for non-U.S. residents similar national regulations) for the care and use of laboratory animals (NIH publication #85-23 Rev.1985) have been observed.

Results

Physicochemical characterization of calcium phosphate ceramics

X-ray analysis of PDP- β -TCP ceramics revealed that it is composed of monetite, hydroxyapatite and β -TCP. β -TCP ceramics consist of a single phase of β -TCP (Fig.2). The pore diameter and accumulated pore volume increased with PDP treatment compared to the untreated ceramics (Fig.3).

Release of BMP-2

Fig.4 shows the *in vivo* decrement patterns of the remaining radioactivity at the skin of the back of ¹²⁵I-labeled BMP-2 combined ceramics blocks. The ceramics incorporated with ¹²⁵I-labeled BMP-2 showed decreased residual radioactivity with implantation time. The highest amounts of BMP-2 retained were observed in fg-HAp/BMP-2 ceramics in all duration and $t_{1/2}$ of BMP-2 loaded was 14 days after implantation. The $t_{1/2}$ of BMP-2 in PDP- β -TCP/BMP-2 ceramics was day 7. In contrast, within 1 day after implantation, more than 50% of BMP-2 was released

Table 2 Histomorphometrical analysis at day 21 after implantation

BMP-2 (μg)	fg-HAp/ BMP-2 ceramics	β -HAP/ BMP-2 ceramics	β -TCP/ BMP-2 ceramics	PDP- β -TCP/ BMP-2 ceramics
	BT	BT	BT	BT
0.0	0.0	0.0	0.0	0.0
0.05	0.0	0.0	0.0	0.0
0.1	0.0	0.0	0.0	0.0
0.5	3.2 $\pm$ 4.9	0.0	0.2 $\pm$ 0.3	10.7 $\pm$ 2.2 ****
1.0	6.7 $\pm$ 6.1	0.0	12.1 $\pm$ 11.1	12.3 $\pm$ 3.8 *
5.0	36.3 $\pm$ 12.7**	3.0 $\pm$ 2.6	32.5 $\pm$ 9.0*	25.2 $\pm$ 8.7

The total volume is 100%, Values are mean standard deviation, N=3
* p < 0.05 relative to b-HAp/BMP-2 ceramics, ** p < 0.04 relative
to b-HAp/BMP-2 ceramics, *** p < 0.02 relative to b-HAp/BMP-
2 ceramics, **** p < 0.01 relative to β -TCP/BMP-2 ceramics

from both b-HAp/BMP-2 and β -TCP/BMP-2 ceramics. At day 1, BMP-2 retention in fg-HAp/BMP-2 ceramics was significantly higher than the other ceramics (p<0.01). PDP- β -TCP/BMP-2 ceramics and b-HAp/BMP-2 ceramics showed significant difference at day 1 (p<0.01). At day 3, fg-HAp/BMP-2 and β -TCP/BMP-2 ceramics showed significant differences compared to b-HAp/BMP-2 ceramics (fg-HAp/BMP-2 ceramics: p<0.04, β -TCP/BMP-2 ceramics: p<0.03). Significant differences were observed between fg-HAp/BMP-2 ceramics and b-HAp/BMP-2 ceramics on day 7 (p<0.04) and between PDP- β -TCP/BMP-2 ceramics and β -TCP/BMP-2 ceramics on day 14 (p<0.04).

BMP-2 dose-response of bone formation

Histomorphometrical analysis

Table 1 shows the presence of ectopic bone formation in fg-HAp/BMP-2 (0.5, 1.0, 5.0 μg), β -TCP/BMP-2 (0.5, 1.0, 5.0 μg) and PDP- β -TCP/BMP-2 (0.5, 1.0, 5.0 μg) ceramics at 21 days after implantation. In b-HAp/BMP-2 ceramics, bone formation was observed only in 5.0 μg BMP-2 group. Cartilage formation was not found in all ceramics at day 21. The morphometric results are shown in Table 2. In 5.0 μg BMP-2 group, fg-HAp/BMP-2 (p<0.04) and β -TCP/BMP-2 (p<0.05) ceramics showed significant differences compared to b-HAp/BMP-2 ceramics. In PDP- β -TCP/BMP-2 ceramics, the 1.0 μg (p<0.04) and 0.5 μg BMP-2 (p<0.02) groups showed significant differences compared to b-HAp/BMP-2 ceramics. Furthermore, PDP- β -TCP/BMP-2 ceramics showed significant difference compared to β -TCP/BMP-2 ceramics in 0.5 μg BMP-2 group (p<0.01).

Histological evaluation

Histological examination of the de novo induced bone (Fig.5, Fig.6 and Fig.7) confirmed interesting differences in each ceramics. Numerous bone including bone marrow and body fluid permeation into the ceramics were observed in fg-HAp/BMP-2, β -TCP/BMP-2 and PDP- β -TCP/BMP-2 ceramics (Fig.5 (b), (c),

(d), Fig.6 and Fig.7). On the other hand, body fluid permeation in b-HAp/BMP-2 ceramics could hardly be observed in the mass of b-HAp (Fig.5 (a)). The fg-HAp was phagocytosed by osteoclasts from the inside of the mass of r-HAp and fg-HAp. Osteoblasts formed new bone on the surface of r-HAp and in the mass of fg-HAp (Fig.5 (c)). r-HAp or reprecipitated- β -TCP (r- β -TCP) crystals were observed on the ceramic surfaces as densely stained areas (Fig.5 (c), Fig.7 (b), (c)). Nano-microcracks were observed in fg-HAp/BMP-2 ceramics (Fig.5 (b), (c), (d)). On the other hand, extensive mosaic appearances were confirmed in PDP- β -TCP/BMP-2 ceramics compared to β -TCP/BMP-2 ceramics (Fig.6, Fig.7 (b), (c)). The apparent histological changes were observed in ceramics treated with PDP (fg-HAp/BMP-2 and PDP- β -TCP/BMP-2 ceramics) compared to the untreated ceramics.

Discussion

PDP treatment consisted of two engineered processes. The first treatment of etching the ceramic surface formed nano-microcracks which might have contributed to the invasion of cells and body fluid. The subsequent treatment of precipitation increased the surface area and formed nano-microcrystals capable of retaining growth factor on the ceramic surface as well as on the nano-microcracks. Furthermore, the ratio of r-HAp/b-HAp from the surface layer of pore walls to the mass of b-HAp in the nano-micropores matrix decreased forming gradations in both crystallinity and grain size of HAp. SEM photographs of fg-HAp showed spherical moss-like grains, 1-2 μ m in size forming needle-like nano-microcrystals of 10 nm in length. In contrast, b-HAp before PDP treatment showed large and angular grains of 1-2 μ m in size with flat surface (Fig.8). The PDP- β -TCP retained plate aggregated crystal with small grain of 0.1 μ m in size (Fig.1). By PDP treatment, grain size of the ceramics became smaller and the surface areas became larger. After PDP treatment, surface areas increased remarkably and the apparent differences in fg-HAp and PDP- β -TCP ceramics were observed in SEM photographs. Interestingly, the x-ray analysis showed that PDP- β -TCP ceramics consisted of mixed phases of monetite, hydroxyapatite and β -TCP (Fig.2). The PDP treatment could have changed the composition of the ceramic surface. Changes in microstructure of the ceramics before and after PDP treatment might have influenced the release of BMP-2, osteoinduction and body fluid permeation. The results showed that fg-HAp/BMP-2 ceramics retained much BMP-2 than other ceramics in all durations. In fg-HAp/BMP-2 and PDP- β -TCP/BMP-2 ceramics, the $t_{1/2}$ of BMP-2 was prolonged for about 14 and 7 times respectively compared to the ceramics without PDP treatment. Statistical analysis showed significant differences of BMP-2 retention between fg-HAp/BMP-2 ceramics and b-HAp/BMP-2 ceramics at day 1 ($p<0.01$), 3 ($p<0.04$) and 7 ($p<0.04$). Similarly, PDP- β -TCP ceramics showed significant difference of BMP-2 retention compared to β -TCP/BMP-2 ceramics at day 14

($p<0.04$) (Fig.5). Thus, PDP treatment improved BMP-2 retention significantly in each composition. Numerous data from literature indicated the importance of the ability of a carrier to retain sufficient BMP to enhance bone formation^{16, 17}. It is suggested that controlled application of BMP-2 would allow constant and prolonged release which renders it effective over time and helps to form a chemotactic gradient necessary for cell response¹⁸. Numerous studies reported the efficacy of drug delivery system (DDS) for bone regeneration¹⁸⁻²¹. We previously investigated the efficacy of slow release of growth factors using polymer materials¹⁹⁻²². Hydrogels of different biodegradable profiles from gelatin were prepared and successfully induced the biological activities of basic fibroblast growth factor (bFGF)¹⁹ and BMP-2^{20, 21} by incorporating the growth factors in the hydrogels. The time profiles of *in vivo* bFGF and BMP-2 retention in the hydrogel depended on the degradation of the hydrogel. Slow degradation of the hydrogel will prolong the release period, resulting to a more enhanced bone formation. It should be noted that gelatin hydrogels of low water content maintained high cellularity for a longer period than those with higher water content. This prolonged release possibly gives a continuous stimulus for osteoblast proliferation which is responsible for the prolonged retention of osteoblast number at a high level. Thus, release curves of the growth factors showed a close correlation with bone formation.

In this study, the improvement in the release of BMP-2 might have enhanced osteoinduction significantly *in vivo* at 21 days after implantation. In fg-HAp/BMP-2 (0.5, 1.0, 5.0 μ g) and PDP- β -TCP/BMP-2 (0.5, 1.0, 5.0 μ g) ceramics, bone formation was observed in all implanted groups (3/3). In b-HAp/BMP-2 ceramics, bone formation was observed only in 5.0 μ g BMP-2 group. The β -TCP/BMP-2 (0.5 μ g) ceramics could induce new bone only in one third (1/3) of the implanted groups (Table 1). In the fg-HAp/BMP-2 (5.0 μ g) ceramics and PDP- β -TCP/BMP-2 (0.5 μ g) ceramics, significant differences in bone areas were observed compared to b-HAp/BMP-2 ($p<0.01$) ceramics and β -TCP/BMP-2 ceramics ($p<0.01$) respectively at day 21 (Table 2).

In this study, no significant difference in the retention of BMP-2 at 21 days after implantation was obtained, while significant differences in bone area were observed at the same time. Therefore, the effect of BMP-2 release must have occurred during the early days after implantation. Many reports supported this hypothesis²³⁻²⁵. Morphogenetically, it is essential that BMP-2 is biologically active during the late phase of the inflammatory reaction, which peaks 2 to 3 days after surgery. The correct timing of BMP-2 action with respect to wound healing may be more important than delayed release²³. The dose-response and time-course study using rat ectopic bone formation assay revealed that implantation of BMP-2 with demineralized matrix carrier resulted to early bone formation. At day 5, many immature and some hypertrophic cartilage cells were present in BMP-containing implants, but no

mineralizing cartilage was detected. After day 7, the chondrocytes were hypertrophic and the cartilage was mineralized ²⁴). The *in vitro* study showed osteoblast-like cells surrounding the microspheres within day 8 after culture ²⁵).

Structural parameters in the design of ceramics are thought to be critical factors in the osteoinductive potential by BMP-2.

Osteogenic cells recognize important material surface properties, like surface texture, and accordingly adjust their attachment. Studies on the response of cells to microgrooved surfaces showed that, while the response is cell type-dependent, overall, the cell elongates in the direction of the groove and then travels guided by the grooves. This phenomenon has become known as 'contact guidance' ²⁶). To support cellular attachment, spreading and growth, and improved cellular function, a lot of reports have been published about roughened implant surface, as well as on controlled microtopography ²⁷). We reached the assumption that by taking account the results of BMP-2 release and the properties of the ceramics including the surface structures and surface areas. The fg-HAp and PDP- β -TCP/BMP-2 ceramics retained much molecules of BMP-2 by the three dimensional structures of reprecipitated HAp (r-HAp) and β -TCP (r- β -TCP) than b-HAp and β -TCP/BMP-2 ceramics with flat surfaces at implanted time. At day 7, most BMP-2 molecules were detached from the flat surfaces of b-HAp and β -TCP ceramics. On the other hand, in fg-HAp and PDP- β -TCP/BMP-2 ceramics, more than half of BMP-2 molecules were retained on the ceramic surfaces. The three dimensional structure of reprecipitated ceramic particles might have contributed to BMP-2 retention *in vivo* (Fig.9, 10). Furthermore, effects of PDP treatment were confirmed by measuring pore size distribution of β -TCP and PDP- β -TCP ceramics. Because of PDP treatment, pore diameter and volume increased evidently (Fig.3). This phenomenon implies that the blood vessel and body fluid easily permeated inside the PDP- β -TCP ceramics. Viable bone tissue consists of ~10% body fluid, 20% organic materials, and 70% minerals including hydroxyapatite (HAp). Cells never live in dry conditions and cannot invade dry and non-nutrient areas ²⁸). Moreover, implanted biomaterials necessarily contact body fluid and living cells in the body. We believe that body fluid permeation into the structure is essential to the cells. Previous *in vivo* study showed densely stained areas and nano-microcracks in the mass of fg-HAp. This phenomenon could not be confirmed in the b-HAp/BMP-2 ceramics. The changes might have caused by PDP treatment. We detected albumin immunohistochemically in the densely stained areas and confirmed body fluid permeation into the mass of fg-HAp ⁸). Albumin, a globular protein with distinct hydrophobic moieties, represents over 50% of the protein in serum, and is an efficient carrier-protein of fatty acids and sterols. In addition, albumin supplies nutrient to cells, and controls colloid osmotic pressure ²⁹). In this study,

we confirmed densely stained areas and nano-microcracks in fg-HAp/BMP-2 ceramics (Fig. 5 (b), (c), (d)). Similarly, the PDP- β -TCP/BMP-2 ceramics showed densely stained areas with mosaic appearance while those areas were not observed in β -TCP/BMP-2 ceramics (Fig.7). Considering the previous studies in fg-HAp/BMP-2 ceramics, it can be inferred that PDP treatment changed the β -TCP (Osferion®) to PDP- β -TCP ceramics allowing permeation of abundant body fluid. This change might have affected the release of BMP-2 and the dose response. The body fluid permeation into the ceramics might have helped cellular activity on the mass of the ceramic.

Conclusion

In conclusion, PDP treatment of the ceramics allowed gradual release of BMP-2 and enhanced osteoinduction and body fluid permeation. By using this method, we can improve commercial bioceramics for more effective biomaterials for bone regenerative therapy or bioengineering.

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References

1. Boyne PJ, Marx RE and Nevins M. A feasibility study evaluating rhBMP-2/absorbable collagen sponge for maxillary sinus floor augmentation. *Int J Periodontics Restorative Dent* 17:11-25, 1997
2. Friess W, Uludag H, Foskett S, Biron R and Sargeant C. Characterization of absorbable collagen sponges as rhBMP-2 carriers. *Int J Pharm* 187:91-99, 1999
3. Marion M, Frederic C, Pierrre M, Cinderella B, Alain M and Delphine LA. Bovine BMP osteoinductive potential enhanced by functionalized dextran-derived hydrogels. *Biomaterials* 26:5085-5092, 2005
4. Uludag H, Gao T, Porter TJ, Friess W and Wozney JM. Delivery systems for BMPs: factors contributing to protein retention at an application site. *J Bone Joint Surg* 83:128-135, 2001
5. Uludag H, Norrie B, Kousinioris N and Gao T. Engineering temperature-sensitive poly (N-isopropylacrylamide) polymers as carriers of therapeutic proteins. *Biotechnol Bioeng* 73:510-521, 2001
6. Tabata Y, Hijikata S and Ikada Y. Enhanced vascularization and tissue granulation by basic fibroblast growth factor impregnated in gelatin hydrogels. *J Controlled Release* 31:189-199, 1994
7. Murata M, Akazawa T, Tazaki J, Ito K, Sasaki T, Yamamoto M, Tabata Y and Arisue M. Blood permeability of a novel

- scaffold for bone morphogenetic protein-2. *J Biomed Mater Res* 81:469-475, 2007
8. Akazawa T, Kobayashi M. Characterization of differently prepared apatites by adsorption behavior of albumin. *J Ceram Soc Jpn* 104(4):284-290, 1996
9. Akazawa T, Murata M, Sasaki T, Tazaki J, Kobayashi M, Kanno T, Nakamura K and Arisue M. Biodegradation and bioabsorption innovation of the functionally graded cattle-bone-originated apatite with blood permeability. *J Biomed Mater Res* 76:44-51, 2006
10. Tazaki J, Akazawa T, Murata M, Yamamoto M, Tabata Y, Yoshimoto R and Arisue M. BMP-2 dose-response and release studies in functionally graded HAp. *Key Engineering Materials* 309-311:965-968, 2006
11. Livingston T, Ducheyne P and Garino J. *In vivo* evaluation of a bioactive scaffold for bone tissue engineering. *J Biomed Mater Res* 62:1-13, 2002
12. Shimaoka H, Dohi Y, Ohgushi H, Ikeuchi M, Okamoto M, Kudo A, Kirita T and Yonemasu K. Recombinant growth/differentiation of marrow mesenchymal stem cells in porous hydroxyapatite ceramic. *J Biomed Mater Res* 68A:168-176, 2004
13. Greenwood FC, Hunter WM and Gglover TC. The preparation of ^{131}I -labeled human growth hormone of high specific radioactivity. *Biochem J* 89: 114-123, 1963
14. Wangenstein D, Weibel ER. Morphometric evaluation of chorioallantoic oxygen transport in the chick embryo. *Respir Physiol* 47:1-20, 1982
15. Weibel ER. Stereological methods. practical methods for biological morphometry. New York: Academic Press; 1980.
16. Uludag H, D'Augusta D, Golden J, Li J, Timony G, Riedel R and Wozney JM. Implantation of recombinant human bone morphogenetic proteins with biomaterial carriers: A correlation between protein pharmacokinetics and osteoinduction in the rat ectopic model. *J Biomed Mater Res* 50:227-238, 2000
17. Winn S, Uludag H and Hollinger JO. Sustained release emphasizing recombinant human bone morphogenetic protein-2. *Adv Drug Deliv Rev* 31:303-318, 1998
18. Sykaras N, Triplett RG, Nunn ME, Lacopino AM and Opperman LA. Effect of recombinant human bone morphogenetic protein-2 on bone regeneration and osseointegration of dental implants. *Clin Oral Implant Res* 12:339-349, 2001
19. Tabata Y, Yamada K, Miyamoto S, Nagata I, Kikuchi H, Aoyama I, Tamura M and Ikada Y. Bone regeneration by basic fibroblast growth factor complexed with biodegradable hydrogels. *Biomaterials* 19:807-815, 1998
20. Yamamoto M, Tabata Y and Ikada Y. Ectopic bone formation induced by biodegradable hydrogels incorporating bone morphogenetic protein. *J Biomater Sci Polymer Edn* 9:439-458, 1998
21. Yamamoto M, Takahashi Y and Tabata Y. Controlled release by biodegradable hydrogels enhances the ectopic bone formation of bone morphogenetic protein. *Biomaterials* 24:4375-4383, 2003
22. Tabata Y. The importance of drug delivery system in tissue engineering. *PSTT* 3:80-89, 2000
23. Yong ML, Sung HN, Yang JS, Tae LK, Seung JL, Young K, In CR, Chong PC, Soo BH and Sang MC. Enhanced bone augmentation by controlled release of recombinant human bone morphogenetic protein-2 from bioabsorbable membranes. *J Periodontol* 74:865-872, 2003
24. Wang EA, D'Alessandro JS, Bauduy M, Cordes P, Harada T, Israel DI, Hewick RM, Kerns KM, Lapan KP, Luxenberg DP, Mcquaid D, Moutsatsos IK, Nove J and Wozney JM. Recombinant human bone morphogenetic protein induces bone formation. *Proc Natl Acad Sci USA* 87:2220-2224, 1990
25. Matsuzaka K, Walboomers F, Yoshinari M, Inoue T and Jansen JA. The attachment and growth behavior of osteoblast-like cells on microtextured surfaces. *Biomaterials* 24:2711-2719, 2003
26. Weiss P. Experiments on cell and axon orientation in vitro, the role of colloidal exudates in tissue organization. *J Exp Zool* 100:353-386, 1945
27. Chehroudi B, McDonnell D and Brunette DM. The effect of micromachined surfaces on formation of bonelike tissue on subcutaneous implants as assessed by radiography and computer image processing. *J Biomed Mater Res* 34:279-290, 1997
28. Cormack DH. Ham's Histology. Philadelphia: Lippincott 6-8: 264-320, 1987
29. Martin DW, Mayes PA. Harper's Review of Biochemistry. Tokyo, Japan: Maruzen, Lange 561, 1983