Tissue Engineering and Regenerative Medicine, Vol. 5, No. 3, pp 488-497 (2008)

| Original Paper |

The Effects of Synthetic Peptide Derived from hBMP-2 on Bone Formation in Rabbit Calvarial Defect

Young-Joon Cho[1], Shin-Il Yeo[1], Jin-Woo Park[1], Hong-In Shin[2], Yong-Chul Bae[3] and Jo-Young Suh[1,*]

[1]*Dept of Periodontology, School of Dentistry, IHPR, Kyungpook Nat'l Univ., 188-1, Samdeok 2Ga, Jung-Gu, Daegu 702-412 Korea*
[2]*Dept of Oral Pathology, College of Dentistry, IHPR, Kyungpook Nat'l University, 188-1, Samdeok 2Ga, Jung-Gu, Daegu 702-412 Korea*
[3]*Dept of Oral Anatomy and Neurobiology, College of Dentistry, Kyungpook Nat'l Univ., 188-1, Samdeok 2Ga, Jung-Gu, Daegu 702-412 Korea*

(Received: July 22th, 2008; Accepted: Aug. 5th, 2008)

Abstract : The bone morphogenic proteins(BMPs) are multifunctional growth factors that belong to the TGF-β superfamily. Among BMPs, BMP-2 is known as the most effective cytokine. The present study evaluated effects of anorganic bovine bone graft material coated with synthetic peptide which corresponds to residues 73-92 of the knuckle epitope of hBMP-2 on bone formation in rabbit calvarial defects. The residues 73-92 of hBMP-2 was absorbed on anorganic bone mineral(ABM) by incubating ABM for 24 hrs. The rabbit calvarial defects were not filled at all(control group), and were filled ABM only(ABM group), ABM with residues 73-92 derived from hBMP-2(ABM/hBMP-2P group) and ABM combined with hBMP-2(ABM/rhBMP-2 group). The groups were evaluated using histologic observation and histomorphometrc analysis at 4 and 8 weeks after healing. The higher osteoconductivity of the ABM/hBMP-2P and ABM/rhBMP-2 groups than the control and ABM only groups. Compared to the control group at 4 weeks after healing, the defects grafted with ABM/hBMP-2P and ABM/rhBMP-2 showed more active bone formation with a statistical significance(p<0.05). And ABM/hBMP-2P and ABM/rhBMP-2 showed a higher percentage bone length from the defect margins than control and ABM only groups at 4 weeks of healing with a statistical significance(p<0.05). ABM/hBMP-2P and ABM/rhBMP-2 may involve mainly initial bone healing and residues 73-92 of hBMP-2 could attribute to new bone formation. Based on the results of this study, anorganic bovine bone maybe a good carrier for peptide and anorganic bovine combined with residues 73-92 of hBMP-2 can increase the new bone formation in early stage in rabbit calvaria.

Key words: *rhBMP-2, bone formation, calvarial defects*

1. Introduction

The bone morphogenic proteins(BMPs) was introduced by Urist in 1965[1] and recombinant BMPs were developed by Wozney in 1988.[2] Among BMPs, BMP-2 is known as the most effective cytokine, while recombinant human BMP-2(rhBMP-2) is useful in treating bony defects when combined with an adequate carrier system or matrix. The functional form of hBMP-2 contains 114 amino acids and signals by oligomerizing type I and type II receptor serine-kinase in cell membranes. The binding epitopes of hBMP-2 are the "wrist epitope" and the "knuckle epitope". The wrist epitope binds BMP receptor I and the knuckle epitope binds the BMP receptor II.[4-6] BMPs and the receptor complex have many biological activities such as the elevation of alkaline phosphatase(ALP), the induction of cartilage or bone, cell differentiation and proliferation through Smad signaling pathways.[7]

In recent years, research about BMPs has become a field in which advances are consistently and constantly being made due to possibility in substitution for bone graft. However, we need to take caution in using BMPs for clinical treatments due to the unwanted effects such as organogenesis, apoptosis, immunogenecity, cell differentiation or cell proliferation.[3] In addition, the long term effects and the releasing patterns of BMPs are still currently unknown. To overcome these concerns, much research about biomimetic materials including short peptide is being carried out. The use of a short peptide is advantageous over the use of the long chain of native proteins. The short peptide

*Tel: +82-53-420-5955; Fax: +82-53-427-3263
e-mail: jysuh@knu.ac.kr (Jo-Young Suh)

sequences are relatively more stable during the modification process. They can be massively synthesized in laboratories more economically as well as being used as a tissue engineering scaffold and can be utilized as a tool to elucidate cellular behavior.[8]

Although the precise BMP signaling pathways have not yet been clarified, it has been well studied on the whole. The cytoplasmic domain of the BMP-bound BMP receptor type II phosphorylates the BMP receptor type I. The phosphorylated BMP receptor type I then phosphorylates Smad1, which is a cytoplasmic signaling molecule specially mediating the action of BMP-2.[9] Phosphorylated Smad1 moves into the nucleus and promotes osteoblastic differentiation by controlling the expression of several genes.[10] Therefore, scientists have experimented to test a plausible hypothesis that synthetic peptide containing knuckle epitope of BMP-2 which binds with BMP-2 receptor type II may promote osteoblastic differentiation since Akiyama *et al.*[11] reported that active BMP type I receptors induced osteoblast phenotypes in the absence of BMP-2. Suzuki *et al.*[12-14] reported that ectopic bone formation in rat calf muscle was observed in alginate hydrogel linked with BMP-2-derived oligopeptide(synthetic peptide NSVNSKIPKACCVPTELSAL, corresponding to residues 68-87 of BMP-2). Saito *et al.*[15] reported that synthetic peptide KIPKASSVPTELSAISTLYL, corresponding to residues 73-92 of BMP-2 and in which Cys78, Cys79, and Met89 are changed to Ser, Ser, and Thr respectively, induced higher alkaline phosphatase activity than does the 68-87 peptide in murine multipotent mesenchymal cell line. Saito *et al.*[16,17] also reported that 73-92 peptide-conjugated alginate gel induced prolonged ectopic calcification in rat calf muscle and induced differentiation of osteoblast precursor cells into osteoblasts, and activated osteoblasts to promote the repair of rat tibial bone defects.

In this report, we synthesized a synthetic peptide which corresponds to residues 73-92(KIPKA SSVPT ELSAI STLYL) of the knuckle epitope of hBMP-2. We then attached this synthetic peptide to anorganic bovine bone material which is widely used for the treatment of bony defects due to its highly biocompatible and osteoconductive properties. The goal of the present study was to evaluate the biologic effects of the anorganic bovine bone graft material combined with synthetic peptide derived from hBMP-2(ABM/hBMP-2P) through assessing the possibility of anorganic bovine bone as a carrier of synthetic peptide and comparing the effects of bone regeneration among anorganic bovine bone(ABM), anorganic bovine bone combined with synthetic peptide derived from hBMP-2(ABM/hBMP-2P) and anorganic bovine bone combined with hBMP-2(ABM/rhBMP-2) on the bone formation in rabbit calvarial defect.

2. Materials and Methods

2.1 Reagents

We used bovine bone derived porous HA(OsteoGrafN-300: Densply Friadent Manheim, Germany) in a particulate form with a particle size of 250-420 μm as anorganic bone mineral (ABM). The rhBMP-2 was purchased from R & D Systems(R & D Systems Inc. Minneapolis, USA.) Peptide KIPKA SSVPT ELSAI STLYL, corresponding to residues 73-92 of hBMP-2, was synthesized by the Peptide Institute(Daegeon, Korea) to a purity of 95%, as determined by reverse-phase high-performance liquid chromatography[HPLC; on a Shiseido Capcell Pak C_{18} column with a 30-60% acetonitrile gradient(10 min) in 0.1% trifluoracetic acid(TFA)-water, at a flow rate 1 mL/min, with detection at 220 nm].

2.2 Preparation of ABM/rhBMP-2 & ABM/residues 73-92 of hBMP-2

The rhBMP-2 was absorbed on ABM by incubating ABM for 24 h in the ratio of 100 mg ABM: 100 μL phosphate-buffered saline(PBS) containing 180 ng rhBMP-2. The residues 73-92 of hBMP-2 was absorbed on ABM by incubating ABM for 24 h in the ratio of 100 mg ABM: 100 μL dimethyl sulfoxide (DMSO) containing 180 μg residues 73-92 of hBMP-2. The present experiment which used 180 ng of rhBMP-2 and 180 μg of residues 73-92 of hBMP-2 was based on the reports of Suzuki *et al.*[14] and Saito *et al.*[15] The incubation was carried out at 4 with gentle shaking to ensure equilibration of the peptide with all exposed surfaces of the microporous ABM. ABM/ rhBMP-2 and ABM/residues 73-92 of hBMP-2 powder were collected and dried after freezing in a freeze dryer. These procedures were established in sterilized conditions.

2.3 Management of Animals

Twelve adult male New Zealandwhite rabbits weighing 3.0 to 3.5 kg were used in this study. They were kept in standard laboratory conditions of a light-dark schedule and relative humidity, were fed a standard rabbit diet and were isolated in separate cages. After administrating an anesthesia of ketamine hydrochloride, 44 mg/kg of body weight(Ketara, Yuhan Corporation, Seoul Korea) and xylazine, 7 mg/kg of body weight (Rumpun, Bayer Korea, Seoul Korea), six rabbits were sacrificed

by a heart perfusion over a period of 4 weeks and while the remaining six rabbits were sacrificed by a heart perfusion at 8 weeks after healing.

2.4 Surgical Procedure

The experimental animals were anesthetized preoperatively with an intramuscular injection of ketamine hydrochloride, 44 mg/kg of body weight(Ketara, Yuhan Corporation, Seoul Korea) and xylazine, 7 mg/kg of body weight (Rumpum, Bayer Korea. Seoul Korea). The dorsal part of the cranium was shaved and prepared aseptically for surgery and 2% lidocaine(contained epinephrine 1:80,000, Yuhan, Pharm, Korea) was injected for local anesthesia and bleeding control in the midline of the cranium. A thirty millimeter long incision was made in the scalp along the sagittal suture. Skin, musculature and periosteum were reflected, and the parietal bones and frontal bones were exposed. Two full thick skull defects were made in parietal bones with a 8-mm trephine bur(3i implant innovation, USA). A trephine bur with 7 mm internal diameter was used to create the defects under copious irrigation with sterile physiologic saline to prevent overheating of the bone edges. The circular bone plugs were gently removed and extreme care was exercised to avoid injury to the duramater and its fibrous attachments to the inner table of the cranial bone. The defects were rinsed with sterile saline and then either filled with 100 mg graft materials or left empty. The periosteum was repositioned and the incision was closed. The defects were evaluated at 4 and 8 weeks after implantation. We devided the experimental sites into four groups as follows(Table 1 and Fig. 1): (a) Control group: defects were not filled at all, (b) ABM only group: defects were filled with OsteoGrafN-300, (c) ABM/hBMP-2P group: defects were filled with ABM/residues 73-92 of hBMP-2, and (d) ABM/rhBMP-2 group: defects were filled with ABM/rhBMP-2. After the surgery, each animal was injected intramuscularly with antibiotics(Baytril, Bayer Korea Seoul Korea) at a dose of 0.2 ml/kg and analgesics (Nobin, Bayer Korea, Seoul Korea) at a dose of 0.44 mg/kg once daily for 1 week.

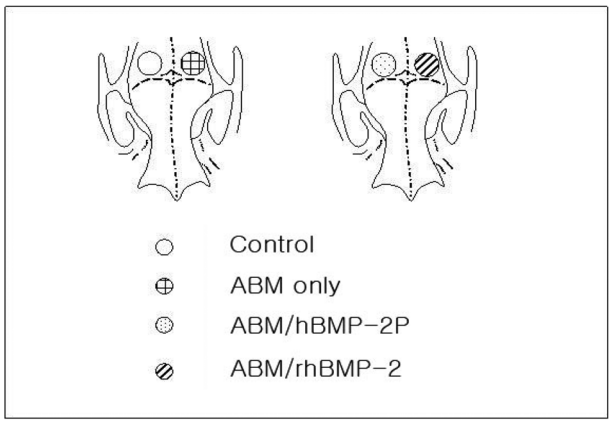

Figure 1. Schematic drawing showing rabbit calvarial defect model. ABM/hBMP-2P; ABM combined with residues 73-92 of hBMP-2, ABM/rhBMP-2; ABM combined with rhBMP-2.

2.5 Histological Evaluation

All rabbits were sacrificed by heart perfusion and block biopsy specimens including the defect and surrounding tissue were taken. The specimens were fixed with the mixture of 4% paraformaldehyde in 0.1 M PBS. After demineralization with 10% ethylene diamine tetra-acetic acid(EDTA), the specimens were dehydrated with a graded series of ethanol and embedded in paraffin. The 3 most central sections of each sample were selected at intervals of 20 μm and sectioned at a final thickness of 3 μm with microtome. The slices were stained by Masson's trichrome.

2.6 Histomorphometric Analysis

Histologic observation and histomorphometric analysis were carried out using a light microscope with image analysis system (i-Solution®, iMTechnology, Inc. Korea). The three most central sections were used for the quantification of the amount of new bone and percent bone length. These specimens were

Table 1. Summary of Experimental Design.

	Control	ABM only	ABM/hBMP-2P	ABM/rhBMP-2
Experimental materials	Non-graft	OsteoGrafN-300	OsteoGrafN-300 + hBMP-2P	OsteoGrafN-300 + rhBMP-2
No. of sacrificed animal at 4 weeks	3	3	3	3
No. of sacrificed animal at 8 weeks	3	3	3	3

ABM/hBMP-2P; ABM combined with residues 73-92 of hBMP-2
ABM/rhBMP-2; ABM combined with rhBMP-2
hBMP-2P; Residues 73-92 of hBMP-2
OsteoGraf®N-300; Dentsply Friadent CeraMed Co, Lakewood, CO, USA

sectioned anterior-posteriorly(i.e. in the parasagittal plane). Histomorphometric analysis was performed first by viewing the Masson's trichrome stained section under the light microscope (Axioskop, Carlzeiss, Germany) at ×40, ×100 and ×200 magnification to ensure visualization of the entire original defect.

Percent bone length was calculated. Percent bone length was defined as the ratio of continuously connected new bone length around ABM particles from both defect's margins, to the distance of total defect. The image was then captured using a digital camera(PL-A662, Pixelink, USA) attached to the microscope and displayed on a computer monitor. At first the total area of the defect was outlined creating the “area of interest”; then the tissue of interest was traced, producing a second area. The data was exported to Micro Excel(Microsoft Corp, Redmond, WA, USA) and, once complied, the various areas measured were expressed as a percentage of the total defect area. The following parameters were measured in terms of the percentage of the defect area they occupied: the total defect area, the area of soft tissue, the area of new bone and the area of particle.

2.7 Statistical Analysis

Histomorphometric results were analyzed using the SAS system. In this investigation, statistical analysis was carried out using one-way ANOVA with Turkey test as a post hoc test among all the groups. Statistical significance was established at $p<0.05$.

3. Results

3.1 Clinical Observation

All animals remained healthy during the observation period and all sites of implantation healed uneventfully. There were no signs of infection, edema or the extrusion of implant materials in all groups.

3.2 Histologic Findings

3.2.1 Control group

At 4 weeks of healing, new bone formation originating from the defect margins was found and some isolated bony islands of round and oval shapes in the middle area of the defect were

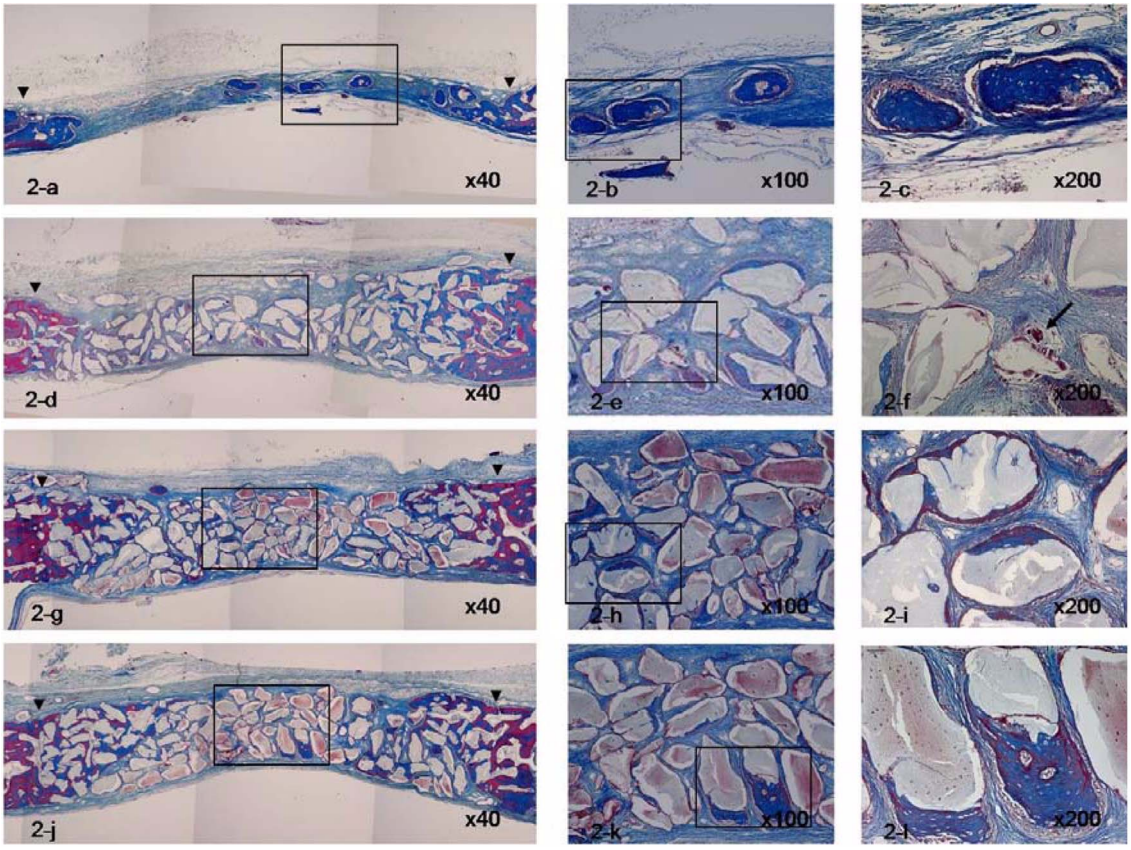

Figure 2. Histologic finding of control (a, b, c), ABM (d, e, f), ABM/hBMP-2P (g, h, i) and ABM/rhBMP-2 (j, k, l) groups at 4 weeks after healing.

seen. The rest of defect was packed with fibrous connective tissue(Fig. 2).

At 8 weeks of healing, newly formed bone grew from the margin to the upper portion of the defect's center, but there was no complete union. Hematopoietic bone marrow was formed in newly formed bony islands. New bone at the margins of the defect was thicker and more matured. Fibrous connective tissues were denser than in the specimen at 4 weeks after healing(Fig. 3).

3.2.2 ABM only group

At 4 weeks of healing, the defect volume was supported by graft materials. Newly formed bone was found at the margin of the defect. Almost all ABM particles were surrounded by fibrous connective tissue and some new bone was formed in central portion of the defect. Some portions of ABM particles resorbed by multinucleated giant cells were seen(Fig. 2).

At 8 weeks of healing, the volume of newly formed bone in the margin of the defect was more than in the specimen at 4 weeks after healing. Small newly formed bone surrounding the grafted ABM particle was seen. However, complete lamella bone formation and complete bridge as fused bone were not shown. Fibrous connective tissues were also denser than in the specimen at 4 weeks after healing(Fig. 3).

3.2.3 ABM/hBMP-2P group

At 4 weeks of healing, newly formed bone grew from the margin to the center of defect. The defects grafted with ABM/hBMP-2P showed more active bone formation in the middle areas of the defect as well as in the margin of the defect. The center of the defect was filled with small amounts of newly formed bone(Fig. 2).

At 8 weeks of healing, the new bone formation pattern around the central ABM particles was similar to ABM only group of 8 weeks after healing, but the bone maturation of the central portion was more definite. The center of the defect was filled with small amounts of newly formed bone similar to histologic appearance at 4 weeks after healing. The volume and density of the new bone was more than in the specimen at 4 weeks after healing(Fig. 3).

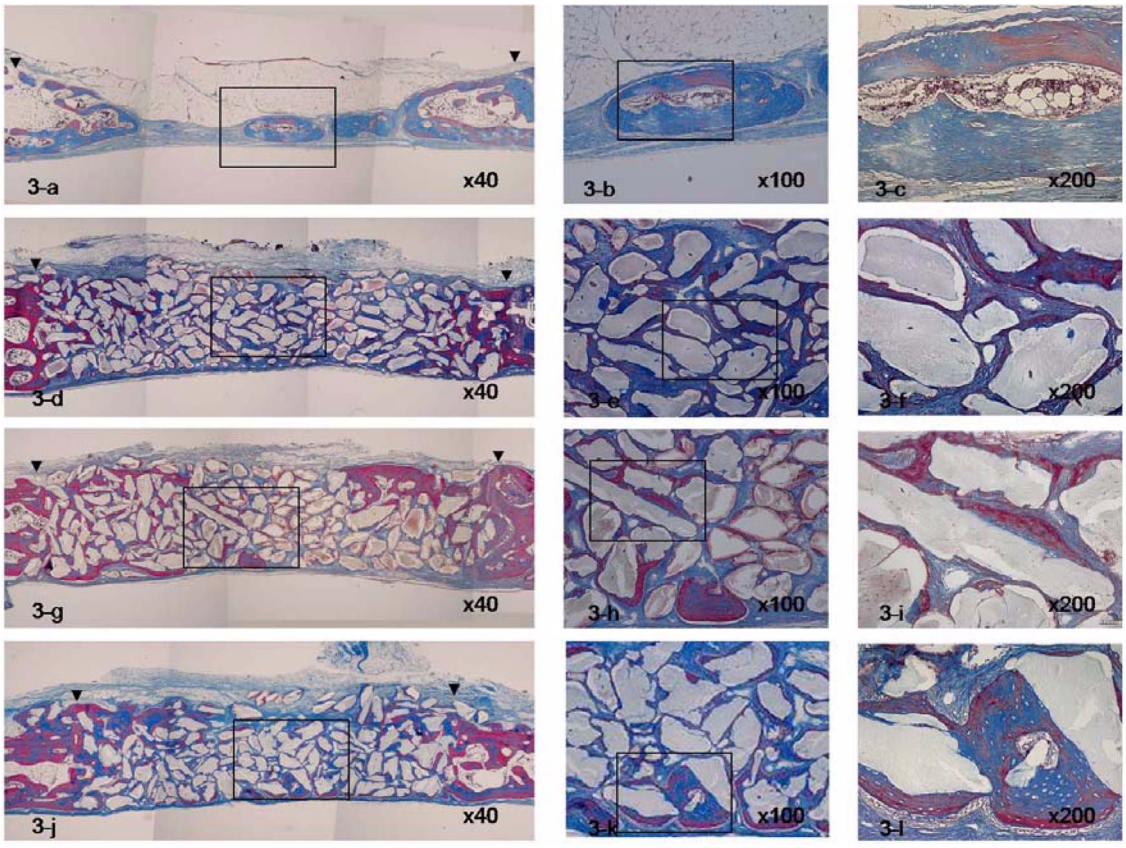

Figure 3. Histologic finding of control (a, b, c), ABM (d, e, f), ABM/hBMP-2P (g, h, i) and ABM/rhBMP-2 (j, k, l) groups at 8 weeks after healing.

3.2.4 ABM/rhBMP-2 group

At 4 weeks of healing, newly formed bone at the margin of the defect grew into the center of defect. The new bone formation pattern around the central ABM particles was similar to the ABM/hBMP-2P group of 4 weeks after healing(Fig. 2).

At 8 weeks of healing, the volume and density of the newly formed bone was more than in the specimen at 4 weeks after healing. Athough the new bone formation pattern around the central ABM particles was similar to the histologic appearance of ABM/hBMP-2P group at 8 weeks after healing, bony bridge has come into being formation almost all below periostem in defect(Fig. 3).

3.3 Histomorphometric Findings

Histomorphometric analysis of the mean volume fractions of newly formed bone, ABM particles, and soft tissue occupying the defects are presented in Fig. 4. The mean percent surface area of new bone for each group is presented in Table 2 and Fig. 5. At 4 weeks of healing, the percentage of new bone formation (mean±SD) in ABM/rhBMP-2, ABM/hBMP-2P, ABM only and control group were 17.76±0.89%, 14.25±1.0%, 11.11±2.55% and 8.32±1.28%, respectively.

At 8 weeks of healing, the corresponding percentage of new bone formation was 25.47±3.2%, 20.41±4.2%, 17.64±3.0% and 17.84±3.6%, respectively. There were more new bone formation in the both ABM/rhBMP-2 group and ABM/hBMP-2P group when compared with control and ABM only group. The new bone formation was increased on all groups according to time lapse. There was a statistically significant difference between 4 weeks and 8 weeks after healing in the control group and ABM/rhBMP-2 group(p<0.05). The new bone area of ABM/hBMP-2P group and ABM/rhBMP-2 group at 4 weeks after healing was statistically significantly increased compared with that of control group and the new bone area of ABM/rhBMP-2 group at 4 weeks after healing was statistically significantly different compared with ABM only group at 4 weeks after healing(p<0.05).

The mean percent bone length of new bone from the defect margin for each group is presented in Fig. 4. The mean percent bone lengths of ABM/hBMP-2P group and ABM/rhBMP-2 group were statistically significantly different compared with those of control group and ABM only groups at 4 weeks after healing(p<0.05). The mean percent bone lengths of ABM/hBMP-2P and ABM/rhBMP-2 groups were statistically significantly different compared with that of control group at 8 weeks

Table 2. Percent of newly formed bone area on each 4 groups as shown by histomorphometric analysis (mean(%)).

Group	4 weeks	8 weeks
	Mean(Range)	Mean(Range)
Control	8.32(6.95~9.47)	17.84(13.76~20.57)★
ABM only	11.11(9.27~14.2)	17.64(14.50~20.53)
ABM/hBMP-2P	14.25(13.06~14.95)	20.41(17.50~25.20)▲
ABM/rhBMP-2	17.76(17.07~18.76)	25.47(23.36~29.10)★▲

(ABM/hBMP-2P; ABM combined with residues 73-92 of hBMP-2)
★Statistically significant difference between 4 weeks and 8 weeks after healing (p<0.05).
▲Statistically significant difference compared with control group at 4 weeks after healing (p<0.05).
§Statistically significant difference compared with ABM only group at 4 weeks after healing (p<0.05).

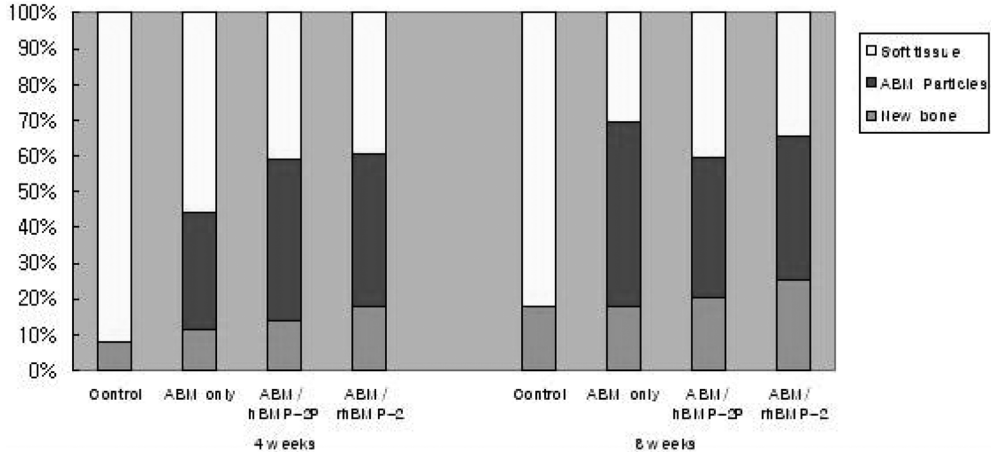

Figure 4. Histomorphometric analysis of the mean volume fractions of newly formed bone, ABM particles and soft tissue occupying the defects were observed. (ABM/hBMP-2P; ABM combined with residues 73-92 of hBMP-2).

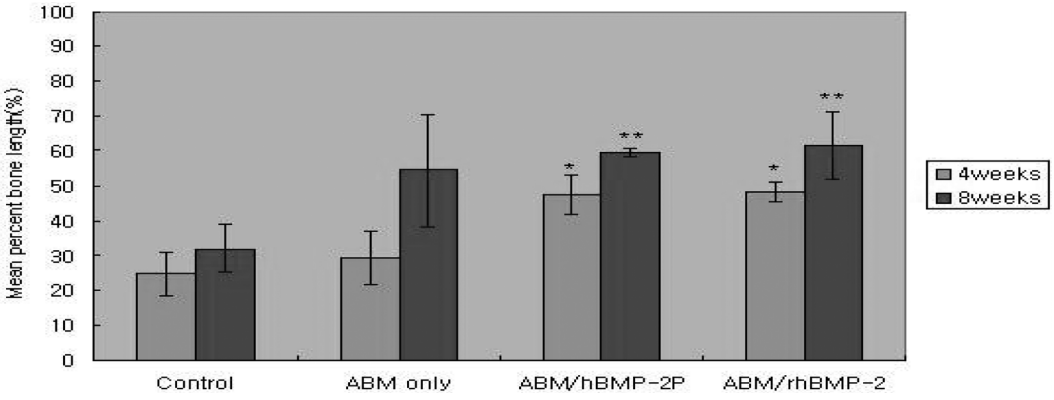

Figure 5. Histomorphometric analysis of percent bone length from the defect margin on each 4 groups(ABM/hBMP-2P; ABM combined with residues 73-92 of hBMP-2).
* : Statistically significant difference compared with control and ABM only group at 4 weeks after healing (p<0.05).
** : Statistically significant difference compared with control group at 8 weeks after healing (p<0.05).

after healing(p<0.05).

4. Discussion

Tissue engineering applications using active short peptides which are attached on biocompatible scaffolds have a great potential for improving the performance of biomaterials. Bone regeneration using bone graft materials combined with peptide is one of the challenging fields in tissue engineering. Although many researches have proved the clinical efficacy of anorganic bovine bone due to its high biocompatibility and osteoconductivity, there still remains any necessity of bovine bone with osteoconductivity.[18,19] Recently however, scientists are endeavoring to overcome this problem. Sigurdsson *et al.*[20] reported that bovine deorganified crystalline bone matrix performed well as a carrier for rhBMP-2-driven periodontal regeneration, although other impediments to their clinical uses exist. Lee *et al.*[21] reported that the immobilizing tetra-cell adhesion molecule(T-CAM) on ABM enhanced the capability of bone substitute to serve as an effective habitat for bone forming cells in vivo. Many researches[22-25] have reported that PepGen P-15^{TM} produced significantly greater vital bone as compared to other graft materials. PepGen P-15^{TM} is a representative FDA (US) approved tissue engineering material which is composed of OsteoGrafN-300 combined with synthetic 15-residue peptide(^{766}GTPGPQGIAGQRGVV^{780}) for enhancing cell attachment. We used OsteoGrafN-300 as carrier of hBMP-2P and rhBMP-2 in this study.

Recent approaches of carriers for synthetic peptide of hBMP-2 have been merely focused on adequate releasing patterns.[26] Those researches have used collagen gel, alginate hydrogel and α-tricalcium phosphate(TCP) as carriers.[12-14,16,17,27] Thereby, the present study evaluated the possibility of anorganic bovine bone material as an effective bone substitute when combined with synthetic peptide, as well as the carrier of synthetic peptide.

In this study, we experimented with “residues 73-92 of hBMP-2” instead of P-15 to find another new bone graft material with tissue engineering. We chose “residues 73-92 of hBMP-2” which is a BMP receptor type II-related peptide among BMP-2 derived peptides[15] because it has a more definite bone inducing effects than P-15 and has many advantages as a short peptide like P-15.[8]

The present study evaluated effects of anorganic bovine bone graft material coated with synthetic peptide which corresponds to residues 73-92 of the knuckle epitope of hBMP-2 on bone formation in rabbit calvarial defects. The experimental model used in this study was based on a report by Takagi and coworkers.[28] Adult rabbit calvariums which had many similarities to the maxillofacial region were selected to use in this study. The calvaria consists of two cortical plates with regions of intervening cancellous bone which is similar to the mandible.[29] The cortical bone in the calvaria physiologically resembles an atrophic mandible.[30] Bone regeneration in the rabbits occurs at a rate of 3 to 4 times faster than in humans. Therefore, rabbits remain an excellent experimental model for investigating calvarial repair during an abbreviated period.[31] The amount of healing that would occur in a bony defect was in large part dependent upon the wound size.[32] An experimental bony wound used to access repair should, therefore, be large enough to preclude spontaneous healing. An experimental

bony wound of this nature may be termed a critical size defect. A critical size defect may be defined as the smallest size intraosseous wound in a particular bone and species of animal that will not heal spontaneously during the lifetime of the animal.[33] It is previously reported that a 15 mm diameter defect was the critical size defect in New Zealand White rabbits.[29] Nevertheless, the 8 mm diameter calvaria defects were created in the present study and were not meant to be critical size defects. Because two critical sized defects cannot be created without encroachment of the cranial sutures in the rabbit cranium as it is too small. Kramer *et al*.[34] reported that a partial bony bridge containing soft tissue bridging the rest of the defect occurred in New Zealand White rabbits with 8 mm diameter calvaria defects up to 16 weeks. These findings indicated that an 8 mm diameter defect was enough to evaluate the effects on the bone formation in rabbits calvaria in this animal model. In our study, a complete bridge did not occur in all groups as expected.

The bone grafting material must get credit for regeneration. Hammerle *et al*.[35] reported that Osteograf N-300 did not alter the basic pattern of bone formation and could initially accelerate new bone formation during guided bone regeneration by the increased recruitment of osteoblasts on the rabbits' skull. However, Al Ruhaimi[36] reported that Osteograf did not reveal active bone healing in the condyle of the rabbit tibia. In histological examination of our study, new bone formation in ABM only group was seen only at the margin of the defect of 4 weeks after surgery and small amounts of newly formed bone surrounding with grafted particles were seen(Figs. 2 and 3). All groups grafted with ABM particles showed that the length of newly formed bone was more than in the control group and in turn the length of newly formed bone in ABM/hBMP-2P and ABM/rhBMP-2 groups was more than in the control and ABM only groups with statistical significance($p<0.05$).In addition, there was little soft tissue invasion near the periosteum and the defect volume was supported by graft materials without a barrier membrane to protect the invasion of soft tissue. Furthermore, ABM particles themselves may increase bone heightor bone thickness when compared to the control group. These results indicated that ABM particles have biocompatible and osteoconductive effects although they did not reveal active bone formation as expected.

Another reason for using anorganic bovine bone material as carrier of residues 73-92 of hBMP-2 instead of a-TCP[27] is rates of resorption The slow resorption of ABM particles has previously been reported on the surface of anorganic bovine bone mineral, without being associated with the characteristic resorption lacunae observed in normal remodelling of native bone.[37,38] In this study, some portions of ABM particles were only resorbed by multinucleated giant cells(Fig. 2c). This means that ABM particles have a very excellent osteoconductive effect to provide enough time to reconstruct bony defects.

In histomorphometric analysis(Fig. 4), ABM/hBMP-2P and ABM/rhBMP-2 groups showed a higher percentage of bone length from the defect margin than control and ABM only group at 4 weeks of healing with statistical significance ($p<0.05$). This means the higher osteoconductivity of ABM with hBMP2P and rhBMP-2. In addition, the amount of bone formation in ABM/hBMP-2P group showed more than in control or ABM only groups, but showed less than in ABM/ rhBMP-2 group(Figs. 2 and 3). Compared to control group at 4 weeks after healing, the defects grafted with ABM/hBMP-2P and ABM/rhBMP-2 groups showed more active bone formation with statistical significance($p<0.05$). In addition, compared to control group at 8 weeks of healing, although the defects grafted with ABM/hBMP-2P and ABM/rhBMP-2 groups also showed more active bone formation in the middle areas of the defect as well as in the margin of the defect, there was no statistically significant difference($p>0.05$). This result indicated that ABM/ hBMP-2P and ABM/rhBMP-2 may involve mainly initial bone healing and residues 73-92 of hBMP-2 could attribute to early new bone formation.

Suzuki *et al*.[14] reported that ectopic bone formation in rat calf muscle was observed in alginate hydrogel linked with 68-87 residues of BMP-2. Saito *et al*.[16,17] reported that 73-92 peptide-conjugated alginate gel particles significantly promoted the repair of rat tibial bone defects and showed prolonged ectopic calcification in rat calf muscle. In our study, theosteoinductive effect of ABM/hBMP-2P and ABM/rhBMP-2 groups was not apparent because small amounts of isolated newly formed bone were found(Figs. 2 and 3). It was considered that the role of alginate hydrogel as a carrier is more effective than anorganic bovine bone because the osteoinductive effect of ABM/hBMP-2P or ABM/rhBMP-2 was not apparent. Although alginate hydrogel is a better carrier than ABM, there are several disadvantages, such as little osteoconductive property and insufficient spacemaintaining effect for clinical uses. Additionally, we used anorganic bovine bone combined with very low dosage of synthetic peptide and rhBMP-2. Therefore, further investigations are also required to find adequate dosage of the synthetic peptide for effective osteoinduction.

Within the limit of our study, it can be concluded that anorganic

bovine bone can be a good carrier for peptide and anorganic bovine combined with residues 73-92 of hBMP-2 can increase the new bone formation in early stage in rabbit calvaria. These new biomimetic strategy may be applied in treatment of bony defects of periodontal or implant therapy.

Acknowledgements: The authors wish to thank Dentsply Friadent, Germany, for providing the anorganic bone mineral (OsteoGrafN-300) used in this study. It's work was supported by basic research grant of MOST(M 10646020002-07N4602-00210).

References

1. MR Urist, Bone formation by autoinduction, *Science*, **150**, 893 (1965).
2. JM Wozney, Novel regulators of bone formation: molecular clones and activities, *Science*, **242**, 1528 (1988).
3. AH Reddi, Role of morphogenetic proteins in skeletal tissue engineering and regeneration, *Nat Biotechnol*, **16**, 247 (1998).
4. T Kirsch, J Nickel, W Sebald, BMP-2 antagonists emerge from alterations in the low-affinity binding epitope for receptor BMPR-II, *EMBO J*, **19**, 3314 (2000).
5. T Kirsch, W Sebald, MK Dreyer, Crystal structure of the BMP-2-BRIA ectodomain complex, *Nat Struct Biol*, **7**, 492 (2000).
6. P Knaus, W Sebald, Cooperativity of binding epitopes and receptor chains in the BMP/TGFbeta superfamily, *Biol Chem*, **382**, 1189 (2001).
7. D Chen, M Zhao, GR Mundy, Bone morphogenetic proteins, *Growth Factors*, **22**, 233 (2004).
8. H Shin, S Jo, AG Mikos, Biomimetic materials for tissue engineering, *Biomaterials* **24**, 4353 (2003).
9. PA Hoodless, T Haerry, M abdollah, MADR1, a MAD-related protein that functions in BMP2 signaling pathways, *Cell*, **85**, 489 (1996).
10. JW Jang, KS Park, SH Kim, *et al*., Tissue engineered bone regeneration using DBP-loaded PLGA scaffold in rabbit model, *Tissue Eng Regen Med*, **2(1)**, 34 (2005).
11. S Akiyama, T Katagiri, Constitutively active BMP type I receptors transduce BMP-2 signals without the ligand in C2C12 myoblasts, *Experimental Cell Res*, **235**, 362 (1997).
12. Y Suzuki, Y Nishimura, M Tanihara, Evaluation of a novel alginate gel dressing: Cytotoxicity to fibroblasts *in vitro* and foreign-body reaction in pig skin *in vitro*. *J Biomed Mater Res*, **39**, 317 (1998).
13. Y Suzuki, M Tanihara, Y Nishimura, *In vivo* evaluation of a novel alginate dressing, *J Biomed Mater Res*, **48**, 522 (1999).
14. Y Suzuki, M Tanihara, K Suzuki, Alginate hydrogel linked with synthetic oligopeptide derived from BMP-2 allows ectopic osteoinduction *in vivo*, *J Biomed Mater Res*, **50**, 405 (2000).
15. A Saito, Y Suzuki, S Ogata, Activation of osteo-progenitor cells by a novel synthetic peptide derived from the bone morphogenetic protein-2 knuckle epitope, *Biochemica Biophysica Acta*, **1651**, 60 (2003).
16. A Saito, Y Suzuki, S Ogata, Prolonged ectopic calcification induced by BMP-2-derived synthetic peptide, *J Biomed Mate Res*, **70A**, 115 (2004).
17. A Saito, Y Suzuki, S Ogata, Accelerated bone repair with the use of a synthetic BMP-2-derived peptide and bone-marrow stromal cells, *J Biomed Mate Res*, **72A**, 77 (2005).
18. DG Smiler, PW Johnson, JL Lozada, Sinus lift grafts and endosseous implants. Treatment of the atrophic posterior maxilla, *Dent Clin North Am*, **36**, 151 (1992).
19. MB Hurzeler, A Kirsch, KL Ackemann, Reconstruction of the severly resorbed maxilla with dental implants in the augmented maxillary sinus: a 5-year clinical investigation, *Int J Oral Maxillofac Implants*, **11**, 466 (1996).
20. G Khang, MS Kim, SH Cho, *et al*., Natural scaffolds biomaterials for tissue regeneration, *Tissue Eng Regen Med*, **1(1)**, 9 (2004).
21. JH Lee, JW Park, BJ Choi, Anorganic bone mineral coated with tetra-cell adhesion molecule enhance bone formation in rabbit calvarial defects, *Key Eng Mater*, **981**, 309 (2006).
22. DM Thompson, MD Rohrer, HS Prasad, Comparison of bone grafting materials in human extraction sockets: clinical, histologic, and histomorphometric evaluations, *Implant Dent*, **15**, 89 (2006).
23. K Yuan, JS Huang, CW Hsu, A mineralization-associated membrane protein plays a role in the biological functions of the peptide-coated bovine hydroxyapatite, *J Periodontal Res*, **42**, 420 (2007).
24. JT Krauser, MD Rohrer, SS Wallace, Human histologic and histomorphometric analysis comparing OsteoGraf/N with PepGen P-15 in the maxillary sinus elevation procedure: a case report, *Implant Dent* **9**, 298 (2000).
25. RA Yukna, JT Krauser, DP Callan, Multi-center clinical comparison of combination anorganic bovine-derived hydroxyapatite matrix (ABM)/cell binding peptide (P-15) and ABM in human periodontal osseous defects. 6-month results, *J Periodontol*, **71**, 1671 (2000).
26. YK Ko, SH Kim, HJ Ha, *et al.*, Tissue engineered biodisc using human intervertebral disc cells and PLGA/DBP scaffold, *Tissue Eng Regen Med*, **4(1)**, 67 (2007).
27. A Saito, Y Suzuki, Repair of 20-mm long rabbit radial bone defects using BMP-derived peptide combined with an α-tricalcium phosphate scaffold, *J Biomed Mater Res*, **77**, 700 (2006).
28. K Takagi, MR Urist, The reaction of the dura to bone morphogenic protein (BMP) in repair of skull defects, *Ann Surg*, **196**, 100 (1982)
29. JW Frame, A convenient animal model for testing bone substitute materials, *J Oral Surg*, **38**, 176 (1980).
30. MJ Shon, SY Lee, TH Kim, *et al*., Effect of growth factors and dexamethasone on proliferation of bone marrow stromal cell, *Tissue Eng Regen Med*, **3(4)**, 457 (2006).
31. IH Chung, PH Choung, YY Jo, Bioengineering of the tooth and jaw, *Tissue Eng Regen Med*, **2(2)**, 86 (2005).
32. E Hjorging-Hansen, and JO Andreasen, Incomplete bone healing of experimental cavities in dog mandible, *Br J Oral Surg*, **9**, 33 (1971).
33. JP Schmitz, JO Hollinger, The critical size defect as an experimental model for craniomandibulofacial nonunions, *Clin Orthop Relat Res*, **205**, 299 (1986).

34. IR Kramer, HC Kelly, HC Wright, A histological and radiological comparison of the healing of defects of the rabbit calvarium with and without implanted heterogeneous anorganic bone, *Arch Oral Biol*, **13(9)**, 1095 (1968).
35. CHF Hammerle, AJ Olah, J Schmid, The biological effect of natural bone mineral on bone neoformation on the rabbit skull, *Clin Oral Implants Res*, **8**, 198 (1997).
36. JW Jang, KS Park, SH Kim, *et al*., Tissue engineered bone regeneration using DBP-loaded PLGA scaffold in rabbit model, *Tissue Eng Regen Med*, **2(1)**, 34 (2005).
37. CHF Hammerle, GC Chiantella, T Karring, The effect of a deproteinized bovine bone mineral on bone regeneration around titanium dental implants, *Clin Oral Implants Res*, **9**, 151 (1998).
38. S Jensen S, N Broggini, E Hjorting-Hansen, Bone healing and graft resorption of autograft, anorganic bovine bone and β-tricalcium phosphate: A histologic and histomorphometric study in the mandibles of minipigs, *Clin Oral Implants Res*, **17**, 237 (2006).