Ectopic bone formation
associated with rhBMP-2
using Biphasic calcium phosphate block
as a carrier in a rat subcutaneous assay model

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2007년 7월

저자 씀

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ABSTRACT

Ectopic bone formation
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The carrier for the delivery of BMPs should serve as a scaffold for new bone. In addition, predictable bone formation in terms of volume and shape should be guaranteed. The purpose of this study is to evaluate the ectopic bone formation of recombinant human bone morphogenetic protein-2(rhBMP-2) using micro macroporous biphasic calcium phosphate (MBCP: mixture of β−TCP and HA) block as a carrier in a rat subcutaneous assay model.

Subcutaneous pockets were created on the back in 40 male Sprague-Dawley rats. In the pockets rhBMP-2/MBCP and MBCP alone were implanted. Blocks were evaluated by histological and histometric parameters following a 2 weeks(each 10 rats; MBCP and rhBMP-2/MBCP) or 8 weeks (each 10 rats; MBCP and rhBMP-2/MBCP) healing interval.

Shape and volume of the block were maintained stable over the healing time. Histologic bone forming activity could not found in the MBCP alone sites at 2 weeks. At 8 weeks show minimal new bone formation. New bone formation in the macropores of the block was evident in the rhBMP-2/MBCP sites. The new bone area of the 8 weeks was greater than the 2 weeks . The quantity of the new bone with a more advanced stage of remodeling had increased further.

These results implicated that MBCP block could serve as a carrier system for predictiable bone tissue engineering using rhBMPs.

Key Words : bone regeneration; carrier; bone morphogenetic protein-2; rat subcutaneous pockets; micro macroporous biphasic calcium phosphate

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Ⅰ. INTRODUCTION

Since Urist[1] demonstrated ectopic bone and cartilage formation following intramuscular implantation of demineralized bone matrix in rats and later named BMPs, several BMPs have been shown to have significant osteoinductive activity[2-8]. It was reported that rhBMP by itself is sufficient to induce bone formation; however, the rapid diffusion of the water-soluble protein rhBMP from the implant site will reduce its onsteoinductive effect. Therefore, carrier system for rhBMPs is essential[9-12].

In our previous study[13], the carrier for BMPs should serve as a scaffold for bone forming cells while providing a space in which bone formation can occur, and resist soft tissue compression during healing period. For clinically successful use of BMPs, the carrier also should have easy to manipulate and sterilize, make into intended shape. It is also important that

shape and volume of newly formed bone is kept. If satisfy these requirements of carrier, predictable bone regeneration would possible and have enough value to tissue engineering side. There were a lot of studies about carrier[14-17], but carrier that satisfy these requirements perfectly is not yet.

MBCP consists of an intimate mixture of 40% β−TCP and 60% HA [18,19]. Besides well-documented osteoconductive effect[20-22], osteoinductive effect of the MBCP is recommended for use as an alternative or additive to autogenous bone for orthopedics, traumatology, odontology and dental applications[23-28].

Porous structure, low resorption rate, favorable osteroconductive and bioactive property of MBCP can be used enough by rhBMPs carrier. Easy manipulation and easy making the intended shapes, especially block type used, are additional merits of using this biomaterial. But, research against the carrier for BMPs is the condition which is almost lacking[29,30].

Because rat's subcutaneous tissue are more poor conditions compare with muscle or bony site or other animal, success this region can foresee better success in other region.

The purpose of this study was to evaluate the ectopic bone formation of rhBMP-2 using MBCP block as a carrier in a rat subcutaneous assay model.

Ⅱ. MATERIALS AND METHODS

2.1. Animals

40 male Sprague-Dawley rats (weight 250-300 g) were used. Rats were maintained in plastic cages in a room with a 12 h-day/night cycle and an ambient temperature of 21°C, with *ad libitum* access to water and standard laboratory pellets. Animal selection and management, surgical protocol, and preparation were in accordance with the routines approved by the Institutional Animal Care and Use Committee, Yonsei Medical Center, Seoul, Korea.

2.2. rhBMP-2 Implant Construction

Disc-shaped MBCP implants* (3mm height and 8mm diameter) were manufactured. rhBMP-2† was reconstituted and diluted in a buffer to produce a concentration of 0.025 mg/ml. For the rhBMP-2/MBCP implants, MBCP implants were loaded with 0.2 ml of the rhBMP-2 solutions for one hour before surgery. For the MBCP implants, MBCP implants were loaded with 0.2 ml of a buffer solutions **(*Figure 1*)**.

* Biomatlante,Vigneux de Bretagne, France

† R&D Systems Inc., Minneapolis, MN, USA

2.3. Surgical Procedures

The animals were anaesthetized by an intramuscular injection (5 mg/kg body wt.) of a 4:1 solution of ketamine hydrochloride‡:Xylazine.§ The surgical site was shaved and scrubbed with iodine. A vertical incision was made in the skin of the back. After flap reflection a subcutaneous pocket was prepared by blunt instrument. Each animal received 1 of 2

experimental treatments: MBCP carrier control and rhBMP-2/MBCP. The periosteum and skin were closed and sutured with absorbable monofilament suture. ||

‡ Ketalar®, Yuhan Co., Seoul, Korea

§ Rompun®, Bayer Korea, Seoul, Korea

|| Monosyn®, Aesculap AG Co. KG., Tuttlingen, Germany.

2.4. Histologic and Histometric Procedures

The animals were sacrificed by CO_2 asphyxiation at 2 and 8 weeks post-surgery. Block sections were removed and fixed in a 10% neutral buffered formalin solution for 10 days. Samples were decalcified by 5% formic acid for 14 days and embedded in paraffin. Serial sections 7 um in thickness were prepared at intervals of 80 um, stained with hematoxylin/eosin (H-E), and examined using a light microscope. The most central sections from each block were selected for histologic and histometric evaluation.

Computer-assisted histometric measurements were obtained using an automated image analysis system coupled with a video camera on a light microscope. Sections were examined at magnifications of x 20 and x 100. Histometric parameters were defined as follows.

- Total augmented area (mm^2): the area of implanted MBCP zone.
- New bone area (mm^2): the area of newly formed bone within the total augmented area.

2.5. Statistical Analysis

Histometric recordings from the samples were used to calculate means and standard deviations (m±SD). For comparisons between the two groups, a paired or unpaired t-test was used. The interactions between the healing times were examined using two way analysis of variance. A p-value <0.01 was considered significant.

Ⅲ. RESULTS

1. Clinical observation

Wound healing was generally uneventful and there were no signs and symptoms of infection or inflammation

2. Histologic Observations

2.1 MBCP control group***(Figure 2).***

At 2 weeks, histological markers of inflammation or foreign body reactions were not generally found. MBCP blocks were surrounded by loose connective tissue. Macropores of the block were filled with connective tissue. Osteogenic activity could not found histologically. At 8 weeks post surgery, the implanted block was covered with dense, fibrous connective tissue. The minimal amount of new bone formation was observed adjacent to the margins of the block. No significant resorption of the MBCP block was observed during the healing time.

2.2 MBCP/ rhBMP-2 group***(Figure 3).***

At 2 weeks, the macropores in the periphery of the block were filled with new bone. Evidence of osteogenic activity, such as dense osteoblast-like cell lining, osteoid and bone apposition along the surface of macropores was observed. Macropores in the center part were usually filled with loose fibrous connective tissue and bone forming activity was rare.

At 8 weeks, the quantity of new bone was greater than that observed at 2 weeks, and the specimens showed a more advanced stage of remodeling and consolidation. Some macropores with fibrous connective tissue could be also found in the central part of the block. The newly

formed bone consisted of woven and lamellar bone, and showed cement lines that were separated earlier from the more recently deposited bone. There was no evidence of cartilage formation.

3. Histometric Analysis

Tables 1 and 2 show the results of histometric analysis. In the rhBMP-2/MBCP group, the quantity of the new bone was greater at 8weeks compared to 2 weeks ($p < 0.01$).

Table-1. Total augmented area (group means ±SD,mm^2 , n=10)

	2 weeks	8 weeks
MBCP	21.9 ± 2.6	19.9 ± 2.8
rhBMP-2/MBCP	23.7 ± 1.9	23.5 ± 2.4

No significant difference when compared to all groups ($P > 0.01$)

Table-2. New Bone area (group means±SD,mm^2, n=10)

	2 weeks	8 weeks
MBCP	0.8 ± 0.2	2.1 ± 1.1*
rhBMP-2/MBCP	3.9 ± 0.7¶	5.8 ± 2.4*¶

*: Statistically significant difference compared to 2 weeks ($p < 0.01$)

¶: Statistically significant difference compared to MBCP group ($p < 0.01$)

Ⅳ. DISCUSSION

The purpose of this study was to evaluate the ectopic bone formation of rhBMP-2 using MBCP block as a carrier in a rat subcutaneous assay model. Because rat's subcutaneous tissue are more poor conditions compare with muscle or bony site or other animal, success this region can foresee better success in other region. Results were evaluated by histologic and histometric analysis following a 2- and 8-weeks of healing interval.

In our previous study[13] using same method of the present study, we demonstrated that rhBMP-2, when impregnated in ACS and β-TCP, provoked osteoinductive activity in rat subcutaneous tissue at 2 weeks. However, one of the interesting findings of the previous study was that at 8 weeks neither ACS nor the newly formed bone observed at 2 weeks was found in the rhBMP-2/ACS sites, while more expanded bone maturation was observed in the rhBMP-2/ β-TCP sites.

This result indicated that ACS did not exert its capacity as a carrier for rhBMP-2 in this model. The lack of space-maintaining capacity of ACS may be one of the major factors responsible for the resorption of the newly-formed bone that was observed in the 2-week sections after implanting. It could be concluded that the carrier for the delivery of BMPs should serve as a scaffold for bone forming cells while providing a space in which bone formation can occur. Although favorable ectopic bone formation could be obtained using β-TCP particle, questions arise as to whether clinical application is possible, since there is clinical difficulty, in its nature of particulated form, in manipulation and maintenance for the intended shape of bone. Since predictable bone tissue regeneration could be one of the major goals of the clinical therapeutic results. a carrier system for rhBMPs should provide the stability over the time in

terms of volume and shape.

MBCP was developed 20 years ago By Daculsi and coworkers, consists of an intimate mixture of 40% β−TCP and 60% HA, is obtained when a synthetic calcium deficient apatite is sintered at temperature above 700˚C. It is now available in blocks, particulates and as an injectable material in a polymer carrier[18,19]. MBCP have shown excellent biocompatibility and bioactivity, this is related to porous structure[20-22]. The micropore less than 10㎛ allow fluid circulation, leading to the dissolution and degradation of the biomaterial. The macropore more than 100㎛ act as a scaffold for bone cells, thus allowing centripetal bone ingrowth[23]. The newly formed bone is in direct contact with the biomaterial surface, making this biomaterial osteoconductive. Besides well-documented osteoconductive effect, osteoinductive effect of the MBCP is recommended for use as an alternative or additive to autogenous bone for orthopedics, traumatology, odontology and dental applications[24,25].

Although osteoinduction by synthetic biomaterials in ectopic sites has been widely observed[26], its mechanism is still largely unknown. Ectopic bone formation by biomaterials have been shown material-dependent. Material properties such as composition, geometry, porosity, size and microstructure have been reported as critical parameters for ectopic bone induction[27]. Osteoinduction by biomaterials also seems to animal-dependent[28] and so far, it has only been observed in the muscles of large animals. Several hypothesis about osteoinduction have been proposed. Synthetic materials implanted in vivo could absorb proteins from surrounding body fluids. Some growth factors that differentiate undifferentiated cells into osteogenic lineage would be concentrated onto synthetic materials. Calcium phosphate ceramics may concentrate bone growth factors from body fluids, which will trigger stem cells

to form bone tissue. Micropores combined with the high solubility of TCP and stability of HA induce the precipitation of biological apatite crystals on to biphasic calcium phosphate ceramics, and the osteoprogenitor cells might in turn recognize the bone-like apatite layer formed in vivo by dissolution-reprecipitation on the material and produce mineralized bone.

In the present study, MBCP alone site at 8 weeks have been shown minimal ectopic bone formation. This results coincides with previous reported study.

Porous structure, low resorption rate, favorable osteoconductive and bioactive property of MBCP can be used enough by rhBMPs carrier. The porous structure of MBCP could be retain and slow releasing of rhBMPs, and provide the appropriate scaffold which would allow cells and newly formed tissues to migrate into it. For clinical aspect, due to MBCP block type is manipulated easily and make the intended shape simply, this is very interesting biomaterial at using for BMP’s carrier. But, research against the carrier for BMPs is the condition which is almost lacking[29,30].

In the present study new bone formation in the macropores of the block was evident in the rhBMP-2/MBCP sites. The new bone area of the 8 weeks was greater than the 2 weeks . The quantity of the new bone with a more advanced stage of remodeling had increased further. And total augmented area was stable during the healing periods. In rat’s subcutaneous tissue, our favorable results showed that MBCP block is a good carrier system for tissue engineering side using rhBMPs.

Ⅴ. CONCLUSION

Using block type of MBCP as a carrier for rhBMP-2 is effective in new bone formation. New bone induced by rhBMPs/MBCP block system was maintained stable during observed healing period. These results indicate that MBCP block system could be recommendable carrier system for tissue engineering using rhBMPs

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FIGURE LEGENDS

Figure 1. Block type MBCP implant used in this study.

Figure 2. Representative photomicrographs of the MBCP control group at 2 weeks(A and C) and 8 weeks (B and D) (arrow head: defect margin, NB: new bone; H-E stain; original magnification A and B x20, C and D x100).

Figure 3. Representative photomicrographs of the rhBMP-2/MBCP group at 2 weeks (A and C) and 8 weeks (B and D) (arrow head: defect margin, arrow: cement lines**,** NB: new bone; H-E stain; original magnification A and B x20; C and D x100).

Figures

Figure 1

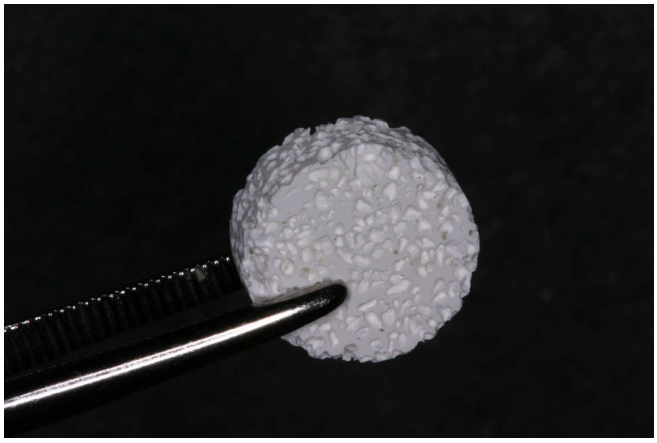


Figure 2

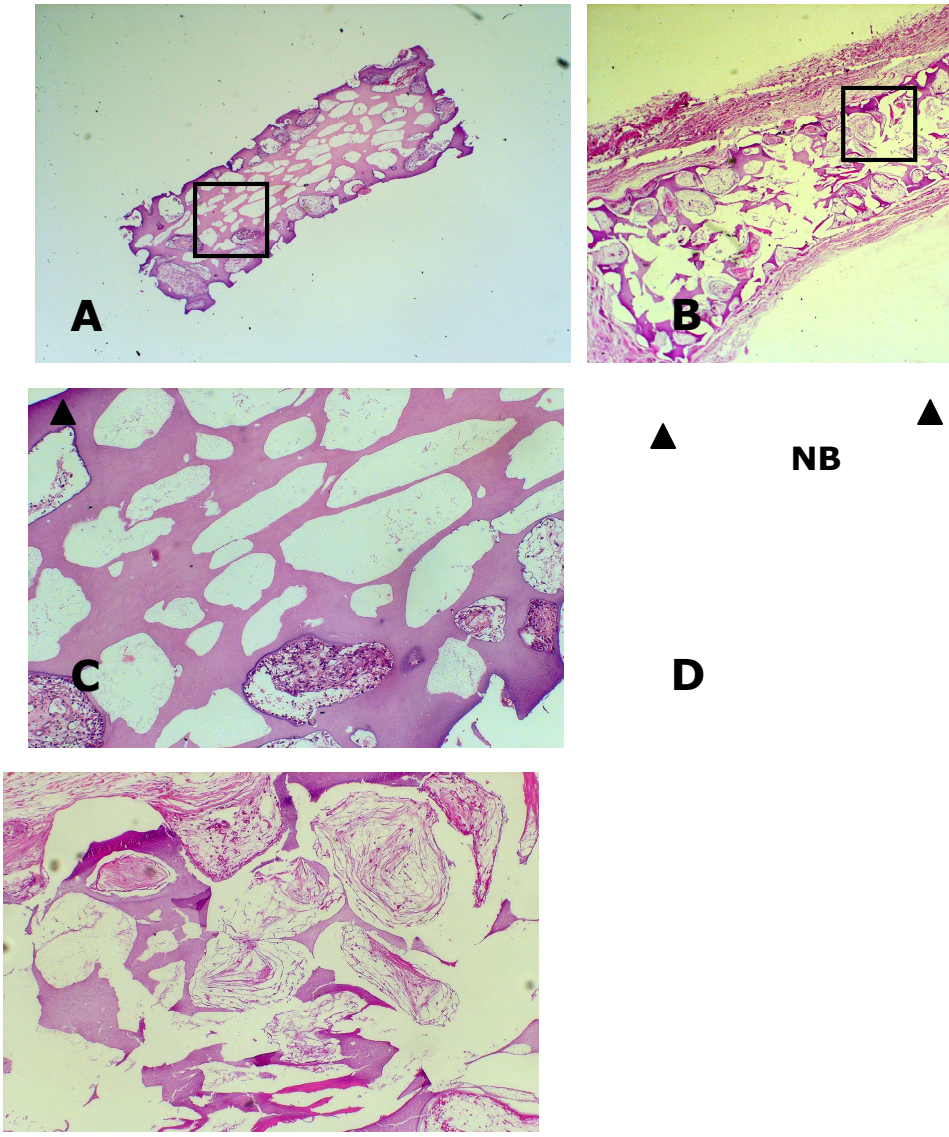
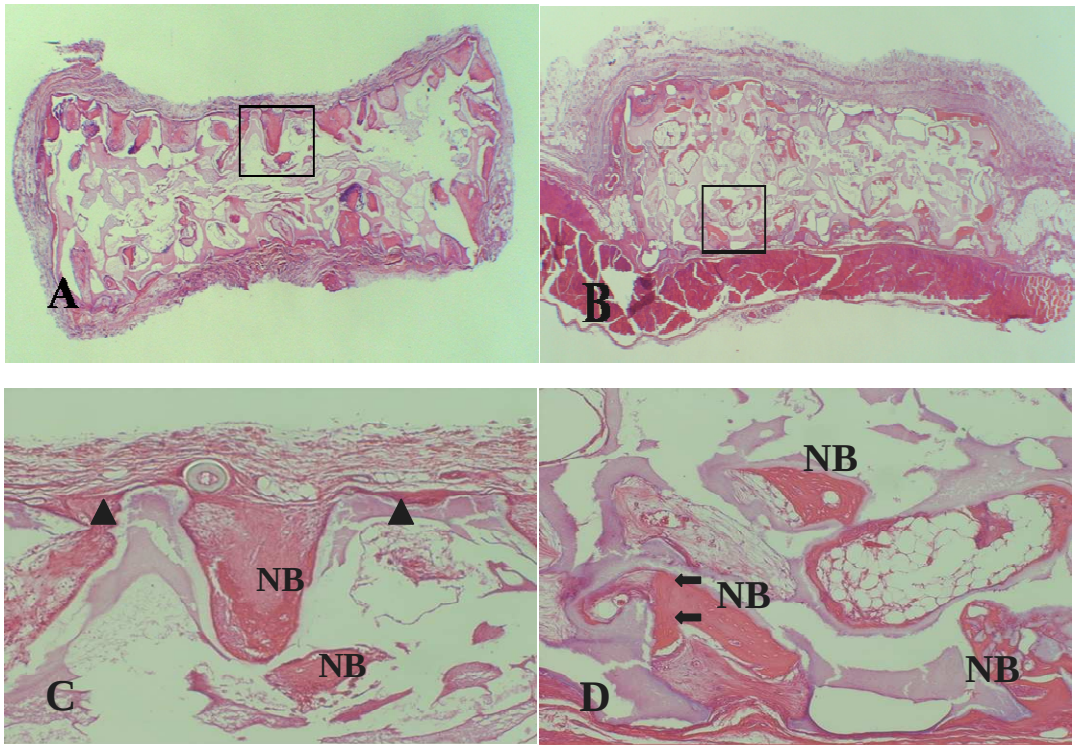


Figure 3



국문요약

백서 피하 조직에서 bone morphogenetic protein-2와 Biphasic calcium phosphate-block 운반체에 의한 이소성 골형성 효과

연세대학교 대학원 치의학과 김준일 [지도교수 조규성]

BMP 의 전달을 위한 운반체는 새로운 골형성을 위한 기질로서의 역할을 해야 한다. 더욱이 일정 기간동안 매식체의 부피와 형태가 유지될 수 있어야 예지성 있게 신생골 형성을 기대할 수 있다. 이 연구에서는 백서 피하 조직에서 bone morphogenetic protein-2와 Micro macroporous biphasic calcium phosphate-block (MBCP block) 운반체에 의한 이소성 골 형성 효과를 평가하고자 한다.

40마리의 웅성 백서의 등부 피하조직에 낭을 형성하였고 rhBMP-2/MBCP 또는 MBCP 단독을 매식하였다. 40마리의 웅성 백서를 4군으로 나누어 각 10마리씩 술후 2주와 8주로 나누어 실험 동물을 희생하였으며 조직학적, 조직계측학적으로 비교 관찰하였다.

매식체의 부피와 형태는 치유기간 동안 안정되게 유지되었으며 조직학적으로 MBCP 단독으로 매식한 부위 2주에서는 새로운 신생골 형성이 나타나지 않았으나, 8주에서는 약간의 신생골 형성이 관찰되었다. rhBMP-2/MBCP 의 매식부위에서는 block 내의 macropore 내에서 신생골 형성이 관찰되었으며 신생골의 면적은 2주에 비해 8주에서 월등하게 증가되는 양상이 관찰되었다. 8주에는 신생골의 골성숙이 관찰되었다.

이상의 결과에서 볼 때 MBCP block 은 rhBMP 를 이용한 조직공학에 있어 예지성 있는 운반체로서 이용될 수 있을 것으로 사료된다.

핵심되는 말: 골재생; 운반체; 골형성유도 단백질-2; 백서 피하조직낭; micro macroporous biphasic calcium phosphate