Bone formation by *Escherichia coli* –expressed recombinant human bone morphogenetic protein-2 in a rat calvarial defect and ectopic subcutaneous model

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감사의 글

본 논문이 완성되기까지 부족한 저를 항상 격려해 주시고 사랑과 관심으로 이끌어 주신 조규성 교수님께 깊은 감사를 드립니다. 그리고, 많은 조언과 따뜻한 관심으로 지켜봐 주신 김종관 교수님, 채중규 교수님, 최성호 교수님, 정의원 교수님과 심사를 맡아주신 정한성 교수님, 김창성 교수님, 윤정호 교수님, 그리고 3년간의 연구강사 기간 동안 많은 가르침을 주시고 진심 어린 충고를 아끼지 않으신 문익상 교수님께도 감사드립니다.

연구 내내 많은 도움을 준 채경준, 정성원, 김민수, 임현창 선생님과 최은영 연구원을 비롯한 치주과 의국원들께도 감사와 애정을 전합니다.

그리고, 늘 조건 없는 사랑을 주시고 말없이 저를 믿어 주시는 사랑하는 부모님과 언니에게 진정으로 사랑과 고마움의 마음을 전합니다. 모든 분들께 진심으로 감사드립니다.

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Abstract

Bone formation by *Escherichia coli*-expressed recombinant human bone morphogenetic protein-2 in a rat calvarial defect and ectopic subcutaneous model

rhBMP-2 produced in prokaryotic system has been studies as an alternative to rhBMP-2 produced eukaryotic mammalian cells. The aim of present study was to investigate bone formation of *E.coli*-expressed rhBMP-2(ErhBMP-2) dose in a rat calvarial defect and ectopic subcutaneous model quantitatively.

rhBMP-2 was obtained from RNA extracted from the human osteoblast cell (U2O2) and reverse transcribed into cDNA encoding the mature form of the BMP-2 protein, which was refolded and purified in vitro. Calvarial defect of 8 mm in diameter and subcutaneous pouch was created in one hundred forty four male Sprague-Dawley rats, which were divided into 6 groups and each group received one of following experimental conditions: 1) a Sham-surgery control, 2) ACS control, 3) 2.5μg ErhBMP-2/ACS, 4) 5μg ErhBMP-2/ACS, 5) 10μg ErhBMP-2/ACS, or 6) 20μg ErhBMP-2/ACS and were evaluated by histologic and histometric analysis following 2- or 8- weeks healing interval.

For calvarial defect model, enhanced bone formation could be observed in ErhBMP-2 treated groups whereas only limited new bone formation was found in control groups. For ectopic subcutaneous model, bone formation was obvious in

every animal treated with ErhBMP-2 at 2 weeks. At 8 weeks, however, the number of animals showing new bone formation was smaller even though degree of bone remodeling and amount of new bone was advanced.

Bone healing was affected by observation period and anatomic sites but not by selected dose difference. ErhBMP-2 could induce bone formation in the rat calvarial defect and subcutaneous model. Therefore, ErhBMP-2 has a potential to be an alternative to rhBMP-2 produced from mammalian cells and the efficacy appears to be comparable or similar to that.

Key Words: Bone morphogenetic protein; Ectopic bone formation; Bone regeneration; Bone tissue engineering; *Escherichia coli*

Bone formation by *Escherichia coli* –expressed recombinant human bone morphogenetic protein-2 in a rat calvarial defect and ectopic subcutaneous model

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I. Introduction

Bone morphogenetic protein (BMP) has come into the spotlight in the area of orthopedics and dentistry because of its autoinduction capacity(Urist and Mikulski 1979). In 1965, Urist found that nonviable bone extract could induce bone formation and named the factor within the bovine bone responsible for bone induction as BMP(Urist 1965). Development of recombinant technology could make it possible to clone the coding sequence (c-DNA) of BMPs, resulting in successful synthesis of recombinant human BMP(rhBMP) and in turn, providing the opportunity for the production of highly purified BMP preparation(Wozney et al. 1988). Since then, a

number of studies about rhBMP have employed rhBMP-2 and demonstrated the potential to be a safe and effective alternative of autogenous bone graft(Nevins et al. 1996; Zegzula et al. 1997; Kim et al. 2005; Hong et al. 2006; Zheng et al. 2006).

Most rhBMPs have been obtained from mammalian cell such as Chinese hamster ovary (CHO) cell so far(Israel et al. 1992). However, low yields (ng/ml range) production of rhBMPs at high cost in this well-established eukaryotic protein expression system is considered as problematic for clinical application. In order to become a viable commercial product, acquisition cost is also an issue. There has been many trials for the production and evaluation of biologically active rhBMPs expressed in *E.coli* as an alternative to rhBMPs produced in mammalian cells(Zhao et al. 1994; Kubler et al. 1998; Bessho et al. 2000; Vallejo et al. 2002; Long et al. 2006). In spite of the fact that prokaryotic expression systems lack the executive capacity of the correct posttranslational modifications unlikely in mammalian cells, the biologic activity has been restored by in vitro purification and refolding process(Zhao et al. 1994; Vallejo et al. 2002; Long et al. 2006). However, the efficacy of rhBMPs expressed in *E.coli* was evaluated as inferior to that of rhBMPs expressed in mammalian cells(Zhao and Chen 2002). In addition, studies with rhBMPs expressed in *E.coli* are just on the initial stage of development: almost of studies has been done in vitro and a few studies, if any, done in the ectopic site of small animals and thought to be insufficient to prove the quantitative efficacy in orthotopic site. If rhBMPs

expressed in *E.coli* could have biologic activity comparable to eukaryotic system, a large quantity of rhBMPs might be provided at low cost.

The aim of present study was to evaluate bone formation of *E.coli*-expressed rhBMP-2 (ErhBMP-2) dose in a rat calvarial defect and ectopic subcutaneous model quatitatively.

Ⅱ. Materials & Methods

1. Animals

One hundred forty four male Sprague-Dawley rats (body weight 200-300g) were used. Animals were maintained in plastic cages in a room with 21-hour day/night cycles, an ambient temperature of 21℃, and ad libitum access to water and a standard laboratory pellet diet. Animal selection and management, surgical protocol, and preparation followed routines approved by the Institutional Animal Care and Use committee, Yonsei Medical Center, Seoul, Korea.

2. Expression of rhBMP-2 in *E.coli*

The ErhBMP-2 was produced at the research institute, Cowellmedi Co. LTD, Pusan, Korea, as follows.

Total RNAs of the human osteosarcoma cells (U2OS) were reverse transcribed with reverse transcriptase (Gibco BRL, NY, USA) into cDNA encoding the mature peptide of the BMP-2 protein, which was amplified by polymerase chain reaction (PCR). The cDNA of mature hBMP-2 was subcloned into a pRSET(A) vector

(Invitrogen, UK). The plasmid obtained was named as pRSET(A)/hBMP-2. The pRSET(A)/hBMP-2 was used to transform *E. coli* BL21(DE3) strain.

The high-cell-density cultivation was accomplished using a bioreactor (KoBioTec, Incheon, Korea) as described by Tabandeh (Tabandeh et al. 2004). Transformed *E. coli*, cultured at a 5L fermenter (KoBioTec, Incheon, Korea) with 3L of the defined medium (batch culture; yeast extract 1g/L, peptone 2g/L) was inoculated with pre-culture (10% of batch culture volume). The cultivation was performed at 30°C in 250rpm (Stirrer speed) for 48h, adding pre-autoclaved nutrient medium (Glucose 33.3 g/L, peptone 10 g/L, yeast extract 5 g/L, MgSO4 1 g/L, FeSO4 8.0 g/L, CaCl2 0.048 g/L, ZnSO4 0.0176 g/L, CuSO4 0.008 g/L).

The cultured biomass was harvested and suspended a sodium phosphate buffer (50mM, pH7.0) at a 220mg wet weight/L. The harvested cell was crushed twice through a French pressure and centrifuged. The pellet was resuspended at a 25 mg wet weight/ml in a suspension buffer (20 mM Tris-HCl (pH 8.5), 0.5 mM EDTA, 2% (v/v) Triton X-100) and was centrifuged. The inclusion bodies (pellets) were resuspended in a solubilization buffer (6M Guanidine-HCl, 0.1M Tris-HCl (pH 8.5), 0.1M DTT, 1mM EDTA) and incubated overnight at room temperature with constant stirring and centrifuged in order to remove insoluble part.

For *in vitro* dimerization, the solubilized rhBMP-2 was incubated in a renaturation buffer (0.5 M Guanidine-HCl, 50 mM Tris-HCl(pH 8.5), 0.75 M CHES, 1 M NaCl, 5 mM EDTA, 3 mM total Glutathione) for 72h. Purification of the active rhBMP-2 (dimer) was performed using Heparin Sepharose 6 Fast Flow column (GE healthcare, USA). The bounded protein was eluted by a continuous NaCl gradient (0.1M ~1.5M). After elution profile was confirmed, active rhBMP-2 protein was eluted and separated by a stepped NaCl gradient (0.15 M, 0.3 M and 0.5 M)

3. ErhBMP-2 implants

ErhBMP-2 was reconstituted and diluted in buffer to produce a concentration of 0.025, 0.05, 0.1 and 0.2mg/ml. For the calvarial defect model, sterile 8mm diameter ACS (Collatape, Calcitek, Carlsbad. CA, USA) was then loaded with 0.1 ml of ErhBMP-2 solutions to produce an implanted dose/defect of 2.5, 5, 10, 20 μg, respectively. For the control experiments, the buffer was loaded alone. The ErhBMP-2 and control implants were fitted into the calvarial defect following a 5-minute binding period.

For the ectopic subcutaneous model, a sterile ACS strap of 2.5 cm x1.0 was inserted to the prepared subcutaneous pouch after 0.3ml of each concentration ErhBMP-2 solution was loaded for 5 minutes.

4. Surgical procedures

The animals were anesthetized by an intramuscular injection (5mg/kg body wt.) of a 4:1 solution of ketamine hydrochloride (Ketalar®, Yuhan Co., Seoul, Korea): Xylazine (Rompun®, Bayer Korea, Seoul, Korea). The surgical site was shaved and scrubbed with iodine. For calvarial defect model, an incision was made in the sagittal plane across the cranium and a full thickness flap was reflected exposing the calvarial bone. A standardized, circular, transosseous defect, 8 mm in diameter, was created on the cranium using trephine drill (3i Implant innovation, Palm Beach Gardens, FL, USA) with copious saline irrigation. After removal of the trephined calvarial disk, ErhBMP-2 and control treatments were applied to the defect sites. For ectopic subcutaneous model, a vertical incision was made in the skin of the back; using blunt dissection, a subcutaneous pocket was prepared.

According to different ErhBMP-2 concentration, the animals were divided into 6 groups of 24 animals each and let them heal for 2 or 8 weeks. For calvarial defect model, each group received one of 6 experimental conditions: 1) a Sham-surgery

control, 2) ACS control, 3) 2.5μg ErhBMP-2/ACS, 4) 5μg ErhBMP-2/ACS, 5) 10μg ErhBMP-2/ACS, or 6) 20μg ErhBMP-2/ACS. For ectopic subcutaneous model, only ErhMBP-2 treatment conditions were given. All of surgical site were sutured for primary closure with 4-0 Monosyn® (Glyconate absorbable monofilament, B-Braun, Aesculap, INC., PA, USA).

5. Histological processing and histometric measurements

Block sections including the surgical sites were removed at sacrifice. The sections were rinsed in sterile saline and fixed in 10% buffered formalin for 10 days. After rinsing in water, the sections were decalcified in 5% formic acid for 14 days and embedded in paraffin. Serial sections, 5μm thick, were cut through the center of the circular calvarial defects as well as the subcutaneous sites. From each block, the two most central sections were selected and stained with hematoxylin and eosin (H&E), and examined by a binocular microscope (Leica DM LB, Leica Microsystems Ltd., Wetzlar, Germany) equipped with a cemera (Leica DC300F, Leica Microsystems Ltd., Heerburgg, Switzerland). Images of the slides were taken and saved as figure files.

After conventional microscopic examination, computer-assisted histometric measurements of the newly formed bone were obtained using an automated image

analysis system (Image-Pro Plus, Media Cybernetics, Silver Spring, MD, USA) in the calvarial defect model. Three parameters were measured: defect closure, new bone area, and bone density. Defect closure (%) was determined by measuring the distance between the defect margin and the new bone margin and was expressed as a percentage of the total defect width. New bone area (mm^2) was measured as newly formed mineralized bone, excluding marrow and fibrovascular tissue. Bone density (%) was determined by the percentage of new bone area in the augmented area, which means all tissues within the boundaries of newly formed bone, i.e., mineralized bone, fibrovascular tissue, bone marrow and residual biomaterial used as carrier. Calculations are shown in Figure 1.

6. Statistical analysis

Histomorphometric recordings from the samples in calvarial defects were used to calculate group means (±SD). A two-way analysis of variance (ANOVA) was used to analyze the effect of time and experimental conditions. The post-hoc Scheffe test was employed to analyzed the difference between the groups ($P<0.05$).

Ⅲ. Results

The postoperative period was generally uneventful for all animals except only one animal, which belonged to the group of 5μg ErhBMP-2/ACS with healing period of 2 weeks and died after surgery with unknown reason. Thirteen specimens of calvarial defect model were excluded due to technical complications in the histologic processing (Sham surgery control, two at 2 weeks and one at 8 weeks; ACS control, two at each 2 and 8 weeks; 2.5μg ErhBMP-2/ACS, two at 2 weeks and one at 8 weeks; 5μg ErhBMP-2/ACS, one at 8 weeks; 10μg ErhBMP-2/ACS, one at 8 weeks; 20μg ErhBMP-2/ACS, one at 2 weeks). Finally, 130 specimens of calvarial defect model in total were investigated histomorphometrically.

1. Calvarial defect model

In Sham surgery and ACS control, only limited amount of new bone could be observed at the defect margins regardless of healing time. Defect was filled with thin, loose connective tissue. In the ACS control, some collagen used as carrier was hardly found.

In the ErhBMP-2 treated groups, all defects exhibited remarkable bone bridging, irrespective of dose of ErhBMP-2 even though maturity and defect closure were more advanced at 8 weeks than 2 weeks. At 2 weeks, ACS carrier was embedded in newly formed bone, as well as still detectable in the center of the remaining defect. Chondrocytic cells were associated with newly formed immature bone, which can infer that bone formation was involved in endochondral ossification (Fig. 3d). At 8 weeks, the defect was completely filled with the newly formed bone and the original defect margin was hardly demarcated from the new bone. New bone area showed a few osteocytes, cemental line and mature marrow space. No remnants of ACS could be detected (Fig. 4).

In the quantitative terms, ErhBMP-2 implantation resulted in 80% to 100% of bony bridging of the defects, while values for control groups were in the range 10 to 20% (Table 1). There was no significant difference in comparison of 2 weeks and 8 weeks group, respectively. New bone area was significantly greater in ErhBMP-2 treated groups than in the untreated controls, irrespective of dose. In the ErhBMP-2 treated groups, new bone area was significantly greater at 8 weeks than at 2 weeks within each amount of ErhBMP-2 (Table 2). In the aspect of bone density, the results showed the similar outcomes with new bone area: at 2 weeks, ErhBMP-2 treated groups showed significantly greater bone density than ACS control and bone density was significantly greater at 8 weeks than at 2 weeks (Table 3). These results showed

that irrespective of dose, implantation of ErhBMP-2 resulted in more significant bone formation than the untreated controls.

2. Ectopic subcutaneous model

At 2 weeks after ErhBMP-2 implantation, all animals in four ErhBMP-2 treated groups showed new bone formation regardless of the dose even though the amount of newly formed bone was very small. Histologically, newly induced woven bone was observed at the periphery of implant. The core of implant consisted of ACS remnants, abundant loose connective tissue. The new bone or bone matrix was immature and lined with cells expected to be osteoblastic (Fig. 5). There was no evidence of significant adverse reactions.

Unlikely at 2 weeks, the number of animals showing new bone formation at 8 weeks was smaller, and positively proportional to the dose of ErhBMP-2 (Table 4). At that time, ACS was completely resorbed and marrow tissue occupied most of implant. The quantity of the new bone was greater than that observed at 2 weeks even though it was not measured histomorphometrically and histological observation revealed that newly formed bone showed a few osteocytes and cement line separated previously formed bone from more recently deposited bone as a manifestation of advanced remodeling.

IV. Discussion

The objective of present study was to investigate the osteogenic efficacy of ErhBMP-2 using the well-established experimental bone healing model in rat, which has been employed for evaluation of rhBMP-2 expressed in mammalian cell system (Pang et al. 2004; Hyun et al. 2005; Kim et al. 2005; Hong et al. 2006). In the present study, the osteogenic efficacy of ErhBMP-2 in both orthotopic and ectopic sites of rat was investigated quantitatively. Histologically, the ErhBMP-2 loading didn't result in any obvious adverse tissue reaction except mild inflammation associated with ACS carrier that presumably could be attributed to the nature of collagen sponge. There was definitely remarkable new bone formation, especially on orthotopic site in the ErhBMP-2 treated group. In the ectopic site, the new bone could be observed on all rats at 2 weeks, which means ErhBMP-2 when loaded on ACS carrier could provoke the osteoinduction in rat subcutaneous tissue initially. However, some of them disappeared at 8 weeks despite the maturity and amount of new bone was advanced. This result could be ascribed to the lack of space-maintenance capacity of ACS and anatomic characteristics of ectopic site. There are several studies with rhBMP-2 derived in CHO cell showing results compatible with this study(Kubler et al. 1998; Kim et al. 2005). However, the higher dose was used, the greater number of animals showing the evidence of new bone there was at 8 weeks. This could be thought as that

a high dose might overcome or compensate the lack of space providing capacity of ACS carrier.

In order to investigate the dose dependency of ErhBMP-2, we used the different dose that is 2.5, 5, 10, and 20μg by factor 2 for each rat calvarial defect at 2- and 8 weeks observation interval. In the design used in this study, it was obvious that ErhBMP-2 induced similar amount of new bone and degree of defect closure, independent on dose. Dose dependency can be affected by various factors such as a function of carrier, species, experimental site, and observation interval(Hasegawa et al. 2008). There have been many researches to figure out the dose dependency but it is still a controversial issue. Of studies using ErhBMP-2, although no direct comparison to this study was possible, Kimura et al. showed application of ErhBMP-2 by a factor of 10 i.e. 0.1, 1 and 10 μg on the rat mandible defect resulted in defect closure of 48 to 64% and failed to show dose response. In the other hand, Kubler et al. used 0.4, 4 and 40 μg of ErhBMP-2 and exhibited that higher concentrate of rhBMPs could induce larger amount of new bone formation in ectopic site. These conflicting results might be caused mainly by effect of different experimental site i.e., orthotopic versus ectopic. We failed to show dose dependency on the calvarial defect. This result might attribute to a narrow range of doses and the characteristics of orthotopic site. In the other hand, dose dependent manner was observed at 8 weeks in the ectopic site which lacks the inherent osteogenic potential. In the present study, 2.5 μg or higher

dose of ErhBMP-2 showed similar bone formation in calvarial defects. To determine the threshold dose in this study design, additional studies using lower dose than 2.5 μg is thought to be necessary.

The efficacy of ErhBMP-2 in comparison to rhBMP-2 produced in CHO cell or animal cell, was evaluated as about one order of magnitude less(Zellin and Linde 1999). This agrees with results from in vitro experiments(Zhao and Chen 2002), in which it was found that the activity of ErhBMP-2 was about five to ten times less active than rhBMP-2 produced in CHO cells. However, the study by Bessho et al. compare ErhBMP-2 with CHO cell-derived rhBMP-2 in vitro and in vivo and concluded that the bone-inducing activity of ErhBMP-2 was similar to that of CHO cell-derived rhBMP-2 and ErhBMP-2 effectively induces bone formation under some conditions(Bessho et al. 2000). In comparison with the study using rhBMP-4 and rat calvarial model(Pang et al. 2004), it was found that the degree of defect closure and bone density was comparable to the results of the present study. Inherently, the post-translational modifications of BMP-2 do not occur in *E.coli*, which is essential for the synthesis of functional BMP-2 protein. The post-translational modification includes glycosylation, dimerization and cleavage of the precursor protein into the mature form(Wang et al. 1990; Scheufler et al. 1999; Long et al. 2006). Due to the inefficient refolding process of the BMP-2 protein after expression in *E.coli*, ErhBMP-2 was thought to be poorly-effective in early study(Zhao and Chen 2002). In recent years,

the success in restoring of the biologic activity after rhBMP-2 expression in *E.coli* made it possible to produce the functional BMP-2 from *E.coli*(Vallejo et al. 2002; Bessa et al. 2008; Choi et al. 2008).In addition, post-translational glycosylation is not critical for functional ErhBMP-2 because the basic glycosylated N-terminal domains of rhBMP-2 are not obligatory for receptor activation(Ruppert et al. 1996). In this study, a non-glycosylated rhBMP-2 was obtained in form of inclusion bodies and refolded to its biologically active form of dimer in vitro. This ErhBMP-2 was proved as biologically active and osteoinductive in vivo, as well as quantitatively efficient in the present investigation.

In conclusion, it may be inferred that ErhBMP-2 is osteoinductive under control in vivo, and there is definite possibility to be an alternative to rhBMP-2 produced in mammalian CHO cells for clinical use. Further experiments with smaller dose of ErhBMP-2 and in higher animals should be conducted in order to figure out its threshold dose and clinical efficacy.

V. Conclusion

In order to evaluate the osteogenic efficacy of ErhBMP-2 histolotically and histomorphometrically, calvarial defects and ectopic subcutaneous pouches were created in 144 Sprague-Dawley male rats. Those defects were treated as follows. 1) a Sham-surgery control, 2) ACS control, 3) 2.5μg ErhBMP-2/ACS, 4) 5μg ErhBMP-2/ACS, 5) 10μg ErhBMP-2/ACS, or 6) 20μg ErhBMP-2/ACS. After 2- and 8 weeks healing period, animals were sacrificed and specimens were investigated via light microscope for histologic and histomorphometric evaluation.

1. For calvarial defect model, all experimental groups showed results superior to control groups irrespective to dose of ErhBMP-2 in all aspect of measurements.
2. For ectopic subcutaneous model, new bone formation was observed in all animals after 2 weeks healing, in the other hand, only some animals showed the evidence of new bone in which the characteristics of mature and remodeled bone were found.
3. ErhBMP-2 is inferred to be osteoinductive under control in vivo, and there is definite possibility to be an alternative to rhBMP-2 produced in mammalian CHO cells for clinical use.

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Legends

Figure 1. Schematic drawing of showing the histometric analysis of the calvarial defect model.

Figure 2. Representive photomicrographs of defect site receiving the Sham surgery at 2 weeks(a), 8 weeks(b), and ACS carrier control at 2 weeks(c), 8 weeks(d). In all cases, thin, loose connective tissue can be observed between the defect margins. The ACS remnants can hardly be found out in ACS control. (arrow heads=defect margin; H&E stain, original magnification X10)

Figure 3. Representive photomicrographs of calvarial defect sites receiving ErhBMP-2/ACS (a,b: 2.5μg, c,d: 5μg, e,f: 10μg, g,h: 20μg) at 2 weeks postsurgery. Irrespective to the dose of ErhBMP-2 loaded, it is observed that immature newly formed bone and embedded ACS remnants. On the center of specimen, chondrocytic cells associated with new bone area also can be seen (d,f). (arrow heads=defect margin; NB=new bone, OB=original bone, CH=chondrocytic cell; H&E stain, original magnification a,c,e and g X10, b,d,f and h X100)

Figure 4. Representive photomicrographs of calvarial defect site receiving ErhBMP-2/ACS (a,b: 2.5μg, c,d: 5μg, e,f: 10μg, g,h: 20μg) at 8 weeks postsurgery. Newly formed bone was integrated to original bone, which made it difficult to discriminate the location of original defect margin. The defect was almost completely filled with the new bone, which was more mature form than that observed at 2 weeks. (arrow heads=defect margin; NB=new bone, OB=original bone; H&E stain, original magnification a,c,e and g X10, b,d,f and h X100)

Figure 5. Representive photomicrographs of ectopic site receiving ErhBMP-2/ACS (a,b: 7.5μg, c,d: 15μg, e,f: 30μg, g,h: 60μg) at 2 and 8 weeks postsurgery. At 2 weeks (a,c,e, and g), at the periphery of implant, newly formed bone was observed, which was lined with cells expected to be osteoblastic. The core of implant consisted of abundant ACS remnants. At 8 weeks (b,d,f, and h), ACS remnants completely disappeared and fatty marrow tissue occupied the core of implant. Osteocytes, reversal line, and osteoblastic lining also were observed. (NB=new bone, M=marrow tissue; H&E stain, original magnification X100)

Tables

Table 1. Defect Closure (group mean ±SD %; N=number of specimens)

Group	2 Weeks (N)	8 Weeks (N)
Sham surgery control	13.5±3.5 (10)	18.3±8.7 (11)
ACS control	18.0±7.7 (10)	21.9±8.3 (10)
2.5 μg ErhBMP-2/ACS	84.2±24.8*† (10)	98.4±.3.1*† (11)
5 μg ErhBMP-2/ACS	100.0±0.0*† (11)	100.0±0.0*† (11)
10 μg ErhBMP-2/ACS	85.9±16.3*† (12)	100.0±0.0*† (11)
20 μg ErhBMP-2/ACS	92.2±11.3*† (11)	100.0±0.0*† (12)

* Statistically significant difference compared to sham surgery control group ($P < 0.05$)

† Statistically significant difference compared to ACS control group ($P < 0.05$)

Table 2. New Bone Area (group mean ±SD mm^2; N=number of specimens)

Group	2 Weeks (N)	8 Weeks (N)
Sham surgery control	0.18±0.05 (10)	0.80±0.27 (11)
ACS control	0.47±0.14 (10)	0.80±0.37 (10)
2.5 μg ErhBMP-2/ACS	1.55±0.72* (10)	5.13±0.91*† (11)
5 μg ErhBMP-2/ACS	2.65±0.46*† (11)	6.18±1.29*† (11)
10 μg ErhBMP-2/ACS	1.74±0.65*† (12)	6.28±1.09*† (11)
20 μg ErhBMP-2/ACS	1.92±1.26*† (11)	5.75±1.11*† (12)

* Statistically significant difference compared to sham surgery control group ($P < 0.05$)

† Statistically significant difference compared to ACS control group ($P < 0.05$)

Table 3. Bone Density (group mean ±SD %; N=number of specimens)

Group	2 Weeks (N)	8 Weeks (N)
Sham surgery control	83.4±10.3† (10)	83.4±10.3 (11)
ACS control	13.6±3.1* (10)	89.5±7.0 (10)
2.5 μg ErhBMP-2/ACS	46.1±10.4*† (10)	73.3±7.0† (11)
5 μg ErhBMP-2/ACS	43.1±5.9*† (11)	70.6±10.8† (11)
10 μg ErhBMP-2/ACS	36.9±13.0*† (12)	74.8±6.7† (11)
20 μg ErhBMP-2/ACS	37.3±13.5*† (11)	73.1±7.4† (12)

* Statistically significant difference compared to sham surgery control group ($P < 0.05$)

† Statistically significant difference compared to ACS control group ($P < 0.05$)

Table 4. Bone formation two and eight weeks after implantation of ErhBMP-2 expressed in *E.coli* (number of animals containing bone induction/total number of animals involved in histologic observation)

Group	2 Weeks	8 Weeks
7.5 μg ErhBMP-2/ACS	12/12	1/12
15 μg ErhBMP-2/ACS	11/11	6/12
30 μg ErhBMP-2/ACS	12/12	8/12
60 μg ErhBMP-2/ACS	12/12	9/12

Figures

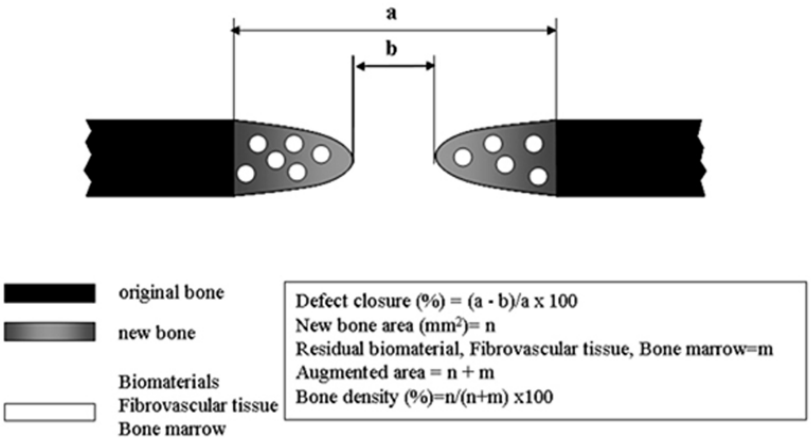

Figure 1

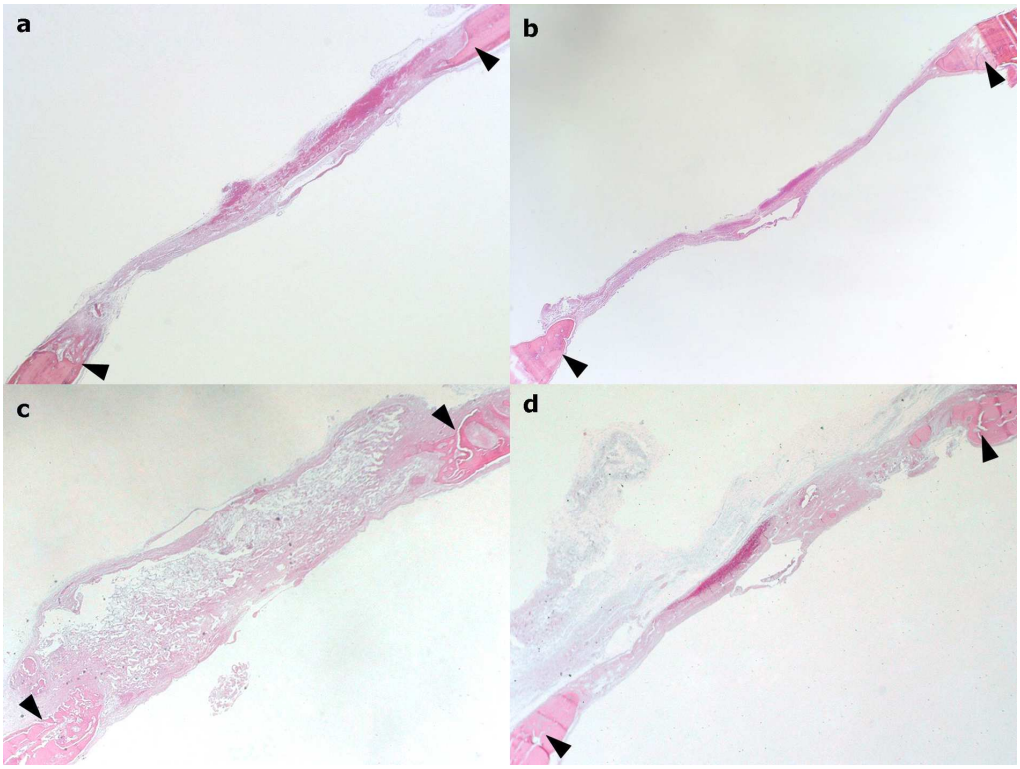

Figure 2

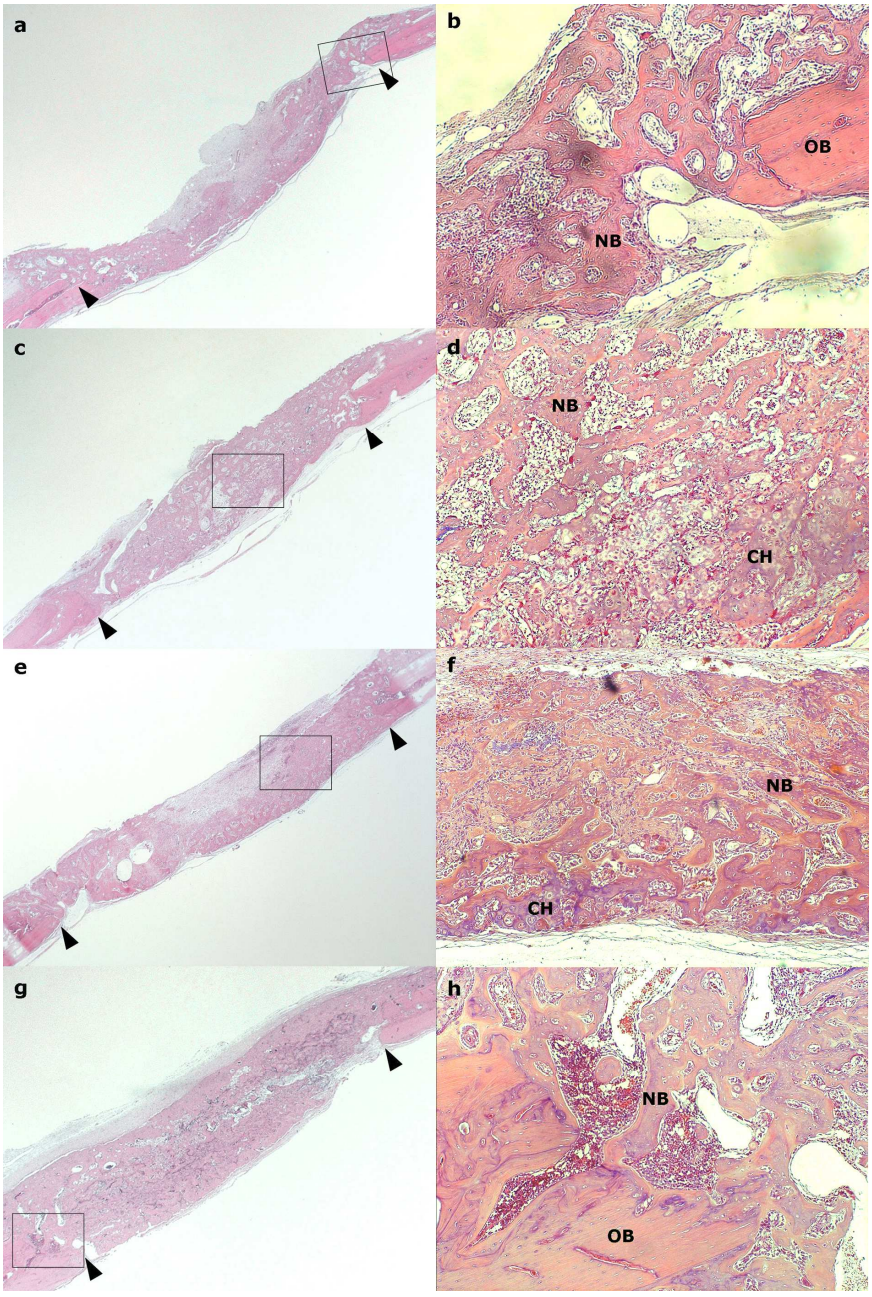

Figure 3

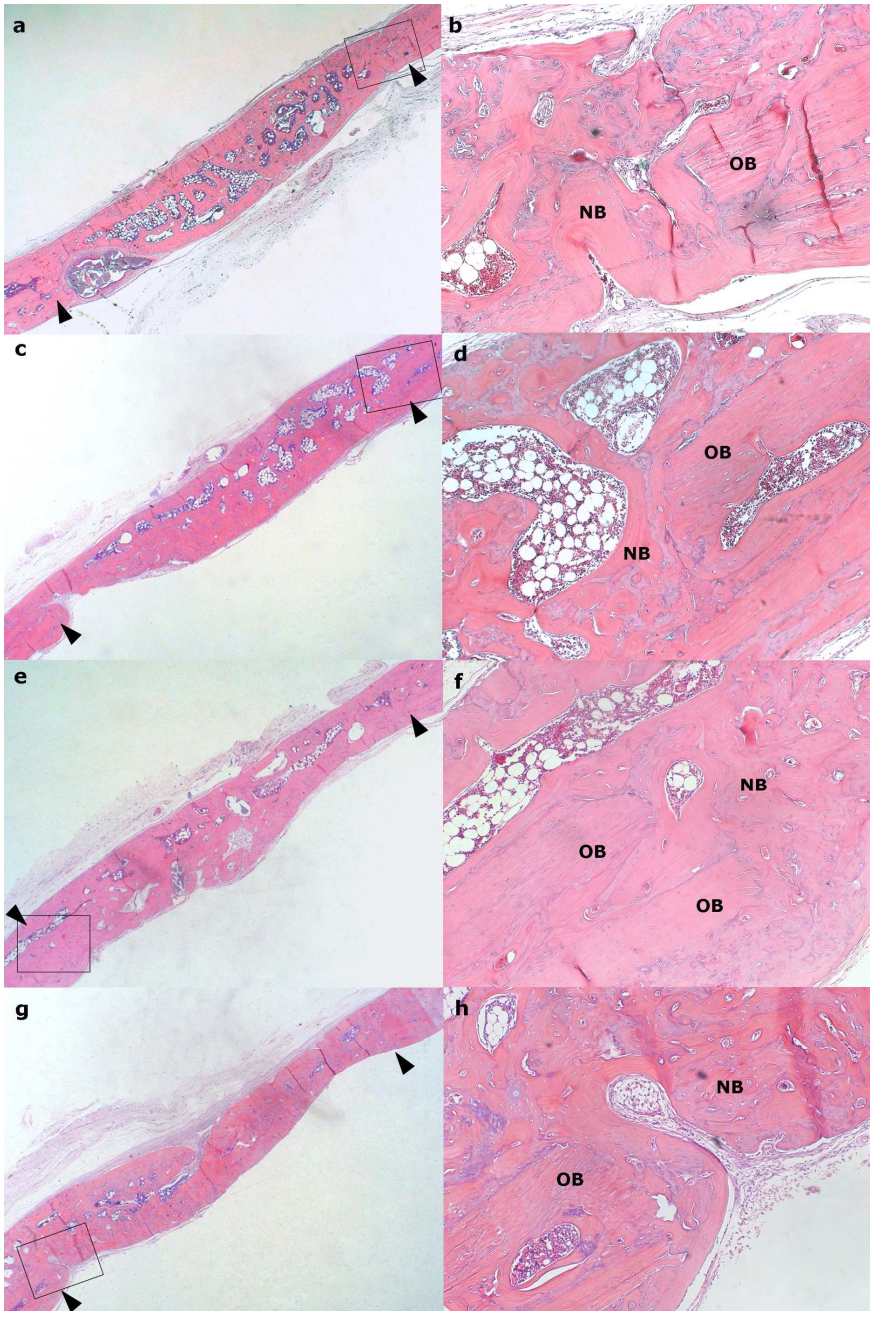

Figure 4

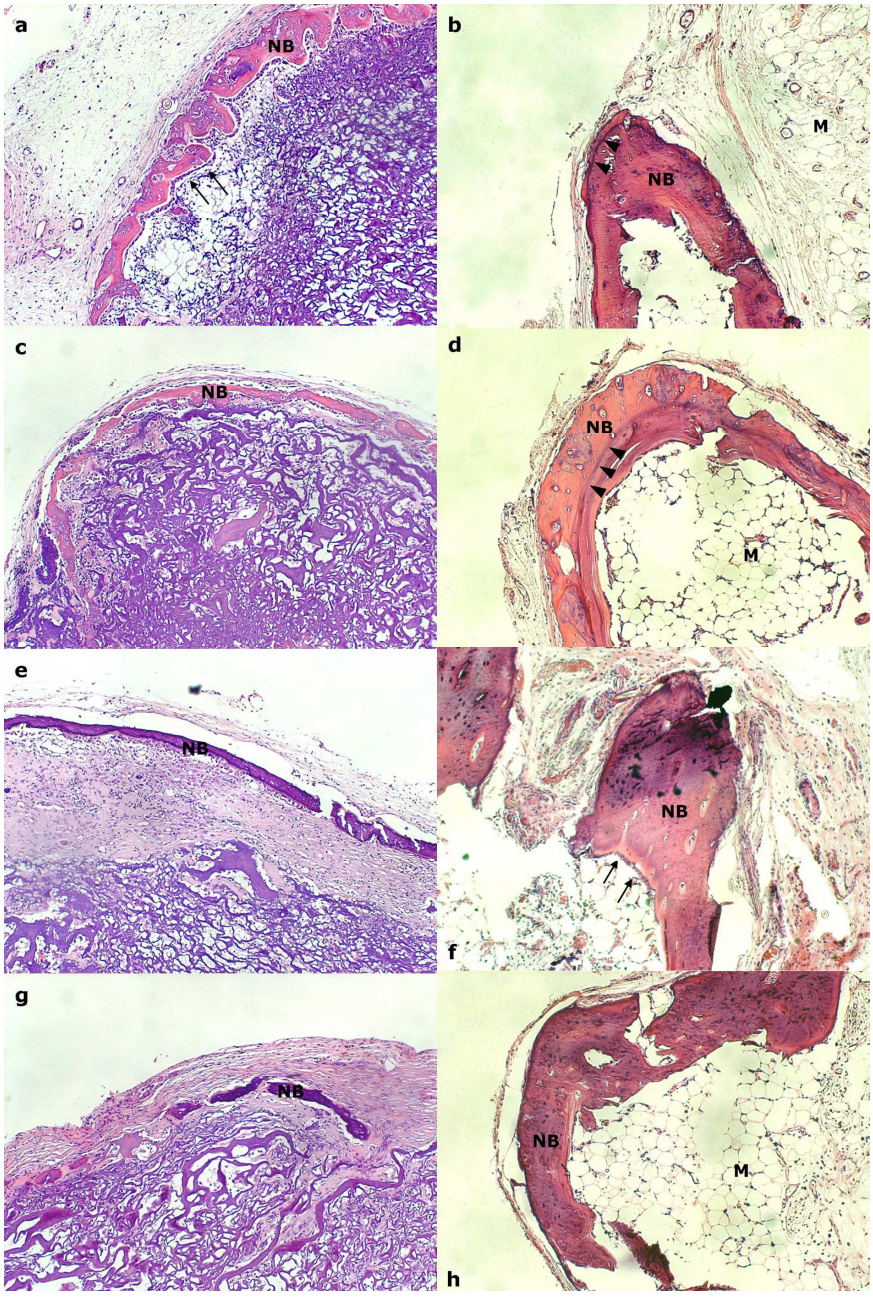

Figure 5

국문요약

백서 두개골 결손부와 피부하조직에서, *E.coli*에서 생산된 recombinant human bone morphogenetic protein-2의 골형성효과

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이 지 현

골형성유도단백질(Bone morphogenetic protein:BMP)는 골재생치료를 위한 효과적인 성장인자단백질로 정형외과 및 치과 영역에서 각광받고 있다. 지금까지 rhBMP 는 동물세포인 Chinese Hamster Ovary cell(CHO cell)에서 recombination technology 를 이용해 생산되어 왔으나, 높은 비용이 임상적 용도로 사용되기에 장애물로 작용하였다. 이를 대체하기 위해 *E.coil* 에서 생산한 rhBMP 에 관한 연구가 1994 년 Zhao 에 의해 처음 보고 되었으며 이 방법으로 생산된 rhBMP 가 기존의 rhBMP 를 대체할 수 있을 것으로 생각하였다. 본 연구에서는 *E.coli* 를 이용하여 rhBMP-2(ErhBMP-2)를 안정적으로 대량 생산하여 이를 백서의 두개골 결손부와 피부하조직에 이식하여 농도에 따른 골형성을 관찰하고, 정량적으로 분석하였다.

총144마리의 Sprague-Dawley rat를 사용하였으며, 6군으로 나누어 실험을

시행하고, 2주 8주의 치유기간을 두었다. 모든 동물에서 두개골 부위 직경 8mm의 골결손부를 형성하고 1) a Sham-surgery control, 2) ACS control, 3) 2.5μg ErhBMP-2/ACS, 4) 5μg ErhBMP-2/ACS, 5) 10μg ErhBMP-2/ACS, or 6) 20μg ErhBMP-2/ACS 을 처치하였다. 실험군 동물의 등에 피부하주머니를 형성해 1) 7.5 μg ErhBMP-2/ACS 2) 15 μg ErhBMP-2/ACS 3) 30 μg ErhBMP-2/ACS 4) 60μg ErhBMP-2/ACS 를 처치하였다. 두개골결손부의 경우, 희생 후 정량분석을 시행하였다.

두개골 결손부의 경우, 대조군에 비해 실험군에서 통계적으로 유의성 있는 신생골 형성이 관찰되었으며, 2주에 비해 8주에서 골량 및 골의 성숙도가 더 높은 것으로 나타났으나, 실험군에서 농도에 따른 차이는 통계적으로 유의차가 없었다. 피부하 결손부의 경우, 2주째에 모든 실험군 동물에서 신생골 형성을 보였으나 8주째에는 농도가 낮은 군에서는 대부분 소실되고 농도가 높은 군에서만 신생골 형성을 확인할 수 있었다.

이 실험을 통하여, *E.coli*에서 생산된 rhBMP-2는 생체내에서 골생성능력을 가지며, 이를 통해 ErhBMP-2가 동물 세포에서 생산된 rhBMP-2를 임상적으로 대체할 수 있으리라 예상할 수 있다

핵심되는 말 : 골형성유도단백질; 골재생; *E.coli* expression system; 백서 두개골결손부; 이소성골형성