

Effect of Porcine Bone Morphogenetic Protein on Healing of Bone Defect in the Rabbit Radius

Nam-Hyun Kim, Kyu-Hyun Yang, Hwan-Mo Lee and Sang-Hwan Oh¹

Segmental long bone defects due to infection or trauma is a difficult problem to manage in patients. We studied the effect of porcine bone morphogenetic protein (pBMP) on healing of defects in the rabbit radius. Porcine BMP was separated and purified from the tibia and femur of pigs by repeated solubilization and precipitation of the protein with different concentrations of urea and CuHCl. The osteoinductive activity of pBMP was confirmed by bioassay using No. 615 mice. In rabbits, about a 15 mm length of radii were removed and 20 mg of pBMP was implanted in the defected area with fibrin sealant (FS), while only FS was implanted in controls. Union of the affected area was observed in 6 weeks in the experimental side. There was no definite evidence of bone bridging across the affected area in the controls. This suggests that pBMP has a bone forming activity in other species and the clinical use of pBMP in treating patients with segmental bone defects is promising.

Key Words: Bone morphogenetic protein, porcine, purification, bone defect, radius, rabbit

Bone tissue is distinguished for its outstanding capacity for regeneration. The success of purification of bone morphogenetic protein (BMP) from bone matrix, which can induce differentiation of undifferentiated mesenchymal cells into cartilage and bone cells, is a great advance in the field of orthopedic surgery, especially in the treatment of non-union and bone defect. After the bone autoinduction phenomenon was discovered by Urist in 1965, numerous reports confirmed the fact that heterotopic implantation or explantation of demineralized bone matrix induced local cartilage and bone differentiation from muscle-derived connective tissue cells (Harakas, 1984). It has long been suggested that factors in bone matrix have an effect of bone inductive capacity. Bone-inductive protein was first isolated by Urist in 1979 and has been

purified from rat, rabbit, bovine, porcine, and human bones (Hanamura et al. 1980; Mizutani and Urist. 1982; Urist et al. 1983; Urist et al. 1983b; Urist et al. 1984; Takahashi et al. 1987; Wu and Hu, 1988; Ko et al. 1990) and also from murine and human osteosarcoma (Takaoka et al. 1980; Bauer and Urist, 1981; Takaoka et al. 1982). The proteins with osteoinductive potential were characterized and partially purified by many authors, and it is clear that proteins with similar inductive ability have the same osteoinductive capacity in other various species.

Segmental long bone defects due to infection or trauma are difficult to manage in patients, because this condition significantly affects their lives for many years. Segmental long bone defects may be resistant to treatment despite the various choices of modern surgical techniques. A hypothesis assumes that BMP induces differentiation of pericytes of periosteum, bone marrow stroma cells, and endosteum into osteoprogenitor cells (Urist et al. 1983a). The authors report on the healing effect of porcine BMP purified by repeated solubilization and precipitation technique (Wu and Hu, 1988; Kim et al. 1991) on defects in the rabbit radius.

Received October 28, 1991

Accepted February 10, 1992

Departments of Orthopaedic Surgery and Biochemistry,
Yonsei University College of Medicine, Seoul, Korea

This study is sponsored by the CMB-YUHAN No. 1989-2.

Address reprint requests to Dr. N H Kim, Department of
Orthopaedic Surgery, Yonsei University College of Medicine,
CPO Box 8044, Seoul, Korea, 120-752

MATERIALS AND METHODS

Separation and purification of pBMP

In one cycle, 5 kg of fresh cortical bone was obtained from the tibias and femurs of pigs within 6 hours after sacrifice. These were stored in a deep freezer (-70°C), and were crushed into pieces using a bone crusher (Korea Crusher Inc, Seoul, Korea), washed with distilled water, and pulverized into 1-2 mm³ particles. The bony particles were defatted in 1:1 chloroform and methanol, demineralized in 0.6N HCl for 48 hours, and washed with NaN₃. The particles were then converted into 580g of insoluble bone matrix gelatine (BMG) after putting the particles in 2M CaCl₂, 0.5M EDTA, and 8M LiCl solutions. Porcine BMP was separated from BMG with 6M urea, 0.5M CaCl₂, and 1 mM phenylmethyl sulphonyl fluoride (PMSF) with 24 hours of periodic stirring. The CaCl₂-urea soluble proteins were desalted and precipitated with 0.5M urea for 24 hours. The precipitate was centrifuged at 12,000 rpm for 40 minutes and lyophilized. The weight of the precipitate was 0.9g and called as P1. It was resolubilized with 6M urea, 0.5M CaCl₂,

and 10 mM ethylmaleimide(NEM) with magnetic stirring and was centrifuged at 3,000 rpm for 20 minutes. The supernatant was dialyzed against 0.5M urea and centrifuged at 12,000 rpm for 40 minutes. The precipitate obtained was purified again by resolubilizing in 200cc of 4M GuHCl, reprecipitated by dialyzing against 0.5M GuHCl for 24 hours, centrifuged at 12,000 rpm for 150 minutes, and finally lyophilized. The precipitate was 39 mg in weight and was labeled as P2. A total 15 cycles were performed to complete this study.

P2 was resolubilized in 6M urea, 0.01M NaOAc, and 3 mM NaN₃ (pH 7.91) and was applied to Sephadex G-150 (Pharmacia, Uppsala, Sweden) column. The column had been equilibrated with the same buffer solution as above and eluted at a flow rate of 9 ml/hr. The column effluent was collected in 5ml fractions and monitored at 280 nm with an ultraviolet spectrophotometer. The fractions of each peak were pooled, desalted by dialysis, and lyophilized. They were labeled as P3 (3.5 mg), P4 (2.0 mg), P5 (12.5 mg), and P6 (9.8 mg)(Fig. 1). Of the fractions of P2, only P3 was turned out to have the osteoinductive activity, and the molecular weight of P3 was determined as 19-20 KDa by the analysis with sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

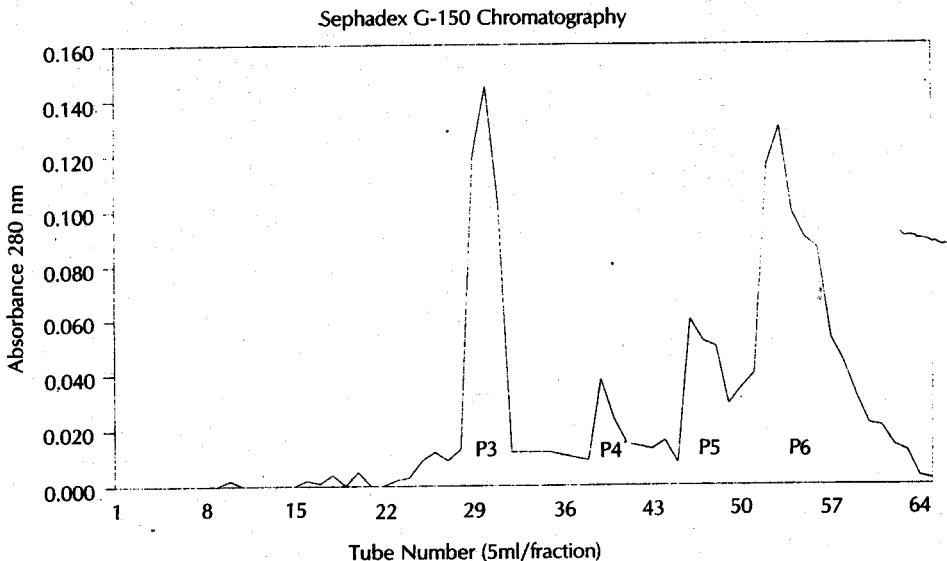


Fig. 1. Absorbance pattern of Sephadex G-150 chromatography showing separation of P2 into four protein fractions (P3, P4, P5, and P6).

Bioassay of bone inducing activity

Seventy five No. 615 strain mice were used. These were divided into seven groups. Six groups were implanted with fractions of proteins (P1-P6) covered with fibrin sealant (Tissel ImmunoAG, Vienna, Austria). The implants were in a muscle pouch in the thigh muscle. The fractions of proteins were used after sterilization with ethylene oxide gas. One group was implanted with bovine serum albumin as a control. Three to four animals were killed from each group at 7, 14, and 21 days after the operation. The specimens were fixed with 10% formalin and imbedded in paraffin then sectioned in $5\ \mu\text{m}$, and studied with microscope after hematoxylin-eosin stain.

Implanting of pBMP in the defect of rabbit radii

Twenty New Zealand white rabbits, weighing from 2.7 kg to 3.5 kg, were used for this experiment. The rabbits were caged under identical conditions with a normal diet (Samyang Co, Seoul, Korea). Kanamycin (0.25 mg/kg) was given postoperatively as an infection prophylaxis. The animals were anesthetized by intravenous injection with nembutal and were operated upon under sterile conditions.

The skin was opened on the radial side of both forelimbs with about a 3 cm incision. After careful dissection, each radial diaphysis was removed with periosteum about 15 mm in length. The pBMP(P2), 20 mg in weight, was implanted in a periosteum-free bone defect in the right radius with fibrin sealant coating. In the left side only fibrin sealant was implanted. No internal fixation or external splinting was used.

Interval roentgenograms were taken at one, three, six, and ten weeks in all surviving animals. The rabbit was positioned supine and the leg was held against the film cassette (magnification was about 4 %) Each animal was held in the same standardized fashion for each film. On the radiograph, the area of the callus was estimated by planimeter on the lateral film, and was expressed for each time interval as a ratio between the area of the defect treated with pBMP and that of control side. The percentage of the defects which had been bridged by callus after each time interval was noted. Five rabbits were killed with overdose of nembutal at each of the four time intervals. Each radius was dissected, inspected grossly, and submitted for histologic sections. histologic sections were prepared from the

adjacent parts of the host and soft tissues. Each specimen was fixed in 10% neutral formalin for at least three days. Decalcification was done with 5% formic acid for two weeks. Specimens were embedded in paraffin, sectioned and stained with hematoxylin-eosin stain.

RESULTS

Bone inducing activity

The weight and bone forming activity of the fractions are listed in Table 1. From the 5 kg of cortical bone, we obtained 580 g of BMG. From this 900 mg of P1 was obtained and only 39 mg of P2 and 3.5 mg of P3 were obtained. P2 and P3 proved to have the bone forming activity and a part of P1 also had bone inducing activity. Bone forming capacities were not detected in the animals with P4, P5, P6, and bovine serum albumin implantation.

Microscopic findings

Histologically, dilatation of capillaries and proliferation of mesenchymal cells were found at the margin of pBMP on the seventh day of implantation. Giant cells and epithelioid cells, indicating foreign body reactions, were not noted. The nuclei of the mesenchymal cells were large and round in shape. After 14 days, chondroblasts and chondroid were observed in the muscle pouch in the P2 group (Fig. 2). New bone tissue with osteocytes was seen in the P3 group. On the 21st day after the operation, mature new bone with prominent lacunae and osteocytes was seen with osteoid (Fig. 3). Similar findings were noted in group P1 except in two animals. No new bone formation was noted in the other groups.

Table 1. Yields of pBMP and its products and bone formation capacity

Product	Weight	Incidence of bone formation
Porcine cortical bone	5 Kg	
Bone matrix gelatine	580 g	
P1	0.9 g	8/10
P2	39 mg	13/13
P3	3.5 mg	9/ 9
P4	2.0 mg	0/10
P5	12.5 mg	0/11
P6	9.8 mg	0/10

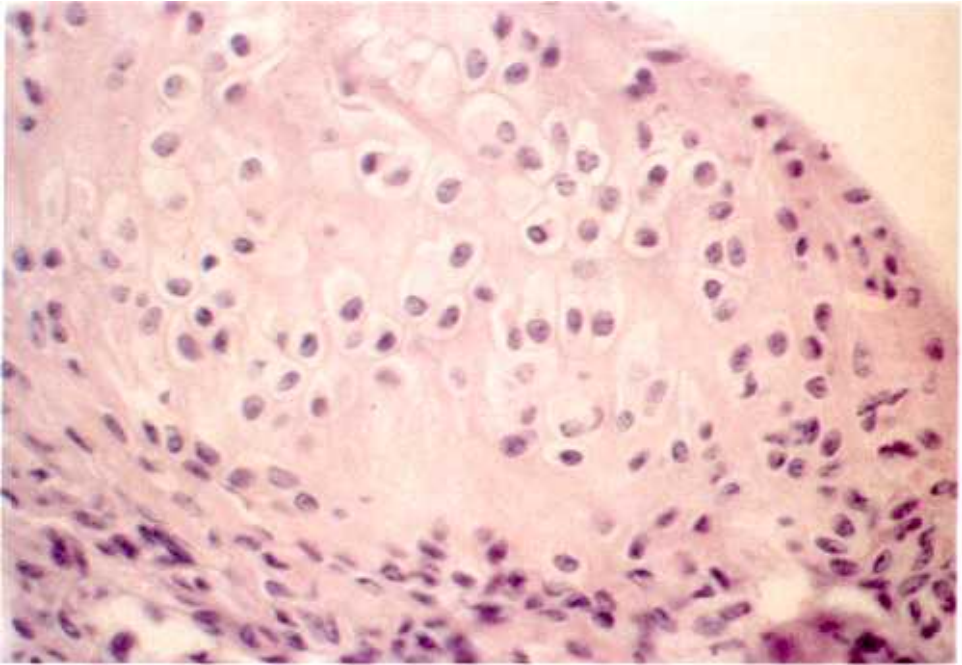


Fig. 2. Photomicrograph showing appearance of chondroid and chondroblast 14 day after implantation of P2 (H-E stain \times 400).

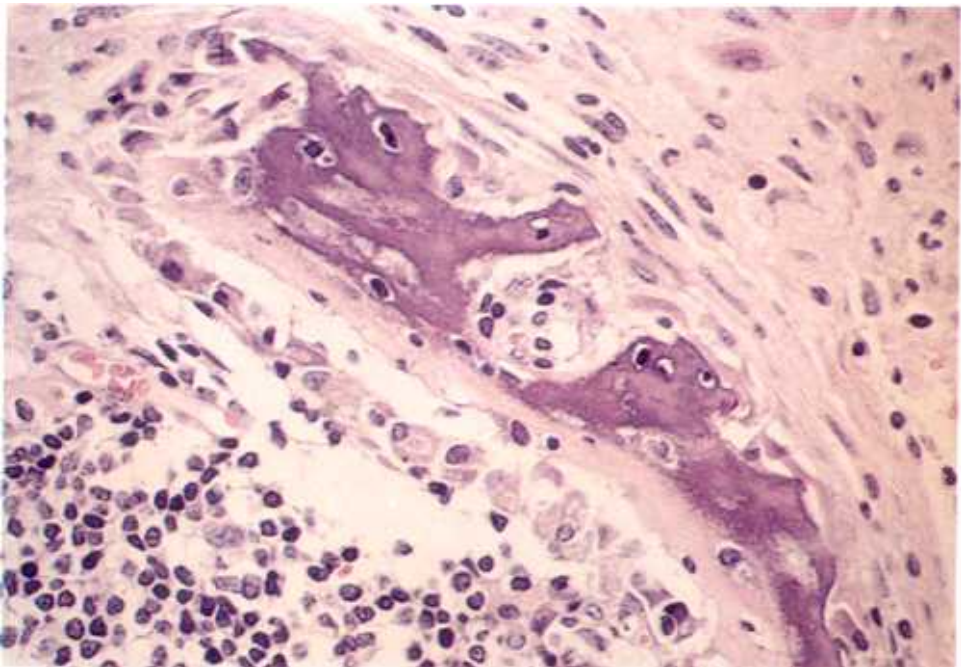


Fig. 3. Photomicrograph of new bone formation with osteoid in muscle pouch with P3 by day 21 (H-E stain \times 400).

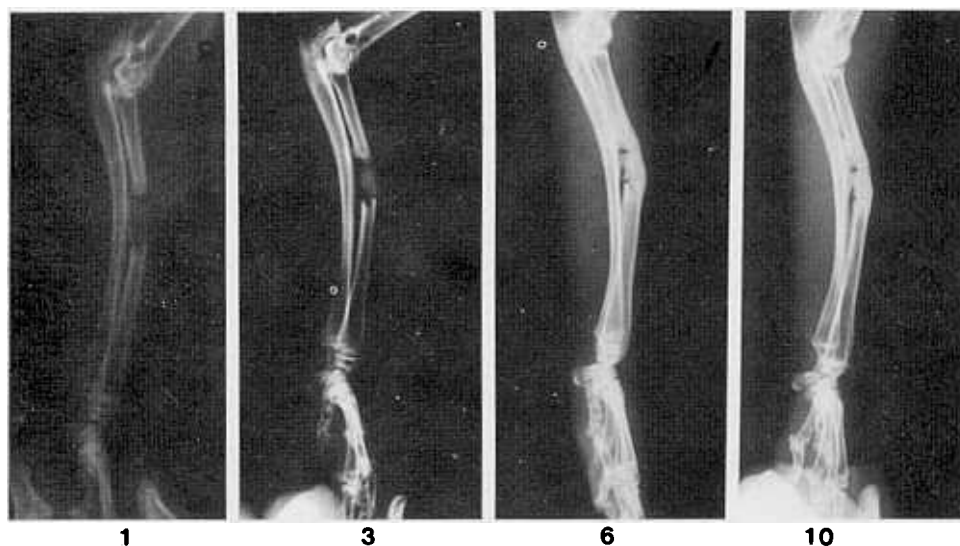


Fig. 4. A typical series of radiography, taken after 1, 3, 6 and 10 weeks, of radial resections implanted with 20 mg of porcine BMP/FS, showing bridging of the defect by mineralized callus.

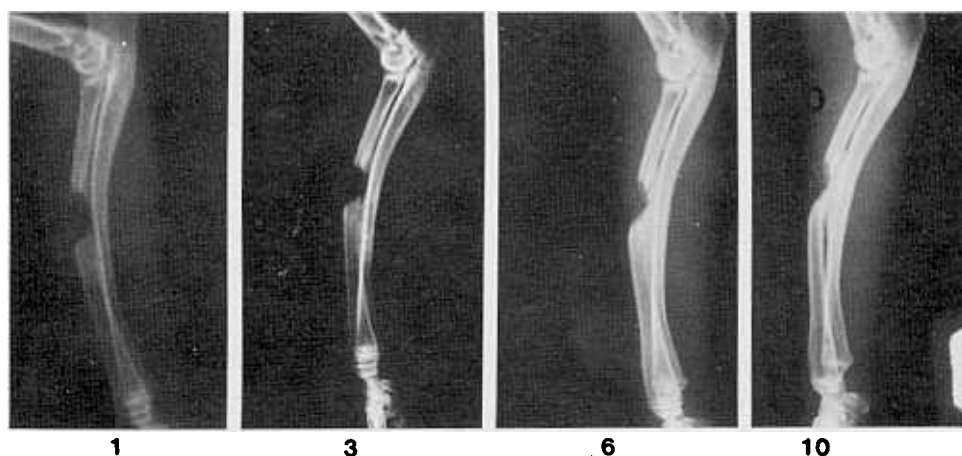


Fig. 5. Radiographs to show a control defect in a rabbit's radius at 1, 3, 6 and 10 weeks after resection and implantation of FS. An incomplete union has developed.

Radiologic Observations

Periodic radiographs revealed the fact that the porcine bone morphogenetic protein/fibrin sealant (pBMP/FS) was superior to control in bone forming

activity. At one week after the operation, there was no definite appearance of callus formation. There was new bone formation within three weeks on the experimental side, but there was no evidence of callus or new bone formation on the control side. The pBMP/FS implant bridged the defect in about

Table 2. Results of morphometry of radius defects

Time since operation (weeks)	N.	Ratio of area of new bone BMP/control	Percent of defect	
			BMP	control
	5	1.1 ± 0.4	3 ± 0.5	2 ± 0.4
3	5	11.3 ± 2.1	56 ± 12	7 ± 2
6	5	3.2 ± 1.9	94 ± 6	43 ± 10
10	5	3.0 ± 1.5	97 ± 9	45 ± 11

Ratio and percentage are given mean \pm standard deviation.

BMP: bone morphogenetic protein



Fig. 6. Gross findings of experimental (A) and control (b) radii. The defect was filled with hard osseous tissue in the experimental side, whereas incomplete filling with fibrous tissue was seen in the control side.

six weeks with a prominent amount of new bone, while a small amount of periosteal new bone formation was observed in the defect of the left radius. Definite evidence of bone union with bridging of the defect was noted at ten weeks. However, the gap was filled with a small amount of new bone and the remaining portion was a fibrous union in the control (Fig. 4, Fig. 5). In general, healing was rapid in the defects treated with pBMP/FS, bridging by a fusiform callus being completed in 6 weeks. There was a significant statistical difference be-

tween the two groups in the healing of the segmental defect of the radii ($p < 0.05$) (Table 2).

Macroscopic observations

In most animals, the soft tissues around the wounds were hyperemic until three weeks after the operation. In later stages there was no abnormality of the tissues.

There were no definite differences between two groups in early stages. There was some soft tissue

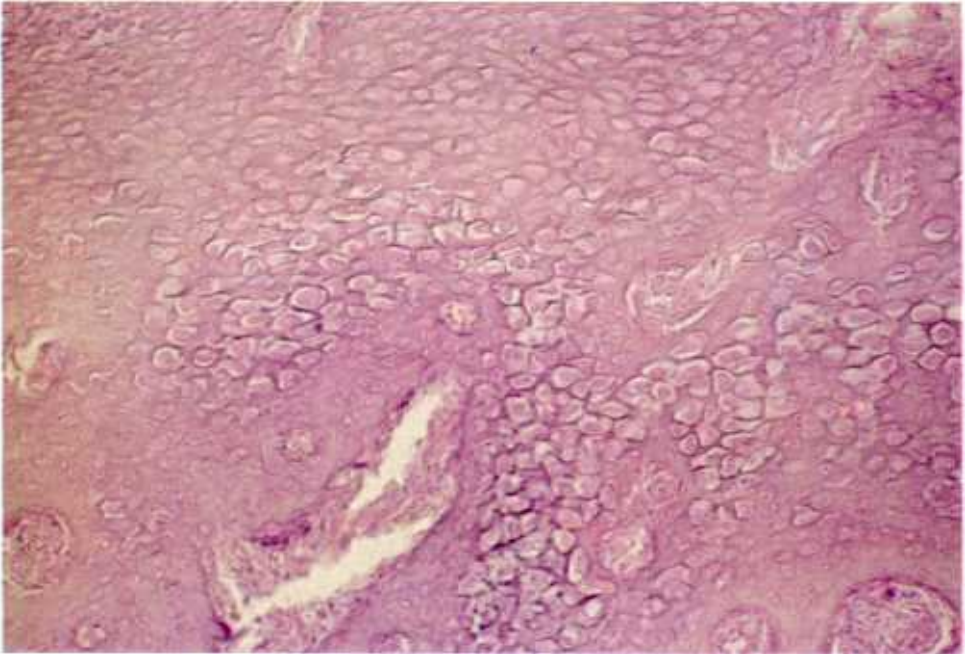


Fig. 7. Photomicrograph taken at one week after implantation of BMP/FS in the defect of the rabbit radius. Numerous chondrocytes and chondroid tissue were noted in the center of the mesenchymal cells (H-E stain $\times 200$).

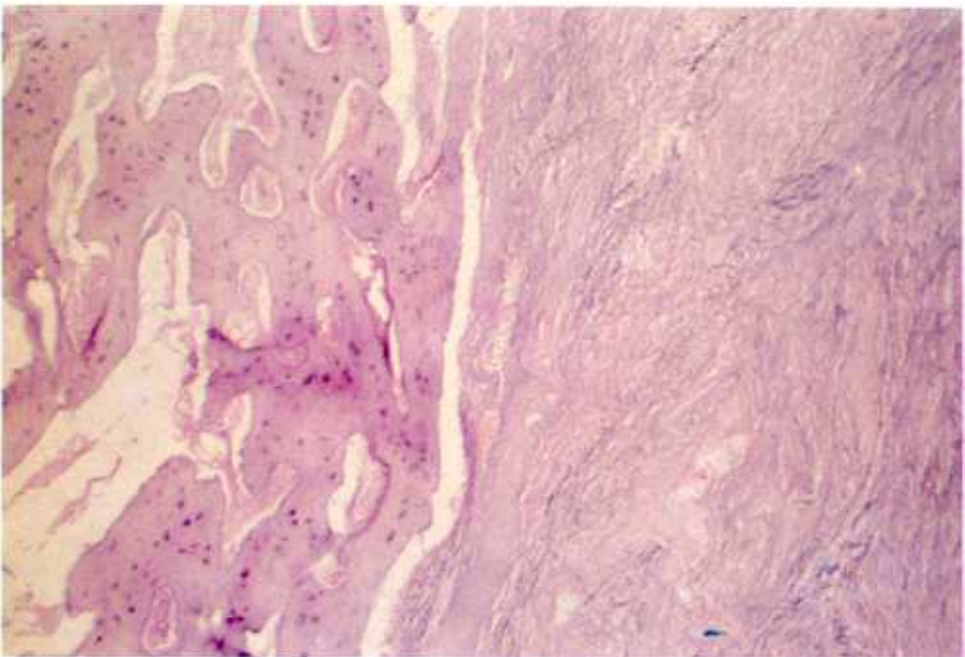


Fig. 8. Six weeks after implantation of BMP/FS. Note the reconstruction of lamellar bone on the periphery of the defect in the radius (H-E stain $\times 200$).

swelling in the implanted site. But, at six weeks, definite evidence of bone formation and bridging between the proximal and the distal fragments were noted in the experimental side, whereas only fibrous tissue was found in the defect of the control side. The ten week specimen demonstrated that the gap was filled with glistening osseous tissue that was hard in consistency in the right side. However, some osseous tissue combined with fibrous tissue incompletely filled the defects of the left side (Fig. 6).

Microscopic findings of bone defect in the rabbit radius

At one week after the operation, there was definite evidence of chondrocytes in the center of defect with some proliferation of chondroblast and chondroid in the experimental side (Fig. 7), but in the left radius, only hemorrhage and fibroblasts were found. The most marked differences between the two groups were noted at three weeks. In the experimental side, the defect was filled with a central spherical mass of lacy trabecular bone and interstitial mesenchymal tissue, surrounded by a shell of densely woven cancellous bone. In the control side, there was no evidence of chondroid formation or chondrocytes. At six weeks after the operation, the woven bone was being remodelled and replaced by lamellar bone and definite evidence of osteoid and cancellous bone formation were found (Fig. 8). On the contrary, only chondrocyte and chondroid tissue were noted in a small part of the defect in the control side. Finally, the defect was filled with new lamellar bone with few chondrocytes and a small amount of chondroid in the right side. The continuity of the cortex had been restored by new lamellar bone in BMP/FS implanted site, whereas the defect in the control side at that stage showed relatively small bands of woven bone and little cartilage in it.

DISCUSSION

The potential for BMP is its capacity to initiate the process of bone induction from the onset of development. Hypothetically, BMP may induce bone marrow stroma cells and perivascular connective tissue cells from the host to grow across the defect and thereby supplement the ingrowth of previously differentiated osteoprogenitor cells (Sato and Urist, 1985). Many kinds of purification techniques have been reported from many institutes. using collagen-

ase (Urist *et al.* 1979), hydroxyapatite chromatography (Urist *et al.* 1984), equilibrium density gradient centrifugation (Takahashi *et al.* 1987), repetition of solubilization and precipitation with different concentrations of urea and guanidine hydrochloride (GuHCl), and gene encoding (Wozney *et al.* 1988). We used different concentrations of urea and GuHCl for repeated solubilization and precipitation (Wu and Hu, 1988) for partial purification of porcine BMP. Because pigs are large vertebrates that are slaughtered for dietary purposes, their bones are the most suitable source to supply large quantities of BMP in Korea,

In the previous report (Kim *et al.* 1991), the authors confirmed that the molecular weight of pBMP was about 19,000-20,000 and it caused the recruitment and proliferation of mesenchymal cells at one week. After the implantation of pBMP in the mouse muscle pouch, the chondroid tissue appeared at the margin of BMP in second week and new bone formation was observed in the third week.

In this study, we observed the healing capacity of pBMP in a large diaphyseal defect of long bone. This model is similar to a defect type nonunion frequently encountered in orthopedic traumatology. In this kind of experimental model two aspects had to be considered. First there was heterogeneity of the source of BMP in the experimental animal, BMP is a noncollagenous glycoprotein located in the bone matrix. Sampath and Reddi (1983) studied the bone forming ability of extracellular bone matrix and partially purified BMP from the human, monkey, bovine and rat. Heterogeneous bone matrix implantation failed to form new bone in rat muscle pouches. Only allogeneic implantation showed evidence of new bone formation. This signifies species specificity of the bone matrix protein, chiefly collagen fibers. In contrast, heterogenic implantation of the partially purified BMP showed new bone formation in all species. This implies that, there was homology in the bone inductive proteins from human, monkey, bovine and rat extracellular bone matrices. In this study, we used pBMP for the evaluation of healing ability in the rabbit. Due to their homology, new bone formation was uniformly observed in rabbits without a severe inflammatory reaction.

The second was the carrier of BMP. A critical local concentration of BMP for a certain period of time was necessary to induce new bone formation. Sato and Urist (1984) reported optimum exposure time (5 hours) and concentration of BMP (10 μ g/40 mg) in their tissue culture model. For an *in vivo*

study, some kind of carrier must be used, because water solubility of BMP is increased after high purification. Gelatin capsule (Ferguson et al. 1987), insoluble noncollagenous protein (Kawamura and Urist, 1988b), polyacetic polyglycolic acid polymer (Johnson, et al. 1988a, 1988b), collagenase or pepsin treated collagens (Yasui et al. 1980), and fibrin glue (Kawamura and Urist, 1988a) were used as a carrier of BMP. Kawamura and Urist (1988a) reported high yield of new bone after implantation of BMP and human fibrin composite into the mouse muscle pouch. Osteogenic activity of the fibrin sealant is still controversial. However, its role in wound healing is well defined in many tissues. In this study, we used human fibrin sealant as a carrier, and observed uniform new bone formation in the defects.

Recalcitrant nonunion of long bone fracture is a challenging problem (Johnson et al. 1988a, 1988b). For the treatment of a large defect in the long bone, various kinds of electric stimulation are not indicated and bone grafts (autogeneic, allogeneic) and bone substitutes also have high failure rates. Until now, the autogeneic bone graft seems to be the treatment of choice. However, its high failure rate of about 30% motivates many researchers to search for better treatment modalities. Johnson et al. (1988b) performed human BMP(allogeneic) and autogeneic cancellous grafts for the treatment of large segmental defects of the tibia. They reported no adverse effects and an average union time of 5.7 months. Nilsson et al. (1986) reported excellent bone repair induced by b-BMP in ulnar defects in dogs. The rate of restoration of bone continuity was about the same as in the autograft control side. The bone forming capacity of BMP was improved with autogenic cancellous bone and autogenic bone marrow. It was probably due to a good supply of the osteoprogenitor cell which will eventually differentiate into the osteoblasts.

In the near future, hopefully, nonunion and defects of long bones could be successfully treated by highly purified human BMP or certain fractions of animal BMP which has a great similarity in terms of the peptide sequence to human BMP.

REFERENCES

- Bauer FC, Urist MR: Human osteosarcoma-derived soluble bone morphogenetic protein. *Clin Orthop* 154: 291, 1981
- Ferguson D, Davis WL, Urist MR, Hurt WC, Allen EP: Bovine bone morphogenetic protein fraction induced repair of craniotomy defects in the rhesus monkey. *Chin Orthop* 219: 251, 1987
- Hanamura H, Higuchi Y, Nakagawa M, Itawa H, Urist MR: Solubilization and purification of bone morphogenetic protein from Dunn osteosarcoma. *Clin Orthop* 153: 232, 1980
- Harakas NK: Demineralized bone-matrix-induced osteogenesis. *Clin Orthop* 188: 239, 1984.
- Johnson EE, Urist MR, Finerman GAM: Bone morphogenetic protein augmentation grafting of resistant femoral nonunion. A preliminary report. *Clin Orthop* 230: 257, 1988a
- Johnson EE, Urist MR, Finerman GAM: Repair of segmental defects of the tibia with cancellous bone grafts augmented with human bone morphogenetic protein. A preliminary report. *Clin Orthop* 236: 249, 1988b
- Kawamura M, Urist MR: Human fibrin is a physiologic delivery system, for bone morphogenetic protein. *Clin Orthop* 235: 302, 1988a
- Kawamura M, Urist MR: Induction of callus formation by implants of bone morphogenetic protein and associated bone matrix non collagenous proteins. *Clin Orthop* 236: 240, 1988b
- Kim NH, Yang KH, Lee HM, Oh SH: Purification of porcine bone morphogenetic protein. *J Kor Orthop Asso* 26: 232, 1991
- Ko L, Ma G, Gao H: Purification and chemical modification of porcine bone morphogenetic protein. *Clin Orthop* 256: 229, 1990
- Mizutani H, Urist MR: The nature of bone morphogenetic protein fractions derived bovine bone matrix gelatine. *Clin Orthop* 171: 213, 1982.
- Nilsson OS, Urist MR, Dawson EG, Schmalzried TP, Finerman GAM: Bone repair induced by bone morphogenetic protein in ulnar defects in dogs. *J Bone Joint Surg* 28B: 635, 1986
- Sampath TK, Reddi AH: Homology of bone induced proteins from human, monkey, bovine and rat extracellular matrix. *Pro Natl Acad Sci USA* 80: 6591, 1983.
- Sato K, Urist MR: Bone morphogenetic protein induced cartilage development in tissue culture. *Clin Orthop* 184: 180, 1984
- Sato K, Urist MR: Induced regeneration of calvaria by bone morphogenetic in dogs. *Clin Orthop* 197: 301, 1985
- Takahashi S, Iwata H, Hanamura H: Nature of bone morphogenetic protein from decalcified rabbit bone matrix. *J Jpn Orthop Asso* 61: 197, 1987
- Takaoka K, Ono K, Amitani K, Kishimoto R, Nakata Y: Solubilization and concentration of a bone-inducing substance from a murine osteosarcoma. *Clin Orthop* 148: 274, 1980
- Takaoka K, Yoshikawa H, Shimizu N, Ono K, Amitani K,

- Nakata Y: Partial purification of bone-inducing substance from a murine osteosarcoma. *Clin Orthop* 164: 265, 1982
- Urist MR: Bone formation by autoinduction. *Science* 150: 893, 1965
- Urist MR, DeLange R, Finerman GAM: Bone cell differentiation and growth factors: Induced activity of chondro-osteogenetic DNA. *Science* 220: 680, 1983a
- Urist MR, Huo Y, Brownell A, Hohl W, Buyske J, Lietze A, Tempst P, Hunkapillar M, DeLange R: Purification of bovine bone morphogenetic protein by hydroxyapatite chromatography. *Proc Natl Acad Sci USA* 81: 371, 1984
- Urist MR, Mikulski A, Lietze A: Solubilized and insolubilized bone morphogenetic protein. *Proc Natl Acad Sci USA* 76: 1828, 1979
- Urist MR, Sato K, Brownell A, Malinin T, Lietze A, Huo Y, Prolo D, Oklund S, Finerman GAM, DeLange R: Human bone morphogenetic protein. *Proc Soc Exp Biol Med* 173: 194, 1983b
- Wozney JM, Rosen V, Celeste AJ, Mitsock LM, Whitters MJ, Kriz RW, Hewick RM, Wang EA: Novel regulators of bone formation: Molecular clones and activities. *Science* 242: 1528, 1988
- Wu Z, Hu X: Separation and purification of porcine bone morphogenetic protein. *Clin Orthop* 230: 229, 1988
- Yasui N, Ochi T, Takaoka K, Ono K, Nakata Y: Osteogenesis by factors isolated from mouse osteosarcoma cells in combination with collagen. *Biken J* 23: 83, 1980