Repair of Ulnar Segmental Defect by Recombinant Human Bone Morphogenetic Protein-2 in Dogs

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ABSTRACT. The efficacy of recombinant human bone morphogenetic protein-2 (rhBMP-2) combined with poly D, L lactic-co-glycolic acid (PLGA)/gelatin sponge complex (PGS) as a carrier on the repair of segmental long-bone defects was evaluated using an ulnar model in dogs. The defect was 2 cm in length and was fixed with bone plating. After implantation of PGS with or without rhBMP-2, the repair process of the defect was evaluated by serial radiography until 16 postoperative weeks. All defects treated with 160 µg or 640 µg of rhBMP-2/PGS revealed bone union radiographically by 12 postoperative weeks, whereas all defects treated with PGS alone revealed no radiographic evidence of healing throughout the experimental period. In defects treated with 40 µg of rhBMP-2/PGS, new bone appeared partially at the defects but did not accomplish union. Bone mineral contents at the defect sites after harvest at 16 weeks postoperatively were significantly (p<0.05) higher in those treated with 160 µg or 640 µg of rhBMP-2 than in those treated with 40 µg of rhBMP-2 or PGS alone. Histologically, defects radiographically diagnosed as having achieved union showed the appearance of cortical bone and bone marrow cells. These findings suggest the use of rhBMP-2/PGS as a potential bone graft substitute in reconstructive surgery in dogs.

KEY WORDS: canine, rhBMP-2, ulnar defect.

Bone grafting is commonly used in patients with nonunion fracture or large bone loss caused by tumor resection or chronic infection. Although various bone graft techniques have been established [18–20], there are disadvantages to each such as additional surgical invasion and limited bone source in autograft, immunomediated rejection and transmission of infectious agents in allograft or xenografts, and technical difficulty in vascularized grafts. Recently, therefore much interest has been focused on the application of bone morphogenetic protein (BMP) as a bone graft substitute in reconstructive orthopedic surgery [4, 5, 9, 11, 15, 22].

BMP induces differentiation of mesenchymal cells into osteoblasts and/or chondroblasts when implanted in extraskeletal sites in rodents. BMP was first postulated by Urist in 1965 [23], and was subsequently extracted chemically from demineralized bone matrix [17, 24]. Wozney et al. [27] employed recombinant DNA technology to produce the proteins in 1988. To date, more than 12 human BMPs have been successfully produced by recombinant methods [3, 15, 27]. All these recombinant human BMPs (rhBMPs) except rhBMP-1 are classified as members of the transforming growth factor-beta (TGF-β) superfamily [3, 27]. rhBMP-2, -4, -5 and -7 express osteogenic activity in rodents [16], and promising results using rhBMP-2 or rhBMP-7 in bone defect healing have been reported in dogs [4, 22].

BMP requires a carrier for expression of its biological activity in vivo [12, 13]. The carrier can determine the shape and size of induced bone, which is important to the induction of new bone at confined sites such as bone defect or nonunion fracture sites. In previous reports on bone healing by rhBMPs [4, 5, 22], insoluble bone matrix (IBM) was used as a carrier of rhBMP. However, IBM may possibly contain some unknown factors and xenogeneic IBM restricts the clinical application of rhBMP [5, 7]. Poly D, L lactic-co-glycolic acid (PLGA)/gelatin sponge complex (PGS) was developed as a new carrier of rhBMP-2 and this combination induced periodontal regeneration in dogs [8]. In the present study, we evaluated the effect of rhBMP-2/PGS in defect healing using a canine segmental ulnar defect model.

MATERIALS AND METHODS

The study was performed in accordance with the guidelines of the Animal Care Committee of Faculty of Agriculture, The University of Tokyo.

Animals: Nine adult beagles (3 males and 6 females) with a mean age of 2.7 years (range 1.8 to 2.5 years) and a mean weight of 12.9 kg (range 9.2 to 14.8 kg) were used. The results of physical, hemotological and blood chemistry examinations before the experiment were all within normal ranges.
Preparation of implants: rhBMP-2 and PGS were provided by Yamanouchi Pharmaceutical Co., Ltd. (Tokyo, Japan). The size of PGS used in this study was 0.8 × 1 × 2 cm. PGS was soaked in rhBMP-2 solution and kept at room temperature for more than 30 min for incorporation of rhBMP-2. The same solution without rhBMP-2 was used for the carrier control.

Experimental design: A 2-cm segmental defect was made and fixed with a bone plate at the bilateral ulnae in 8 dogs. PGS with or without rhBMP-2 was then implanted into the defect. In a preliminary study using 2 beagles, 2-cm defects without implantation failed to achieve union and this defect was therefore regarded as of sufficient size for the purpose of this experiment. The doses of rhBMP-2 were 0, 40, 160 and 640 µg (0, 25, 100 and 400 µg/cm³ PGS, respectively), with 4 ulnae allotted to each 4 group. After implantation, serial radiography and blood examinations were performed until euthanasia at 16 postoperative weeks. After euthanasia by administration of pentobarbital sodium (100 mg/kg, IV), ulnae were removed and bone mineral content was measured, and histological examination was performed.

Another dog undergoing unilateral implantation (rhBMP-2, 640 µg) was euthanatized at 6 postoperative weeks to examine earlier histological changes.

Operation: Fifteen min after administration of atropine sulfate (0.05 mg/kg, SC), anesthesia was induced with thiopental sodium (16–18 mg/kg IV) and maintained by inhalation of isoflurane and oxygen. The shaft of the ulna was exposed by caudal incision along the ulna, and a seven-hole bone plate was placed on the caudomedial surface of the intact ulna. Two holes at the proximal and distal portions were drilled through the cortices and tapped for insertion of a 2.7-mm cortical screw. The plate was then removed and a 2-cm segmental osteoperiosteal defect was created with an oscillating saw. The ends of the ulna were then stabilized by replacement of the plate. After irrigation with saline to remove free marrow cells, PGS with or without rhBMP-2 was implanted into the defect. The musculature and skin were closed routinely. The contralateral ulna was serially treated by the same surgical procedure.

Before extubation, 1 mg of butorphanol was administered (IM) as an analgesic. All dogs received 20 mg/kg of ampicillin (SC, BID) for 5 days after the operation.

Physical and blood examinations: Physical conditions and gait were recorded after surgery. Blood examination was performed weekly until 8 postoperative weeks and then every 2 weeks until 16 postoperative weeks. Plasma concentrations of blood urea nitrogen (BUN), creatinine, alkaline phosphatase (ALP) and alanine aminotransferase (ALT) were determined by dry chemistry (Dry-Chem, Fuji Medical System, Tokyo, Japan).

Radiography: Radiographic investigation was done at the same postoperative periods as blood examination. The radiographs were interpreted blindly by 2 observers and were graded on a 0- to 5-point scale as shown in Table 1. When the grading differed between the 2 observers, the radiographs in dispute were evaluated by a 3rd observer. After euthanasia at 16 postoperative weeks, all treated bones were harvested. Radiographs were taken after removal of plates, and bone mineral contents at the implanted sites of the harvested bone were measured by dual-energy X-ray absorptiometry (XR-26; Norland Co., Wisconsin, U.S.A).

Histological evaluation: The bones including implanted sites were fixed in 10% formalin. Decalcified sections with 4.5-µm thickness were stained with hematoxylin-eosin and observed by light microscopy. Sections were obtained parallel to the long axis of the bone and extending over the length of the defect.

Statistical analysis: Kruskal-Wallis test was used to evaluate differences among the groups. Parameters evaluated included radiographic grades in each period and bone mineral contents. When significant differences between the groups were observed, the differences were analyzed by Mann-Whitney’s u-test for radiographic grades or with unpaired Student’s t-test for bone mineral content. A p value of less than 0.05 was considered significant.

RESULTS

All the dogs tolerated operation well and radiography taken immediately after operation revealed good plate position.

Physical and blood examinations: All the dogs completed the 16-weeks protocol without any continuous disturbances of the limb. On physical examination, mild lameness was observed during the 1st 2 weeks postoperatively in both the BMP-160 and -640 groups. In contrast, these signs were milder or not observed in the BMP-40 or control group. In limbs with transient lameness, mild swelling was also noted. These signs disappeared by 3 postoperative weeks and no functional disturbance was noted thereafter.

A mild increase in plasma ALP concentration compared to preoperative values was observed after 1 week in all dogs (176.2 ± 33.3 vs. 71 ± 20.0 IU/ml). However, these values decreased to the normal range by 3 postoperative weeks (94.0 ± 18.8). Plasma concentrations of BUN, creatinine and ALT were within normal limits throughout the experimental period. Hematological examinations also...
reveal no remarkable abnormalities.

Radiographic findings: In the BMP-160 and -640 groups, complete healing of defects was confirmed on radiographical investigation. Figures 1A-1D show the healing process of a defect after implantation of 160 µg of rhBMP-2. The earliest change to BMP on radiography was partial appearance of the callus. The amount of the callus was greater overlying the plate than at the defect (Fig. 1B). In addition, this outside callus was larger in BMP-640 group than in BMP-160 group. After 4 weeks, density at the defect increased (Fig. 1C), whereas that overlying the plate gradually decreased thereafter by remodeling (Fig. 1D). Figure 2 shows radiographs of all the treated ulnae after removal of the plates at 16 postoperative weeks, demonstrating union of the defects in both the BMP-160 and -640 groups and nonunion in both the control and BMP-40 groups.

Changes in radiographic grade in each group are shown in Fig. 3. All defects in the BMP-160 and -640 groups were scored as grade 4 (radiographic union) by 12 postoperative weeks, whereas no remarkable changes in scores were observed in the control group throughout the experimental period. In the BMP-40 group, partial callus formation was observed in 3 of 4 defects by 4 weeks and one of the defects was scored as grade 2. However no further increase in score was noted thereafter.

Gross findings: Gross findings of the implanted sites at euthanasia were closely consistent with the radiographic images. In the BMP-160 and -640 groups, the plates were covered with various amounts of firm bone, with the amount covering the plate being greater in the BMP-640 group. At removal of the plate, all defects that had been confirmed to show radiographic union were filled with firm bone, whereas the cut ends in the control group were connected with soft fibrous tissue. In the BMP-40 group, none of the defects were bridged completely, although bone tissue was palpable within the defect.

Bone mineral content: Bone mineral content at the implanted site of ulnae harvested at 16 weeks postoperatively in each group is shown in Fig. 4. The values of bone mineral content in the BMP-160 or -640 group were significantly higher than those in the control or BMP-40 groups. Values in the BMP-160 group were higher than in BMP-640 group, but there was no significant difference between the 2 groups.

Histological findings: Histological bony union was confirmed in all defects that had been regarded as showing radiographic union. Cortical bone was observed at the periphery of the induced bone, and marrow cells were seen in the cavity surrounded by the induced bone tissue. In the section harvested at 6 postoperative weeks, the implanted site was replaced by the immature trabecular bone with a large number of new capillaries, and PGS was completely absorbed (Fig. 5A). Compared to this immature trabecular bone at 6 postoperative weeks, the peripheral bone at 16 postoperative weeks was considerably thicker (Fig. 5B), suggesting conversion from trabecular to cortical bone and the progression of remodeling. All defects in control group were filled with fibrous connective tissue, while in the BMP-40 group, complete bridging of bone was not seen in any of the defects.

DISCUSSION

The results of this study demonstrated that rhBMP-2 combined with PGS could restore segmental long bone defects in dogs. Among graft materials currently available for clinical use, autogeneous cancellous bone is the most effective in stimulating bone healing response. However, autografting requires additional surgical procedures resulting in increased morbidity and operative time [4, 11, 22], and in some cases, the quantity of bone available for harvest is
insufficient. Vascularized autografts need special techniques for microvascular tissue transfer [11, 20]. Although the use of allogeneic or xenogeneic bone grafts can minimize these concerns, the lower osteogenic potential, inferior
Bone defect healing induced by BMP-2

revascularization, and possibility of biocompatibility mismatch or infectious transmission limit the effectiveness of these grafts [4, 11, 20, 22]. The ideal substance for reconstruction of bone defects would be reliable, biocompatible, and easy to obtain and use, and would be able to induce rapid growth of the host bone.

In non-vascularized bone grafts, the process of graft revascularization and replacement by new host bone is quite slow [14, 20]. Necrotic allograft bone still remains even after 45.5 months [19]. During the slow process of incorporation, complications such as graft fracture, nonunion, and infection can occur [19, 20]. In the present study, PGS was completely resorbed and replaced by new immature bone tissues by at least 6 postoperative weeks, and sections at 16 postoperative weeks indicated the progression of remodeling. This earlier replacement by host bone may be of great advantage in the application of rhBMP-2 when compared to bone grafts.

Since osteoinduction by demineralized bone matrix (DBM) was first reported by Urist in 1965, the potency of DBM or extracted BMPs as a bone graft substitute have been well investigated in rodents [17, 21, 24]. However, the effect of DBM in non-rodent mammals is uncertain, with failure of osteoinduction using DBM having been reported in both dogs [18] and monkeys [1]. Griffon et al. [6] reported that bone-inducing agent derived from human osteosarcoma cells, which contained BMPs and other osteoinductive factors, induced ectopic bone in mice, but not in dogs even in intraskeletal sites, suggesting that dogs have much lower sensitivity to BMP than rodents. Partially purified BMPs extracted from DBM have been reported to be useful for the repair of canine nonunion [7] or segmental defect models [14]. In this study, no ulnar defects treated with 40 µg of rhBMP-2 reached union, whereas only 0.93 µg of rhBMP-2 was reported to induce a 70% union rate in femoral defects in rats [12]. These results indicate that a greater amount of BMP may be needed for osteoinduction in dogs than in rodents, and that the amount of BMPs in DBM could be insufficient for osteoinduction in dogs.

The major limitation to the clinical use of partially purified BMPs extracted from DBM is the considerably small quantity of BMPs obtained from a large amount of whole bone. In addition, partially purified BMPs may be contaminated with several osteoinductive factors other than BMPs or with other unknown factors. rhBMPs are free of other growth factors and can be produced in unlimited quantity, and species-specificity of BMP-2 is thought to present less of a problem in clinical use [2, 5, 9, 22, 25, 28]. Heckman and Boyan [7] reported that canine BMPs combined with bovine DBM carrier were also ineffective, suggesting that purity of the carrier is also an important factor for osteoinduction. PGS in this study is a synthetic material with less immunogenicity and is thought to be suitable for clinical use.

Several reports have appeared on the effects of rhBMPs on the repair of canine segmental defects. Cook et al. [4] used a canine ulnar defect model to examine the effects of rhBMP-7 (osteogenic protein-1) with bovine IBM as a
carrier and reported that 625, 1,200, and 2,500 µg of rhBMP-7 induced healing of a 2.5-cm defect. The radiographic healing process induced by 1,200 µg of rhBMP-7 in that study was closely similar to that induced by 160 µg of rhBMP-2 in the present study. Toriumi et al. [22] reported that 250 µg of rhBMP-2 with canine IBM could induce the repair of a 3-cm mandibular defect in dogs. Although the size and site of the defect differed among these studies, the dose of rhBMP-2 needed for defect healing was lower than that of rhBMP-7, indicating that rhBMP-2 has more osteoinductive potency than rhBMP-7 in dogs, as in rodents [26].

In the present study, a transient increase in plasma ALP concentration was detected in all dogs. Since there were no sham-operated dogs, it could not be determined whether the increased ALP value was due to the implanted rhBMP-2 or to the surgical invasion of ostectomy. However, the levels of ALP decreased by 3 weeks, and are thought to be of no

Fig. 5. (A) Photomicrographs of the implanted sites at 6 weeks after implantation of 640 µg of rhBMP. PGS was completely resorbed and replaced by newly induced trabecular bone. (B) Photomicrographs of the implanted sites 16 weeks after implantation of 640 µg of rhBMP. The bone tissue at the periphery is thicker compared to that at 6 weeks after implantation. CE; Cut end of ulna.
clinical significance. The transient clinical symptoms observed during the first 2 weeks after surgery were apparently elicited by the implanted rhBMP-2. This period corresponds to the phase from mesenchymal cell proliferation to subsequent differentiation into osteogenic cells [17, 25, 28]. These marked responses of host tissues might be considered as clinical signs resembling inflammatory responses. No other functional disturbances were noted, however, the higher dose of rhBMP-2 seemed to induce a larger amount of bone in tissues surrounding the defects. Ossification of adjacent tissue has the potential to disturb vital structures such as nerves or muscles [10]. Since it may be difficult to completely avoid extraskeletal bone formation, the choice of the optimal dosage of rhBMP-2 is important to minimize undesirable effects.

The present results indicated that the lowest dose of rhBMP-2 needed for defect healing was between 40 and 160 µg (25 and 100 µg/cm³ PGS, respectively). Bone mineral content measurement showed no significant difference between 160 and 640 µg of rhBMP-2, suggesting a ceiling effect on higher doses of rhBMP-2. A larger amount of plate-covering bone in the 640-µg group, removed before the measurement, might be associated with a smaller amount of bone mineral content. However, the optimal dose of rhBMP-2 in dogs still cannot be decided, because the number of bones examined was insufficient in this study and the biomechanical properties of the treated ulna were not tested. Furthermore, in clinical use, soft tissues surrounding the defects might be deficient or atrophic with trauma, inflammation, and sometimes infection, which will markedly reduce their osteoinductive potency [10]. Further studies will be needed to determine the optimal dose of rhBMP-2 in various type of patients.

In conclusion, rhBMP-2/PGS has potent osteoinductive activity in dogs and may be useful as a bone graft substitute for orthopedic reconstruction. Further studies will be needed to clarify the mechanical property of the induced bone and to select the optimal dose of rhBMP-2.

REFERENCES


