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Bone regeneration using recombinant human bone morphogenetic protein-2 (rhBMP-2) in alveolar defects of primate mandibles

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SUMMARY. **The efficacy of bone morphogenetic protein (BMP) for bone reconstruction has been widely studied in numerous animal experiments, but insufficient information exists about its ability to regenerate bone in primates. The purpose of this study was to evaluate the effects of recombinant human BMP-2 (rhBMP-2) on bone formation in alveolar bone defects in the mandibles of young primates. Marginal bone defects were created in the mandibles of nine 5-year-old rhesus monkeys and rhBMP-2 permeated in a polylactic-co-glycolic acid-coated gelatin sponge (PGS) was implanted into the bone defects. The resected bone treated with rhBMP-2 regenerated completely at 12 weeks postoperatively, and remodelling and consolidation of new bone were seen histologically. This study provides evidence of considerable bone regeneration in alveolar defects after surgical implantation of rhBMP-2 in non-human primates. This technique may be an effective alternative to autogenous bone grafts for reconstructive surgery in clinical practice. © 2001 The British Association of Oral and Maxillofacial Surgeons**

INTRODUCTION

Reconstruction of defects in the alveolar ridge caused by severe periodontal disease, loss of teeth, injury, or resection of tumour is necessary for dental restoration, but remains problematic. The supply of enough bone is essential for the successful rehabilitation of oral function using dental implants or dentures. A number of materials have been used to restore alveolar ridge defects, although autogenous bone is currently the preferred material. However, the limited availability of donor tissue as well as morbidity at the donor site are the constraining factors associated with this type of graft. Consequently, different material is highly desirable.

Bone morphogenetic protein (BMP) was first discovered by Urist in 1965.[1] Wozney *et al*.[2] subsequently succeeded in cloning a cDNA of BMP that enabled mass production of recombinant BMP. BMP is the most promising osteoinductive substance and is expected to be applied clinically for bone reconstruction. rhBMP-2 has restored critical-size bone defects in numerous animal experiments.[3–8] We have successfully repaired an alveolar bone defect in a canine mandible by the implantation of rhBMP-2, but the demonstration of bone formation by rhBMP-2 in primates is a prerequisite for its clinical application. The use of rhBMP-2 to repair mandibular bone defects in old Japanese monkeys produced inconclusive results, although the combination of rhBMP-2 and bone marrow grafts was successful.[9] BMP alone may induce insufficient bone formation in higher species, which have long lifespans and slow metabolic rates.[10] Another reason for insufficient BMP-induced bone formation in primates might be a reduced response to BMP due to aging. Fleet *et al*.[11] showed that aging blunted rhBMP-2-induced bone formation in rats, possibly in part because of a decrease in the number of mesenchymal stem cells in older rats or a change in the response of these target cells to rhBMP-2. We therefore attempted to examine the effectiveness of rhBMP-2 for bone reconstruction in young primates using an alveolar bone defect model that resembles the clinical condition and involves a simple and reproducible surgical procedure.

MATERIALS AND METHODS

Animals

Nine healthy 5-year-old male rhesus monkeys (*Macaca mulatta*), weighing about 5 kg, were used in this study. Surgical techniques and animal care conformed to *The principles of laboratory animal care* (NIH publication No. 85-23, revised in 1985) and to *Guiding principles for the care and use of animals in the field of physiological*

Sciences (The Physiological Society of Japan 1988). The Animal Care Committee of Tokyo Medical and Dental University approved the protocol of the study.

Preparation of implant materials

Both rhBMP-2 (produced by Genetic Institute Cambridge, MA, USA) and polylactic-co-glycolic acid (PLGA) coated gelatin sponge (PGS) were provided by Yamanouchi Pharmaceutical Co., Ltd (Tokyo, Japan). PGS was used as the carrier of rhBMP-2 and has the following properties: 30000 MW; a 1 : 1 molar ratio of lactic to glycolic acid; a 4 : 1 weight ratio of PLGA to gelatin, and 90% porosity. First, 2 mg of rhBMP-2 was dissolved in 1 ml of 5 mmol sodium glutamate, 2.5% glycine, 0.5% sucrose, and 0.01% Tween 80, pH 6.5. Subsequently, 1.4 ml of the rhBMP-2 solution (2 mg rhBMP-2/ml, total 2.8 mg rhBMP-2) was injected into the PGS and kept at room temperature for 30 min before implantation to allow for absorption. The same solution without rhBMP-2 was used for the control.

Surgical procedure

All premolar and molar teeth in the primates' mandibles had been extracted three months before the implantation of rhBMP-2 and the extraction sockets had healed completely. General anaesthesia was induced by an intramuscular injection ketamine hydrochloride of 20 mg/kg and maintained by isoflurane. The mandible was exposed by an intraoral approach through a full thickness flap including the periosteum. A 20-mm wide and 15-mm high portion of the mandible was resected in the region of the molar using a surgical saw (Fig. 1A). A PGS block with or without rhBMP-2 was implanted into the bone defect (Fig. 1B), or the defect was left empty. The sponge-like form of the blocks allowed for them to be fitted closely and positioned securely in the defects. We repositioned the mucoperiosteal flap and closed using 4 0 nylon sutures. Cefotetan 0.5 g was given for one week postoperatively. Butorphanol tartrate 0.2 mg/kg was injected intramuscularly as an analgesic throughout the observation period. After operation, the monkeys were fed a soft diet to maintain their nutritional status.

Experimental design

The mandibles were resected on the left side only in 7 of the 9 animals and a PGS block containing rhBMP-2 was implanted into the bone defect. In the other two animals, bilateral mandibular resections were performed and a PGS block with rhBMP-2 was implanted on the left side and PGS without rhBMP-2 was implanted or the defect left empty on the right side. We harvested 4 experimental specimens 6 weeks after operation, and 2 control and 5 experimental specimens 12 weeks after operation. The experimental design is summarized in Table 1.

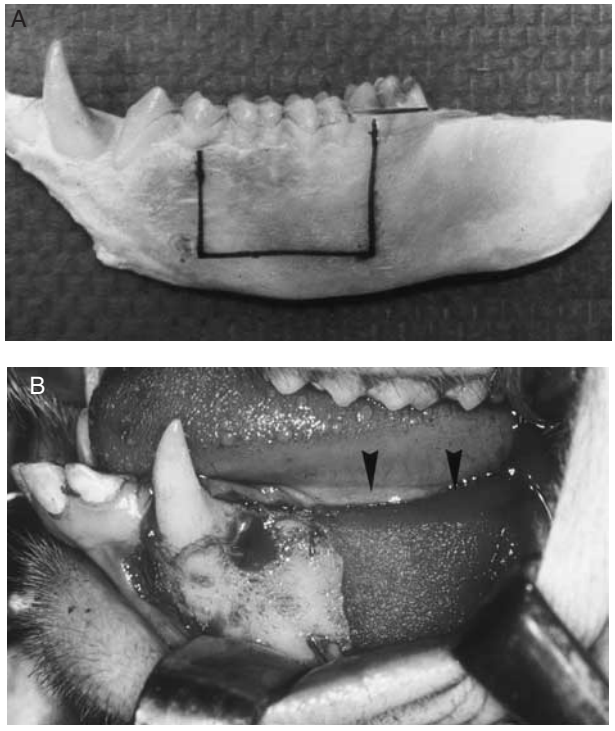

Fig. 1 Resected areas of mandible (A). A 20-mm wide and 15-mm high section of mandible was resected using a surgical drill. A rhBMP-2/PGS block (indicated with arrows) was implanted into the artificial bone defects (B).

Table 1 Number of implants harvested at 6 or 12 weeks

	6 weeks	12 weeks
Control no implant	0	1
PGS alone	0	1
rhBMP-2/PGS	4	5

Time course analysis of bone formation

Intraoral radiographs of the surgical sites were taken to document progressive bone formation at 1, 2, 4, 6, 8, 10, and 12 weeks after operation, using a Venus Dental X-ray Unit (Yoshida Seiko, Japan) operating at 60 kVp and 10 mA with an exposure time of 0.1 s. We used ultraspeed dental film (Occlusal, Kodak, Eastman Kodak, Rochester, NY, USA).

Tissue harvest and radiological analysis

Anaesthetized animals were killed at 6 and 12 weeks after operation, 4 animals at 6 weeks and 5 at 12 weeks. Experimental sites were recovered as a block and immediately placed in 70% ethanol. Radiographs of the harvested

specimens were taken using a DH-158 HM X-ray unit (Hitachi, Japan) operating at 42～45 kVp and 500 mA with an exposure time of 1 min with ultrahigh contrast mammography film (X-OMATL-II, Kodak, Eastman Kodak, Rochester, NY, USA).

Histology

The specimens were fixed in 70% ethanol, dehydrated with a graded series of ethanol and embedded in methyl-methacrylate. The block was sectioned to about 50 μm thick using the cutting-grinding system (EXACT, Hamburg, Germany), and the sections were stained with toluidine blue for light microscopy. The block was also sectioned to about 10 μm thick using a microtome (model 2050, Reichert Jung, Germany) and the sections were stained with Goldner's trichrome to observe new bone formation at high magnification.

Peripheral quantitative computed tomographic (pQCT) analysis

pQCT was made using an XCT-960A (Norland Stratec, Birkenfeld, Germany) to estimate the quality of the newly formed bone. Five images with slices 1.2 mm thick taken at the same intervals were obtained from each specimen. The bone mineral density (BMD, mg/cm^3) and the total area (mm^2) of new bone were measured from pQCT images. The colour spectrum scales indicated the degree of BMD. Calcified tissue with BMD over 213 mg/cm^3 was defined as bone. The bone mineral content (BMC) was calculated from the following measurements:

BMC (mg)
= BMD (mg/cm^3) × total new bone area (cm^3)
× slice thickness (0.12 cm).

RESULTS

Postoperative course

All animals recovered well and survived for the duration of the study. There was slight swelling at all resection sites, with the swelling apparent for longer in the BMP implanted sites than in the control sites, but it subsided within 2 weeks. All the monkeys lost weight, between 7% and 15% of their total body weight, but they regained normal body weight by 5–8 weeks after operation. There was a slight difference in weight loss between the animals that underwent unilateral and bilateral mandibular resections. No other complications were observed.

Macroscopic findings

Macroscopic examination at 6 weeks postoperatively showed that the newly formed bone had not yet developed to the top of the alveolar ridge (Fig. 2A). The consistency was not sufficiently hard 6 weeks postoperatively, but the defect had completely regenerated with rhBMP-2-induced bone 12 weeks postoperatively (Fig. 2B).

Time course of bone formation

Figure 3 shows serial radiographic observations for the time course of bone formation. Initial radio-opacity was

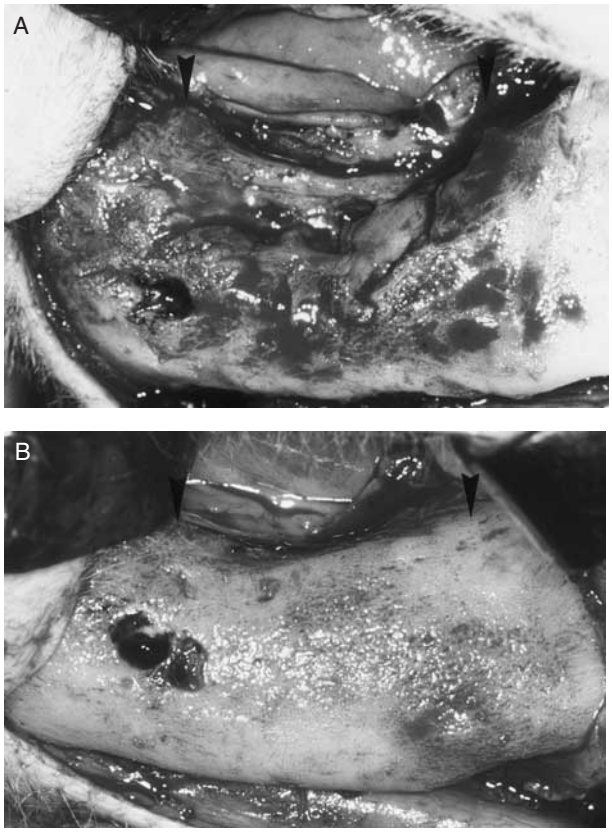

Fig. 2 rhBMP-2 regenerated mandibles. rhBMP-2-induced bone had not developed to the top of the alveolar ridge at 6 weeks postoperatively (A), but had completely filled the resected area at 12 weeks (B). Arrows indicate edges of the defects.

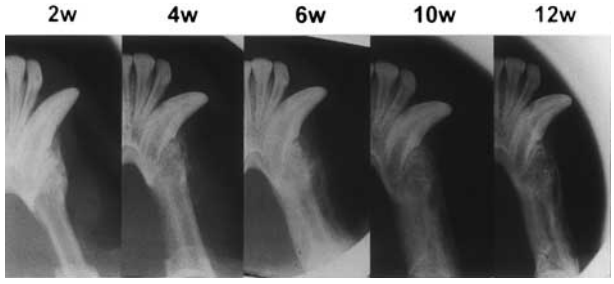

Fig. 3 Time course of bone formation using rhBMP-2. Radiographs taken at 2, 4, 6, 10 and 12 weeks after implantation of rhBMP-2. Initial radiopacity at 4 weeks postoperatively, which increased by 10 weeks. The defects were reconstituted with new bone at 12 weeks.

detected at 4 weeks postoperatively. The radiodensity of the new bone continued to increase over 10 weeks. The new bone was indistinguishable from the host bone on radiographic observation at 12 weeks postoperatively. The newly formed bone had a smooth surface and its configuration was exactly like that of the resected bone. In contrast, almost no radio-opacity was seen in either of the controls during the observation period (data not shown).

Radiological evaluation

The radiograph of the specimen harvested at 6 weeks postoperatively showed a slight radiopaque shadow (Fig. 4A), which had extended to fill the experimental defect at 12 weeks (Fig. 4B). On the other hand, the open defect was of lower density than the experimental defect (Fig. 4C) and PGS alone showed little radiopaque structure (Fig. 4D) even 12 weeks after surgery.

Histological evaluation

Six weeks after rhBMP-2 implantation, the defects were not restored completely and residual PGS was still visible. Although trabeculae of the new bone extended toward the internal regions of the defects, it was still thin and primitive (Fig. 5A). The specimens stained with Goldner's trichrome showed that thick osteoid seams, stained as red bands, covered trabeculae of the new bone. Numerous plump osteoblasts lined the surface of the thick osteoid seams (Fig. 6A). These observations indicated active bone formation. There was no evidence of an inflammatory response to the carrier in any specimens.

Twelve weeks after operation, histological observations confirmed bone regeneration in rhBMP-2-implanted defects, but the extent of bone regeneration varied among individual animals. In three of five animals that had been given a rhBMP-2 graft, the newly formed bone had taken on the configuration of the original unresected mandible, and the regenerated bone was of adequate height and width. In the other two animals, the regenerated bone had insufficient width but sufficient height to reach the top of the alveolar ridge. No residual PGS was found in any of the 5 experimental and 2 control cases. The new bone in the defect was a mixture of woven and mature lamellar bone. At 12 weeks postoperatively, the lamellar structure had developed well in the newly formed bone, and the trabecular bone was thicker than it was at 6 weeks (Fig. 5B). Remodelling and consolidation of the new bone were also seen within the defect. The bone marrow space was filled with marrow elements of normal appearance including numerous fat cells. The osteoids were thinner than those seen at 6 weeks after surgery. Osteoblasts on the bone surface were mainly flat and elongated (Fig. 6B). These observations indicate that there was a decrease in active bone formation. Little new bone formation was seen in the defects of the controls (Fig. 5C&D). Dense fibrous tissue filled the defects and new bone formation was limited to the cut edges of the defect in both controls.

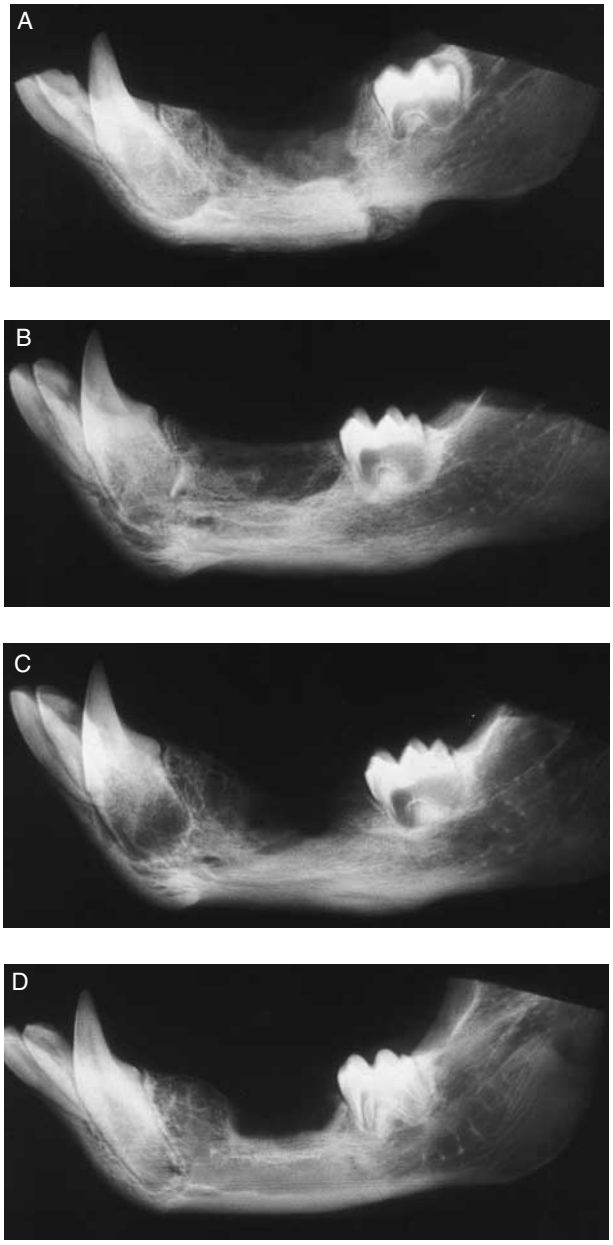

Fig. 4 Radiographs of the harvested specimens. A radiopaque shadow in the bone defects with implantation of rhBMP-2 showed slightly higher density than the controls at 6 weeks postoperatively (A). Entire area of defect was visualized as radiopaque 12 weeks (B). Defects in two kinds of controls, no implantation (C) and PGS alone (without rhBMP-2) (D), showed little bone formation at 12 weeks.

pQCT analysis

Figure 7 shows a representative scanning image by pQCT. The surface of new bone was irregular, and excessive bone formation with low BMD was observed

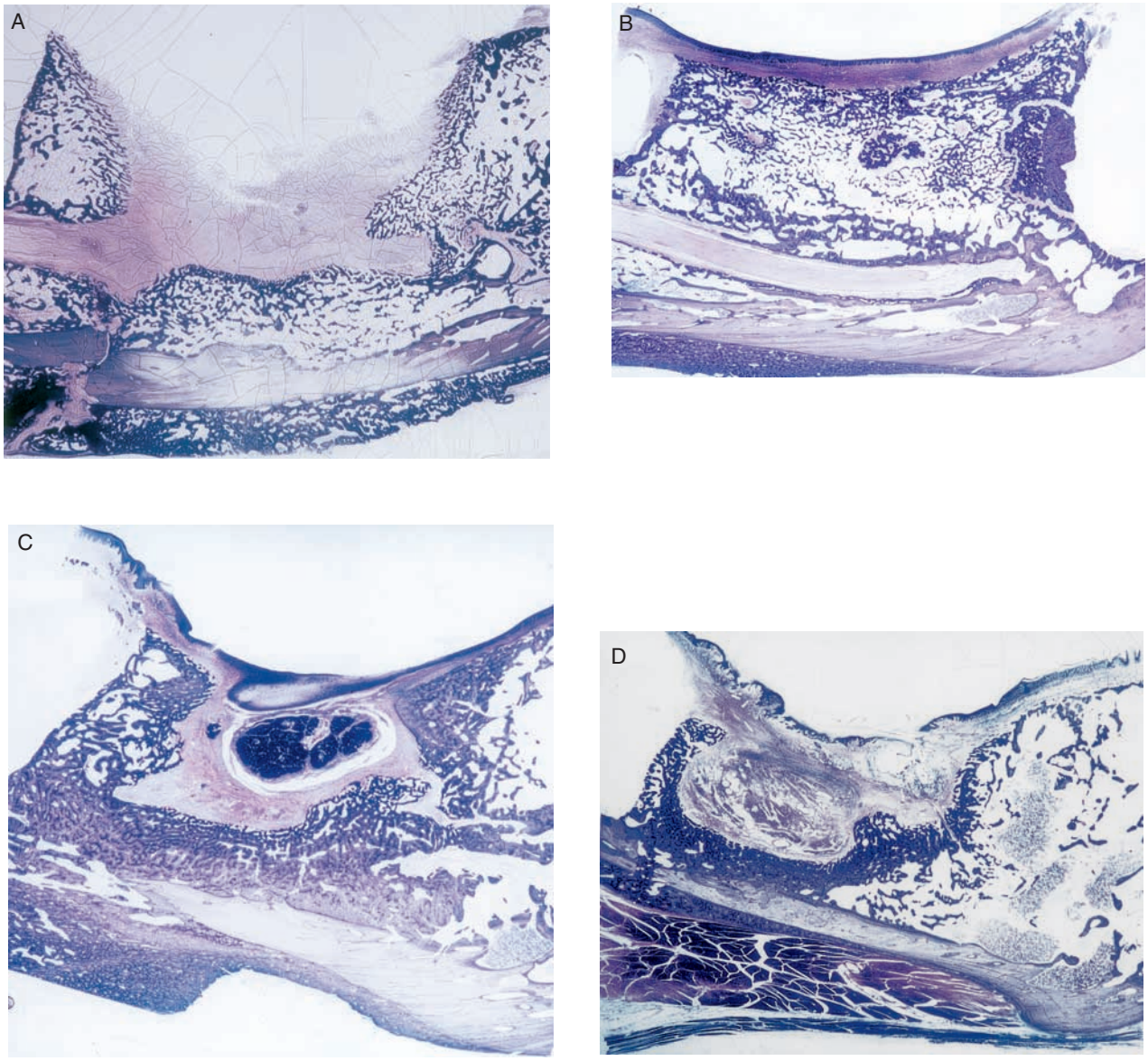

Fig. 5 Photomicrographs of histological sections at low magnification (toluidine blue, original magnification ×1). With implantation of rhBMP-2, defects were not restored completely at 6 weeks, but showed trabeculae of the new bone extending toward the internal regions of the defects (A). Twelve weeks after implantation of rhBMP-2, the newly developed bone had extended to contact with the host bone. Remodelling and consolidation of the new bone within the defect were seen (B). Both the open defect (C) and PGS alone (without rhBMP-2) (D) resulted in limited bone formation at 12 weeks.

on the lateral side of the mandible 6 weeks after implantation of rhBMP-2. The surface of the induced bone became smooth 12 weeks after implantation of rhBMP-2. The BMD of the inner region of the newly formed bone was low, but that of the outer region gradually increased to the level of the host bone. The cut edges of the defects in the two controls were completely healed, and the scanning images of two controls showed high BMD. However, mandibular height in the controls reached only half that in the experimental animals at 12 weeks postoperatively.

The mean total area of new bone (mm^2) and mean BMC (mg) are shown in Table 2. The mean (SD) total area of new bone in the rhBMP-2 implanted sites increased from 20.4 (14.2) mm^2 to 57.0 (17.6) mm^2 between 6 and 12 weeks postoperatively. The mean (SD) of the total area of new bone induced by rhBMP-2 at 12 weeks (57.0 (17.6) mm^2) was 3.5 times larger than that of the controls (16.2 mm^2 PGS alone; 18.2 mm^2 no implant). The results of the mean BMC were similar to those of the mean total area of new bone.

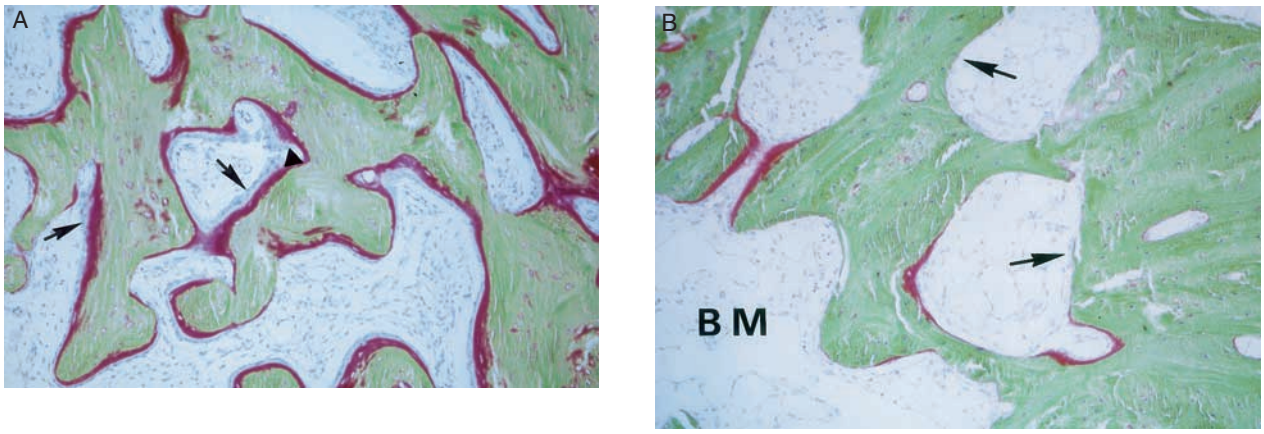

Fig. 6 Photomicrographs of histological sections at high magnification. (Goldner's trichrome, original magnification × 25). Six weeks after rhBMP-2 implantation, active bone formation was detectable (A). Trabeculae of the new bone were covered by thick osteoid seams with red bands (triangle). The surface of thick osteoid seams was lined with numerous plump osteoblasts (arrows). Twelve weeks after implantation, osteoids were thinner than at six weeks (B). Osteoblasts (arrows) on the bone surface were mainly flat and elongated. Bone marrow space (BM) was densely packed with normal-looking marrow elements including numerous fat cells.

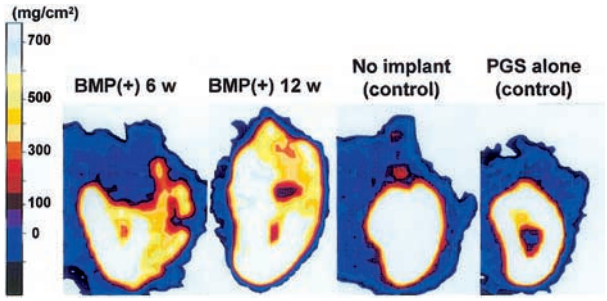

Fig. 7 Cross-sectional scanning images in the central region of the resected mandibles by pQCT. Six weeks after implantation of rhBMP-2, there was excessive lateral bone formation, but new bone had not extended in height to contact the host bone. Twelve weeks after implantation, the surface of the induced bone was smooth and BMD of the outer mandibular region gradually increased to match the BMD of the host bone. Scanned images of both controls – no implantation and PGS alone (without rhBMP-2) – showed minimal bone formation at 12 weeks.

Table 2 Total area and bone mineral content of newly formed bone. Numbers are mean (SD)

	No of observations	Total area (mm^2)	Bone mineral content (mg)
BMP (6 weeks)	4	20.4 (14.2)	1.14 (0.85)
BMP (12 weeks)	5	57.0 (17.6)	3.62 (0.90)
No implant (12 weeks)	1	18.2	1.11
PGS alone (12 weeks)	1	16.2	0.98

DISCUSSION

BMP is reported to have induced successful bone reconstruction in primates,[12–18] but further evidence about jaw bone reconstruction is still required. Among these reports, Boyne *et al.*[12] successfully restored bone in large defects created by hemimandibulectomy using rhBMP-2. However, we showed that rhBMP-2 alone did not repair mandibular bone defects sufficiently in old Japanese monkeys, but was successful when used in combination with bone marrow.[9] As one possible reason for the discrepancy in results may be a reduced response to BMP as a result of aging,[11] we examined the efficiency of rhBMP-2 in alveolar defects of young non-human primates in this study.

The formation of rhBMP-2-induced bone in primates seems to take longer than in other experimental animals. For example, it takes only 2–4 weeks for BMP to repair bone defects in rats.[3,19,20] Despite the same experimental conditions, we found that bone formation in monkeys was slower than in dogs. Cells that migrate into the BMP implants in primates, which have longer lifespans and slower metabolic rates,[10] are thought to respond less to BMP than those cells in rats or dogs. Histologically, rhBMP-2-induced bone in the canine mandible has extremely dense trabecular bone without a distinct medullary cavity even at 24 weeks postoperatively.[21] On the other hand, in monkeys, the rhBMP-2-induced bone had a medullary cavity, and had undergone extensive remodelling and consolidation of new bone after 12 weeks. Therefore, bone formation induced in monkeys was of better quality and was more mature than that induced in dogs, although the rate of bone formation was slower. The differential responses to rhBMP-2 in various animals will be an important consideration when deciding whether rhBMP-2 is ready for clinical trials.

pQCT analysis confirmed the maturation of induced bone in the present study, with new bone density essentially the same as the surrounding cortical bone at 12 weeks postoperatively, associated with normal sponge bone in the inner region. We think that regenerated bone using rhBMP-2 can provide adequate strength, because bone strength is closely related not only to bone mass

but also to trabecular bone microstructure.[22,23] The carrier for BMP plays an important part in BMP-induced bone induction and we selected the specifically developed BMP carrier PGS in this study.[24–26] PGS is biocompatible, bioresorbable, and adapts easily to the shape of defects because of its sponge-like form. However, it is not strong enough to maintain augmented contours. The induced bone at the alveolar ridge was thin in two of the five monkeys at 12 weeks postoperatively, which may be the result of the collapse of the PGS. Additional studies are therefore required to identify more effective carrier materials that will maintain the augmented shape and maximize uniform delivery of rhBMP-2 to the surgical site.

We used only two control animals for the following reasons: first, our previous studies, as well as those of other workers, have indicated that the defect size (20×15 mm), which is about one-third of the size of the mandible, is critical size for the healing of the defect.[9,27,28] Secondly, we designed the experiment in which one animal received both control and BMP implants to allow direct comparison between the effect of BMP and the control, and the results showed the superior bone regeneration in the experimental side. Finally, we wanted to reduce the number of animals used because of the limited availability of the monkeys.

We used 2.8 mg of rhBMP-2 for each animal. It is not yet clear whether this is the optimal dose of rhBMP-2. Previous studies reported a dose-dependent increase in bone induction by rhBMP-2.[3,4,29] However, a high dose of BMP may have adverse pharmacological effects rather than the desired physiological effects. For instance, increased dosage may lead to increased vascularity that causes excessive tissue oedema. To minimize the effective dose of BMP, a combination graft with bone marrow[9] or other growth factors such as TGF-β[30] should be considered. The optimum concentration of implanted rhBMP-2 will probably vary according to species and the experimental site chosen. These dosing requirements must be established before clinical trials are considered.

The regeneration of bone defects using rhBMP-2 holds promise as a future treatment for implant dentistry and reconstructive surgery. The purpose of mandibular reconstruction is not only to recover the shape of mandible, but also to regain normal oral functioning including mastication, deglutition, and speech. Accordingly, additional studies should examine the functional results and long-term observation of rhBMP-2-induced bone regeneration.

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