Effects of Dose of Recombinant Human BMP-2 on Bone Formation at Palatal Sites in Young and Old Rats

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This study was designed to examine the effects of dose of recombinant human BMP-2 (rhBMP-2) on bone formation at palatal sites in 10-week-old (10w) and 70-week-old (70w) rats, when combined with a polylactate-polyglycolate copolymer/gelatine sponge (PGS).

New bone formation was observed at six weeks after implantation. In the 10w rats, thickness of new bone (TNB) increased as the dosage increased from 0 μ g to 4 μ g, and decreased significantly as the dosage increased from 8 μ g to 24 μ g. In contrast, in the 70w rats, TNB increased as the dosage increased from 0 μ g to 16 μ g, and did not significantly change as the dosage increased from 16 μ g to 24 μ g.

These results suggest that the most effective dosage of rhBMP-2 for induction of bone formation varies according to age.

Keywords: Dose of rhBMP-2, Bone formation, Aging

INTRODUCTION

It has been demonstrated that recombinant human BMP-2 (rhBMP-2) could induce bone formation and enhance periodontal regeneration¹⁻¹²⁾. Use of rhBMP-2 *in vivo* needs a carrier material. On this account, polylactate-polyglycolate copolymer/gelatine sponge (PGS) — an improved sponge-type carrier material — has been reported to be an effective carrier of rhBMP-2^{2,4,6,7)}.

To evaluate the effects of rhBMP-2 on bone formation, it is necessary to use old animals as well as young animals. This is because the age profile of most people suffering from periodontitis or alveolar bone loss reflects the aged and elderly. We examined the effects of aging on bone formation induced by rhBMP-2 at palatal subperiosteal sites using 10-, 30-, and 70-week-old rats and found that the volume of new bone declined with aging.

In the abovementioned studies^{4,5)}, it was not investigated at which concentration did rhBMP-2 induce maximal bone formation at a particular age. Against this backdrop, we investigated the effects dose of rhBMP-2 on bone formation using 30-week-old rats as adult age⁶⁾. Nonetheless, it is still necessary to extend the investigation to rats of other age groups, to the end of unraveling the corresponding concentrations of rhBMP-2 which would induce maximal bone formation.

The purpose of the present study, therefore, was

to examine the effects of dose of rhBMP-2 on bone formation at palatal subperiosteal sites in young and old rats.

MATERIALS AND METHODS

Animals

Forty-one 10-week-old (10w) and 42 70-week-old (70w) male Wister rats were used in this experiment. All procedures involving rats were performed in compliance with the guidelines for the care and use of laboratory animals of the School of Dentistry and Graduate School of Dental Medicine, Hokkaido University.

Combination of rhBMP-2 with carrier material

RhBMP-2 and PGS were donated by Astellas Pharmaceutical Co. Ltd. (Tokyo, Japan). RhBMP-2 was dissolved in LF6 buffer (5 mM sodium glutamate, 2.5% glycine, 0.5% sucrose, and 0.01% Tween 80; pH 4.5). PGS, composed of polylactate-polyglycolate copolymer (molar ratio at 50 : 50; molecular weight at 30,000) and gelatine sponge, was used as the absorbable carrier (Fig. 1). Weight ratio of polylactate-polyglycolate copolymer to gelatin sponge was 4:1, and porosity was approximately 90%. PGS was cut into pieces of 4.0 $\times$ 0.5 $\times$ 1.0 mm (mean weight: 250 μ g), rhBMP-2 (0.0, 4.0, 8.0, 16.0, or 24.0 μ g) was added to PGS, and then the resultant product lyophilized.

Implantation

Both 10w and 70w rats were divided into five groups according to the dosage of rhBMP-2 contained in the PGS and which was implanted: 0 μ g, 4 μ g, 8 μ g, 16 μ g, and 24 μ g.

Surgical procedures following the method of Matsumoto *et al.*⁵⁾ were performed under intra-peritoneal anesthesia with pentobarbital sodium (40 μ g/g body weight) and local infiltration anesthesia with 2% lidocaine hydrochloride containing 1:80,000 epinephrine. After full-thickness flap was raised, implantation was done at palatal subperiosteal sites (Fig. 2).

Nylon fibers, obtained by unraveling nylon surgical sutures, were placed on the palatal bone surface as markers to distinguish new bone from original bone. PGS was implanted over the nylon fibers. The incision was then sutured (Fig. 3). At six weeks after implantation, the animals were euthanized by overdose inhalation of ether.

Fig. 1 Polylactate-polyglycolate copolymer/gelatin sponge (PGS) on a sterilized sheet.

Fig. 2 Palatal subperiosteal site with full-thickness flap raised.

Histological procedures and histometric evaluations

Tissue blocks including palate and molar teeth were removed, and fixed with 10% buffered formalin. Fixed tissue blocks were demineralized using Plank-Rychlo solution, and then embedded in paraffin. Serial sections of 5 μ m thickness were cut in the frontal plane throughout the entire mesiodistal extension, and stained with hematoxylin and eosin (H&E).

Distance between the new bone surface and the most nasal position of nylon fibers was measured as the thickness of new bone using a grid (Fig. 4). Thickness of new bone (TNB) was determined by averaging the thickness of new bone of five sections at intervals of 100 μ m in every specimen.

Statistical analysis

The Tukey-Kramer test was used for TNB comparisons for the same group of 10w rats and 70w rats. The Mann-Whitney rank sum test was used for TNB comparisons between groups.

●: nylon fiber

Fig. 3 Implant on the bone surface (cross section). Nylon fibers, as markers to distinguish new bone from original bone, were placed on the bone surface after a full-thickness flap was raised. Then, the implant was implanted over the nylon fibers.

●: nylon fiber

Fig. 4 Measurement of the thickness of new bone. Distance between the new bone surface and the most nasal position of nylon fiber was measured as the thickness of new bone.

RESULTS

Histological observation

In every specimen, neither infected region nor atypical cell proliferation was observed. Implants had disappeared, leaving only the nylon fibers. New bone formation continued at the site of original bone (Figs. 5 and 6).

Histometric analysis

Table 1 shows the TNB of every group. In the 10w rats, TNB increased as the dosage increased from 0 μg to 4 μg , reached the maximal level at 4 μg ,

and decreased significantly as the dosage increased from 8 μg to 24 μg . In contrast, in the 70w rats, TNB increased as the dosage increased from 0 μg to 16 μg , reached the maximal level at 16 μg , and did not significantly change as the dosage increased from 16 μg to 24 μg .

There were significant differences in TNB between 10w and 70w rats at dosages of 0 μg , 4 μg , and 8 μg . However, there were no significant differences at dosages of 16 μg and 24 μg . The maximal TNB of 10w rats ($203 \pm 91 \mu\text{m}$ at 4 μg) was significantly greater than that of 70w rats ($128 \pm 45 \mu\text{m}$ at 16 μg).

Fig. 5 Photomicrographs of group : (a) 0 μg ; (b) 4 μg ; (c) 8 μg ; (d) 16 μg ; and (e) 24 μg of 10w rats (H&E staining).

Growth of new bone (NB) continued at the site of original bone (OB). Nylon fibers (arrow head) were surrounded by bone tissue (bar = 100 μm).

Fig. 6 Photomicrographs of group : (a) 0 µg; (b) 4 µg; (c) 8 µg; (d) 16 µg; and (e) 24 µg of 70w rats (H&E staining). Growth of new bone (NB) continued at the site of original bone (OB). Nylon fibers (arrow head) were surrounded by bone tissue (bar=100µm).

DISCUSSION

The objective of the present study was to examine the effects of dose of rhBMP-2 on bone formation at palatal sites in 10- and 70-week-old male Wister rats.

Wister rats become reproductive adults at 9-14 weeks of age, and remain so until approximately 60 weeks of age. Their average life span is about two and a half years. Considering this background, we selected 10- and 70-week-old rats to represent young and old rats, respectively.

To apply BMP to bone augmentation or periodontal therapy, it is necessary to perform the investi-

gation at submucosal sites adjacent to the jawbone, alveolar or palatal bone. This is because there is a fair possibility that implants might drop out in the case of subgingival implantation. The chief cause lies in the difficulty to suture sulcular incisions, as interproximal spaces in rats are very small. Thus we decided to use palatal subperiosteal sites in this study.

It is important to select the appropriate carrier material for BMP. It has been demonstrated that PGS was effective as a carrier of BMP, and that BMP combined with PGS could enhance periodontal regeneration^{2,7}. Therefore, we selected PGS as the

Table 1 Thickness of new bone (TNB) of every group

	Group				
	0 μ g	4 μ g	8 μ g	16 μ g	24 μ g
10w rats					
TNB (μ m)	87 $\pm$ 53 ^a	203 $\pm$ 91 ^b	189 $\pm$ 80 ^b	135 $\pm$ 63 ^{c,*}	113 $\pm$ 67 ^{a,c,*}
Number of sites	8	9	8	8	8
70w rats					
TNB (μ m)	18 $\pm$ 17 ^d	69 $\pm$ 35 ^e	101 $\pm$ 39 ^{e,f}	128 $\pm$ 45 ^{f,*}	111 $\pm$ 46 ^{f,*}
Number of sites	9	9	8	8	8

Same alphabets indicate no significant differences according to Tukey-Kramer test ($P>0.05$).

Same symbol (*) indicates no significant differences between the 10w and 70w rats of the same group according to Mann-Whitney rank sum test ($P>0.05$).

carrier of rhBMP-2 in this study.

It has been reported that at palatal subperiosteal sites, the implantation of rhBMP-2 combined with PGS resulted in new bone formation continuing at the site of original bone^{4,7}. However, it is necessary to distinguish the new bone from the original bone histometrically.

The nylon fibers used in this study appeared to be effective markers for the histometric evaluation of new bone formation continuing at the site of original bone in decalcified specimens. This was chiefly because they seemed to have neither stimulative nor inhibitory effect on bone formation.

For histometric evaluation of new bone, an adequate postoperative observation period is necessary. In the case of short-term observations, the total amount of new bone might not be estimated because further bone formation could occur until the implanted material disappears completely. It has been demonstrated that the implantation of rhBMP-2 combined with PGS disappeared at six weeks after implantation at palatal subperiosteal sites in rats^{4,6,7}.

Iwasaka *et al.* reported the implants disappeared at six weeks after implantation even in the case of rats with diabetes mellitus⁷. In this study, six weeks seemed to be adequate because of the evident disappearance of the implants.

In the 10w rats, TNB significantly increased as the dosage increased from 0 μ g to 4 μ g, and significantly decreased as the dosage increased from 8 μ g to 24 μ g. In contrast, in the 70w rats, TNB increased as the dosage increased from 0 μ g to 16 μ g, and did not significantly change as the dosage increased from 16 μ g to 24 μ g. These results indicated that in both 10w and 70w rats, the implantation of rhBMP-2 combined with PGS was capable of enhancing bone formation. However, the most effective dosage of rhBMP-2 differed between the 10w and 70w rats.

Maximal TNB of the 10w rats was significantly greater than that of the 70w rats. This suggested that the 70w rats required a greater dosage of

rhBMP-2 than the 10w rats, and that responsiveness to rhBMP-2 or the number of BMP-producing cells decreased with aging. These results were consistent with those of previous studies which showed that the number and function of osteoblast cells decreased with aging^{13,14}.

It has been reported that BMP caused a dose-dependent increase in bone resorption *in vitro*¹⁵⁻²². Kanatani *et al.* examined the effects of BMP-2 on osteoclastic activity in a mouse bone cell culture¹⁵. They found that BMP-2 caused increase in bone resorption and stimulated mature osteoclast function. Putting these *in vitro* studies¹⁵⁻²² and our results together, it could be said that an effective dose of rhBMP-2 would enhance bone formation, whereas an overdose of rhBMP-2 would lead to inhibitory effect on bone formation.

In conclusion, the present results indicate that at palatal subperiosteal sites, the most effective dosage of rhBMP-2 for induction of bone formation varies according to age.

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