Accelerators of Osteogenesis by Recombinant Human Bone Morphogenetic Protein-2

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Abstract: Bone morphogenetic protein (BMP) appears to be one of the most promising cytokine and for clinical use in reconstructive surgery for bony defects and augmentation. To evaluate the effect of basic fibroblast growth factor (bFGF), FK506, elcatonin, and hyperbaric oxygenation (HBO) on osteoinduction by recombinant human bone morphogenetic protein-2 (rhBMP-2), 2 or 5 µg of rhBMP-2 was implanted into intramuscular sites of rats. At 21 days after implantation, the osteoinductive activity in the treatment group and control group was compared radiographically, biochemically, and histologically. The amount of new bone in the treatment group was significantly greater than that in the control group. The alkaline phosphatase activity and calcium content in the treatment group were significantly higher than those in the control group. These results suggest that bFGF, FK506, elcatonin, and HBO accelerated the activity and rate of osteoinduction by rhBMP2. These results may be useful when BMP is applied clinically in near future.

Keywords: FK506, Basic fibroblast growth factor (bFGF), elcatonin, hyperbaric oxygenation (HBO), Recombinant human bone morphogenetic protein.

Introduction

Bone morphogenetic protein (BMP) appears to be one of the most promising biomaterial and for clinical use in reconstructive surgery for bony defects and augmentation. Therefore, BMP is noted in the field of bone reconstructive surgery. Since recombinant human bone morphogenetic protein-2 (rhBMP-2) has become available, many animal studies on osteoinduction by rhBMP-2 have been performed (Fujimura et al.1995; Okubo et al. 1999; Okubo et al. 2000). However for clinical application of rhBMP-2 to tissue with low blood supply tissue, e.g. scarred tissue or irradiated tissue, it is necessary to evaluate the factors that enhance osteoinduction by rhBMP-2.

In the present study, the basic mechanism of osteoinduction by rhBMP-2 and preclinical studies are discussed and reviewed mainly referring to our previous research regarding accelerators of osteogenesis and related studies.

Effect of basic Fibroblast Growth Factor (bFGF)

FGF has various effects on cellular proliferation and it has a strong proliferative affected on endothelial cells, osteocytes and chondrocytes (Connolly et al. 1987; Gospodarowicz et al. 1987; Globus et al. 1988). In addition, FGF and transforming growth factor β (TGFβ) are co-active on proliferating chondrocytes and osteoblasts (Iwamoto et al. 1989; Inoue et al. 1989; Nakamura et al. 1995). We evaluated the effect of FGF on the osteoinductive activity by rhBMP-2. BMP-2 (Genetics Institute, MA) was provided by Yamanouchi Pharmaceutical Co. Ltd. (Tokyo, Japan). It was dissolved in a buffer (pH 4.5) containing 5 mM glutamic acid, 2.5% glycine, 0.5% sucrose and 0.01% Tween 80, and stored at −80°C. FGF was provided by Kaken Pharmaceutical Co. Ltd. (Tokyo, Japan). Type I collagen solution (3 mg/ml, pH 3.0) (Cellmatrix LA®; Nitta Gelatin Inc., Osaka, Japan) was used as the carrier for BMP-2 and FGF-2. This collagen was purified from fresh porcine skin, and the telopeptide was removed by proteolytic digestion. BMP-2 (2 µg) and 0, 16, 80 and 400 ng, and 2, 10 and 50 µg of FGF-2 (n = 10
in each group) were mixed with 3 mg of type I collagen. The mixtures were lyophilized (0.04 Torr) (Eyela® type FDU-830; Tokyo Rika Inc., Tokyo, Japan) and shaped into discs (4 mm diameter; 1.5 mm thickness).

Seventy male 10-week-old Wistar rats weighing 240–260 g were used. They were divided into seven groups. All the rats were anaesthetized with an intraperitoneal injection of sodium pentobarbital (4.0 mg solidus 100 g body weight). After disinfecting the operative region and incising the skin, a disc containing BMP-2 (2 µg), bFGF (0, 16, 80 and 400 ng, and 2, 10 and 50 µg in each) and type I collagen (3 mg) were implanted into the right calf muscles of the rats. These seven groups (n = 10 in each group) consisted of group 1 (control), BMP-2 + FGF (0 ng) + type I collagen; group 2, BMP-2 + FGF (16 ng) + type I collagen; group 3, BMP-2 + FGF (80 ng) + type I collagen; group 4, BMP-2 + FGF (400 ng) + type I collagen; group 5, BMP-2 + FGF (2 µg) + type I collagen; group 6, BMP-2 + FGF (10 µg) + type I collagen; and group 7, BMP-2 + FGF (50 µg) + type I collagen. They were fed rodent chow (Certified diet MF; Oriental Koubo Inc., Tokyo, Japan) for the period of the study. Three weeks after the operation, all the animals were killed with an intraperitoneal injection of excess sodium pentobarbital. The specimens with peripheral tissues were fixed in 10% formalin neutral buffer solution (pH 7.4), demineralized in EDTA, and embedded in paraffin. They were cut into 4 µm-thick sections and stained with hematoxylin and eosin. The samples for quantitative analysis were weighed and then homogenized in 0.25 M sucrose in a Polytron homogenizer (Bio-Mixer; type ABM, Nissei Inc., Osaka, Japan). The sediment was demineralized in 0.5 N HCl, and the calcium (Ca) content of the soluble fraction was determined by the orthocresolphthalein complexone method. The alkaline phosphatase (ALP) activity and total protein in the resultant supernatant were determined by the 4-nitrophosphatase method. The Ca content (µg/mg of tissue) and the ALP activity (IU/mg of protein) were used as indices of bone formation. The treatment of each animal was conducted according to the 1988 guidelines for animal experiments at Kyoto University.

Three weeks after implantation, ALP was increased in the 16, 80 and 400 ng FGF-2-treated groups but decreased in the 50 µg FGF-2-treated group. Histological examination revealed increased bone formation in the 16, 80 and 400 ng FGF-2-treated groups (Table 1). These results show that combined treatment with FGF-2 and BMP-2 has a biphasic effect on osteoinductive activity, i.e. it increases with low doses of FGF-2 and decreases with high doses of FGF-2 (Fujimura et al. 2002).

Effect of FK506
FK506 has generally been used as an immunosuppressant for organ transplantation. We evaluated the effect of FK506 on osteoinduction by rhBMP-2. One hundred and twenty male Wistar rats (10 weeks old and weighting 230–250 g) were randomly divided into the following four groups of 30 rats each: 1) The short-term FK506 group (SFG) received a daily intramuscular (i.m.) injection of 0.1 ml of FK506 (1 mg/kg) from 2 days before the implantation of lyophilized specimens until the day of implantation. Then the animals received a daily injection of 0.1 ml of saline i.m. from the day of implantation until sacrifice. 2) The medium-term FK506 group (MFG) received a
Accelerators of Osteogenesis by rhBMP-2

daily injection of 0.1 ml of FK506 (1 mg/kg i.m.) from 2 days before implantation until 7 days after implantation. Then a daily injection of 0.1 ml of saline i.m. was given for the next 7 days until sacrifice. 3) The long-term FK506 group (LFG) received a daily injection of 0.1 ml of FK506 (1 mg/kg i.m.) from 2 days before implantation until sacrifice. 4) The control group (CG) received a daily injection of 0.1 ml of saline i.m. from 2 days before implantation until sacrifice.

rhBMP-2 was obtained from W. Sebald (Würzburg University, Germany, Ruppert et al. 1996). Atelopeptide type-I collagen (CL) (pH 3.0 was used as the carrier). rhBMP-2 (5 µg) was mixed with 3 mg of CL and was lyophilized (EYELA FDU-830; Tokyo Rikakikai Inc., Tokyo, Japan). Then the material was compressed in a syringe to form discs (4 mm in diameter and 1.5 mm thick).

Rats were anesthetized with intraperitoneal sodium pentobarbital (5.0 mg per 100 g of body weight) and lyophilized disc specimens were implanted into the right calf muscle. After implantation, the fascia and skin were sutured.

FK506 (Tacrolimus; Fujisawa Pharmaceutical Co., Ltd., Osaka, Japan) was suspended in saline and injected into the left calf muscle of each rat (1 mg/kg/day). This dose has already been given intramuscularly in rat organ transplantation models (Akahane et al. 1999).

In the radiographic findings, the area of the shadows at 21 days after implantation was in the order of CG > MFG > SFG > LFG. In the histological findings, fourteen days after implantation, new bone surrounded by immature mesenchymal-type cells was present at border of the implant around almost the entire circumference in every group. However, cartilage was still observed in the LFG. The immature new bone area was larger in the SFG than in the other groups. Twenty-one days after implantation, the trabecular bone area was increased in the CG, but not in the MFG. In the SFG and LFG, it was decreased at the border of the implant, and fatty marrow occupied most of the marrow tissue. Twenty-one days after implantation, the ALP activity was 6.37 ± 0.37 in the CG, 3.34 ± 0.19 in the SFG, 5.40 ± 0.46 in the MFG, and 1.30 ± 0.24 in the LFG. The values in the SFG, and LFG were significantly lower than in the CG and MFG. Twenty-one days after implantation, the Ca content was 33.81 ± 3.44 in the CG, 18.43 ± 1.94 in the SFG, 24.11 ± 2.61 in the MFG, and 15.24 ± 1.96 in the LFG. Values in the SFG and LFG were significantly lower than in the CG (Table 2). These findings demonstrate that short-term administration of FK506 promotes early osteoinduction. However, long-term administration accelerates both bone formation and bone resorption, and insufficient oxygen supply leads to failure of bone matrix maturation, resulting in poor bone formation (Kaihara et al. 2002).

### Effect of Elcatonin

Elcatonin is a derivative of eel calcitonin synthesized by substituting an ethylene bond for the disulfide bond (Morikawa et al. 1976; Otani et al. 1978; Orimo et al. 1990). It has also been reported from in vivo and in vitro studies that elcatonin suppresses bone resorption (Orimo et al. 1990; Yamamoto I et al. 1981). In the clinical field, elcatonin is used currently for the treatment of Paget’s disease, and osteoporosis. However, the role of this hormone in producing an anabolic effect on osteoblasts is not yet fully understood. We evaluated the effect of elcatonin on osteoinduction by rhBMP-2, especially the anabolic effect on osteoblasts.

### Table 2.

| FK506 | Ca content µg/mg tissue | ALP activity IU/mg protein |
|-------|-------------------------|---------------------------|
|       | day 7       | day 14       | day 21       | day 7      | day 14      | day 21      |
| SFG   | 0.15 (0)    | 33.5 (1.1)* | 18.4 (1.9)*  | 2.4 (0.4)* | 1.9 (0.2)   | 3.3 (0.2)*  |
| MFG   | 0.14 (0)    | 24.4 (3.1)  | 24.1 (2.6)   | 2.3 (0.2)* | 2.4 (0.3)   | 5.4 (0.3)   |
| LFG   | 0.13 (0)    | 13.3 (1.0)* | 15.4 (1.9)*  | 2.4 (0.3)* | 3.1 (0.3)   | 1.3 (0.4)*  |
| CG    | 0.1 (0)     | 23.4 (2.9)  | 33.8 (3.4)   | 1.8 (0.4)  | 2.7 (0.4)   | 6.3 (0.4)   |

Data are means (SD).

*Significant difference at p < 0.05, compared with CG.
Twenty Wistar rats (male; 10 weeks old; weight 240–260 g) were used. Four groups, consisting of a high elcatonin group (HEG), medium elcatonin group (MEG), low elcatonin group (LEG) and control group (CG), were established with 5 rats in each group.

rhBMP-2 derived from E. coli was obtained from W. Sebald (Würzburg University, Germany, Rupport et al. 1996). CL (pH 3.0) was used as a carrier. Five µg of rhBMP-2 mixed with 3 mg of CL was lyophilized (EYELA FDU-830; Tokyo Rikakikai Inc., Tokyo, Japan). The material was compressed in the injection syringe to discal form (4 mm in diameter, 1.5 mm in thickness).

As the pharmacological agent, 14-day doses of elcatonin (Elcitonin®; Asahi Chemical Industry Co., Ltd., Tokyo, Japan) were prepared, 80 U for HEG, 8 U for MEG, and 0.8 U for LEG. The total volume of the elcatonin agent in physiological saline solution was 0.2 ml for each rhBMP-2 implanted group. The elcatonin solution was placed into a mini-osmotic pump (alzet® model 2002; ALZA Co., CA), that would pump out the solution continuously at a constant rate of 0.5 µl/hour for 14 days. For CG, only 0.2 ml of physiological saline was placed into the mini-osmotic pump.

All rats were anesthetized with intraperitoneal administration of sodium pentobarbital. The lyophilized discal specimens were implanted into a right calf muscle. After the implantation, the fascia and skin were sutured. A one-cm-long incision was made in the paramedian abdominal wall, including the skin, muscle, and the peritoneum and the mini-osmotic pump, which had been previously prepared for each group, was inserted into the peritoneal space. The abdominal wall was then closed by suturing layer by layer.

Twenty-one days after the implantation, all rats were sacrificed with an overdose of sodium pentobarbital. The lyophilized discal specimens were implanted into a right calf muscle. After the implantation, the fascia and skin were sutured. A one-cm-long incision was made in the paramedian abdominal wall, including the skin, muscle, and the peritoneum and the mini-osmotic pump, which had been previously prepared for each group, was inserted into the peritoneal space. The abdominal wall was then closed by suturing layer by layer.

Twenty-one days after the implantation, all rats were sacrificed with an overdose of sodium pentobarbital. The implanted region was excised with the surrounding tissue and a radiograph was taken. Each excised specimen was removed and then cut into 2 halves, one for histological analysis and the other for biochemical analysis.

The soft tissue radiographs revealed opaque shadows morphologically identical to the implanted specimens. These opaque shadows were observed in each of the specimens of all groups.

In HEG, there was a relatively vigorous trabecular bone on the outermost edge of the implanted material. Lining osteoblasts were observed around the trabecular bone. Bone marrow, including angioid tissue, was rich at the central side of the trabecular bone. Fatty marrow occupied a major part of the marrow tissue. In MEG, there was trabecular bone on the outermost edge of the implanted material. The trabecular bone was thinner than that in HEG. Bone marrow included fatty tissue. Collagen fibers remained at the center of the implanted material. In LEG, less trabecular bone and marrow were observed compared to the respective amounts in MEG and HEG. At the central side of the newly formed trabeculae, a small area of bone marrow was observed. In CG, especially, the amount of trabecular bone was clearly less than in the other groups and few osteoblasts were observed. The values of ALP activity on day 21 were 5.87 ± 0.43 (mean ± SD IU/mg protein) in CG, 6.41 ± 0.37 in LEG, 7.10 ± 0.37 in MEG, and 7.37 ± 0.50 in HEG (Table 3). The value was highest in HEG and lowest in CG. The values of Ca content on day 21 were 25.0 ± 1.61 (mean ± SD µg/mg tissue) in CG, 26.6 ± 0.96 in LEG, 29.0 ± 0.60 in MEG, and 31.3 ± 1.56 in HEG. The ALP activity and Ca content in HEG were highest and lowest in CG. In HEG and MEG, the values of ALP activity and Ca content were significantly lower than in CG and LEG (p < 0.01). These results suggested that elcatonin is effective in enhancing osteoinduction by rhBMP-2, and that elcatonin has an anabolic effect on osteoblasts in addition to an anti-resorptive effect (Okubo et al. 2000).

**Effect of Hyperbaric Oxygenation (HBO)**

Hyperbaric oxygen (HBO) therapy is an oxygenation method used to treat anoxia by increasing dissolved oxygen. HBO therapy has been shown to increase collagen synthesis, capillary ingrowth (Hunt et al. 1972), neovascularization, and osteogenesis (Nilson et al. 1988). Recently, the use of

| Elcatonin | Ca content µg/mg tissue | ALP activity IU/mg protein |
|-----------|------------------------|---------------------------|
| LEG       | 26.6 (1.0)             | 6.4 (0.4)                 |
| MEG       | 29.0 (0.6)*            | 7.1 (0.4)*                |
| HEG       | 31.3 (1.6)*            | 7.3 (0.5)*                |
| CG        | 25.0 (1.6)             | 5.9 (0.4)                 |

Data are means (SD).

*Significant difference at p < 0.01, compared with CG.
HBO therapy to improve the rate of bone healing in conjunction with surgery for dental implant, osteomyelitis and osteonecrosis has increased. We compared osteoinduction by rhBMP-2 with and without HBO therapy. Thirty Wistar rats were randomly assigned to an HBO group and a control group of 15 rats each. CL was used as a carrier. Five µg of rhBMP-2 mixed with 3 mg of CL was lyophilized (EYELA FDU-830; Tokyo Rikakikai Inc., Tokyo, Japan). The material was compressed in an injection syringe to discal form (4 mm in diameter, 1.5 mm in thickness).

All rats were anaesthetized by intraperitoneal administration of sodium pentobarbital (5.0 mg per 100 g of body weight). Following disinfection of the operative region, the lyophilized discal specimens were implanted into a right calf muscle pouch. The fascia and skin were sutured.

The rats in the HBO group were placed in a pressure chamber (KHO-100; Kawasaki Engineering Inc., Hyogo, Japan) and exposed to a pressure of 2.0 ATA 100% inspired flow oxygen for 60 minutes everyday for 3, 7, and 21 days. During the first 15 minutes of HBO therapy, the pressure was increased to 2.0 ATA, and decompression proceeded for 15 minutes after the treatment.

Three, 7, and 21 days after the implantation, the rats were sacrificed by an overdose of sodium pentobarbital. Then the implanted region was excised together with the surrounding tissue and soft X-rayed. Each excised specimen was removed and cut into 2 halves, one for histological analysis and the other for biochemical analysis.

On day 21, soft X-ray revealed opaque shadows morphologically identical to the implanted specimens in both groups. The oval shadows in the HBO group were larger with slighter high radio-opacity than those in the control group. On day 7 after the implantation, in the HBO group, cartilage tissue was induced at the outer edge of implanted material. In the control group, no cartilage or chondrocytes were detected in these findings. On day 21 after the implantation, new bone formation was found in both groups. Around the trabecular bone, lining osteoblasts and a few osteoclasts were observed in both groups. In the control group, trabecular bone tissue was observed at the outer edge of the implanted material. In the HBO group, the trabecular area was greater than that in the control group. The bone marrow area in the HBO group, including fatty marrow in part, was wider than that in the control group. The trabecular area bone in the HBO group was wider than that in the control group. The results of the micrograph analysis of the trabecular area and the percentage of the trabeculum occupying the overall area are summarized in Table 4.

The ALP activity and Ca content of the HBO group and the control group are shown in Table 3. The ALP activity and the Ca content in the HBO group were significantly higher than those in the control group on days 7 and 21.

The present results suggest that HBO therapy accelerates the activity and rate of osteoinduction by rhBMP-2, since hyperbaric oxygenation may enhance the effects of rhBMP-2 on the differentiation from immature mesenchymal cells to osteoblasts (Okubo et al. 2001).

Conclusions
In skeletal reconstruction using BMPs, lower amount of BMPs had better induce more bony tissue. Therefore, some materials have been studied in vivo for the promotion of osteoinduction. To date, FGF, FK506, elcatonin, HBO, and prostaglandin E1 and other materials have been studied as the accelerators in our group. These results may be useful when BMP is applied clinically in near future.

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