Prostaglandin E_1 and recombinant bone morphogenetic protein effect on strength of hydroxyapatite implants

Ichiro Ono,[1] Tohru Tateshita,[1] Yoshinori Kuboki[2]
[1]*Division of Plastic and Reconstructive Surgery, Department of Dermatology, Fukushima Medical University, School of Medicine, Hikarigaoka-1, Fukushima 960-1295, Japan*
[2]*Department of Biochemistry, School of Dentistry, Hokkaido University, North-13, West-7, Kitaku, Sapporo 060, Japan*

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Abstract: Although combinations of hydroxyapatite (HAP) and bone morphogenetic protein (BMP) are expected to provide potent alternatives to autogenous bone grafts, it is still anticipated that substances that act synergistically with BMP will be found because the inducing potential of purified BMP in bone is not strong enough. We already have shown that prostaglandin (PG) E_1 has a strong and dose-dependent synergistic effect on the osteoinductive activity induced by recombinant human (rh) BMP and that it enhances osteoconduction even when used alone. In this study, porous HAP rods were treated as follows: (1) without PGE_1 or rhBMP (control group); (2) with varying concentrations of PGE_1; and (3) with varying concentrations of PGE_1 combined with 1 μg of rhBMP-2. The rods were subperiosteally implanted on the cranial bone of rabbits to evaluate the effect of these treatments on the mechanical strength of the implanted HAP rods. The HAP rods were removed 3, 6, or 9 weeks after implantation and subjected to mechanical strength determinations. The control group (no addition of BMP to the rods) showed no significant increase in three-point bending strength or in compression strength compared to pre-implantation. On the other hand, PGE_1 combined with rhBMP had a strong and dose-dependent effect on the mechanical strength of HAP, increasing it significantly, especially compression strength. PGE_1 also increased mechanical strength even when used alone. Histological examination revealed that PGE_1, whether or not it was combined with rhBMP, increased bone formation into the pores of HAP and consequently increased the mechanical strength of porous HAP. © 1999 John Wiley & Sons, Inc. J Biomed Mater Res, 45, 337–344, 1999.

Key words: bone substitute; hydroxyapatite ceramics; bone morphogenetic protein; prostaglandin E_1; osteogenesis; mechanical strength

INTRODUCTION

There are a large number of fundamental and clinical studies about hydroxyapatite ceramics (HAP).[1–6] Since porous HAP is a ceramic and thus has the disadvantage of being fragile, impact resistance remains the greatest problem in the clinical application of this material.[6–10] On the other hand, bone morphogenetic protein (BMP), a bone-inducing material, has attracted attention in recent years,[11–16] and since the cloning of BMP, basic studies on this substance have been carried out using recombinant human (rh) BMP-2.[17–19] Although porous HAP implants combined with rhBMP-2 have been effective in producing orthotopic bone formation[20] and increased mechanical strength of the implanted HAP, it can be anticipated that other biomolecules synergistically may enhance the activity of rhBMP in this system. Until now, however, there have been few in vivo studies aimed at finding such synergistic substances. Several of the candidates that have been suggested are insulin-like growth factor,[21] basic fibroblast growth factor (FGF),[22–25] and transforming growth factor (TGF) β,[26–28] and these all have been found to have a synergistic effect on rhBMP in in vivo studies. Other substances that might be useful in this regard are the prostaglandin (PG)s, especially PGE_1 or PGE_2.[29–33] In a previous study using our animal model, we added rhBMP-2 to porous HAP ceramic pellets, implanted them subperiosteally on the cranial bone of rabbits, and observed the degree of osteogenesis[20,25,34–37] and alterations of mechanical strength.[36] It clearly was demonstrated that rhBMP-2 has strong bone induction properties in our animal model and when combined with HAP[37] increases the mechanical strength of the HAP significantly.[36] We also showed that PGE_1 has a strong dose-dependent effect on the osteoconductive activity induced by rhBMP and that it promotes osteoinduction even

Correspondence to: I. Ono

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when used alone.[38] The present experiments were designed to prove the hypothesis that the use PGE_1 with or without rhBMP-2 improves the mechanical strength of HAP. In this study, porous HAP rods were treated with rhBMP-2 and PGE_1 in various concentrations, subcutaneously fixed to the craniums of rabbits, and then removed and subjected to strength testing after implantation periods ranging from 3 to 9 weeks. Strength values and histological findings were compared with those obtained before implantation.

MATERIALS AND METHODS

We previously described our technique by which HAP pellets are grafted as an onlay beneath the cranial periosteum.[20,34,36,37] We used the same procedures with Japanese white rabbits weighing 3–4 kg, which, under pentobarbital (Nembutal, Abbott Laboratories, North Chicago, Illinois) anesthesia, were secured to an operating table. A skin incision was made in the head, and the cranial periosteum was raised from the cranium to create a pocket beneath. The porous HAP ceramic used was Apaceram (Asahi Optical Co., Ltd., Tokyo, Japan) cylindrical rods 5 mm in diameter, 10 mm in length, and with a porosity of 70% and a pore diameter of 50–500 μm [Fig. 1(a,b)].[36,37] After the HAP rods were subjected to one of several treatments, as described below, they were subperiosteally implanted directly on the cranial bone. Complete hemostasis then was confirmed and the skin was sutured. The rhBMP-2 was produced by genetic bioengineering techniques (Genetic Institute, Cambridge, Massachusetts, supplied by Yamanouchi Pharmaceutical Co. Ltd., Tokyo, Japan)[20,25] and synthetic PGE_1 was used (Ono Pharmaceutical Co. Ltd., Osaka, Japan).[38]

Rabbits weighing over 3–4 kg and skeletally mature were obtained from Sampei Animal Center, Fukushima, Japan and were acclimatized to our laboratory conditions for at least 7 days prior to use. They were maintained on standard laboratory chow and water *ad libitum* in the animal care facility of Fukushima Medical University and exposed to regular light periods (12 h of light/12 h of dark) and isothermal conditions (22°–24°C) under the regulations of the university. This study was carried out under the control of the Animal Research Committee in accordance with the Guidelines on Animal Experiments of Fukushima Medical University and of the Japanese Government Animal Protection and Management Law (No. 105).

One hundred thirty five Japanese white rabbits were divided into nine groups, as indicated below. The control group received rods treated only with 0.1 mL of phosphate-buffered saline (PBS) prior to implantation on the cranial bone. The low-dose BMP group received rods treated with 1 μg of rhBMP-2, and the high dose BMP group received rods treated with 5 μg of rhBMP-2 as a positive control.[20] The low-dose, medium-dose, and high-dose PGE_1 groups received rods treated with 1 μg, 10 μg, and 30 μg of PGE_1, respectively. In addition to those groups, groups receiving rods treated with varying doses of PGE_1 (1 μg, 10 μg, and 30 μg) combined with 1 μg of rhBMP-2 also were evaluated. Each group further was divided into three subgroups to examine time-dependent effects. Each subgroup contained five rabbits with two rods implanted in each rabbit, one for three-point bending strength determinations and the other for compression strength determinations. In addition, 20 rods were used for control measurements, 10 under dry conditions and 10 immersed in 0.1 mL of saline.

The animals in these subgroups were kept for 3, 6, and 9 weeks, respectively, and were sacrificed by administering excess pentobarbital. The implanted HAP rods surgically were isolated from the subcutaneous layer and kept in cooled saline until the strength determinations were performed, followed by histological analysis. They were examined with three-point bending strength and compression strength tests using a Shimadzu Autograph (DSS-5000; Shimadzu Co., Tokyo, Japan). The three-point bending strength was determined with a cross-head speed of 0.5 mm/min and a load cell of 100 kgf with 8 mm intervals between the three stress points; and the compression strength was deter-

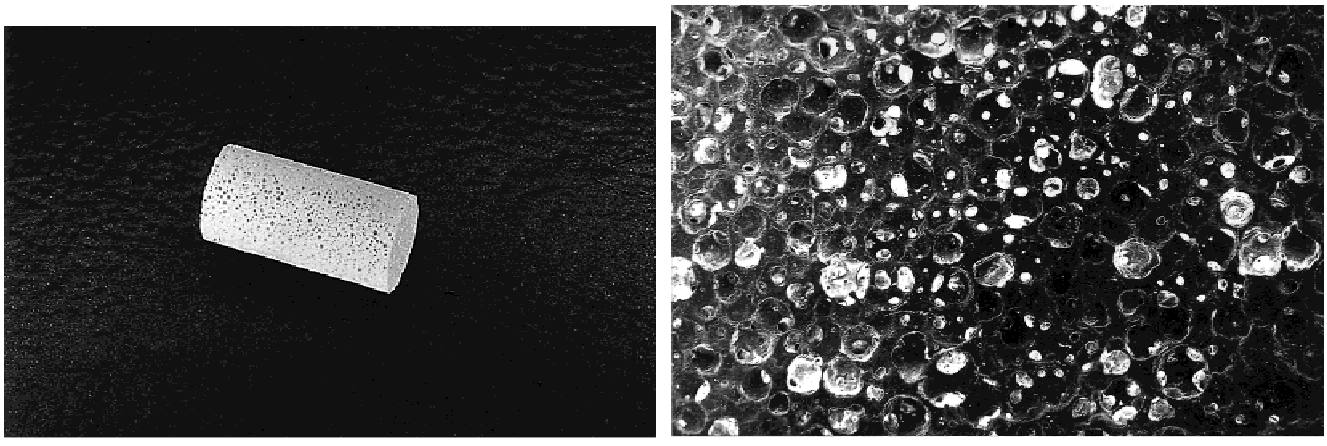

Figure 1. The porous HAP rod used. (a) A molded cylindrical rod 5 mm in diameter and 10 mm in length; (b) SEM image (original magnification ×20). The open structure of interconnected pores serves to increase its bone conduction capacity. The porosity is 70% and the pore diameter is 50–500 μm.

mined with a cross-head speed of 0.5 mm/min and a load cell of 100 kgf. Strength was defined as the load value immediately before the rod broke.[36] Histological examinations were performed on all samples after strength determinations. After the specimens were decalcified they were embedded in paraffin and stained with hematoxylin and eosin (H&E).

All values are expressed as means ± standard errors (SE), and statistical analysis was performed by analysis of variance (ANOVA) followed by Fisher's comparison test using Statview-J 4.5 (Abacus Concepts, Inc., Berkeley, California).

RESULTS

The three-point bending strength before implantation was 38.5 ± 2.9 kg/cm^2 and 32.3 ± 2.6 kg/cm^2 in dry and wet conditions, respectively, which was not a significant difference. The three-point bending strength did not increase in the controls. On the other hand, in both the low- and high-dose BMP groups, increases in three-point bending strength were found as early as 3 weeks. The low-dose group showed significant increases at 6 weeks ($p < 0.005$) and 9 weeks ($p < 0.0001$) compared with the controls, and the high-dose group showed significant increases at 3 ($p < 0.0005$), 6 ($p < 0.0001$), and 9 weeks ($p < 0.0001$). Thus, since a significant difference was noted between the low and high dose groups at all times, there was a dose-dependent effect of rhBMP. In the PGE_1-treated groups, there was a tendency to increased three-point bending strength. In the high dose PGE_1 group, the three-point bending strength was 92.9 ± 13.3 kg/cm^2 at 9 weeks, which was significantly higher ($p < 0.05$) than that of the control group (49.2 ± 2.3 kg/cm^2) and not significantly different from that of the low-dose BMP-treated group (126.9 ± 22.4 kg/cm^2). There was no significant increase in three-point bending strength from the combined use of PGE_1 plus rhBMP-2 compared to the low-dose BMP group (Fig. 2).

The compression strength values before implantation were 29.7 ± 1.1 kg/cm^2 and 27.1 ± 1.0 kg/cm^2 in dry and wet conditions, respectively, which also was not significantly different. In the control group, which had received untreated rods, the strength of the rods did not change significantly after implantation. As in the three-point bending strength experiments, both the low- and high-dose BMP groups showed significant increases in resistance to compression from 6 weeks after implantation. Increases in the high-dose BMP group at 6 ($p < 0.0001$) and 9 weeks ($p < 0.0005$) were significant compared with the control group; and in the low-dose BMP group, the values were significant at 6 ($p < 0.05$) and 9 weeks ($p < 0.0001$) compared to the control group. No significant difference was seen between the low- and high-dose groups at 9 weeks, and no dose-dependent effect was noted. However, at 6 weeks the values of compression strength of the high-dose BMP group were significantly higher than those of the low-dose BMP group. In the PGE_1-treated groups there were tendencies toward increased resistance to compression strength. In the high-dose PGE_1 group, resistance to compression strength was 111.6 ± 20.2 kg/cm^2 at 9 weeks, which was significantly higher ($p < 0.0001$) than that of the control group (38.8 ± 3.0 kg/cm^2) but not significantly different from that of both the low-dose and high-dose BMP-treated groups. Use of combined high PGE_1 and BMP revealed a significantly stronger effect on the resistance to compression strength at 9 weeks compared to both the low-dose BMP group ($p < 0.005$) and the high-dose BMP group ($p < 0.0005$) (Fig. 3).

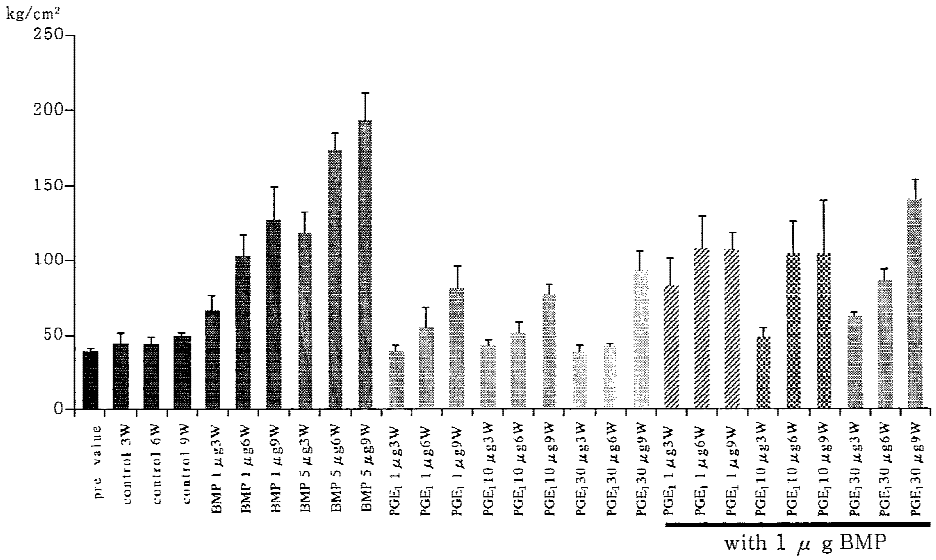

Figure 2. Effects of the rhBMP–PGE_1–HAP combination on the three-point bending strength ($n = 5$; mean ± SE).

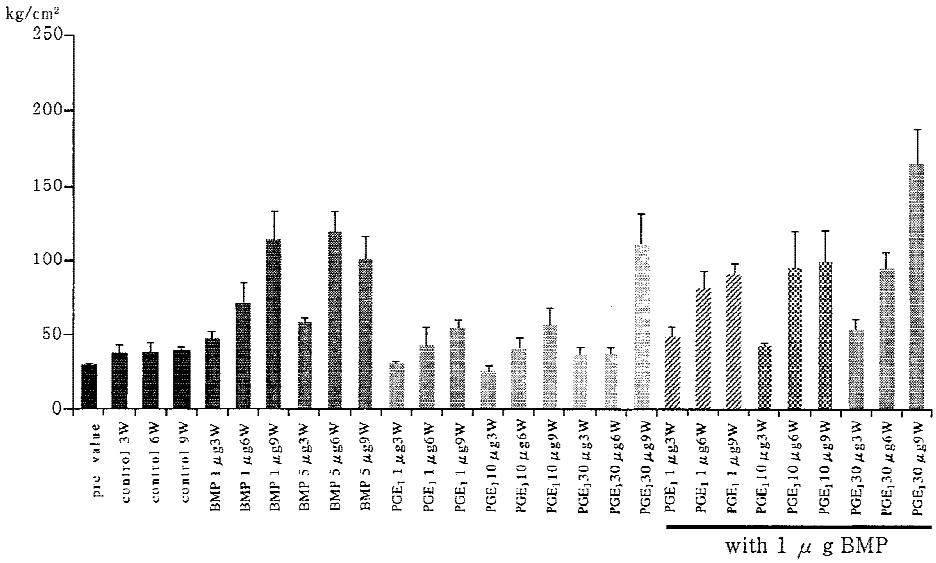

Figure 3. Effects of the rhBMP–PGE_1–HAP combination on the compression strength (n = 5; mean ± SE).

Histological examinations of the control group 3 weeks after implantation revealed a small degree of bone formation only at the surface of the porous HAP rod in contact with the cranial bone. There was only a very small increase in bone formation at 6 weeks and also at 9 weeks (Fig. 4). On the other hand, in the low-dose BMP group, whose rods had been treated with 1 μg of rhBMP-2, bone formation first progressed around the rod as well as into the pores beginning at 3 weeks, which was not observed in the controls. Bone formation into the pores proceeded in a time-dependent manner; it was noted at the margins of the pores at 6 weeks and had progressed markedly further into the pores at 9 weeks. However, complete filling of the pores with bone was not observed at that time (Fig. 5). In the high-dose BMP group, which had received 5 μg of rhBMP-2 with the implant, bone formation proceeded around and into the porous rod beginning at 3 weeks, further expanded around the pore margins by 6 weeks, and was confirmed in most of the spores at 9 weeks (Fig. 6). In the PGE_1-treated groups, especially in the high-dose group, at 3 and 6 weeks there was little bone formation as compared to the control groups, and osteogenesis was limited to the side in contact with the cranial bone (Fig. 7). On the other hand, with the combined use of high PGE_1 and BMP, bone had formed to some extent around the pellets as early as 3 weeks after insertion, and the degree of osteogenesis was almost the same as in the high-dose BMP group and apparently more than in the low-dose PGE_1–BMP and the low-dose BMP groups. In this group, however, osteoconduction from the surface of the cranial bone was extensive even 3 weeks after insertion. Subsequently, osteogenesis within the pellets progressed very rapidly, and after 9 weeks bone occupied almost the entire volume of the pores. The extent of bone formation in this group also was comparable to that of the high-dose BMP group at 9 weeks after insertion and was much greater than that of the other reference groups [Fig. 8(a,b)].

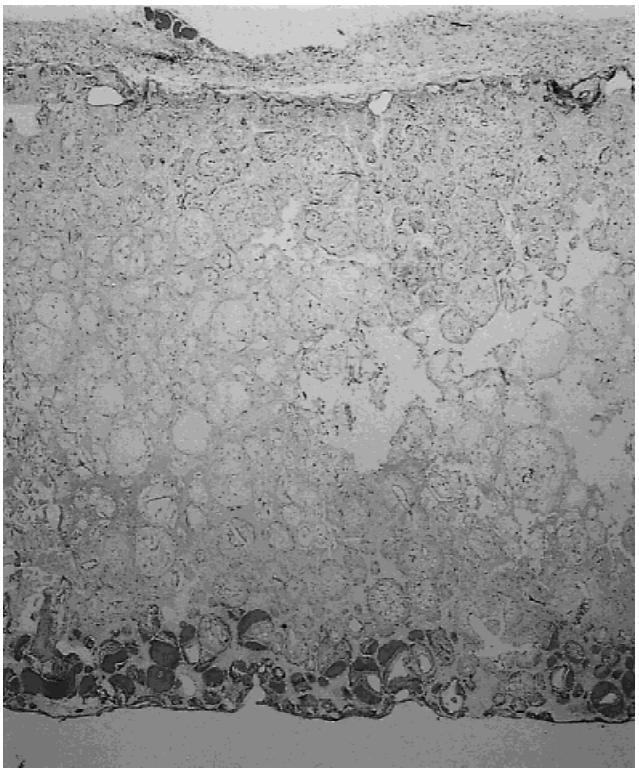

Figure 4. Histological findings of the control group at 9 weeks (H&E, original magnification ×15). Although a small degree of bone formation was seen on the HAP surface contact with the cranial bone, bone formation in the other part of the pores was minimal.

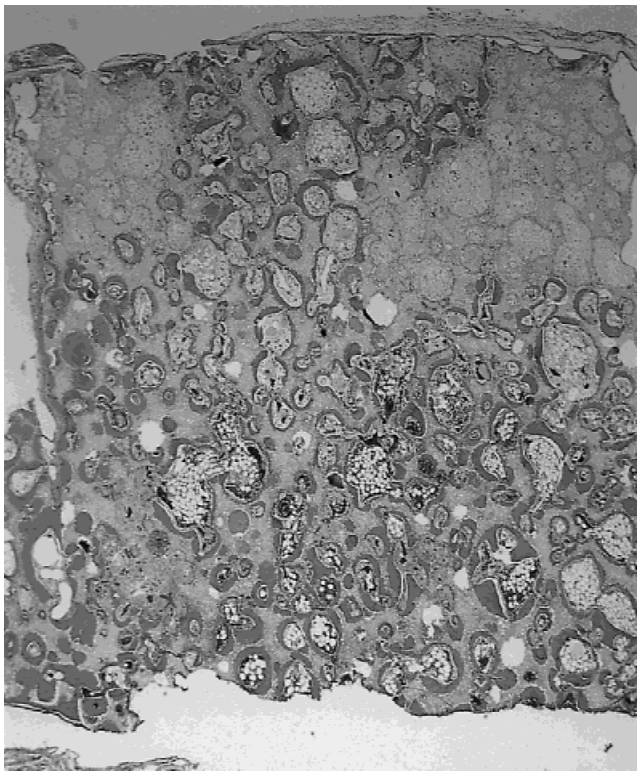

Figure 5. Histological findings of the 1 μg rhBMP-2 group at 9 weeks (H&E, original magnification ×15). Although bone formation is clearly visible within some of the pores, it had not extended into all pores.

DISCUSSION AND CONCLUSIONS

In recent years, much attention has been focused on HAP as a bone substitute, replacing autogenous free bone grafts in the fields of plastic surgery, orthopedic surgery, and dentistry. Already it is being used widely clinically.[1–6] However, in addition to improvements in the structure of HAP itself,[36] substances that can be used together with HAP to induce bone formation more efficiently and actively, to bind tightly to the surrounding bone tissue, and to increase the mechanical strength of the material long have been sought.[11] Studies on BMP have received considerable attention since the reports by Urist et al.,[15,16,39] who found that when insoluble materials remaining after hydrochloride decalcification of bovine bone were implanted subcutaneously, bone tissue formed around the grafted material. Subsequently, many research groups have carried out basic experiments focused on BMP extraction, isolation, and purification.[12,18,40] Now rhBMP can be produced by genetic bioengineering techniques,[19] and both basic studies and clinical trials have been initiated with it. As a result, clinical application of rhBMP combined with bone substitutes such as HAP can be anticipated in the near future.[37] Previously we investigated the results of bone formation using computer image analysis and found that the group treated with a rhBMP–collagen–HAP complex exhibited significantly greater bone induction and also that the treatment increased the mechanical strength of the HAP significantly.[25,36,37] In those experiments we showed that rhBMP induced osteogenetic activity and that collagen synergistically enhanced the effect of rhBMP, although not to a great extent.[34,37] Therefore, investigations to find substances that have a stronger synergistic effect than collagen on the osteogenetic activity of rhBMP will continue in order to make the use of this protein as practical as possible, both clinically and economically.

Recent studies show that prostaglandins, including PGE_1, E_2, and I_2, play a role in both osteogenesis and bone resorption as well as in the healing of bone fractures and in the wound healing process itself.[29–33] Moreover, in terms of skeletal physiological processes, prostaglandins have been known to increase skeletal turnover and bone formation.[30] We also found that PGE_1 has a strong and dose-dependent synergistic effect on the osteoinductive activity of rhBMP and that it increases osteoconduction even when used alone.[38] Although little is known about the effect that administration of prostaglandin combined with other sub-

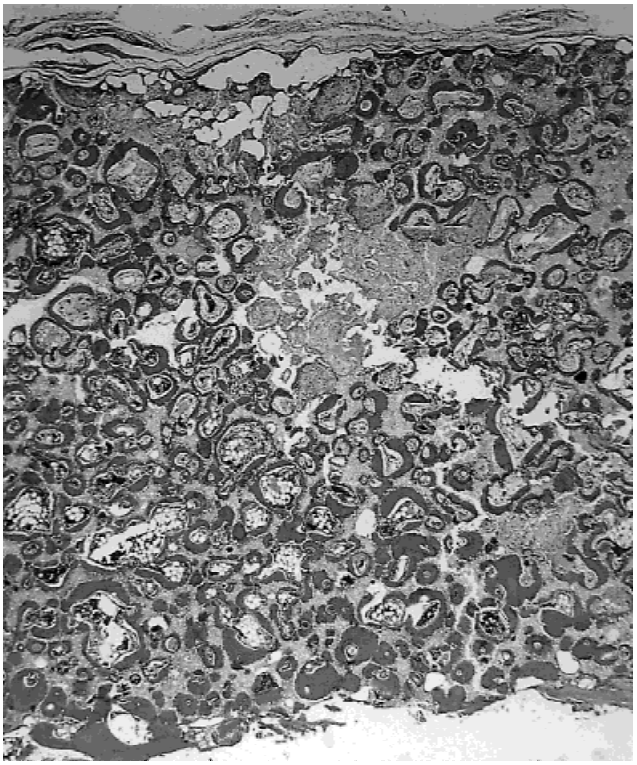

Figure 6. Histological findings of the 5 μg rhBMP-2 group at 9 weeks (H&E, original magnification ×15). Bone formation was found in most of the HAP pores, and bone formation was greater than in the 1 μg rhBMP-2 group of the same week.

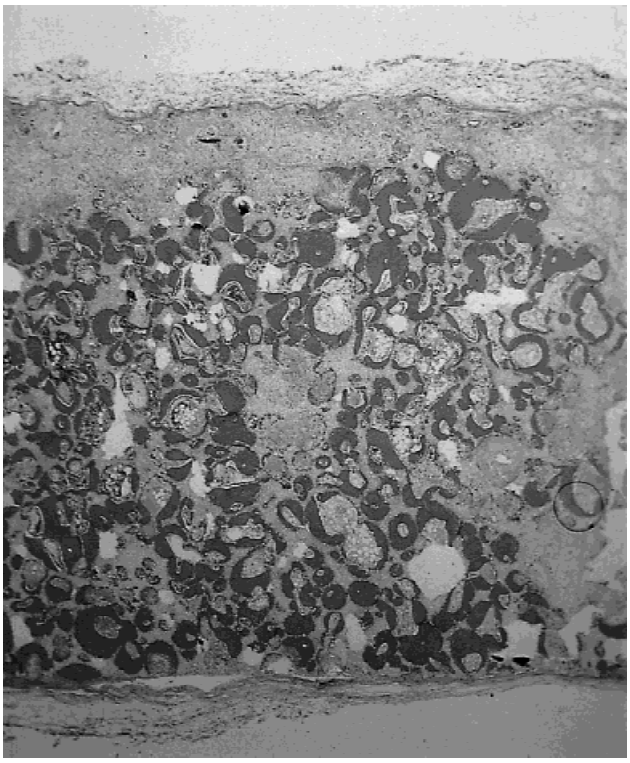

Figure 7. Histological findings of the 30 μg PGE_1 group at 9 weeks (H&E, original magnification ×15). Bone formation was found in most of the HAP pores, and bone formation was almost same as in the 1 μg rhBMP-2 group of the same week. In this group, it is evident that bone ingrowth progressed in an osteoconduction manner.

stances, such as BMP, might have on osteoinduction in vivo, from our evaluations PGE_1 has a rather strong osteoconductive effect when used alone, and when in combination it promotes osteoinduction by BMP. In that study,[38] we analyzed the outcome of using two different concentrations of PGE_1, 1 μg and 10 μg, in addition to a relatively small amount of rhBMP (1 μg), and found that a high dose of PGE_1 (10 μg) has a very strong effect on osteogenetic activity when combined with a low dose (1 μg) of rhBMP. It also was noteworthy that the high dose of PGE_1 (10 μg) had a very strong osteoconductive effect when used alone with HAP pellets. The mechanism of this phenomenon is not clear, but we speculate that administration of PGE_1 may result in stimulation of the production of certain cytokines, such as TGFβ or interleukin (IL)-6, which may affect the osteogenic activity of BMP.[41,42]

In the present study, we compared the resistance to breakage of HAP rods after implantation and found that PGE_1 combined with rhBMP-2 or by itself has a direct effect on increasing mechanical strength. In the control group, no evident reduction in strength was seen at 9 weeks after implantation, indicating that HAP provides strong stability as a bone-supporting material. We clearly demonstrated that addition of rhBMP-2 to HAP significantly increases the mechanical strength values obtained in both the three-point bending and compression strength tests. At 6 or 9 weeks, the three-point bending strength and compression strength values in the 1 μg and 5 μg rhBMP-2 groups, respectively, increased about 2.5- and 4-fold compared to those obtained before implantation.[36] Histological examination, however, revealed earlier and greater bone formation in the 5 μg group than in the 1 μg group, so we concluded that immediate and continuous ossification requires the use of larger amounts of rhBMP-2, that is, as much as 5 μg per rod. We also concluded that combining rhBMP-2, even in a small dose, with a porous ceramic material increases the mechanical strength of the material as early as 3 weeks after implantation and offers a potentially useful substitute for autogenous-free bone grafts.

In this study, porous HAP rods also were treated with 1 μg, 10 μg, or 30 μg of PGE_1 with or without 1 μg of rhBMP-2 and subperiosteally implanted on the cranial bone of rabbits to evaluate the alterations of mechanical strength of the implanted HAP rods. The PGE_1 in combination with BMP had a strong and dose-dependent effect on increasing the mechanical strength, especially compression strength, and it also increased mechanical strength even when used alone. Histological examination revealed that PGE_1, alone or combined with BMP, increased bone formation into the pores of HAP and thus increased the mechanical strength of porous HAP. In earlier experiments, 10 μg of PGE_1 exhibited enough osteoinduction histopathologically while 30 μg of PGE_1 were required to show significant mechanical strength differences both alone and in combination with BMP. The reason for the difference in required dose is not clear, but we speculate that the areas of contact of the implanted HAP with the cranial bone were different because the shapes of the implants were different. In any case, it is evident from our results that PGE_1 enhanced the efficacy of the HAP-rhBMP complexes and enabled us to reduce the BMP requirement without using collagen of animal origin. It enhanced mechanical strength, which is one of the very important properties one would expect of compatible substitutes for autogenous-free bone grafts, such as from iliac bone. Furthermore, from the histopathological examinations, the structure of the HAP ceramic which we used was shown to have been significantly improved and highly suitable for facilitating both bone induction and conduction when used in conjunction with BMP and PGE_1.

The rhBMP was donated by Yamanouchi Pharmaceutical Co., Tokyo, Japan, the PGE_1 was donated by Ono Pharmaceutical Co., Japan, and the hydroxyapatite ceramic rods were donated by Asahi Optical Company Ltd., Tokyo, Japan. We would like to express our sincere appreciation to Mr. Takehiko Nakajima from Asahi Optical Company Ltd., Tokyo, Japan.

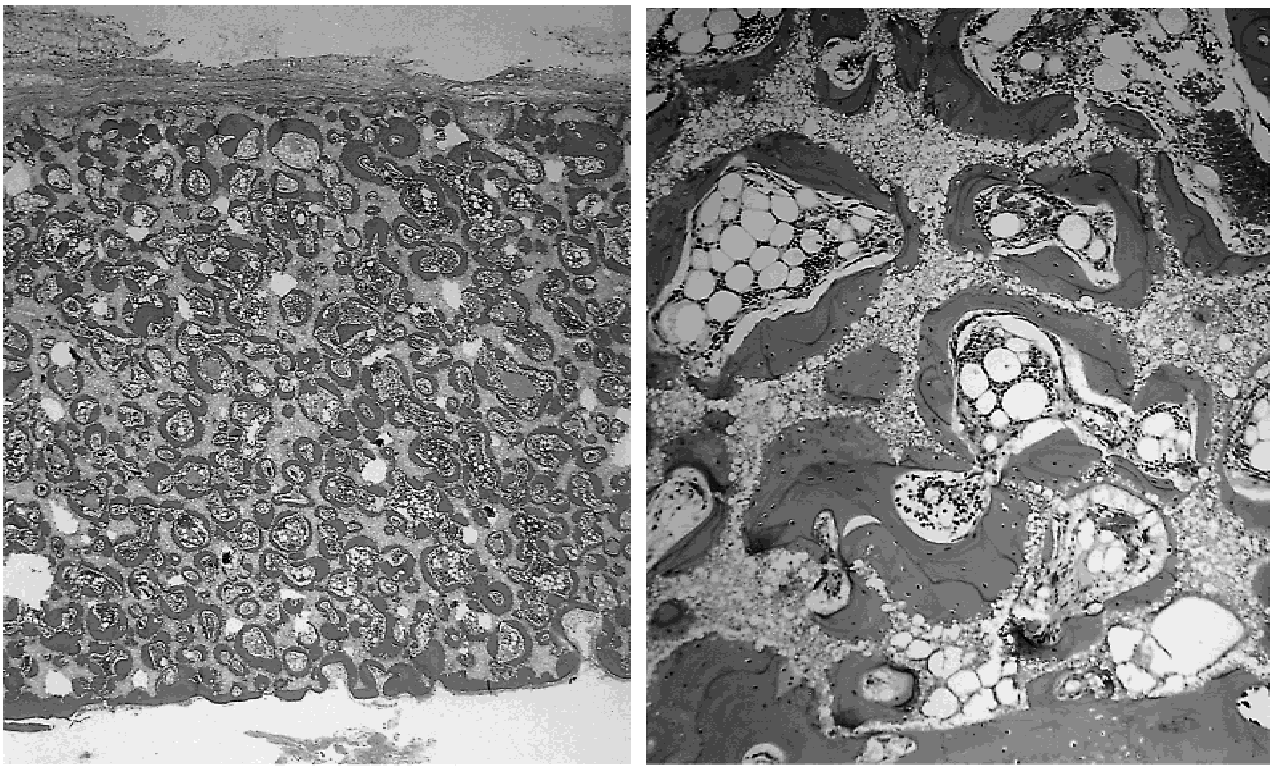

Figure 8. Histological findings of the 30 μg PGE_1 plus 1 μg rhBMP-2 group at 9 weeks (H&E original magnification ×15). Bone formation was found in most of the HAP pores, and bone formation was greater than in the 1 μg rhBMP-2 group and compatible to that of the 5 μg rhBMP-2 group in the same week. (b) Highly magnified image of the same sample (H&E original magnification ×60). Newly formed bone as well as osteocytes and bone-marrow-like structure is observed in the pores.

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