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Ectopic bone formation in mice associated with a lactic acid/dioxanone/ethylene glycol copolymer–tricalcium phosphate composite with added recombinant human bone morphogenetic protein-2

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Abstract

A new putty-like material with bone-inducing capacity was made by combining a block copolymer of poly D,L-lactic acid with randomly inserted *p*-dioxanone and polyethylene glycol (PLA-DX-PEG) and beta-tricalcium phosphate (*β*-TCP) powder with added recombinant human bone morphogenetic protein-2 (rhBMP-2). To optimize the material's efficacy for bone formation, we formulated the optimal composition ratio of the respective constituent that gives the greatest osteoinductive efficacy in a mouse model of ectopic bone formation.

In this series of studies, we investigated the size of ectopic bone mass induced 3 and 6 weeks after implantation of the materials composed of 30 mg of PLA-DX-PEG with 2 μg of rhBMP-2 and 0, 15, 30, or 60 mg of *β*-TCP powder. An additional experiment was designed to investigate how content ratios of *β*-TCP powder in 30 mg-putty implants (0%, 16.7%, 33.3%, 50%, 66.7%, 83.3%, or 100%) for a fixed dose (5 μg) of the rhBMP-2 altered the size of the induced ossicle.

The results from the first experiment indicated that the bone yields were linearly dependent on the amount of additional *β*-TCP powder. In the second experiment, the largest ossicles induced by 5 μg of rhBMP-2 were obtained when the polymer/*β*-TCP ratio was 1/2 in mice. The data provide important insights into the fabrication of implants that provide efficacious delivery of rhBMP-2. The new putty-like material may be valuable for repairing or regenerating bone in a clinical setting.

Keywords: Bone morphogenetic protein (BMP); Biodegradation; Bone; Bone repair; Drug delivery

1. Introduction

Bone morphogenetic proteins (BMPs) produced by DNA recombination technology (rhBMP-2 and rhBMP-7/OP-1) are now used to induce new bone formation for spinal fusion and the repair of non-union fractures in the absence of bone grafting [1–4]. Bio-absorbable collagen from animal sources has been used routinely as a carrier for BMPs [5–8]. However, this type of collagen has significant limitations when it is used as the delivery system for rhBMPs: (1) there is a potential risk for disease transfer, e.g. variants of Creutzfeldt-Jacob disease (vCJD) or other prion-related diseases, (2) immunological reactions from hosts, and (3) limited mechanical strength and difficulty to mold [9–11].

In order to overcome these problems, we have developed a biodegradable synthetic polymer composed of poly D, L-lactic acid with randomly inserted *p*-dioxanone-polyethylene glycol block co-polymer (PLA-DX-PEG) [12,13]. The advantages of this polymer as a carrier material are (1) no risk of disease transfer and (2) adjustable molecular size to control biodegradability, which in turn regulates the release of rhBMPs. In terms of the efficacy of the polymer as a carrier material, we determined the optimal content of the rhBMP-2 in the polymer by using a mouse model of ectopic bone formation [14]. Unfortunately, the highly sticky nature of the polymer presented difficulties in handling and challenges to fabrication.

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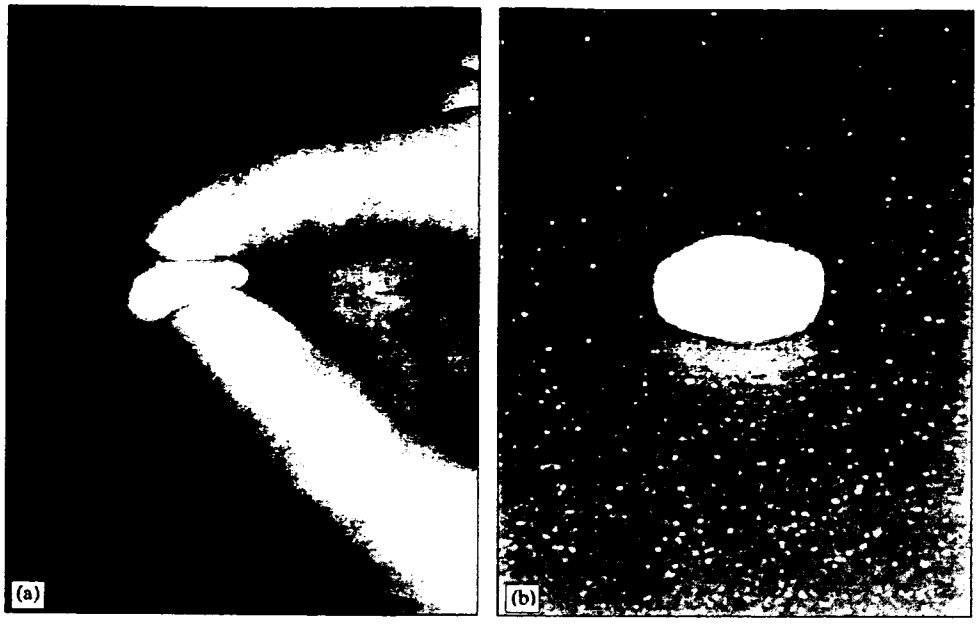

Fig. 1. Gross appearance of (a) a new bone-inducing putty implant comprised of PLA-DX-PEG, β-TCP powder, and rhBMP-2, (b) a sample of the test implants in Study 2. This disk-shaped implant is comprised of PLA-DX-PEG block co-polymer (10 mg), β-TCP powder (20 mg), and rhBMP-2 (5 μg).

In an attempt to increase the plasticity of the polymer for ease of handling, beta-tricalcium phosphate (β-TCP) powder was added to change the sticky polymer gel into a putty-like material (Fig. 1). We have further shown that successful spinal fusion can be achieved by using rhBMP-2-retaining putty implant in an animal model [15]. However, the optimal ratio of putty implant components that gives the greatest osteoinductive efficacy has not been examined. In this series of experimental studies, therefore, we (1) examined the effect of adding the β-TCP powder on the bone-inducing capacity of fixed low dose of rhBMP-2 in the polymer and (2) determined the optimal ratio of the β-TCP powder in the polymer that would give the maximum bone yield by rhBMP-2 in the putty implant.

2. Materials and methods

2.1. RhBMP-2 and carrier materials

RhBMP-2 was produced by Wyeth (Cambridge, MA) and donated to us through Astellas Pharma Inc., (Tokyo, Japan). The rhBMP-2 was supplied in a buffer solution (5 mmol/L glutamic acid, 2.5% glycine, 0.5% sucrose, and 0.01% Tween-80) at a concentration of 3.52 μg/μl after filter sterilization. Poly D,L-lactic acid-*p*-dioxanone-polyethylene glycol block copolymer (PLA-DX-PEG) (MW; 9800, PLA/DX/PEG molar ratio; LA/DX/EO = 42/14/44), was synthesized and provided to us by Taki Chemical Co., Ltd. (Kakogawa, Japan). Details of the physicochemical characteristics of this biodegradable polymer have been reported previously [12]. β-TCP powder (particle size; less than 100 μm in diameter) was manufactured and provided to us by Olympus Biomaterial Corp., (Tokyo, Japan).

2.2. Preparation of test implants

2.2.1. Pellet implants to examine the effect of adding β-TCP powder on a fixed amount of polymer and rhBMP-2 (Study 1)

To examine the effect of adding β-TCP powder on the bone yield arising from a fixed amount of BMP, 30 mg polymer implants with 2 μg rhBMP-2 were fabricated and mixed with 0, 15, 30, or 60 mg of β-TCP powder. The approximate volumes of the implants with 0, 15, 30, and 60 mg of additional β-TCP powder increased from 36 to 54, 72, and 108 mm^3, respectively. Twelve test implants with each of these respective amounts of β-TCP powder were prepared. The details covering implant composition and assignment in each respective group are listed in Table 1.

Table 1
Implant assignment in Study 1

Group	rhBMP-2 (μg)	PLA-DX-PEG (mg)	β-TCP (mg)	Ratio of β-TCP (%)
P30	2	30	0	0
P30/T15	2	30	15	33.3
P30/T30	2	30	30	50
P30/T60	2	30	60	66.7

2.2.2. Pellet implants to examine the effects of changing the β-TCP/polymer ratio in the putty implants with a fixed dose of rhBMP-2 (Study 2)

In order to determine the optimal β-TCP/polymer ratio in the rhBMP-2-retaining putty implants with respect to the induced bone yields, 30 mg-putty implants comprised of varying amounts of polymer and β-TCP powder with a fixed dose (5 μg) of rhBMP-2 were prepared. The content ratio of β-TCP powder in these test implants was 0%, 16.7%, 33.3%, 50%, 66.7%, 83.3%, or 100% (Table 2). The volume of the implants, regardless of composition, was calculated to be approximately 36 mm^3. Twelve test implants at each of the above β-TCP powder percentage content values were prepared and stocked in a freezer at −30 °C until use.

2.3. Animals and surgery

One hundred and thirty-two closed colony male SPF (ICR) mice (Japan SLC Inc., Hamamatsu, Japan), at 6 weeks of age were housed and acclimated in cages with free access to food and water for 1 week.

Experiments were carried out in strict accordance with the Institutional Guidelines for the Care and Use of Laboratory Animals of Osaka City University.

The mice were anesthetized with a subcutaneous injection of ketamine and xylazine, and the rhBMP-2-containing implants were surgically implanted into the left dorsal muscle pouches (one implant per animal). Three or 6 weeks after the surgery, 6 mice from each respective group were euthanized and the BMP-induced ectopic ossicles formed at the implanted sites were harvested and evaluated.

2.4. Evaluation methods

All harvested tissues were fixed with 10% neutral formalin and then radiographed with a soft X-ray apparatus (Sofron Co., Tokyo, Japan). The approximate volume of each rhBMP-induced ossicle with an oval shape was calculated by a formula, $V = ABC\pi/6$ (A = long diameter, B = short diameter, C = thickness).

For histological analysis, fixed specimens were decalcified with a K-CX solution (Astellas Pharma Inc., Tokyo, Japan), dehydrated in a gradient ethanol series, and embedded in paraffin wax. The mid-vertical section and two additional sections, each 1.5 mm apart from the mid-vertical section, were cut into 4 μm thicknesses in each plane, stained with hematoxylin-eosin, and observed under a light microscope. The percentage of new bone area including bone marrow in ossicles containing β-TCP was determined from images captured with a digital camera (DP 70, Olympus Corporation, Tokyo, Japan) using image analysis software (Micro Analyzer, Nihon Poladigital K. K., Tokyo, Japan). The average value of three percentages of new bone area in an ossicle was expressed as a percentage bone area.

Table 2
Implant assignment in Study 2

Group	rhBMP-2 (μg)	PLA-DX-PEG (mg)	β-TCP (mg)	Ratio of β-TCP (%)
P30/T0	5	30	0	0
P25/T5	5	25	5	16.7
P20/T10	5	20	10	33.3
P15/T15	5	15	15	50
P10/T20	5	10	20	66.7
P5/T25	5	5	25	83.3
P0/T30	5	0	30	100

2.5. Statistical analysis

Statistically significant differences in these studies were determined by one way tailed Student's t-test and post hoc testing using the Scheffe method. When P values were less than 0.05, the difference was considered significant.

3. Results

3.1. Effects of adding β-TCP powder on a fixed amount of polymer and rhBMP-2 (Study 1)

Fig. 2 shows the soft X-ray pictures of ossicles harvested 3 and 6 weeks after implantation. Fig. 3 presents the mean volumes and percentage bone areas of ossicles induced by the putty implants comprised of the 30 mg of PLA-DX-PEG with 2 μg rhBMP-2 and 0, 15, 30, or 60 mg of β-TCP. The ossicle sizes harvested at 3 and 6 weeks in each group indicated no distinct difference in each group, although the radio-densities of the ossicles appeared to be reduced during the period between weeks 3 and 6 post-implantation. The volume of the induced ossicles appeared to increase linearly, depending on the amount of added β-TCP powder with no significant difference observed in the mean percentage bone area within the ossicles from each respective group.

On histological evaluation of the ossicles harvested at 6 weeks from each group, new bone with hematopoietic marrow formation was consistently detected almost evenly on the section of the pellet, although residual β-TCP was noted in ossicles by an implant added containing 60 mg of β-TCP powder (Fig. 4).

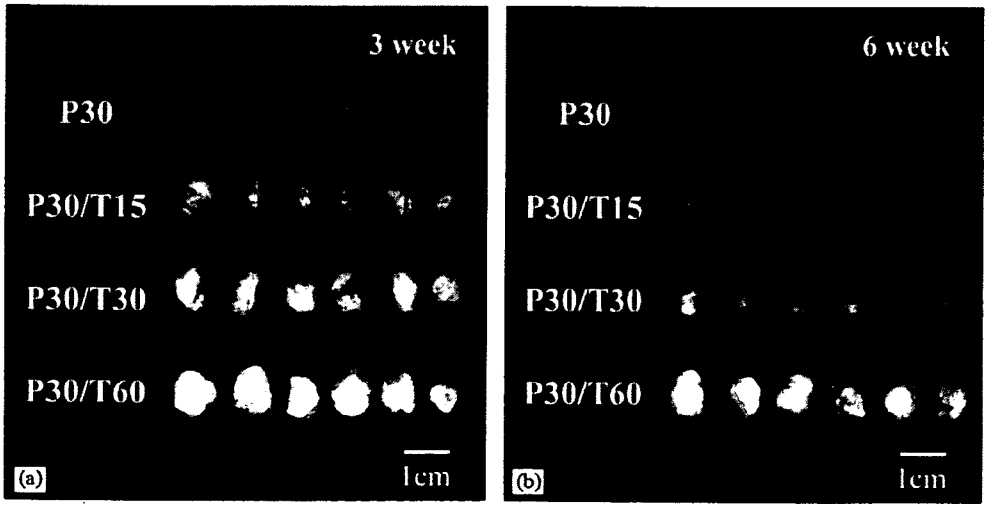

Fig. 2. Study 1: Soft X-ray photographs of ectopically induced ossicles by 2 μg of rhBMP-2. P30 indicates the amount of polymer (30 mg), and /T15-60 indicates the original amount of β-TCP powder (15–60 mg) added to the 30 mg of polymer. (a) Ossicles harvested 3 weeks after implantation and (b) 6 weeks after implantation.

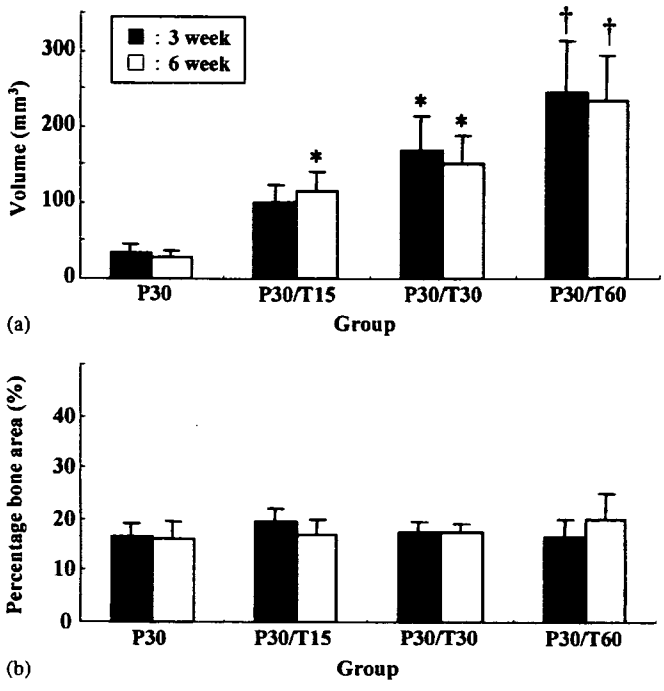

Fig. 3. Study 1: (a) Volumes of harvested ossicles induced by 2 μg of rhBMP-2 retained in implants comprised of 30 mg of polymer and 0–60 mg amounts of β-TCP powder. (b) Percentage bone areas on histological sections of induced ossicles from each group. $^{*}p<0.05$ (compared to group P30), $\dagger p<0.05$ (compared to group P30, P30/T15, and P30/T30). The volume of the induced ossicles appeared to increase linearly depending on the amount of added β-TCP powder with no significant difference observed between the groups in terms of the mean percentage bone areas in the ossicles based on histology.

3.2. Effects of changing the β-TCP/polymer ratio in the putty implants with a fixed dose of rhBMP-2 (Study 2)

Fig. 5 shows the soft X-ray pictures of ossicles harvested at 3 and 6 weeks after implantation of the putty pellets with various β-TCP/polymer ratios. Fig. 6 presents mean volumes and percentage bone areas of ossicles induced by 5 μg of rhBMP-2 in putty pellets having the various combination ratios of polymer and β-TCP powder. The mean induced-ossicle volume increased with the increase of β-TCP powder amount up to 66.7% and then decreased in the groups with a higher β-TCP powder ratio; however, there was no significant difference in the percentage bone area on histology in each group. According to these results, the putty implants containing 66.7% of β-TCP powder produced the greatest volume of new bone mass.

Upon histological analysis of the ossicles induced by the implants with lower ratios of β-TCP powder, most of the β-TCP powder was absorbed and little if trace of this material remained 3 weeks after implantation. Significant amounts of β-TCP remained in the ectopic new bone from groups of P15/T15, P10/T20, P5/T25, and P0/T30. Six weeks after implantation, bone mass sizes remained constant but the amounts of residual β-TCP were reduced (Fig. 7).

4. Discussion

The efficacy of the PLA-DX-PEG polymer as a carrier material for the rhBMP-2 has been reported previously and

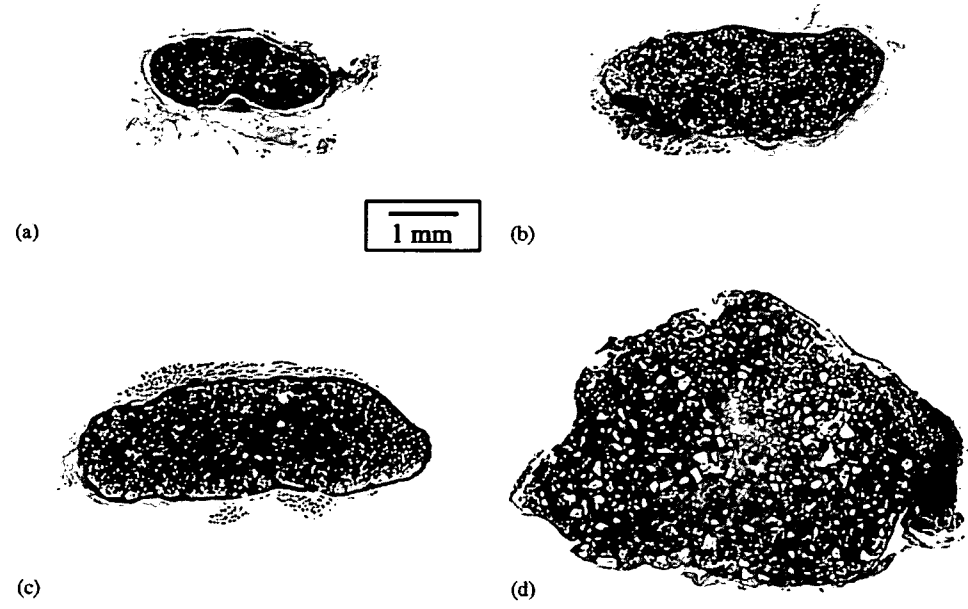

Fig. 4. Decalcified histological sections of ossicles stained with hematoxylin-eosin. Ectopic ossicles induced in 6 weeks after implantation by 2 μg of rhBMP-2 retained in 30 mg of polymer (a), 30 mg polymer and 15 mg (b), 30 mg (c), or 60 mg (d) of β-TCP powder. New bone with hematopoietic marrow formation was detected consistently and almost evenly on sections from the pellets in each group. Remnants of β-TCP powder are seen within the ossicle induced by an implant containing 60 mg of β-TCP powder (d).

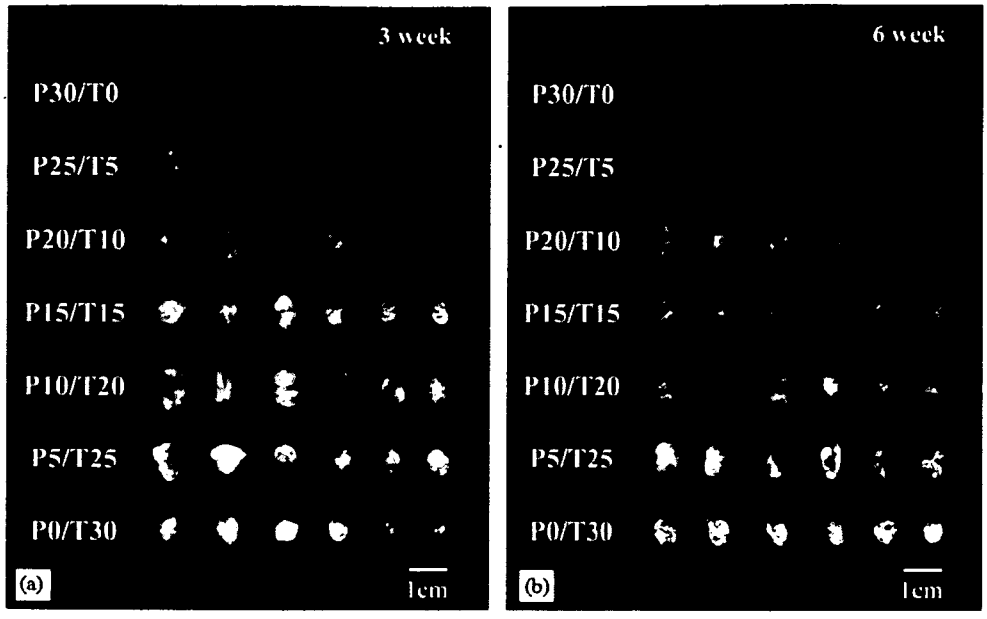

Fig. 5. Study 2: Soft X-ray photographs of ectopic ossicles induced by 5 μg of rhBMP-2 retained in 30 mg of polymer/β-TCP composite carrier. P30/T0 indicates a polymer (30 mg) with no β-TCP (mg). P25/T5 indicates the carrier material was comprised of 25 mg of polymer and 5 mg of β-TCP in the implants and so on. (a) Ossicles harvested 3 weeks after implantation and (b) 6 weeks after implantation. Relatively larger ossicle sizes are noted in the P15/T15 and P10/T20 groups.

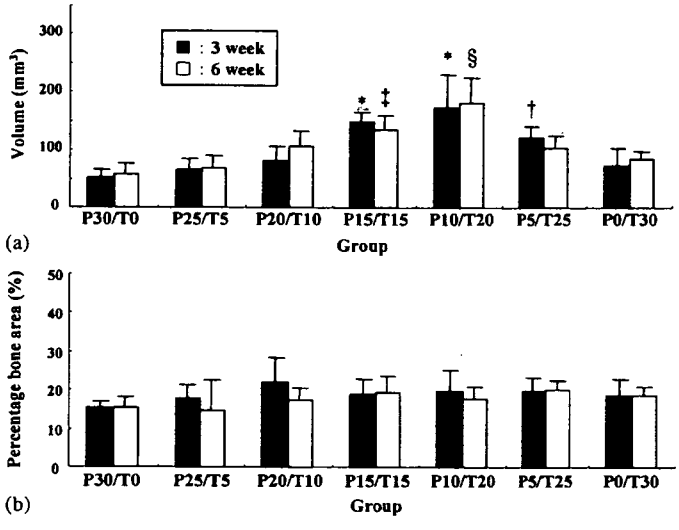

Fig. 6. Study 2: (a) Volumes of ossicles and (b) percentage bone areas on the histological sections of the ossicles harvested from each respective group at 3 and 6 weeks after implantation. $^{*}p<0.05$ (compared to group P30/T0, P25/T5, P20/T10, and P0/T30). $\dagger p<0.05$ (compared to group P30/T0), $\ddagger p<0.05$ (compared to group P30/T0, P25/T5), $\S p<0.05$ (compared to group P30/T0, P25/T5, P20/T10, P5/T25, and P0/T30). The maximum ossicle size induced in mice by 5 μg of rhBMP-2 was obtained by group P20/T10. There was no significant difference both in terms of the volumes and percentage bone areas between those at 3 weeks and at 6 weeks within the respective groups.

the optimal dose of rhBMP-2 in terms of bone yield has also been determined [14,16–18]. Through the addition of the β-TCP powder to the BMP-retaining polymer, the aims of this study were to (1) overcome the difficulties in handling and fabrication due to the very sticky gel nature of the polymer and (2) increase the induced bone yields by volume expansion of the rhBMP-2 containing polymer implants and subsequent improvement in performance of the rhBMP-2.

The results of the study indicated that both of these problems could be resolved by adding β-TCP powder in an amount equal to or 2 times greater than the amount of rhBMP-2 containing polymer. The amounts of β-TCP used in these studies converted the polymer to putty-like material that was easy to handle and mold, and also increased the BMP-induced bone yield. There were few adverse effects of β-TCP addition on the bone-inducing capacity of rhBMP-2 because new bone formation was consistently noted despite the effective dilution of the rhBMP-2 content in the implants by β-TCP addition. The sizes of the β-TCP including bone mass increased, although the bone areas excluding β-TCP areas on histological sections did not change.

The experiments to optimize the ratio of β-TCP to polymer/rhBMP-2 composite with a fixed dose (5 μg) of rhBMP-2 showed that bone yields could be increased by use of both the polymer and β-TCP; the experiments also showed that the optimized β-TCP/polymer ratio was 1/1 or 2/1.

Previously, we had shown that the rhBMP-2 dose required to elicit 1 cm^3 of new bone in mice was approximately 48 μg when use in conjunction with the polymer alone [14]. In this study, with the β-TCP/polymer carrier, the maximal bone mass induced by 2 μg of rhBMP-2 was approximately 230 mm^3; that is, the rhBMP-2 dose required to elicit 1 cm^3 of new bone was calculated to be approximately 9 μg. These data indicate that there was

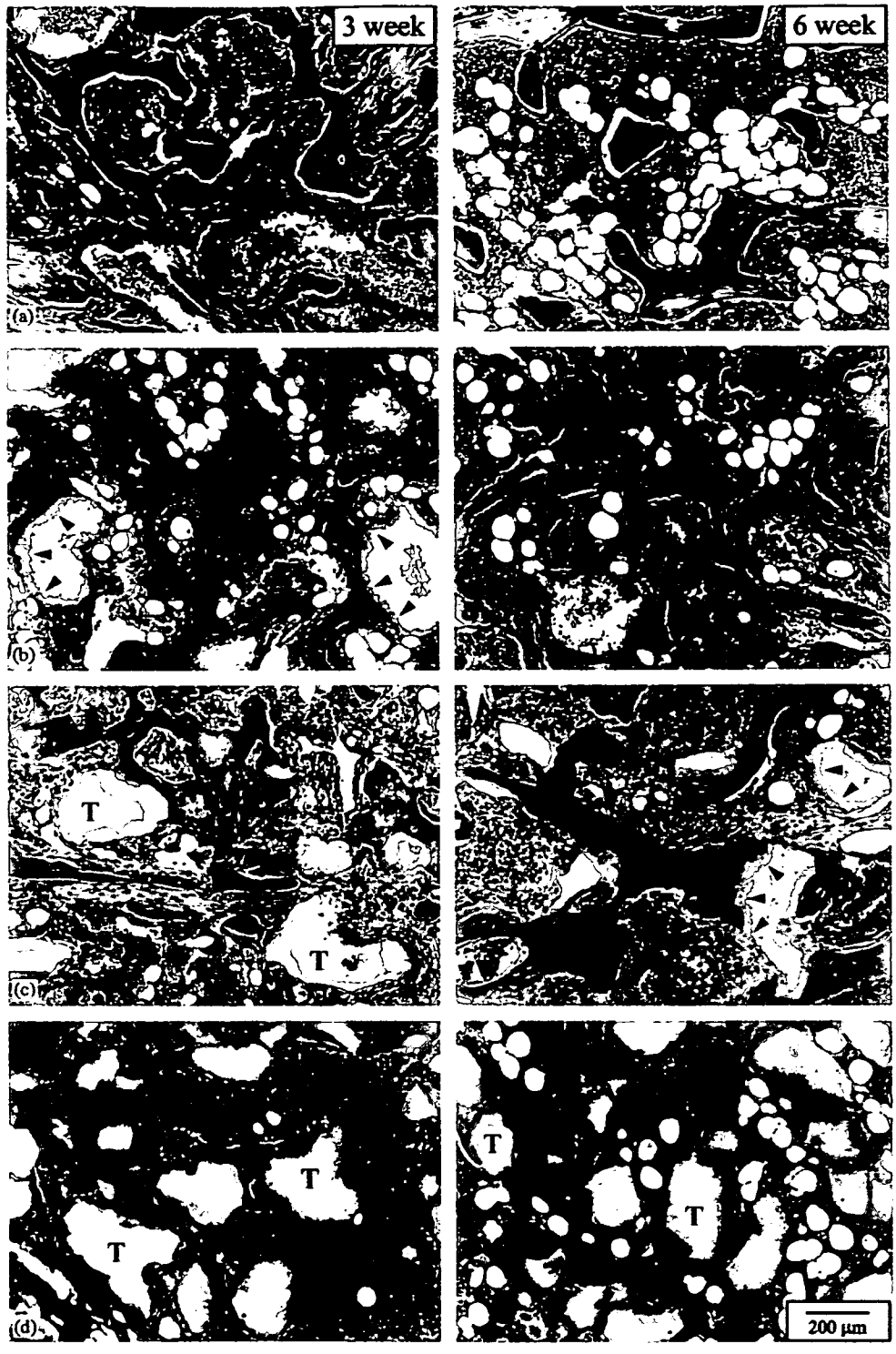

Fig. 7. Histology of ossicles (hematoxylin-eosin stain) induced ectopically by 5 μg of rhBMP-2 in combination with 30 mg of carrier with various composition ratios (polymer/β-TCP): (a) P30/T0, (b) P20/T10, (c) P10/T20, and (d) P0/T30. Left side: ossicles harvested at 3 weeks, and right side: at 6 weeks. New bone formation was detected consistently in all groups. At 3 weeks, traces of β-TCP powder remained in (b) and more β-TCP remained within the ossicles (c and d) where higher ratios of β-TCP were contained. At 6 weeks, the amounts of residual β-TCP were reduced in the ossicles harvested by group P20/T10 (b) and P10/T20 (c). B: bone marrow, T: β-TCP, Arrow head: the remaining β-TCP.

improved performance of rhBMP-2. However, the results from the current study could not be extrapolated to humans due to the low responsiveness to rhBMP-2 in highly evolved species including humans [19,20]. To determine the optimal dose of rhBMP-2, experiments in primates as well as safety checks of the delivery system will be necessary before this new bone-inducing biomaterial can be used in the clinic.

During the period between 3 and 6 weeks after implantation, radiological and histological analyses

indicated that the amount of β-TCP contained within ossicles decreased. The degradation of the β-TCP appeared to be accelerated when implanted with rhBMP-2. We speculate that this might be due to the recruitment of osteoclasts from the precursor cells residing in bone marrow, which was formed in the BMP-induced ossicles as shown previously [21]. The osteoclasts, stimulated by BMP, may be responsible for the enhanced resorption of the β-TCP [22,23]. This enhanced resorption, when used with rhBMP-2, might be an additional advantage of the bone-inducing putty implant. Remodeling and restoration of new bone with normal anatomy and mechanical strength might occur more rapidly under these conditions.

The malleable nature of the putty implant material makes it easier to apply at sites of bone reconstruction or repair. This may be particularly useful in situations where there is a need to re-establish as quickly as possible the normal anatomy and load-bearing capacity of bone. Surgeries involving bone and joint replacement are clearly situations where restoration of mechanically competent bone, often in the presence of prosthesis, is the desired clinical outcome. The expansion properties of the putty-like material could facilitate the integration of new bone growth with those devices.

5. Conclusion

The characteristics of a new bone-inducing putty implant comprised of PLA-DX-PEG block co-polymer, β-TCP powder, and low dose of rhBMP-2 are described in this paper. The addition of the β-TCP powder to the polymer resulted in the formation of a putty-like material with superior handling, fabrication, and osteoinductive property. The optimal ratio of β-TCP/polymer ranged from 1/1 to 2/1 in order to obtain the maximal bone yield from a fixed low dose of rhBMP-2. The rhBMP-2-bearing putty implant is potentially valuable in future clinical settings.

Acknowledgments

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Reference

[1] Wozney JM, Rosen V, Celeste AJ, Mitsock LM, Whitters MJ, Kriz RW, et al. Novel regulators of bone formation: molecular clones and activities. Science 1988;242:1528–34.

[2] Wang EA, Rosen V, D'Alessandro JS, Bauduy M, Cordes P, Harada T, et al. Recombinant human bone morphogenetic protein induces bone formation. Proc Natl Acad Sci USA 1990;87:2220–4.

[3] Takaoka K, Yoshikawa H, Hashimoto J, Miyamoto S, Masuhara K, Nakahara H, et al. Purification and characterization of a bone-inducing protein from a murine osteosarcoma (Dunn type). Clin Orthop Relat Res 1993;292:329–36.

[4] Takaoka K, Yoshikawa H, Hasimoto J, Masuhara K, Miyamoto S, Suzuki S, et al. Gene cloning and expression of a bone morphogenetic protein derived from a murine osteosarcoma. Clin Orthop Relat Res 1993;294:344–52.

[5] Govender S, Csimma C, Genant HK, Valentin-Opran A, Amit Y, Arbel R, et al. Recombinant human bone morphogenetic protein-2 for treatment of open tibial fractures: a prospective, controlled, randomized study of four hundred and fifty patients. J Bone Joint Surg Am 2002;84-A:2123–34.

[6] Burkus JK, Dorchak JD, Sanders DL. Radiographic assessment of interbody fusion using recombinant human bone morphogenetic protein type 2. Spine 2003;28:372–7.

[7] Geesink RG, Hoefnagels NH, Bulstra SK. Osteogenic activity of OP-1 bone morphogenetic protein (BMP-7) in a human fibular defect. J Bone Joint Surg Br 1999;81:710–8.

[8] Johnsson R, Stromqvist B, Aspenberg P. Randomized radiostereometric study comparing osteogenic protein-1 (BMP-7) and autograft bone in human noninstrumented posterolateral lumbar fusion: 2002 Volvo Award in clinical studies. Spine 2002;27:2654–61.

[9] Bach FH, Fishman JA, Daniels N, Proimos J, Anderson B, Carpenter CB, et al. Uncertainty in xenotransplantation: individual benefit versus collective risk. Nat Med 1998;4:141–4.

[10] Butler D. Last chance to stop and think on risks of xenotransplants. Nature 1998;391:320–4.

[11] DeLustro F, Dasch J, Keefe J, Ellingsworth L. Immune responses to allogeneic and xenogeneic implants of collagen and collagen derivatives. Clin Orthop Relat Res 1990;260:263–79.

[12] Saito N, Okada T, Horiuchi H, Murakami N, Takahashi J, Nawata M, et al. A biodegradable polymer as a cytokine delivery system for inducing bone formation. Nat Biotechnol 2001;19:332–5.

[13] Saito N, Takaoka K. New synthetic biodegradable polymers as BMP carriers for bone tissue engineering. Biomaterials 2003;24:2287–93.

[14] Kato M, Toyoda H, Namikawa T, Hoshino M, Terai H, Miyamoto S, et al. Optimized use of a biodegradable polymer as a carrier material for the local delivery of recombinant human bone morphogenetic protein-2 (rhBMP-2). Biomaterials 2006;27:2035–41.

[15] Namikawa T, Terai H, Suzuki E, Hoshino M, Toyoda H, Nakamura H, et al. Experimental spinal fusion with recombinant human bone morphogenetic protein-2 delivered by a synthetic polymer and beta-tricalcium phosphate in a rabbit model. Spine 2005;30:1712–6.

[16] Saito N, Okada T, Horiuchi H, Murakami N, Takahashi J, Nawata M, et al. Biodegradable poly D,L-lactic acid-polyethylene glycol block copolymers as a BMP delivery system for inducing bone. J Bone Joint Surg Am 2001;83-A:S92–8.

[17] Saito N, Okada T, Toba S, Miyamoto S, Takaoka K. New synthetic absorbable polymers as BMP carriers: plastic properties of poly D,L-lactic acid-polyethylene glycol block copolymers. J Biomed Mater Res 1999;47:104–10.

[18] Murakami N, Saito N, Takahashi J, Ota H, Horiuchi H, Nawata M, et al. Repair of a proximal femoral bone defect in dogs using a porous surfaced prosthesis in combination with recombinant BMP-2 and a synthetic polymer carrier. Biomaterials 2003;24:2153–9.

[19] Valentin-Opran A, Wozney J, Csimma C, Lilly L, Riedel GE. Clinical evaluation of recombinant human bone morphogenetic protein-2. Clin Orthop Relat Res 2002;395:110–20.

[20] McKay B. Commercial approval of rhBMP-2 in spinal fusions: bringing the product to the market. In: Shandell LJ, Grodininsky AJ, editors. Tissue engineering in musculoskeletal clinical practice. USA: American Academy of Orthopaedic Surgeons; 2004. p. 61–71.

[21] Matsushita N, Terai H, Okada T, Nozaki K, Inoue H, Miyamoto S, et al. A new bone-inducing biodegradable porous beta-tricalcium phosphate. J Biomed Mater Res A 2004;70A:450–8.

[22] Kanatani M, Sugimoto T, Kaji H, Kobayashi T, Nishiyama K, Fukase M, et al. Stimulatory effect of bone morphogenetic protein-2 on osteoclast-like cell formation and bone-resorbing activity. J Bone Miner Res 1995;10:1681–90.

[23] Kaneko H, Arakawa T, Mano H, Kaneda T, Ogasawara A, Nakagawa M, et al. Direct stimulation of osteoclastic bone resorption by bone morphogenetic protein (BMP)-2 and expression of BMP receptors in mature osteoclasts. Bone 2000;27:479–86.

骨粗鬆症性椎体骨折後偽関節例に対するCPCを用いた椎体形成術

—バルーンと内視鏡の応用—

星野雅俊[1)]，中村博亮[2)]，小西定彦[2)]，寺井秀富[1)]，辻尾唯雄[1)]，高岡邦夫[1)]

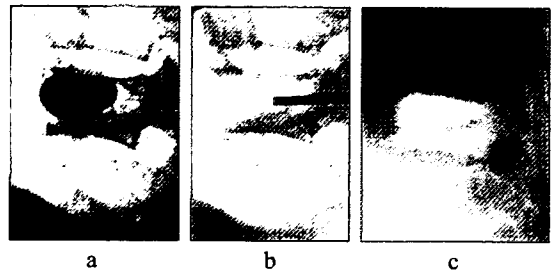
我々は，骨粗鬆症性椎体骨折の偽関節例に対して，椎体内壊死組織の掻爬と充分なcalcium phosphate cement（CPC）挿入腔の形成を目的として，ウロマチックバルーンと内視鏡を応用した椎体形成術を行ってきた[1)]．今回は，この術式の適応と手術方法を紹介し，その治療成績，特に術後1年以上経過観察が可能であった症例群のX線学的所見を報告する．

対象および方法

2003年3月より2006年1月までに当院で椎体形成術を行った骨粗鬆症性椎体骨折後偽関節症例14例15椎体を対象とした．男性は3例で，女性11例であり，平均年齢は73歳（65～87歳）であった．椎体骨折発生から手術までの期間は平均7ヵ月（3～13ヵ月），罹患椎はTh12が8椎体，L2が3椎体，L1が2椎体，Th9，L4がそれぞれ1椎体であった．適応は，明らかな麻痺がなく，遷延する強い腰背部痛のためADLを高度に制限されている例とした．偽関節の画像診断は，動態レントゲン側面像，特に臥位後屈像と立位前屈像とで前方椎体高に明らかな差のあるもの，MRI T2強調画像において椎体内および高輝度を示す液体成分の貯留の確認ができるものとした．

臨床的検討項目は，手術時間，出血量，合併症，VASおよび歩行状態の術前後の変化とした．また，1年以上経過を追跡できた7例8椎体に対して，X線学検討を行った．%椎体高を，圧迫骨折椎前縁の椎体高を上下正常椎体高の平均で除した割合で求め，術前，術直後，術後1週，1ヵ月，3ヵ月，6ヵ月，1年の推移を検討した．また，椎体周囲の骨形成確認時期と，隣接椎骨折の発生を調べた．

手術手技は，全身麻酔下で行い，両側椎弓根上に小皮切（2cm）を加える．イメージ下に経椎弓根的にウロマチックバルーンを椎体内へ挿入して膨らませ，空隙を形成する（図1）．膝用30°あるいは70°斜視鏡下に，鉗子で偽関節腔内組織の掻爬を行う（図2）．これを両側椎弓根孔から行い，造影剤で椎体外への漏れがないことを確認した後，CPCを注入する．粉液比は4とし，粘度をあげる．



a　b　c

図1　a，b　バルーンによる椎体内の空隙作成
c　CPC充填

a　b

図2　a　内視鏡視下椎体内結合組織切除　b　内視鏡視像　結合組織を認める

Vertebroplasty for pseudoarthrosis following osteoporotic vertebral fracture—Use of urinary balloon catheter and endoscope— : Masatoshi HOSHINO et al. (Department of Orthopaedic Surgery, Osaka City University Graduate School of Medicine)

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Key words : Vertebroplasty, Endoscope, Balloon

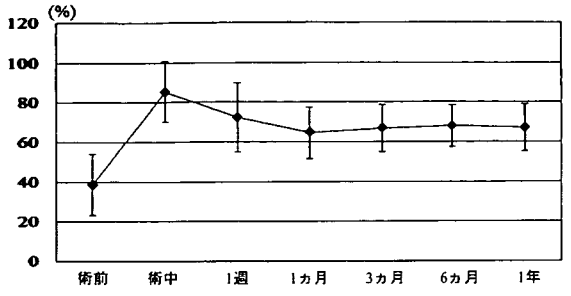

図3 %椎体高の推移

結　　果

手術時間は平均173分（102～215分），出血量は平均100ml（10～250ml）．肺塞栓症状や術中低血圧などの全身合併症は認めらなかったが，操作中に骨脆弱性のため椎弓根内側壁を穿破した症例が1例あり，セメント注入時に，この部からの脊柱管へのセメント漏出がみられた．椎弓切除を追加しCPCを摘出した．その他，感染や再手術は認めなかった．疼痛のVisual Analogue Scaleは術前平均85が術後平均19に有意に減少した．ADLは，術前10例が車イス移動あるいはねたきりの歩行不能で，4例が介助歩行（杖あるいは歩行器）であったが，術後，6例において独歩が可能となり，8例において介助歩行が可能となった．X線的検討で%椎体高は平均で，術前38%，術中85%，術後1ヵ月61%，術後1年で61%であり，矯正損失を術後1ヵ月まで認めたが，その後変形は進行せず安定化した（図3）．椎体周囲の骨形成はレントゲン上，8椎体中7椎体に確認できた．うち3椎体は罹患椎の前壁に限局した骨形成が平均12ヵ月で認められ，4椎体は隣接椎との骨性架橋形成が平均20ヵ月で認められた．隣接椎骨折は8例中2例に認めたが，いずれも腰背部痛は軽度で保存治療で治癒した．

考　　察

偽関節腔内には線維組織を主体とした不良肉芽組織が存在した[2]．関節鏡を応用し鏡視下に椎体偽関節内壊死組織の掻爬を行うことで，生体親和性の良いCPCと母床骨を直接結合させれば，早期に椎体安定性が獲得でき，矯正の保持に有利と考える．海外では，特殊なballoonが開発され，balloon kyphoplastyとして広く臨床適応されているが，その使用は欧米，韓国などでのみ使用が許可されており，本邦では使用が不可能である[3)~5)]．我々は，汎用されているウロマチックバルーンカテーテルを主に内視鏡視用の空隙の作成を目的に使用した．矯正損失を術後1ヵ月まで認めたがその後変形は進行せず安定化した．矯正損失は主に母床骨の圧潰のためであり，CPC硬化体の形状はほぼ維持されていた．本法では，特殊な器具を用いず既存の器具の応用で椎体内の挿入腔の形成と椎体内の掻破を安全に行うことができた．椎体内に充分量のCPCを挿入でき，高い硬化強度が得られたと考える．

ま　と　め

本法は骨粗鬆症性椎体骨折偽関節例に対して有用な手技であった．椎体の安定化は1年以上にわたって維持された．

文　　献

1) Hoshino M, Nakamura H, Konishi S, et al. Endoscopic vertebroplasty for the treatment of chronic vertebral compression fracture. J Neurosurg Spine In press.
2) Hasegawa K, Homma T, Uchiyama S, et al. Vertebral pseudoarthrosis in the osteoporotic spine. Spine 1998；23：2201-2206.
3) Lieberman IH, Dudeney S, Reinhardt MK, et al. Initial outcome and efficacy of "kyphoplasty" in the treatment of painful osteoporotic vertebral compression fractures. Spine 2001；26：1631-1638.
4) Coumans JV, Reinhardt MK, Lieberman IH. Kyphoplasty for vertebral compression fractures: 1-year clinical outcomes from a prospective study. J Neurosurg Spine 2003；99：44-50.
5) Kasperk C, Hillmeier J, Noldge G, et al. Treatment of painful vertebral fractures by kyphoplasty in patients with primary osteoporosis: a prospective nonrandomized controlled study. J Bone Miner Res 2005；20：604-612.

A Biodegradable Delivery System for Antibiotics and Recombinant Human Bone Morphogenetic Protein-2: A Potential Treatment for Infected Bone Defects

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ABSTRACT: To produce an osteogenic and bacteriocidal biomaterial for the treatment of infected nonunions or bone defects, a synthetic degradable block copolymer of poly-D,L-lactic acid segments with randomly inserted *p*-dioxanone and polyethylene glycol (PLA-DX-PEG) segments was mixed with recombinant human BMP-2 (rhBMP-2) and antibiotics at high concentration. We then examined the in vitro elution profile of an antibiotic (teicoplanin) from the polymer, the effects of antibiotics on the bone-inducing capacity of rhBMP-2 or on ectopic new bone formation induced by the rhBMP, and the ability of the polymer to repair bone in a rat cranial defect model. Approximately 40% of teicoplanin was released within the first 24 h, with the remaining amount released steadily over 21 days with no loss of antibacterial activity. The polymer had disappeared by degradation in the phosphate buffered saline (pH 7.4) at the end of the incubation period. The in vivo performance of pellets with antibiotics and rhBMP-2 revealed no significant change in bone yield within the ossicles after 3 weeks. Also, antibiotics had no inhibitory effect on the ability of rhBMP2 to repair cranial defects. Indeed, when the defect was filled by a polymer disc loaded with rhBMP-2 with or without teicoplanin, the defect was repaired by new bone, and normal anatomy was restored within 6 weeks. In conclusion, the PLA/DX/PEG polymer appears to work as effectively for antibiotics as it does for rhBMP-2. Additionally, the biological activity of rhBMP-2 was retained irrespective of the presence of antibiotics. © 2006 Orthopaedic Research Society. Published by Wiley Periodicals, Inc. J Orthop Res 24:327–332, 2006

Keywords: biodegradable delivery system; antibiotics; human bone protein; bone defects

INTRODUCTION

High-energy injuries to the extremities often result in open comminuted fractures. This injury is often associated with severe soft tissue damage, bone defects, infection, and ultimately nonunion. Standard treatment for these cases includes removal of the abscess and necrotic tissue including necrotic bony fragments, local and/or systemic administration of antibiotics sensitive against the infectious micro-organism, and reconstruction of the damaged bone or nonunion by bone grafting, bone transport, shortening of the bone, or implantation of biomaterials. However, this treatment course can take several months to years, and the outcome is often unsatisfactory. This may be due to difficulty in controlling the infection, to delay in reconstruction of the damaged bone (which usually is undertaken only after complete eradication of the infection), or to slow new bone or callus formation during the repair of the damaged bone. Therefore, more effective methods of eradicating infection while promoting local new bone formation could improve treatment.

The selection and concentration of the antibiotics used to treat the infection are important. The

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bacteriocidal or bacterostatic effect of the antibiotic generally depends on the local concentration of the antibiotics. Therefore, a local delivery system enabling a sustained high concentration of antibiotics might be an effective way to control the infection. The treatment of infected hip or knee replacment cases with polymethylmethacrylate (PMMA) cement beads bearing a high dose of antibiotics has proven useful.[1–3] However, PMMA comes with a potential risk for irritating host tissue and for reestablishment of new infection[4] due to its limited biocompatiblity and nonresorbable nature. To overcome this difficulty, a system that can deliver antibiotics locally and that is biodegradable and compatible will be required.

Another major goal in the treatment of infected fractures with bone defects or nonunion is the promotion of new bone or callus formation. Bone morphogenetic proteins (BMPs) are now well established as efficacious bone-inducing molecules. In particular, BMP-2 and BMP-7 (OP-1) synthesized by DNA recombination (recombinant BMP-2 and -7 of human type; rhBMP-2 and rhBMP-7) are available to promote local new bone formation in clinical practice. Collagen is used with these proteins as a carrier material despite the potential risks of the collagen donor source eliciting an immunological response from the host or transferring diseases. To avoid those risks, we recently developed a new biodegradable hydrogel polymer, consisting of poly-D,L-lactic acid with randomly inserted *p*-dioxanone/polyethylene glycol to form a (PLA-DX-PEG) block copolymer, that works effectively as carrier material for the BMP. We speculated that this polymer, which has good biocompatibility and degradation characteristics, might also be useful for the sustained local delivery of high dose antibiotics. Furthermore, by concomitant addition of the rhBMP, a new bioactive material with antibiotic and osteogenic capacity would result. To develop this biomaterial further, we asked the following questions: (1) what is the release profile of antibiotics from the polymer? (2) How do the antibiotics affect the osteogenic capacity of the BMP? and, (3) Is the antibiotic/rhBMP/polymer composite implant able to repair a bone defect?

MATERIALS AND METHODS

PLA-DX-PEG Polymer, Antibiotics, and Recombinant Human BMP-2

Poly-D,L-lactic acid–*p*-dioxanone–polyethylene glycol block copolymer (PLA-DX-PEG; MW; 9800, PLA/DX/PEG molar ratio; 5/1/3) was synthesized and provided to us by Taki Chemicals Co. (Kakogawa, Japan). The polymer has a hard gel character at room temperature, turns to a soft gel at 50°C, and degrades in buffered saline over 2 to 3 weeks at 37°C. The chemical and biological characteristics of this polymer have been described previously.[5]

RhBMP-2 was produced by the Genetics Institute (Cambridge, MA) and donated to us through Yamanouchi Pharmaceutical Co. (Tokyo, Japan). The rhBMP-2 was supplied in a buffer solution (5 mmol/L glutamic acid, 2.5% glycine, 0.5% sucrose, and 0.01% Tween-80) at a concentration of 1 μg/μL after filter sterilization.

Antibiotics for medical use were obtained commercially: cefazolin (CEZ), ampicillin (ABPC), dibecacin (DKB), vancomycin (VCM), teicoplanin (TEIC), and minocycline (MINO).

Release Profile of Antibiotics from the Degradable Polymer

Teicoplanin was used to investigate how an antibiotic is released from the PLA-DX-PEG polymer at 37°C under in vitro conditions. The PLA/DX/PEG polymer block (30 mg) containing 4 μg of teicoplanin was prepared as descrived above. The composite was placed in a siliconized plastic vial tube containing 1 mL of PBS, and incubation started at 37°C. The vial tube was centrifuged and decanted to obtain supernatant for the measurement of the teicoplanin that had been eluted into PBS. One milliliter of PBS was then added to the vial tube with polymer precipitate, and incubation continued at 37°C. This procedure was repeated at scheduled time points (0, 0.5, 1, 3, 7, 10, 14, and 21 days) after starting incubation. Supernatant samples collected at each time point were bioassayed to estimate the amount and bacteriocidal action of teicoplanin released from the polymer.

To quantify the amount of released teicoplanin and to confirm its bacteriocidal activity, the concentrations in the supernatant samples were measured by bioassay.[6] Briefly, *Bacillus subtilis* ATCC 6633 spore suspensions (3.7×10^8 CFU/mL) were seeded on gel sheet culture media in assay plates, and 30 μL of the sample added to small wells made in the culture gel. Plates were incubated at 35°C for another 18 h, and the area of bacterial growth inhibition around each respective well was calculated using fine calipers. The teicoplanin content in 30 μL of sample in each well was determined from a standard curve using a log scale. Values were obtained for all samples and plotted in a time sequence.

Bone Inducing Capacity of Antibiotics/rhBMP-2/Polymer Composite Implants

To prepare a single implant, 30 mg of the PLA-DX-PEG polymer was softened by heating to 50°C and mixed with the rhBMP-2 solution (5 μg/5 μL to each implant)

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Table 1. Antibiotics and the 90% Minimal Inhibitory Concentration (MIC_{90}) for Methicillin Sensitive *Staphyloococcus aureus*

Antibiotics	Ampicillin	Cefazolin	Dibekacin	Vancomycin	Teicoplanin	Minocycline
Abbreviation	ABPC	CEZ	DKB	VCM	TEIC	MINO
Family	Penicilin	1st Cefem	Aminoglicoside	Peptide	Peptide	Tetracyclin
MIC_{90} (μg/mL)	6.25	0.78	0.78	0.78	1.56	0.39

and an antibiotic at a concentration equivalent to 1×10^4 MIC_{90} of *Staphyloococcus aureus* (Table 1).[7] The mixture was then cooled and fabricated into a disc (30 mg, 6 mm diameter). Rh/BMP-2/polymer composite implants without antibiotics were made in the same manner and used as controls. All procedures were carried out under sterile conditions.

The effects of the antibiotics on the bone-inducing capacity of rhBMP-2 were examined in a model of ectopic bone formation by implanting the rhBMP retaining discs into the dorsal muscles of mice. Forty-nine male ICR mice (6 weeks old; Nippon SLC, Hamamatsu, Japan) were divided equally into seven groups to test six antibiotics (Table 1). The mice were anesthetized with an intramuscular injection of ketamine (30 mg/kg) and xylazine (10 mg/kg). The rhBMP-2 retaining PLA-DX-PEG polymer discs with or without antibiotics were surgically implanted into the left dorsal muscle pouches (one pellet per animal). Three weeks after surgery, the mice were sacrificed, and the implants harvested to examine for bone formation.

All harvested tissues were radiographed with a soft X-ray apparatus. The bone mineral content (BMC) (milligrams per ossicle) of each ossicle was measured by dual-energy X-ray absorptiometry (DXA) using a bone mineral analyzer (ALOCA DCS 600EX Tokyo, Japan). Following this procedure, the ossicles were then processed for histological examination.

All experiments were carried out in strict accordance with the Institutional Guidelines for the Care and Use of Laboratory Animals of Osaka City University.

Cranial Bone Defect Model in Rats

Twenty male Wistar rats (8 weeks old; Nippon SLC, Hamamatsu, Japan) were used to examine the capacity of the rhBMP-2/antibiotics (teicoplanin)/polymer composite implant to repair a bone defect. The rats were divided equally into four groups. The rats were anesthetized by intramuscular injection of ketamine (30 mg/kg) and xylazine (10 mg/kg). Through a midline skin incision, the parietal skull was exposed, and a cranial bone defect of 8 mm in diameter was created using a trephine punch and surgical air tome.[8] To each animal in the BMP/teicoplanin (BMP-TP) group, a polymer implant (30 mg) containing rhBMP-2 (5 μg) and teicoplanin 4 μg (corresponding to 1×10^4 of MIC_{90} dose in the polymer) was implanted into the defect. For the BMP and teicoplanin group rats, polymer pellets containing rhBMP (5 μg) alone or teicoplanin alone were implanted (BMP group and TP group, respectively). In one group, polymer implants alone with no BMP or teicoplanin were implanted (P group). After surgery, all rats were housed in cages with free access to food and water. At the end of a 6-week period, the rats were sacrificed, and the calvarias dissected to evaluate the degree of the defect repair.

Repair was evaluated on plain radiograms, CT scan images, and histological sections. Radiograms of the calvariae were taken with a soft X-ray apparatus (Softex, Sofron Co., Ltd., Tokyo, Japan). Repaired on nonrepaired areas were calculated using Scion image software (Scion Co., Frederick, Maryland). CT images, each representing 1 mm slice thickness, were obtained (GE Yokogawa medical system, Tokyo, Japan) and used to construct three-dimensional images to visualize the repaired skull surfaces. The calvariae from each group were then fixed in neutralized 10% formalin, decalcified with Plank-Rychlo's solution, and embedded in paraffin wax. Sections of 4 μm thickness were prepared, stained with hematoxylin-eosin, and observed under a light microscope.

Statistical Analysis

Data are presented as mean ± SE. Statistical analysis was performed by using STATVIEW 5.0 (Abacus Concepts, Berkeley, CA) by unpaired nonparametric Mann-Whitney tests at a 95% confidence level. An associated probability (p-value) of <0.05 was considered significant.

RESULTS

Release Profile of the Teicoplanin

Figure 1 shows the profile of the time-bound changes in concentration of teicoplanin released into 1 ml of PBS (Fig. 1a) and the cumulative percent dose released from a PLA-DX-PEG polymer with an original weight of 30 mg (Fig. 1b) over 21 days. The dose of teicoplanin released was plotted in log scale (Fig. 1a). The concentration of teicoplanin in PBS maintained a level higher than the MIC_{90} for *S. aureus* for up to 14 days. Approximately 40% of the teicoplanin incorporated into

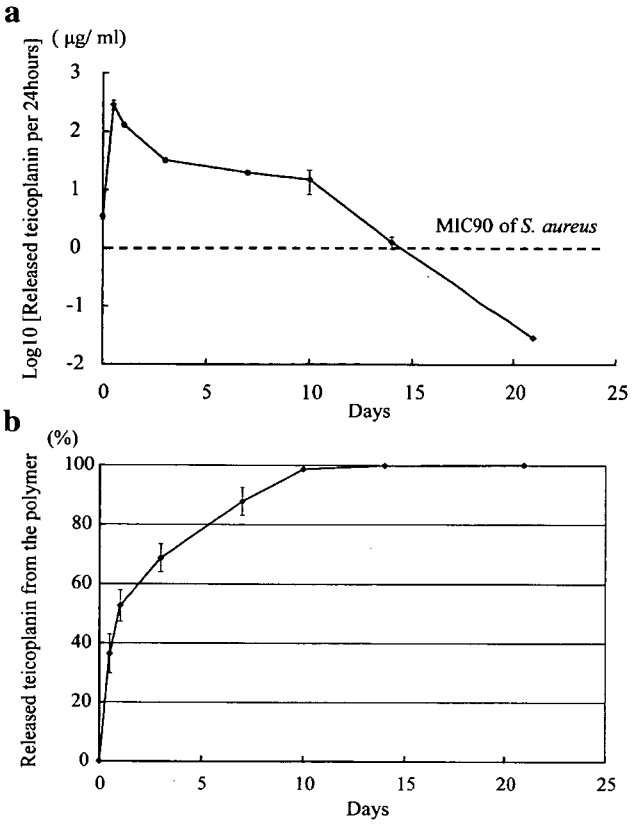

Figure 1. (a) The amount of teicoplanin released in 24 h over a series of collection intervals. Note the vertical axis is a log scale. The dotted line shows the MIC_{90} of *S. aureus*. (b) Total percentage of released teicoplanin at several time points.

PLA-DX-PEG was released in the initial 24 h (Fig. 1b). The remaining teicoplanin was gradually released from the degrading polymer after a further 3 weeks. By this point the polymer had essentially disappeared.

Ectopic Bone Formation

Hard tissue pieces were harvested consistently from implanted sites where rhBMP-2/polymer composite implants with or without antibiotics had been placed 3 weeks earlier. On soft X-ray radiographs, these samples revealed a calcified network encased within a shell-shaped calcified layer (Fig. 2a).

On DXA analysis, no significant difference in terms of mineral content in the ossicles was noted among the antibiotic treatment and control groups. None of the antibiotics examined appeared to have any adverse effects on the bone-inducing capacity of rhBMP-2 (Fig. 2b).

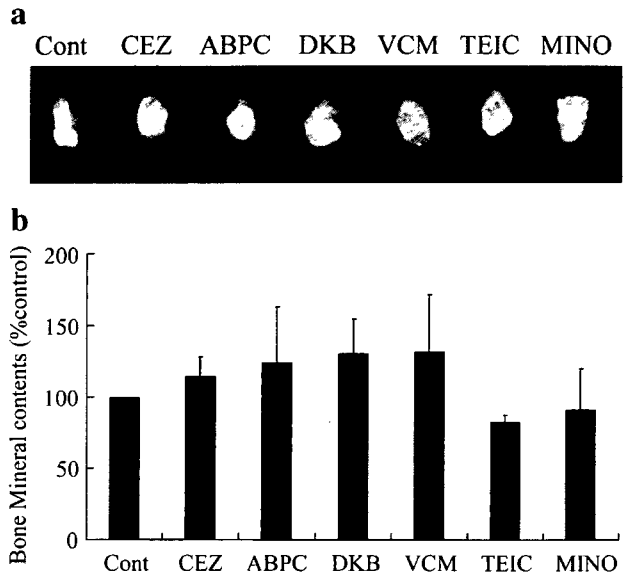

Figure 2. (a) Soft X-ray photograph of the ossicles harvested at 3 weeks after implantation. (b) The bone mineral content of the ossicles at 3 weeks after implantation. No difference was found among the groups.

Cranial Bone Defect Repair

The representative radiographs and CT images of parietal cranial defects at 6 weeks after defect generation and implantation of polymer implants (30 mg) with 5 μg of rhBMP-2 (BMP and BMP-TP group) or without the rhBMP-2 (TP and P groups) are shown in Figure 3. The repaired area in each group was expressed as a percentage of the original defect area (Fig. 4). The percentage repaired areas were significantly higher in BMP and BMP-TP groups ($88.0 \pm 14\%$ and $95.5 \pm 8\%$, respectively) than the areas measured in other groups ($24.1 \pm 15\%$, $13.6 \pm 8\%$ in TP and P group, respectively). No significant difference was noted between the BMP and BMP-TP groups, so no adverse effect of teicoplanin on the BMP specific bone-inducing capacity was found.

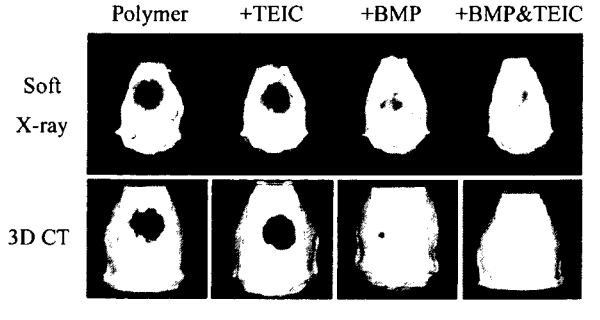

Figure 3. Soft X-ray and 3D CT image of a rat cranial defect 6 weeks after treatment. The defect area healed only after treatment with rh-BMP2.

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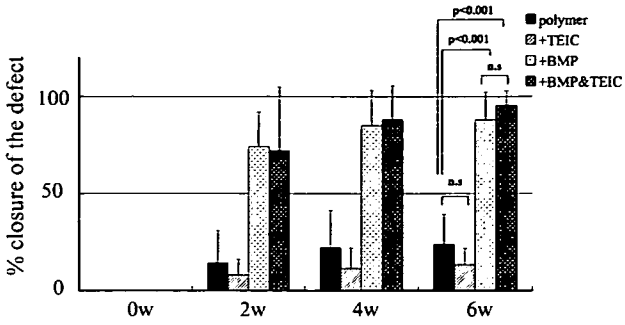

Figure 4. The closure size of critical defect on 3D CT image. The defect area was reduced up to 70% after treatment with rh-BMP2.

In the group containing no rh-BMP2, a small amount of new bone formation was observed at the periphery of the defect (Fig. 5). However, the defect was occupied with a layer of fibrous tissue (Fig. 5a, b, e, and f). In the group containing rh-BMP2 (BMP and BMP-TP groups), the defects were repaired by newly formed bone, and the original histology of the parietal skull with hematopoietic marrow tissue in the diploe was ultimately restored (Fig. 5c, d, g, and h).

DISCUSSION

The degradable PLA-DX-PEG block copolymer utilized in this study was originally synthesized as a carrier material for BMP to elicit bone formation.[5] The exact molecular structure, molecular size, and molar ratio of PLA, DX, and PEG segments were optimized for the bone-inducing activity of rhBMP-2.[5,9,10] Saito et al.[5] demonstrated that the polymer could deliver rhBMP-2 more effectively than collagen in vivo. In this study, the capacity of the specific polymer for the continuous local release of antibiotics was examined prior to use in combination with rhBMP-2.

Our results indicate that under in vitro conditions, a sustained release of antibiotics (teicoplanin) occurred from the degradable PLA-DX-PEG block copolymer in two phases; an initial rapid release phase followed by a gradual slow release phase. In the initial phase, aproximately 40% of the total dose of the hydrophilic teicoplanin was released. The direct elution of teicoplanin is most likely due to the hydrophilic character of the polymer. The amount of the antibiotic remaining most likely reflects an affinity for the polymer molecules. The slow release of the residual antibiotic follows the progressive hydrolysis of the polymer. However, the mechanism by which antibiotics are slowly released from the polymer is not well understood.

The bacteriocidal activity of the antibiotics released from the polymer was not altered during the experimental period by contact with the

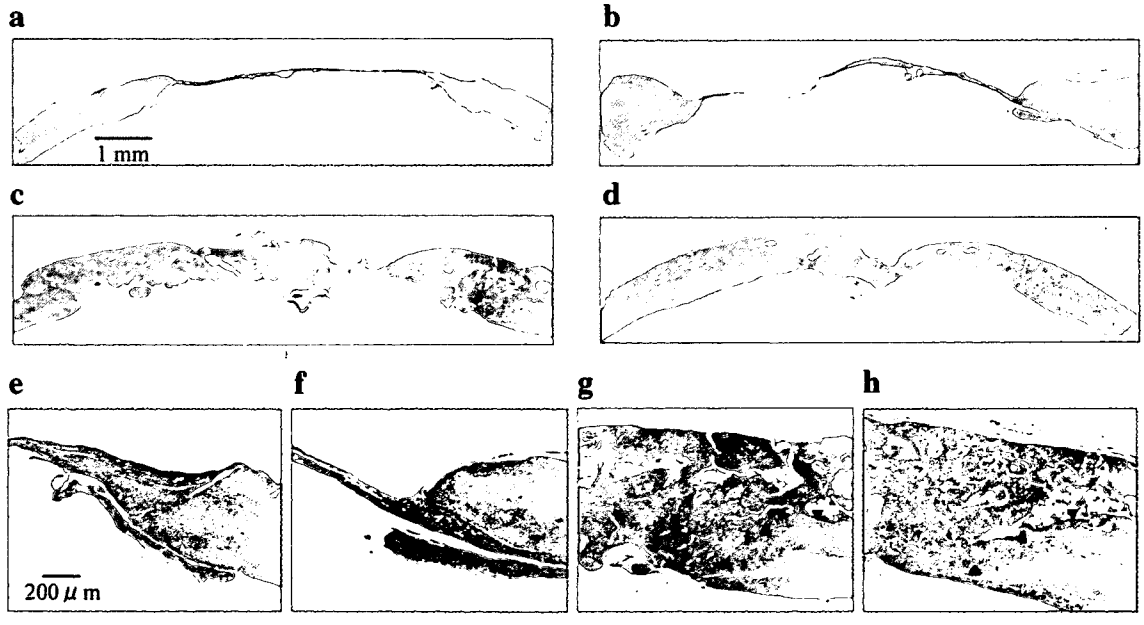

Figure 5. Histology of rat calvarial specimens 6 weeks after treatment with only polymer (a,e), with teicoplanin (b,f), with rh-BMP2 (c,g), with rh-BMP2 and teicoplanin (d,h). Figures e through h are higher (original) magnification (×10) images of the edge between the defect area and normal calvaria (arrow head) in (a)–(d).

polymer and incubation, as shown by the bioassay data for teicoplanin. Antibiotic durability might be an essential requirement for local treatment of refractory infectious lesions such as osteomyelitis.[11]

An ectopic bone formation model was used to evaluate the effects of different antibiotics on the bone-inducing capacity of rhBMP-2. No evidence was found to suggest that the presence of antibiotics had an inhibitory action on the osteoinductive properties of the BMP. The size and bone mineral content of the BMP-induced ossicles were not altered by addition of those antibiotics. Additionally, the parietal cranial bone defects of critical size were consistently repaired by new bone formation when the defects were filled with polymer containing both rhBMP-2 and antibiotics at high concentration (1×10^4 MIC_{90} for *S. aureus*). These results support the potential efficacy of rhBMP-2 in combination with antibiotics for the treatment of infected nonunion fractures and prevention of infection at sites undergoing reconstruction. We chose the cranial defect model instead of a long bone defect because of difficulty in keeping the shape of the polymer-only group because of its gel characteristics in body temperature. Recently, Yoneda et al.[12] resolved the problem by using beta tricalcium phosphate and succeeded in repair of long bone critical defects. Further study must be undertaken using long bone defects before clinical use.

In a recent study, Chen et al.[13] reported the usefulness of BMP-7 in a collagen carrier for the treatment of an infected bone defect in a rat model. However, the amount of rhBMP-7 required for bone regeneration was five times greater than that needed to repair the bone defect without infection. Clearly, in the absence of antibiotics, the high dose and resultant high cost of BMP treatment presents a challenge for the repair of infected bone using osteoinductive proteins. The local delivery of antibiotics concomitantly with rhBMP-2 in the polymer carrier might be a useful approach to reduce the dose and cost of the repair. Further studies are underway in our laboratory to examine the efficacy of this system in an infected bone defect model.

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REFERENCES

1. Becker PL, Smith RA, Williams RS, et al. 1994. Comparison of antibiotic release from polymethylmethacrylate beads and sponge collagen. J Orthop Res 12:737–741.
2. DiMaio FR, O'Halloran JJ, Quale JM. 1994. In vitro elution of ciprofloxacin from polymethylmethacrylate cement beads. J Orthop Res 12: 79–82.
3. Buchholz HW, Elson RA, Engelbrecht E, et al. 1981. Management of deep infection of total hip replacement. J Bone Joint Surg Br 63-B:342–353.
4. McLaren AC. 2004. Alternative materials to acrylic bone cement for delivery of depot antibiotics in orthopaedic infections. Clin Orthop 427:101–106.
5. Saito N, Okada T, Horiuchi H, et al. 2001. A biodegradable polymer as a cytokine delivery system for inducing bone formation. Nat Biotechnol 19:332–335.
6. Erickson RC, Hildebrand AR, Hoffman PF, et al. 1989. A sensitive bioassay for teicoplanin in serum in the presence or absence of other antibiotics. Diagn Microbiol Infect Dis 12:235–241.
7. Yoshida I, Kimura Y, Higashiyama I, et al. 2003. Surveillance of susceptibility of clinical isolates of various bacterial species to antibacterial agents—antimicrobial activity against Gram-positive cocci and anaerobic bacteria isolated in 2000. Nippon Kagaku Ryohou Gakkai Zasshi 51: 179–208.
8. Schmoekel H, Schense JC, Weber FE, et al. 2004. Bone healing in the rat and dog with nonglycosylated BMP-2 demonstrating low solubility in fibrin matrices. J Orthop Res 22:376–381.
9. Saito N, Okada T, Horiuchi H, et al. 2001. Biodegradable poly-D,L-lactic acid-polyethylene glycol block copolymers as a BMP delivery system for inducing bone. J Bone Joint Surg Am 83-A(Suppl 1):S92–S98.
10. Saito N, Okada T, Toba S, et al. 1999. New synthetic absorbable polymers as BMP carriers: plastic properties of poly-D,L-lactic acid-polyethylene glycol block copolymers. J Biomed Mater Res 47:104–110.
11. Gursel I, Korkusuz F, Turesin F, et al. 2001. In vivo application of biodegradable controlled antibiotic release systems for the treatment of implant-related osteomyelitis. Biomaterials 22: 73–80.
12. Yoneda M, Terai H, Imai Y, et al. 2005. Repair of an intercalated long bone defect with a synthetic biodegradable bone-inducing implant. Biomaterials 26:5145–5152.
13. Chen X, Kidder LS, Lew WD. 2002. Osteogenic protein-1 induced bone formation in an infected segmental defect in the rat femur. J Orthop Res 20:142–150.

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Bone morphogenetic protein activities are enhanced by 3′,5′-cyclic adenosine monophosphate through suppression of Smad6 expression in osteoprogenitor cells

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Abstract

Bone morphogenetic proteins (BMPs) belong to the transforming growth factor (TGF)-β superfamily, and some display potent osteogenic activity both in vivo and in vitro. The BMP signaling cascade involving BMP receptors at the cell membrane and intracellular messengers (Smads) has been elucidated, but the regulatory mechanisms of BMP signaling have not been clarified. We previously found that pentoxifyline (PeTx), a nonspecific inhibitor of phosphodiesterase (PDE), and rolipram, a PDE-4-specific inhibitor, enhance BMP-4-induced osteogenic differentiation of mesenchymal cells, probably through the elevation of intracellular cyclic adenosine monophosphate (cAMP) accumulation and modulation of BMP signaling pathways as enhanced BMP-4 action was reproduced by addition of dibutylyl-cAMP (dbcAMP). However, the precise mechanisms underlying the enhancing effects of those agents on BMP signaling were not completely revealed. As already reported, BMPs utilize a specific intracellular signaling cascade to target genes via R-Smads (Smad1,5,8), Co-Smad (Smad4) and I-Smads (Smad6,7). One possibility for cAMP-mediated effects on BMP signaling might be suppression of I-Smads expression since these proteins form a negative feedback loop in BMP signaling. To examine this possibility, changes in I-Smad (Smad6) expression on addition of dbcAMP or PeTx were examined in a bone-marrow-derived osteogenic cell line (ST2). Alkaline phosphatase activity in ST2 cells was consistently induced by BMP-4 treatment (300 ng/ml), and Smad6 mRNA expression was also induced by BMP-4 treatment. Although concurrent treatment of ST2 cells with BMP-4 and dbcAMP elicited further activation of alkaline phosphatase, addition of dbcAMP reduced BMP-4-induced Smad6 expression in a dose-dependent manner. Furthermore, detection of phosphorylated Smad1/5/8 on Western blotting analysis was prolonged, suggesting prolonged kinase activity of BMP receptors through suppressed expression of Smad6. Elevated intracellular cAMP might thus enhance BMP signaling by suppressing Smad6 induction and prolonging intracellular BMP signaling.

Keywords: Cyclic adenosine monophosphate; Osteoprogenitor cells; Bone morphogenetic protein; Smad6; Pentoxifyline

Introduction

The bone-inducing activity of bone morphogenetic proteins (BMPs) was originally identified in bone matrix through ectopic bone formation after implanting decalcified bone matrix into rodent muscle. A group of BMP molecules belonging to the transforming growth factor (TGF)-β superfamily has since been identified and recognized as multi-functional growth factors.

In bone biology, BMPs are considered crucial in bone and cartilage formation for embryonic development, postnatal bone metabolism and repair of damaged bone. From a clinical perspective, BMPs and related molecules regulating BMP activity are expected to offer powerful tools for the treatment of systemic or local skeletal disorders like osteoporosis and repair of fracture or bone defects associated with bone tumor excision or trauma. For clinical purposes, BMP-2 and BMP-7/osteogenic protein

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(OP)-1 have been synthesized using DNA recombination and utilized in a limited number of human cases in combination with local delivery systems [1]. Gene therapy using BMP genes has also been attempted in experimental animals [2], but efficacious and safe vectors for delivering BMP genes in gene therapy have remained problematic for gene therapy.

When considering the efficient use of BMPs either systemically or locally, one basic and important problem is the low responsiveness of human mesenchymal cells to BMPs [3]. Large doses (in the order of milligrams) of costly BMP are thus required for local lesions in each patient. To overcome such problems and enable more widespread and effective use of BMP molecules, additional agents or methods that intensify BMP activity are desirable. In this context, we screened the phosphodiesterase (PDE) inhibitors pentoxifyline (PeTx, a non-specific inhibitor of PDE) and rolipram (a PDE-4-specific inhibitor), revealing increases in BMP-2-induced bone mass following systemic daily injection of these agents [4,5]. However, the precise pharmacological basis of these effects was uncertain. Elevation of intracellular levels of cyclic adenosine monophosphate (cAMP) may have been involved and might intensify the intracellular BMP signaling cascade. One possible mechanism for cAMP to intensify BMP signaling would be interference with the negative feedback mechanism in BMP signaling formed by inhibitory Smads (I-Smads). The present study investigated changes in mRNA expression of major I-Smads, Smad6 and phosphorylated receptor-regulated Smads (R-Smads) levels after treatment with BMP-4 and dibutylyl-cAMP (dbcAMP), a cell-membrane permeable analog of cAMP, in lined murine osteogenic ST2 cells.

Materials and methods

Reagents

PeTx (1-(5-oxohexyl)-3,7-dimethylxanthine) and dbcAMP were purchased from Sigma Chemical Co. (St. Louis, MO, USA). PeTx and dbcAMP were prepared at 90 mM and 100 mM, respectively, as stock solutions in culture medium.

As a source of mouse BMP-4, conditioned medium from Chinese hamster ovary (CHO) cells transfected with murine BMP-4 (mBMP-4) cDNA was used. Details of the CHO cell have been described in our previous publication [6]. Briefly, cells transfected with mBMP-4 cDNA or mock vector (for controls) were inoculated at a density of 1.0×10^6 cells/100 mm plastic dish (Falcon #3003; Becton Dickinson Labware, Tokyo, Japan) in 10 ml of α-minimal essential medium (α-MEM; Sigma Chemical Co.) with 10% (vol/vol) heat-inactivated fetal bovine serum (FBS; Gibco, Grand Island, NY, USA) and antibiotics/antimycotics (100 U/ml penicillin; 100 μg/ml streptomycin; and 0.25 μg/ml amphotericin B, Sigma Chemical Co.) at 37°C in 5% CO_2 atmosphere. Conditioned medium was collected after 5 days incubation, filtered through a membrane filter (Corning, NY, USA; pore size 0.22 μm) and stored at 4°C. Under these conditions, judging from the induction of alkaline phosphatase (ALP) activity in osteoprogenitor cells, a 5% mixture of conditioned medium corresponded to approximately 100 ng/ml of recombinant human BMP-2 (generously provided by Yamanouchi Pharmaceutical, Tokyo, Japan) (data not shown). For experiments, conditioned medium from cells transfected with mock vector was used for negative controls.

Cell culture

Mouse mesenchymal cell line C3H10T1/2 [7], mouse bone marrow stromal cell line ST2 [8] and mouse osteoblastic cell line MC3T3-E1 [9] were obtained from the Riken Cell Bank (Ibaraki, Japan). The C3H10T1/2 and ST2 cell lines are widely regarded as osteogenic precursors as cells go on to exhibit osteoblastic phenotypes under the control of BMPs [10,11]. Cells were seeded at a density of 3×10^5 cells/100-mm plastic dish and cultured in α-MEM containing 10% FBS and antibiotics/antimycotics at 37°C in 5% CO_2 humidified air. Upon reaching confluence, cells were used in the following experiments.

Determination of alkaline phosphatase activity

Alkaline phosphatase activity was measured as a marker of osteoblastic differentiation in response to BMP-4 or other agents. Cells were seeded at a density of 1×10^5 cells/well in 48-well plates ($n = 4$ per group). Upon achieving confluence, medium was replaced by fresh medium containing 5% FBS and pre-incubated for 1 h. BMP-4 and other chemicals were then added to the cultures, and incubation was continued for 3 more days. Cells were pre-incubated with or without PeTx/dbcAMP for 15 min before treatment with BMP-4. After 3 days of incubation, medium was removed, and cells were washed 3 times with saline then lysed in 250 μl of 0.2% Triton-X (Sigma Chemical Co.) with saline. Plates were frozen at −80°C and dissolved again then sonicated for 40 s. After centrifugation, supernatant was collected and used for ALP assay and protein assay. For ALP assay, samples were incubated with 0.5 mM *para*-nitrophenol phosphate, 0.5 mM $MgCl_2$ and 0.5 M Tris–HCl for 30 min at 37°C. To stop the reaction, a quarter volume of 1 N NaOH was added, and then absorbance was read at 405 nm. As a standard, 5 mM *para*-nitrophenol was used. Protein concentration was measured using a DC protein assay kit (Bio-Rad Laboratories, CA, USA). Experiments were performed independently in triplicate.

Determination of intracellular cAMP

When cultures maintained in 24-well plates ($n = 4$ per group) reached confluence, medium was replaced using fresh medium of the same composition. After 1 h of pre-incubation, BMP-4 and other chemicals were added to the cultures, and incubation was continued for 5 or 15 min. The medium was then removed, and cells were overlaid with 250 μl of 0.1 N HCl for 10 min at room temperature, collected by scraping and then centrifuged. Supernatant was then collected and used for cAMP assay, which was performed using a cAMP Kit (R&D Systems, Minneapolis, USA) according to the instructions of the manufacturer. The cAMP assay is based on the competitive binding technique, in which cAMP present in a sample competes with a fixed amount of ALP-labeled anti-cAMP rabbit polyclonal antibody.

Northern blot analysis

Cells were seeded in 100-mm diameter dishes and cultured until confluence. Upon achieving confluence, medium was replaced by fresh medium containing 5% FBS and cultured for 1 h. Cells were then treated with BMP-4 (300 ng/ml) or dbcAMP (2 mM) for 24 h. Cells were pre-incubated with or without dbcAMP for 15 min before treatment with BMP-4.

Total RNA was isolated from cells using Isogen (Wako Pure Chemical Industries, Osaka, Japan), and poly(A)$^+$ RNA was obtained using Poly(A) Quik mRNA Isolation Kit (Stratagene, CA, USA) according to the instructions of the manufacturer. Poly(A)$^+$ RNA (2 μg) was electrophoresed in 1.0% agarose gels in the presence of 1.1 M formaldehyde and blotted to Hybond N membranes (Amersham Bioscience, NJ, USA). The complete coding regions of mouse Smad6 cDNA were labeled by [α-^{32}P]-dCTP using a Random Primer Labeling Kit (Takara Biochemicals, Otsu, Japan). Hybridization was performed in a solution containing 0.5 M phosphate buffer, 1 mM ethylene diamine tetra-acetic acid (EDTA) and 7% sodium dodecyl sulfate (SDS) at 65°C overnight, and the filter was washed at 65°C with 6× standard saline citrate (SSC), 0.1% SDS for 15 s, 2× SSC and 0.1% SDS for 10 min twice. Filters were stripped using boiled distilled water containing 0.1% SDS and rehybridized. Amounts of mRNA were verified by hybridizing filters with a glyceraldehyde 3-phosphate dehydrogenase (GAPDH) probe. Signals were detected using a BAS-2500 image analysis system (Fuji Photo Film Co., Tokyo, Japan).

Smad6 cDNA was generously provided by T. Imamura (Cancer Institute of the Japanese Foundation for Cancer Research, Tokyo, Japan), and GAPDH cDNA was generated by reverse transcription polymerase chain reaction (RT-PCR) using specific primer sets as follows: for GAPDH, sense primer 5′-ATGGTGAAGGTCGGTGTGAA-3′ and anti-sense primer 5′-CTCTTGCTCAGTGTCCTTGCT-3′.

Quantitative real-time reverse transcription polymerase chain reaction

When cultures maintained in 6-well plates ($n = 3$ per group) became confluent, medium was replaced by fresh medium containing 5% FBS and cultured for 1 h, then BMP-4 and other chemicals were added to cultures. In the case of combined treatment, BMP-4 was added 15 min later than other chemicals. Total RNA was isolated from cells treated with each chemical for each time period using NucleoSpin RNA II (Macherey-Nagel, Duren, Germany) according to the instructions of the manufacturer. A total of 1 μg of total RNA was reverse-transcribed into first-strand cDNA with an oligo-dT primer using Superscript II reverse transcriptase (Invitrogen, CA, USA). Real-time RT-PCR was performed according to the instructions of the manufacturer (Applied Biosystems, Foster City, CA, USA). TaqMan fluorogenic probes for Smad6 were purchased from Applied Biosystems. Real time RT-PCR for GAPDH was performed using SYBR Green Supermix (Bio-Rad Laboratories). Experimental samples were matched to a standard curve generated by amplifying serially diluted products using the same PCR protocol. Amplified GAPDH cDNA was used for internal control. To correct for variability in RNA recovery and efficiency of reverse transcription, GAPDH cDNA was amplified and quantitated in each cDNA preparation. Normalization and calculation steps were performed as described by Pfaffl [12]. Experiments were performed on 3 independent occasions, and each experiment comprised of 3 samples.

Western blot analysis

Cells were plated at a density of 2×10^4 cells/well on 6-well plates and cultured until confluence. At 1 h after replacing with fresh medium containing 5% FBS, BMP-4 (300 ng/ml) and dbcAMP (2 mM) were added to the media, and cells were cultured for designated periods. Dibutylyl cyclic AMP (dbcAMP) was added 15 min prior to BMP-4. The resultant sample was boiled for 5 min in 20 μl of sample buffer for SDS polyacrylamide electrophoresis (SDS-PAGE) as described previously with minor modifications [13]. Equal amounts of protein samples were applied and run on each lane of SDS 5–20% acrylamide gels (40 mA, low voltage, 90 min) and ultimately blotted to ECL™ nitrocellulose membrane (Amersham Bioscience). Membranes were developed using ECL™ plus reagent (Amersham Bioscience). Signal intensities were measured according to staining of each band using a Chemi Doc XRS-J digital densitometer (Bio-Rad Laboratories).

Statistical analysis

Data are expressed as mean ± SD for each group. Differences between treatment groups were analyzed using

Fisher's PLSD test. Values of $P < 0.05$ were considered statistically significant.

Results

ALP induction by BMP-4, PeTx and dbcAMP

PeTx, a methylxanthine-derived nonspecific inhibitor of PDEs, had no effect on ALP activity in ST2 cells at PeTx concentrations of 0.225–0.9 mM. In contrast, PeTx enhanced BMP-induced ALP activity in a dose-dependent manner (Fig. 1A), indicating a specific enhancing effect of PeTx on BMP-2 activity. Intracellular levels of cAMP increased about 7-fold in 15 min after the addition of PeTx (0.9 mM). Levels of cAMP were unaffected by addition of BMP-4 (50 ng/ml) with PeTx (Fig. 1B).

The potentiating effect of PeTx on BMP action in ST2 cells was mimicked by the addition of dbcAMP (Fig. 1C). Similar results were obtained using C3H10T1/2 and MC3T3-E1 cells (data not shown).

Smad6 mRNA induced by BMP-4 stimulation

Fig. 2 shows dose- and time-dependent changes in Smad6 mRNA expression following BMP-4 treatment, as determined using quantitative real-time RT-PCR methods. In ST2 cells, Smad6 mRNA expression was significantly up-regulated by mBMP-4 treatment in a dose-dependent manner over the BMP-4 dose range from 50 ng/ml to 300 ng/ml in 6 h (Fig. 2A). Up-regulated expression of Smad6 mRNA peaked in 3 h and lasted up to 24 h after BMP treatment (300 ng/ml) then gradually reduced to the baseline level within 72 h (Fig. 2B).

Suppression of Smad6 expression by cAMP

In order to elucidate how cAMP accelerates osteoblastic differentiation in ST2 cells, the expression of Smad6 mRNA after treatment with dbcAMP and BMP-4 was analyzed. Smad6 mRNA expression was induced by BMP-4 stimulation, but addition of dbcAMP significantly ameliorated BMP-4-induced Smad6 expression (Fig. 3).

Quantitative real-time RT-PCR of Smad6 mRNA expression treated with or without BMP-4 (300 ng/ml) and various doses of dbcAMP (100–2000 μM) at 12 h revealed that dbcAMP reduced BMP-4-induced Smad6 expression in a dose-dependent manner (Fig. 4).

Results of quantitative real-time RT-PCR for Smad6 mRNA expression at 6, 12, 24 and 72 h are shown in Fig. 5. At 6, 12 and 24 h, dbcAMP reduced the expression of Smad6 mRNA that was induced by BMP-4 (Figs. 5A–C). Although level of Smad6 mRNA induced by BMP-4 returned to

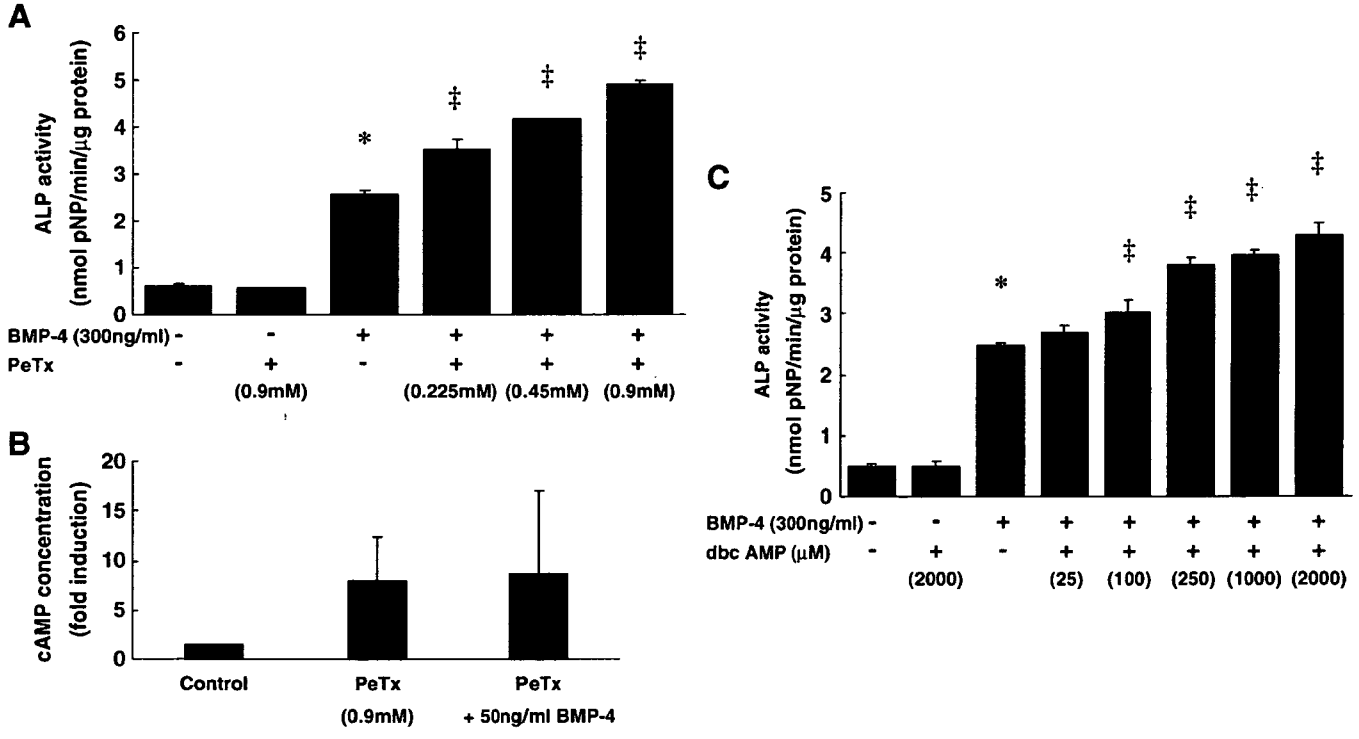

Fig. 1. (A, C) PeTx and dbcAMP intensified BMP-4-induced ALP activation in a dose-dependent manner. ST2 cells were cultured in 24-well plates until confluence then treated with BMP-4, PeTx (A) and dbcAMP (B). After 3 days, ALP activity was measured. (B) Concentration of cAMP in ST2 cells treated with BMP-4 and PeTx for 15 min. Bars and lines represent mean ± SD for 4 wells. *$P < 0.05$, cells treated with BMP-4 compared with untreated cells. ‡$P < 0.05$, cells treated with BMP-4/PeTx or dbcAMP compared with BMP-4-treated cells.

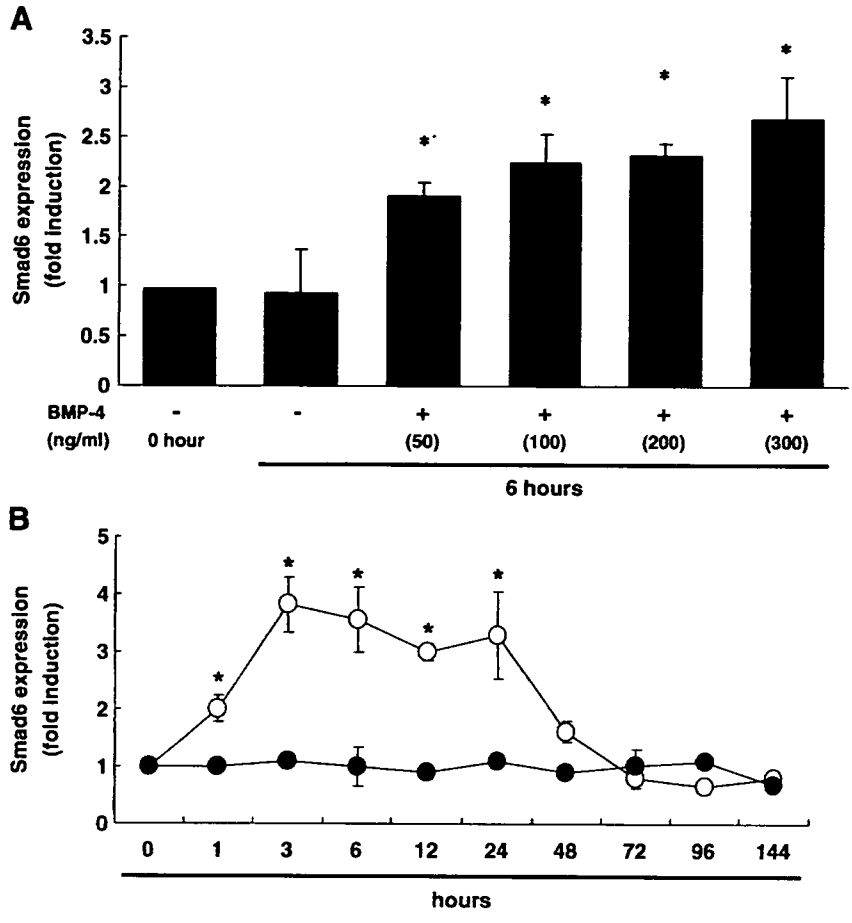

Fig. 2. Effects of BMP-4 on expression of Smad6 in ST2 cells. ST2 cells were cultured in 6-well plates to confluence then treated with BMP-4. After 6 h (A) or other designated times (B), total RNA was extracted, and levels of Smad6 expression were analyzed using real-time RT-PCR, normalized to GAPDH expression and presented as the expression level relative to that in control untreated cells. BMP-4 induced Smad6 expression in a dose-dependent manner at 6 h after stimulation (A). In panel B, closed circles represent Smad6 expression at each time point in untreated cells, and open circles represent expression in cells treated with BMP-4 (300 ng/ml). Bars and lines represent mean ± SD for 3 wells. Similar results were obtained in 3 independent series of studies. $^{*}P < 0.05$, cells treated with BMP-4 compared with untreated cells.

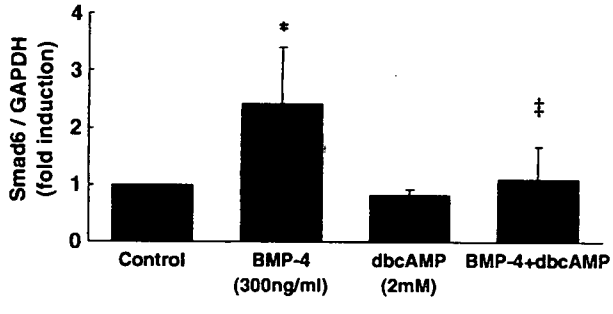

Fig. 3. Cyclic AMP reduced BMP-4-induced Smad6 expression in ST2 cells. Results of Northern blotting. After 24 h of treatment using BMP-4 (300 ng/ml), dbcAMP (2000 μM) or both, mRNA was extracted and analyzed. Messenger RNA obtained using a Poly(A) Quik mRNA Isolation Kit was electrophoresed in agarose-formaldehyde gels, blotted to Hybond N membranes and hybridized with Smad6 and GAPDH probes. Showing the summary of 3 independent experiments. Bars and lines represent mean ± SD for 3 experiments. $^{*}P < 0.05$, cells treated with BMP-4 compared with untreated cells. $^{\ddagger}P < 0.05$, cells treated with BMP-4/dbcAMP compared with BMP-4 treated cells.

baseline by 72 h, dbcAMP still repressed Smad6 mRNA levels (Fig. 5D).

Prolonged phosphorylation of BMP-specific R-Smads by dbcAMP

Phosphorylation of BMP-specific R-Smads by BMP-4 was analyzed by immunoblotting using phospho-Smad1/5/8-specific antibody with or without dbcAMP treatment (Fig. 6). Phosphorylation of Smad1/5/8 after BMP-4 stimulation started from 1 h after BMP-4 stimulation and became undetectable after day 3. Addition of dbcAMP did not exhibit significant effects at 1 or 24 h after treatment, but phosphorylated Smad1/5/8 were detected until day 5.

Discussion

The cyclic nucleotide PDE family includes a large numbers of PDE isomers, which are encoded in at least 13