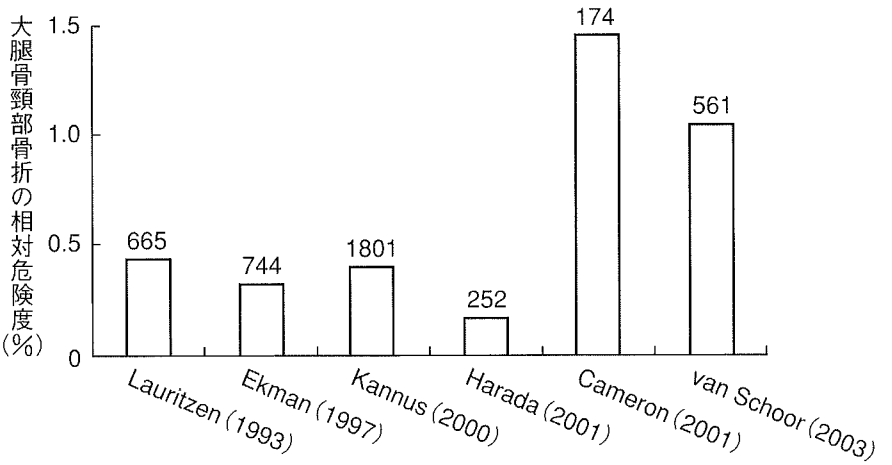

図2　ヒッププロテクターが大腿骨頸部骨折発生率に与える影響
名前と年は発表者と報告年。バーの上の数字は対象者数。

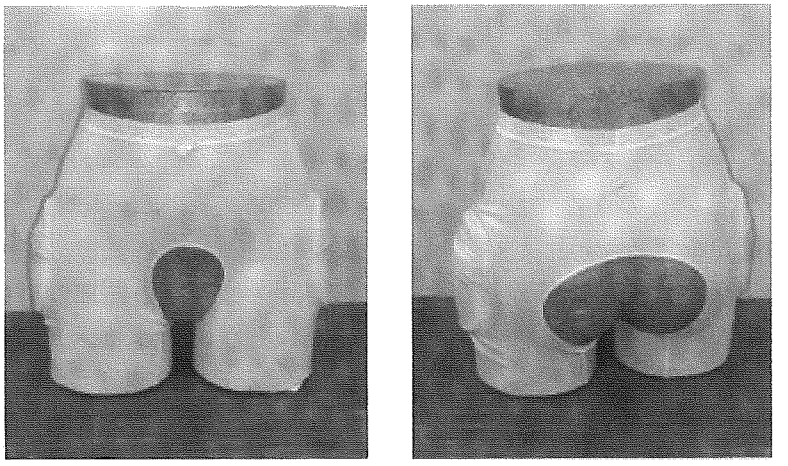

図3　改良型股割れパンツ
トイレ動作時に脱がずにすむように設計を行った。この上に下着を着用する。

群の4群比較を行った。

2　対象と方法

研究目的および方法を4施設の入所者に行い，参加を承諾した歩行可能な65歳以上の女性103名（年齢81.2±7.5歳）を無作為に4群に分け，6ヵ月間の装着率および転倒骨折率の調査を行った。本人あるいは家族から書面によるインフォームドコンセントをえた。従来型パンツか股割れ型パンツかの選択に関しては，クラスターごとに無作為に分け，シェルのあるなしに関しては封筒法による無作為化を行った。開始時に移動能力や歩行速度および嗜好品などについてのアンケート調査を行い，また，観察開始1ヵ月の時点で，施設介護職員に対するアンケート調査も行った。

3　結　　果

各群間に，年齢・身長・体重に差は認めなかった(**表1**)。また，20歳時と比較しての身長低下，歩行速度，移動能力，合併症，閉経時期，アルコール・コーヒー・牛乳飲用量，睡眠薬服用頻度

表1　対象者と群分け

パンツ	シェル	人数	年齢（歳）	身長（cm）	体重（Kg）
従来型	あり	27	79.2 ± 9.0	145.3 ± 7.6	45.9 ± 9.0
従来型	なし	27	80.0 ± 6.8	145.9 ± 7.4	46.4 ± 9.4
股割れ型	あり	26	81.7 ± 6.0	146.3 ± 6.9	46.9 ± 9.0
股割れ型	なし	23	84.2 ± 7.1	145.2 ± 6.3	43.8 ± 7.0

平均±標準偏差

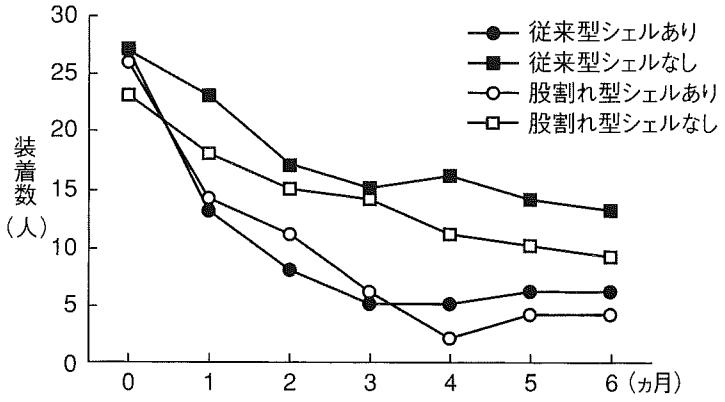

図4　装着者数変化

に関しても，各群間に差を認めなかった。

観察期間中，両群ともに大腿骨頸部骨折は1例も生じなかった。他の部位の骨折も観察されなかった。転倒回数は，シェルあり群9回，シェルなし群3回，従来型パンツ群7回，股割れ型パンツ群5回で各群間に差を認めなかった。

装着率変化を，**図4**に示す。各群ともに，開始直後より装着率は急激に低下したが，低下率はシェルあり群でより急激であり，従来型パンツと股割れ型パンツ群の間では，シェルのあるなしにかかわらず差を認めなかった。

1ヵ月時点での介護者に対するアンケート調査では，どの群においても対象者に対する指導頻度が少ないことが判明したが，指導を行えば対象者の装着率が向上することも判明した（**図5**）。

4　考　　察

大腿骨頸部骨折の発生要因は単一のものではなく，種々の要因が絡み合って形成されている。多くの大腿骨頸部骨折は，骨量が骨折閾値以下に低下した高齢者に発生する。しかし，骨量だけでは将来の頸部骨折を予測することはできないとされており，大腿骨頸部骨折発生に関しては転倒というイベントが重要な意味をもつ。実際，大腿骨頸部骨折の90%以上は転倒にともなって発生する。そのため転倒要因と骨強度規定因子のバランスを理解することが重要である。

したがって，頸部骨折を予防するためには理論上，以下のような方策が考えられる。まず，転倒そのものを防ごうとするもので，転倒要因で改善可能なものを対象とする。種々の運動療法や生活環境改善などがここに含まれる。また，骨の脆弱性を改善し骨折予防を行おうとするものには，骨粗鬆症の治療方法が食事療法などもあわせてすべて含まれる。薬物療法においては，ビスフォスフォネート製剤が骨量を著明に増加させ，頸部骨折発生率を50%程度抑制することが大規模臨床試験で証明されている。ところが，骨量増加のみで頸部骨折の発生を抑制しようとすると，20%以上の骨量増加が要求される。これはビスフォスフォネート製剤をもってしても

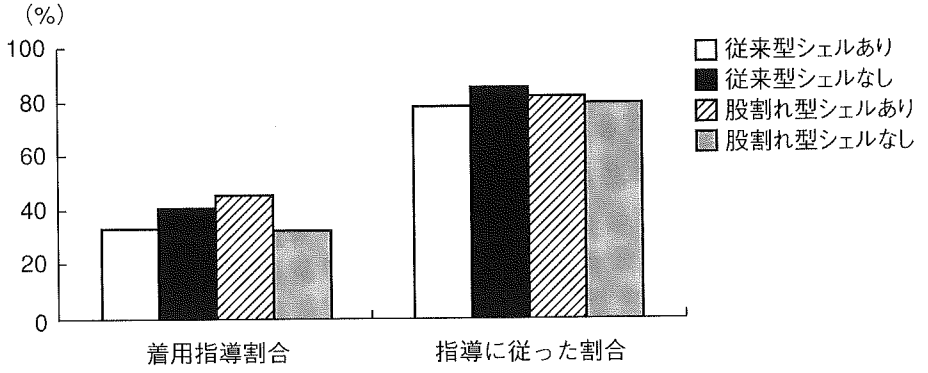

図5　施設職員に対するアンケート
開始1ヵ月後に実施。対象者に対して，毎日着用を指導したかとの問いと，その指導に対して対象者が従ったか否かに関する質問。各群間に差を認めない。

到達できないレベルである。そこで，骨が弱くて転倒しても骨が折れない方法として，ヒッププロテクターが考案された。

ヒッププロテクターには大きく分けて二つの種類がある。衝撃分散型（energy-shunting）と衝撃吸収型（energy-absorbing）で，前者は硬くて軽いシェル構造を，後者は柔らかくて重いジェル構造をしている。転倒して病院に運ばれた306名のうち，頸部骨折を起こした206名と起こさなかった100名の転倒様式を調査した研究[1]によると，骨折者の76%が側方への転倒で，56%が大転子上に血腫を認めたと報告されている。一方，非骨折者は側方への転倒が少なく（63%），手を伸ばすような防御反応が42%に認められた（骨折群では17%）。したがって，プロテクターを大転子外側に設置し，転倒時の大転子への衝撃を減弱させれば，頸部骨折を予防できると推測される。

臨床試験での成績は，1993年にLauritzenらによって報告されて以来，いずれの報告でも大腿骨頸部骨折発生の相対危険率を50%以下に抑制することに成功している[2~6]（**図2**）。ただし，最近では，後述する装着率の低さを含めて，ヒッププロテクター効果に関する否定的な結果も報告されている[7]。前述のように，ヒッププロテクター装着は大腿骨頸部骨折発生を抑制しうるが，それは当然のことながらヒッププロテクターを正しく装着していた場合のみである。これまでの研究においても，脱落症例が多いことが問題となっている。対象者はさまざまな理由でヒッププロテクターを装着しない。シェル型は硬くて痛みをともなうことが多く，ジェル型は柔らかい代わりに重くてかさばる。不快感（プロテクターがきつい，暑い，装着そのものに対する拒否反応）や，見栄え（腰回りが膨らむ），あるいは不自由さ（トイレ動作時の煩雑さ）を理由にヒッププロテクターを着けないことが多く，特に夜間の装着率は著しく低下する。

そこで，われわれはトイレ動作などに便利なように股割れ型のヒッププロテクターを開発したが，今回の調査で，われわれの改良は装着率向上に貢献しないことが判明した。対象者への聞き取り調査では，シェルあり群ではやはり疼痛が一番多い不満であり，股割れ群ではかぶれなどのこれまでに報告されていないような訴えも認められた。

ヒッププロテクターは正しく装着されれば，大腿骨頸部骨折発生率を有意に減少させることができる。特に，施設入所者などで転倒のコントロールが難しいと思われるような対象者には最適の装具と思われる。問題点である装着率の低さを改善するために，今後もスタイルの変更などを模索すべきであるが，シェルそのものの構造にも改良を加える必要がある。さらに，より重要なことは，介護する側の意識を高め，転倒骨折を防ぐ努力を日々の業務に取り入れていくことであると考えられた。

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A prostanoid receptor EP4 agonist enhances ectopic bone formation induced by recombinant human bone morphogenetic protein-2

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A prostanoid receptor EP4 agonist enhances ectopic bone formation induced by recombinant human bone morphogenetic protein-2☆

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Abstract

The anabolic effects of prostaglandin E_2 on bone are effected through the activation of EP4, a G protein-coupled receptor. In the present study, we examined the effects of a prostanoid receptor-selective agonist (ONO-4819) in an experimental system of ectopic bone formation using recombinant human bone morphogenetic protein-2 (rhBMP-2). Collagen pellets containing rhBMP-2 were implanted onto the back muscles of mice and then treated with ONO-4819 administered every 8 h by subcutaneous injection. The ossicles elicited ectopically by rhBMP-2 in mice treated with 30 μg/kg ONO-4819 were significantly larger in size and had a higher bone mineral density and bone mineral content when compared to the controls. We also noted that the anabolic effect of ONO-4819 was seen only in the early phase of the rhBMP-2-induced bone-forming process. These experimental results indicate that the EP4 receptor agonist enhances the rhBMP-2-induced bone formation through a selective effect on early stage mesenchymal cells, which in turn may result in increased responsiveness of the host animals to rhBMP-2.

Keywords: Prostaglandins; EP4 agonist; BMP; Bone morphogenetic protein; Bone formation; Osteoblast; Mesenchymal cell

Bone morphogenetic proteins are physiological agents responsible for the inherent regenerating potential of bone. These proteins promote the differentiation of early stage mesenchymal cells into chondrogenic and osteogenic lineages to support new bone formation. Because of the specific biological activity of BMPs and the successful generation of synthetic BMPs by DNA recombination, there is tremendous interest in using these proteins for bone repair and reconstructive surgery in the clinic. However, one of the major problems with the clinical application of rhBMP-2 is that milligrams of protein is required to elicit new bone formation in humans. The lower responsiveness of humans to BMP results in a high cost for the clinical use of the BMP. Therefore, in order to improve the cost-effectiveness of this protein, the use of additional drugs to enhance BMP action might be a possible solution.

Prostaglandins produced by osteocytes or cells of the osteoblastic lineage have been shown to play an important role in both bone formation and bone resorption [1–3]. Among the PGs, prostaglandin E (PGE) exhibits significant anabolic effects on bone formation when administered systemically or locally to the skeleton [4–6]. PGE_2 exerts its effects through the interaction with specific G-protein-coupled surface receptor subtypes, EP1, EP2, EP3, and EP4. These receptors are encoded by distinct genes, are expressed differentially depending on the tissue, and are responsible for the tissue-specific actions of PGE_2 [7–9]. A previous study using mice that lack each of the four receptor subtypes revealed that EP4 is the only receptor that mediates the PGE_2-induced anabolic action in bone formation in

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rodents [10–13]. Further studies indicated that an EP2-selective agonist could enhance bone healing in beagle dogs or rats [14,15]. Taken together, these data suggest that BMP-elicited bone formation could be enhanced with the use of PGE_2 receptor-specific agonists.

To test this hypothesis, we examined the effect of an EP4 subtype-specific agonist on BMP-dependent ectopic bone formation in vivo.

Materials and methods

Mice. One hundred and ten male closed colony SPF (ICR) mice (Charles River Japan, Tokyo), four weeks of age, were housed and acclimated in cages with free access to food and water for one week. Experiments were carried out in strict accordance with the Institutional Guidelines for the Care and Use of Laboratory Animals of Osaka City University.

Preparation of BMP-containing collagen pellet implants. RhBMP-2 was produced by the Genetics Institute (Cambridge, MA) and donated to us through Yamanouchi Pharmaceutical (Tokyo, Japan). The rhBMP-2 was provided 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. To prepare one implant sample, 5 μL (5 μg of rhBMP-2) of the rhBMP-2 solution was added to 20 μL of 0.01 N HCl solution and blotted onto a collagen sponge disk (6 mm diameter, 1 mm thickness) fabricated from commercially available bovine collagen sheets (Helistat, Integra Life Sciences, Plainsboro, NJ), freeze-dried, and kept at −20 °C until implantation into mice. All procedures were carried out under sterile conditions.

EP4-receptor-selective agonist (ONO-4819). The prostanoid receptor EP4-selective agonist (ONO-4819), methyl 7-[(1*R*,2*R*,3*R*)-3-hydroxy-2-[(*E*)-(3*S*)-3-hydroxy-4-(*m*-methoxymethylphenyl)-1-butenyl]-5-oxocyclopentyl]-5-thiaheptanoate (Patent Cooperation Treaty published No. WO 00/03980), was obtained from Ono Pharmaceutical (Osaka, Japan) and dissolved in physiological saline prior to subcutaneous injection into mice.

Experimental design. The mice were anesthetized with diethyl ether before surgery and the rhBMP-2 containing collagen pellet was implanted into the left dorsal muscle pouches (one pellet per animal). To determine the dose-dependent effects of the EP4 agonist, the mice were divided into four groups (10 mice per group). Vehicle alone (0 μg/kg), or 10, 30, and 100 μg/kg ONO-4819 were injected subcutaneously every 8 h for 21days after surgical implantation of the pellets. In order to examine the phase-dependent effect of the EP4 agonist during the BMP-induced bone forming process, another group of mice was divided into four sub-groups (10 mice per group) and injected with ONO-4819 subcutaneously (30 μg/kg body weight) every 8 h for 7 days during the pre- and post-implantation phases. The phases were divided as follows; −1–0w (day −7 to day −1), 0–1w (day 0 to day 6), 1–2w (day 7 to day 13), and 2–3w (day 14 to day 21) after surgery. Three weeks after surgery, the mice were sacrificed, the implants were harvested and evaluated by morphological, radiological, and histological methods.

Evaluation of rhBMP-2-induced ectopic bone. The size of each ossicle was determined based on three mutually orthogonal measurements (*a*, *b*, and *c*) by using a caliper. A formula, $V = abc\pi/6$, was used to calculate ossicle volumes. All harvested tissues were radiographed with a soft X-ray apparatus (Sofron, Tokyo, Japan). To evaluate bone quality and the quantity of the ossicles, the BMD (milligrams per square centimeter) and BMC (milligrams per ossicle) of each ossicle were measured by dual-energy X-ray absorptiometry (DXA) using a bone mineral analyzer (DCS-600EX, Aloka, Tokyo). The ossicles or tissue mass from each group was then fixed in neutralized 10% formalin, decalcified with K-CX (Fujisawa Pharmaceutical, Japan), and embedded in paraffin wax. Sections of 3 μm in thickness were cut, stained with hematoxylin–eosin, and observed under a light microscope.

Bone metabolic markers in mice. In order to investigate the effects of ONO-4819 on systemic bone metabolism, blood samples were collected from mice lacking implants in the control and ONO-4819 (30 μg/kg ONO-4819; 5 mice per group) groups following one, two, and three weeks of drug treatment. The blood samples were stored at −80 °C until required for biochemical measurements. Serum osteocalcin was measured by immunoradiometric assay (IRMA) using a commercial kit (Immutopics, San Clemente, CA). Total alkaline phosphatase (ALP) activity, calcium (Ca) and phosphate (P) in serum were also measured in each group with commercially available kits.

Statistical Analysis. Data are presented as means ± SE. The degree of significance was determined by post hoc testing using the Bonferroni method. An associated probability (P value) of <0.05 was considered significant.

Results

Effects of ONO-4819 on rhBMP-2-induced ectopic bone

Administration of ONO-4819 at the dose of 30 μg/kg percutaneously every 8 h for 3 weeks produced large ectopic ossicles (Fig. 1) (Control 45.0 ± 8.1 mm^3, 10 μg/kg 48.7 ± 4.4 mm^3, 30 μg/kg 86.5 ± 10.1 mm^3, and 100 μg/kg 55.7 ± 8.9 mm^3). There were no significant differences between the control group and 10 or 100 μg/kg ONO-4819 treatment groups.

Radiographic examination revealed that the calcified mass area in 30 and 100 μg/kg group was larger than the mass observed in control group (Fig. 2). The trabeculae in the ossicles of the 30 and 100 μg/kg group appeared more densely packed than those in the control ossicles based on histological examination (Figs. 3A–D).

The systemic administration of ONO-4819 increased the bone mineral content (BMC) and bone mineral density (BMD) of rhBMP-2-induced bone (Fig. 4). In terms

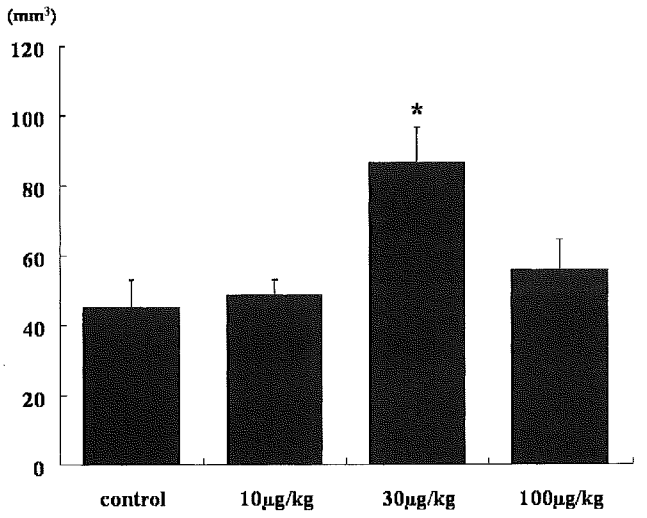

Fig. 1. Volume of harvested tissues ($n = 10$ each). RhBMP-2-induced ossicles with 30 μg/kg ONO-4819 treatment were significantly larger than the controls. Data expressed as means ± SE. *Significantly different from controls ($P < 0.05$).

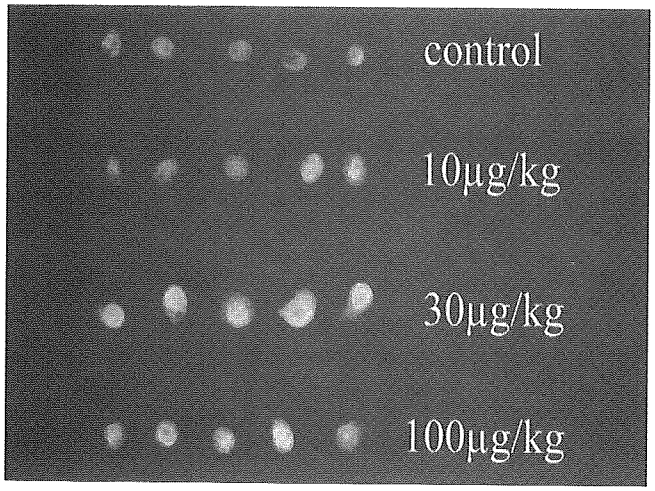

Fig. 2. Soft X-ray photograph of the ossicles harvested at 3 weeks after implantation. A typical implant from each group is shown (control, ONO-4819 10, 30, and 100 μg/kg). The radio-opaque areas of the ONO-4819 treatment groups were larger than those observed in the control group.

of BMC and BMD, the values of 30 μg/kg group (BMC 21.57 ± 4.13 mg, BMD 33.79 ± 1.64 mg/cm^2) were significantly higher than those of control group (BMC 7.91 ± 1.27 mg, BMD 18.68 ± 1.30 mg/cm^2). For BMD, the values of 100 μg/kg group (BMC 13.47 ± 2.26 mg, BMD 30.61 ± 1.92 mg/cm^2) were significantly higher than those of control group. There were no significant differences between 10 μg/kg group (BMC 12.87 ± 2.19 mg, BMD 22.39 ± 2.03 mg/cm^2) and control group. The evaluation of the effect of ONO-4819 on BMC and BMD of tibiae in mice that did not receive implants without BMP implantation showed no significant systemic effect at the dose of ONO-4819 used in the study (Fig. 5).

Phase-dependent effects of ONO-4819

Fig. 6 shows the effect of ONO-4819 on early, intermediate, and late phases of BMP-induced new bone formation. ONO-4819 enhanced rhBMP-2-induced bone formation for the first week after implantation and

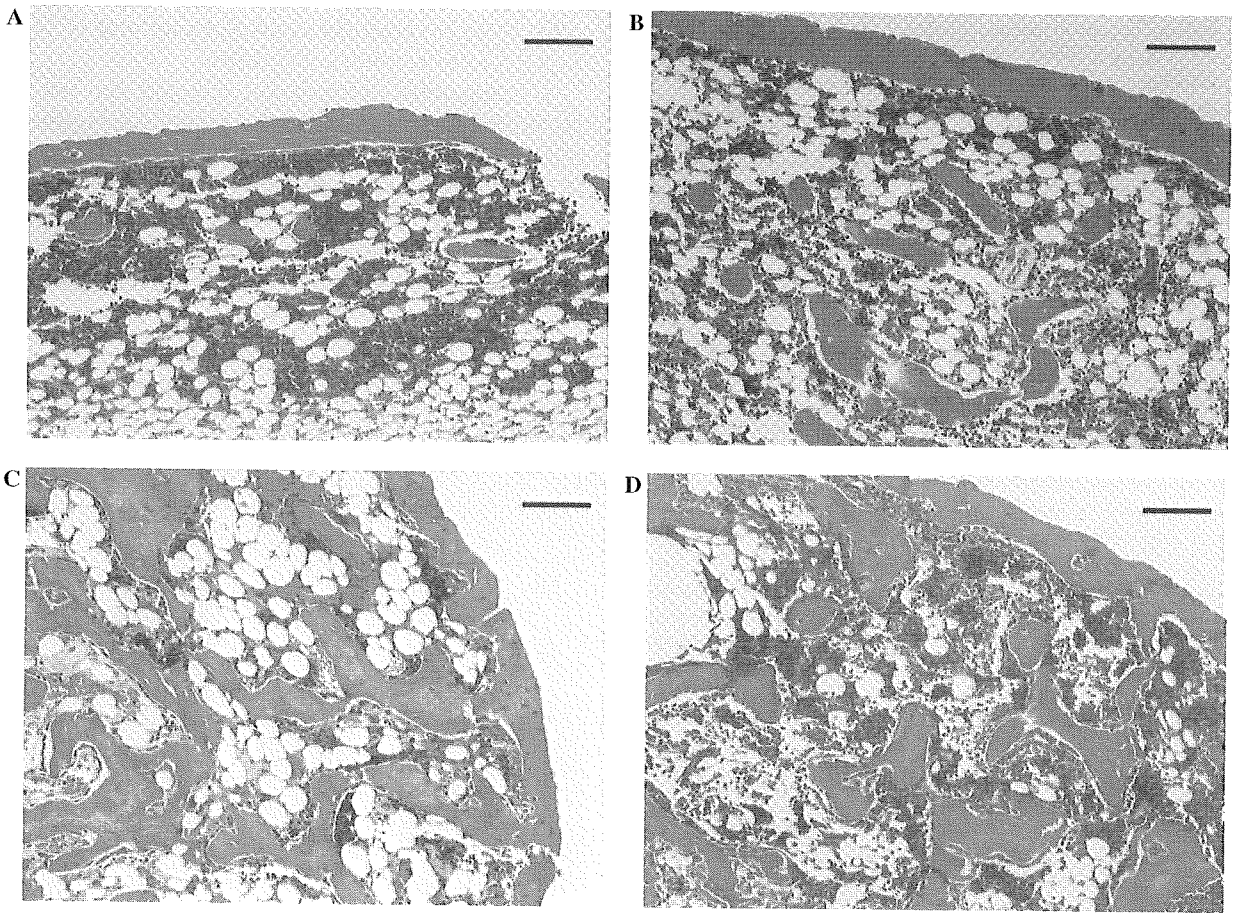

Fig. 3. Histological sections of the ossicles at 3 weeks after implantation are shown (hematoxylin–eosin stain; original magnification 100×, bar = 100 μm). (A) Control, (B) ONO-4819 10 μg/kg, (C) ONO-4819 30 μg/kg, and (D) ONO-4819 100 μg/kg. New bone formation with hematopoietic marrow and bony trabeculae were visible in the rhBMP-2-induced ossicles. In the 30 and 100 μg/kg ONO-4819 treatment groups, there were visible increases in the number and thickness of bony trabeculae when compared with the control group.

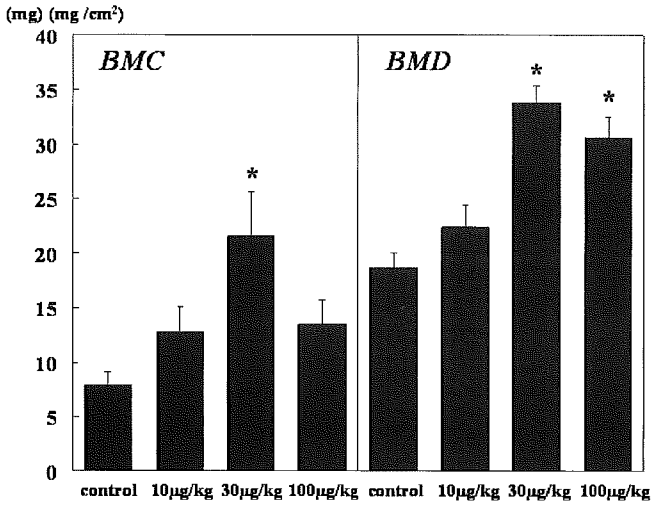

Fig. 4. The bone mineral content (BMC) and density (BMD) of the ossicles at 3 weeks after implantation. There were significant differences between the control group and the 30 μg/kg ONO-4819 group in terms of BMC and BMD. The BMD from 100 μg/kg ONO-4819 group was significantly higher than that of control group. Data are expressed as means ± SE. *Significantly different from controls ($P < 0.05$).

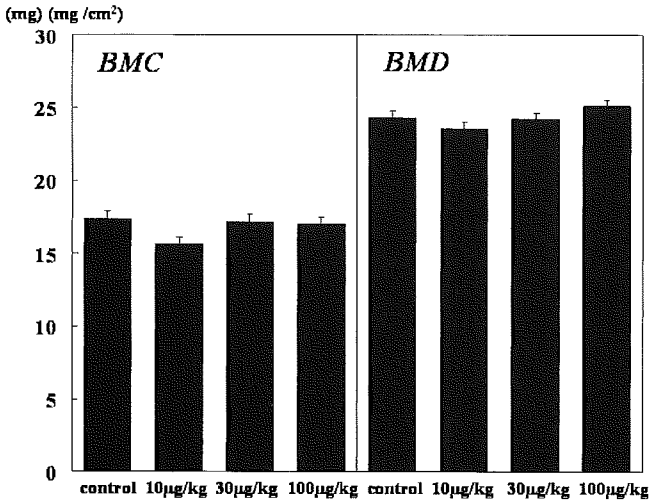

Fig. 5. The effect of ONO-4819 on the systemic skeletal system was examined by recording the values for bone mineral content (BMC) and density (BMD) in the tibia in mice that did not receive rhBMP2 pellets. There were no significant differences between the treatment groups and the control groups.

BMC and BMD in that group were significantly higher (BMC 16.51 ± 3.24 mg, BMD 29.75 ± 2.23 mg/cm^2) than the control group. There was no significant difference among other groups, −1–0 week (BMC 9.66 ± 1.79 mg, BMD 17.90 ± 2.02 mg/cm^2), 1–2 week (BMC 10.14 ± 1.17 mg, BMD 24.04 ± 1.24 mg/cm^2), 2–3 week groups (BMC 8.04 ± 1.08 mg, BMD 17.40 ± 1.20 mg/cm^2), and control group (BMC 7.91 ± 1.26 mg, BMD 18.68 ± 1.30 mg/cm^2).

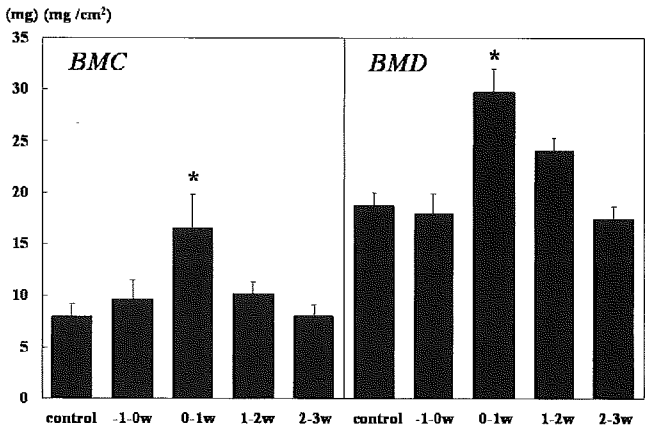

Fig. 6. The phase-dependent effect of ONO-4819 on rhBMP-2-induced ectopic bone formation was examined at the level of bone mineral content (BMC) and density (BMD) of the ossicles. The BMC and BMD from the initial phase (0–1 week) treatment group were significantly higher than the values recorded for the control group. There were no significant differences between pre-(−1–0w), middle-(1–2w) or late-(2–3w) phase and the control group. Data are expressed as means ± SE. *Significantly different from controls ($P < 0.05$).

Systemic effects of ONO-4819

The side effects of ONO-4819 administration were represented by changes in body weight of mice in each group receiving this drug. All of the experimental groups that received ONO-4819 injections for 3 weeks showed suppressed body weight gain compared to control group. A marked retardation in body weight increase was observed in 100 μg/kg group (Fig. 7).

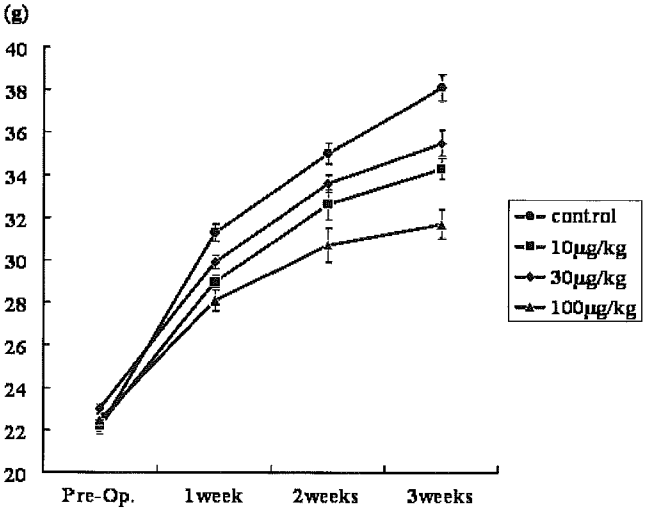

Fig. 7. Mice that received a systemic injection of ONO-4819 for 3 weeks showed a significant decline in body weight gain when compared to the control group. There was a marked retardation in body weight gain in the 100 μg/kg ONO-4819 treatment group.

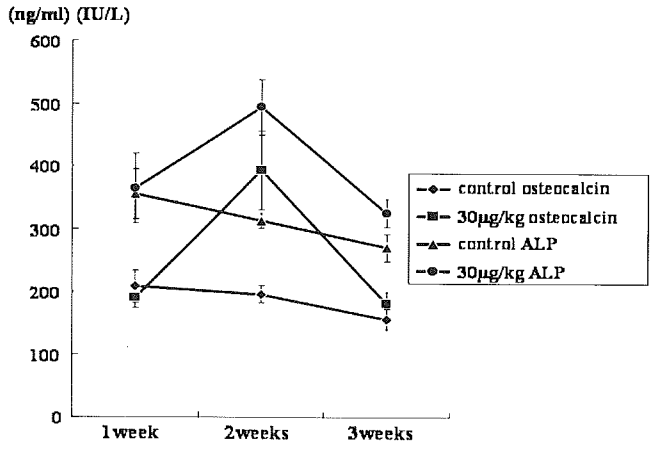

Fig. 8. Two weeks after the start of drug treatment, the serum osteocalcin level and total ALP activity from the 30 μg/kg ONO-4819 group that did not receive rhBMP-2 pellets were increased significantly when compared to the control groups.

Bone metabolic markers in mice

After two week administration of ONO-4819, both serum osteocalcin (393 ± 62.5 ng/ml) and ALP activity (494 ± 44.2 IU/L) levels increased significantly compared to controls (osteocalcin 196 ± 13.9 ng/ml, ALP activity 313 ± 12.1 IU/L). However, there were no significant differences in these parameters at 1 week and 3 weeks after administration (Fig. 8). There was no significant increase in serum calcium and phosphate levels in any of the groups at any time point (data not shown).

Discussion

The experimental results presented above indicated that the EP4 agonist enhanced rhBMP-2-induced ectopic new bone formation in mice. Therapeutic agents that enhance bone formation have been described in previous studies. Daily subcutaneous injection of 1–34 or 1–84 parathyroid hormones (PTH) is known to enhance systemic bone formation and is currently utilized for the treatment of osteoporosis [16]. In addition, it has been shown that a daily systemic injection (20 mg/kg) of the phosphodiesterase-4 (PDE-4)-selective inhibitor, Rolipram, can enhance BMP-2-dependent ectopic new bone formation in mice [17]. All of these agents elevate intracellular cyclic nucleotides (cyclic AMP or GMP) via the G-protein-coupled receptors specific to the respective ligand. However, the exact mechanisms by which BMP-dependent bone formation is enhanced after the elevation of intracellular cAMP in bone forming cells remain unclear and further studies are required.

PGE_2 elevates intracellular cAMP and it produces this effect on cells through four receptor subtypes, EP1, EP2, EP3, and EP4. The anabolic effect of PGE_2 on bone was effected through the activation of EP2 or EP4 and consequent elevation of intracellular cAMP level [18]. In a previous animal study, the activation of the EP4 receptor by continuous administration of an EP4-selective compound agonist was reported to enhance fracture repair. [13]. Paralkar et al. [14] also reported that an EP2-selective compound agonist (CP-533,536) enhanced bone healing in critical sized bone defects of the canine ulnar and tibial osteotomy model. Clearly, given the data from all of these studies, there is strong evidence to conclude that PGE_2-selective receptor agonists have anabolic effects on bone metabolism. These findings prompted us to use EP4 receptor-selective agonist (ONO-4819) to improve the performance of BMP-dependent bone formation. In this study, the systemic administration of ONO-4819 (30 μg/kg) at 8 h intervals resulted in a significant increase of both BMC and BMD in the BMP-induced ectopic ossicles. However, higher doses (100 μg/kg) of ONO-4819 resulted in slight increase of BMD and no increase in BMC with suppression of body weight increase. These data would suggest that at higher doses, the systemic administration of the EP4 agonist may cause adverse events, an important observation in terms of the practical application of this reagent.

Another explanation for the anabolic effect of the EP4 agonist on the BMP-induced bone formation comes from the studies involving cyclooxygenase (COX). Cyclooxygenase is the major enzyme that produces prostaglandins. COX-2 is one of the isoforms highly expressed in osteoblasts [19]. Zhang et al. [20] showed the complementary effect of BMP-2 in $COX\text{-}2^{-/-}$ marrow cell culture and suggested that BMP signaling events might replace PGE_2 effects. Chikazu et al. [21] reported that in osteoblasts, BMP-2 transcriptionally induces COX-2 expression which in turn regulates, via the cbfa-1 binding site, the production of PGE2 and promotion of osteoblastic differentiation. From these data, a conclusion might be that BMP and PGE_2 might have complementary or cooperative anabolic effects on bone metabolism. These results indicate that ONO-4819 and BMP work cooperatively on mesenchymal cells to stimulate the early phase of osteoblastic differentiation.

In the BMP-induced ectopic bone forming reaction, undifferentiated mesenchymal cells in contact with BMP proliferate and differentiate to chondrocytes in the initial phase. The resultant cartilage tissue is resorbed and replaced by bone through the vascular invasion by osteoblasts and marrow cells in the subsequent phases. In order to identify the time phase when ONO-4819 exerts its pharmacological effects, the EP4 agonist was administered for one week over the pre-(−1–0 week), initial-(0–1 week), middle-(1–2 week) or late-(2–3 week) phase, respectively. The anabolic effect of the EP4 agonist was seen in mice that received the EP4 agonist during the initial phase. This result would indicate the EP4 agonist acted upon the proliferating mesenchymal cells at the interface with the BMP-retaining pellets.

The anabolic effects of ONO-4819 administered systemically appeared fairly specific to the BMP-induced ossicles, since the BMD of the host mouse skeleton of the host mice was unchanged. Serum osteocalcin and alkaline phosphatase levels were significantly increased at two weeks after ONO-4819 injection but the levels returned to baseline during the period of treatment with the EP4 agonist.

In summary, the intermittent systemic administration of ONO-4819, a selective agonist for the EP4 prostanoid receptor, was shown to have an anabolic effect on ectopic ossicles induced by rhBMP2. The doubling of ossicle size by 30 μg/kg BW of ONO-4819 indicates that this agent enhances the action of BMP. The potentiation of BMP action by the ONO-4819 compound could potentially reduce the dose of BMP required to form an adequate mass of new bone. Higher doses of the EP4 agonist resulted in adverse catabolic effects, a finding that may influence the route for administration of this drug. In order to avoid these effects, the local administration of a low and optimized dose of the drug in combination with BMP will achieve the goals of enhancing the BMP action by the drug and reducing the dose of BMP for new bone formation. The development of a practical delivery system to enable the coadministration of BMP and an EP4 agonist is currently underway in our laboratory.

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Enhancement of recombinant human bone morphogenetic protein-2 (rhBMP-2)-induced new bone formation by concurrent treatment with parathyroid hormone and a phosphodiesterase inhibitor, pentoxifylline

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Abstract We investigated the enhancement of new bone formation elicited ectopically by recombinant human bone morphogenetic protein-2 (rhBMP-2), using parathyroid hormone (PTH) and a phosphodiesterase inhibitor (PDEi), pentoxifylline (PTX), in an animal model. Collagen sponge sheet discs containing rhBMP were implanted onto the back muscles of mice. PTX alone (200 mg/kg body weight [BW]), PTH(1–34) (10 μg/kg BW), PTX plus PTH (200 mg/kg BW and 10 μg/kg BW, respectively), or vehicle (control) were injected subcutaneously daily for 3 weeks after implantation. At the end of this period, rhBMP-2-induced ectopic ossicles were harvested from each group of animals. Ossicles from the PTX-treated group were significantly larger in size, with unchanged bone mineral density (BMD), as compared with the ossicles from the controls. In contrast, the ossicles from the PTH-treated group had significantly higher BMD, but showed no difference in size when compared with those from the control animals. The ossicles of the PTX + PTH treatment group were significantly larger than those of the control and PTH treatment groups. In addition, the BMD of the harvested tissues from the PTX + PTH treatment group was significantly higher than that of tissues from the control and PTX treatment groups. Although the calcium content of ossicles was significantly higher in the PTX-, PTH-, and PTX + PTH-treated groups than in the control group, the Ca content of ossicles from the PTH + PTX-treated group was highest (two times that of controls), followed by the PTH- and PTX-treated groups.

Key words bone morphogenetic protein (BMP) · ectopic bone formation · parathyroid hormone · phosphodiesterase inhibitor (PDEi)

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Introduction

Parathyroid hormone (PTH) is a major regulator of osteoblastic function through its specific G-protein-coupled receptor and the protein kinase A (PKA) kinase cascade [1,2]. A number of animal studies have demonstrated that intermittent PTH injection in animals exerts anabolic effects on bone formation [3–7], although the precise mechanism of this anabolic action remains unknown. From a clinical perspective, the anabolic action of this hormone highlights its potential value in the treatment of bone diseases associated with suppressed bone formation or bone loss, such as osteoporosis and non-union fractures.

Phosphodiesterase inhibitors (PDEis), e.g., pentoxifylline (PTX) and rolipram, have also been shown to enhance bone-forming potential in in vivo situations [8–10], probably through elevating intracellular levels of cyclic adenosine monophosphate (cAMP) in osteoblasts and enhancing the responsiveness of young mesenchymal cells to BMP-2 or BMP-4 [11,12].

The intracellular level of cAMP is upregulated by G protein-coupled adenylcyclase [13] and degraded by phosphodiesterases (PDEs) [14], a family of enzymes that catalyze the hydrolysis of cAMP and cyclic granosine monophosphate (cGMP). Thus, both PDEis and PTH may lead to the elevation of intracellular cAMP following activation via the PKA cascade. However, based on previous experimental studies, the mechanisms of action of these agents appear to be different. Pentoxifylline (PTX), a non-selective PDE inhibitor, promoted larger bone morphogenetic protein (BMP)-induced ossicles [9] with identical bone mineral density (BMD) when compared to non-treated control ossicles. In contrast to the effects of the PDEi, the BMP-induced ossicles from PTH-treated animals had an identical size and higher BMD when compared with the controls. These experimental data invoke the possibility that concurrent treatment with PDEi and PTH may act

in a complementary manner to maximize BMP-induced new bone mass. Clearly, this could have significant therapeutic value in the clinical setting. The present experimental study was designed to address this hypothesis.

Materials and methods

Preparation of BMP-containing collagen pellets

Recombinant human (rh)BMP-2 was produced by the Genetics Institute (Cambridge, MA, USA) and donated to us through Yamanouchi Phamaceutical (Tokyo, Japan). The rhBMP-2 was provided in a buffer solution (5 mM glutamic acid, 2.5% glycine, 0.5% sucrose, and 0.01% Tween 80) at a concentration of 1 μg/1 μl after filter sterilization. To prepare one collagen disc implant sample, 5 μl (5 μg of rhBMP) of the rhBMP-2 solution was added to 20 μl of 0.01 N HCl solution and blotted into a porous collagen disc (6 mm in diameter, 1 mm in thickness) fabricated from commercially available bovine collagen sheets (Helistat; Integra Life Sciences, Plainsboro, NJ, USA), freeze-dried, and kept at −20°C until implantation into mice. All procedures were carried out under sterile conditions.

Pentoxifylline and PTH(1–34)

Pentoxifylline (PTX) was obtained from Research Biochemical International (Natick, MA, USA) and dissolved in physiological saline prior to use. Human (h)PTH(1–34) was synthesized and donated to us by Asahi Chemical (Tokyo, Japan). The hPTH(1–34) was dissolved in vehicle containing 0.1% bovin serum albumin (BSA).

Experimental protocols

Forty male ddy mice, at 4 weeks of age, were purchased from Nippon SLC (Shizuoka, Japan). The mice were housed in cages with free access to food and water from 1 week before the start of the experiment until 3 weeks after implantation. The mice were randomly assigned to four groups (10 mice for each group): (1) control group: vehicle alone, (2) PTX 200 mg/kg (body weight) BW, injected daily, (3) PTH 10 μg/kg BW, injected daily, and (4) PTX 200 mg/kg BW + PTH 10 μg/kg BW, injected daily. The doses of PTH and PTX were optimized based on data from our previous study [9] and preliminary experiments.

Before surgery to implant the samples, the animals were anesthetized with diethyl ether. The uniformly-sized implants were placed into the left dorsal muscle pouches (one implant per animal).

The implants were harvested and processed for radiological, chemical, and histological examinations. All of the harvested tissues were measured and then radiographed with a soft X-ray apparatus (Sofron; Sofron, Tokyo, Japan) and fixed in 10% neutral pH formalin solution. The volume of each ossicle was determined based on three mutually orthogonal measurements (A,B,C) of the ossicle, done with a caliper, and the ossicle volume was calculated by the formula, $V = ABC \times \pi/6$ [10,15,16]. The BMD of each ossicle (g/mm^2) was measured by single-energy X-ray absorptiometry (SXA), using a bone mineral analyzer (DCS-600R; Aloka, Tokyo, Japan). After measurement of these parameters, three of the ten samples from each group were defatted with chloroform, decalcified with 10% EDTA, and embedded in paraffin wax. Sections 5 μm in thickness were cut, stained with hematoxylin and eosin (H&E), and examined under a light microscope. The remaining seven samples from each group were decalcified in 5 ml of 0.6 N HCl in separate sealed glass bottles and stirred for 48 h. The calcium content of each bottle was measured by the orthocresolphthalein complexone (OCPC) method (Calcium C-test kit, wako; Wako Pure Chemical Industries, Osaka, Japan), as previously described [9,17,18]. All the animal experiments were carried out in compliance with the requirements of the Institutional Animal Care Committee at Shinshu University.

Statistical analysis

The data were assessed by Kruskal-Wallis analysis of variance and the Mann-Whitney *U*-test. A P value of 0.05 was used to delineate significant differences between groups.

Results

Volume of the harvested tissue

Figure 1 shows the mean volumes of the harvested tissue from each of the four groups 3 weeks after implantation. The mean volume of the harvested tissue of the

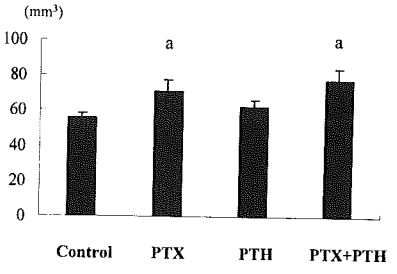

Fig. 1. The ossicles of the PTX alone and PTX + PTH groups were significantly larger than those of the control group. However, there was no significant difference between the volumes of the PTH-alone and control groups (n = 10). Data values are means ± SD. [a] $P < 0.05$ vs control

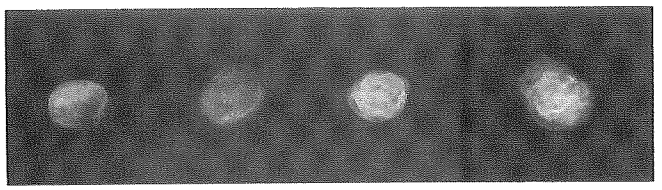

Fig. 2. Soft X-ray photographs of the ossicles at 3 weeks after implantation. A typical ossicle from each group is shown

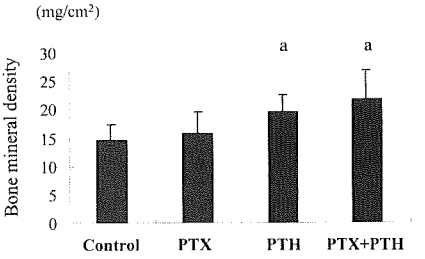

Fig. 3. The bone mineral density (BMD) values of the harvested tissues from the PTH-alone and PTX + PTH group were significantly higher than that in the control group (n = 10). Data values are means ± SD. $^{a}P < 0.05$ vs control

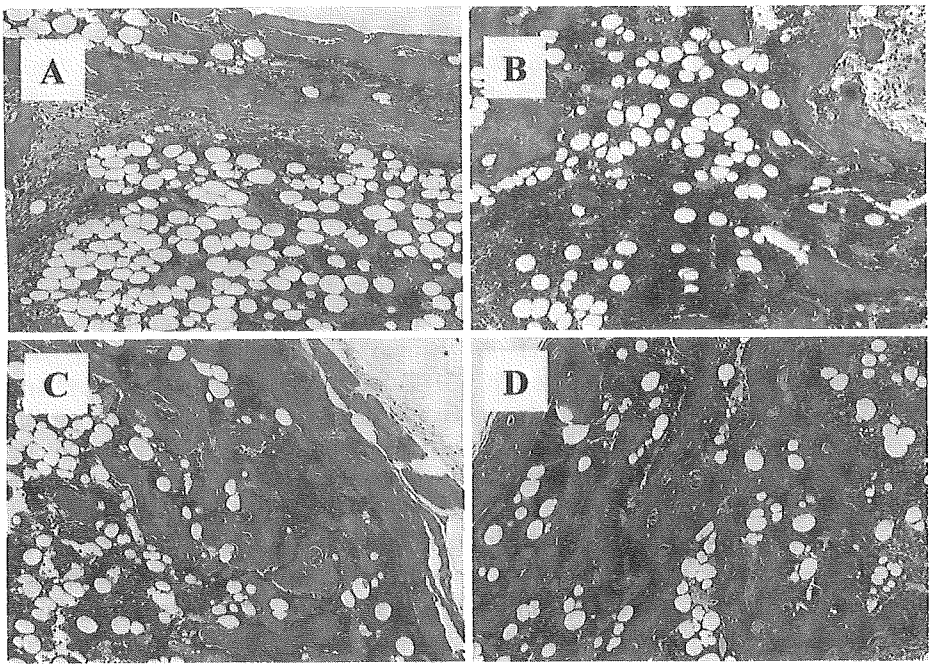

Fig. 4. Photomicrographs of the harvested tissues at 3 weeks after implantation. **A** Control; **B** PTX; **C** PTH; **D** PTX + PTH. New bone formation with hematopoietic bone marrow and trabeculae is visible in the tissue. In the PTH-alone treatment group and the PTX + PTH treatment group, there were visible increases in the number and thickness of bony trabeculae when compared to the PTX and control group. **A–D** H&E, ×100

control group was 55.6 ± 2.5 mm³; that of the PTX group was 70.9 ± 6.3 mm³; and that of the PTX + PTH group, 77.3 ± 6.5 mm³. The values for the PTX and PTX + PTH groups were significantly higher than that recorded for the control group ($P < 0.05$). However, there was no significant difference between the controls and the PTH group (62.1 ± 3.5 mm³).

Radiographic findings

The radiographic appearances of the harvested tissues 3 weeks after implantations are shown in Fig. 2. All of the pellets harvested 3 weeks after implantation showed radiographic evidence of calcification and calcified trabeculae.

Bone mineral density (BMD) of the ossicles

Figure 3 shows the mean BMD of ossicles from each group, measured with SXA. There was no significant difference in BMD between the PTX group (15.7 ± 3.8 mg/cm²) and the control group (14.5 ± 2.7 mg/cm²). However, the BMDs of the PTH group (19.4 ± 3.1 mg/cm²) and the PTX + PTH group (21.8 ± 5.2 mg/cm²) were significantly higher than those of the control group and the PTX group.

Histology

Ossicles from all of the four groups revealed normal bone histology, with hematopoietic marrow and bony trabeculae (Fig. 4). Small amounts of collagen carrier remnants were seen in the centers of all the ossicles.

Calcium content in the ossicles

The mean calcium content in ossicles harvested from each group is shown in Fig. 5. The calcium content in

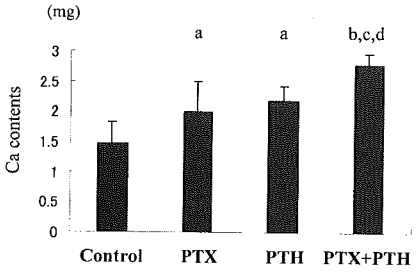

Fig. 5. Ca content of the harvested tissues at 3 weeks after implantation. Ca content was measured by OCPC method (Calcium C test kit; Wako) ($n = 7$). The Ca content of the ossicles from the PTX group and the PTH group was significantly higher than that in the control group. The Ca content of the ossicles from the PTX + PTH concurrent treatment group was significantly higher than that in the ossicles from the control, PTX-alone, and PTH-alone treatment groups. Data values are means ± SD. [a] $P < 0.05$ vs control; [b] $P < 0.01$ vs control; [c] $P < 0.05$ vs PTX group; [d] $P < 0.05$ vs PTH group

ossicles from the PTX (1.99 ± 0.51 mg), PTH (2.18 ± 0.24 mg), and PTX + PTH groups (2.78 ± 0.18 mg) was significantly higher than the value in the control group (1.46 ± 0.36 mg). The Mean calcium content in the ossicles of the PTX + PTH group was significantly higher than that in the PTH and PTX groups ($n = 7$).

Discussion

The results of the present experimental study revealed additive anabolic effects of the daily injection of active fragment of human PTH and a nonselective PDEi (PTX) on endochondral ossification elicited ectopically by rhBMP-2 in mice. The additive and complementary effects of both agents on endochondral bone formation were postulated from the results of our previous studies using the same model, indicating two aspects of the enhancement of bone formation, i.e., increased mass of the new bone and increased density of the new bone. Daily injection of PTX increased the mass of BMP-induced ectopic bone without changing the BMD [9], and PTH increased the BMD without changing the bone mass. In the initial phase of new ectopic bone formation in the BMP-retaining collagen pellets, the pellet is resorbed and replaced by a cartilage shell at the periphery of the pellet. This shell is then thought to be replaced by new bone through endochondral ossification, with the shell determining the final size of the BMP-induced ossicle [19]. In previous studies of ours, it was radiographically determined that the calcified rings in tissues harvested from mice treated for 1 week with PTX and other PDEi agents were larger than those observed in the control group [9,10]. Because the chondro-osseous differentiation of undifferentiated mesenchymal cells is initiated when these cells come into contact with BMP at the periphery of the BMP-retaining disk, this increase in the bone mass may indicate greater sensitivity and earlier initiation of the response to BMP-2 induced by PTX compared with the control. PTX therefore appears to have a stimulatory effect on the early stage of bone formation during BMP-induced osteogenesis. In an in vitro study, PTX has been shown to enhance BMP effects predominantly in less differentiated cells which have the potential for osteogenic or chondrogenic differentiation [20]. PTX may target undifferentiated mesenchymal cells that come into contact with BMP in the initial phase of ossification and may increase their responsiveness to BMP. This, is turn, may lead to the earlier initiation of chondrogenic differentiation, resulting in a larger cartilagenous anlage to be replaced later by new bone tissue, and leading to an increase in implant size. However, our study did not find an increase in the size of the harvested tissue as a result of PTH treatment. This indicates that PTH may not affect the cartilage formation phase in this model. The tissues harvested from mice treated with PTH for only the first week were not significantly different from the control, either in size or in BMD (data not shown). The BMD of the tissue harvested from the PTH-treatment group at 3 weeks after implantation was significantly higher than that of the tissue from the control group and the group treated with PTX alone. These findings suggest that PTH may affect a late rather than an early phase of this BMP-induced endochondral ossification process. The mechanisms of the anabolic effect of PTH remain unknown, however. Several studies have reported that the receptor for PTH is expressed in the cells of both chondrocytes and osteoblasts [21,22]. However, this claim does not seem to support the findings of the present study, in that the anabolic effects of PTH were observed at a later phase of the BMP-induced endochondral ossification process. Further studies to clarify this point therefore appear to be necessary.

Thus, PTX and PTH appear to act on distinct phases of the osteogenic process. Therefore, we hypothesized that the two agents would work additively when used concurrently. Our data seemed to confirm this hypothesis, with concurrent PTX + PTH treatment resulting in the induction of new ossicles of a larger size and with a higher BMD than the controls.

Several studies have reported that the cortical area of the tibia or femoral diaphysis increased significantly after injections of 50–200 μg/kg BW per day of PTH(1–34) [23–28]. However, other studies found that injection at doses of 1.5–10 μg/kg BW had an anabolic effect on bone formation [29–30]. We performed preliminary experiments in the model used in the present study. The injection of 4, 10, and 40 μg/kg BW per day of PTH (1–

34) resulted in a significant increase in the BMD of the harvested tissues compared with that produced by the vehicle alone. However, it should be noted that the increase in BMD generated by the 40 μg/kg-dose (18.5 ± 3.9 mg/cm²) was less than that resulting from the doses of 4 μg/kg (20.5 ± 3.3 mg/cm²) and 10 μg/kg (21.0 ± 3.5 mg/cm²). The dose of 10 μg/kg was therefore considered optimal, and a low dose of PTH(1–34) seemed to have an effect on this ectopic bone-formation model.

The mechanisms underlying the anabolic effects of PTH and PTX on bone formation are not fully understood. PTH appears to increase the bone-forming activity of osteoblasts, and it may increase the rate of maturation of pre-osteoblasts into osteoblasts, or it may increase the bone-forming activity of osteoblasts [31]. Bone mass can be increased by intermittent PTH administration, but the mechanism of this phenomenon is not known. In the study presented here, PTH treatment was found to increase the BMD and the calcium content of BMP-2 induced ectopic new bone, but it did not increase bone volume. These results indicate that intermittent administration of PTH is likely to have an anabolic effect on BMP-2-induced ectopic new bone formation. Further studies are needed, however, to clarify the mechanisms involved.

The exact mechanism by which a PDEi stimulates BMP-induced bone formation also awaits elucidation. Elevation of intracellular cAMP level by a PDEi, coupled with intracellular signaling through the PKA cascade by PTH, may stimulate bone formation [20]. For the future, it will be important to study the crosstalk between BMP, BMP receptors, Smads, Cbfa-1, and the PKA signaling cascade. There is considerable evidence in the literature to suggest that the anabolic effects of PTH are mediated by cAMP [1,2,32] and, by extrapolation, PDEs [11,20].

A recent study aimed at further understanding of the anabolic actions of PTX on bone formation has also implicated crosstalk between BMP signaling and PKC signaling cascades [12]. However, that report mentioned that PDEis, including PTX, could promote osteoblast differentiation by a mechanism independent of PKA activation. We speculate that this mechanism may be one of the reasons why PTH and PDEi have different effects on osteoblast differentiation. Future studies to investigate the molecules and signal pathways by which PTH and PDEis mediate osteoblast differentiation should contribute to an understanding of their anabolic effect on bone.

In conclusion, the present study has confirmed that daily injections of PTH and PTX enhance rhBMP-2 induced endochondral new bone formation in an additive and complementary manner in an animal model of bone induction. These agents may provide a new approach to enhancing the clinical efficacy of BMP-mediated new bone formation for the treatment of fracture and the correction of bone defects.

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A new bone-inducing biodegradable porous β-tricalcium phosphate

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Abstract: A new type of degradable biomaterial with bone-inducing capacity was made by combining porous β-tricalcium phosphate (β-TCP) with a delivery system for recombinant human bone morphogenetic protein-2 (rhBMP-2). The BMP delivery system consisted of a block copolymer composed of poly-D,L-lactic acid with random insertion of *p*-dioxanone and polyethylene glycol (PLA-DX-PEG), a known biocompatible and biodegradable material. The efficacy of this biomaterial in terms of its bone-inducing capacity was examined by ectopic bone formation in the dorsal muscles of the mouse. In the β-TCP implants coated with the PLA-DX-PEG polymer containing more than 0.0025% (w/w) of rhBMP-2, new ectopic bone tissues with marrow were consistently found on the surface of implants. The radiographic density of β-TCP was diminished in a time-dependent manner. On histological examination, numerous multinucleated osteoclasts with positive tartrate-resistant acid-phosphatase (TRAP) staining were noted on the surface of the β-TCP. These experimental results indicate that β-TCP implants coated with synthetic rhBMP-2 delivery system might provide effective artificial bone-graft substitutes with osteoinductive capacity and biodegradable properties. In addition, this type of biomaterial may require less rhBMP-2 to induce significant new bone mass. © 2004 Wiley Periodicals, Inc. J Biomed Mater Res 70A: 450–458, 2004

Key words: BMP; β-TCP; synthetic delivery system; osteoinductive; biodegradable

INTRODUCTION

Repair of bone fractures or defects is achieved by local new bone formation. However, the regenerative repair of bone is often impaired when the damage is severe as seen in comminuted open fractures or large bone defects associated with bone tumor resection. In these cases, autogenous bone grafting is routinely indicated to reactivate the regenerative potential and promote local bone formations because of its demonstrated efficacy.[1] The osteogenic potential of autogenous bone graft is due to the retention of osteogenic precursor cells with the ability to proliferate and differentiate to osteoblasts. Additionally, the grafted bone is resorbed and replaced by newly formed bone, thereby reestablishing a level of structural integrity at the grafted site. However, there are a couple of disadvantages associated with autogenous bone grafting. These include a limited source of donor bone coupled with donor site morbidities. To avoid these problems, new bone graft substitutes that exhibit bone-inducing capacity together with absorbability would be desirable. To date, there have been no absorbable materials with both osteoinductive and osteoconductive properties that have been proven as ideal substitutes for autogenous bone grafts, although a variety of biocompatible and osteoconductive materials have been reported.[2]

Porous beta-tricalcium phosphate (β-TCP) is well known as a biodegradable material with good osteoconductive capacity and demonstrated clinical efficiacy.[3] Some researchers have attempted to add bone-inducing capacity to β-TCP by combining this material with recombinant human bone morphoge-

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netic protein-2 (rhBMP-2) to accelerate bone healing.[4–7] Most of these studies have showed successful results by using β-TCP itself as a BMP carrier.

Meanwhile, considerable efforts have been focused on finding ways to reduce the minimum dose of rhBMP-2 that is essential for adequate bone regeneration. One of the difficulties when using rhBMP-2 clinically is the significant amount of this protein required for complete bone healing in humans. Therefore, the development of a carrier system that controls the release of rhBMP-2 is very important to reduce the dosage of rhBMP-2. We have already developed a biodegradable delivery system for rhBMP-2 that has been shown to enhance bone formation.[8–12]

In this study, we attempted to make a new biodegradable bone-inducing material by adding osteoinductive capacity of rhBMP-2 to porous β-TCP granules using a newly developed delivery system for rhBMP-2. Our goal was to test whether this approach could enhance bone formation using lower doses of rhBMP-2. The efficacy of this new bone graft substitute was examined in terms of its bone-inducing capacity and degradability in an experimental mouse model as a first step to further study in the clinic.

MATERIALS AND METHODS

Materials

As a rhBMP-2 delivery system, a block copolymer composed of poly-D,L-lactic acid with random insertion of *p*-dioxanone and polyethylene glycol (PLA-DX-PEG) was synthesized and provided by Taki Chemical (Kakogawa, Japan). The details of physicochemical properties of this polymer have been reported previously elsewhere.[10] RhBMP-2 was produced at Genetics Institute (Cambridge, MA) and donated to us through Yamanouchi Pharmaceutical Company (Ibaraki, Japan). Porous β-TCP (OSferion®, coarse granule, approximately 3 mm in particle diameter and 5 mg in weigh, from 100 to 400 μm in pore size, porosity of 75%, 1050° sintering temperature) was manufactured by Olympus (Tokyo, Japan) and donated to us for the purpose of these studies.[3,13]

Preparation of porous β-TCP granules combined with BMP delivery system

To prepare implants, 600 mg of β-TCP together with 200 mg of PLA-DX-PEG and various amounts of rhBMP-2 (0, 1.25, 5, 20, or 100 μg in 200 μL of 0.01 N HCl) were mixed in 3 mL of distilled acetone in glass vials. The resultant mixtures were then placed in a vacuum for a few seconds to replace air in the pores of the β-TCP with solvent. Acetone was then removed from the β-TCP granules by evaporation with a centrifuge evaporator. The glass vials were shaken several times during evaporation so that rhBMP-2 delivery material thoroughly impregnated the β-TCP granules. The resultant dried β-TCP granules coated with rhBMP-2 delivery system were stocked in a freezer at −30°C until use. A total of eight experimental groups including controls were prepared (Table I). The surface of porous β-TCP granule was observed by scanning electron microscopy (SEM; Hitachi 4700SI) to examine a structural characteristic of the PLA-DX-PEG coating.

TABLE I
Contents of rhBMP-2/PLA-DX-PEG in 600 mg of β-TCP and Bone Formation at 3 and 6 Weeks

	rhBMP-2 (μg)	PLA-DX-PEG (mg)	Concentration of rhBMP-2 (wt %)	Bone Formation	
				3 Weeks	6 Weeks
1	100	200	0.0125	+	++
2	20	200	0.0025	−	+
3	5	200	0.000625	−	−
4	1.25	200	0.000156	−	−
5	0	200	0	−	−
6	100	0	0.0167	+	+
7	20	0	0.0033	−	+
8	0	0	0	−	−

Materials from each group were divided into 20 implants (five to six coarse granules), respectively, and implanted into the back muscle pouch. Bone formation was rated in three grades by the pattern of newly formed bone induced around the implant. (++; uniformly covered by new bone +; partially covered by new bone −; no bone formation, assessed in soft X-ray radiographs)

Experimental protocol

One hundred sixty male ddY mice at 5 weeks of age, weighing 25–30 g, were used (20 per group) for this experiment in strict accordance with the institutional guidelines for the care and use of laboratory animals. The implants were aseptically placed into the left dorsal muscle pouch of mouse under anesthesia with diethyl ether. Approximately 30 mg of β-TCP granules including PLA-DX-PEG and rhBMP-2 (5–6 granules per animal) were implanted. Five animals from each group were sacrificed at 1, 2, 3, and 6 weeks after surgery, and the implants were harvested together with surrounding soft tissues. Harvested specimens were fixed in 10% neutral-buffered formalin solution and processed for radiological and histological examinations.

Radiographic and histological examination

The samples were radiographed with a soft X-ray apparatus (SOFRON®, Tokyo, Japan). For histological examination, samples were decalcified in 10% formic acid, dehydrated in a gradient ethanol series, mounted in paraffin, sectioned in 4 μm thickness and stained with hematoxylin-eosin. To detect osteoclasts, tartrate resistant acid phosphatase (TRAP) was stained by use of a histochemical method.